Cotreatment with Vorinostat (Suberoylanilide Hydroxamic Acid) Enhances Activity of Dasatinib (BMS-354825) against Imatinib Mesylate–Sensitive or Imatinib Mesylate–Resistant Chronic Myelogenous Leukemia Cells

Warren Fiskus,1 Michael Pranpat,1 Maria Balasis,1 Purva Bali,1 Veronica Estrella,1 Sandhya Kumaraswamy,1 Rekha Rao,1 Kathy Rocha,1 Bryan Herger,1 Francis Lee,2 Victoria Richon,3 and Kapil Bhalla1

Abstract

Purpose: We determined the effects of vorinostat [suberoylanilide hydroxamic acid (SAHA)] and/or dasatinib, a dual Abl/Src kinase (tyrosine kinase) inhibitor, on the cultured human (K562 and LAMA-84) or primary chronic myelogenous leukemia (CML) cells, as well as on the murine pro-B BaF3 cells with ectopic expression of the unmutated and kinase domain-mutant forms of Bcr-Abl.

Experimental Design: Following exposure to dasatinib and/or vorinostat, apoptosis, loss of clonogenic survival, as well as the activity and levels of Bcr-Abl and its downstream signaling proteins were determined.

Results: Treatment with dasatinib attenuated the levels of autophosphorylated Bcr-Abl, p-CrkL, phospho-signal transducer and activator of transcription 5 (p-STAT5), p-c-Src, and p-Lyn; inhibited the activity of Lyn and c-Src; and induced apoptosis of the cultured CML cells. Combined treatment of cultured human CML and BaF3 cells with vorinostat and dasatinib induced more apoptosis than either agent alone, as well as synergistically induced loss of clonogenic survival, which was associated with greater depletion of Bcr-Abl, p-CrkL, and p-STAT5 levels. Cotreatment with dasatinib and vorinostat also attenuated the levels of Bcr-AblE255K and Bcr-AblT315I and induced apoptosis of BaF3 cells with ectopic expression of the mutant forms of Bcr-Abl. Finally, cotreatment of the primary CML cells with vorinostat and dasatinib induced more loss of cell viability and depleted Bcr-Abl or Bcr-AblT315I, p-STAT5, and p-CrkL levels than either agent alone.

Conclusions: As shown here, the preclinical in vitro activity of vorinostat and dasatinib against cultured and primary CML cells supports the in vivo testing of the combination in imatinib mesylate–sensitive and imatinib mesylate–resistant CML cells.

The fusion oncogene bcr-abl encoded Bcr-Abl tyrosine kinase activates many pro-growth and cell survival mechanisms, which confer resistance to apoptosis (1, 2). These include increased phosphorylation and transactivation by signal transducer and activator of transcription 5 (STAT5), which leads to increased expression of the antiapoptotic Bcl-xL and Pim-2 protein (3–5), as well as increased Ras/Raf/mitogen-activated protein kinase/extracellular signal–regulated kinase (ERK) kinase/ERK1/2, AKT, and nuclear factor-κB (NF-κB) activity (1, 2, 6, 7). Through deregulated AKT activity, Bcr-Abl inhibits the forkhead transcription regulator FOXO3a, which leads to depletion of the cyclin-dependent kinase-2 inhibitor p27 and the BH3 domain–containing proapoptotic Bim protein (8–10). Collectively, these molecular perturbations promote cell proliferation and survival and contribute to Bcr-Abl-mediated leukemia transformation of bone marrow progenitor cells. Clinical studies have shown that Bcr-Abl tyrosine kinase remains a therapeutic target in all phases of chronic myelogenous leukemia (CML; ref. 11). Although highly active in inducing clinical and cytogenetic complete remissions in many CML patients, resistance to imatinib mesylate (IM; Gleevec) is an increasing clinical problem in CML, especially in the accelerated phase and blast crisis phase where only short-term responses are observed (11). The major mechanisms of resistance to IM include mutations in the kinase domain of bcr-abl, amplification of the bcr-abl gene, as well as Bcr-Abl-independent mechanisms of resistance (12–16). Within the Bcr-Abl kinase domain, close to 40 known point mutations have been described. These have been linked to IM resistance in CML (17). The mutations are of two broad categories: those that directly interfere with the ability of IM to bind to the kinase...
domain (e.g., T315I) and those that impair the ability of Bcr-Abl to achieve inactive conformation required for binding to IM (e.g., E255K, P-loop mutation; ref. 18). Bcr-Abl mutations impart varying degrees of resistance to IM. Some remain susceptible to higher concentrations of IM whereas others that interfere directly with the binding of Bcr-Abl to IM (e.g., T315I, involving the gatekeeper threonine residue) confer the highest form of resistance to IM (19). These findings highlight the need to develop and test novel anti-Bcr-Abl agents that are more potent than IM and/or are able to override the resistance to IM due to either mutations or amplifications of Bcr-Abl.

BMS-354825 (dasatinib) is a synthetic, small-molecule, thiazole-based, orally bioavailable, ATP-competitive, dual Abl/Src kinase inhibitor (20). Dasatinib has been shown to inhibit the activity of the Src kinase family members c-Src and Lyn (21, 22). Dasatinib is able to bind the active and inactive conformations of Abl and inhibits the tyrosine kinase activity of Bcr-Abl (23, 24). Dasatinib is ~325-fold more potent than IM in inhibiting the activity of Bcr-Abl (25, 26). CrkL is a 39-kDa, tyrosine-phosphorylated adaptor protein, which is involved in hematopoietic and leukemia cell signaling and is an important substrate of Bcr-Abl (27). Inhibition of Bcr-Abl activity in CML cells has been gauged by the decline in the levels of phosphorylated CrkL (13). Importantly, in vitro studies have shown that dasatinib is also able to inhibit most clinically significant IM-resistant mutant isoforms of Bcr-Abl, but is ineffective against Bcr-AblT315I due to steric hindrance caused by the side chain of the isoleucine (23, 26). Dasatinib prolongs the survival of mice with IM-resistant, Bcr-Abl-dependent leukemia, but the drug was ineffective against tumors expressing the mutant Bcr-AblT315I (23). In phase I and early phase II studies, dasatinib has been reported to induce complete hematologic and cytogenetic responses in patients with IM-resistant or IM-intolerant chronic phase of CML (28, 29). However, the responses are significantly lower in patients with more advanced phases of CML. Taken together, these findings suggest that to override primary or acquired mechanisms of resistance in advanced phases of CML, dasatinib would have to be combined with novel agents that are not only active against IM-resistant mutant forms of Bcr-Abl but would also be effective in overriding non-Bcr-Abl-dependent mechanisms of IM resistance (30).

Vorinostat [suberoylanilide hydroxamic acid (SAHA)] is a hydroxamic acid–based polar histone deacetylase inhibitor (31). Treatment with hydroxamic acid analogue histone deacetylase inhibitors leads to increased levels of genes involved in cell cycle regulation such as p21 and p27, generation of reactive oxygen species, induction of TNF-related apoptosis-inducing ligand and its death receptors, as well as up-regulation of the levels of the pro-death proteins (e.g., Bax, Bak and Bim; refs. 32–38). These agents are also known to deplete the levels of antiapoptotic proteins (e.g., Bcl-2, Bcl-xL, X-linked inhibitor of apoptosis, survivin, AKT, and Pim-2) in human leukemia cells (32–38). Collectively, these effects inhibit cell cycle growth, lower the threshold to apoptotic stimuli, and induce apoptosis of CML cells. Recent studies from our laboratory have shown that treatment with the hydroxamic acid analogue histone deacetylase inhibitors alone (e.g., vorinostat, LAQ824, and LBH589) also depleted Bcr-Abl, as well as induced apoptosis and sensitized Bcr-Abl-expressing leukemia cells to apoptosis induced by IM (35–37). By inducing acetylation of heat shock protein 90 (hsp90) through inhibition of histone deacetylase 6, treatment with hydroxamic acid analogue histone deacetylase inhibitors was shown to inhibit the ATP-binding and chaperone function of hsp90 (39). This led to polyubiquitination, proteasomal degradation, and depletion of hsp90 client proteins, including Bcr-Abl, c-Raf, and AKT (36, 37, 39). Significantly, our studies also showed that treatment with hydroxamic acid analogue histone deacetylase inhibitors reduced the levels of the highly IM-refractory Bcr-AblT315I and induced apoptosis of primary IM-refractory CML blast crisis cells (36, 37). Based on the strong rationale generated by these observations, we determined the combined effects of dasatinib and vorinostat against cultured and primary, IM-sensitive or IM-resistant, human CML cells, including those that expressed Bcr-AblT315I. We also determined the effects of the combination against mouse pro-B BaF3 cells with ectopic expression of the unmutated Bcr-Abl or Bcr-AblE255K and Bcr-AblT315I.

Materials and Methods

Reagents and antibodies. Dasatinib (BMS-354825) was kindly provided by Bristol-Myers Squibb (Princeton, NJ). Vorinostat (SAHA) was kindly provided by Merck (Boston, MA). Monoclonal c-Abl antibody, polyclonal anti-STAT5A/B, polyclonal anti-Lyn, polyclonal anti-c-Src, monoclonal c-Myc, and goat polyclonal anti-Pim-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-p-STAT5, monoclonal anti-p27, monoclonal anti-Bcl-xL, and monoclonal anti-phosphotyrosine were purchased from BD Biosciences (San Diego, CA). Polyclonal p-ART was purchased from Biosource, Inc (Camarillo, CA). Anti–phospho-c-Src and anti–phospho-Lyn were purchased from Cell Signaling Technology (Beverly, MA). Antibodies for the immunoblot analyses of p21, p-CrkL, CrkL, AKT, Bim, Bcl-xL, and ERK1/2 were obtained as previously described (35–40).

Creation of BaF3/Bcr-Abl, BaF3/Bcr-AblE255K, and BaF3/Bcr-AblT315I cell lines. Mutant Bcr-Abl-containing plasmids were generated by site-directed mutagenesis of pSTARP210Bcr-Abl or pSVNeoBcr-Abl, as previously described (36). Briefly, the p210Bcr-AblE255K and p210Bcr-AblT315I constructs were created by site-directed mutagenesis of a Bcr-Abl-containing pSVneo construct using a QuikChange II XL kit (Strategene, Cedar Creek, TX) according to the recommendations of the manufacturer, and the resulting clones were sequenced to confirm the point mutation (41, 42). For nucleofection of the p210 Bcr-Abl constructs into BaF3 cells, 5 × 10^6 BaF3 cells in 100 μL of Nucleofector solution V (Amaxa, Gaithersburg, MD) were mixed with 5 μg of either p210 Bcr-Abl wild type, p210 Bcr-Abl (T315I), or p210 Bcr-Abl (E255K) in a cuvette and nucleofected using program G-16. Following nucleofection, the cells were incubated at a concentration of 1 × 10^6/mL in complete RPMI 1640 supplemented with 10% WEHI medium as the source of IL-3, overnight, to recover. Stable transfectants of BaF3 cells expressing the wild-type or mutant form of Bcr-Abl (i.e., T315I or E255K) were maintained in RPMI 1640 supplemented with 10% serum, 1.0 unit/mL penicillin, 1 μg/mL streptomycin, and 0.75 mg/mL G418. Stably expressing cells were then further selected by removal of IL-3. After confirmation of Bcr-Abl expression by immunoblot analysis, cells were used for the studies described below.

Cell lines and cell culture. Bcr-Abl-expressing CML LAMA-84 and K562 cells were obtained and maintained in culture as previously described (35–37). Logarithmically growing cells were exposed to the designated concentrations of dasatinib and/or vorinostat. Following these treatments, cells or cell pellets were washed free of the drug(s) before the conduction of the studies.

Primary CML cells. CML cells from the peripheral blood and/or bone marrow of 11 patients who had met the clinical criteria of Cancer Therapy: Preclinical
IM-resistant advanced phase of CML were harvested and purified as previously described (35–37). Informed consents were signed by all patients to allow use of their cells for these experiments, as part of a clinical protocol approved by the University of South Florida Institutional Review Board.

**Colony growth inhibition.** Following treatment with the designated concentrations of dasatinib and/or vorinostat for 48 hours, untreated and drug-treated cells were washed in RPMI 1640. Then, 200 cells treated under each condition were plated in duplicated wells in a 12-well plate containing 1.0 mL of Methocult medium (Stem Cell Technologies, Inc., Vancouver, Canada) per well according to the protocol of the manufacturer. The plates were placed in an incubator at 37°C with 5% CO2 for 7 days. Following this incubation, colonies consisting of ≥50 cells in each well were counted with an inverted microscope and the percent colony growth inhibition compared with the untreated control cells was calculated.

**Cell lysis and protein quantitation.** Untreated or drug-treated cells were centrifuged and the cell pellets were resuspended in 200 μL of lysis buffer [1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 1 μg/mL pepstatin A, 2 μg/mL aprotinin, 20 mmol/L L-3-nitrophenyl phosphate, 0.5 mmol/L sodium orthovanadate, and 1 mmol/L 4-(2-aminoethyl) benzenesulfonylfluoride hydrochloride] and incubated on ice for 30 minutes. The cell lysates were centrifuged and an aliquot of each cell lysate was diluted 1:10 and protein quantitated with a bichinonic acid protein quantitation kit (Pierce, Rockford, IL) according to the protocol of the manufacturer.

**Western blot analysis.** Western blot analyses of Bcr-Abl, p-STAT5, p-CrkL, p-Lyn, p-Src, STAT5, CrkL, c-Src, Lyn, p21, p27, Bcl-xL, α-tubulin, and β-actin were done on total cell lysates using specific antisera or monoclonal antibodies, as previously described (35–40). The expression level of either β-actin or α-tubulin was used as the loading control for the Western blots.

**Immunoprecipitation of Src and Bcr-Abl and immunoblot analysis.** Following designated treatments, cells were lysed with the lysis buffer as described above. Bcr-Abl was immunoprecipitated from total cell lysates of untreated and drug-treated cells with a monoclonal anti-c-Abl antibody from Santa Cruz Biotechnology and incubated at 4°C for 1 to 2 hours on a rotator. Precarried protein-G beads were added to the lysate mixture and incubated overnight at 4°C on a rotator. The immunoprecipitates were washed four times with lysis buffer and eluted from the agarose beads by boiling with 6× SDS sample buffer before immunoblot analysis. Total c-Src was immunoprecipitated from untreated or untreated total cell lysates with a rabbit polyclonal antibody from Santa Cruz Biotechnology and incubated at 4°C for 1 to 2 hours on a rotator. Prewashed protein-A beads were added to the lysate mixture and incubated overnight at 4°C on a rotator. The immunoprecipitates were washed and eluted for SDS-PAGE as described above.

**Src kinase assay.** K562 or LAMA-84 cells (107) were treated with dasatinib for 24 hours. One milligram of total cell lysate was used for immunoprecipitation of either Src or Lyn. For immunoprecipitation, 3 μg of anti-v-Src monoclonal antibody (Calbiochem, San Diego, CA) or 5 μg of anti-Lyn (Santa Cruz Biotechnology) were incubated for 3 hours with the total cell lysate on a rotator at 4°C (22, 43). Protein-G beads were added to the lysate-antibody mixture and incubated on a rotator overnight at 4°C. The following day, the agarose beads were washed six times with 500 μL of lysis buffer and once with 500 μL of 1× kinase buffer [100 mmol/L Tris-HCl (pH 7.2), 125 mmol/L MgCl2, 5 mmol/L MnCl2, 2 mmol/L EGTA, 2 mmol/LDTT, and 250 mmol/L Na3VO4]. For the kinase reaction, the beads were resuspended in 30 μL of 1× kinase buffer. Ten micromolars of glutathione S-transferase–purified Gab1CT and 10 μCi of [γ-32P]ATP (Perkin-Elmer, Boston, MA) were added and the reaction was incubated at 30°C for 15 minutes (43). The kinase reactions were terminated by adding 5 μL of 6× SDS sample buffer and boiling the samples for 5 minutes. The boiled samples were centrifuged briefly to pellet the agarose beads. The proteins were separated by SDS-PAGE, transferred to nitrocellulose, and visualized by autoradiography.

**Fig. 1.** Treatment with dasatinib diminishes activity of Bcr-Abl and Src family proteins, attenuates the levels of downstream Bcr-Ab1 targets, and induces apoptosis in cultured CML blast crisis cells K562 and LAMA-84. A, K562 and LAMA84 cells were treated with the indicated concentrations of dasatinib for 24 hours. Then, Bcr-Abl was immunoprecipitated from total cell lysates and Western blot analysis was done for tyrosine-phosphorylated Bcr-Abl. The blot was stripped and probed for total Bcr-Abl levels. Immunoblot analysis was done for Bcr-Abl, p-STAT5, p-CrkL, p27, Bcl-xL, p-Src (Tyr416), Lyn, and c-Src from the same lysates. The level of β-actin served as the loading control. B, K562 cells were treated with the indicated concentrations of dasatinib for 24 hours. Then, c-Src and Lyn were immunoprecipitated separately from the same cell lysates. Src and Lyn kinase assays were done in the presence of [γ-32P]ATP. After the kinase reactions, proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Autophosphorylation of Src or Lyn and phosphorylation of an exogenous substrate (Gab1CT) were visualized by autoradiography. C, K562 and LAMA-84 cells were treated with the indicated concentrations of dasatinib for 48 hours. Following treatment, the percentages of Annexin V–stained apoptotic cells were measured by flow cytometry. Columns, mean of three experiments; bars, SE.
Analysis of cell cycle status. K562 or LAMA-84 cells were treated with dasatinib and/or vorinostat for 24 hours. Cells were washed and fixed in 70% ethanol overnight. Fixed cells were stained with propidium iodide and subjected to flow cytometry and analyses with ModFit 3.0 (35–37).

Assessment of apoptosis by Annexin V staining. Untreated or drug-treated cells were stained with Annexin V (PharMingen, San Diego, CA) and propidium iodide and the percentage of apoptotic cells was determined by flow cytometry (35–37). To analyze synergism between dasatinib and vorinostat in inducing apoptosis, K562 cells were treated with dasatinib (1-5 nmol/L) and vorinostat (1-5 μmol/L) at a constant ratio of 1:1,000 for 48 hours. The percentage of apoptotic cells was determined by flow cytometry.

Assessment of percentage of nonviable cells. Cells were stained with trypan blue (Sigma, St. Louis, MO). The numbers of nonviable cells were determined by counting the cells that showed trypan blue uptake in a hemocytometer and reported as percentage of untreated control cells (36, 37).

Results

Dasatinib inhibits Bcr-Abl, c-Src, and Lyn tyrosine kinase activities and induces apoptosis of K562 and LAMA84 cells. As a dual Abl/Src kinase inhibitor, dasatinib is a significantly more potent inhibitor of unmutated Bcr-Abl than Imatinib (IM) and is able to inhibit the activity of nearly all of the mutant forms of Bcr-Abl (23, 25, 26). Although dasatinib has been shown to inhibit ectopically expressed unmutated and mutated Bcr-Abl, the kinase inhibitory effects of dasatinib have not been documented against endogenous Bcr-Abl and c-Src in human CML cells. Therefore, we first determined the kinase inhibitory, cell cycle, and apoptotic effects of dasatinib in the cultured CML K562 and LAMA-84 cells. Figure 1A and B clearly shows that exposure to low nanomolar concentrations of dasatinib inhibits the autophosphorylation of Bcr-Abl and c-Src, as well as depletes the kinase activity of the immunoprecipitated c-Src and Lyn, as measured by the decrease in the phosphorylation of the Gab1CT substrate in the in vitro kinase assay (22). Concomitantly, dasatinib attenuated the intracellular levels of p-STAT5, p-CrkL, p-Src, and p-Lyn in a dose-dependent manner. This was accompanied by a striking increase in p27 but a decline in the levels of c-Myc and Bel-α, which are transactivated by STAT5. No significant change occurred in the levels of Bcr-Abl and c-Src or of Lyn, STAT5, and CrkL. Exposure to 2.0 to 10 nmol/L dasatinib also induced apoptosis of K562 and LAMA-84 in a dose-dependent manner, with ~90% apoptosis of LAMA-84 and 50% apoptosis of K562 cells at the 10 nmol/L concentration of dasatinib (Fig. 1C).

Cotreatment with vorinostat and dasatinib exerts synergistic effects in K562 and LAMA-84 cells. In a previous report, we showed that treatment with vorinostat attenuates Bcr-Abl, AKT, and c-Raf levels and induces cell cycle growth arrest and apoptosis of CML cells. Additionally, vorinostat was shown to sensitize CML cells to apoptosis induced by IM (35). Next, we determined the effects of the combined treatment with
Figure 2D shows that cotreatment with dasatinib and vorinostat on the colony growth of K562 cells. We next determined the effects of treatment with vorinostat and dasatinib also attenuated Bcr-Abl levels more than treatment with vorinostat alone (Fig. 2A). This was accompanied by marked accumulation of the cells in the G₁ phase and concomitant decline in the percent of cells in the S phase of the cell cycle (Fig. 2B), again more with the combination than with either agent alone. Combined treatment with vorinostat and dasatinib also induced synergistic apoptotic effects against K562 cells, as determined by the median dose-effect isobologram analysis described by Chou and Talalay (Fig. 2C). For dasatinib and vorinostat, the combination index values were <1.0 in all cases. The combination index values were 0.65, 0.55, 0.48, 0.51, and 0.56, respectively. A similar effect was noted against LAMA-84 cells (data not shown). The effects of dasatinib and vorinostat were also determined against normal bone marrow progenitor cells. Whereas dasatinib (up to 20 nmol/L) had no effect, exposure to 2.0 μmol/L vorinostat induced loss of survival of 15.9% of normal bone marrow progenitor cells (mean of two samples with experiments done in duplicate). This loss of survival was not augmented by cotreatment with 20 nmol/L dasatinib (data not shown). We next determined the effects of dasatinib and/or vorinostat on the colony growth of K562 cells. Figure 2D shows that cotreatment with dasatinib and vorinostat caused significantly more inhibition of colony growth than treatment with either drug alone (P < 0.01). We also determined whether cotreatment with vorinostat enhances the effects of dasatinib on Bcr-Abl activity and on the downstream pro-growth and pro-survival signaling in K562 and LAMA-84 cells. As shown in Fig. 3, compared with treatment with either agent alone, cotreatment with vorinostat and dasatinib produced a greater decline in the levels of p-CrkL in K562 and LAMA-84 cells. Combined treatment was also highly effective in lowering the levels of Bcl-xL, p-Src, and p-Lyn. Whereas vorinostat alone induced p21, cotreatment with dasatinib abrogated vorinostat-induced p21 levels.

Dasatinib and vorinostat induce apoptosis in BaF3 cells expressing unmutated or mutant Bcr-Abl. Next, we determined the effects of dasatinib and/or vorinostat in BaF3 cells with ectopic expression of either the unmutated Bcr-Abl or the point mutants Bcr-AblE255K or Bcr-AblT315I. Similar to the effects seen in K562 and LAMA-84 with endogenous expression of Bcr-Abl, treatment with 5.0 to 20 nmol/L dasatinib induced apoptosis in BaF3/Bcr-Abl cells in a dose-dependent manner (Fig. 4A). Following treatment with 20 nmol/L dasatinib, >70% of cells showed apoptosis. In contrast, BaF3/Bcr-AblE255K cells were relatively less sensitive to dasatinib-induced apoptosis, showing a dose-dependent increase in apoptosis at higher concentrations of dasatinib (20-2,000 nmol/L; Fig. 4A). BaF3/Bcr-AblT315I cells were resistant to IM up to levels as high as 10 μmol/L (data not shown). BaF3/Bcr-AblT315I-expressing cells were also resistant to apoptosis induced by high levels of dasatinib (2.0 μmol/L). Treatment with dasatinib alone did not lower the levels of Bcr-Abl in BaF3 cells with ectopic expression of either the unmutated Bcr-Abl or the point mutants Bcr-AblE255K or Bcr-AblT315I (Fig. 4B and C). Whereas exposure to 5.0 nmol/L dasatinib markedly inhibited autophosphorylation of the unmutated Bcr-Abl, higher concentration of dasatinib (20 nmol/L) achieved a similar effect against Bcr-AblE255K (Fig. 4B). Similarly, the higher dasatinib levels were required to attenuate p-STAT5 and p-CrkL levels in BaF3/Bcr-AblE255K versus BaF3/Bcr-Abl cells (Fig. 4B). Dasatinib did not appreciably inhibit autophosphorylation of Bcr-Abl (data not shown) or lower p-STAT5 and p-CrkL levels in BaF3/Bcr-AblT315I cells (Fig. 4C). On the other hand, in a dose-dependent manner, vorinostat induced apoptosis not only of BaF3/Bcr-Abl and BaF3/Bcr-AblE255K but also of BaF3/Bcr-AblT315I cells (Fig. 4D). Exposure to 2.0 μmol/L vorinostat resulted in apoptosis of ~40% of BaF3/Bcr-AblE255K cells (Fig. 4D). Notably, treatment with ≥1.0 μmol/L vorinostat induced more apoptosis of BaF3/Bcr-AblT315I than of BaF3/Bcr-Abl cells. This is consistent with previous reports showing that cells expressing the IM-resistant T315I point mutant are more sensitive to hydroxamic acid analogue histone deacetylase inhibitors than unmutated Bcr-Abl-expressing cells (36, 37).

Cotreatment with vorinostat enhances dasatinib-induced apoptosis of IM-resistant BaF3 cells including those expressing Bcr-AblT315I. We also determined the effects of treatment with vorinostat on the levels of Bcr-Abl and downstream signaling targets in BaF3/Bcr-Abl, BaF3/Bcr-AblE255K, and BaF3/Bcr-AblT315I cells. Treatment of the BaF3 transfectants with vorinostat depleted Bcr-AblT315I > Bcr-AblE255K > unmutated Bcr-Abl levels (Fig. 5A). In contrast, exposure to up to 2.0 μmol/L dasatinib had no effect on the unmutated or mutant forms of Bcr-Abl (Fig. 5A, and data not shown). Next, we determined the effects of cotreatment with vorinostat and dasatinib on cell cycle status, apoptosis, and clonogenic survival of K562 and LAMA-84 cells. As compared with the treatment with vorinostat or dasatinib alone, cotreatment with vorinostat and dasatinib caused more depletion of the levels of autophosphorylated Bcr-Abl (Fig. 2A).

**Fig. 3.** Cotreatment with dasatinib and vorinostat enhances anti-Bcr-Abl activity and attenuates levels of Bcr-Abl and p-CrkL greater than either agent alone in K562 and LAMA-84 cells. Following treatment with 2 nmol/L dasatinib and/or 2 μmol/L vorinostat for 24 hours, Western blot analysis of p-STAT5, STAT5, p-CrkL, CrkL, p21, Bcl-xL, p-Src, p-Lyn, and c-Src was done on total cell lysates from K562 and LAMA-84. The levels of β-actin served as the loading control.
dasatinib on the levels of Bcr-Abl in BaF3/Bcr-Abl, BaF3/Bcr-AblE255K, and BaF3/Bcr-AblT315I cells. As compared with treatment with either agent alone, combined treatment with vorinostat and dasatinib markedly depleted the levels of both of the mutant forms of Bcr-Abl in BaF3 cells. Based on the sensitivity to apoptosis with dasatinib noted in Fig. 4A, we used higher concentrations of dasatinib in the combination against BaF3/Bcr-AblE255K and BaF3/Bcr-AblT315I cells (Fig. 5A). Cotreatment with vorinostat and dasatinib, more than either agent alone, also depleted the levels of p-STAT5 and p-CrkL in BaF3 cells with ectopic expression of unmutated or of Bcr-AblE255K or Bcr-AblT315I (Fig. 5A). The mechanism underlying the striking decrease in p-STAT5 levels without any change in STAT5 levels is not obvious. However, it may be that cotreatment with the high levels of dasatinib employed in this experiment might render the upstream tyrosine kinase (responsible for phosphorylation of STAT5) more susceptible to depletion due to vorinostat-mediated disruption of the chaperone function of hsp90 for the tyrosine kinase. Additionally, similar to the effects seen in K562 and LAMA-84 cells with endogenous expression of Bcr-Abl, cotreatment with vorinostat and dasatinib versus treatment with either agent alone induced significantly more apoptosis of BaF3/Bcr-Abl cells (Fig. 5B; P < 0.05). Notably, combined treatment with vorinostat and dasatinib was also more effective than either agent alone in inducing apoptosis of BaF3/Bcr-AblT315I and BaF3/Bcr-AblE255K cells (Fig. 5C and D). The combination induced apoptosis of ~90% of BaF3/Bcr-AblE255K and 60% of BaF3/Bcr-AblT315I cells (Fig. 5C and D).

Cotreatment with dasatinib and vorinostat exerts superior antileukemia activity against primary IM-resistant CML cells. We next determined the antileukemia effects of dasatinib and/or vorinostat against primary IM-resistant CML cells isolated from the peripheral blood and/or bone marrow from 11 patients who had relapsed with IM-resistant CML blast crisis cells. Two of these samples were documented to express Bcr-Abl/T315I (samples 10 and 11). In the remaining samples of IM-resistant primary CML cells (samples 1-9), due to inadequate sample size, the mutational status of Bcr-Abl could not be determined. Table 1 indicates that in the samples 1 to 9, dasatinib induced loss of cell viability in a dose-dependent manner. Exposure to 2.0 μmol/L vorinostat alone also induced loss of cell viability in all of the samples, including samples 10 and 11. Notably, whereas in samples 1 to 9, cotreatment with dasatinib and vorinostat induced more loss of cell viability, in samples 10 and 11, addition of dasatinib did not augment the loss of cell viability due to treatment with vorinostat alone. One sample (CML #4) yielded sufficient cells for immunoprecipitation of Bcr-Abl to evaluate the effect of dasatinib and/or vorinostat on autophosphorylation and levels of Bcr-Abl, as well as on levels of p-STAT5 and p-CrkL. As was observed in K562 cells, dasatinib inhibited autophosphorylation of Bcr-Abl without affecting Bcr-Abl levels (Fig. 6A). Treatment with dasatinib also attenuated the levels of p-STAT5 and p-CrkL, with little effect on STAT5 and CrkL levels (Fig. 6A). Whereas exposure of sample no. 4 cells to 2.0 μmol/L vorinostat alone depleted the levels of Bcr-Abl, p-STAT5, and p-CrkL, cotreatment with
dasatinib (2 nmol/L) and vorinostat (2.0 μmol/L) was even more effective than treatment with either agent alone in attenuating Bcr-Abl, p-STAT5, and p-CrkL (Fig. 6B). These findings are consistent with the increased lethality exerted against sample no. 4 cells by the combination of dasatinib (2 nmol/L) and vorinostat (2.0 μmol/L; Fig. 6B). Treatment with dasatinib was clearly less effective in depleting the levels of p-STAT5 in the primary versus cultured CML cells. Although the mechanisms underlying this disparity have not been elucidated, it is possible that in primary cells, STAT5 phosphorylation may be mediated by the activity of Src as well as another tyrosine kinase(s), which is not inhibited by dasatinib.

**Discussion**

Previous reports have separately described the activity of the pan-histone deacetylase inhibitor vorinostat and the dual Abl/Src tyrosine kinase inhibitor dasatinib against Bcr-Abl-expressing human CML cells (25, 26, 35). Here we show for the first time that the combination of dasatinib and vorinostat is even more effective than treatment with either agent alone in attenuating Bcr-Abl, p-STAT5, and p-CrkL. These findings are consistent with the increased lethality exerted against sample no. 4 cells by the combination of dasatinib (2 nmol/L) and vorinostat (2.0 μmol/L; Fig. 6B). Treatment with dasatinib was clearly less effective in depleting the levels of p-STAT5 in the primary versus cultured CML cells. Although the mechanisms underlying this disparity have not been elucidated, it is possible that in primary cells, STAT5 phosphorylation may be mediated by the activity of Src as well as another tyrosine kinase(s), which is not inhibited by dasatinib.

**Table 1. Dasatinib and/or vorinostat induces loss of viability of primary CML blast crisis cells**

<table>
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<tr>
<th>Sample no.</th>
<th>Untreated</th>
<th>2 nmol/L, dasatinib</th>
<th>5 nmol/L, dasatinib</th>
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Note: Peripheral blood or bone marrow from nine IM-resistant or refractory patients and two samples with mutant Bcr-AblT315I (*) were treated with the indicated doses of dasatinib and/or vorinostat for 48 hours. Then, the percentages of nonviable cells for each drug alone or drug combination were determined by trypan blue uptake in a hemocytometer. Values represent the percentage of nonviable cells from each condition as compared with untreated cells.
Bcr-Abl, c-Raf, and AKT (37, 39). In turn, this promotes the association of hsp90 with its client proteins, including binding and chaperone function of hsp90, thereby disrupting the acetylation of hsp90 (39, 40, 45). This inhibits the ATP to inhibit the activity of histone deacetylase 6, which induces deacetylase inhibitors, including vorinostat, has been shown Bcr-AblE255K or Bcr-AblT315I, as well as against IM-resistant cells with ectopic expression of Bcr-Abl or its mutant isoforms. Notably, the combination is also more active against BaF3/Bcr-Abl and human CML cells to dasatinib-induced apoptosis. That the combination of vorinostat and dasatinib induces more apoptosis of Bcr-Abl transformed cells is also consistent with several previous reports where cotreatment with a hydroxamic acid analogue histone deacetylase inhibitor and a tyrosine kinase inhibitor, targeting a tyrosine kinase that is a bona fide hsp90 client protein (e.g., Bcr-Abl, Her-2, and FLT-3), was shown to induce more apoptosis than treatment with either agent alone (35, 50, 51). Our findings show that, as compared with treatment with vorinostat alone, cotreatment with dasatinib mediates more depletion of unmutated or mutant Bcr-Abl. Although we do not have any experimental evidence to clarify the mechanism underlying this effect, it is possible that inhibition of Bcr-Abl activity and autophosphorylation may make it more susceptible to polyubiquitylation and proteasomal degradation due to vorinostat-mediated disruption of the chaperone function of hsp90.

Recent studies have suggested that treatment with IM may be ineffective against CML stem cells (17), and acquired resistance to IM due to mutations in the Bcr-Abl kinase domain is a common occurrence (12–15, 17). Amplification and increased expression of Bcr-Abl in CML progenitors may also confer IM resistance (12–14, 30). Dasatinib is clearly a more potent inhibitor than IM of the unmutated Bcr-Abl tyrosine kinase activity, and dasatinib is also able to inhibit most of the mutant forms of Bcr-Abl (23, 26–30). Vorinostat is shown here to not only deplete the levels of the unmutated and mutant forms of Bcr-Abl but also induces apoptosis of CML cells through Bcr-Abl-independent mechanisms. This is important because IM resistance may also be due to the dependence of CML cells for their growth and survival on signaling kinases other than Bcr-Abl (e.g., Lyn or mitogen-activated protein kinase; refs. 16, 52, 53). Therefore, the combination of vorinostat with dasatinib has the potential of overriding many, if not all, of the IM resistance mechanisms in CML progenitor cells. Additionally, based on the multiple mechanism of activity noted above, the combination may also be able to override Bcr-Abl-independent and Bcr-Abl-dependent resistance mechanisms prevalent in the CML stem cells (31, 36, 54). Mutations in the kinase domain of Bcr-Abl conferring IM resistance fall into two main groups: those that inhibit contact with IM and those that prevent Bcr-Abl from achieving the inactive conformation required for the binding of IM to Bcr-Abl. The point mutants Bcr-AblE255K and Bcr-AblT315I have been recognized as the important and common examples of two groups of mutations that confer IM resistance fall into two main groups: those that inhibit contact with IM and those that prevent Bcr-Abl from achieving the inactive conformation required for the binding of IM to Bcr-Abl.

Fig. 6. The effect of dasatinib and/or vorinostat on primary CML blast crisis cells. A, primary CML cells were treated with the indicated concentrations of dasatinib for 24 hours. Bcr-Abl was immunoprecipitated from the total cell lysates and immunoblotted for tyrosine-phosphorylated Bcr-Abl. Blots were stripped and probed for total Bcr-Abl levels. Western blot analysis was done for Bcr-Abl, p-STAT5, STAT5, p-CrkL, and CrkL on total cell lysates from the same cells. The levels of β-actin served as the loading control. B, CML blast crisis cells were treated with dasatinib and/or vorinostat for 24 hours. After this, Western blot analysis was done for Bcr-Abl, p-STAT5, STAT5, p-CrkL, and CrkL on the total cell lysates. The level of β-actin served as the loading control.

time that combined treatment with vorinostat and dasatinib is significantly more active than either agent alone against human CML cells with endogenous expression of the unmutated Bcr-Abl. Notably, the combination is also more active against BaF3 cells with ectopic expression of Bcr-Abl or its mutant isoforms Bcr-AblE255K or Bcr-AblT315I, as well as against IM-resistant human primary CML cells. Treatment with pan-histone deacetylase inhibitors, including vorinostat, has been shown to inhibit the activity of histone deacetylase 6, which induces the acetylation of hsp90 (39, 40, 45). This inhibits the ATP binding and chaperone function of hsp90, thereby disrupting the association of hsp90 with its client proteins, including Bcr-Abl, c-Raf, and AKT (37, 39). In turn, this promotes polyubiquitylation and proteasomal degradation of the client proteins (46). Vorinostat has also been shown to down-modulate the levels of the Bcl-2 and the inhibitor of apoptosis family member proteins (35, 40). Additionally, treatment with vorinostat up-regulates the levels of Bim, a protein induced by the forkhead family of transcription factors that are repressed by phosphorylation by AKT (8, 10). By attenuating c-Raf and in turn inhibiting the activity of the ERK, which is known to phosphorylate Bim and diminish its association with Bcl-2 and Bcl-xL, vorinostat also promotes the proapoptotic effects of Bim (47, 48). Collectively, these effects lower the threshold and promote vorinostat-induced apoptosis (49). The reported findings also explain why cotreatment with vorinostat sensitizes BaF3/Bcr-Abl and human CML cells to dasatinib-induced apoptosis. That the combination of vorinostat and dasatinib mediates more apoptosis of Bcr-Abl transformed cells is also consistent with several previous reports where cotreatment with a hydroxamic acid analogue histone deacetylase inhibitor and a tyrosine kinase inhibitor, targeting a tyrosine kinase that is a bona fide hsp90 client protein (e.g., Bcr-Abl, Her-2, and FLT-3), was shown to induce more apoptosis than treatment with either agent alone (35, 50, 51). Our findings show that, as compared with treatment with vorinostat alone, cotreatment with dasatinib mediates more depletion of unmutated or mutant Bcr-Abl. Although we do not have any experimental evidence to clarify the mechanism underlying this effect, it is possible that inhibition of Bcr-Abl activity and autophosphorylation may make it more susceptible to polyubiquitylation and proteasomal degradation due to vorinostat-mediated disruption of the chaperone function of hsp90.
Dasatinib and Vorinostat against CML

5. Melo JV, Deininger MW. Biology of chronic myelogenous leukemia crisis cells. A similar effect may explain the superior activity of signaling initiated by Bcr-Abl, thus augmenting the growth and apoptosis, as was reported in the primary CML blast crisis cells (35). Cotreatment with vorinostat may override these mechanisms and sensitize leukemia cells to dasatinib-induced growth arrest and apoptosis, as was reported in the primary CML blast crisis cells (35). Additionally, cotreatment with vorinostat and dasatinib not only attenuates Bcr-Abl but also depletes the downstream pro-growth and pro-survival signaling molecules, including p-AKT, p-ERK1/2, and p-STAT5. Accordingly, vorinostat not only depletes the Bcr-AblE255K and Bcr-AblT315I levels in dasatinib-treated BaF3/Bcr-AblE255K and BaF3/Bcr-AblT315I cells, respectively.

In early clinical trials, dasatinib has exhibited promising levels of activity in IM-resistant CML (28, 30). However, a substantial proportion of patients fail to achieve cytogenetic complete remission, especially in patients with more advanced phases of CML (28, 30, 55). Cells from these patients may harbor additional chromosomal abnormalities and genetic perturbations that often involve the recruitment of coexpressors and histone deacetylase activity. This can potentially repress genes involved in differentiation and apoptosis (1, 31, 56, 57). Cotreatment with vorinostat may override these mechanisms and sensitize leukemia cells to dasatinib-induced growth arrest and apoptosis, as was reported in the primary CML blast crisis cells (35). Additionally, cotreatment with vorinostat and dasatinib not only attenuates Bcr-Abl but also depletes the downstream pro-growth and pro-survival signaling molecules, including p-AKT, p-ERK1/2, and p-STAT5. Accordingly, vorinostat may be exerting a “longitudinal” two-step inhibition of the signaling initiated by Bcr-Abl, thus augmenting the growth inhibitory and apoptotic activity of dasatinib against CML blast crisis cells. A similar effect may explain the superior activity of the combination of a mammalian target of rapamycin inhibitor and IM against CML cells (58). Cotreatment with vorinostat and dasatinib also caused more inhibition of p-STAT5, which was associated with more attenuation of the STAT5 target gene products Bcl-xL and c-Myc (3–5). Bcl-2 family members have been shown to act in a complementary manner to promote Bcr-Abl-mediated induction of leukemia (59, 60). Additionally, our findings show that vorinostat-mediated induction of p21 was blocked by cotreatment with dasatinib. This is consistent with previous reports, which have indicated that p21 induction decreases apoptosis induced by histone deacetylase inhibitor, and interruption of histone deacetylase inhibitor–induced p21 potentiates apoptosis due to treatment with histone deacetylase inhibitor (61). Collectively, more dramatic inhibitory effects on several pro-growth and pro-survival signaling molecules may also contribute to the synergistic apoptotic effects of the combination of vorinostat and dasatinib in the cultured and primary CML cells. Because these findings have not been verified in vivo, the clinical significance of these observations remains uncertain.

In the present studies, vorinostat is shown to sensitize BaF3/Bcr-AblE255K and BaF3/Bcr-AblT315I cells to clinically achievable levels of dasatinib. Because, at these levels, dasatinib is able to inhibit the Bcr-Abl tyrosine kinase activity of the mutant Bcr-AblE255K, it is likely that cotreatment with vorinostat enhances this activity of dasatinib not only by depleting the levels of Bcr-AblE255K but also through other downstream mechanisms described above. However, the structural basis for how cotreatment with vorinostat leads to increased activity of relatively high levels of dasatinib (2.0 μmol/L) against the contact inhibitory mutant Bcr-AblT315I is not entirely clear. It is also possible that vorinostat augments Bcr-Abl-independent growth inhibitory and cytotoxic mechanisms of dasatinib against Bcr-AblT315I-expressing cells. However, further studies are needed to characterize these mechanisms. Alternatively, it is conceivable that vorinostat-mediated acetylation and inhibition of hsp90 chaperone function for Bcr-Abl affects its conformation in a manner that allows higher concentrations of dasatinib to interact with and inhibit Bcr-AblT315I (24, 25). In a recent report, the non-ATP-competitive Bcr-Abl kinase inhibitor ON012380 was shown to inhibit the activity of Bcr-AblT315I (62). It would also be important to determine whether cotreatment with vorinostat would further augment the activity of ON012380 against CML cells expressing Bcr-AblT315I.

References

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Cotreatment with Vorinostat (Suberoylanilide Hydroxamic Acid) Enhances Activity of Dasatinib (BMS-354825) against Imatinib Mesylate–Sensitive or Imatinib Mesylate–Resistant Chronic Myelogenous Leukemia Cells

Warren Fiskus, Michael Pranpat, Maria Balasis, et al.


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