

Bcl-xL Is a Dominant Antiapoptotic Protein that Inhibits Homoharringtonine-Induced Apoptosis in Leukemia Cells

Shiliang Yin, Rui Wang, Fan Zhou, Hong Zhang, and Yongkui Jing

Department of Pharmacology, Shenyang Pharmaceutical University, Shenyang, People's Republic of China (S.Y., H.Z.); Shenyang Military General Hospital, Shenyang, People's Republic of China (F.Z.); and Department of Medicine, Tisch Cancer Institute, Mount Sinai School of Medicine, New York, New York (R.W., Y.J.)

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ABSTRACT

Homoharringtonine (HHT) has been reported to be effective in a portion of patients with acute myeloid leukemia (AML) or chronic myeloid leukemia (CML). To investigate its mechanism of action, cell growth inhibition and cytotoxicity of HHT were investigated in three AML cell lines, HL-60, NB4, and U937, and in three CML cell lines, K562, KU812, and KCL22. AML cells were more sensitive than CML cells to HHT-induced cytotoxicity. Using HL-60 cells, it was revealed that HHT decreased the levels of myeloid cell leukemia 1 (Mcl-1), X-linked inhibitor of apoptosis protein (XIAP), survivin, and B-cell lymphoma 2 (Bcl-2)-homology domain 3 (BH3)-only proteins as well as the mitochondrial membrane potential. The levels of Bcl-2, Bcl-2-associated X protein (Bax), and Bcl-2 homologous antagonist/killer (Bak) proteins in HL-60 cells were not changed after HHT treatment. U937, K562, KU812, and KCL22 cells expressed

B-cell lymphoma-extra large (Bcl-xL) and were less responsive to HHT-induced apoptosis than HL-60 cells. Silencing Mcl-1 or Bcl-xL, but not XIAP or survivin, enhanced HHT-induced apoptosis in U937 cells. The levels of HHT-induced apoptosis in K562, KCL22, and KU812 cells were inversely correlated with the levels of Bcl-xL but not those of Bcl-2 or Mcl-1. K562 cells expressing high levels of Bcl-xL but no Bcl-2 were less responsive to HHT-induced apoptosis than KCL22 cells that expressed lower levels of Bcl-xL and higher levels of Bcl-2 protein. In K562 cells, knockdown of Bcl-xL, but not of Mcl-1, enhanced HHT-induced apoptosis. Transfection of Bcl-xL into KCL22 cells attenuated HHT-induced apoptosis. These data suggest that Bcl-xL plays a more important role than Bcl-2 and Mcl-1 in protecting against HHT-induced apoptosis.

Introduction

Homoharringtonine [HHT; 4-methyl-2-hydroxy-2-(4-hydroxy-4-methylpentyl)butanedioate] is a cephalotaxus alkaloid obtained from *Cephalotaxus* species, which are evergreen coniferous shrubs (Grem et al., 1988). Extractions of seeds of this genus are toxic to cancer cells and have been used in traditional Chinese medicine (Huang et al., 1983). HHT has been demonstrated to have antileukemia effects in patients with acute myeloid leukemia (AML) or chronic myeloid leukemia (CML) in China, and several clinical trials have been performed in the United States (Quintás-Cardama et al., 2009). HHT as a single agent has positive therapeutic effects in a

portion of patients with AML or CML (O'Dwyer et al., 1986) and is being incorporated into combination treatments of patients with AML in China (Jin et al., 2006; Tang et al., 2006; Zhang et al., 2008). Although most of the clinical investigations done in China were in patients with AML, clinical studies performed in the United States were focused mainly on patients with CML (Quintás-Cardama et al., 2009). A phase I/II investigation revealed a therapeutic potential in patients with CML after treatment failure with interferon (Quintás-Cardama et al., 2007). Several other studies showed improved therapeutic effects in patients with CML when HHT was given in combination with interferon or cytarabine (Quintás-Cardama et al., 2009; Stone et al., 2009). Because most patients with chronic-phase CML are responsive to imatinib as an initial treatment, HHT has been considered for development as treatment only for patients with

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ABBREVIATIONS: HHT, homoharringtonine; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; Fas, fatty acid synthase; FADD, Fas-associated protein with death domain; c-FLIP, cellular FADD-like interleukin-1 β -converting enzyme-inhibitory protein; Mcl-1, myeloid cell leukemia 1; XIAP, X-linked inhibitor of apoptosis protein; Bcl-xL, B-cell lymphoma-extra large; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; Bak, Bcl-2 homologous antagonist/killer; Rh123, Rhodamine-123; PARP, poly(ADP-ribose) polymerase; DR4, death receptor 4; DR5, death receptor 5; Bim, Bcl-2-like protein 11; Puma, p53 up-regulated modulator of apoptosis; siRNA, small interfering RNA; FITC, fluorescein isothiocyanate; PI, propidium iodide; PBS, phosphate-buffered saline; MMP, mitochondrial membrane potential; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; FACS, fluorescence-activated cell sorting; VDAC, voltage-dependent anion channel.

CML resistant to or relapsed from imatinib treatment or for patients with AML resistant to conventional chemotherapy (Marin et al., 2005; de Lavallade et al., 2007).

HHT has been found to inhibit protein synthesis at both the initiation and the elongation phases of translation and to lead to reduction in the levels of proteins that are essential for survival and proliferation of cancer cells (Huang, 1975; Fresno et al., 1977; Tujebajeva et al., 1989). Although there are two reports showing that HHT and harringtonine can induce differentiation of leukemia cells (Boyd and Sullivan 1984, Zhou et al., 1990), most recent reports indicate that HHT is a potent apoptosis inducer in several types of leukemia cells (Tang et al., 2006; Lou et al., 2007; Kuroda et al., 2008). The apoptosis induction ability of HHT seems to account for its main therapeutic potential in the treatment of patients with leukemia. In the current study, we compared the apoptosis induction abilities of HHT in AML and CML cell lines as well as in primary leukemia cells obtained from patients with AML or CML. We found that AML cells were much more sensitive than CML cells to HHT treatment. The mechanisms of HHT apoptosis induction and the different responses between AML and CML cell lines were investigated by exploration of the intrinsic and extrinsic apoptotic pathways. The roles of extrinsic apoptotic signaling were investigated by measuring the levels of c-FLIP, of death receptors, and of the cleavage of caspase-8 as well as using cells defective in the expression of caspase-8 or FADD. The intrinsic apoptotic pathway in HHT-treated cells was investigated by measuring decreases of the mitochondrial membrane potential and changes in the levels of apoptosis-related proteins. Our data revealed that HHT induced apoptosis mainly through a mitochondria-mediated pathway that was correlated with down-regulation of Mcl-1, survivin, and XIAP protein in AML cells. Higher expression levels of Bcl-xL in CML cell lines than in AML cell lines accounted for the lower responses of CML cells to HHT treatment. Silencing Bcl-xL enhanced HHT-induced apoptosis. HHT barely influenced the levels of Bcl-2, Bax, and Bak but caused conformational change and mitochondrial translocation of Bax. These data provide a novel explanation of different responses of AML and CML cells to HHT treatment.

Materials and Methods

Reagents. HHT was obtained from Beijing Union Pharmaceutical Factory (Beijing, China). Rhodamine-123 (Rh123) and 5,6-carboxy-2',7'-dichlorodihydrofluorescein diacetate were obtained from Invitrogen (Carlsbad, CA). Antibodies to poly(ADP-ribose) polymerase (PARP) were obtained from Roche Diagnostics (Mannheim-Waldhof, Germany). Antibodies to caspase-3, caspase-8, caspase-9, and XIAP were obtained from BD Biosciences (San Jose, CA). Antibodies to c-FLIP, DR4, and DR5 were obtained from Enzo Life Sciences (Plymouth Meeting, PA). Antibodies to Bax, Bid, β -actin, and Mcl-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to Bcl-2 and survivin were obtained from Roche Diagnostics Corp. (Indianapolis, IN). Antibodies to Bak, Bad, Bim, and Puma were obtained from Cell Signaling Technology (Danvers, MA). Antibodies to Noxa and VDAC/porin were obtained from Abcam Inc. (Cambridge, MA).

Cell Lines. HL-60, U937, and K562 cells were obtained from the American Type Culture Collection (Manassas, VA). KU812 cells were obtained from Dr. Y. Honma (Yokoyama et al., 1996), and KCL22 cells were obtained from Dr. K. Ozawa (Ohmine et al., 2003). NB4 cells were obtained from Dr. M. Lanotte (Lanotte et al., 1991).

Jurkat subclone A3, FADD-deficient subclone I 9.1, and caspase-8-deficient subclone I 9.2 cells were obtained from American Type Culture Collection (Lombard et al., 2005). All cell lines were cultured in RPMI-1640 medium supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, 1 mM L-glutamine, and 10% (v/v) heat-inactivated fetal bovine serum.

Primary AML Cells. Peripheral blood (10 ml) was obtained from consenting patients with newly diagnosed AML or CML showing initial percentages of circulating blasts $>80\%$ at Shenyang Military General Hospital, Shenyang China. Mononuclear cells were isolated after Ficoll-Hypaque density gradient centrifugation and were cultured in RPMI-1640 medium supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, 1 mM L-glutamine, and 20% fetal bovine serum. These cells were treated with HHT at several concentrations for 48 h and collected for apoptosis assays as described below.

Stable Transfection of Bcl-xL. KCL22 cells were transfected with a pcDNA3.1 plasmid with or without an encoded Bcl-xL expression sequence using Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions and were incubated in medium containing 0.5 mg/ml G418. The clones resistant to G418 were isolated by limited dilution, and the expression of Bcl-xL was determined by Western blot analysis. Three clones, KB6, KB8, and KB11, with overexpression of Bcl-xL and two vector transfected clones, KV2 and KV3, were selected and used to compare the responses of these clones to HHT treatment.

RNA Interference. Mcl-1 siRNA, Bcl-xL siRNA, survivin siRNA, XIAP siRNA, and negative control siRNA were purchased from Santa Cruz Biotechnology, Inc. siRNA was transfected into K562 or U937 cells by electroporation (Amaza, Gaithersburg, MD) following the manufacturer's instructions. In brief, 2×10^6 cells were electroporated in 100 μ l of nucleofector solution (Amaza Reagent V for K562 cells and Amaza Reagent C for U937 cells) containing 20 pmol of each siRNA using the preselected Amaza Program T-016 for K562 cells and Amaza Program W-001 for U937 cells. siRNA transfected cells were plated in a six-well plate with 2 ml of supplemental RPMI-1640 medium for 15 h and subsequently further treated for 24 h with and without 0.5 μ M HHT (for K562 cells) or 20 nM HHT (for U937 cells). Cells treated with or without HHT were harvested for Western blotting analysis.

Cell Growth Inhibition and Viability Assays. Cells were seeded at 10^5 cells/ml and incubated with various concentrations of HHT for 3 days. The total cell number was determined with the aid of a hemocytometer. The growth-inhibitory ability of HHT treatment was calculated and expressed as the ratio of the cell number in treated cells to that in untreated cells. Cell viability was measured by trypan blue exclusion. In brief, 50 μ l of cell suspension was mixed with 50 μ l of 0.4% trypan blue solution, and the number of cells either stained blue or unstained was determined using a hemocytometer. Viable cells possessing intact cell membranes were able to exclude trypan blue staining, whereas nonviable cells without intact membrane took up the dye. The percentage of unstained cells represents the portion of viable cells.

Quantitation of Apoptotic Cells. Levels of apoptotic cells were determined by sub-G₁ analysis and Annexin V-FITC staining. Cells in the sub-G₁ phase were measured as described previously (Yu et al., 2008). Cells were fixed with ice-cold 70% ethanol overnight at a density of 10^5 cells/ml and treated with 200 μ g/ml RNase for 30 min at 37°C. Propidium iodide (PI) was then added to a final concentration of 50 μ g/ml, and the cells in the sub-G₁ phase were quantitated by flow cytometry with an excitation wavelength of 488 nm and an emission wavelength of 625 nm. Data were analyzed using CellQuest software (BD Biosciences). For the annexin V-FITC staining assay, 10^5 cells were washed twice with PBS and then labeled with annexin V-FITC and PI in binding buffer according to the instructions provided by the manufacturer (Annexin V-FITC Apoptosis Detection Kit; Oncogene, Cambridge, MA). The fluorescent signals of FITC and PI were detected at 518 nm and at 620 nm, respectively, by flow cytometric analysis (Jing et al., 1999).

Measurement of the Mitochondrial Membrane Potential. MMP was assessed by the retention of Rh123, a membrane-permeable fluorescent cationic dye that is selectively taken up by mitochondria. The levels of Rh123 retention in mitochondria are proportional to the MMP (Emaus et al., 1986). In brief, cells (10^6) treated with or without HHT were incubated with $0.1 \mu\text{g/ml}$ Rh123 in the dark for 20 min at room temperature. After washing with PBS, the cells were analyzed by FACSscan (BD Biosciences) with excitation and emission wavelengths of 495 and 535 nm, respectively.

Western Blot Analysis. Protein extracts ($50 \mu\text{g}$) prepared with radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 100 μM leupeptin, and 2 $\mu\text{g/ml}$ aprotinin, pH 8.0) were separated on 8% or 12% SDS-polyacrylamide gels and then transferred to nitrocellulose membranes. The membranes were stained with 0.2% Ponceau S red to assure transfer and equal protein loading. After blocking with 5% nonfat milk, the membranes were incubated with an antibody to a specific protein overnight at 4°C . Immunocomplexes were visualized using enhanced chemiluminescence Western blotting detection reagent (Amersham Biosciences, England, UK) (Chen et al., 2006). Protein quantitation was determined by the Bradford protein binding assay (Bradford, 1976).

Bax Conformational Change. Cells treated with HHT were harvested, washed in PBS, and suspended in CHAPS lysis buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, and 1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate) containing protease inhibitor cocktail. After 30-min incubation at 4°C , the suspension was centrifuged for 15 min at $14,000g$. The obtained supernatant fluid containing 500 μg of protein was precleared with 20 μl of protein A/G plus agarose (Santa Cruz Biotechnology) and then subjected to immunoprecipitation with 3 μg of either normal mouse IgG (Santa Cruz Biotechnology) or anti-Bax (Sigma-Aldrich, St. Louis, MO) at 4°C for 2 h with gentle rotation. Then, 30 μl of protein A/G plus agarose beads were added and incubated overnight to pull down protein-antibody complexes. The beads were obtained by centrifuging and then washed three times with CHAPS lysis buffer. The conformationally changed Bax was determined by Western blot analysis with the use of polyclonal Bax antibody, and the coimmunoprecipitated Bak was determined using anti-Bak polyclonal antibody (Rahmani et al., 2009).

Mitochondrial Translocation. Cytosol and mitochondrial fractions of cells (10^7) treated with or without HHT were isolated using a Cytosol/Mitochondria Kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. The levels of Bax

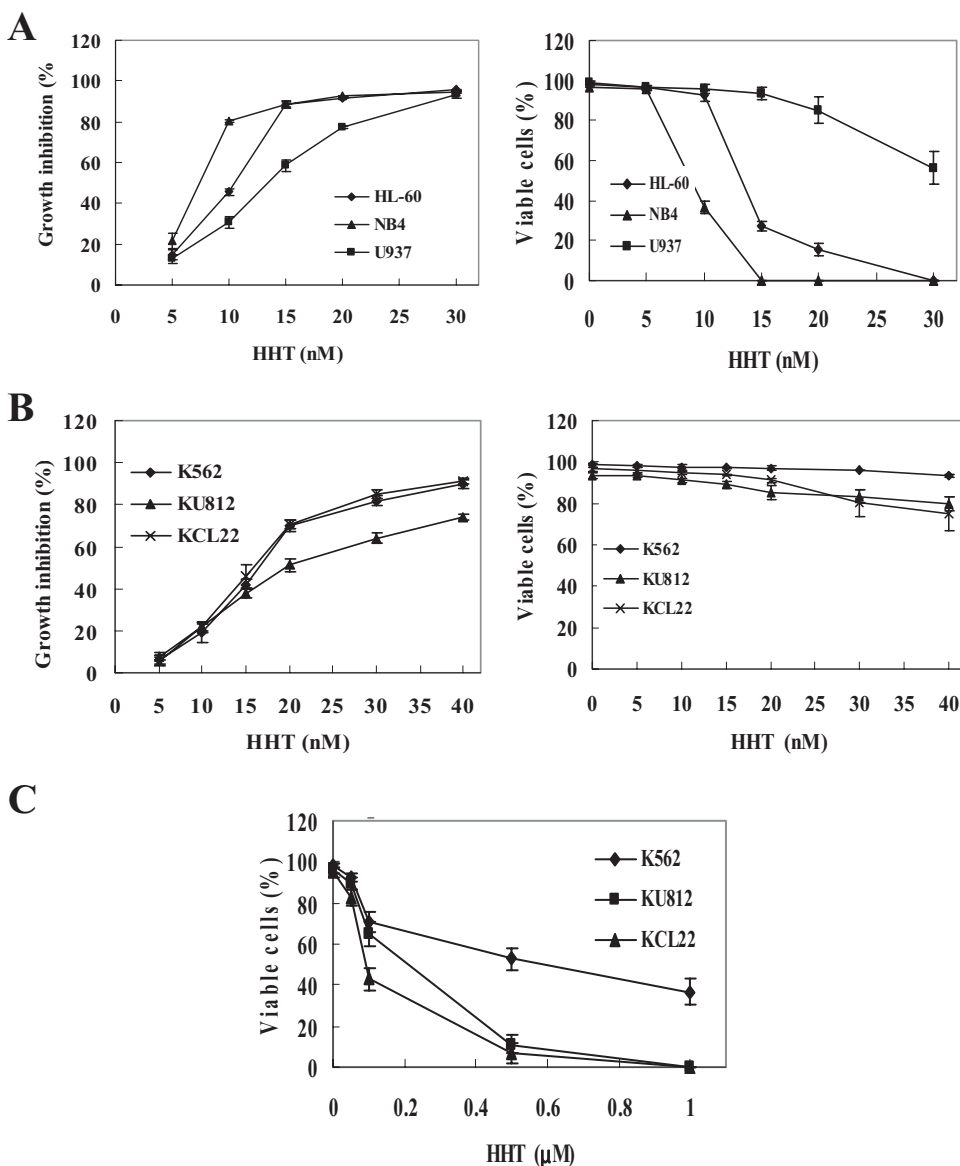


Fig. 1. Cell growth inhibition and cytotoxicity of HHT in AML and CML cell lines. A, cell growth inhibition and viability of AML cell lines. HL-60, NB4, and U937 cells were treated with HHT at the indicated concentrations for 3 days. B, cell growth inhibition and viability of CML cell lines. K562, KU812, and KCL22 cells were treated with HHT at the indicated concentrations for 3 days. C, viability of CML cell lines treated with high concentrations of HHT for 3 days. Total cell number was determined using a hemocytometer. Cell viability was determined by trypan blue exclusion. Values shown are mean \pm S.E. of three independent experiments.

in both cytosol and mitochondrial fractions were determined using polyclonal Bax antibody by Western blot analyses. The purity of the mitochondrial and cytosolic fractions was examined by Western blot analysis of the levels of β -actin and VDAC/porin.

Statistic Analysis. Data were analyzed for statistical significance using the Student's *t* test (Excel; Microsoft Corporation, Redmond, WA). A *p* value of less than 0.05 was considered statistically significant.

Results

AML Cell Lines Are More Sensitive to HHT-Induced Growth Inhibition and Cytotoxicity than CML Cell Lines. Three AML cell lines (HL-60, NB4, and U937) and three CML cell lines (K562, KU812, and KCL22) were used to test the growth inhibition and cytotoxicity due to HHT treatment. All of these cell lines were treated with HHT at a variety of concentrations for 3 days. HHT inhibited growth in all cell lines at concentrations less than 40 nM (Fig. 1A). HHT at a concentration of 5 nM had minimal growth inhibitory effects in all six cell lines. AML cell lines showed greater

responses to HHT treatment at 10 nM than CML cell lines (Fig. 1A). U937 cells were less responsive to HHT-induced growth inhibition than HL-60 and NB4 cells. K562 cells were less responsive to HHT-induced growth inhibition than KU812 and KCL22 cells (Fig. 1B). Cell viability as a result of HHT treatment differed in AML and CML cell lines. More than 80% of NB4 and HL-60 cells lost viability after 3-day treatment with 20 nM HHT (Fig. 1A). U937 cells were less sensitive than NB4 and HL-60 cells in that more than 50 nM HHT was required to induce cytotoxicity in more than 80% of U937 cells (data not shown). CML cells were only slightly sensitive to HHT-mediated cytotoxicity at concentrations lower than 40 nM (Fig. 1B). To obtain significant cytotoxicity, the concentrations of HHT required were greater than 100 nM. Treatment with 500 nM HHT for 3 days induced more than 80% of KU812 and KCL22 cells to lose their viability (Fig. 1C). K562 cells were least sensitive to the cytotoxic effects of HHT (Fig. 1C). These data indicate that AML cells are more responsive than CML cells to the cytotoxic effects of HHT treatment and that HHT may induce cytotoxicity

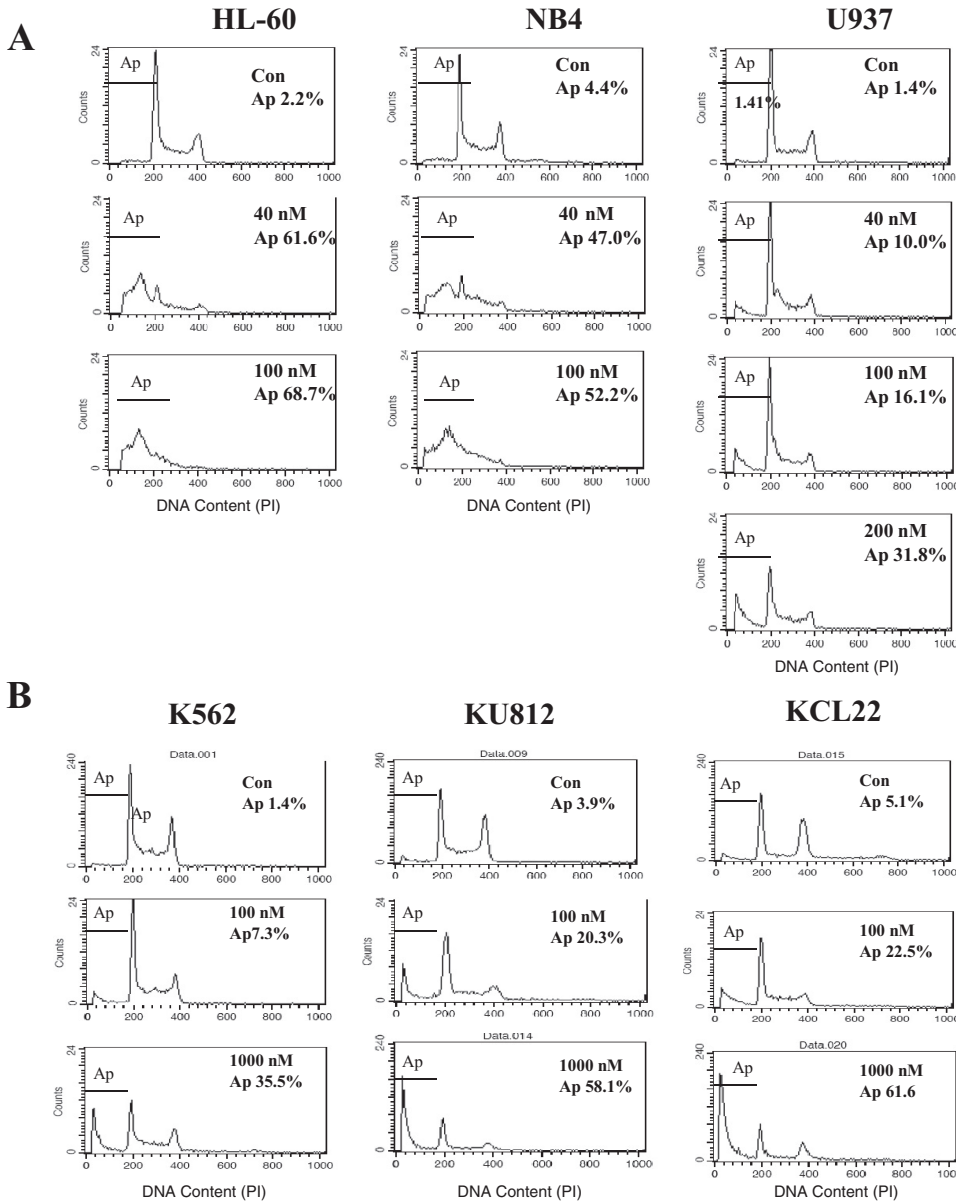


Fig. 2. Apoptosis induction of HHT in AML (A) and CML (B) cell lines. HL-60, NB4, and U937 cells were treated with HHT at the indicated concentrations for 12 h. K562, KU812, and KCL22 cells were treated with HHT at the indicated concentrations for 48 h. The hypodiploid apoptotic peak (sub- G_1) was determined by flow cytometry after staining with PI. Con, control; Ap, apoptotic cells in sub- G_1 phase.

through a mechanism(s) different from that by which it inhibits cell growth.

AML Cells Are More Sensitive than CML Cells to HHT-Induced Apoptosis. Because AML cells were much more sensitive to HHT-induced cell death, we determined the relative levels of HHT-induced apoptosis in the three AML cell lines by measuring relative levels of fragmented DNA using FACS after treatment for 12 h. HL-60 and NB4 cells were more sensitive than U937 cells to HHT-induced apoptosis (Fig. 2A). Treatment with HHT at a concentration of 40 nM for 12 h induced apoptosis of 61.6, 47.0, and 10.0% in HL-60, NB4, and U937 cells, respectively (Fig. 2A). Increasing the HHT concentration to 0.2 μ M induced apoptosis in 31.8% of U937 cells (Fig. 2A). Unlike AML cells, CML cells were not sensitive to HHT treatment at low concentrations. HHT at a concentration of 100 nM did not induce evident apoptosis in CML cells after treatment for 24 h (data not shown), but it induced 7.3, 20.3, and 22.5% of K562, KU812, and KCL22 cells, respectively, to undergo apoptosis after treatment for 48 h (Fig. 2B). When the concentration was increased to 1 μ M for 48 h, HHT induced 35.5, 58.1, and 61.6% of K562, KU812, and KCL22 cells, respectively, to undergo apoptosis (Fig. 2B).

AML Primary Cells Are More Responsive to HHT-Induced Apoptosis than CML Primary Cells. Eleven

blood samples were collected from patients with leukemia: seven AML samples and four CML samples. Mononuclear cells were isolated from these samples and then treated with HHT at various concentrations for 48 h. Apoptosis as a result of HHT treatment was determined by analyzing cells in the sub- G_1 phase. HHT induced significant apoptosis in AML samples at a concentration of 40 nM, but significant apoptotic cells in CML cells could be detected only after treatment with 1 μ M HHT (Fig. 3). HHT at 100 nM induced 60% of AML cells to undergo apoptosis, but induced apoptosis in only 20% of CML cells (Fig. 3). These data suggest that the cytotoxic effects of HHT observed in AML and CML cells were mediated through induction of apoptosis and that CML cells may express some proteins that attenuate HHT-induced apoptosis.

HHT Decreases the MMP and the Levels of Antiapoptotic Proteins. The membrane permeability transition of mitochondria is controlled by the MMP, and MMP disruption plays a central role in mitochondrially mediated apoptosis and caspase-9 activation. The levels of MMP were analyzed using flow cytometry in HL-60 cells after treatment with HHT subsequent to staining with the cationic dye Rh123. A significant decrease of MMP was observed in HL-60 cells after 4-h treatment with 30 nM HHT (Fig. 4A).

Decreases in the levels of MMP lead to activation of

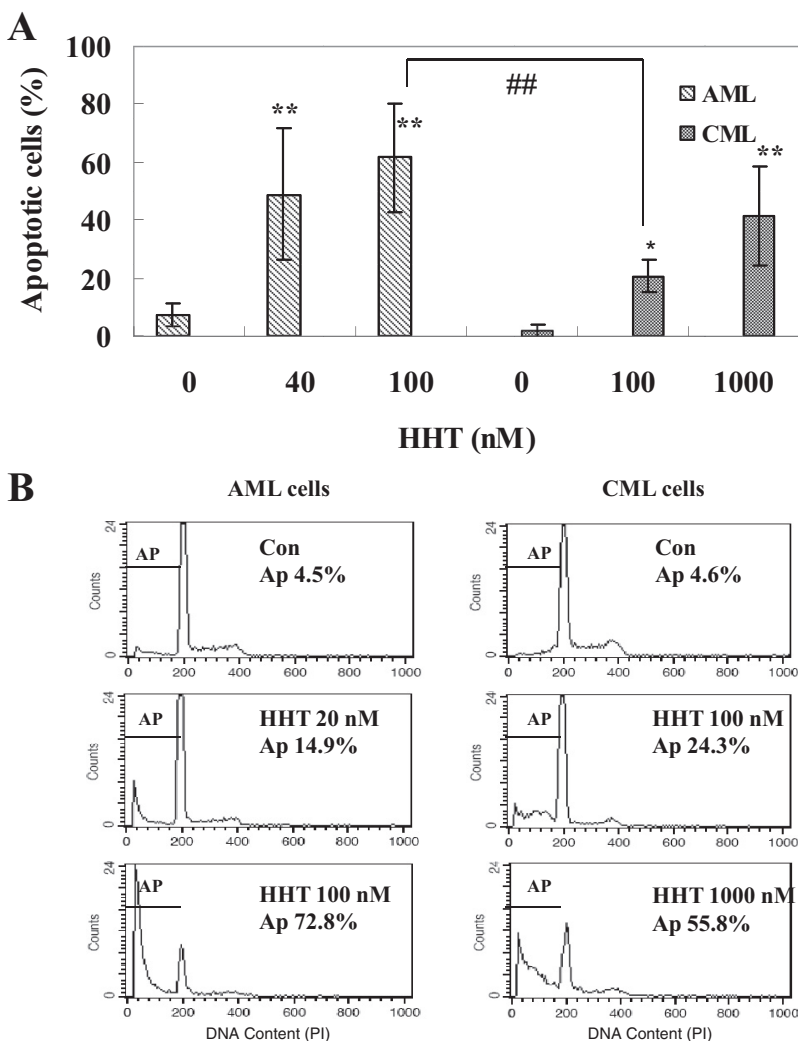


Fig. 3. Apoptosis induction of HHT in cultured primary leukemia cells obtained from patients with AML or CML. A, comparison of HHT-induced apoptosis in primary AML and CML cells. Leukemia cells isolated from seven AML patient samples and four CML patient samples were put into culture in the presence of HHT at the indicated concentrations for 48 h. Apoptotic cells were determined by detection of the hypodiploid apoptotic peak (sub- G_1) by flow cytometry after staining with PI. Values shown are the means \pm S.D. **, $p < 0.01$; *, $p < 0.05$ compared with the cells without treatment; ##, $p < 0.001$, compared with AML cells treated with HHT at 100 nM. B, a representative sample from a patient with AML and one representative sample from a patient with CML.

caspase-9 and -3. These decreases are regulated by the proapoptotic, antiapoptotic, and BH3-only proteins of the Bcl-2 family (Kuroda and Taniwaki 2009). The levels of proapoptotic proteins Bax and Bak were not regulated by HHT treatment. The levels of the antiapoptotic proteins Bcl-2, Bcl-xL, and Mcl-1 were differentially regulated by HHT treatment. HL-60 cells did not express Bcl-xL. HHT treatment decreased the levels of Mcl-1 and only slightly decreased the levels of Bcl-2. The levels of BH3-only proteins Bid, Bim, Noxa, and Puma were decreased after HHT treatment (Fig. 4B). HHT induced PARP cleavage and decreased the levels of pro-caspase-3 and -9 at concentra-

tions of 20 and 30 nM, suggesting that caspase-3 and -9 are activated (Fig. 4B). The activities of caspase-3 and -9 are inhibited by both XIAP and survivin. Therefore, the protein levels of XIAP and survivin were also determined after HHT treatment. The levels of both XIAP and survivin proteins were found to be decreased (Fig. 4B). The levels of viability and the levels of Mcl-1, Noxa, Puma, XIAP, and survivin protein were determined in HL-60 cells after treatment with 20 nM HHT for various times. HL-60 cells treated with 20 nM HHT for 8 h did not lose their viability (Fig. 4C), but the levels of these proteins were significantly decreased (Fig. 4D). These data suggest that down-

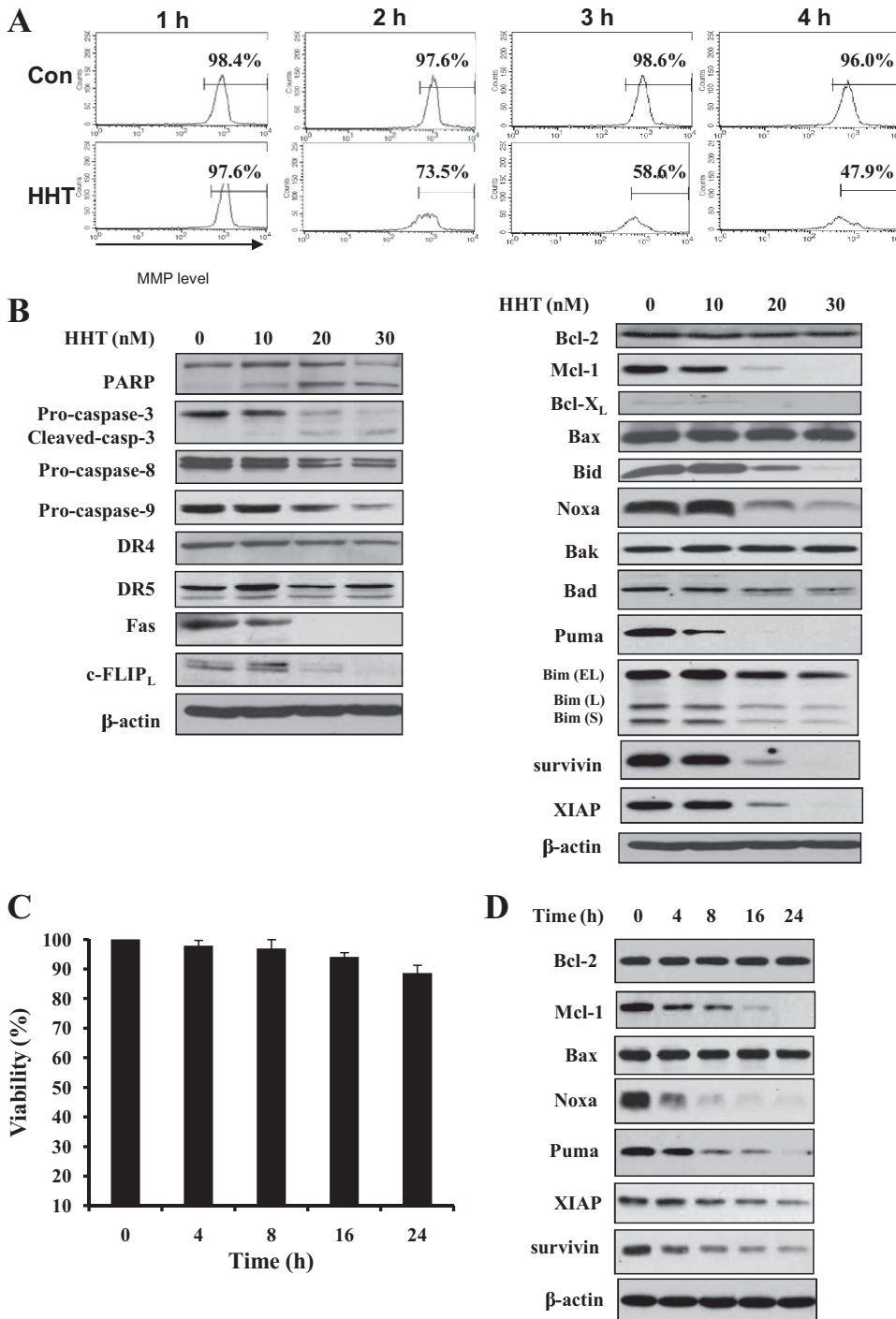


Fig. 4. HHT decreases the levels of MMP and antiapoptotic proteins. A, effects of HHT on MMP levels. HL-60 cells were treated with 30 nM HHT for the indicated times. The levels of MMP were determined according to changes of fluorescence density after Rh123 staining. The shift to the left of the peak indicated a loss of MMP. B, effects of HHT on the levels of proteins regulating apoptosis. HL-60 cells were treated with HHT at the indicated concentrations for 24 h, and the levels of the indicated proteins were determined by Western blot analyses. C, effects of HHT on viability. HL-60 cells were treated with HHT at 20 nM for the indicated times, and the viability was determined by the trypan blue exclusion assay. D, time-dependent effects of HHT on the levels of several anti- and proapoptotic proteins. HL-60 cells were treated with HHT at 20 nM for the indicated times, and the relative levels of the indicated proteins were determined by Western blot analysis.

regulation of these proteins is not secondary to cell death and should be a primary target for HHT treatment. The levels of Bcl-2, Bcl-xL, Mcl-1, XIAP, and survivin were compared in AML NB4, HL-60, and U937 cells and in CML K562, KU812, and KCL22 cells. The levels of Bcl-xL, Mcl-1, and survivin in HL-60 cells were lower than those in U937 cells. The level of Bcl-xL in U937 cells was higher than that in HL-60 cells but lower than those in CML cells (Fig. 5A). Silencing Bcl-xL and Mcl-1, but not XIAP or survivin, sensitized U937 cells to HHT-induced apoptosis (Fig. 5B). These data suggest that the levels of Bcl-xL and Mcl-1 may determine the sensitivities of the three AML cell lines to HHT-induced apoptosis.

To determine whether a death receptor-mediated pathway is involved in HHT-induced apoptosis, the levels of DR4, DR5, Fas, c-FLIP, pro-caspase-8, and Bid were investigated in HL-60 cells after treatment with HHT. The levels of Fas, c-FLIP, pro-caspase-8, and Bid were decreased after HHT

treatment. The levels of DR4 and DR5 were not or only weakly decreased (Fig. 4B).

Jurkat Cells Deficient in Caspase-8 or FADD Are Sensitive to HHT-Induced Apoptosis. To determine whether a death receptor-mediated pathway plays an important role in HHT-induced apoptosis, a subclone of Jurkat cells lacking caspase-8 (I 9.2) and another subclone lacking FADD (I 9.1) were used (Fig. 6A). I 9.1 cells were as sensitive as the parental subclone A3 cells to HHT-induced apoptosis. I 9.2 cells had a decreased response to HHT-induced apoptosis compared with that of A3 cells (Fig. 6A). The levels of Bcl-xL and Mcl-1 were decreased by HHT treatment in all three cell lines (Fig. 6B). I 9.2 cells did not express caspase-8 and I 9.1 cells did not express FADD, which suggests that caspase-8 could be only partly involved in HHT-induced apoptosis. The apoptosis induction of HHT in cells lacking caspase-8 is probably initiated via the mitochondrial pathway resulting from the decrease in the protein levels of Mcl-1 and Bcl-xL (Fig. 6B).

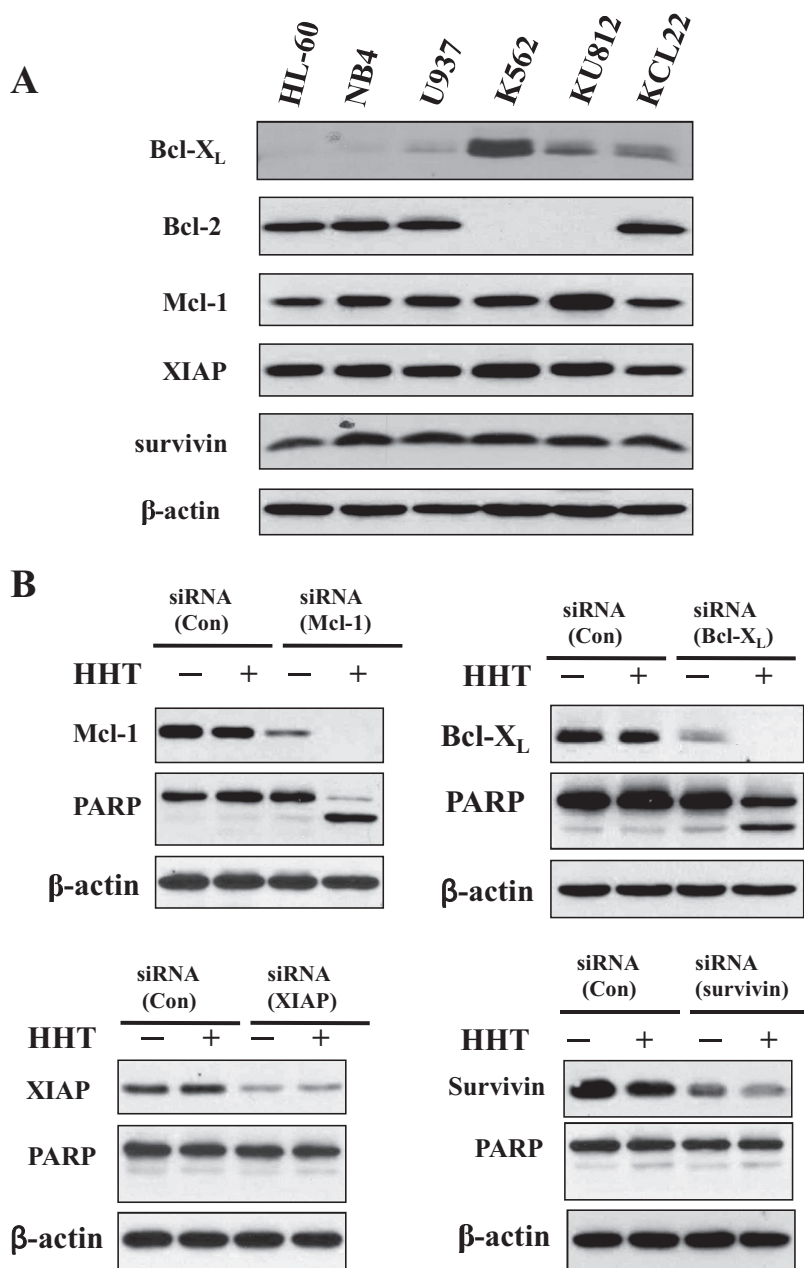


Fig. 5. Knockdown of Mcl-1 and Bcl-xL, but not XIAP or survivin, enhances HHT-induced apoptosis in U937 cells. A, the basal levels of Bcl-xL, Bcl-2, Mcl-1, XIAP, and survivin in NB4, HL-60, U937, K562, KU812, and KCL22 cells. B, the effects of Mcl-1, Bcl-xL, XIAP, and survivin siRNA on HHT-induced apoptosis in U937 cells. U937 cells were incubated with control siRNA or Mcl-1 siRNA, Bcl-xL siRNA, XIAP siRNA, or survivin siRNA for 15 h and then treated with or without 20 nM HHT for 24 h. Total protein was isolated and subjected to Western blot analysis to determine the levels of PARP, Bcl-xL, Bcl-2, Mcl-1, XIAP, and survivin proteins using the specific antibodies as described under *Materials and Methods*.

The High Levels of Bcl-xL in CML Cells Mediate the Reduced Response to HHT-Induced Apoptosis. CML cells expressed higher levels of Bcl-xL than AML cells, and HHT induced apoptosis only at high concentrations or after longer periods of treatment in CML cell lines (Figs. 2B and 5A). We compared the apoptosis induction in the three CML cell lines after treatment with HHT for 24 h based on PARP cleavage and down-regulation of the antiapoptotic proteins. HHT at 0.5 and 1 μ M induced apoptosis in KU812 and KCL22 cells, but not, or only weakly, in K562 cells based on the cleavage of PARP (Fig. 7A). Because the results obtained in AML cells suggest that HHT-induced apoptosis occurs mainly via a mitochondrial-mediated pathway, the levels of Bax, Bcl-2, Bcl-xL, Mcl-1, survivin, and XIAP proteins were also determined in these three CML cell lines after treatment with HHT at concentrations of 0.5 and 1 μ M. Bcl-2 was detected in KCL22 cells, but not in K562 or in KU812 cells (Figs. 5A and 7A). Mcl-1 was present, and its level was completely decreased by HHT in all three cell lines. On the basis of levels of parental PARP, the levels of survivin and

XIAP were also reduced by HHT treatment (Fig. 7A). KCL22 cells expressing Bcl-2 were the most sensitive to HHT-induced apoptosis among the three CML cell lines (Fig. 7A). Because HL-60 cells, which express high levels of Bcl-2, were sensitive to HHT-induced apoptosis (Fig. 4B), it seems that Bcl-2 is not the main antiapoptotic protein mediating apoptosis resistance to HHT treatment. Both KU812 and KCL22 cells express lower levels of Bcl-xL than K562 cells. The levels of Bcl-xL were inhibited by HHT treatment in both KU812 and KCL22 cells, which were responsive to HHT-induced apoptosis at the tested concentrations. Although the expression of Bcl-xL was also inhibited by HHT treatment in K562 cells, a certain amount of Bcl-xL was still present and at levels similar to the basal levels detected in KU812 and KCL22 cells (Fig. 7A). These data suggest that Bcl-xL is a key antiapoptotic protein and that it would be important to determine the sensitivity of CML cells to HHT treatment. It has been shown that Bcl-xL is more active than Bcl-2 in binding to Bax and in blocking its activation (Leber et al., 2010). HHT treatment induced conformational change of Bax as determined with a specific antibody (Fig. 7B) as well as its binding to Bak as determined by a coimmunoprecipitation assay (Fig. 7B). Moreover, Bax translocated from cytosol to mitochondria after HHT treatment at a concentration of 1 μ M, which induced apoptosis in KCL22 cells (Fig. 7C). These data suggest that Bax activation plays an important role in HHT-induced apoptosis and that this role may be attenuated by the expression of Bcl-xL.

To further determine the role of Bcl-xL in HHT-induced apoptosis, Bcl-xL siRNA was used to silence Bcl-xL in K562 cells. K562 cells pretreated with Bcl-xL siRNA have decreased levels of Bcl-xL and became more sensitive to HHT-induced apoptosis as determined by measuring PARP and pro-caspase-9 cleavage (Fig. 8A) and by FACS analysis of annexin V staining (Fig. 8B). In contrast, although Mcl-1 siRNA pretreatment decreased the basal levels of Mcl-1 in K562 cells, it did not increase the levels of HHT-induced apoptosis as determined by PARP cleavage (Fig. 8C) and by the levels of annexin V-positive cells (Fig. 8D). Silencing either XIAP or survivin did not enhance HHT-induced apoptosis as determined by measurement of PARP cleavage (Fig. 8, E and F). Taking these data together with the fact that K562 cells do not express Bcl-2, Bcl-xL levels seem to control HHT-induced apoptosis in CML cells.

Overexpression of Bcl-xL Blocks HHT-Induced Apoptosis in KCL22 Cells. KCL22 cells expressed higher levels of Bcl-2 and lower levels of Bcl-xL than K562 cells and were more sensitive to HHT-induced apoptosis (Figs. 5A and 7A). To further confirm the role of Bcl-xL in HHT-induced apoptosis, we generated stable clones of KCL22, KB6, KB8, and KB11 cells with overexpression of Bcl-xL. The responses of KB6, KB8, and KB11 cells to HHT-induced apoptosis was compared in two subclones of KCL22 transfected with an empty vector, KV2 and KV3. As shown in Fig. 9A, HHT-induced apoptosis in KB6, KB8, and KB11 cells was inhibited by overexpression of Bcl-xL. Western blot analysis revealed that KB6, KB8, and KB11 cells expressed higher levels of Bcl-xL than KV2 and KV3 cells (Fig. 9B). The levels of Mcl-1 and XIAP were equally expressed in these subclones regardless of transfection with Bcl-xL (Fig. 9B). However, the levels of Bcl-2 were relatively lower in the KB6, KB8, and KB11 cells compared with those in KV2 and KV3 cells (Fig. 9B).

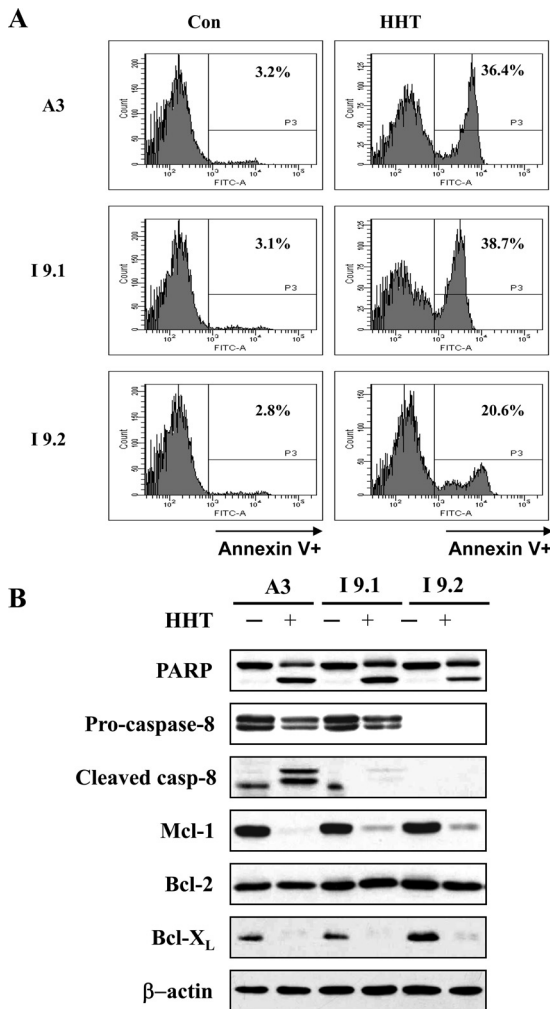


Fig. 6. HHT-induced apoptosis in Jurkat subclones lacking caspase-8 or FADD expression. **A**, apoptosis induction. A3, I 9.1, and I 9.2 cells were treated with 90 nM HHT for 24 h. Apoptotic cells were determined by flow cytometry after staining with annexin V-FITC. **B**, Western blot analysis of PARP, caspase-8, Mcl-1, Bcl-2, and Bcl-xL proteins. Cells were incubated with or without 90 nM HHT for 24 h. The levels of each indicated protein were detected using specific antibodies as described under *Materials and Methods*.

Further Western blot analysis of apoptosis-related proteins was performed in KV2 and KB8 cells after HHT treatment. The results revealed that HHT at 2 μ M activated caspase-3 and -9 in KV2 cells, but not in KB8 cells (Fig. 9C). HHT treatment decreased the levels of Mcl-1, survivin, and somewhat that of XIAP in both KV2 and KB8 cells (Fig. 9C). These data suggest that Bcl-xL is indeed more active than Bcl-2 or Mcl-1 in blocking HHT-induced apoptosis.

Discussion

Although HHT has been reported to be an effective treatment in a portion of patients with AML or CML, the reasons for these different responses are not understood (Grem et al., 1988). Although the mechanisms of action of HHT are not fully understood, apoptotic cells have been detected in HHT-treated patient samples (Visani et al., 1997). Using cell lines derived from malignant hemopoietic cells, it has been found that HHT induced apoptosis in several types of malignant cells (Tang et al., 2006; Jie et al., 2007; Lou et al., 2007). Using AML and CML cell lines and primary cells, we have observed different apoptosis responses to HHT treatment (Figs. 2 and 3). These different responses may account for the variety of in vivo responses to HHT treatment.

Apoptotic pathways mediated by mitochondria (intrinsic pathway) and death receptors (extrinsic pathway) have been well characterized. Caspases have been shown to play crucial roles in the initiation and execution of apoptosis in both pathways. Using AML cell lines we have investigated both the intrinsic and the extrinsic apoptosis pathway-related events. HHT treatment induced apoptosis in HL-60 cells at

concentrations of 20 to 40 nM (Fig. 2). Correlated with this apoptosis induction, we observed decreases of the MMP (Fig. 4A). MMP can be regulated by the extrinsic pathway via cleaved Bid as a result of caspase-8 activation and by the intrinsic pathway as a result of different expressions of antiapoptotic proteins, principally Bcl-2, Bcl-xL, and Mcl-1. Using Western blot analysis, we found the levels of Bid and pro-caspase-8 to be decreased after HHT treatment, suggesting that caspase-8 may be activated. Caspase-8 can be activated by increased levels of death receptors (Elrod and Sun 2008), and its activity is inhibited by c-FLIP. The levels of both Fas and c-FLIP were decreased after HHT treatment (Fig. 4B). To further explore the role of caspase-8 activation in HHT-mediated apoptosis, Jurkat cells lacking expression of caspase-8 or FADD were used. Cells lacking caspase-8 had decreased responses to HHT treatment, but cells lacking FADD were as responsive as parental cells to HHT treatment (Fig. 6A). Only one third of HHT apoptosis induction ability was decreased in cells lacking caspase-8, which suggests that the caspase-8 activation does not play a major role in HHT-induced apoptosis and that activation of caspase-8 could be due to decreases in the levels of c-FLIP.

Mitochondrial membrane potential is controlled by levels of proapoptotic proteins, antiapoptotic proteins, and BH3-only proteins of Bcl-2 family (Kuroda and Taniwaki 2009). We found that HHT treatment did not change the levels of the proapoptotic proteins Bax and Bak. HHT treatment did not change the levels of Bcl-2 protein, but it did decrease the levels of Mcl-1 protein (Fig. 4B). Bcl-xL was not detected in HL-60 cells either before or after HHT treatment. Although

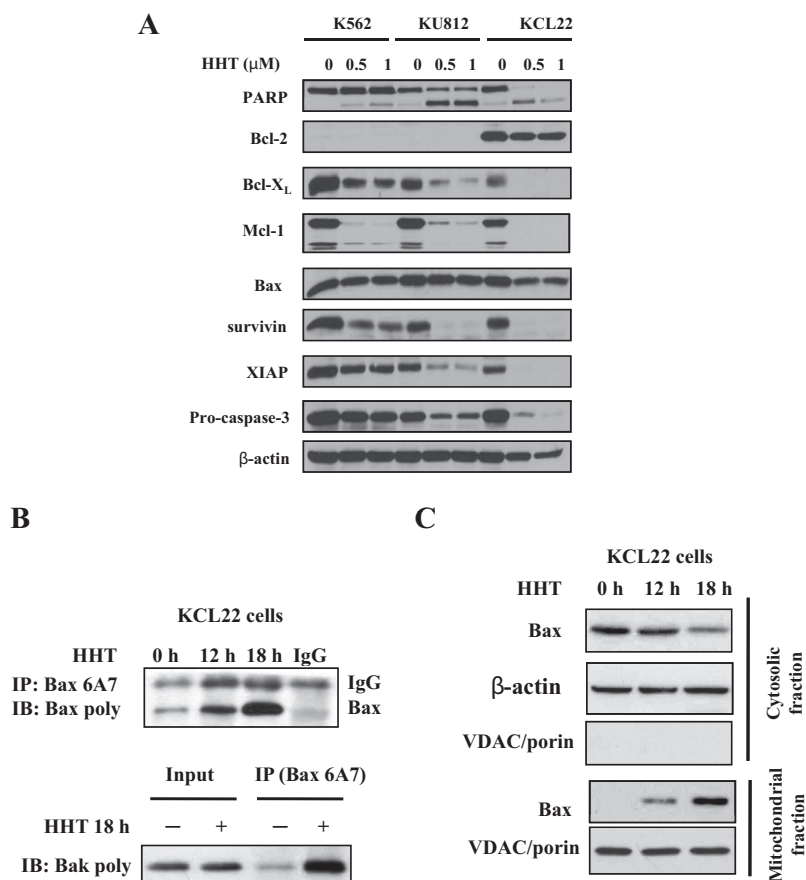


Fig. 7. Apoptosis induction and antiapoptotic protein regulation by HHT in CML cell lines. A, Western blot analysis of the levels of PARP cleavage and proteins regulating apoptosis. K562, KU812, and KCL22 cells were treated with HHT at the indicated concentrations for 24 h. The levels of each indicated protein were detected using specific antibodies as described under *Materials and Methods*. B, conformational change of Bax. KCL22 cells were treated with HHT at 1 μ M for the indicated times and lysed in buffer containing 1% CHAPS. Conformationally changed Bax protein was immunoprecipitated with the anti-Bax 6A7 antibody and probed using poly anti-Bax and anti-Bak Ab-1 antibodies. C, mitochondrial translocation of Bax. KCL22 cells were treated with HHT at 1 μ M for the indicated times. The cytosol and mitochondrial fractions were separated as described under *Materials and Methods*. VDAC/porin was used as the standard for mitochondrial isolation.

BH3-only proteins have been found to be induced in leukemia cells after treatment with chemotherapeutic agents, HHT treatment decreased the levels of the BH3-only proteins Bim, Noxa, and Puma (Fig. 4B). Because the activities of caspase-3 and -9 are inhibited by XIAP and survivin, decreases in their levels have been found to result in apoptosis (Pennati et al., 2007; Dubrez-Daloz et al., 2008). HHT treatment decreased the levels of XIAP and survivin in HL-60 cells (Fig. 4B). Because Bcl-xL is not expressed in HL-60 cells, decreases in the levels of Mcl-1, XIAP, and survivin rather than increases in the levels of BH3-only proteins seem to contribute to HHT-mediated apoptosis in HL-60 cells. These data are consistent with previous reports showing that down-regulation of Mcl-1, XIAP, and survivin are correlated with HHT-induced apoptosis in AML cells and myeloma cells (Tang et al., 2006; Kuroda et al., 2008). AML cells did not express, or

expressed only lower levels of, Bcl-xL, and the levels of Mcl-1 were completely reduced by HHT treatment (Figs. 4B and 5A). Therefore, Bcl-2 levels may not play a critical role in controlling the sensitivity of cells to HHT-induced apoptosis because cells with high levels of Bcl-2 were responsive to HHT-induced apoptosis (Figs. 4B and 7A). There is a report showing that HHT induced apoptosis via down-regulation of Mcl-1 in AML cells, but our data suggest that down-regulation of Mcl-1 is not sufficient for HHT-induced apoptosis once Bcl-xL is expressed. The expression of the Mcl-1 protein was completely inhibited by HHT treatment even in cells undergoing or resistant to HHT-induced apoptosis (Figs. 4B, 6B, 7A and 9C). U937 cells expressing higher levels of Bcl-xL than NB4 and HL-60 cells (Fig. 5A) were less sensitive to HHT-induced apoptosis (Fig. 2A). Jurkat subclones that expressed Bcl-xL were less sensitive to HHT treatment than

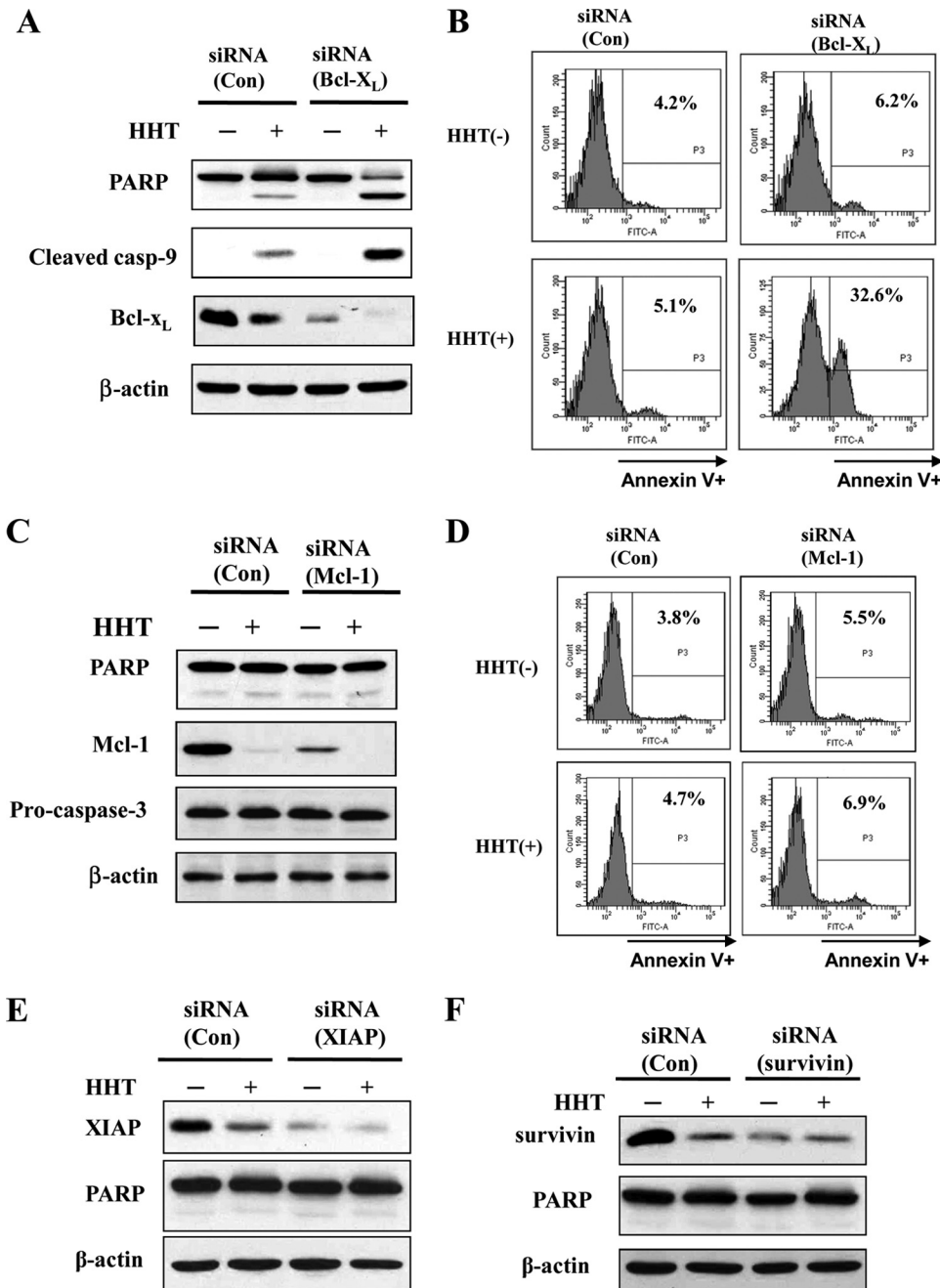


Fig. 8. Silencing Bcl-xL, but not Mcl-1, XIAP, or survivin, enhanced HHT-induced apoptosis in K562 cells. **A**, the influence of Bcl-xL siRNA on HHT-induced PARP cleavage. **B**, the influence of Bcl-xL siRNA on HHT-induced apoptosis. **C**, the influence of Mcl-1 siRNA on HHT-induced PARP cleavage. **D**, the influence of Mcl-1 siRNA on HHT-induced apoptosis. **E**, the influence of XIAP siRNA on HHT-induced PARP cleavage. **F**, the influence of survivin siRNA on HHT-induced PARP cleavage. K562 cells were incubated with control siRNA or the siRNA as indicated for 15 h and then treated with and without 0.5 μ M HHT for 24 h. The cells were collected for FACS analysis for determining annexin V-positive cells (**B** and **D**) or for total protein isolation to analyze the levels of PARP, caspase-9, Bcl-xL, Mcl-1, XIAP, or survivin using Western blot analysis (**A**, **C**, **E**, and **F**).

HL-60 cells (Fig. 6). Bcl-xL silencing sensitized U937 cells to HHT-induced apoptosis (Fig. 5B). These data imply that the basal levels of Bcl-xL control the sensitivity of the cells to HHT-induced apoptosis.

CML cells were not sensitive to HHT-induced cytotoxicity at low concentrations (Fig. 1). We found that all three CML cell lines tested contained higher levels of Bcl-xL (Figs. 5A and 7A) and were less sensitive to HHT-induced apoptosis compared with the three AML cell lines (Fig. 2). To obtain a significant apoptotic effect in CML cells after 24 h of treatment, 0.5 μ M HHT was required for KU812 and KCL22 cells and greater than 1 μ M HHT was required for K562 cells. By comparing the levels of Bcl-2, Bcl-xL, and Mcl-1 with the apoptosis induction ability of HHT treatment in the three CML cell lines, we found that HHT induced cleavage of PARP and pro-caspase-3 in KCL22 and KU812 cells, but not, or weakly, in K562 cells at concentrations of 0.5 and 1 μ M (Fig. 7). Intriguingly, although apoptosis was not observed in K562 cells, the levels of Mcl-1 were dramatically reduced by HHT treatment (Fig. 7A). Although KCL22 cells expressed Bcl-2, they were more sensitive to HHT-induced apoptosis than K562 cells, which did not express Bcl-2 (Figs. 5A and 7A). Therefore, neither Bcl-2 expression nor down-regulation of Mcl-1 correlated with the sensitivity of the three CML cell lines to HHT-induced apoptosis. It is noteworthy that the basal levels of Bcl-xL were inversely correlated with HHT-induced apoptosis in the three CML cell lines. KCL22 cells

expressed the lowest levels of Bcl-xL and were the most sensitive to HHT-induced apoptosis (Figs. 5A and 7A). The levels of Bcl-xL were significantly decreased by HHT treatment in KCL22 cells, which then underwent apoptosis. Although the levels of Bcl-xL in K562 cells were also decreased after HHT treatment, a certain amount of Bcl-xL protein was detected that was higher than the basal level present in KCL22 cells (Fig. 7A). Again, these data suggest that Bcl-xL, but not Bcl-2, is a key inhibitor of HHT-induced apoptosis. The silencing by siRNA of Bcl-xL, but not of Mcl-1, XIAP, or survivin, sensitized K562 cells to HHT-induced apoptosis (Fig. 8). To further test the role of Bcl-xL, we generated KCL22 cells with overexpression of Bcl-xL and found that these cells became resistant to HHT-induced apoptosis even though they had decreased levels of Bcl-2 compared with the vector alone-transfected cells (Fig. 9). The reason for the decrease in the levels of Bcl-2 in Bcl-xL-transfected KCL22 cells is unclear. AML cell lines contain high levels of Bcl-2 either without or with lower levels of Bcl-xL (Fig. 5A). K562 cells have high levels of Bcl-xL without expression of Bcl-2, but KCL22 cells express high levels of Bcl-2 with low levels of Bcl-xL (Fig. 5A). These data suggest that there is an inverse regulation machinery between the expression of Bcl-2 and Bcl-xL. It has been shown that Bcl-xL is more active than Bcl-2 in binding to Bax and in inhibiting Bax activation (Leber et al., 2010). The Bax conformation was changed, and Bax was translocated into mitochondria after HHT treat-

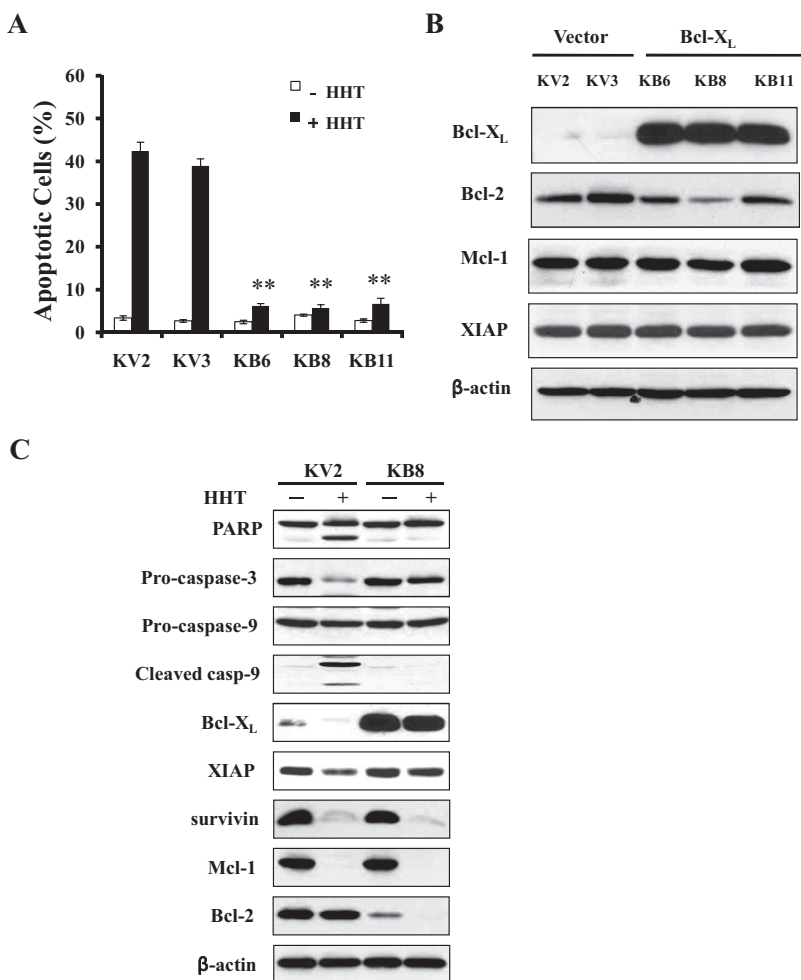


Fig. 9. Overexpression of Bcl-xL decreases HHT-induced apoptosis in KCL22 cells. **A**, apoptotic cells determined by FACS after annexin V-FITC staining. The data shown are the mean \pm S.D. of annexin V-positive cells of three tests. **, $p < 0.01$ compared with apoptotic cells in KV2 or KV3 cells treated with HHT. Cells were treated with and without 2 μ M HHT for 36 h. **B**, the basal levels of Bcl-xL, Bcl-2, Mcl-1, XIAP, and survivin in the subclones of KCL22 cells transfected with the Bcl-xL expression vector (KB6, KB8, and KB11) or an empty vector (KV2 and KV3). **C**, Western blot analyses of PARP, caspase-3, caspase-9, XIAP, survivin, Mcl-1, Bcl-2, and Bcl-xL proteins. KV2 and KB8 cells were treated with and without 2 μ M HHT for 36 h. The levels of each indicated protein were detected using specific antibodies as described under *Materials and Methods*.

ment (Fig. 7, B and C). These data suggest that HHT induces apoptosis through activation of Bax and that Bcl-xL is a dominant antiapoptotic protein required for blocking HHT-induced apoptosis.

Overall, our data revealed that HHT treatment decreased the levels of several proteins, including BH3-only proteins and antiapoptotic proteins. The down-regulation of Mcl-1, but not survivin or XIAP, contributes to HHT-induced apoptosis in AML cells, which contain low levels of Bcl-xL. However, this down-regulation was not sufficient for HHT to induce apoptosis in CML cells which express high levels of Bcl-xL (Figs. 5, 7, and 8). CML cells express the BCR-ABL fusion protein caused by a (9;22) chromosomal translocation (Deininger et al., 2000). Transfection of BCR-ABL into non-CML cells has been shown to increase the expression of Bcl-xL (Amarante-Mendes et al., 1998; de Groot et al., 2000). Patients with CML in chronic phase, but not in blast crisis, are responsive to imatinib (O'Hare et al., 2008). However, some patients with CML become resistant to imatinib treatment as a result of mutation of BCR-ABL. Our data suggest that HHT plus an inhibitor of Bcl-xL might be effective for the treatment of resistant and/or relapsed patients with CML.

Authorship Contributions

Participated in research design: Yin, Wang, and Jing.

Conducted experiments: Yin, Wang, Zhang, and Jing.

Contributed new reagents or analytic tools: Zhou.

Performed data analysis: Yin, Wang, and Jing.

Wrote or contributed to the writing of the manuscript: Yin, Wang, and Jing.

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Address correspondence to: Yongkui Jing, Department of Medicine, Mount Sinai School of Medicine, New York, NY 10029. E-mail: yongkui.jing@mssm.edu