Synergistic Interactions between Vorinostat and Sorafenib in Chronic Myelogenous Leukemia Cells Involve Mcl-1 and p21^{CIP1} Down-Regulation

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Abstract

Purpose: Interactions between the multikinase inhibitor sorafenib (Bay 43-9006) and the histone deacetylase inhibitor vorinostat were examined in chronic myelogenous leukemia (CML) cells sensitive and resistant to imatinib mesylate.

Experimental Design: K562, LAMA 84, and primary CML patient-derived CD34$^+$ mononuclear cells were exposed to vorinostat followed by sorafenib, after which effects on cell viability and various survival signaling pathways were monitored by flow cytometry, clonogenic assays, and Western blotting. Real-time reverse transcription-PCR was used to monitor gene expression, and the functional contribution of p21^{CIP1} and Mcl-1 down-regulation were determined in cells transfected with corresponding constructs.

Results: Pretreatment (24 h) with vorinostat followed by sorafenib optimally induced mitochondrial injury and cell death in Bcr/Abl$^+$ cells (e.g., K562 and LAMA 84). Similar results were obtained in imatinib mesylate–resistant cells expressing activated Lyn as well as in primary CD34$^+$ bone marrow cells obtained from CML patients. This regimen also markedly inhibited CML cell colony formation. Combined but not individual treatment of CML cells with vorinostat and sorafenib triggered pronounced mitochondrial dysfunction (i.e., cytochrome *c*, Smac, and AIF release), caspase activation, poly(ADP-ribose) polymerase cleavage, and down-regulation of Mcl-1. Sorafenib also blocked vorinostat-mediated induction of p21^{CIP1}. Down-regulation of Mcl-1 was caspase and transcription independent, whereas p21^{CIP1} down-regulation was partially caspase and transcription dependent. Enforced expression of p21^{CIP1} and particularly Mcl-1 significantly attenuated vorinostat/sorafenib-mediated lethality.

Conclusions: These findings suggest that combined treatment with vorinostat and sorafenib synergistically induces apoptosis in CML cells through a process that involves Mcl-1 down-regulation and inhibition of p21^{CIP1} induction.

Chronic myelogenous leukemia (CML) is a clonal disease characterized by the 9:22 translocation, giving rise to the Bcr/Abl fusion protein, which is pathognomonic for this disorder (1). The Bcr/Abl kinase is constitutively active and signals downstream to a variety of survival pathways, including AKT, signal transducers and activators of transcription 5, nuclear factor-κB, and extracellular signal-regulated kinase (ERK) 1/2 (2–4). Collectively, the survival signals transduced by the Bcr/Abl kinase provide CML progenitor cells with a survival advantage over their normal counterparts while at the same time conferring resistance to various chemotherapeutic agents (5–7). The treatment of CML has been revolutionized by the introduction of the Bcr/Abl kinase inhibitor imatinib mesylate (Gleevec), which has proven highly active in CML, particularly in the setting of chronic-phase disease (8–10). However, it is less active in the accelerated and blast crisis phases of CML; moreover, patients become resistant to this agent through various mechanisms, including increased Bcr/Abl expression or, more commonly, the development of mutations in the Bcr/Abl kinase, which prevent drug binding and inhibitory activity (11–13). Recently, second-generation Bcr/Abl kinase inhibitors, such as dasatinib and nilotinib, have been introduced, which are active against most Bcr/Abl kinase mutations conferring resistance to imatinib mesylate (14, 15). However, these agents are largely inactive against the T315I Bcr/Abl mutation in the Bcr/Abl kinase "gatekeeper" region. Consequently, the development of alternative strategies capable of eradicating resistant CML cells remains a high priority.

Histone deacetylase inhibitors (HDACI) represent a class of agents that act by blocking histone deacetylation, thereby modifying chromatin structure and gene transcription. HDACIs, along with histone acetyltransferases, reciprocally regulate

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the acetylation status of the positively charged NH_2-terminal histone tails of nucleosomes. In general, HDACIs promote histone acetylation and neutralization of positively charged lysine residues on histone tails, allowing chromatin to assume a more relaxed, open conformation, which favors gene transcription (16). However, HDACIs also induce acetylation of many other nonhistone targets, actions that may have important biological consequences (17). Notably, HDACIs induce apoptosis of numerous neoplastic cell types, including those of leukemic origin, through multiple mechanisms, including induction of oxidative injury, disruption of checkpoint control, and up-regulation of death receptors (18–20). Vorinostat (suberoylanilide hydroxamic acid) is a hydroxamic acid HDACI that has shown preliminary evidence of activity in acute myelogenous leukemia (21). Recently, attention has focused on the capacity of vorinostat and other hydroxamic acid HDACIs to acetylate and disrupt the function of heat shock proteins, such as Hsp90 (22). Because oncogenic fusion proteins may be highly dependent on chaperone function for survival (23), this finding may have particular significance for diseases such as CML. Indeed, HDACIs have been shown to enhance the activity of Bcr/Abl kinase inhibitors, such as imatinib mesylate, through a process that may involve down-regulation of Bcr/Abl and/or disruption of signaling pathways (24). Recently, enhanced lethality of a regimen combining dasatinib and HDACIs toward CML cells has been described (25).

Sorafenib (Bay 43-9006) is a multikinase inhibitor that was originally developed as an inhibitor of Raf-1, but which was subsequently shown to inhibit multiple other kinases, including platelet-derived growth factor, vascular endothelial growth factor receptors 1 and 2, and FLT3 (26). The Ras/RAF/mitogen-activated protein kinase (MEK1/2)/ERK kinase (ERK1/2) is frequently dysregulated in human cancers, including those of hematopoietic origin (27), and therefore represents a logical target for pharmacologic intervention. However, several groups, including our own, have shown that sorafenib kills human leukemia cells through a mechanism that involves down-regulation of the antiapoptotic Bcl-2 family member Mcl-1 (28, 29), which is known to play a critical role in the survival of malignant hematopoietic cells (30). Sorafenib-mediated Mcl-1 down-regulation occurs through a translational rather than a transcriptional or posttranslational process (28). Notably, sorafenib has been shown to kill Bcr/Abl$^+$ leukemia cells *in vitro* (28, 29) at concentrations below those achievable in the plasma (e.g., 15-20 μmol/L; ref. 31).

Evidence that HDACIs promote the antileukemic activity of certain tyrosine kinase inhibitors, particularly in CML cells (24), as well as reports that Mcl-1 expression represents a potential determinant of HDACI lethality (32) raised the possibility that sorafenib and vorinostat might cooperate to kill Bcr/Abl$^+$ leukemia cells. Here, we report that sorafenib and vorinostat interact synergistically to kill Bcr/Abl$^+$ cells, including cells resistant to imatinib mesylate, through a process that involves down-regulation of Mcl-1 and $p21^{CIP1}$, and most likely independent of Bcr/Abl inhibition. Collectively, these findings suggest that a strategy combining sorafenib and vorinostat warrants further attention in Bcr/Abl$^+$ hematologic malignancies.

Materials and Methods

Cells. LAMA 84 cells were purchased from the German Collection of Microorganisms and Cell Cultures. K562 cells, including imatinib mesylate–resistant cells exhibiting activated Lyn, were obtained as described previously (33). BaF/3 cells expressing wild-type or T315I-mutant Bcr/Abl were kindly provided by Dr. Brian Druker (Oregon Health and Sciences University Cancer Center, Portland, Oregon) and have been described in detail previously (34). All cells were cultured in RPMI 1640 supplemented with sodium pyruvate, MEM essential vitamins, L-glutamate, penicillin, streptomycin, and 10% heat-inactivated FCS (Hyclone). CD34$^+$ cells were obtained with informed consent from the bone marrow of four patients with chronic-phase CML, two of whom were imatinib mesylate naive and two who had progressed while receiving imatinib mesylate therapy, undergoing a routine diagnostic bone marrow aspiration, and from two patients undergoing routine diagnostic aspirations for a nonmyeloid hematologic disorder (thrombocytopenia). These studies have been sanctioned by the Institutional Review Board of Virginia Commonwealth University/Medical College of Virginia. Blood was collected into heparinized syringes, diluted 1:3 with RPMI 1640, and transferred as an overlayer to centrifuge tubes containing 10 mL of Ficoll-Hypaque (specific gravity, 1.077-1.081; Sigma). After centrifugation at room temperature for 30 min, the interface layer, containing mononuclear cells, was extracted with a sterile Pasteur pipette, suspended in RPMI 1640, and washed thrice. CD34$^+$ cells were then isolated using a Miltenyi microbead separation system (Miltenyi Biotech) as per the manufacturer's instructions. The CD34$^+$ cells were then diluted into RPMI 1640 containing 10% FCS at a concentration of 2×10^5/mL and exposed to drugs as described for K562 cells.

Reagents. Sorafenib (Bay 43-9006; Bayer) was provided by the Cancer Treatment and Evaluation Program, National Cancer Institute/NIH (Bethesda, MD). Vorinostat was provided by Merck. Sodium butyrate was supplied by Calbiochem. BOC-fmk was purchased from Enzyme Products Ltd. All drugs were formulated in sterile DMSO. 7-Aminoactinomycin D (7-AAD) was purchased from Sigma-Aldrich and formulated as per the manufacturer's instructions. All transient transfection reagents were procured from Amaxa, Germany, through their U.S. distributor in Rockville, Maryland. Trypan blue working solution was purchased from Invitrogen.

Experimental format. Logarithmically growing cells were placed in sterile plastic T-flasks (Corning) to which the designated drugs were added. The flasks were then placed in a 5% CO_2, 37°C incubator for various intervals. At the end of the incubation period, cells were transferred to sterile centrifuge tubes, pelleted by centrifugation at $400 \times g$ for 10 min at room temperature, and prepared for analysis as described below.

Assessment of apoptosis. Apoptotic cells are evaluated by Annexin V/propidium iodide staining and, in some cases, verified by Wright-Giemsa staining as described previously (24). In brief, morphologically apoptotic cells were identified by classic features (i.e., nuclear condensation, cell shrinkage, and formation of apoptotic bodies in slides). Alternatively, the Annexin V/propidium iodide assay was carried out as per the manufacturer's instructions (BD PharMingen) using a Becton Dickinson FACScan flow cytometer. In general, the results of morphologic assessment and Annexin V/propidium iodide staining yielded highly concordant results.

Assessment of cell viability. Drug effects on cell viability were monitored by flow cytometry using 7-AAD as the staining dye as described previously (35). Briefly, cells were stained with 25 μmol/L 7-AAD solution at room temperature in regular culture medium and analyzed in the FL2 channel using the Becton Dickinson flow cytometer. Cells were also analyzed for viability using the ViaCount reagent in conjunction with a Guava PCA instrument using CytoSoft software as per the manufacturer's instruction. Results were also verified by trypan blue staining and enumeration of trypan blue–excluding cells using a hemocytometer. Results for each method were found to be in good agreement.

Preparation of S-100 fractions and assessment of cytochrome c, Smac, and AIF release. K562 cells were harvested after drug treatment and cytosolic S-100 fractions were prepared as described in detail previously (24). Western blot analysis assessing cytochrome *c*, Smac, and AIF release was done as described below.

Immunoblot analysis. Immunoblotting was done as described previously (24). The source of primary antibodies was as follows: AIF, cytochrome *c*, phosphorylated c-Jun NH_2-terminal kinase, c-Jun NH_2-terminal kinase 1, phosphorylated ERK, ERK, Mcl-1, Bak, Bid, and Bcl-x_L were from Santa Cruz Biotechnology; phosphorylated Bcr/Abl, cleaved caspase-8, cleaved caspase-3, Crkl, and phosphorylated Crkl were from Cell Signaling Technology; $p21^{CIP1}$ and MEK1 were from Transduction Laboratories; poly(ADP-ribose) polymerase (C-2-10) was from Biomol Research Laboratories; Smac was from Upstate Biotechnology; caspase-8 was from Alexis; and tubulin was from Oncogene.

Plasmids. The construct pcDNA3.1-Mcl-1 was a generous gift from Dr. R.W. Craig (Dartmouth Medical School, Hanover, NH). Plasmids encoding enhanced green fluorescent protein under the transcriptional control of the human cytomegalovirus immediate-early promoter (pEGFP-C2) were obtained from Clontech Laboratories, and the human $p21^{CIP1}$ cDNA was a kind gift from Dr. Stephen Elledge (Harvard University Medical School, Boston, MA). The entire $p21^{CIP1}$ cDNA was inserted in frame into the (COOH-terminal) multiple cloning site of pEGFP-C2. The full-length $p21^{CIP1}$ cDNA in the fusion construct was sequenced and the reading frame was confirmed.

Transient transfections. Transient transfection of K562 cells was carried out using an Amaxa Nucleofector apparatus as per the manufacturer's protocol. Optimized protocols for each cell line were used using transfection kit V. Briefly, cells were cultured for 2 days before transfection but not allowed to grow beyond a concentration of 5×10^5/mL before transfection. One million cells were pelleted by centrifugation and resuspended in 100 μL of transfection reagent of kit V, after which 2.5 μg DNA was added. Resuspended cells in medium containing DNA were transferred to cuvettes and transfected using the Nucleofector using a cell-specific optimized protocol (T-16). Transfected cells were immediately transferred to regular medium (5 mL) and exposed to the indicated drugs after 24 h.

Quantitative real-time PCR. K562 cells were left untreated or treated (with either 7.5 μmol/L sorafenib, 2.0 μmol/L vorinostat alone, or the combination), after which cells were lysed and total RNA was extracted by using RNeasy Mini kit (Qiagen). Quantitative real-time PCR analysis was carried out on the ABI Prism 7900 Sequence Detection System (Applied Biosystems) using the Taqman One-Step PCR Master Mix Reagents kit (polynucleotide: 4309169) as per the recommendation of the manufacturer. Details of the Mcl-1 primers used are explained previously (28). Premixed primer-probe sets of $p21^{CIP1}$ were purchased from Applied Biosystems and used as per the manufacturer's instructions. The probes were labeled at the 5′-end with 6-carboxyfluoresceine and at the 3′-end with 6-carboxytetramethyrhodamine. rRNA (18S rRNA) was used as endogenous control. Each sample was analyzed in triplicate and mRNA concentration was normalized against 18S rRNA.

Reporter gene assay. K562 cells were transfected with 2.5 μg of $p21^{CIP1}$ promoter-luciferase plasmid along with β-galactosidase (for normalizing luciferase activity) using Amaxa Nucleofector apparatus as explained above. Cells were then collected and lysed in promoter lysis buffer (from Promega) and the protein content of the sample was normalized. Luciferase activities of the samples were measured with a dual-luciferase reporter assay kit using a luminometer (model vector 3, Perkin-Elmer). β-Galactosidase content of the cells was also measured using a β-Galactosidase Enzyme Assay kit (from Promega) and used to normalize luciferase activity.

Statistical analysis. The significance of differences between experimental conditions was determined using the two-tailed Student's *t* test. Characterization of synergistic and antagonistic interactions was done using median dose effect analysis in conjunction with a commercially available software program (CalcuSyn, Biosoft; ref. 36).

Results

Sequential treatment with vorinostat followed by sorafenib synergistically induces cell death in K562 and LAMA 84 cells. Interactions between sorafenib and vorinostat were first examined using different schedules of drug administration. Sequential exposure of K562 cells to minimally toxic concentrations of vorinostat (24 h; 1.0-2.0 μmol/L) followed by addition of a modestly toxic concentration of sorafenib (i.e., 5-10 μmol/L) for an additional 24 h resulted in a marked increase in apoptosis (Fig. 1A). Median dose effect analysis of agents administered at a fixed ratio (sorafenib/vorinostat, 3.75:1) revealed combination index values considerably less than 1.0, indicating a synergistic interaction (Fig. 1A, *inset*). Essentially identical results were obtained in LAMA 84 cells (Fig. 1B). Simultaneous exposure of cells to sorafenib and vorinostat resulted in a slightly less pronounced increase in apoptosis, whereas the sequence sorafenib followed by vorinostat did not lead to increased cell killing (data not shown). Consequently, for all subsequent studies, the optimal sequence of vorinostat followed by sorafenib was used.

To determine whether these findings were restricted to vorinostat, parallel studies were done with the HDACIs trichostatin A and sodium butyrate. Pretreatment (24 h) with trichostatin A or sodium butyrate followed by sorafenib (24 h) resulted in a marked increase in lethality (Fig. 1C). A time course analysis revealed that, in K562 cells preexposed to vorinostat, enhanced lethality became apparent 12 h after sorafenib exposure and increased thereafter (Fig. 1D). Finally, clonogenic assays indicated that, whereas individual exposure of K562 cells to vorinostat or sorafenib modestly reduced colony formation, sequential exposure strikingly increased lethality (Fig. 1E).

Sorafenib potentiates vorinostat lethality in imatinib mesylate–resistant cells and in primary CML cell samples. Attempts were then made to extend these findings to include imatinib mesylate–resistant CML cells as well as primary CML cell samples. As shown in Fig. 2A, sequential treatment with 2.0 μmol/L vorinostat (24 h) followed by sorafenib (10 μmol/L; 24 h), exposures that were only modestly toxic by themselves, resulted in a marked increase in apoptosis in CML cells exhibiting a Bcr/Abl-independent form of resistance associated with Lyn activation (33). To determine whether this drug combination exhibited activity toward resistant CML cells expressing a mutant form of Bcr/Abl (T315I) conferring resistance to imatinib mesylate and second-generation inhibitors, such as dasatinib or nilotinib (14, 15), BaF/3 cells expressing wild-type or T315I-mutant Bcr/Abl were exposed to the combination of vorinostat and sorafenib as above. As shown in Fig. 2B, each agent administered individually exhibited only minimal toxicity, whereas a pronounced increase in lethality was observed with combined treatment. Significantly, lethality was equivalent in cells expressing wild-type versus those expressing T315I-mutant Bcr/Abl. In addition, sorafenib (7.5 μmol/L) was essentially nontoxic toward bone marrow $CD34^+$ cells obtained from four patients with CML, including two patients who did not have prior exposure to imatinib mesylate and two patients who had progressed while receiving this agent, whereas vorinostat administered alone exhibited modest to moderate lethality (Fig. 2C). However, combined treatment resulted in a pronounced increase in apoptosis ($P < 0.02$-0.05 versus vorinostat alone). In contrast, sorafenib failed to increase vorinostat lethality normal bone marrow cells obtained from patients undergoing routine diagnostic procedures for nonmalignant hematopoietic disorders ($P > 0.05$ versus vorinostat alone; Fig. 2C).

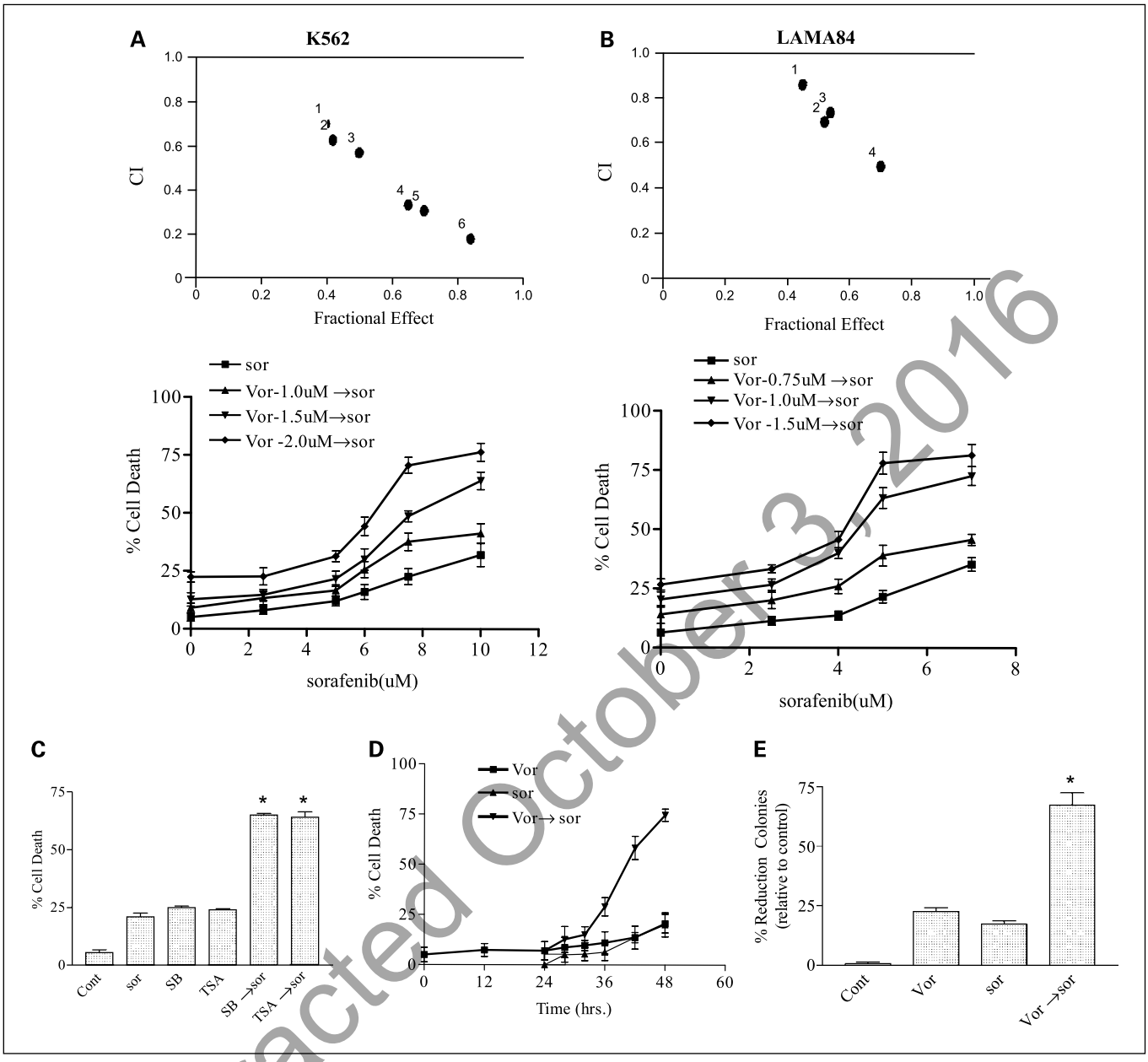

Fig. 1. Sequential treatment of vorinostat followed by sorafenib synergistically induces cell death in K562 and LAMA 84 cells. *A,* K562 cells were exposed to 1.0 to 2.0 μmol/L vorinostat (*Vor*) for 24 h. *B,* LAMA 84 cells were exposed to 0.75 to 1.5 μmol/L vorinostat for 24 h followed by the indicated concentrations of sorafenib (*sor*) for an additional 24 h. The extent of cell death at the end of drug exposure was monitored by flow cytometry with 7-AAD staining. Insets, K562/LAMA 84 cells were sequentially exposed to a range of vorinostat and sorafenib concentrations alone and in combination at a fixed concentration ratio (e.g., 2:7.5 for K562 and 1:5 for LAMA 84 cells). At the end of this period, cell death was monitored as above for each condition. Fractional effect values were determined by comparing results with those of untreated controls, and median dose effect analysis was used to determine combination index (*CI*) values using CalcuSyn software. Combination index values <1.0 denote a synergistic interaction. Two additional studies yielded equivalent results. *C,* K562 cells were treated with 1.0 mmol/L sodium butyrate (*SB*) or 300 nmol/L trichostatin A (*TSA*) for 24 h followed by 7.5 μmol/L sorafenib for an additional 24 h. The extent of cell death at the end of drug exposure was monitored by flow cytometry with 7-AAD staining. *D,* K562 cells were either left untreated or exposed to 2.0 μmol/L vorinostat, 7.5 μmol/L sorafenib, or the combination administered sequentially as indicated in (*A*). The extent of apoptosis for the indicated intervals was monitored by 7-AAD staining as above. *E,* K562 cells were treated with 2.0 μmol/L vorinostat for 24 h followed by 7.5 μmol/L sorafenib for an additional 24 h. At the end of drug treatment, cells were washed and plated in soft agar as described in Materials and Methods. Colonies, consisting of groups ≥50 cells, were scored at day 10. Values for each condition were expressed as a percentage of control colony formation. Columns, mean of triplicate determinations; bars, SD. *, $P < 0.01$, significantly greater than values for cells exposed to HDACIs (sodium butyrate, trichostatin A, and vorinostat) alone.

Sorafenib promotes vorinostat-mediated mitochondrial injury and caspase activation. The effects of sequential exposure of cells to vorinostat and sorafenib were then examined in relation to mitochondrial injury. For these studies, analysis was done 8 h after addition of sorafenib to vorinostat-treated cells (i.e., before the dramatic onset of apoptosis as shown in Fig. 1D). Whereas treatment with vorinostat or sorafenib either had no effect or had a minimal effect on release of cytochrome *c*, AIF, or Smac/DIABLO into the cytoplasmic compartment, cells sequentially exposed to these agents showed a marked increase in mitochondrial injury (Fig. 3A). Consistent with these findings, combined but not individual treatment resulted in a

pronounced increase in cleavage of caspase-3 and caspase-8 and degradation of poly(ADP-ribose) polymerase (Fig. 3B).

Addition of sorafenib to vorinostat-pretreated cells results in the marked down-regulation of ERK1/2 and Mcl-1. The effects of sequential exposure of K562 cells to these agents were then examined in relation to effects on various survival signal proteins. As in the previous studies, analysis was done in vorinostat-pretreated cells 10 h after administration of sorafenib (i.e., before the extensive onset of apoptosis observed at later intervals; Fig. 1D). As observed in the Western blots, individual or sequential treatment did not induce discernible changes in the expression of Bax, Bid, Bak, XIAP, or Bcl-x_L (Fig. 3C). Bcl-2 levels were very low in these cells as previously reported (33) and did not change with treatment (data not shown). In marked contrast, cells exposed to 7.5 μmol/L sorafenib, with or without vorinostat pretreatment, displayed a dramatic reduction in Mcl-1 expression, in accord with previous reports (28, 29). These agents, alone or in combination, had no effect on total Bcr/Abl levels and minimally reduced expression of phosphorylated Bcr/Abl. Consistent with this finding, no significant changes in the level of total or phosphorylated Crkl were observed (Fig. 3C), arguing against inhibition of Bcr/Abl as the basis for synergism between these agents. Expression of total c-Jun NH_2-terminal kinase or phosphorylated c-Jun NH_2-terminal kinase was also not altered by drug treatment. In addition, cells exposed to vorinostat alone exhibited a modest decline in phosphorylated ERK1/2 levels compared with untreated controls. However, exposure of either untreated or vorinostat-pretreated cells to sorafenib abrogated phosphorylated ERK1/2 levels completely, as anticipated.

As previously reported (37), exposure of cells to vorinostat robustly induced $p21^{CIP1}$ expression in K562 cells (Fig. 3C). However, induction of $p21^{CIP1}$ was essentially abrogated following exposure of cells to sorafenib. Lastly, a time course analysis (Fig. 3D) revealed that, in vorinostat-treated cells, addition of sorafenib markedly reduced or abrogated expression of $p21^{CIP1}$ and Mcl-1 within 2 to 6 h of drug exposure (i.e., before the extensive onset of apoptosis; Fig. 1D). Together, these findings indicate that exposure of vorinostat-pretreated cells to sorafenib is associated with the pronounced inactivation

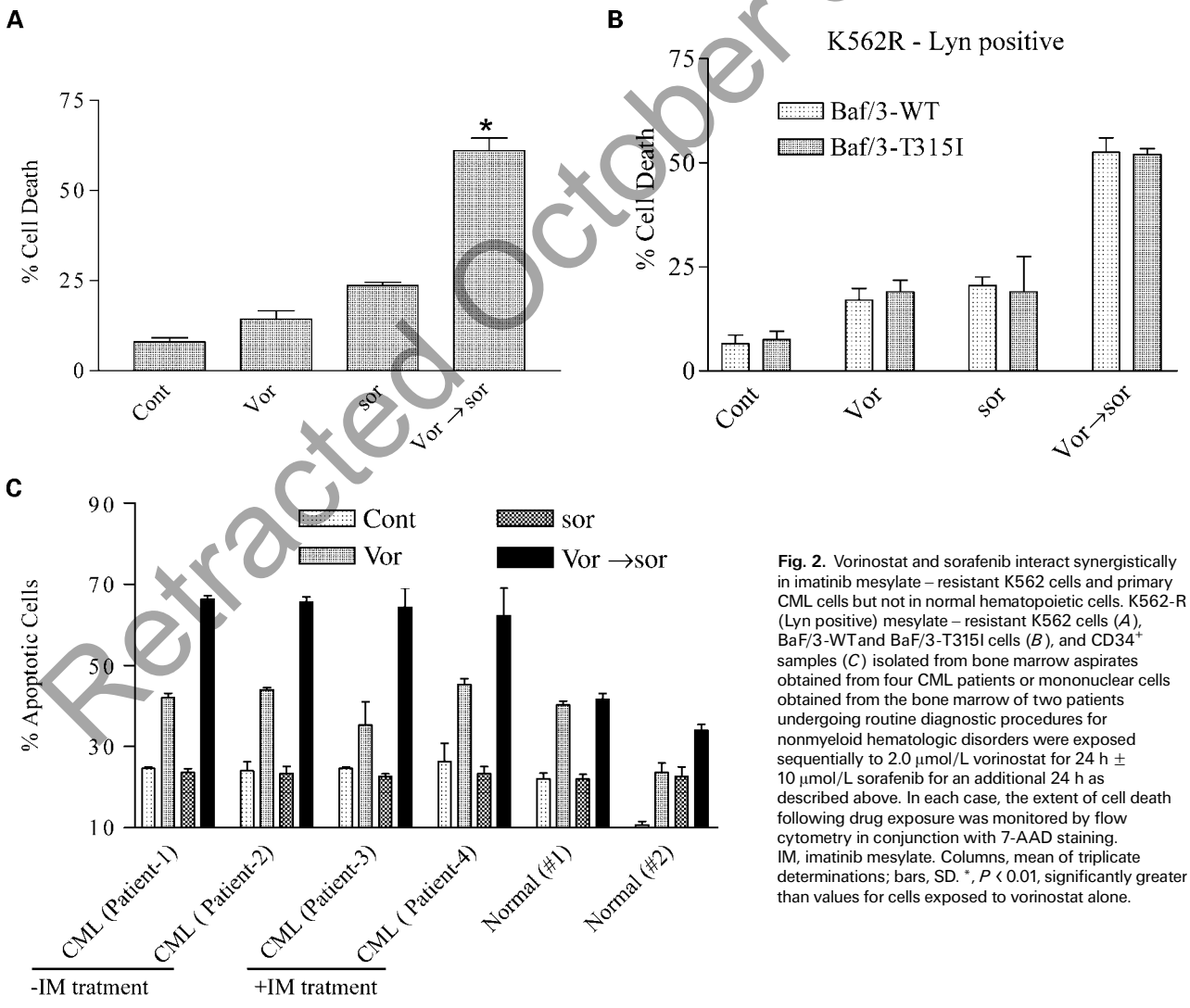

Fig. 2. Vorinostat and sorafenib interact synergistically in imatinib mesylate – resistant K562 cells and primary CML cells but not in normal hematopoietic cells. K562-R (Lyn positive) mesylate – resistant K562 cells (*A*), BaF/3-WT and BaF/3-T315I cells (*B*), and $CD34^+$ samples (*C*) isolated from bone marrow aspirates obtained from four CML patients or mononuclear cells obtained from the bone marrow of two patients undergoing routine diagnostic procedures for nonmyeloid hematologic disorders were exposed sequentially to 2.0 μmol/L vorinostat for 24 h ± 10 μmol/L sorafenib for an additional 24 h as described above. In each case, the extent of cell death following drug exposure was monitored by flow cytometry in conjunction with 7-AAD staining. IM, imatinib mesylate. Columns, mean of triplicate determinations; bars, SD. *, $P < 0.01$, significantly greater than values for cells exposed to vorinostat alone.

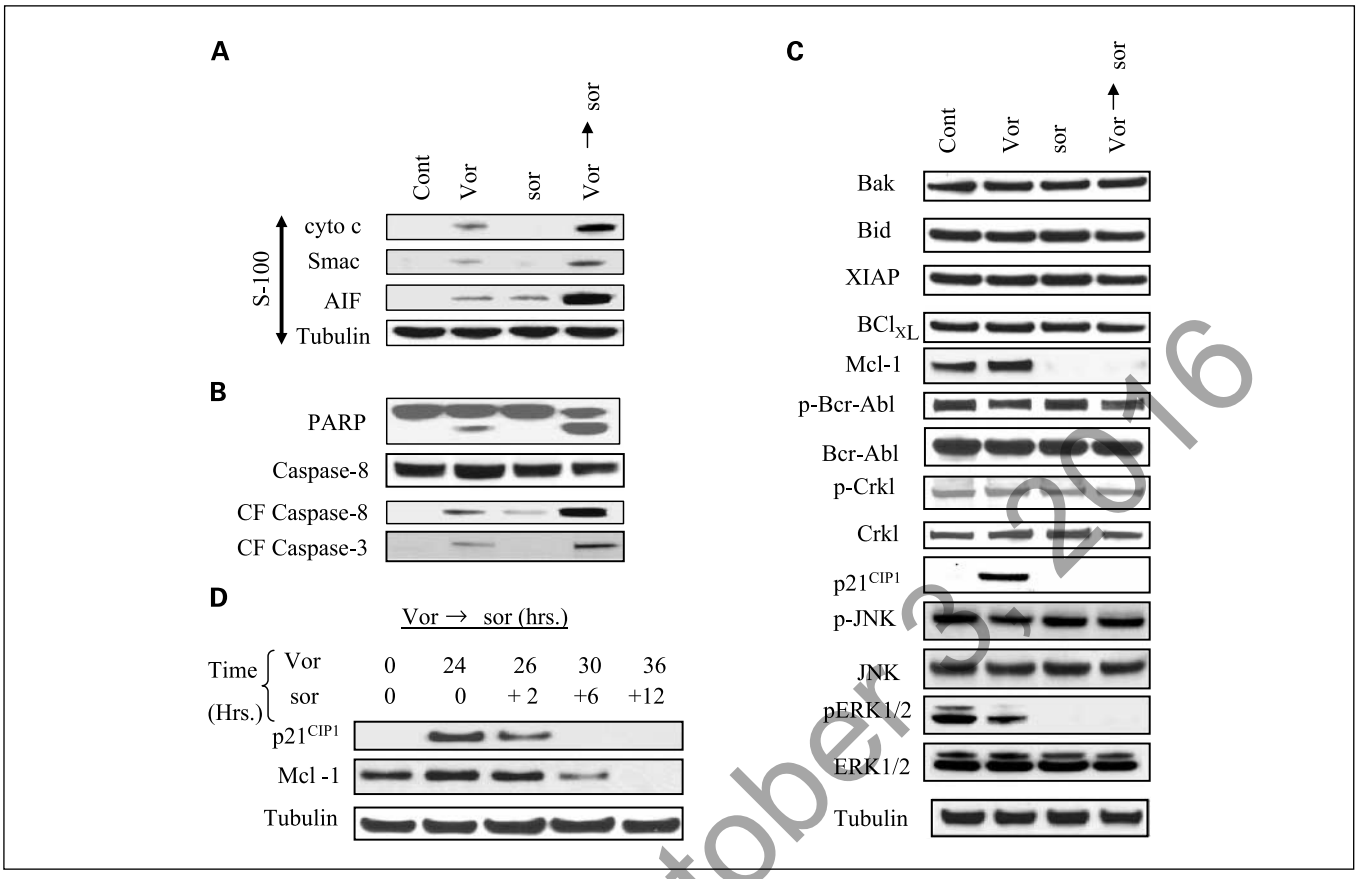

Fig. 3. Combined exposure of K562 cells to vorinostat and sorafenib triggers mitochondrial injury, caspase activation, down-regulation of Mcl-1, and attenuation of p21^{CIP1} induction. K562 cells were treated with either 2.0 μmol/L vorinostat for 34 h, 7.5 μmol/L sorafenib for 10 h, or 2.0 μmol/L vorinostat for 24 h followed by 7.5 μmol/L sorafenib for an additional 10 h. *A,* cytosolic (S-100) fractions were obtained as described in Materials and Methods, and expression of cytochrome *c* (*cyto c*), AIF, and Smac/DIABLO was monitored by Western blot. *B* and *C,* at the end of drug exposure, cells were lysed, sonicated, protein denatured, and subjected to Western blot analysis using the indicated primary antibodies. *D,* K562 cells were either left untreated or treated with 2.0 μmol/L vorinostat or 7.5 μmol/L sorafenib alone or sequentially as described above. Samples were collected at the designated intervals and cells were lysed, sonicated, and subjected to Western blot analysis using the indicated primary antibodies and the proteins were denatured. Blots were stripped and reprobed with anti-tubulin antibodies to ensure equal loading and transfer of protein. Results are representative of three separate studies.

of ERK1/2 and down-regulation of p21^{CIP1} and Mcl-1 and that these events precede the dramatic increase in cell death.

Sorafenib-mediated down-regulation of p21^{CIP1} is partially caspase dependent, whereas Mcl-1 down-regulation is caspase independent. To determine whether alterations in signaling proteins represented primary or secondary events, parallel studies were done in the presence of the pan-caspase inhibitor BOC-fmk. As shown in Fig. 4A, down-regulation of p21^{CIP1} in sorafenib/vorinostat-treated cells was slightly diminished in the presence of BOC-fmk, whereas Mcl-1 down-regulation was unaffected. This suggests that caspase-mediated cleavage may contribute to the reduction in p21^{CIP1} expression, at least in part, but is unlikely to be involved in Mcl-1 down-regulation.

Potentiation of vorinostat lethality by sorafenib involves factors other than or in addition to ERK1/2 inactivation. To investigate whether the ability of sorafenib to promote vorinostat lethality involved inactivation of the MEK1/2/ERK1/2 pathway, parallel studies were done with the MEK1/2 inhibitor PD184352 (38). PD184352 mimicked the capacity of sorafenib to abrogate expression of phosphorylated ERK1/2 in vorinostat-treated cells (Fig. 4B). However, despite the similarities in this response, the vorinostat/sorafenib regimen was very significantly more lethal to K562 cells than the vorinostat/PD184352 regimen ($P < 0.005$; Fig. 4B). This finding argues that factors other than or in addition to MEK1/2/ERK1/2 inactivation contribute functionally to the lethality of the sorafenib/vorinostat regimen.

Inhibition of transcription contributes to down-regulation of p21^{CIP1} but not Mcl-1. Previous studies indicated that sorafenib-mediated down-regulation of Mcl-1 occurred primarily at the translational level (28). To determine whether a similar mechanism was operative in the case of cells treated with vorinostat/sorafenib, real-time reverse transcription-PCR analysis was used. These results indicated that exposure of K562 cells to vorinostat or sorafenib, alone or in combination, had no effect on Mcl-1 mRNA levels (Fig. 5A). However, different results were obtained in the case of p21^{CIP1}. In accord with previous findings, vorinostat alone strikingly increased p21^{CIP1} mRNA levels (Fig. 5B), consistent with evidence that acetylation of the p21^{CIP1} promoter augments transcription (39). However, coadministration of sorafenib dramatically reduced p21^{CIP1} mRNA levels ($P < 0.005$, compared with vorinostat alone). Luciferase reporter assays showed that coadministration of sorafenib significantly diminished p21^{CIP1} reporter activity in

these cells ($P < 0.01$, compared with vorinostat alone; Fig. 5C). Together, these findings suggest that sorafenib-mediated inhibition of transcription may contribute to $p21^{CIP1}$ but not Mcl-1 down-regulation in cells exposed to the sorafenib/vorinostat regimen.

Down-regulation of Mcl-1 and down-regulation of $p21^{CIP1}$ play functional roles in vorinostat/sorafenib-mediated lethality. To determine whether the observed down-regulation of Mcl-1 and down-regulation of $p21^{CIP1}$ play significant functional roles in enhanced lethality of the vorinostat/sorafenib regimen, K562 cells ectopically expressing these proteins were used. To this end, K562 cells expressing empty vector or an Mcl-1–encoding construct were exposed to vorinostat followed by sorafenib as described previously. K562/Mcl-1 cells expressed significantly more Mcl-1 than their control counterparts, and although levels were clearly reduced following drug treatment, they remained significantly higher than those observed in control cells (Fig. 6A). Notably K562/Mcl-1 cells were very significantly less sensitive to the vorinostat/sorafenib regimen than controls ($P < 0.005$; Fig. 6B). Parallel studies were done in K562 cells stably transfected with a GFP-$p21^{CIP1}$ construct. In both control and K562-GFP-$p21^{CIP1}$ cells, endogenous $p21^{CIP1}$ was induced by vorinostat, an effect blocked by sorafenib (Fig. 6C). However, in GFP-$p21^{CIP1}$ cells, basal and vorinostat-induced expression of GFP-$p21^{CIP1}$ was higher in cells transfected with the construct, and sorafenib was less effective in blocking induction of GFP-$p21^{CIP1}$. Notably, cells stably expressing the GFP-$p21^{CIP1}$ construct were modestly but significantly less sensitive to the vorinostat/sorafenib regimen than cells transfected with the empty vector ($P < 0.05$; Fig. 6D). Together, these findings suggest that down-regulation of Mcl-1 and down-regulation of $p21^{CIP1}$ both play significant functional roles in potentiation of vorinostat lethality by sorafenib.

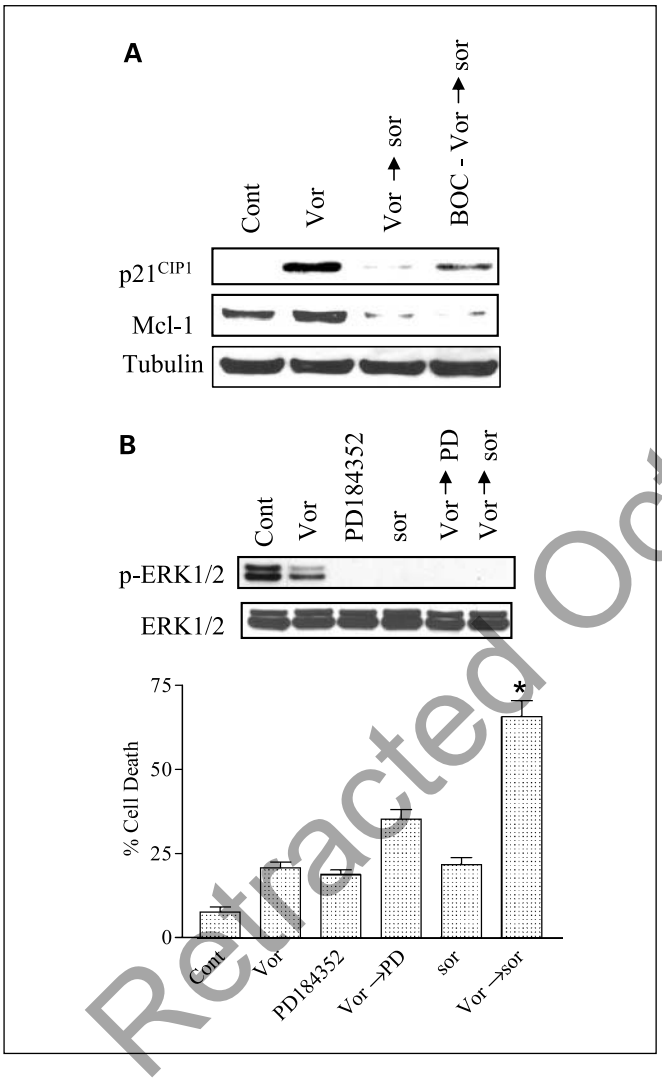

Fig. 4. Sorafenib/vorinostat interactions involve both caspase-dependent and caspase-independent events and are not mimicked by MEK1/2 inhibitors. *A,* K562 cells were left untreated or pretreated with 10 μmol/L BOC-fmk for 30 min followed by treatment with 2.0 μmol/L vorinostat for 34 h, 7.5 μmol/L sorafenib for 10 h, or the sequence of vorinostat followed by sorafenib, after which cells were lysed, sonicated, and subjected to Western blot analysis using the indicated primary antibodies. *B,* K562 cells were either left untreated or treated with 2.0 μmol/L vorinostat for 48 h and 7.5 μmol/L sorafenib or 2.5 μmol/L PD184352 for 24 h or sequentially with vorinostat followed by sorafenib or PD184352. The extent of cell death at the end of drug exposure was monitored by flow cytometry in conjunction with 7-AAD staining. Inset, levels of phosphorylated and total ERK1/2 expression were monitored in K562 cells by Western blot. Columns, mean of triplicate determinations; bars, SD. *, $P < 0.02$, significantly greater than values for cells exposed to vorinostat followed by PD184352.

Discussion

The results of the present study indicate that sorafenib and vorinostat interact in a highly synergistic manner to induce apoptosis in Bcr/Abl^+ human leukemia cells and that this process involves multiple mechanisms, including down-regulation of Mcl-1 and $p21^{CIP1}$. Although efforts to develop novel strategies in CML, particularly in the setting of imatinib mesylate–resistant disease, have recently focused on newer and more effective Bcr/Abl kinase inhibitors (e.g., dasatinib, nilotinib, and MK0457; refs. 14, 15, 40), an alternative and potentially complementary strategy is to use agents, or combination regimens, which operate downstream or independently of the Bcr/Abl kinase. In this regard, HDACIs are of particular interest in that they kill leukemic cells through diverse mechanisms and can down-regulate/inactivate Bcr/Abl by interfering with Hsp90 function, leading to proteasomal degradation of the protein (22). In addition, development of the multikinase and Raf inhibitor sorafenib may be of particular interest given evidence of involvement of ERK1/2 in Bcr/Abl-associated survival signaling (2). In fact, previous studies suggest that MEK1/2 inhibitors can potentiate the lethality of HDACIs in Bcr/Abl^+ human leukemia cells (38). Notably, sorafenib has recently been shown to be highly active in chronic eosinophilic leukemia cells displaying the T674I mutation, which is pathognomonic for this disease, primarily through inhibition of the FIP1L1-platelet-derived growth factor receptor-α kinase (41). However, in the current studies, inhibition of the Bcr/Abl kinase by sorafenib, either alone or in combination with vorinostat, exerted very modest effects on Bcr/Abl expression or activation, reflected by minimal down-regulation of phosphorylated Bcr/Abl or its downstream target Crkl. These findings suggest that the sorafenib/vorinostat regimen kills Bcr/Abl^+ leukemia cells through a mechanism other than inactivation of Bcr/Abl or its downstream effectors.

It is likely that Mcl-1 down-regulation by sorafenib contributed significantly to potentiation of vorinostat lethality in these cells and to the resulting synergistic antileukemic interactions.

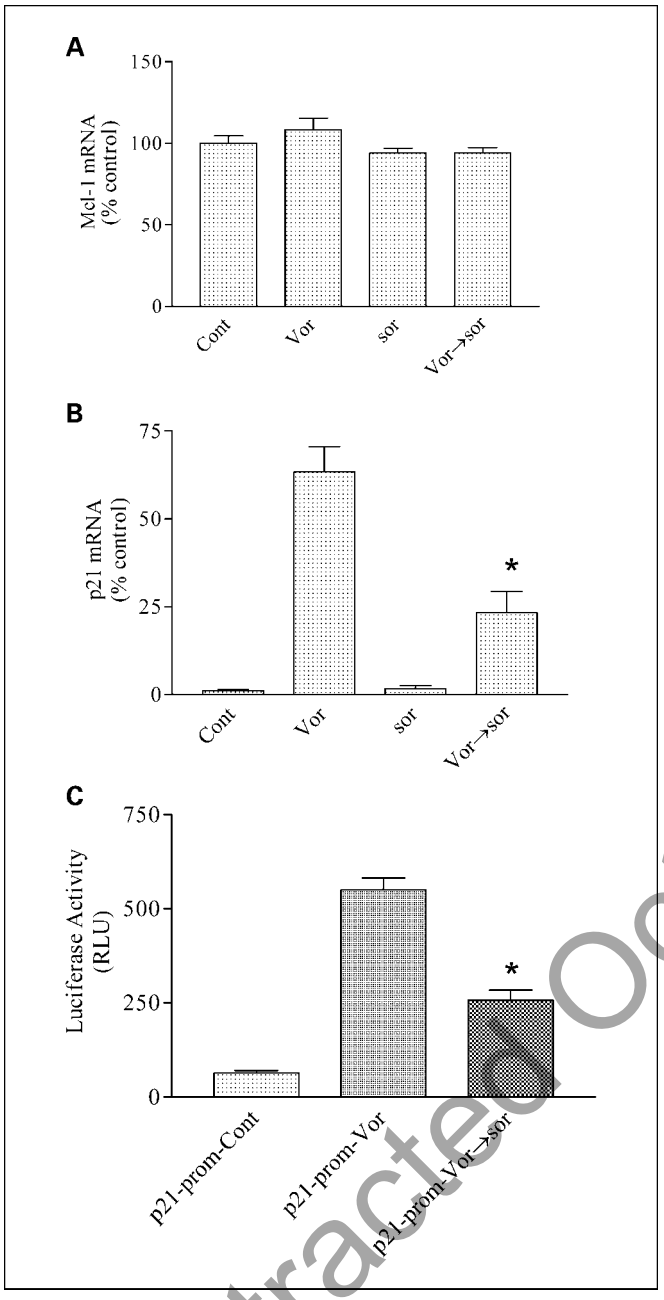

Fig. 5. Mcl-1 down-regulation in vorinostat/sorafenib-treated cells is transcription independent, whereas attenuation of $p21^{CIP1}$ induction is partially transcription dependent. *A,* K562 cells were left untreated or treated with 2.0 μmol/L vorinostat for 34 h, 7.5 μmol/L sorafenib for 10 h, or the two agents in sequence. At the end of drug exposure, total RNA was isolated and mRNA levels of Mcl-1 (*A*) and $p21^{CIP1}$ (*B*) were determined by quantitative real-time PCR as described in Materials and Methods. *C,* K562 cells were transfected with a pGL2-$p21^{CIP1}$ (full-length 2.4 kb) promoter construct with β-galactosidase cDNA and maintained in culture medium for 24 h, after which they were exposed to agents as described above. Following drug exposure, cells were harvested in reporter lysis buffer and luciferase activity was measured and normalized against β-galactosidase as described in Materials and Method. RLU, relative luciferase unit. Columns, mean of triplicate experiments; bars, SD. **, $P > 0.05$, not significantly different from control values; *, $P < 0.01$, significantly less than values for vorinostat-treated cells.

Mcl-1 is a multidomain antiapoptotic member of the Bcl-2 family that acts through several mechanisms to block mitochondrial outer membrane permeabilization and apoptosis (42). For example, Mcl-1 has been shown to cooperate with $Bcl\text{-}x_L$ to tether the proapoptotic multidomain protein Bak and prevent it from interacting with Bax to promote mitochondrial injury (43). The pronounced ability of the vorinostat/sorafenib regimen to induce release of proapoptotic proteins (e.g., cytochrome *c*) is consistent with this view. Several studies have shown that Mcl-1 plays a particularly important functional role in promoting survival in malignant hemapoietic cells, including leukemia and myeloma cells (30). In addition, Mcl-1 is a downstream target of Bcr/Abl in both primary CML cells and Bcr/Abl-dependent cell lines and is down-regulated by Bcr/Abl kinase inhibitors, such as imatinib mesylate (44). Significantly, down-regulation of Mcl-1 (i.e., by antisense oligonucleotides) diminished the survival of Bcr/Abl^+ leukemia cells and lowered the threshold for imatinib mesylate lethality (44). Mcl-1 expression has also been shown to be an important determinant of HDACI lethality and, under some circumstances, is down-regulated by these agents (45). Because Mcl-1 is a short-lived protein (half-life, 2-4 h; ref. 46), it is very susceptible to down-regulation by agents that disrupt transcription or other components of the biosynthetic pathway (e.g., cyclin-dependent kinase inhibitors such as flavopiridol or seliciclib) that disrupt the pTEBb transcription elongation complex (47, 48). In this context, we and other groups have shown that sorafenib kills human leukemia cells, including Bcr/Abl^+ cells, through a mechanism involving Mcl-1 down-regulation (28, 29). A similar phenomenon has been reported in hepatoma cells exposed to this agent (49). Notably, this phenomenon reflected inhibition of Mcl-1 translation associated with diminished phosphorylation of the eiF4E translation initiation factor and was independent of MEK1/2/ERK1/2 inactivation (28). These findings, along with the failure of sorafenib to reduce Mcl-1 reporter activity, suggest that interactions between HDACIs and sorafenib differ fundamentally from those involving cyclin-dependent kinase inhibitors, such as flavopiridol, in which Mcl-1 down-regulation primarily stems from inhibition of transcription (47). Such observations, along with the ability of ectopic expression of Mcl-1 to protect Bcr/Abl^+ leukemic cells from the vorinostat/sorafenib regimen, argue that Mcl-1 down-regulation plays a significant role in the lethality of this drug combination. Whether inhibition of Mcl-1 translation by sorafenib offers advantages over transcriptional repression (i.e., by cyclin-dependent kinase inhibitors) in this setting remains to be determined.

The present findings also suggest that down-regulation of the endogenous cyclin-dependent kinase inhibitor $p21^{CIP1}$ contributed to synergistic interactions between these agents. The ability of HDACIs to acetylate the $p21^{CIP1}$ promoter and induce $p21^{CIP1}$ expression has been described in numerous cell types (37). Furthermore, disruption of $p21^{CIP1}$ expression has been shown to enhance HDACI lethality in various malignant cell types, including those of leukemic origin (50). This phenomenon may stem from disruption of $p21^{CIP1}$-mediated G_1 arrest, interference with its direct antiapoptotic actions (e.g., inhibition of caspase-3 or c-Jun NH_2-terminal kinase activation; refs. 51–53), or a combination of these events. These considerations, in conjunction with the finding that enforced expression of $p21^{CIP1}$ significantly reduced vorinostat/sorafenib lethality, support the notion of a functional role for $p21^{CIP1}$ dysregulation in the observed antileukemic synergism. Furthermore, down-regulation of $p21^{CIP1}$ by sorafenib was at least partially attributable to diminished transcription, a finding that may

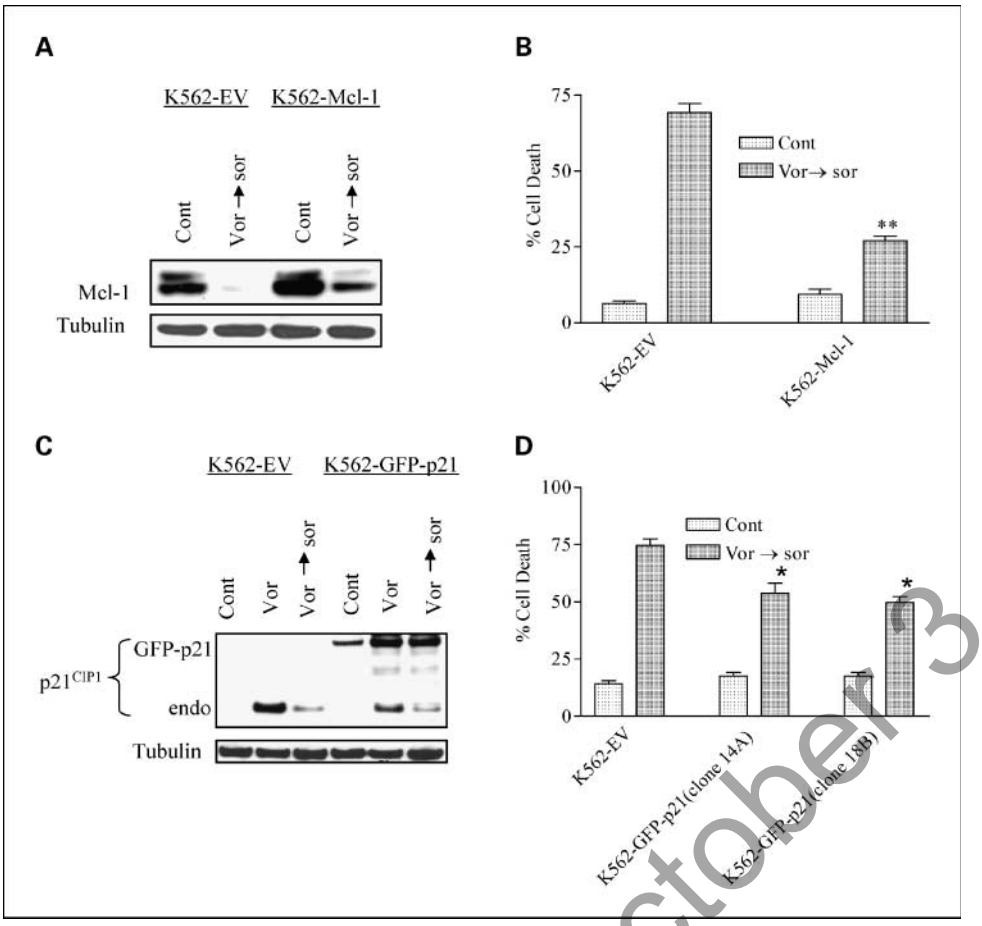

Fig. 6. Enforced expression of Mcl-1 or $p21^{CIP1}$ significantly attenuates the lethality of the vorinostat/sorafenib regimen. *A,* K562 cells were transiently transfected with a pcDNA3.1-Mcl-1 construct or empty vector, and treated with 2.0 μmol/L vorinostat for 24 h followed by addition of 7.5 μmol/L sorafenib for 10 h. After drug exposure, Western blot analysis was used to monitor the effect of drugs on expression of the indicated proteins. Blots were stripped and reprobed with anti-tubulin antibodies to ensure equal loading and transfer of protein. *B,* K562 cells transiently transfected as in (*A*) were exposed to 2.0 μmol/L vorinostat for 24 h followed by addition of 7.5 μmol/L sorafenib for 24 h. Cell viability was then determined by monitoring 7-AAD uptake as described above. *C,* K562 cells were stably transfected with a GFP-$p21^{CIP1}$ construct, and treated with 2.0 μmol/L vorinostat for 24 h followed by addition of 7.5 μmol/L sorafenib for an additional 10 h. After drug exposure, Western blot analysis was used to monitor the effect of drugs on expression of the indicated proteins. Blots were stripped and reprobed with anti-tubulin antibodies to ensure equal loading and transfer of protein. For (*A*) and (*B*), two additional studies yielded equivalent results. *D,* K562 clones stably transfected with a GFP-$p21^{CIP1}$ construct were sequentially exposed to 2.0 μmol/L vorinostat (24 h) followed by 7.5 μmol/L sorafenib for an additional 24 h. Viability was then determined by 7-AAD uptake as explained in Materials and Methods. Columns, mean of triplicate experiments; bars, SD. **, $P < 0.01$, significantly less than values for K562-empty vector (*K562-EV*) cells; *, $P < 0.05$.

reflect inhibition of the MEK1/2/ERK1/2 pathway. For example, $p21^{CIP1}$ transcription is ERK1/2 dependent (54), and MEK1/2 inhibitors have been reported to block HDACI-mediated $p21^{CIP1}$ induction in leukemia cells (38). Thus, sorafenib, by inhibiting Raf, and its downstream targets MEK1/2 and ERK1/2, may mimic the actions of MEK1/2 inhibitors in blocking $p21^{CIP1}$ induction. However, despite similar effects on the MEK1/2/ERK1/2 pathway, sorafenib was significantly more effective than MEK1/2 inhibitors in promoting vorinostat lethality (Fig. 4B), suggesting that other sorafenib actions (e.g., Mcl-1 down-regulation) very likely contribute to its actions. Finally, inasmuch as $p21^{CIP1}$ is a relatively short-lived protein, it is subject to regulation at both the transcriptional and translational/posttranslational levels (55). Consequently, the possibility that sorafenib-mediated translation inhibition (28) contributes to $p21^{CIP1}$ down-regulation cannot be excluded.

That the sorafenib/vorinostat regimen killed leukemia cells exhibiting imatinib mesylate resistance most likely stems from actions that are independent of Bcr/Abl. Although HDACIs have previously been reported to down-regulate expression of Bcr/Abl (56), this phenomenon was not observed with the vorinostat concentrations used in the present study. In addition, although sorafenib has recently been shown to be a potent inhibitor of the FIP1L1-platelet-derived growth factor receptor-α kinase, dysregulation of which is responsible for chronic eosinophilic leukemia (41), sorafenib does not seem to act directly at the level of the Bcr/Abl kinase. Consistent with this view, coadministration of sorafenib with vorinostat had little or no effect on absolute levels or phosphorylation of Bcr/Abl or its downstream target, Crkl (57). In addition, the vorinostat/sorafenib regimen remained active against imatinib mesylate–resistant cells that have lost their dependence on Bcr/Abl (33) or which are resistant due to the existence of the T315I mutation in the Bcr/Abl gatekeeper region, which confers a high degree of resistance to imatinib mesylate as well as newer kinase inhibitors that remain active against other Bcr/Abl mutations (e.g., T351M and E255K; refs. 12, 14, 15). It is likely that the vorinostat/sorafenib regimen induces apoptosis in imatinib mesylate–resistant or imatinib mesylate–independent cells by disrupting pathways downstream of or unrelated to Bcr/Abl, thereby circumventing the need to disrupt Bcr/Abl signaling directly.

In summary, the present findings suggest that a strategy combining the HDACI vorinostat with the multikinase inhibitor sorafenib effectively induces apoptosis in Bcr/Abl^+ leukemia cells through an Mcl-1–dependent and $p21^{CIP1}$-dependent mechanism. The *in vivo* relevance of these findings will depend on several factors, including whether effective plasma concentrations of these agents can be obtained and whether selectivity is achieved. With respect to the first point, peak plasma vorinostat concentrations in excess of 1 μmol/L have been observed in phase I trials (58), and steady-state sorafenib plasma concentrations in excels of 15 μmol/L have

been reported (31). In addition, the failure of sorafenib to enhance the lethality of vorinostat in normal bone marrow cells, in contrast to Bcr/Abl$^+$ cells, suggests that the regimen may not be generically toxic to hematopoietic cells and allows for the possibility of therapeutic selectivity. Collectively, these findings suggest that, in addition to the development of more effective Bcr/Abl kinase inhibitors, including agents such as VX-680, which are active against the T315I Bcr/Abl mutant (40), a strategy combining sorafenib with vorinostat in Bcr/Abl$^+$ hematologic malignancies warrants further examination.

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