Histone Deacetylase Inhibitors Promote STI571-mediated Apoptosis in STI571-sensitive and -resistant Bcr/Abl+ Human Myeloid Leukemia Cells

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ABSTRACT

Interactions between the Bcr/Abl kinase inhibitor STI571 (Gleevec, imatinib mesylate) and histone deacetylase inhibitors (HDIs) have been examined in STI571-sensitive and -resistant Bcr/Abl+ human leukemia cells (K562 and LAMA 84). Cotreatment of K562 cells with 250 nm imatinib mesylate and 2 μM suberoylalanilide hydroxamic acid (SAHA) for 24 h, exposures that were minimally toxic alone, resulted in a marked increase in mitochondrial damage (e.g., cytochrome c, Smac/DIABLO, and apoptosis-inducing factor release), caspase activation, and apoptosis. Similar events were observed in other Bcr/Abl+ cells (i.e., LAMA 84), and in cells exposed to STI571 in combination with the HDI sodium butyrate. Coexposure of cells to HDIs in conjunction with STI571 resulted in multiple perturbations in signaling and cell cycle-regulatory proteins, including down-regulation of Raf, phospho-mitogen-activated protein kinase (MEK), phospho-extracellular signal-regulated kinase (ERK), phospho-Akt, phospho-signal transducers and activators of transcription 5, cyclin D1, and McI, accompanied by dephosphorylation and cleavage of retinoblastoma protein and a striking increase in phosphorylation of c-Jun NH2-terminal kinase. Coexposure of Bcr/Abl+ cells to STI571 also blocked SAHA-mediated induction of p21CIP1 and resulted in down-regulation of Bcr/Abl protein expression. STI571 and SAHA also interacted synergistically to induce apoptosis in STI571-resistant K562 and LAMA 84 cells that display increased Bcr/Abl protein expression. Lastly, inductive expression of a constitutively active MEK1/2 construct significantly attenuated SAHA/STI571-mediated apoptosis in K562 cells, implicating disruption of the Raf/MEK/ERK axis in synergistic antileukemic effects of this drug combination. Together, these findings indicate that combined exposure of Bcr/Abl+ cells to the kinase inhibitor STI571 and HDIs leads to diverse perturbations in signaling and cell cycle-regulatory proteins associated with a marked increase in mitochondrial damage and cell death. They also raise the possibility that this strategy may be effective in some Bcr/Abl+ cells that are resistant to STI571 through increased Bcr/Abl expression.

INTRODUCTION

CML represents a clonal disorder of a primitive hematopoietic stem cell that results in the progressive accumulation of progenitor cells that are impaired in their capacity to undergo maturation (1). From a pathophysiological standpoint, the development of CML represents a consequence of expression of the Bcr/Abl oncogene, which encodes a fusion protein that is found in the cells of 95% of patients with the disease (2). Constitutive activation of the Bcr/Abl tyrosine kinase confers hematopoietic cells with a survival advantage, contributing to leukemic transformation (3). In addition to protecting hematopoietic cells from certain noxious environmental stimuli (e.g., growth factor deprivation), expression of the Bcr/Abl kinase renders cells relatively insensitive to apoptosis induced by cytotoxic drugs (4, 5). Currently, the pathways downstream of Bcl/Abi responsible for apoptosis resistance in CML cells are not known with certainty. However, multiple signaling/survival pathways have been implicated in this phenomenon, including dysregulation of nuclear factor κB, STAT5, MEK/MAPK, Bc1-XL, and Akt, among others (6–10).

Recently, the treatment of CML has been revolutionized by the introduction of STI571 (Gleevec, imatinib mesylate), a p.o. active tyrosine kinase inhibitor that inhibits Bcr/Abl, c-Kit, platelet-derived growth factor, and other kinases (11). STI571 interferes with the growth of Bcr/Abl+ leukemia cells and induces apoptosis in Bcr/Abl+ leukemia cells in vitro (11, 12). Significantly, oral administration of STI571 to CML patients results in clinical responses in >90% patients (13). However, the emergence of STI571 resistance in CML patients initially responsive to this agent (14) and the observation that patients in accelerated phase CML or blast crisis are less likely to respond to imatinib mesylate (15) have prompted the search for additional approaches to the treatment of this disease. Mechanisms of resistance to STI571 include diminished drug uptake, Bcr/Abl amplification, and mutations in the Bcr/Abl kinase domain, among others (16–18). One possible approach to this problem involves the combination of STI571 with other agents that exhibit antileukemic activity. In this regard, increased activity against Bcr/Abl+ leukemic cells has been described when STI571 was combined with conventional cytotoxic drugs (19–21), arsenic trioxide (22), geldanamycin (23), or tumor necrosis factor apoptosis-inducing ligand (24). Most recently, our group has described synergistic interactions between STI571 and pharmacological MEKI1/2 inhibitors (e.g., PD184351 and U0126) or the CDKI flavopiridol in Bcr/Abl+ cells, including those resistant to STI571 due to increased Bcr/Abl protein expression (25, 26).

HDIs, including trichostatin A, short chain fatty acids such as sodium and phenylbutyrate, SAHA, depsipeptide, MS-275, and aphidicidin, among others, represent a novel class of agents that act by promoting histone acetylation, resulting in relaxation of the chromatin structure (27). Chromatin relaxation and uncoupling permit the expression of diverse genes, including those involved in the differentiation process [e.g., p21CIP1 (28)]. In fact, HDIs (e.g., SAHA and SB) have been shown to induce maturation in various human leukemia cell lines (29, 30). Under some circumstances, HDIs induce apoptosis rather than maturation in human leukemia cells (29, 31, 32), although the factors that determine which response predominates remain obscure. HDIs also induce maturation in certain Bcr/Abl+ leukemia cells (e.g., K562), a phenomenon associated with diminished activation of the MAPK pathway (33). In view of evidence that STI571 may modify...
the differentiation response of Bcr/Abl+ cells (34), the possibility arose that combining STI571 with HDIs might lead to enhanced antileukemic activity. To address this issue, we have examined interactions between STI571 and clinically relevant HDIs, i.e., SB and SAHA. Here we report that coadministration of HDIs with STI571 in several CML cell lines (e.g., K562 and LAMA 84) results in disruption of multiple signaling pathways, induction of mitochondrial injury, and a dramatic potentiation of apoptosis. Moreover, this drug combination potently induces cell death in STI571-resistant Bcr/Abl+ cells displaying increased Bcr/Abl expression. Together, these findings suggest that the strategy of combining STI571 with clinically relevant HDIs warrants consideration in CML and related hematological malignancies.

MATERIALS AND METHODS

Cells. K562, HL60, Jurkat, and U937 human leukemia cells were purchased from American Type Culture Collection (Manassas, VA). LAMA 84 cells were purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). All were cultured in RPMI 1640 supplemented with sodium pyruvate, MEM, essential vitamins, t-glutamate, penicillin, streptomycin, and 10% heat-inactivated FCS (Hyclone, Logan, UT). They were maintained in a 37°C, 5% CO2, fully humidified incubator, passed twice weekly, and prepared for experimental procedures when in log-phase growth (cell density ≤ 4 × 10^6 cells/ml).

Multidrug-resistant K562R cells were derived from the parental line by subculturing in progressively higher concentrations of doxorubicin as described previously (35). They were cultured in the absence of doxorubicin before all of the experimental procedures. In addition, STI571-resistant LAMA 84 cells, designated LAMA 84-R-STI, were generated by reseeding LAMA 84 cells in progressively higher concentrations of STI571. These cells are maintained under selection pressure in medium containing 0.5 μM STI571. IC_{50} STI571 values for LAMA 84S and LAMA 84-R-STI are 0.3 and 0.5 μM, respectively. For studies involving the LAMA 84R line, cells were washed free of drug and resuspended in drug-free medium 48 h before experimentation.

Reagents. STI571 was kindly provided by Dr. Elizabeth Buchdunger (Novartis Pharmaceuticals, Basel, Switzerland) and prepared as a 10 mM stock solution in sterile DMSO (Sigma, St. Louis, MO). SB and SAHA were obtained from Calbiochem (San Diego, CA); BOC-fmk and IETD-fmk were purchased from Enzyme Products, Ltd. (Livermore, CA) and formulated in sterile DMSO before use.

Experimental Format. Logarithmically growing cells were placed in sterile plastic T-flasks (Corning, Corning, NY), to which were added the designated drugs, and the flasks were replaced in the incubator for intervals. At the end of the incubation period, cells were transferred to sterile centrifuge tubes, pelleted by centrifugation at 400 × g for 10 min at room temperature, and prepared for analysis as described below.

Assessment of Apoptosis. After drug exposures, cytocentrifuge preparations were stained with Wright-Giemsa and viewed by light microscopy to evaluate the extent of apoptosis (i.e., cell shrinkage, nuclear condensation, formation of apoptotic bodies, and so forth) as described previously (25). For these studies, the percentage of apoptotic cells was determined by evaluating ≥500 cells/condition in triplicate. To confirm the results of morphological analysis, annexin V/PI staining was used. Annexin V/PI (BD PharMingen, San Diego, CA) analysis of cell death was carried out as per the manufacturer’s instructions. Analysis was carried out using a Becton Dickinson FACSScan cytofluorometer (Mansfield, MA). To further confirm the morphology results, TUNEL staining was used. For TUNEL staining, cytocentrifuge preparations were obtained and fixed with 4% formaldehyde. The slides were treated with acetic acid:ethanol (1:2), stained with terminal deoxynucleotidyl transferase reaction mixture containing 1× terminal deoxynucleotidyl transferase reaction buffer (0.25 units/μl terminal deoxynucleotidyl transferase, 2.5 mM CoCl2, and 2 pmol of fluorescein-12-dUTP; Boehringer Mannheim, Indianapolis, IN), and visualized using fluorescence microscopy.

Determination of MMP (ΔΨ_m). MMP was monitored using DiOC6 (36). For each condition, 4 × 10^5 cells were incubated for 15 min at 37°C in 1 ml of 40 mM DiOC6 (Calbiochem) and subsequently analyzed using a Becton Dickinson FACScan cytofluorometer with excitation and emission settings of 488 and 525 nm, respectively. Control experiments documenting the loss of ΔΨ_m were performed by exposing cells to 5 μM carbamoylcyanine m-chlorophenylhydrazone (Sigma; 15 min, 37°C), an uncoupling agent that abolishes the MMP.

Preparation of S-100 Fractions and Assessment of Cytochrome c Release. U937 cells were harvested after drug treatment as described previously (37) by centrifugation at 600 × g for 10 min at 4°C and washed in PBS. Cells (4 × 10^6) were lysed by incubation for 3 min in 100 μl of lysis buffer containing 75 mM NaCl, 8 mM Na2HPO4, 1 mM NaH2PO4, 1 mM EDTA, and 350 μg/ml digitonin. The lysates were centrifuged at 12,000 × g for 5 min, and the supernatant was collected and added to an equal volume of 2× Laemmli buffer. The protein samples were quantified and separated by 15% SDS-PAGE.

Immunoblot Analysis. Immunoblotting was performed as described previously (25). Briefly, after drug treatments, cells were pelleted by centrifugation, lysed immediately in Laemmli buffer [1× Laemmli buffer = 50 mM Tris base (pH 6.8), 2% SDS, 2.88 mM β-mercaptoethanol, and 10% glycerol], and sonicated briefly. Homogenates were quantified using Coomassie Blue protein assay reagent (Pierce, Rockford, IL). Equal amounts of protein (20 μg) were boiled for 10 min, separated by SDS-PAGE (5% stuffer and 10% resolving), and electrophoblotted to nitrocellulose membrane. After blocking in TBS-T (0.05%) and 5% milk for 1 h at 22°C, the blots were incubated in fresh blocking solution with an appropriate dilution of primary antibody for 4 h at 22°C. The sources of antibodies were as follows: Bcl-xL (rabbit polyclonal antibody), Santa Cruz Biotechnology; XIAP (rabbit polyclonal antibody), R&D Systems (Minneapolis, MN); Mc-l (mouse monoclonal antibody), PharMingen (San Diego, CA); cyclin D1 (mouse monoclonal antibody) and p21 (mouse monoclonal antibody), Pharmingen; ERK1/2 (rabbit polyclonal antibody), Cell Signaling Technology (Beverly, MA); phospho-ERK1/2 (Thr21/240), Phospho-JNK (rabbit polyclonal antibody), Cell Signaling Technology; JNK (rabbit polyclonal antibody), Santa Cruz Biotechnology; phospho-JNK (mouse monoclonal antibody), Santa Cruz Biotechnology; phospho-p38 MAPK (rabbit polyclonal antibody), Cell Signaling Technology; phospho-p70S6K (421/424) (rabbit polyclonal antibody), Cell Signaling Technology; phospho-STAT5, Cell Signaling Technology; pRB (mouse monoclonal antibody), PharMingen; underphosphorylated pRB (mouse monoclonal antibody), PharMingen; caspase 3 (mouse monoclonal antibody), Transduction Laboratories (Lexington, KY); PARP (C–2; 10; mouse monoclonal antibody), BioMol Research Laboratories (Plymouth, MA); cytochrome c (mouse monoclonal antibody), Santa Cruz Biotechnology; AIF (mouse monoclonal antibody), Santa Cruz Biotechnology; Smac (rabbit polyclonal antibody), Upstate Biotechnology (Lake Placid, NY); caspase 8 (rabbit polyclonal antibody), Pharmingen; and caspase 9 (rabbit polyclonal antibody), Cell Signaling Technology. Detection was performed using a chemiluminescence detection kit (Pierce, Rockford, IL). Cells were visualized with a MoFLO (DakoCytomation, Fort Collins, CO) cytometer. Cells were gated using forward and side scatter for viable cells and pulse width to remove doublets. Final purity was 95% or greater with respect to GFP.
expression. Viability of this population was also consistently >95%. The cells were then exposed to drugs as indicated and examined for morphological evidence of apoptosis as described above.

Statistical Analysis. The significance of differences between experimental conditions was determined using the two-tailed Student t test. Analysis of synergism and antagonism was performed using median dose effect analysis (38) in conjunction with a commercially available software program (Calcysyn; Biosoft, Ferguson, MO), as we have described previously (25).

RESULTS

To characterize interactions between STI571 and SAHA in K562 cells, dose-response studies were performed (Fig. 1). Exposure of cells for 24 h to STI571 concentrations as high as 300 nM negligibly induced apoptosis, whereas 2.0 μM SAHA administered alone was also minimally toxic (Fig. 1A). However, when cells were exposed to SAHA in combination with 100 nM STI571, a clear increase in apoptosis was observed (i.e., ~20%), and for STI571 concentrations ≥ 250 nM, the majority of cells (i.e., ~75%) were apoptotic. Similarly, when cells were exposed for 24 h to 250 nM STI571 in combination with increasing concentrations of SAHA, a sharp increase in apoptosis was noted at 1.0 μM SAHA, and at SAHA concentrations ≥ 1.5 μM, the majority of cells were apoptotic (Fig. 1B). Median dose effect analysis of apoptosis induction over a range of STI571 and SAHA concentrations yielded CI values > 1.0, corresponding to a synergistic interaction (Fig. 1C). Parallel results were obtained when DNA strand breaks were assayed by TUNEL analysis (data not shown).

Time course studies of K562 cells exposed to 250 nM STI571 ± 2.0 μM SAHA are shown in Fig. 2. Whereas each of these agents administered individually over 48 h minimally induced apoptosis, combined treatment resulted in an increase in apoptosis that was first observed at 12 h and that reached near-maximal levels by 24 h (Fig. 2A). After 48 h of combined treatment, >90% of cells were apoptotic. Similar results were observed when loss of MMP (ΔΨm) was monitored, although STI571 by itself was somewhat more toxic in this regard after 48 h of exposure (Fig. 2B). Together, these findings indicate that combined treatment with STI571 and the HDI SAHA results in early induction of mitochondrial injury and apoptosis in Bcr/Abl+ K562 cells.

To determine whether potentiation of apoptosis in K562 cells treated with STI571 in conjunction with a HDI would be associated with loss of leukemic cell self-renewal capacity, clonogenic assays were performed (Fig. 2C). Whereas a 24-h exposure to 250 nM STI571 or 2.0 μM SAHA alone substantially reduced clonogenicity (i.e., to

Fig. 2. K562 cells were exposed to 2.0 μM SAHA ± 250 nM STI571 for the indicated intervals, after which the percentage of apoptotic cells (A) or the percentage of cells displaying loss of MMP (ΔΨm; B) was determined as described in “Materials and Methods.” C, cells were treated with 2.0 μM SAHA ± 250 nM STI571 for 24 h, washed free of drugs, and plated in soft agar as described in “Materials and Methods.” At the end of a 12-day incubation period, colonies were scored, and survival was expressed as a percentage relative to untreated controls.
25% of control values), combined treatment resulted in a >2-log reduction in colony formation.

The effects of combined exposure of K562 cells to SAHA and STI571 for 24 h were then examined in relation to mitochondrial injury, caspase activation, and expression of apoptosis-regulatory proteins (Fig. 3). Whereas the individual effects of STI571 (250 nM) or SAHA (2.0 μM) were minimal, exposure of cells to a combination of these agents resulted in a striking increase in release of cytochrome c, AIF, and Smac/DIABLO into the cytosolic, S-100 cell fraction. These events were accompanied by a marked increase in caspase-9 cleavage and degradation of caspase-3, caspase-8, PARP, and Bcr/Abl. Interestingly, whereas individual treatment had little effect, combined exposure to STI571 and SAHA resulted in a marked decline in levels of the Bcr/Abl protein. Thus, treatment of Bcr/Abl+ cells with a subtoxic concentration of STI571 in conjunction with the HDI SAHA resulted in a marked increase in release of proapoptotic mitochondrial proteins, activation of the caspase cascade, and reduced expression of Bcr/Abl.

Interactions between SAHA and STI571 were then examined in relation to effects on various signaling-, cell cycle-, and apoptosis-regulatory proteins in K562 cells (Fig. 4). Interestingly, exposure to SAHA alone (16 h) resulted in a clear reduction in expression of Raf, whereas STI571 had little effect (Fig. 4A). Coadministration of STI571 and SAHA resulted in a further diminution in Raf expression. Roughly parallel changes in levels of phospho-MEK1/2 and phospho-ERK1/2 were observed. Combined treatment with STI571 and SAHA also resulted in a marked decrease in phosphorylation of p70S6K on ERK-associated sites (421/424; Ref. 39), as well as markedly diminished expression of phospho-STAT5, a target of Bcr/Abl (9). No changes in the expression of total Akt were noted, although a modest decline in phosphorylated (activated) Akt was observed in cells exposed to both SAHA and STI571. In addition, whereas STI571 and
SAHA individually failed to modify expression of phospho-JNK, combined treatment resulted in a very dramatic increase in JNK activation. A slight increase in phosphorylation of p38 MAPK was observed in SAHA-treated cells, but this did not change with addition of STI571.

Combined treatment with STI571 and SAHA did not alter expression of the antiapoptotic proteins Bcl-xL or XIAP (Fig. 4B). However, STI571 treatment alone induced a small decrease in expression of the antiapoptotic protein Mcl-1, as we have reported previously (25), whereas addition of SAHA, which by itself exerted minimal effects, resulted in a further diminution in Mcl-1 expression.

Interactions between SAHA and STI571 were then examined in relation to expression of several cell cycle-regulatory proteins (Fig. 4C). Treatment with SAHA resulted in a robust induction of p21CIP1, similar to effects noted in Bcr/Ab1 leukemia cells (30). Unexpectedly, coexposure to STI-571 substantially diminished induction of p21CIP1 by SAHA. Despite this action, combined exposure of cells to SAHA and STI571 resulted in a modest increase in expression of underphosphorylated pRb accompanied by cleavage of both total and underphosphorylated protein. Lastly, K562 cells treated with both STI571 and SAHA displayed a clear reduction in levels of cyclin D1, a phenomenon previously linked to induction of apoptosis (40). Collectively, these findings indicate that coexpression of K562 cells to STI571 and SAHA results in perturbations in the expression of multiple signaling-, cell cycle-, and apoptosis-regulatory proteins, including down-regulation of Raf; diminished activation of MEK1/2, ERK1/2, and p70S6K; a striking activation of JNK; reduced expression of Bcr/Ab1, Mcl-1, p21CIP1, and cyclin D1; and dephosphorylation/cleavage of pRb.

To assess the role of caspases in these events, K562 cells were treated for 20 h with STI571 + SAHA in the presence or absence of the pan-caspase inhibitor BOC-fmk or IETD-fmk (Fig. 5A). BOC-fmk markedly inhibited apoptosis, whereas IETD-fmk was minimally effective, suggesting a relatively minor role for the extrinsic pathway in STI571/SAHA-mediated lethality in these cells. However, whereas BOC-fmk was ineffective in blocking cytochrome c release into the cytosol in cells exposed to STI571 + SAHA, it largely blocked Smac/DIABLO release (Fig. 5B), indicating that the latter represents a secondary, caspase-dependent event. As anticipated, BOC-fmk attenuated cleavage of procaspase-3, Bcr/Ab1, pRb, underphosphorylated pRb, Raf-1, Mcl-1, p21CIP1, and cyclin D1. Each lane contained 25 μg of protein; blots were stripped and reprobed for tubulin to ensure equivalent loading and transfer. Two additional studies yielded equivalent results.

Fig. 5. A, K562 cells were exposed to 2.0 μM SAHA + 250 nM STI571 for 24 h in the presence or absence of 25 μM BOC-fmk or IETD-fmk, after which apoptosis was monitored as described above. Values represent the means ± SD for three separate experiments. B, cells were treated with SAHA + STI571 ± BOC-fmk (25 μM), after which release of cytochrome c or Smac/DIABLO into the S-100 cytosolic fraction was assessed as described above. C, cells were treated with SAHA + STI571 ± BOC-fmk, after which Western analysis was used to assess expression of procaspase-3, Bcr/Ab1, pRb, underphosphorylated pRb, Raf-1, Mcl-1, p21CIP1, and cyclin D1. Each lane contained 25 μg of protein; blots were stripped and reprobed for tubulin to ensure equivalent loading and transfer. Two additional studies yielded equivalent results.

Fig. 6. LAMA 84 cells were exposed to 200 nM STI571 ± 1.0 μM SAHA for 24 h, after which the percentage of annexin V/PI+ cells (shown as gated figures) was determined by flow cytometry as described in “Materials and Methods.” Two additional studies yielded equivalent results.
large majority of cells (i.e., 70%) became apoptotic, as reflected by annexin positivity. In contrast, no evidence of synergism was observed when SAHA was combined with several Bcr/Abl-leukemic cell lines, including U937, HL60, NB4, and Jurkat (Table 1). These findings indicate that synergistic interactions between SAHA and STI571 are restricted to human leukemic cells expressing the Bcr/Abl protein.

Concordantly with the results of the preceding studies, Western analysis revealed that combined exposure of LAMA 84 cells to STI571 + SAHA resulted in a marked increase in cytosolic release of cytochrome c, and a corresponding activation of caspases-9, -3, and -8 (data not shown). Furthermore, treatment of LAMA 84 cells with the combination of STI571 and SAHA resulted in down-regulation of Raf, p21^CIP1, cyclin D1, Mcl-1, and phospho-STAT5; enhanced underphosphorylation and cleavage of pRb; and a dramatic increase in JNK phosphorylation, similar to results obtained in K562 cells (data not shown).

Attempts were then made to establish whether such interactions could be extended to include HDIs other than SAHA. To this end, K562 and LAMA 84 cells were exposed to the indicated concentrations of STI571 in the presence or absence of SB (1 or 2 mM), after which the extent of apoptosis was assessed (Table 2A). As shown, coadministration of STI571 with SB resulted in a marked increase in apoptosis in both cell lines. Analogous to results obtained with SAHA, enhanced lethality was associated with increased release of cytochrome c into the cytosol; activation of procaspases-3 and -9; down-regulation of Raf, p21^CIP1, Mcl-1, and cyclin D1; and a marked activation of JNK (data not shown).

In previous studies, we have demonstrated that coadministration of STI571 with either MEK1/2 inhibitors or the CDKI flavopiridol results in enhanced lethality in STI571-resistant K562 cells exhibiting increased expression of Bcr/Abl (25, 26). Parallel studies involving the STI571/HDI regimen were therefore carried out in K562R cells, which were derived from a multidrug-resistant cell line (35), and in STI571-resistant LAMA 84 cells (LAMA 84-R-STI), which were generated by culturing cells in progressively higher concentrations of STI571 (Table 2B). It can be seen that coadministration of STI571 (1.0 or 1.25 M) for 48 h, which resulted in only modest lethality in either cell line, with a minimally toxic concentration of SAHA (i.e., 1.0 or 2.0 M) induced cell death in the majority (e.g., ~65%) of K562R and LAMA 84-R-STI571 cells. Exposure of STI571-sensitive K562 and LAMA 84 cells to these STI571 concentrations for 48 h induced cell death in virtually 100% of cells (data not shown). Essentially identical results were obtained when STI571-resistant cells were exposed to STI571 in combination with SB (data not shown). Such results indicate that coadministration of STI571 with HDIs effectively increases cell death in STI571-resistant Bcr/Abl^+ cells, at least in those displaying increased expression of the Bcr/Abl protein.

Finally, to assess the functional contribution of dysregulation of the Raf/MEK/MAPK axis to synergistic interactions between STI571 and HDIs in Bcr/Abl^+ cells, K562 cells were transiently transfected with a vector expressing either GFP alone or a constitutively active MEK1/2/GFP fusion protein (Fig. 7). Purified cell populations (e.g., >95%) expressing GFP and exhibiting 96% viability were isolated using a Cytomation MoFLO cell sorter and exposed for 24 h to 250 nM STI571 ~2.0 M SAHA. At the end of this period, the extent of apoptosis was monitored as described above. Cells transfected with the constitutively active MEK1/2 were modestly but significantly more resistant to STI571-mediated lethality than controls transfected with GFP alone (P < 0.05), consistent with earlier reports demonstrating potentiation of STI571-induced apoptosis by pharmacological MEK1/2 inhibitors (25). Moreover, transient transfection of cells with mutant MEK1/2 very significantly, albeit partially, protected cells from the lethality of the SAHA/STI571 regimen (P < 0.01). These findings suggest that dysregulation of the Raf/MEK/MAPK cascade in K562 cells exposed to STI571 in conjunction with HDIs plays a significant functional role in the enhanced lethality of this drug combination, although it is likely that other factors contribute to this phenomenon.

**DISCUSSION**

The results of the present study indicate that coadministration of the Bcr/Abl kinase inhibitor STI571 with clinically relevant HDIs results in a dramatic increase in mitochondrial damage and apoptosis in Bcr/Abl^+ human leukemia cells, accompanied by down-regulation of the Bcr/Abl protein. It has long been known that in human leukemia cells, HDIs, presumably by promoting chromatin relaxation, permit the transcriptional activation of genes involved in differentiation and other events (27, 28). In this regard, whereas HDIs such as SB have been shown to induce maturation in leukemic cells, including K562 (41), they may also trigger apoptosis (30), possibly through generation of reactive oxygen species (42). In any event, K562 cells, perhaps due to a generic resistance to apoptosis conferred by constitutive activation of the Bcr/Abl kinase and its downstream cytoprotective targets (3–5), are relatively insensitive to HDI-mediated cell death (43). The enhanced ability of the STI571/HDI regimen to trigger apoptosis in these cells may stem from interference with the actions of one or more

<table>
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<tr>
<th>Cell line</th>
<th>Control</th>
<th>SAHA</th>
<th>STI571</th>
<th>STI + SAHA</th>
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<td>K562</td>
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<td>8.3 ± 3.2</td>
<td>74.3 ± 5.3</td>
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<td>U937</td>
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<td>5.4 ± 1.9</td>
<td>0.9 ± 0.8</td>
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<tr>
<td>Jurkat</td>
<td>0.8 ± 0.4</td>
<td>6.3 ± 3.2</td>
<td>1.1 ± 0.6</td>
<td>7.3 ± 2.0</td>
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<tr>
<td>NB4</td>
<td>1.2 ± 0.5</td>
<td>5.6 ± 2.3</td>
<td>1.8 ± 1.3</td>
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<td>HL60</td>
<td>2.1 ± 0.6</td>
<td>6.4 ± 1.9</td>
<td>3.1 ± 1.3</td>
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Table 1: Effects of SAHA and STI571 on apoptosis

| Bcr/Abl^+ K562 cells and several Bcr/Abl^+ leukemia cell lines, including U937 monocytic leukemia, Jurkat lymphoblastic leukemia, and NB4 and HL60 promyelocytic leukemia cells, were exposed to SAHA ± STI571 for 24 h, after which the percentage of apoptotic cells was determined by examining Wright Giemsa-stained cytospin slides as described in “Materials and Methods.” Concentrations for the individual cell lines were as follows: K562, 2.5 M SAHA; and 250 nM STI571; U937, 1.5 M SAHA and 250 nM STI571; Jurkat, 0.75 M SAHA; and 250 nM STI571; NB4, 1.5 M SAHA; and 250 nM STI571; HL60, 1.0 M SAHA; and 250 nM STI571. In each case, values represent the means ± SD for three separate experiments.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Control</th>
<th>SAHA</th>
<th>STI571</th>
<th>STI + SAHA</th>
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<tr>
<td>A</td>
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<td>5.1 ± 2.2</td>
<td>10.3 ± 3.6</td>
<td>61.2 ± 6.2</td>
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<td>LAMA 84</td>
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<td>6.4 ± 2.8</td>
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<td>55.1 ± 5.4</td>
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Table 2: Effect of STI571 + HDIs in STI571-sensitive and -resistant Bcr/Abl^+ leukemia cells

A, parental K562 and LAMA 84 cells were exposed to the indicated concentrations of STI571 ± SB for 48 h, after which the percentage of apoptotic cells was determined by examining Wright Giemsa-stained cytospin slides as described above. SB concentrations were 2.0 M for K562 and 1.0 M for LAMA 84 cells; the STI571 concentrations were 250 and 100 nM, respectively. B, STI571-resistant K562 (K562R) and LAMA 84 (LAMA 84R) cells were exposed to STI571 ± SAHA as described above, after which the percentage of apoptotic cells was determined as described above. The concentrations of SAHA were 2.0 M for K562R cells and 1.25 M for LAMA 84R cells; the STI571 concentrations were 1.25 and 1.0 M, respectively. In each case, values represent the means ± SD for three separate experiments.
Bcr/Abl downstream cytoprotective targets or, alternatively, from dysregulation of signaling/cell cycle-regulatory pathways (e.g., inactivation of Raf/MEK/ERK, Akt, and STAT5; down-regulation of Mcl-1, p21CIP1, and cyclin D1). Another speculative possibility is that STI571, which has been reported to induce maturation in Bcr/Abl+ cells (20), may, when combined with HDIs, generate conflicting signals that convert a differentiation response to an apoptotic response (44, 45).

Several lines of evidence support the notion that interruption of the Raf/MEK/MAPK cascade in Bcr/Abl+ cells by HDIs contributes to the marked induction of apoptosis by the STI571/HDI regimen. For example, involvement of Raf-1 in Bcr/Abl survival signaling has been reported (46). In addition, perturbations in MEK/MAPK have been noted in HDI-treated Bcr/Abl+ cells, although results have varied. In this context, Rivera and Adunyah (47) reported early activation of ERK in K562 cells exposed to SB, whereas Witt et al. (33) described a correlation between butyrate-induced differentiation in K562 cells and inhibition of ERK. Such disparate results could potentially reflect a biphasic temporal pattern of ERK activation/down-regulation after HDI exposure. In this regard, STI571 has been shown to oppose ERK activation in Bcr/Abl+ cells, at least at early intervals (8). In accord with these findings, we previously observed (25) early inhibition of ERK activation in STI571-treated K562 cells, although this was followed by a late rebound to basal or elevated levels of activity. Significantly, in that study, inhibition of MEK/ERK activation in STI571-treated K562 cells (i.e., by pharmacological MEK1/2 inhibitors) resulted in a very marked degree of mitochondrial damage and apoptosis (25). Taken together with the present finding that constitutive activation of MEK/ERK protected cells from HDI/STI571 lethality, these observations are compatible with the concept that (a) down-regulation of the Raf/MEK/ERK axis, in association with other STI571-mediated perturbations, promotes HDI-induced lethality; or (b) disruption of the Raf/MEK/ERK cytoprotective pathway lowers the threshold for STI571-mediated cell death. In support of the former possibility, we have recently observed that pharmacological MEK1/2/ERK blockade (e.g., by agents such as U0126) results in a marked potentiation of apoptosis in K562 cells exposed to HDIs.4 However, the finding that protection of HDI/STI571-treated cells by constitutive activation of MEK/ERK was incomplete indicates that other factors are involved in the lethality of this regimen.

Combined treatment with STI571 and HDIs also resulted in a striking increase in activation of the stress-related kinase JNK. Although exceptions exist, activation of stress-related kinases such as JNK and p38 MAPK generally favors cell death, whereas activation of MEK/MAPK exerts cytoprotective effects. In fact, the ratio of the net outputs of the JNK and MAPK cascades has been shown to play a key role in survival/cell death decisions (48). Moreover, JNK activation has been implicated in cytochrome c release associated with apoptosis (49). It is therefore tempting to speculate that the dramatic shift from MEK/MAPK to JNK signaling in Bcr/Abl+ cells exposed to the combination of STI571 and HDIs contributed to the marked potentiation of apoptosis.

In addition to disruption of the MEK1/2/ERK pathway, the possibility that dysregulation of the CDKI p21CIP1 may also play a role in synergistic interactions between STI571 and HDIs in Bcr/Abl+ cells appears plausible. For example, interference with p21CIP1 induction (e.g., in cells expressing an antisense construct; Ref. 50) or in cells exposed to the CDKI flavopiridol (51, 52) shows promise for promoting leukemic cell apoptosis after treatment with several differentiation-inducing agents, including phorbol 12-myristate 13-acetate, bryostatin 1, and, most recently, HDIs including SB, SAHA, and ABHA (53–55). This phenomenon may be related to the ability of p21CIP1 to bind to and inhibit caspase-3 (56). The observations that acetylation of histones by HDIs specifically activates the p21CIP1 promoter (57) and that p21CIP1 is regularly induced by HDIs in association with maturation (30) suggest that this CDKI plays a critical role in opposing HDI-mediated cell death. The mechanism by which STI571 blocks p21CIP1 induction in HDI-treated cells is not clear but could stem from disruption of the Raf/MEK/ERK axis, which is known to operate upstream of p21CIP1 (58). The finding that the STI571/HDI regimen induced pRb dephosphorylation was unanticipated, given the ob-

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4 C. Yu and S. Grant, unpublished data.
served down-regulation of p21cip1, but it could reflect reduced expression of cyclin D1 and diminished cyclin-dependent kinase 4/6 activity. A hypothetical model summarizing these and other interactions between HDIs and STI571 is shown in Fig. 8.

The potentiation of STI571 lethality by HDIs in STI571-resistant K562 and LAMA 84 cells was similar if not greater than that which we have previously observed in the case of combination regimens involving pharmacological MEK1/2 inhibitors (25) or, more recently, the CDKI flavopiridol (26). Resistance to STI571 can potentially stem from multiple factors, including diminished cellular uptake, amplification of bcr/abl and increased Bcr/Abl protein expression, pharmacokinetic factors, and mutations in the Bcr/Abl kinase domain (18).

For reasons that are unclear, increased expression of Bcr/Abl is the most common mechanism of resistance in cultured cell lines (17), including those isolated in our laboratory (25, 26). However, in cells obtained from CML patients who have developed in vivo resistance to STI571, increased Bcr/Abl expression is less frequently observed than mutations at the Bcr/Abl kinase contact site (e.g., Thr159 and Tyr253) have been the most widely reported (60). In addition, Corbin et al. (61) have recently used site-directed mutagenesis to identify other mutations in the Bcr/Abl kinase domain that reduce the inhibitory effects of STI571 and could potentially be clinically relevant. The ability of STI571/HD1 regimens to induce apoptosis in otherwise resistant K562 or LAMA 84 cells suggests that this strategy either circumvents the effects of increased Bcr/Abl expression or, alternatively, acts through pathways that operate downstream or independently of Bcr/Abl.

Whereas such a strategy may be effective in cells that display increased Bcr/Abl expression, it remains to be determined whether it would prove active in cells expressing Bcr/Abl mutations. In this context, the recent development of leukemia cell lines exhibiting such mutations (62) will clearly be of considerable value, particularly in helping to determine whether the HDI/STI571 regimen can down-regulate mutant Bcr/Abl protein. Given the recent introduction of several novel HDIs into clinical trials in humans (63), the concept of combining such agents with STI571 for the treatment of patients with CML and related disorders may be feasible in the near future. For these reasons, additional efforts to explore this novel antileukemic strategy are currently under way.

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Histone Deacetylase Inhibitors Promote STI571-mediated Apoptosis in STI571-sensitive and -resistant Bcr/Abl + Human Myeloid Leukemia Cells

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