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Bcl-X_L is a dominant antiapoptotic protein which inhibits homoharringtonine-induced apoptosis in leukemia cells.

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Running title: Bcl-X_L inhibits homoharringtonine-induced apoptosis.

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Abbreviations: AML: acute myeloid leukemia; CML: chronic myeloid leukemia; DCFH-DA: 5,6-carboxy-2',7'-dichlorodihydrofluorescein diacetate; c-FLIP: cellular-FLICE inhibitory protein; CHAPS: 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; DR4: death receptor 4; DR5: death receptor 5; FADD: Fas-associated via death domain; HHT: Homoharringtonine; MMP: mitochondrial membrane potential; PARP: poly-(ADP-ribose)-polymerase (PARP); PI: propidium iodide; PMSF: phenylmethylsulfonyl fluoride; SDS: sodium dodecyl sulfate.

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Abstract:

Homoharringtonine (HHT) has been reported to be effective in a portion of patients with acute myeloid leukemia (AML) and 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 Mcl-1, XIAP, survivin, and BH3-only proteins as well as the mitochondria membrane potential. The levels of Bcl-2, Bax, and Bak proteins in HL-60 cells were not changed after HHT treatment. U937, K562, KU812 and KCL22 cells expressed Bcl-X_L and were less responsive to HHT-induced apoptosis than HL-60 cells. Silencing Mcl-1 or Bxl-X_L, 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-X_L, but not Bcl-2 or Mcl-1. K562 cells expressing high levels of Bcl-X_L but no Bcl-2 were less responsive to HHT-induced apoptosis than KCL22 cells which expressed lower levels of Bcl-X_L and higher levels of Bcl-2 protein. In K562 cells, knock-down of Bcl-X_L, but not of Mcl-1, enhanced HHT-induced apoptosis. Transfection of Bcl-X_L into KCL22 cells attenuated HHT-induced apoptosis. These data suggest that Bcl-X_L plays a more important role than Bcl-2 and Mcl-1 in protecting against HHT-induced apoptosis.

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Introduction:

Homoharringtonine (4-methy-2-hydroxy-4-methylpentyl butanedioate, HHT) 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 as traditional Chinese medicine (Huang, *et al* 1983). HHT has been demonstrated to have anti-leukemia effects in acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) patients in China and several clinical trials have been performed in the United States (Quintas-Cardama, *et al* 2009). HHT as a single agent has positive therapeutic effects in a portion of AML and CML patients (O'Dwyer, *et al* 1986) and is being incorporated into combination treatments of AML patients 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 AML patients, whereas clinical studies performed in USA were mainly focused on CML patients (Quintas-Cardama, *et al* 2009). A phase I/II investigation revealed a therapeutic potential in CML patients after treatment failure with interferon (Quintas-Cardama, *et al* 2007). Several other studies showed improved therapeutic effects in CML patients when HHT was given in combination with interferon or ara-C (Quintas-Cardama, *et al* 2009, Stone, *et al* 2009). Since most CML patients in the chronic phase are responsive to imatinib as an initial treatment, HHT has been considered for development as treatment only for CML patients resistant to, or relapsed from imatinib treatment or for AML patients resistant to conventional chemotherapy (de Lavallade, *et al* 2007, Marin, *et al* 2005).

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 (Fresno, *et al* 1977, Huang 1975, 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 (Kuroda, *et al* 2008, Lou, *et al* 2007, Tang, *et al* 2006). It seems that the apoptosis induction ability of HHT accounts for its main therapeutic potential in the treatment of leukemia patients. In the current study we compared the apoptosis induction abilities of HHT in AML cell lines and in CML cell lines as well as in primary leukemia

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cells obtained from both AML and CML patients. 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 mitochondria membrane potential and changes in the levels of apoptosis-related proteins. Our data revealed that HHT induced apoptosis mainly through a mitochondrial-mediated pathway which was correlated with down-regulation of Mcl-1, survivin, and XIAP protein in AML cells. Higher expression levels of Bcl-X_L in CML cell lines compared to those in AML cell lines accounted for the lower responses of CML cells to HHT treatment. Silencing Bcl-X_L 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 (DCFH-DA) were obtained from Molecular Probes (Eugene, OR). Antibodies to poly-(ADP-ribose)-polymerase (PARP) were obtained from Boehringer Mannheim (Mannheim-Waldhof, DE), to caspase-3, caspase-8, caspase-9, and XIAP from BD Biosciences (San Jose, CA), to c-FLIP, DR4, and DR5 from Alexis Biochemicals (Plymouth Meeting, PA), to Bax, Bid, β-actin, and Mcl-1 from Santa Cruz Biotechology, Inc. (Santa Cruz, CA), to Bcl-2 and survivin from Roche Diagnostics Corp. (Indianapolis, IN), to Bak, Bad, Bim, and Puma from Cell Signaling Technology (Danvers, MA), and to Noxa and VDC/porin from abcam (Cambridge, MA).

Cell lines: HL-60, U937, and K562 cells were obtained from ATCC (Rockville, MD). KU812 cells were obtained from Dr. Y. Honma (Yokoyama, *et al* 1996) and KCL22 cells were obtained

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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 ATCC (Rockville, MD) (Lombard, *et al* 2005). All of these cell lines were cultured in RPMI-1640 medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mM *L*-glutamine, and 10% (v/v) heat-inactivated fetal bovine serum (FBS).

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

Stable transfection of Bcl-X_L: KCL22 cells were transfected with a pcDNA3.1 plasmid with or without an encoded *Bcl-X_L* expression sequence using Lipofectamine Reagent 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-X_L was determined by Western blot analysis. Three clones, KB6, KB8, and KB11, with overexpression of Bcl-X_L and two vector transfected clones, KV2 and KV3, were selected and used to compare their responses to HHT treatment.

RNA interference: Mcl-1 siRNA (sc-35877), Bcl-X_L siRNA (sc-77361), survivin siRNA (sc-29499), XIAP siRNA (sc-37508) and negative control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology, Inc. siRNA was transfected into K562 or U937 cells by electroporation (Amaxa, Gaithersburg, MD) following the manufacturer's instructions. Briefly, 2 x 10^6 cells were electroporated in 100 μL nucleofector solution (Amaxa Reagent V for K562 cells and Amaxa Reagent C for U937 cells) containing 20 pmol of each siRNA using the preselected Amaxa Program T-016 for K562 cells and Amaxa Program W-001 for U937 cells. siRNA transfected cells were plated in a 6-well plate with 2 mL supplemented RPMI-1640 medium for 15 h and subsequently further treated for 24 h with and without 0.5 μM HHT for K562 cells or 20 nM HHT

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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 1.0 x 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. Briefly, 50 μL cell suspension was mixed with 50 μL 0.4% trypan blue solution and the number of cells either stained blue or unstained were determined using a hemocytometer. Viable cells possessing intact cell membranes are able to exclude trypan blue staining while non-viable cells without intact membrane take 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 subG1 analysis and Annexin V-FITC staining. Cells in the subG1 phase were measured as described previously (Yu, *et al* 2008). Cells were fixed with ice-cold 70% ethanol overnight at a density of 1 x 10^5 cells /mL and treated with 200 μg/mL RNase for 30 min at 37ºC. Propidium iodide (PI) was then added to a final concentration of 50 μg/mL and the cells in the subG1-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. For the annexin V-FITC staining assay, 1 x 10^5 cells were washed twice with PBS, then labeled with annexin V-FITC and PI in binding buffer according to the instructions provided by the manufacturer in the 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): MMP was assessed by the retention of Rh123, a membrane-permeable fluorescent cationic dye that is selectively taken up by mitochondria. The levels of MMP are proportional to the MMP (Emaus, *et al* 1986). Briefly, cells (1 x 10^6) treated with or without HHT were incubated with 0.1 μg/mL Rh123 in the dark for 20 min at room temperature. After washing with PBS, the cells were analyzed by FACScan (Becton Dickinson) with excitation and emission wavelengths of 495 and 535 nm, respectively.

Western blot analysis: Protein extracts (50 μg) prepared with RIPA lysis buffer [50 mM

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Tris-HCl, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% NP-40, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 100 μM leupeptin, and 2 μg/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 4ºC. 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, 1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate} containing protease inhibitor cocktail. After 30 min incubation at 4ºC the suspension was centrifuged for 15 min at 14,000×*g*. The obtained supernatant fluid containing 500 μg protein was precleared with 20 μl protein A/G plus agarose (Santa Cruz) and then subjected to immunoprecipitation with 3 μg of either normal mouse IgG (Santa Cruz) or anti-Bax (Sigma) at 4ºC for 2 h with gentle rotation. Then 30 μl 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 using CHAPS lysis buffer. The conformationally changed Bax was determined by Western blot analysis using polyclonal Bax antibody and the co-immunoprecipitated Bak was determined using anti-Bak polyclonal antibody (Rahmani, *et al* 2009).

Mitochondrial translocation: Cytosol and mitochondrial fractions of cells (1×10^7) treated with or without HHT were isolated using a Cytosol/Mitochondria Kit (PIERCE) according to the manufacturer's instructions. The levels of Bax 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

(Microsoft Excel, Microsoft Corporation). A *p*-value of less than 0.05 was considered statistical 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 cells, 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 due to HHT treatment differed between AML and CML cell lines. More than 80% of NB4 and HL-60 cells lost viability after treatment with HHT at 20 nM for 3 days (Fig. 1A). U937 cells were less sensitive than NB4 and HL-60 cells as 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 HHT at 500 nM for 3 days induced more than 80% of KU812 and KCL22 cells to lose their viability (Fig. 1C). K562 cells were the least sensitive cell line 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 through a different mechanism(s) from that by which it inhibits cell growth.

AML cells are more sensitive than CML cells to HHT-induced apoptosis. Since 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

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cells to HHT-induced apoptosis (Fig. 2A). Treatment with HHT at a concentration of 40 nM for 12 h induced apoptosis of 61.5%, 47.0% and 10.0% in HL-60, NB4, and U937 cells, respectively (Fig. 2A). Increasing the HHT concentration to 0.2 μM induced apoptosis in 31.8% of U937 cells (Fig. 2A). Unlike AML cells, CML cells were not sensitive to HTT treatment at low concentrations. HTT 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 μM for 48 h, HTT 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 leukemia patients: seven AML samples and four CML samples. Mononuclear cells were isolated from these samples and then were treated with HHT at various concentrations for 48 h. Apoptosis due to HHT treatment was determined by analyzing cells in the subG1 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 μM HHT (Fig. 3). HHT at 100 nM induced 60% of AML cells to undergo apoptosis, but only induced apoptosis in 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 which attenuate HHT-induced apoptosis.

HHT decreases the MMP and the levels of anti-apoptotic proteins. The membrane permeability transition of mitochondria is controlled by the MMP and MMP disruption plays a central role in mitochondrial-mediated apoptosis and caspase-9 activation. The levels of MMP were analyzed using flow cytometry in HL-60 cells after treatment with HHT after staining with the cationic dye Rh123. A significant decrease of MMP was observed in HL-60 cells after treatment with 30 nM HHT for 4 h (Fig. 4A).

Decreases in the levels of MMP lead to activation of caspase-9 and -3. These decreases are regulated by the pro-apoptotic, anti-apoptotic, and the BH3-only proteins of the Bcl-2 family (Kuroda and Taniwaki 2009). The levels of pro-apoptotic proteins, Bax and Bak, were not

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regulated by HHT treatment. The levels of the anti-apoptotic proteins, Bcl-2, Bcl-X_L, and Mcl-1, were differentially regulated by HHT treatment. HL-60 cells did not express Bcl-X_L. 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 pro-capase-9 at concentrations of 20 and 30 nM, suggesting that caspase-3 and caspase-9 are activated (Fig. 4B). The activities of caspase-3 and caspase-9 are inhibited by both the inhibitor of apoptosis protein, 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 those proteins were significantly decreased (Fig. 4D). These data suggest that down-regulation of those proteins are not secondary to cell death and should be primary targets for HHT treatment. The levels of Bcl-2, Bcl-X_L, 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-X_L, Mcl-1, and survivin in HL-60 cells were lower than those in U937 cells. The levels of Bcl-X_L in U937 cells were higher than that in HL-60 cells, but were lower than those in CML cells (Fig. 5A). Silencing Bcl-X_L 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-X_L and Mcl-1 may determine the sensitivities of the three AML cell lines to HHT-induced apoptosis.

To determine if 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

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(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 to that of A3 cells (Fig. 6A). The levels of Bcl-X_L and Mcl-1 were decreased by HHT treatment in all three cell lines (Fig. 6B). Since I 9.2 cells did not express caspase-8 and I 9.1 cells did not express FADD, it suggests that caspase-8 could only be 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-X_L (Fig. 6B)

The high levels of Bcl-X_L in CML cells mediate the reduced response to HHT-induced apoptosis. CML cells expressed higher levels of Bcl-X_L than AML cells and HHT only induced apoptosis at high concentrations or after longer periods of treatment in CML cell lines (Fig. 5A, 2B). 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 μM induced apoptosis in KU812 and KCL22 cells, but not, or only weakly, in K562 cells based on the cleavage of PARP (Fig. 7A). Since the results obtained in AML cells suggest that HHT-induced apoptosis is mainly via a mitochondrial-mediated pathway, the levels of Bax, Bcl-2, Bcl-X_L, 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 μM. Bcl-2 was detected in KCL22 cells, but not in K562 or in KU812 cells (Fig. 5A, 7A). Mcl-1 was present and its level was completely decreased by HHT in all three cell lines. 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 based on the levels of parental PARP (Fig. 7A). Since 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 anti-apoptotic protein mediating apoptosis resistance to HHT treatment. Both KU812 and KCL22 cells express lower levels of Bcl-X_L than K562 cells. The levels of Bcl-X_L 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-X_L was also inhibited by HHT treatment in K562 cells, a certain amount of Bcl-X_L was still

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present and it was similar to the basal levels detected in KU812 and KCL22 cells (Fig. 7A). These data suggest that Bcl-X_L is a key anti-apoptotic protein and that it would be important to determine the sensitivity of CML cells to HHT treatment. It has been shown that Bcl-X_L is more active than Bcl-2 in binding to Bax and in blocking its activation (Leber, *et al*). 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 co-immunoprecipitation assay (Fig. 7B). Moreover, Bax translocated from cytosol to mitochondria after HHT treatment at a concentration of 1 μM 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-X_L.

To further determine the role of Bcl-X_L in HHT-induced apoptosis, Bcl-X_L siRNA was used to silence Bcl-X_L in K562 cells. K562 cells pretreated with Bcl-X_L siRNA have decreased levels of Bcl-X_L 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. 8E, 8F). Taking these data together with the fact that K562 cells do not express Bcl-2, Bcl-X_L levels appear to control HHT-induced apoptosis in CML cells.

Overexpression of Bcl-X_L blocks HHT-induced apoptosis in KCL22 cells. KCL22 cells expressed higher levels of Bcl-2 and lower levels of Bcl-X_L than K562 cells and were more sensitive to HHT-induced apoptosis (Fig. 5A, 7A). To further confirm the role of Bcl-X_L in HHT-induced apoptosis, we generated stable clones of KCL22, KB6, KB8, and KB11 cells with overexpression of Bcl-X_L. 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-X_L. Western blot analysis revealed that KB6, KB8, and KB11 cells

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expressed higher levels of Bcl-X_L than KV2 and KV3 cells (Fig. 9B). The levels Mcl-1 and XIAP were equally expressed in these subclones regardless of transfection with Bcl-X_L (Fig. 9B) However, the levels of Bcl-2 were relative lower in the KB6, KB8, and KB11 cells compared to those in KV2 and KV3 cells (Fig. 9B). 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 μM 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-X_L indeed is 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 AML and CML patients, 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 (Jie, *et al* 2007, Lou, *et al* 2007, Tang, *et al* 2006). Using AML and CML cell lines and primary cells we have observed different apoptosis responses due to HHT treatment (Figs. 2, 3). These different responses may account for the variety of *in vivo* responses to HHT treatment.

The mitochondria (intrinsic pathway)- and death receptor (extrinsic pathway)-mediated apoptotic pathways 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 20-40 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 due to caspase-8 activation and by the intrinsic pathway due to different expressions of anti-apoptotic proteins, principally, Bcl-2, Bcl-X_L, and Mcl-1. Using Western blot analysis, we found the levels of Bid and pro-caspase-8 were decreased after HHT

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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). Since only one-third of HHT apoptosis induction ability was decreased in cells lacking caspase-8, it 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 pro-apoptotic proteins, anti-apoptotic 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 pro-apoptotic 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-X_L was not detected in HL-60 cells either before or after HHT treatment. Although 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). Since the activities of caspase-3 and 9 are inhibited by XIAP and survivin, decreases in their levels have been found to result in apoptosis (Dubrez-Daloz, *et al* 2008, Pennati, *et al* 2007). HHT treatment decreased the levels of XIAP and survivin in HL-60 cells (Fig. 4B). Since Bcl-X_L is not expressed in HL-60 cells, it seems that decreases in the levels of Mcl-1, XIAP, and survivin, rather than increases in the levels of BH3-only proteins, 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 (Kuroda, *et al* 2008, Tang, *et al* 2006). AML cells did not express, or expressed only lower levels of, Bcl-X_L and the levels of Mcl-1 were completely reduced by HHT treatment (Fig. 4B, 5A). Therefore, it seems that Bcl-2 levels may not play a critical role in controlling the sensitivity of cells to HHT-induced apoptosis since cells with high levels of Bcl-2 were responsive to HHT-induced apoptosis (Fig. 4B, 7A). There is a report showing that HHT

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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-X_L 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, 9C). U937 cells expressing higher levels of Bcl-X_L than NB4 and HL-60 cells (Fig. 5A) were less sensitive to HHT-induced apoptosis (Fig. 2A). Jurkat sublones, which expressed Bcl-X_L, were less sensitive to HHT treatment than HL-60 cells (Fig. 6). Silencing Bcl-X_L sensitized U937 cells to HHT-induced apoptosis (Fig. 5B). These data imply that the basal levels of Bcl-X_L 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-X_L (Fig. 5A, 7A) and were less sensitive to HHT-induced apoptosis compared to three AML cell lines (Fig. 2). To obtain a significant apoptotic effect in CML cells after 24 h of treatment, 0.5 μM of HHT was required for KU812 and KCL22 cells and greater than 1 μM of HHT was required for K562 cells. By comparing the levels of Bcl-2, Bcl-X_L, 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 μM (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 which did not express Bcl-2 (Fig. 5A, 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. Interestingly, the basal levels of Bcl-X_L were inversely correlated with HHT-induced apoptosis in the three CML cell lines. KCL22 cells expressed the lowest levels of Bcl-X_L and were the most sensitive to HHT-induced apoptosis (Fig. 5A, 7A). The levels of Bcl-X_L were significantly decreased by HHT treatment in KCL22 cells which then underwent apoptosis. Although the levels of Bcl-X_L in K562 cells were also decreased after HHT treatment, a certain amount of Bcl-X_L protein was detected which was higher than the basal level present in KCL22 cells (Fig. 7A). Again, these data suggest that

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Bcl-X_L, but not Bcl-2, is a key inhibitor of HHT-induced apoptosis. The silencing by siRNA of Bcl-X_L, but not of Mcl-1, XIAP, or survivin, sensitized K562 cells to HHT-induced apoptosis (Fig. 8). To further test the role of Bcl-X_L we generated KCL22 cells with overexpression of Bcl-X_L and found that these cells became resistant to HHT-induced apoptosis even though they had decreased levels of Bcl-2 compared to the vector alone-transfected cells (Fig. 9). The reason for the decrease in the levels of Bcl-2 in Bcl-X_L-transfected KCL22 cells is unclear. AML cell lines contain high levels of Bcl-2 either without or with lower levels of Bcl-X_L (Fig. 5A). K562 cells have high levels of Bcl-X_L without expression of Bcl-2, but KCl22 cells express high levels of Bcl-2 with low levels of Bcl-X_L (Fig. 5A). These data suggest that there is an inverse regulation machinery between the expression of Bcl-2 and Bcl-X_L. It has been shown that Bcl-X_L is more active than Bcl-2 in binding to Bax and in inhibiting Bax activation (Leber, *et al*). The Bax conformation was changed and Bax was translocated into mitochondria after HHT treatment (Fig. 7B, 7C). These data suggest that HHT induces apoptosis through activation of Bax and that Bcl-X_L seems to be a dominant anti-apoptotic 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-X_L. However, this down-regulation is not sufficient for HHT to induce apoptosis in CML cells which express high levels of Bcl-X_L (Fig. 5, 7, 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-X_L (Amarante-Mendes, *et al* 1998, de Groot, *et al* 2000). CML patients in chronic phase, but not in blast crisis, are responsive to imatinib (O'Hare, *et al* 2008). However, a portion of CML patients become resistant to imatinib treatment due to mutation of BCR-ABL. Our data suggest that HHT plus an inhibitor of Bcl-X_L might be effective for the treatment of resistant and/or relapsed CML patients.

Authorship Contribution

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

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

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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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FIGURE LEGENDS

Fig.1. Cell growth inhibition and cytotoxity 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±SE of three independent experiments.

Fig. 2. Apoptosis induction of HHT in AML and CML 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 (subG1) was determined by flow cytometry after staining with PI. Con, control; Ap, apoptotic cells in subG1 phase.

Fig. 3. Apoptosis induction of HHT in cultured primary leukemia cells obtained from AML and CML patients. (A) Comparison of HHT-induced apoptosis in primary AML and CML cells. Leukemia cells isolated from 7 AML patient samples and 4 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 G1) by flow cytometry after staining with PI. Values shown are the means ± SD. ** $p < 0.01$, * $p < 0.05$, compared to the cells without treatment; ## $p < 0.001$, compared to AML cells treated with HHT at 100 nM. (B) A representative sample from an AML patient and one representative sample from a CML patient.

Fig. 4. HHT decreases the levels of MMP and anti-apoptotic 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 following Rh123 staining. The shift to the left of the peak indicated a loss of MMP. (B) Effects of HHT on the levels of proteins

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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 pro-apoptotic 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.

Fig. 5. Knock-down of Mcl-1 and Bcl-X_L, but not XIAP or survivin, enhances HHT-induced apoptosis in U937 cells. (A) The basal levels of Bcl-X_L, Bcl-2, Mcl-1, XIAP, and survivin in NB4, HL-60, U937, K562, KU812, and KCL22 cells. (B) The effects of Mcl-1, Bcl-X_L, XIAP, and survivin siRNA on HHT-induced apoptosis in U937 cells. U937 cells were incubated with control siRNA or Mcl-1 siRNA, Bcl-X_L 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-X_L, Bcl-2, Mcl-1, XIAP, and survivin proteins using the specific antibodies as described in Materials and Methods.

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-X_L 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 in Materials and Methods.

Fig. 7. Apoptosis induction and anti-apoptotic 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 in Materials

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and Methods. (B) Conformational change of Bax. KCL22 cells were treated with HHT at 1 μM for the indicated times and lysed in buffer containing 1% CHAPS. Conformational changed Bax protein was immunoprecipated 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 μM for the indicated times. The cytosol and mitochondrial fractions were separated as described in the Materials and Methods. VADC/porin was used as the standard for mitochondrial isolation.

Fig. 8. Silencing Bcl-X_L, but not Mcl-1, XIAP, or survivin, enhanced HHT-induced apoptosis in K562 cells. (A) The influence of Bcl-X_L siRNA on HHT-induced PARP cleavage. (B) The influence of Bcl-X_L 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 μM HHT for 24 h. The cells were collected for FACS analysis for determining annexin V-positive cells (B & D) or for total protein isolation to analyze the levels of PARP, caspase-9, Bcl-X_L, Mcl-1, XIAP, or survivin using Western blot analysis (A, C, E, F).

Fig. 9. Overexpression of Bcl-X_L decreases HHT-induced apoptosis in KCL22 cells.

(A) Apoptotic cells determined by FACS after annexin V-FITC staining. The data shown are the mean ± SD of annexin V-positive cells of three tests. ** $p<0.01$, compared to apoptotic cells in KV2 or KV3 cells treated with HHT. Cells were treated with and without 2 μM HHT for 36 h. (B) The basal levels of Bcl-X_L, Bcl-2, Mcl-1, XIAP, and survivin in the subclones of KCL22 cells transfected with the Bcl-X_L 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-X_L proteins. KV2 and KB8 cells were treated with and without 2 μM HHT for 36 h. The levels of each indicated protein were detected using specific antibodies as described in Materials and Methods.

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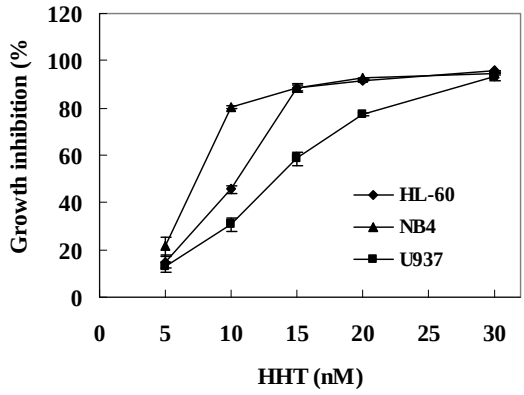
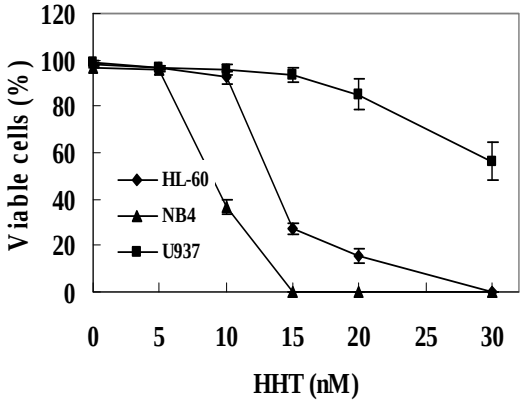
A





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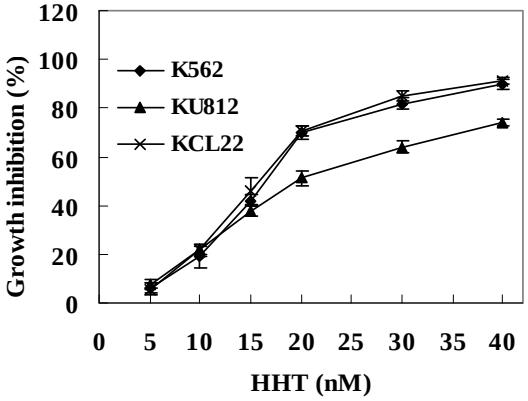

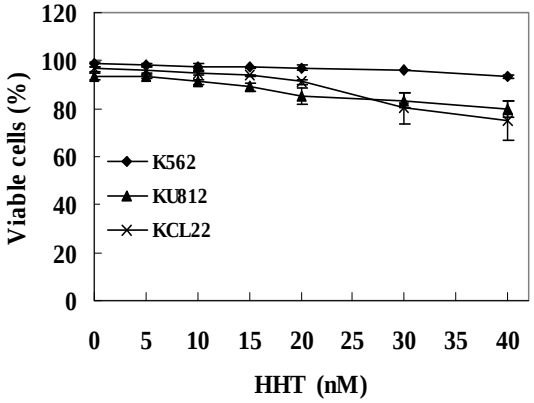

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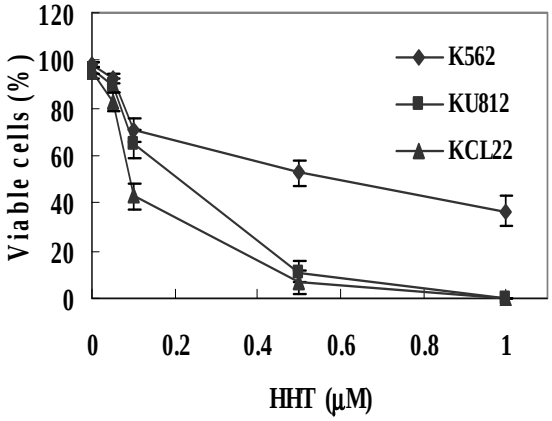

Fig. 1

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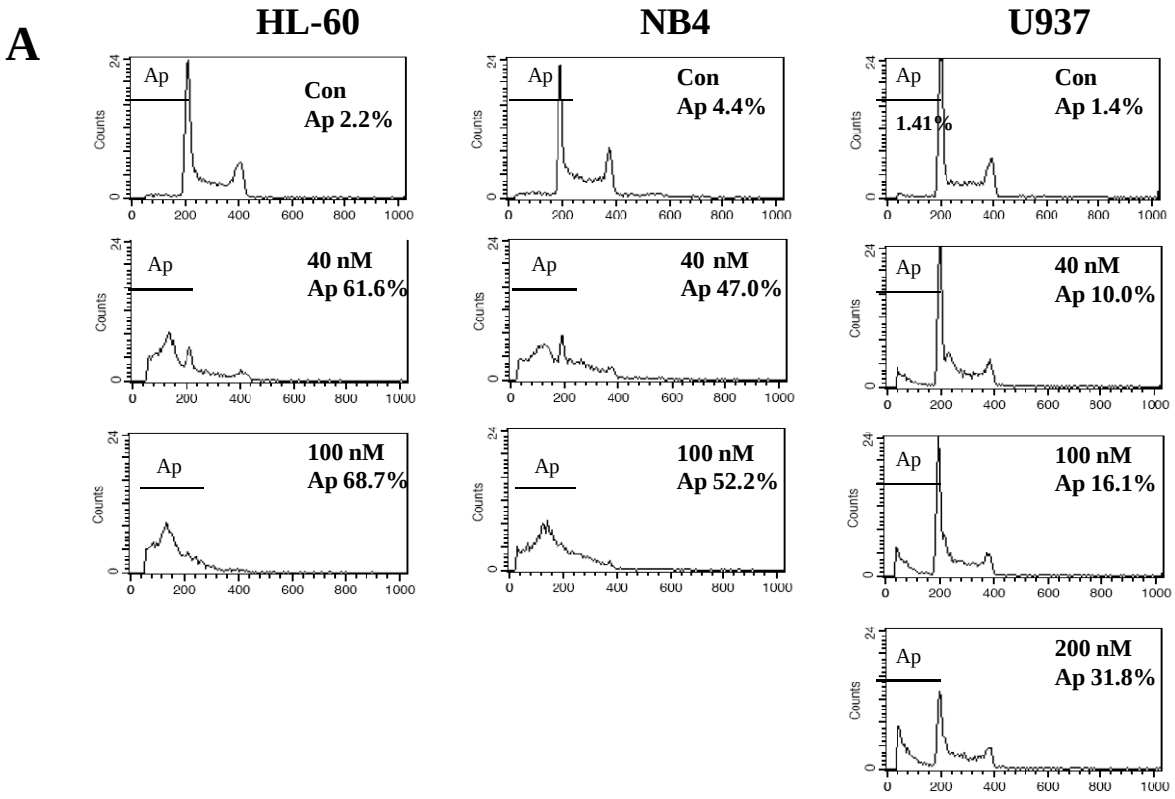

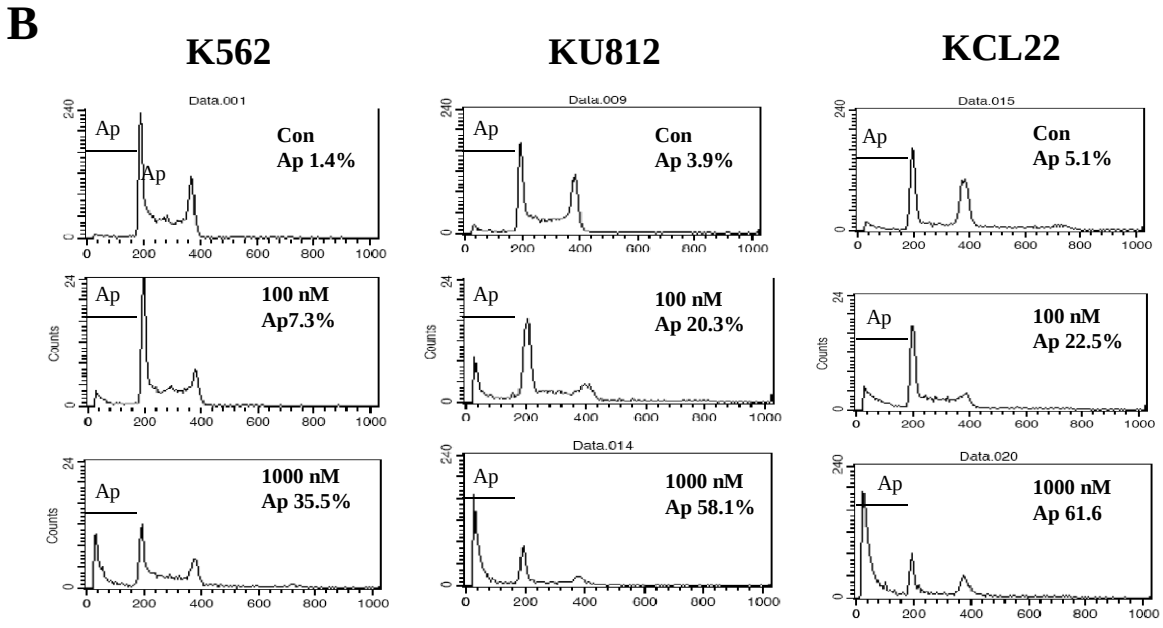

Fig. 2

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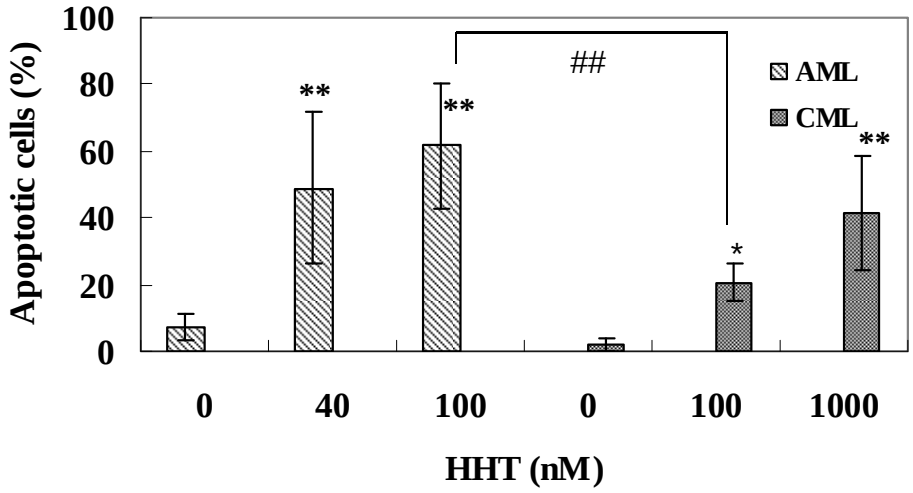

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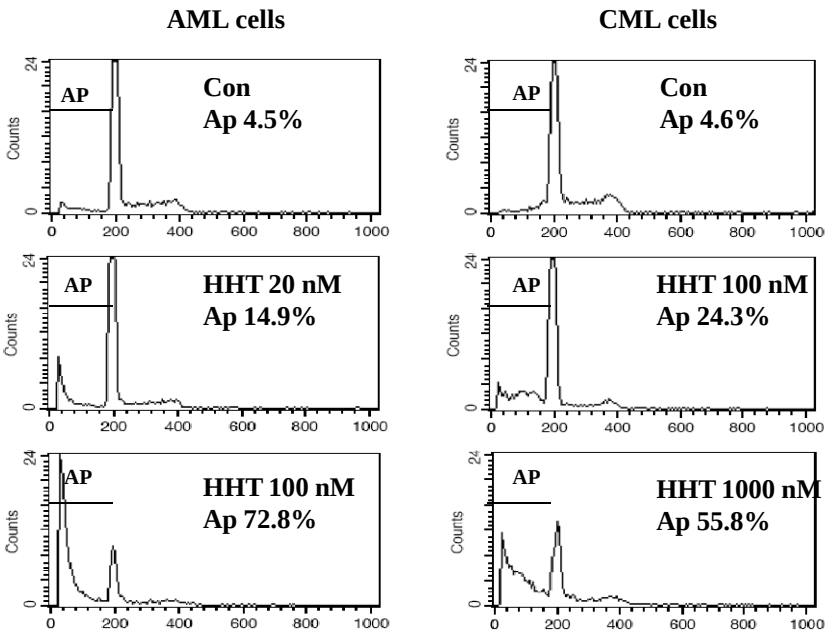

Fig. 3

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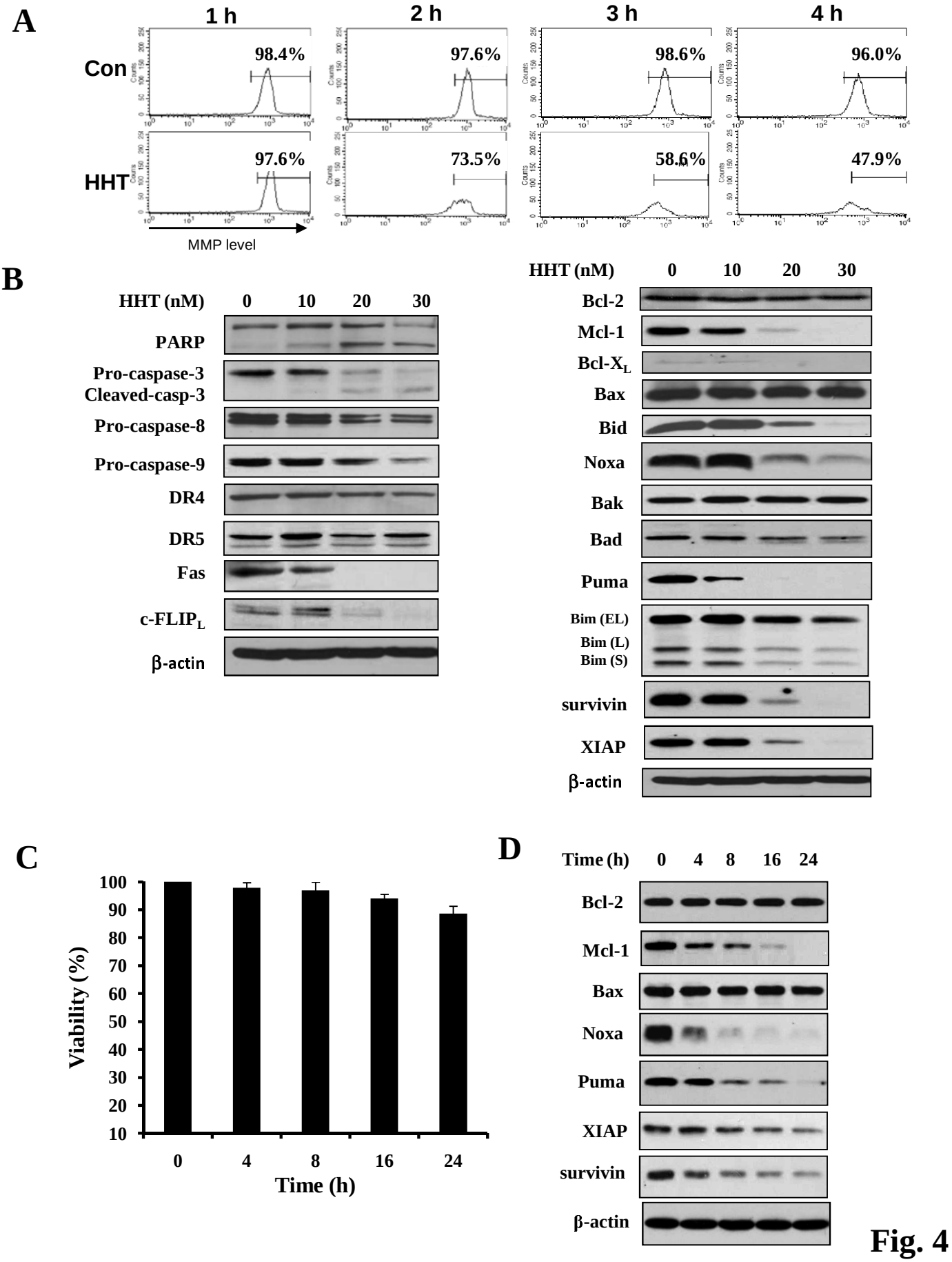

Fig. 4

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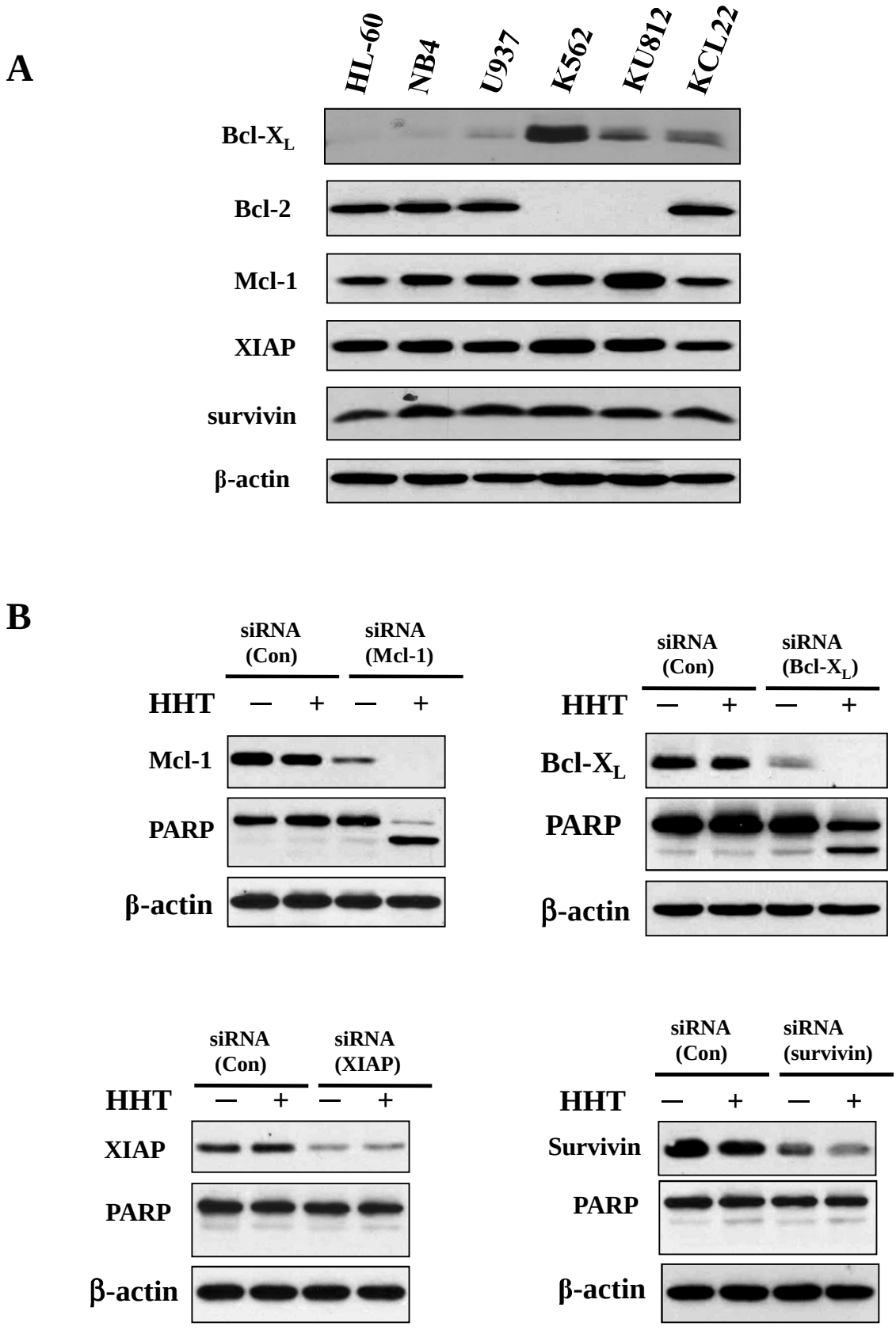

Fig. 5

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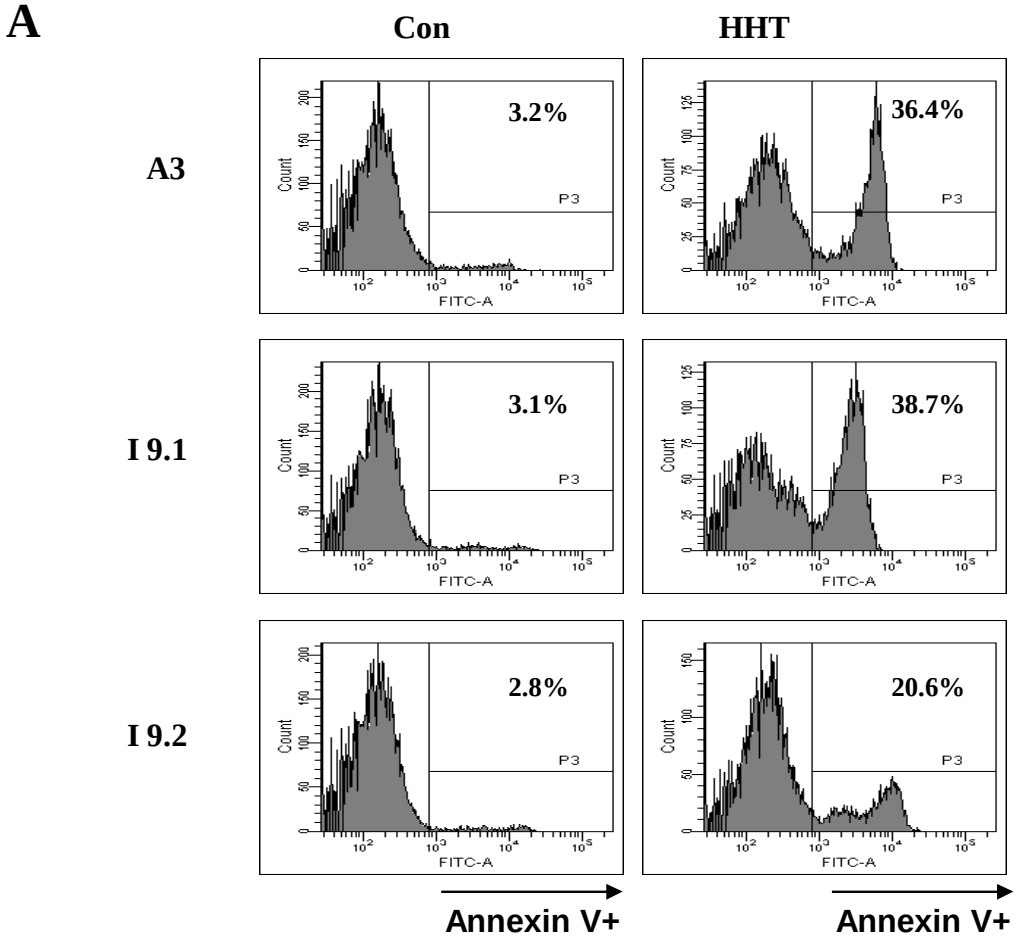

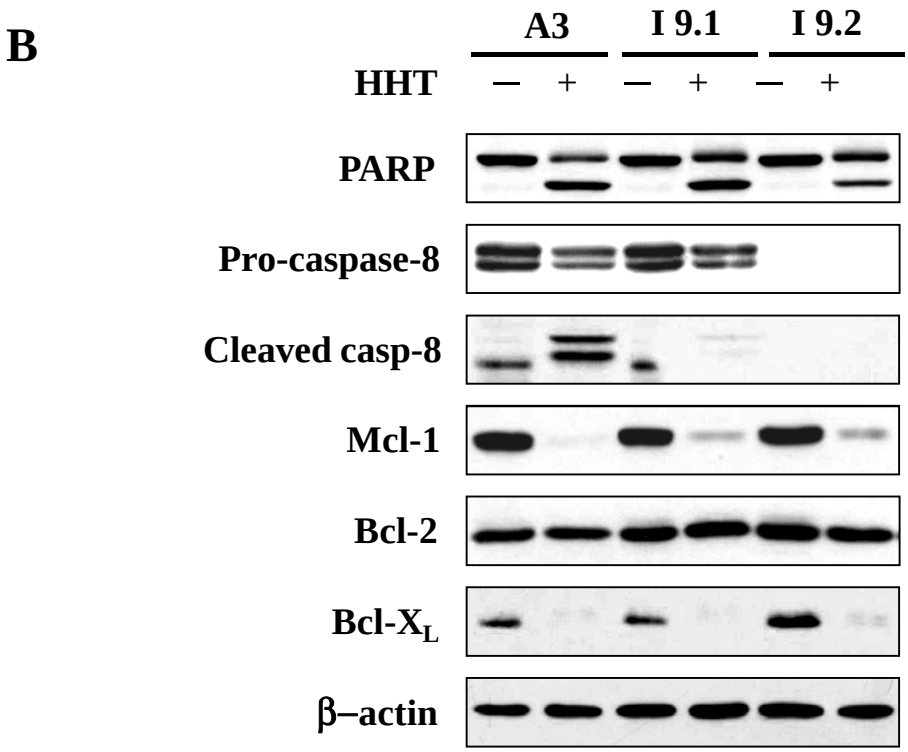

Fig. 6

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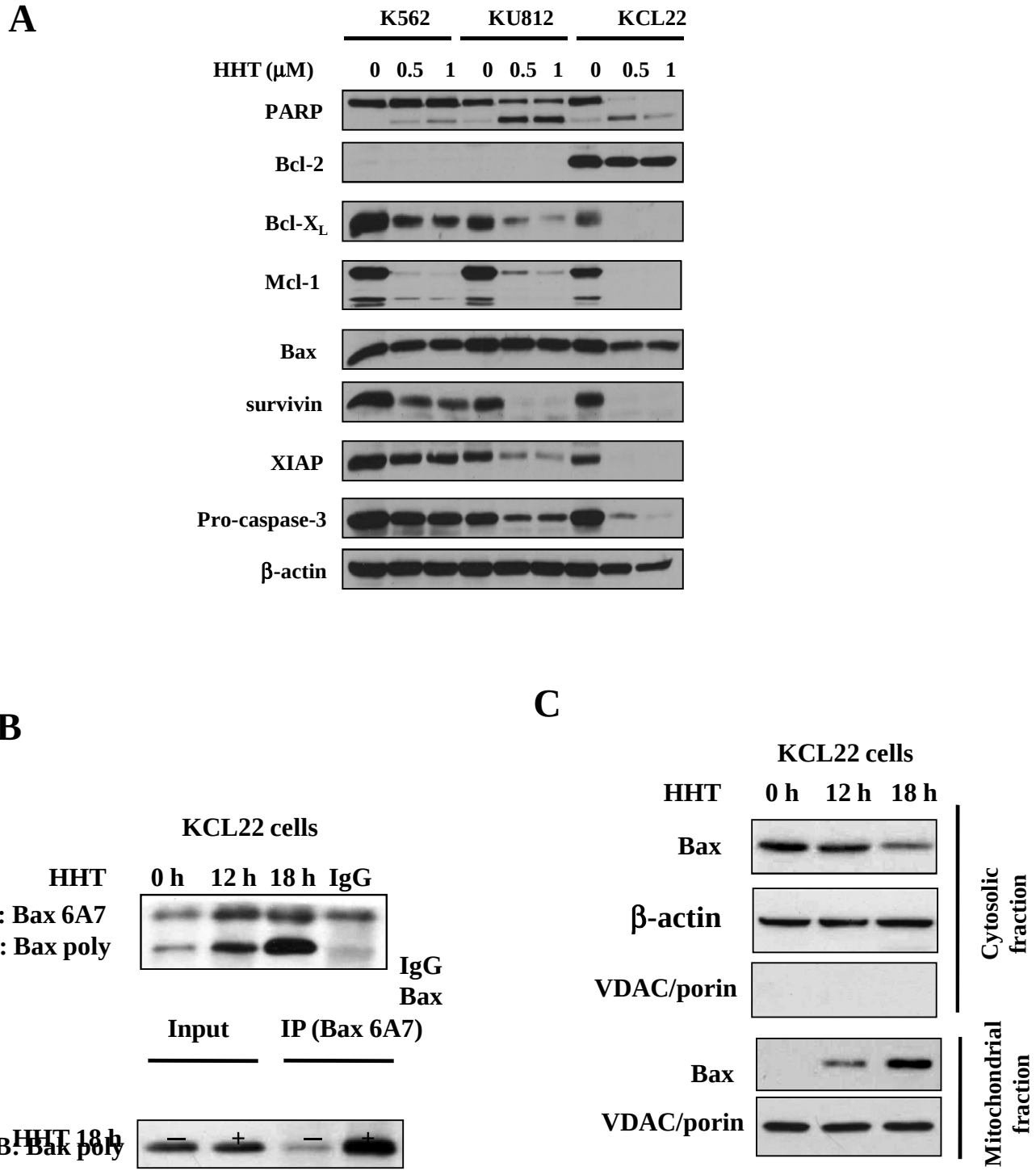

Fig. 7

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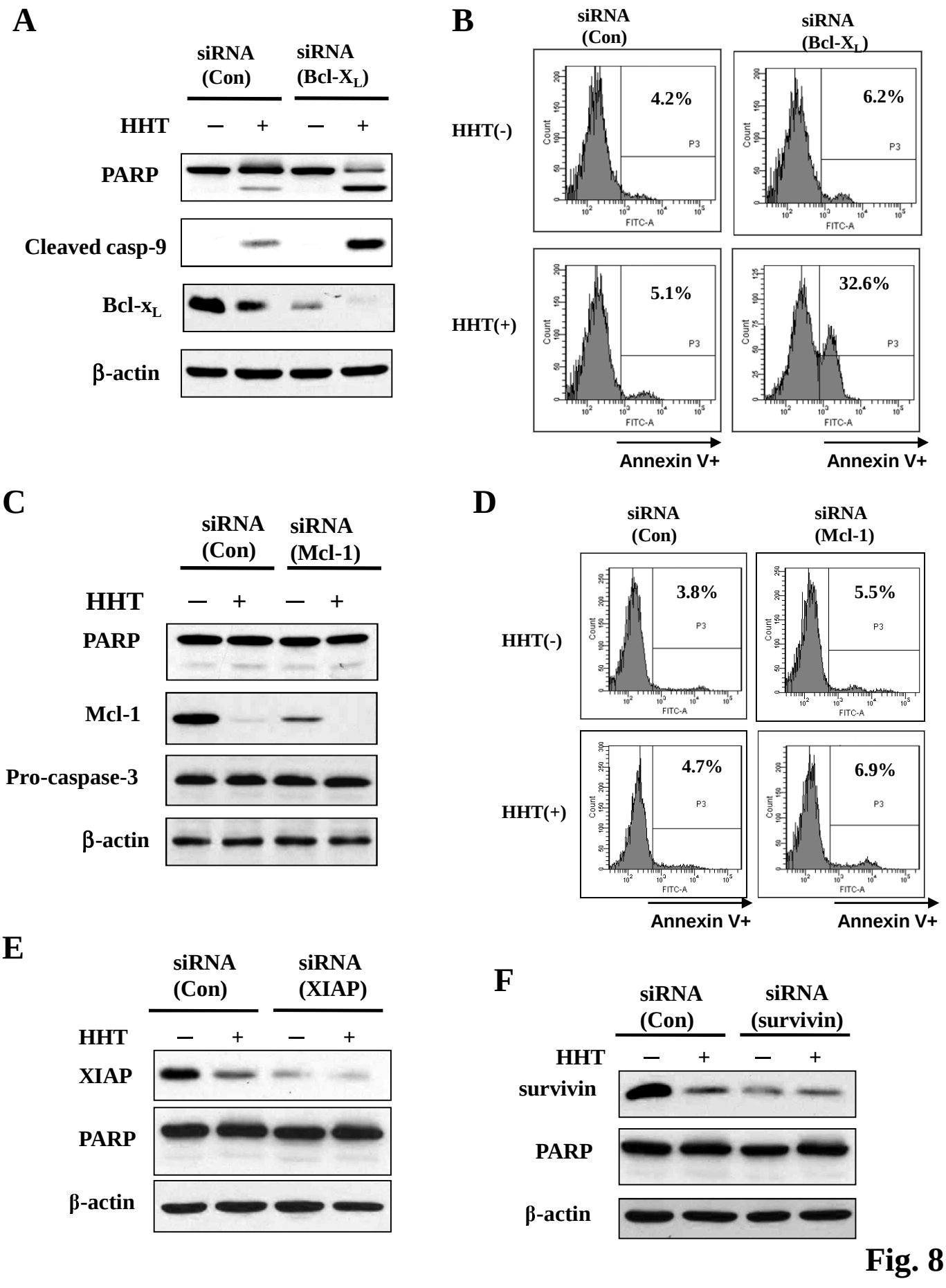

Fig. 8

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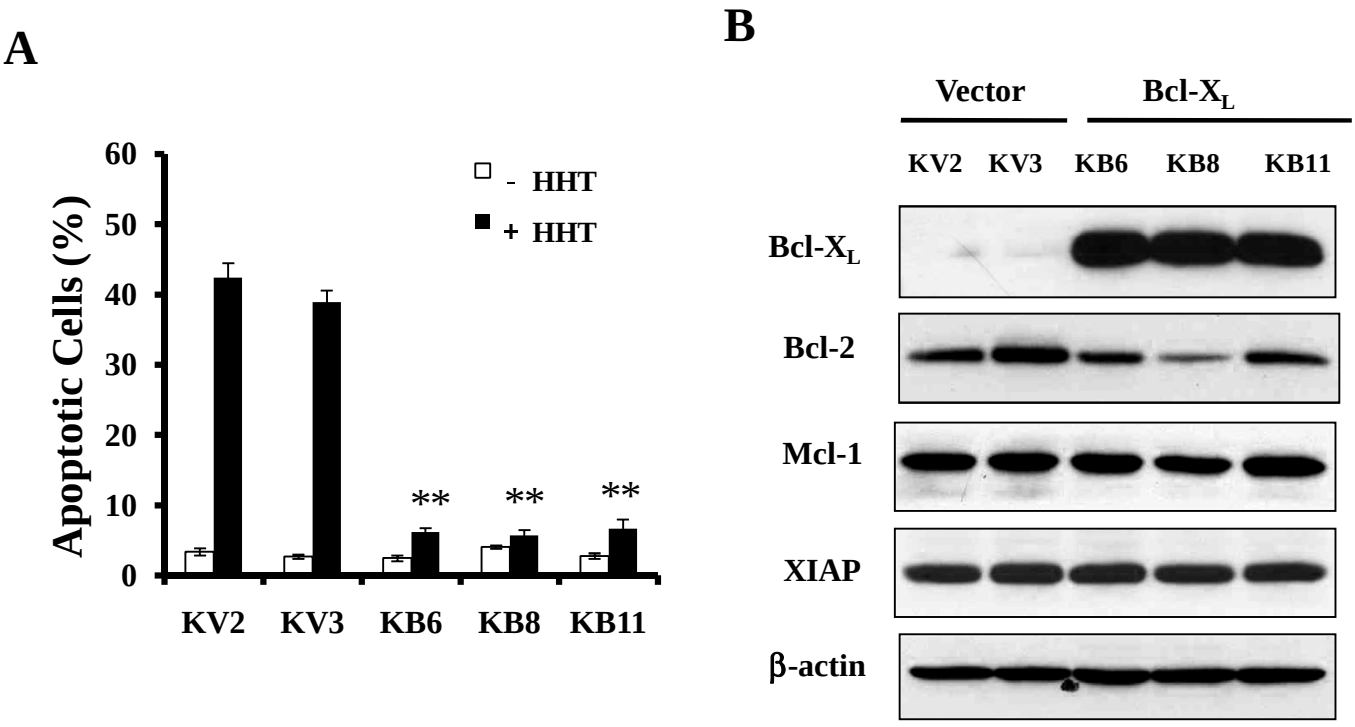

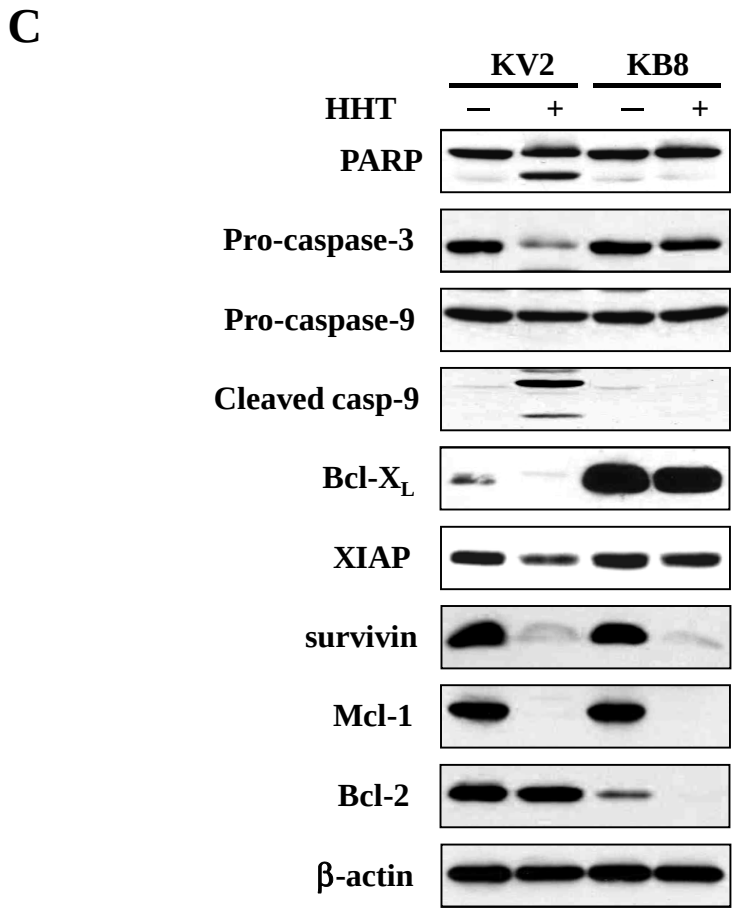

Fig. 9