Cotreatment with STI-571 Enhances Tumor Necrosis Factor α-related Apoptosis-inducing Ligand (TRAIL or Apo-2L)-induced Apoptosis of Bcr-Abl-positive Human Acute Leukemia Cells

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ABSTRACT

Bcr-Abl tyrosine kinase inhibitor STI-571 induces differentiation and apoptosis of HL-60/Bcr-Abl (with ectopic expression of p190 Bcr-Abl) and K562 (with endogenous expression of p210 Bcr-Abl) cells (Blood, *96:* 2246–2253, 2000). Cotreatment with STI-571 partially overcomes the resistance to antileukemic drug-induced apoptosis of HL-60/Bcr-Abl and K562 cells. Tumor necrosis factor (TNF) α-related apoptosis-inducing ligand (Apo-2L/TRAIL), after binding with its signaling death receptors (DR4 and DR5), triggers the intrinsic "mitochondrial" pathway of apoptosis more efficiently in the cancer than do normal cells. In the present studies, we compared the apoptotic effects of Apo-2L/TRAIL, with or without cotreatment with STI-571, in HL-60/neo, HL-60/Bcr-Abl, and K562 cells. As compared with HL-60/neo, HL-60/Bcr-Abl and K562 cells are relatively resistant to Apo-2L/TRAIL-induced apoptosis. In HL-60/Bcr-Abl and K562 *versus* HL-60/neo cells, Apo-2L/TRAIL caused less cytosolic accumulation of cytochrome *c* and the processing of caspase-9 and -3. This was also associated with decreased processing of caspase-8, c-FLIP$_L$ and Bid. Reduced effects of Apo-2L/TRAIL in Bcr-Abl-positive leukemic cells were not attributable to diminished expression of DR4 and DR5, or higher expressions of the decoy receptors DcR1 and -2 or c-FLIP$_L$. Cotreatment with STI-571 significantly enhanced Apo-2L/TRAIL-induced apoptosis ($P < 0.01$) as well as increased the processing of caspase-9 and -3 and XIAP, without affecting the levels of DR4, DR5, decoy receptors, or c-FLIP$_L$. Cotreatment with STI-571 did not enhance Apo-2L/TRAIL-induced apoptosis of HL-60/neo cells. These studies suggest that a combined treatment with STI-571 may be an effective strategy to selectively sensitize Bcr-Abl-positive leukemic blasts to Apo-2L/TRAIL-induced apoptosis.

INTRODUCTION

Apo-2L/TRAIL,[2] also known as Apo-2 ligand, has been shown to induce apoptosis of a variety of tumor cell lines more efficiently than normal cells (1–3). Although in a recent report Apo-2L/TRAIL was demonstrated to induce apoptosis of human hepatocytes, Apo-2L/TRAIL has been shown to actively suppress human mammary adenocarcinoma growth in mice without any significant toxic effects that are seen with the *in vivo* use of TNF and Fas ligand (CD95L; Refs. 3, 4). Apo-2L/TRAIL can bind to DR4, DR5, DcR1, DcR2, and osteoprotegerin (1). DR4 and DR5 contain a cytoplasmic region consisting of a stretch of 80 amino acids designated as the "death domain," which is responsible for transducing the death signal (1). Ligation by Apo-2L/TRAIL recruits the adaptor molecule FADD to the DD of DR4 and DR5 (5). Through its DED, FADD interacts with caspase-8 and -10 (5, 6). Although FADD −/− cells have been shown to be sensitive to apoptosis induced by the ligation of DR4 and DR5 but not that of Fas (7), both caspase-8 and FADD are essential to the function of APO-2L/TRAIL-mediated DISC (5). Once recruited to FADD, caspase-8 drives its autoactivation through oligomerization and subsequently activates the downstream effector caspases such as caspase-3, -6, and -7 (6, 8). Activated and processed caspase-8 can also cleave and activate the BH3 domain containing proapoptotic molecule Bid, which then translocates to the mitochondria triggering the preapoptotic mitochondrial events including the cytosolic release of cyt c (9–11). In the cytosol, cyt c and dATP bind to Apaf-1 and cause its oligomerization (12, 13). Apaf-1, in turn, binds and processes procaspase-9 into an active caspase that recruits, cleaves, and activates the effector caspase-3 (12–14). Activated caspase-3 can proteolytically cleave a number of cellular proteins, *e.g.*, PARP, lamins, DFF45 (ICAD, DNA fragmentation factor), fodrin, gelsolin, PKCδ, Rb (retinoblastoma protein), DNA-PK,

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[2] The abbreviations used are: Apo-2L/TRAIL, TNF α-related apoptosis-inducing ligand; TNF, tumor necrosis factor; DR, death receptor; DcR, decoy receptor; DD, death domain; DED, death effector domain; DISC, death-inducing signaling complex; cyt c, cytochrome *c*; PARP, poly-(ADP-ribose); IAP, inhibitor of apoptosis proteins; TK, tyrosine kinase; CML, chronic myelogenous leukemia; ALL, acute lymphoblastic leukemia; NFκB, nuclear factor κB.

and so forth, resulting in the morphological features and DNA fragmentation of apoptosis (6, 8, 15). The ability of Apo-2L/TRAIL to trigger the intrinsic or mitochondrial pathway to apoptosis is supported by the observation that, although sensitive to Fas L and TNF-α, Apaf −/− cells are relatively resistant to Apo-2L/TRAIL-induced apoptotic signaling (16).

There are several known determinants of Apo-2L/TRAIL-induced apoptotic signaling. Treatment with DNA-damaging anticancer agents can induce p53 and/or NFκB, which, in turn, can up-regulate DR5 and/or DR4 expression, thereby enhancing Apo-2L/TRAIL-induced apoptotic signaling (17, 18). In contrast, DcR1, which is bound to the cell membrane through a glycolipid anchor and lacks DD, as well as the levels of DcR2, which has an incomplete and inactive DD, bind and titrate down Apo-2L/TRAIL and can act as inhibitors of Apo-2L/TRAIL-induced apoptosis (1). Additionally, an endogenous intracellular protein, FLAME-1, (also known as c-FLIP$_L$, CASH, CLARP, MRIT and Usurpin) which has an NH_2-terminal FADD homology and COOH-terminal caspase homology domains without caspase activity, has a dominant negative effect against caspase-8 and can potentially inhibit Apo-2L/TRAIL-induced death signaling (19). Finally, the levels of IAP family members, which include cIAP1, cIAP2, XIAP, and survivin, may also inhibit Apo-2L/TRAIL-induced apoptosis by specifically binding to and inhibiting the activities of caspase-3, -9, and -7 (20–22).

The dysregulated activity of the TK encoded by the Bcr-Abl fusion gene is responsible for the malignant phenotype of the Bcr-Abl expressing CML in blast crisis and ALL blasts (23, 24). The fusion gene either encodes for the p210 or p185 TK implicated in the pathogenesis of CML or ALL, respectively (23, 24). Bcr-Abl-expressing leukemic blasts display resistance to apoptosis, even when exposed to high doses of antileukemic drugs (25–27). Consistent with this, the ectopic or endogenous expression of Bcr-Abl in HL-60/Bcr-Abl or K562 cells, respectively, was demonstrated to block the mitochondrial permeability transition (Δψm) and release of cyt c, thereby inhibiting the activation of the "executioner" caspases and apoptosis (27, 28). Ectopic or endogenous Bcr-Abl expression up-regulates several antiapoptotic mechanisms including the levels of Bcl-x$_L$ as well as the activities of NFκB and Akt kinase (28–31). NFκB transactivates and up-regulates the levels of the IAP family of proteins, which are known to inhibit the activity of caspases-9, -7, and -3 (20, 32). Recent studies have shown that the inhibition of Bcr-Abl TK activity by a relatively specific inhibitor STI-571 induces differentiation and apoptosis as well as causes *in vitro* and *in vivo* eradication of Bcr-Abl-positive human leukemia cells (33–35). Exposure to STI-571 was shown to lower TK activity but not the levels of Bcr-Abl (35). STI-571 also lowered Bcl-x$_L$ levels and Akt kinase and NFκB activities as well as sensitized Bcr-Abl-positive leukemic cells to Ara-cytosine β-D-arabinofuranoside, etoposide-, and doxorubicin-induced apoptosis (35). In the present studies, we compared the Apo-2L/TRAIL-induced molecular steps of apoptosis and its determinants in Bcr-Abl-positive HL-60/Bcr-Abl and K562 *versus* HL-60/neo cells. Our findings demonstrate that, although relatively resistant, cotreatment with STI-571 can significantly sensitize Bcr-Abl-positive leukemic cells to Apo-2L/TRAIL-induced apoptosis.

MATERIALS AND METHODS

Reagents. The recombinant human homotrimeric (Apo-2L/TRAIL; leucine zipper construct was a gift from Immunex Corp (Seattle, WA; Ref. 3). In some experiments, homotrimeric Apo-2L/TRAIL from Genentech (South San Francisco, CA) was also used. Apo-2L/TRAIL from the two sources was equipotent with respect to the biological effects studied here. STI-571 was kindly provided by Novartis Pharma AG (Basel, Switzerland). Monoclonal anti-caspase-8 antibody was purchased from Upstate Biotechnology (Lake Placid, NY) and anti-FADD antibody from Transduction Labs (Lexington, KY). Anti-Bid antisera (9, 13) were kindly provided by Dr. Xiaodong Wang of the University of Texas, Southwestern School of Medicine (Dallas, TX). Monoclonal anti-XIAP and anti-caspase-9 antibody was purchased from StressGen Biotechnologies Corp. (Victoria, B.C., Canada). Polyclonal anti-PARP and monoclonal anti-cIAP1 antibody were purchased from PharMingen Inc. (San Diego, CA). Polyclonal anti-DR4, anti-DcR1 and -2, and anti-Apo-2L/TRAIL antibodies as well as Apo-2L/TRAIL R2 (DR5):Fc were purchased from Alexis Corp (San Diego, CA). Polyclonal anti-DR5 was obtained from Cayman Chemicals Co. (Ann Arbor, MI).

Cell Culture and Cell Growth Inhibition. Human leukemic cells HL-60/neo and HL-60/Bcr-Abl and the erythroid blast crisis CML K562 cells were cultured and passaged as described previously (28, 35). Logarithmically growing cells were exposed to the designated concentrations of either STI-571 for 48 h or Apo-2L/TRAIL for 24 or 48 h. Alternatively, cells were cotreated with Apo-2L/TRAIL (for 24 or 48 h) and STI-571 or were sequentially treated with STI-571 followed by Apo-2L/TRAIL. After these treatments, cells were pelleted and washed free of the drug(s) prior to the performance of the studies described below.

Preparation of S-100 Fraction and Western Analysis of Cytosolic cyt c. Untreated and drug-treated cells were harvested by centrifugation at 1000 × *g* for 10 min at 4°C. The cell pellets were washed once with ice-cold PBS and resuspended with 5 volumes of buffer [20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM DTT, and 0.1 mM phenylmethylsulfonylfluoride], containing 250 mM sucrose. The cells were homogenized with a 22-gauge needle, and the homogenates were centrifuged at 100,000 × *g* for 30 min at 4°C (S-100 fraction; Refs. 12, 28). The supernatants were collected, and the protein concentrations of S-100 were determined by Bradford method (Bio-Rad, Hercules, CA). Twenty to 30 μg of the S-100 fraction was used for Western blot analysis of cyt c, as described previously (36).

Western Analyses of Proteins. Western analyses of DR4, DR5, Apo-2L/TRAIL, caspase-8, caspase-9, caspase-3, Fas, Fas L, Bid, PARP, XIAP, cIAP1, survivin, and β-actin were performed using specific antisera or monoclonal antibodies according to previously reported protocols (16, 36, 37). Horizontal scanning densitometry was performed on Western blots by using acquisition into Adobe Photo Shop (Apple, Inc., Cupertino, CA) and analysis by the NIH Image Program (NIH, Bethesda, MD). The expression of β-actin was used as a control.

Apoptosis Assessment by Annexin-V Staining. After drug treatments, cells were resuspended in 100 μl of staining

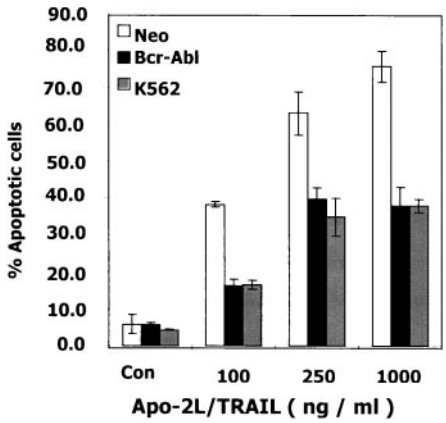

Fig. 1 Apo-2L/TRAIL-induces apoptosis of HL-60/neo but not of HL-60/Bcr-Abl and K562 cells. Cells were exposed to the indicated concentrations of Apo-2L/TRAIL for 24 h. After these treatments, the percentage of apoptotic cells that were untreated controls (*Con*), or treated with Apo-2L/TRAIL, was determined by Annexin-V staining followed by flow cytometry (see "Materials and Methods").

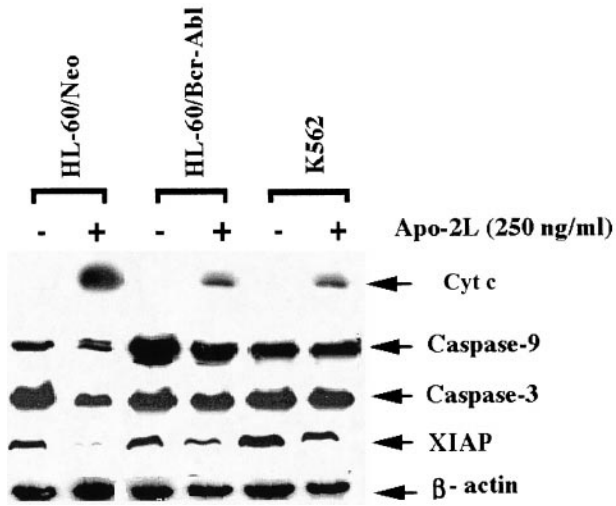

Fig. 2 Apo-2L/TRAIL-induced cytosolic accumulation of cyt-c and processing of caspase-9, -3, and XIAP is inhibited in HL-60/Bcr-Abl and K562 *versus* HL-60/neo cells. Cells were exposed to 250 ng/ml Apo-2L/TRAIL for 24 h. After this, cells were pelleted, and cell-lysates were used for Western analyses of the indicated proteins using specific antibodies (see "Materials and Methods").

solution (containing Annexin-V fluorescein and propidium iodide in a HEPES buffer; Annexin-V-FLUOS Staining Kit; Boehringer-Mannheim, Indianapolis, IN). After incubation at room temperature for 15 min, cells were analyzed by flow cytometry (37). Annexin-V binds to those cells that express phosphotidylserine on the outer layer of the cell membrane, and propidium iodide stains the cellular DNA of those cells with a compromised cell membrane. This allows for the discrimination of live cells (unstained with either fluorochrome) from apoptotic cells (stained only with Annexin-V) and necrotic cells (stained with both Annexin-V and propidium iodide; Ref. 38).

Morphology of Apoptotic Cells. After drug treatment, 50×10^3 cells were washed with PBS (pH 7.3) and resuspended in the same buffer. Cytospin preparations of the cell suspensions were fixed and stained with Wright's stain. Cell morphology was determined by light microscopy (39). In all, five different fields were randomly selected for the counting of at least 500 cells. The percentage of apoptotic cells was calculated for each experiment, as described previously.

Statistical Analysis. Significant differences between values obtained in a population of leukemic cells treated with different experimental conditions were determined by paired *t* test analyses. A one-way ANOVA was also applied to the results of the various treatment groups, and *post hoc* analysis was performed using the Bonferroni correction method.

RESULTS

Apo-2L/TRAIL-induced Apoptosis Is Inhibited in Bcr-Abl-positive Leukemic Cells. We have previously demonstrated that as compared with the control HL-60/neo, HL-60/Bcr-Abl and K562 cells are resistant to high-dose Ara-C, etoposide, and doxorubicin, as well as TNF-α and Fas L-induced apoptosis (28, 35). Because Apo-2L/TRAIL has its unique set of apoptosis-signaling (DR4 and DR5) and nonsignaling DcRs (DcR1 and DcR2), in the present studies, we compared the apoptotic effects of Apo-2L/TRAIL with these cell-types. Fig. 1 demonstrates that exposure to 100, 250, and 1000 ng/ml of Apo-2L/TRAIL for 24 h induced significantly more apoptosis of HL-60/neo *versus* HL-60/Bcr-Abl or K562 cells ($P < 0.01$). A dose-dependent increase in the apoptotic effect of Apo-2L/TRAIL was observed against HL-60/neo cells. But in HL-60/Bcr-Abl and K562 cells, apoptosis attributable to 1000 ng/ml was not significantly different from that observed after exposure to 250 ng/ml of Apo-2L/TRAIL for 24 h ($P < 0.05$; Fig. 1). Apo-2L/TRAIL concentrations that induced apoptosis of 50% of HL-60/neo, HL-60/Bcr-Abl, and K562 cells, was 0.15, 1.5, and 1.6 μg/ml, respectively (mean of three experiments). Apoptosis attributable to a longer exposure interval to Apo-2L/TRAIL (48 h) was also inhibited in HL-60/Bcr-Abl and K562 cells (data not shown).

Apo-2L/TRAIL-induced Molecular Cascade of Apoptosis Is Inhibited in Bcr-Abl-positive Leukemic Cells. Apo-2L/TRAIL-induced DISC activity is known to induce the processing of caspase-8 and c-$FLIP_L$, resulting in the Bid cleavage activity of caspase-8 (5, 40). By inducing the generation of processed and truncated Bid, which translocates to the mitochondria and causes the release of cyt c into the cytosol, Apo-2L/TRAIL can recruit the intrinsic "mitochondrial" pathway to apoptosis (9, 16). This was observed in HL-60/neo but was markedly reduced in Apo-2L/TRAIL-treated HL-60/Bcr-Abl and K562 cells (Fig. 2). Reduced cytosolic accumulation of cyt c also caused a relatively reduced processing of caspase-9 and -3 in Apo-2L/TRAIL-treated HL-60/Bcr-Abl and K562 cells (Fig. 2). As compared with HL-60/neo, HL-60/Bcr-Abl cells expressed considerably higher levels of caspase-9, which showed some processing and decline after treatment with Apo-2L/TRAIL. However, Apo-2L/TRAIL-induced processing of caspase-3 was clearly greater in HL-60/neo *versus* HL-60/Bcr-Abl cells. XIAP has been reported to be processed by the activity of caspase-3 (20), as was evident in HL-60/neo cells

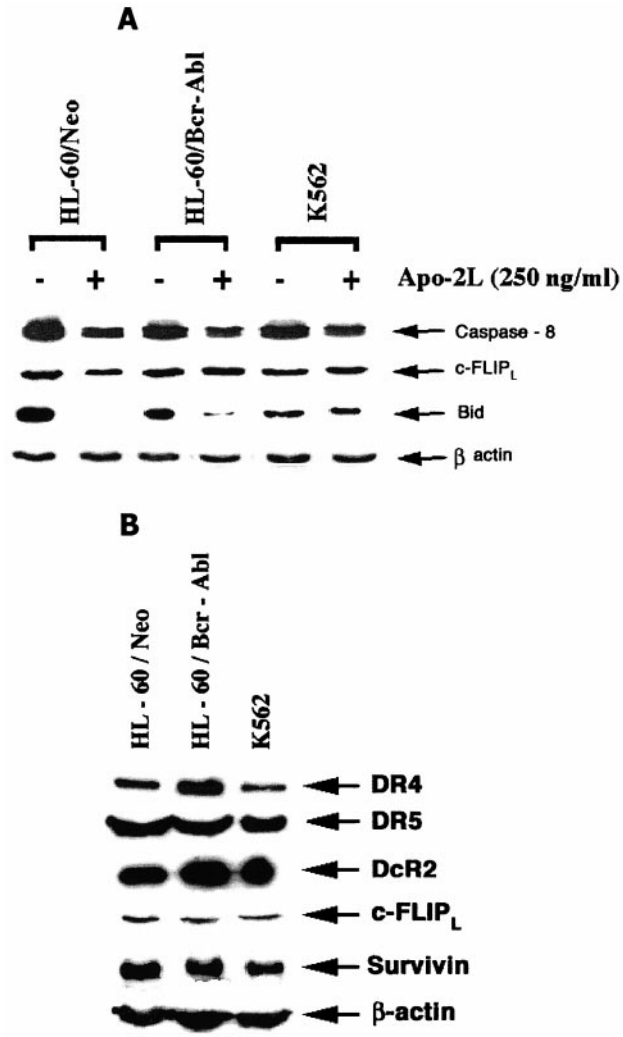

Fig. 3 *A*, Apo-2L/TRAIL-induced processing of caspase-8, c-FLIP$_L$, and Bid is inhibited in HL-60/Bcr-Abl and K562 cells. After exposure of cells to 250 ng/ml Apo-2L/TRAIL for 24 h, cell-lysates were obtained and Western analyses of the indicated proteins were performed using specific antibodies (see "Materials and Methods"). *B*, comparative expression by Western analyses of DR4, DR5, DcR2, c-FLIP$_L$, and survivin in HL-60/neo, HL-60/Bcr-Abl, and K562 cells. β-actin expression was used to control for protein loading.

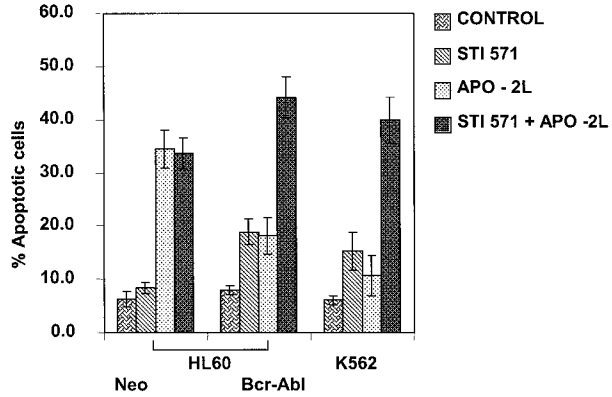

Fig. 4 Cotreatment with STI-571 enhances Apo-2L/TRAIL-induced apoptosis in HL-60/Bcr-Abl and K562 but not in HL-60/neo cells. Cells were exposed to 0.25 μM STI-571 for 48 h, or to 100 ng/ml Apo-2L/TRAIL for 24 h, or to cotreatment with Apo-2L/TRAIL (for the first 24 h) during the 48-h exposure to STI-571. After these treatments, the percentage of apoptotic cells was determined by Annexin-V staining followed by flow cytometry (see "Materials and Methods").

after treatment with Apo-2L/TRAIL. Again, this was clearly inhibited in HL-60/Bcr-Abl and K562 cells (Fig. 2). Lower concentrations of Apo-2L/TRAIL (100 ng/ml) induced less, whereas higher levels of Apo-2L/TRAIL (1000 ng/ml) induced more processing of caspase-9 and -3 and XIAP (data not shown). Together with the data in Fig. 1, which shows inhibition of Apo-2L/TRAIL-induced apoptosis (as represented by lowering of caspase-3-mediated Annexin-V expression), these findings demonstrate that Apo-2L/TRAIL-induced molecular cascade leading to caspase-3 activity is also inhibited in HL-60/Bcr-Abl and K562 cells. Fig. 3*A* shows that exposure to 250 ng/ml of Apo-2L/TRAIL for 24 h caused less down-regulation of Bid and c-FLIP$_L$ in HL-60/Bcr-Abl and K562, as compared with HL-60/neo cells. The difference in Apo-2L/TRAIL-induced processing of caspase-8 was only slight. Because Bcr-Abl expression in K562 cells has been shown not to affect Apo-2L/TRAIL-induced DISC activity (5), the findings in Fig. 3*A* may be attributable to variable feedback processing of Bid by lower activity of caspase-3 generated in K562 and HL-60/Bcr-Abl *versus* HL-60/neo cells (16, 41, 42). Fig. 3*B* demonstrates that, in the three cell-types, there were only modest differences in the protein expressions of DR4, DR5, DcR2, c-FLIP$_L$, and survivin. However, these modest differences could not explain the reduced Apo-2L/TRAIL-induced cytosolic accumulation of cyt-c, or apoptosis of HL-60/Bcr-Abl and K562 *versus* HL-60/neo cells.

Cotreatment with STI-571 Enhances APO-2L/TRAIL-induced Apoptosis of HL-60/Bcr-Abl and K562 Cells. On the basis of our recent findings that cotreatment with STI-571 sensitizes Bcr-Abl-positive leukemic blasts to Ara-C and etoposide-induced apoptosis (35), we determined the effect of STI-571 on the resistance to Apo-2L/TRAIL-induced apoptosis of HL-60/Bcr-Abl and K562 cells. For this, cells were exposed to clinically achievable and effective levels of STI-571 for 48 h, to maximize its inhibitory effect on Bcr-Abl TK and the associated antiapoptotic mechanisms (35, 43), and cotreated with Apo-2L/TRAIL for 24 or 48 h. Fig. 4 demonstrates that although it had no effect in HL-60/neo cells, cotreatment with 0.25 μM STI-571 significantly increased Apo-2L/TRAIL-induced (100 ng/ml) apoptosis of HL-60/Bcr-Abl and K562 cells ($P < 0.01$) to levels equivalent to those observed in HL-60/neo cells. This potentiating effect was also seen with more prolonged exposure to Apo-2L/TRAIL, *e.g.*, 48 h. However, this effect was reduced when the exposure to Apo-2L/TRAIL followed the treatment with STI-571 or when the dose of STI-571 was decreased to 0.1 μM for 48 h (data not shown). Concurrently with this sensitizing effect of STI-571 cotreatment on Apo-2L/TRAIL-induced apoptosis, we also observed greater Apo-2L/TRAIL-induced cytosolic accumulation of cyt c. In conjunction with this, whereas caspase-8 processing was enhanced in both HL-60/Bcr-Abl and K562 cells, the processing of caspase-9 was increased

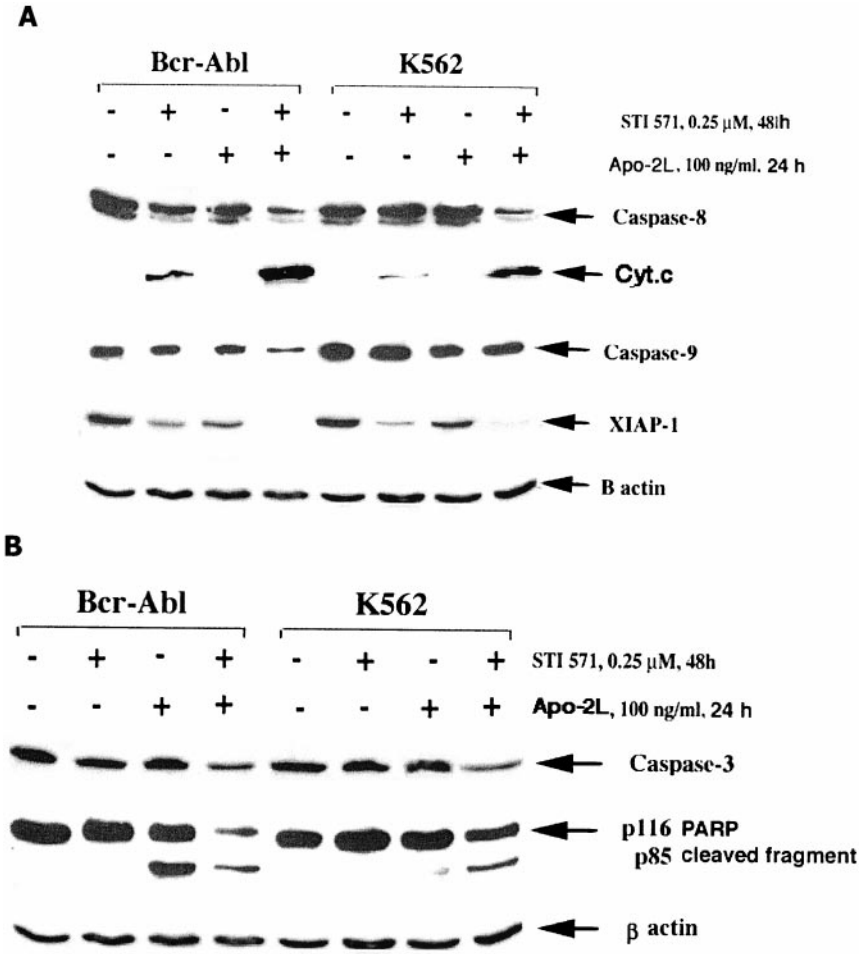

Fig. 5 Cotreatment with STI-571 enhances the cytosolic accumulation of cyt c and processing of caspase-8, -9, and XIAP in HL-60/Bcr-Abl and K562 cells. *A*, after treatment with the indicated dose of STI-571 (48 h), or Apo-2L/TRAIL for 24 h, or cotreatment with Apo-2L/TRAIL for the first 24 h during the exposure to STI-571, cell lysates were obtained. After this, Western analyses of the indicated proteins were performed using the specific antibodies (see text). *B*, cotreatment with STI-571 enhances the processing and activity of caspase-3. After treatment with the indicated dose of STI-571 (48 h) or Apo-2L/TRAIL for 24 h, or cotreatment with Apo-2L/TRAIL for the first 24 h during the exposure to STI-571, cell lysates were obtained. After this, Western analyses of the indicated proteins were performed using the specific antibodies (see "Materials and Methods").

by cotreatment with Apo-2L/TRAIL only in HL-60/Bcr-Abl cells (Fig. 5*A*). In addition, cotreatment with STI-571 increased Apo-2L/TRAIL-induced decline in XIAP levels. Increased processing and PARP cleavage activity of caspase-3 was also observed in HL-60/Bcr-Abl and K562 cells (Fig. 5*B*). For these studies, we used 0.25 μM of STI-571, which has been shown to significantly inhibit Bcr-Abl TK (16). It should be noted that, in these experiments, lower concentration of Apo-2L/TRAIL (100 ng/ml) was less effective than 250 ng/ml in triggering the molecular cascade of apoptosis in Bcr-Abl-positive leukemic cells (Fig. 5 *versus* Fig. 2 and 3*A*). Cotreatment with STI-571 did not affect Apo-2L/TRAIL-induced molecular cascade of apoptosis in HL-60/neo cells (data not shown). We also determined whether STI-571 potentiated the apoptotic effect of Apo-2L/TRAIL by modulating the levels of DR4, DR5, FADD, DcRs, or c-FLIP$_L$. STI-571 did not increase DR4, DR5, or FADD levels, nor did it decrease the expression of DcR2 or c-FLIP$_L$ in any of the cell types (data not shown).

DISCUSSION

Previous reports had documented the relative resistance of Bcr-Abl-positive human leukemic blasts to apoptosis induced by antileukemic drugs and Fas L (25–28). In the present studies, we demonstrate that, although Apo-2L/TRAIL has its unique set of signaling DRs and triggers both the extrinsic and intrinsic pathway of apoptosis (1–5, 16), Apo-2L/TRAIL-induced apoptosis is inhibited in HL-60/Bcr-Abl and K562 cells. Bcr-Abl-positive cells have constituitively increased activities of Akt and NFκB and overexpression of Bcl-x$_L$ (Refs. 20, 28–32; Fig. 6). These antiapoptotic mechanisms would operate to inhibit both the mitochondrial (through Bcl-x$_L$) and the common (through up-regulation of IAPs) pathways of apoptosis (Ref. 28; Fig. 6). Consistent with this, our findings also demonstrate that Apo-2L/TRAIL induces more cytosolic accumulation of cyt c in HL-60/neo than in HL-60/Bcr-Abl and K562 cells. The marked reduction in Apo-2L/TRAIL-induced mitochondrial release of cyt c in HL-60/Bcr-Abl cells seems to be out of proportion to the protection against apoptosis. This may be attributable to a threshold effect for the cytosolic cyt c for mediating caspase-9 and -3 activation, despite the antiapoptotic effects of Bcr-Abl in these cell-types. Consequently, the activity of the "apoptosome" that results in the processing and activity of caspase-3 is also inhibited in the latter cell type. Bcr-Abl expression in K562 is not known to affect Apo-2L/TRAIL-induced DISC (5). However, we observed decreased Apo-2L/TRAIL-induced processing of c-FLIP$_L$ and Bid and not as much processing of

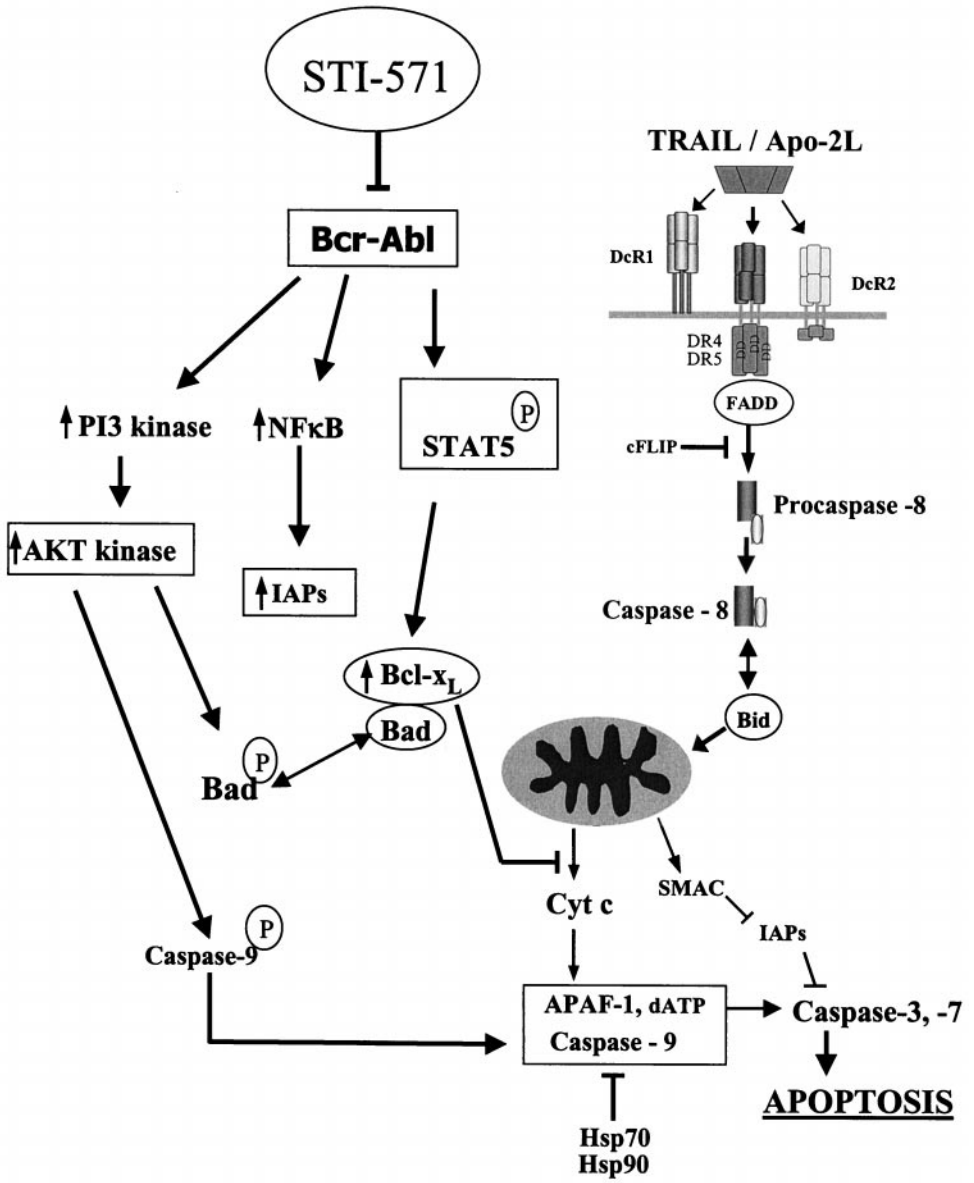

Fig. 6 Molecular mechanisms underlying STI-571-mediated sensitization of Bcr-Abl-positive leukemic cells to Apo-2L/TRAIL-induced apoptosis. By inhibiting Bcr-Abl TK activity, STI-571 inhibits constitutively active PI3 kinase STAT5 and NFκB in Bcr-Abl-positive leukemic blasts. This results in the lowering of Bcl-x_L levels and its antiapoptotic activity. It also has the potential to lower the levels of IAP and improve caspase-9 activity (see text). These effects of STI-571 would promote Apo-2L-TRAIL-induced cyt c and SMAC/DIABLO release from mitochondria and the subsequent activation of the effector caspases-3 and -7, resulting in apoptosis (see "Results").

caspase-8, in HL-60/Bcr-Abl and K562 cells. Previous studies have suggested a feedback processing of caspase-8 and Bid by the activities of the downstream effector caspases (15, 16, 41, 42). Therefore, it is possible that reduced generation of the activities of the caspase-3 and other effector caspases in HL-60/Bcr-Abl and K562 cells may also dampen Apo-2L/TRAIL-induced processing of Bid. It is noteworthy that, in the three cell-types, although there were modest differences in the expression of DR4, DR5, DcR2, c-FLIP$_L$, caspase-8, and Bid, this could not explain the significantly reduced Apo-2L/TRAIL-induced apoptosis of HL-60/Bcr-Abl or K562 cells as compared with HL-60/neo cells. Lack of such a connection has also been reported for other cell types (44, 45). In HL-60/Bcr-Abl and K562 cells, reduced generation of caspase-3 activity attributable to Apo-2L/TRAIL may also be responsible for the reduced processing of XIAP, because XIAP has been shown to be a substrate for caspase-3 (20).

Recent studies have suggested that cotreatment with STI-571 sensitizes HL-60/Bcr-Abl and K562 cells to Ara-C-, etoposide-, and doxorubicin-induced apoptosis (35). This sensitizing effect could be correlated with STI-571-mediated down-regulation of the activities of Bcr-Abl TK and Akt kinases as well as the lowering of Bcl-x_L and XIAP levels, mechanisms that inhibit the intrinsic and common effector pathway of apoptosis (35). Because these antileukemic drugs mostly trigger apoptosis by activating the mitochondrial pathway, which is also engaged by Apo-2L/TRAIL (16, 46), it is not surprising that STI-571 also sensitizes HL-60/Bcr-Abl and K562 cells to Apo-2L/TRAIL-induced apoptosis. However, it should be noted that, although cotreatment with STI-571 clearly increased Apo-2L/

TRAIL-induced apoptosis in both HL-60/Bcr-Abl and K562 cells, caspase-9 processing was increased only in HL-60/Bcr-Abl cells (Fig. 5*A*). This discrepancy may be attributable to different levels of expression and/or activities of heat shock proteins hsp70 and hsp90, and/or SMAC/DIABLO, which regulate caspase-9 processing and the effects of IAPs on caspase-3, respectively (47–49; Fig. 6). Treatment with STI-571 had no affect on the expression of DR4, DR5, caspase-8, or c-FLIP$_L$ (data not shown), which suggests that these molecular determinants did not play a role in the sensitizing effect of STI-571 and Apo-2L/TRAIL. STI-571 has been shown to inhibit the growth and induce differentiation of Bcr-Abl-positive leukemic cells (35, 43). Although this did not abrogate, it may have caused the dampening of the sensitizing effect of STI-571 on Apo-2L/TRAIL-induced apoptosis when Apo-2L/TRAIL was administered after the exposure to STI-571.

STI-571 has been demonstrated to produce a high rate of hematological remissions in CML, but the remissions induced in patients with blast crisis of CML or Bcr-Abl-positive ALL are not durable (50, 51). *In vitro* studies have also demonstrated that resistance to STI-571 is associated with gene amplification and increased expression of Bcr-Abl (52–54). Recent strategies have focused on inhibiting Bcr-Abl expression in addition to lowering its TK activity (55–59). However, novel therapeutic regimens that would test anti-Bcr-Abl strategies in combination with other antileukemic agents, such as Apo-2L/TRAIL may have to be tested and may become a part of the therapeutic armamentarium against Bcr-Abl-positive acute leukemias. The data presented here creates a strong rationale to further investigate STI-571 and Apo-2L/TRAIL as a potential therapeutic strategy in Bcr-Abl-positive human leukemias.

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Editor's Note: Cotreatment with STI-571 Enhances Tumor Necrosis Factor α-related Apoptosis-inducing Ligand (TRAIL or Apo-2L)–induced Apoptosis of Bcr-Abl–positive Human Acute Leukemia Cells

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The editors are publishing this note to alert readers to concerns about this article (1). Figure 5A and B use the same β-actin control panel. The β-actin control panel in Fig. 3A is similar to lanes 2 through 6 in the β-actin panels in Fig. 5A and B. The original data are no longer available for review, and the authors stand by the conclusions of the article.

Reference

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