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THE MULTIKINASE INHIBITOR SORAFENIB INDUCES APOPTOSIS IN HIGHLY IMATINIB MESYLATE-RESISTANT BCR/ABL^{+} HUMAN LEUKEMIA CELLS IN ASSOCIATION WITH STAT5 INHIBITION AND MCL-1 DOWN-REGULATION

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Running title: Sorafenib in imatinib mesylate-resistant CML.

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Abbreviations:

IM: imatinib mesylate; ERK: extracellular regulated kinase; MEK: mitogen-activated extracellular-regulated kinase; STAT5: signal transducer and activator of transcription 5; Mcl-1: Myeloid cell leukemia-1; eIF4E: eukaryotic initiation factor 4E; Bim: Bcl-2-interacting mediator of cell death.

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ABSTRACT

The effects of the multi-kinase inhibitor BAY 43-9006 (sorafenib), an agent previously shown to induce apoptosis in human leukemia cells through inhibition of Mcl-1 translation, have been examined in Bcr/Abl$^+$ leukemia cells resistant to imatinib mesylate (IM). When administered at pharmacologically relevant concentrations (10-15 μM), sorafenib potently induced apoptosis in imatinib mesylate-resistant cells expressing high levels of Bcr/Abl, cells exhibiting a Bcr/Abl-independent, Lyn-dependent form of resistance, and CD34$^+$ cells obtained from imatinib-resistant patients. In addition, Ba/F3 cells expressing mutations rendering them resistant to IM (e.g., E255K, M351T) or to IM, dasatinib, and nilotinib (T315I) remained fully sensitive to sorafenib. Induction of apoptosis by sorafenib was associated with rapid and pronounced down-regulation of Mcl-1 and diminished STAT5 phosphorylation and reporter activity, but only very modest and delayed inactivation of the Bcr/Abl downstream target Crkl. Moreover, transfection with a constitutively active STAT5 construct partially but significantly protected cells from sorafenib lethality. Ba/F3 cells expressing Bcr/Abl mutations were as sensitive to sorafenib-induced Mcl-1 down-regulation and dephosphorylation of STAT5 and eIF4E as wild-type cells. Finally, stable knockdown of Bim with shRNA in K562 cells significantly diminished sorafenib lethality, arguing strongly for a functional role of this pro-apoptotic Bcl-2 family member in the lethalality of this agent. Together, these findings suggest that sorafenib effectively induces apoptosis in highly imatinib-resistant CML cells, most likely by inhibiting or downregulating targets (i.e., STAT5 and Mcl-1) downstream or independent of Bcr/Abl.

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INTRODUCTION

Chronic myelogenous leukemia (CML) is a stem cell disorder characterized by a reciprocal translocation of the long arms of chromosomes 9 and 22, giving rise to the characteristic Bcr/Abl chimeric fusion protein (Walz and Sattler, 2006). Bcr/Abl is a constitutively active tyrosine kinase which signals downstream to multiple survival signaling pathways, including STAT5, MEK1/2/ERK1/2, and NF-κB which collectively conferring on CML cells a survival advantage (Van Etten, 2004). CML treatment has been revolutionized by the development of Bcr/Abl kinase inhibitors, of which imatinib mesylate (IM;Gleevec) is the prototype. IM traps Bcr/Abl in an inactive configuration and potently inhibits Bcr/Abl and other kinases, including c-Kit and PDGF (Buchdunger *et al.*, 2000). Despite its success in chronic phase disease (Druker *et al.*, 2001), CML patients eventually become refractory to IM through various mechanisms, including diminished drug uptake, *bcr/abl* amplification and/or increased expression of Bcr/Abl, or the development/ preexistence kinase domain mutants that prevent drug binding (Walz and Sattler, 2006). The latter mechanism is most commonly encountered in IM-refractory patients (Walz and Sattler, 2006). To circumvent this problem, novel second generation Bcr/Abl kinase inhibitors (e.g., AMN107; nilotinib and BMS-354825; dasatinib) have been developed which are more potent than IM, and active against most mutations in the phosphorylation loop or ATP-binding site (e.g., E255K, M351T) rendering cells resistant to IM (Talpaz *et al.*, 2006). However, they are ineffective against mutations in the "gatekeeper" region (i.e., T315I) (Talpaz *et al.*, 2006). Consequently, the development of new strategies to eradicate such cells represents a high priority.

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The Raf pathway, which activates the MEK1/2(mitogen-activated protein kinase kinase1/2)/ERK1/2 (extracellular signal-regulated kinase1/2) is frequently dysregulated in human cancer (Davies *et al.*, 2002;Rajagopalan *et al.*, 2002). Attention has recently focused on the multi-kinase inhibitor sorafenib (BAY 43-9006), originally developed as a specific inhibitor of C-Raf and B-Raf (Lyons *et al.*, 2001). However, sorafenib inhibits multiple other kinases, including VEGFR-2, VEGFR-3, PDGFR-β, Flt3, and c-Kit (Wilhelm *et al.*, 2004). Sorafenib is well tolerated when administered with continuous dosing on a daily 200 mg BID schedule (Awada *et al.*, 2005;Strumberg *et al.*, 2005) and inactivates ERK1/2 at these doses. Notably, steady state sorafenib plasma levels of 15-20 μM have been reported (Awada *et al.*, 2005;Strumberg *et al.*, 2005). Recently, several groups, including our own, have reported that sorafenib potently induces apoptosis in human leukemia cells, including Bcr/Abl^+ leukemias, through down-regulation of Mcl-1 (Rahmani *et al.*, 2005a;Yu *et al.*, 2005). Mcl-1, a multi-domain member of the Bcl-2 family, promotes the survival of malignant human hematopoietic cells, including multiple myeloma and leukemia cells (Derenne *et al.*, 2002;Moulding *et al.*, 2000). The mechanism by which sorafenib downregulates Mcl-1 expression involves translation inhibition, a phenomenon associated with dephosphorylation of the eIF4E translation initiation factor (Rahmani *et al.*, 2005a). Notably, sorafenib-mediated down-regulation of Mcl-1 is independent of MEK1/2/ERK1/2 (Rahmani *et al.*, 2005a;Yu *et al.*, 2005), suggesting that the pro-apoptotic effects of sorafenib involve actions other than disruption of Raf and downstream signaling pathways.

Currently, information is lacking concerning the activity of sorafenib against imatinib mesylate-resistant CML cells. Resistance to imatinib mesylate has been characterized as either Bcr/Abl-dependent (Walz and Sattler, 2006; Donato *et al.*, 2003) or Bcr/Abl-independent

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(Donato *et al.*, 2003;Dai *et al.*, 2004). For example, mutations in the Bcr/Abl kinase domain (e.g., T315I) induce steric changes in the kinase domain that prevent drug binding and reduce or abrogate activity (Gorre *et al.*, 2001); nevertheless cells remain dependent upon Bcr/Abl for survival. A logical approach to the eradication of such cells would be the use of alternative small molecules to inhibit mutant Bcr/Abl. Indeed, certain aurora kinase inhibitors (e.g., VX-680) inhibit Bcr/Abl displaying the T315I mutation and effectively kill highly IM-resistant cells (Young *et al.*, 2006). On the other hand, IM resistance associated with loss of Bcr/Abl and increased activity of the Src kinase Lyn has been described (Donato *et al.*, 2003;Dai *et al.*, 2004). Because such cells have lost Bcr/Abl dependence, their elimination may require interruption of other survival pathways. The present studies were prompted by a desire to determine whether sorafenib triggers apoptosis in various IM-resistant Bcr/Abl$^+$ leukemic cells, and to elucidate mechanisms underlying these actions. Our results indicate that sorafenib potently induces apoptosis in Bcr/Abl$^+$ cells highly resistant to IM through diverse mechanisms, including expression of the T315I Bcr/Abl mutation. These events are associated with a rapid inactivation of signal transducer and activator of transcription (STAT)5, a transcriptional factor that plays a critical role in myeloid leukemia cells survival, as well as downregulation of the anti-apoptotic multi-domain Bcl-2 family member, Mcl-1.

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MATERIALS AND METHODS

Cells

Human leukemia K562 cells were cultured as previously reported (Rahmani *et al.*, 2005b). STI571-resistant K562 cells designated K562-STI-R were generated by culturing cells in progressively higher concentrations of STI571. These cells, exhibit an STI571 I.C.$_{50}$ ~ 15-fold greater than parental cells. K562 cells ectopically overexpresing constitutively active STAT5 (Flag-tagged pMX-STAT5A-N642H) and their control empty vector pMX-neo, were previously described (Rahmani *et al.*, 2005b). K562 cells which display a marked reduction in Bcr/abl protein levels (K562-Bcr/Abl^{-}) have been described in detail previously. (Dai *et al.*, 2004). Ba/F3 cells expressing wild type Bcr/Abl (Bcr/Abl-wt) and Bcr/Abl bearing 3 major clinically relevant mutations (E255K, T315I, and M351T) have previously been described (La Rosee *et al.*, 2002).
K562 cells stably expressing short hairpin RNA (shRNA) directed against Bim were generated as follows: Two complementary DNA oligonucleotides containing the previously reported targeted sequence (Malhi et al., 2006; 5'-AATTACCAAGCAGCCGAAGAC-3') were synthesized, annealed, and cloned into pSUPER.retro.neo vector (Oligoengine, Seattle, WA) using standard techniques. An shRNA directed against GFP (ggttatgtacaggaacgca) obtained from Ambion (Austin, TX) was cloned into the pSUPER.retro.neo as described above and served as a control of Bim shRNA expressing cells. The constructs were verified by DNA sequencing and transfected into K562 cells using Amaxa nucleofector™. Stable clones were selected in the presence of 400 µg/ml G418 and screened for reduced Bim expression by Western blot analysis.

Isolation of patient-derived leukemic CD34^{+} mononuclear cells

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Bone marrow cells were obtained with informed consent during routine diagnostic procedures from four patients with CML who were treated with IM and displayed progressive disease. Bcr/Abl mutational analysis was performed on two of these samples and did not reveal known mutations. These studies have been sanctioned by the Investigational Review Board of Virginia Commonwealth University/Medical College of Viriginia. Mononuclear cells were isolated by Ficoll-Hypaque density gradient separation. Mononuclear cells from patients with CML were enriched for $CD34^+$ cells using a Miltenyi microbead separation system (Miltenyi BioTech; Auburn, CA) according to the manufacturer's protocol. Leukemic $CD34^+$ mononuclear cells were then diluted into RPMI medium containing 10% fetal calf serum at a concentration of 10^6 cells/ml, and exposed to drugs as described in the case of continuously cultured cell lines.

Reagents

Sorafenib was provided by Bayer Pharmaceuticals Corporation (West Haven, CT) and the National Cancer Institute, NIH (Bethesda, MD). It was dissolved in DMSO and aliquots were maintained at

-80^{o}C.

Assessment of apoptosis

Apoptotic cells were routinely identified by Annexin V-FITC staining as previously described (Rahmani *et al.*, 2002). Briefly, 10^5 cells were collected, washed in cold phosphate-buffered saline (PBS), and then resuspended in binding buffer (10 mM Hepes/NaOH, pH7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) containing Fluorescein-labeled annexin V (PharMingen) and PI. Samples were incubated for 15 min and then analyzed by flow cytometer (Becton Dickinson FACScan).

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Transient transfection and Reporter gene assay

Transient transfection was performed using Amaxa nucleofector™ (Koeln, Germany) as previously described (Rahmani *et al.*, 2005b). To determine the transcriptional activity of STAT5, K562 cells were cotransfected with STAT5-luc or its control counterpart TA-luc plasmids encoding firefly luciferase (Panomix, CA) and pRL-TK-luc plasmid encoding for Renilla luciferase using the Amaxa nucleofector™. Cells were incubated for 6 hr and then treated with indicated agents for an additional 16 hr after which, activity of firefly and Renilla luciferases were measured using the Dual-Luciferase reporter assay system (Promega). Values of firefly luciferase activity were normalized to those obtained for Renilla luciferase activity. Then, the ratios obtained for STAT5-luc were divided by those obtained for TA-luc.

Immunoblotting

Immunoblotting was performed using whole cells lysates prepared as previously described in detail (Rahmani *et al.*, 2002). The primary antibodies used in this study were: Mcl-1 (PharMingen; San Diego, CA). Poly(ADP-ribose) Polymerase (PARP) (Biomol Research Laboratories, Plymouth Meeting, PA). Phospho-STAT5 (Tyr694) and phospho-eIF4E (Ser209, Cell Signaling Technology). α-tubulin (Calbiochem).

Statistical analysis

The significance of differences between experimental conditions was determined using the student's t test for unpaired observations.

RESULTS

Imantinb mesylate-resistant cells exhibiting increased Bcr/Abl expression or a Bcr/Abl-independent form of resistance remain sensitive to Sorafenib

Previous studies indicated that wild-type Bcr/Abl$^+$ cells were susceptible to sorafenib-induced apoptosis (Yu *et al.*, 2005;Rahmani *et al.*, 2005a). Attempts were therefore undertaken to determine whether sorafenib might be active against cells resistant to imatinib mesylate through various mechanisms. To this end, sorafenib dose-response curves were compared in three cell types: wild type K562 cells, K562 cells cultured in progressively higher concentrations of IM as previously described (Yu *et al.*, 2002) and which exhibited approximately a four to five-fold increase in Bcr/Abl protein; and imatinib-resistant K562 cells which displayed a reduction in Bcr/Abl expression accompanied by an increase in Lyn activation (Dai *et al.*, 2004) (Figure. 1A). The latter two cell types have previously been shown to exhibit a marked reduction in IM sensitivity (Yu *et al.*, 2002;Dai *et al.*, 2004). However, dose response curves for sorafenib concentrations of 5-20 μM were identical for the three cell lines ($P > 0.05$ in each case; Figure 1B). Thus, IM-resistant K562 cells displaying increased Bcr/Abl expression or the development of a Bcr/Abl-independent form of resistance remained fully sensitive to sorafenib.

Imatinib mesylate-resistant Bcr/Abl$^+$ cells expressing Bcr/Abl kinase mutations remain sensitive to sorafenib.

To determine whether cells displaying various Bcr/Abl mutations rendering them resistant to imatinib would remain sensitive to sorafenib, Ba/F3 cells transfected with wild-type, Bcr/Abl-E255K, Bcr/Abl-T315I, or Bcr/Abl-M351T were employed. Consistent with previous reports (La Rosee *et al.*, 2002), each of the mutant cell lines was highly resistant to IM (1 μM) administered for 24 or 48 hr (Figure 2A). In contrast, there was little or no cross-resistance to sorafenib administered at concentrations of 2.5-20 μM for 24 (Figure 2B) and 2.5-15 μM for 48 hr (Figure 2C). Consistent with these results, sorafenib (10 μM) exerted similar effects on PARP degradation in each of the cell lines after 24 hr (Figure 2D) or 48 hr drug exposure (data not shown). These findings indicate that Bcr/Abl mutations conferring a high degree of resistance to IM, including the T315I mutation which effectively protects cells from agents such as dasatinib and AMN107 (Talpaz *et al.*, 2006;von Bubnoff *et al.*, 2006), fail to attenuate sorafenib-induced apoptosis.

Sorafenib inhibits STAT5 phosphorylation and activity through a MEK1/2/ERK1/2-independent mechanism

The STAT5 signaling pathway is activated by Bcr/Abl, and plays an important role in Bcr/Abl-mediated leukemogenic actions (de Groot *et al.*, 1999). Furthermore, other Bcr/Abl kinase inhibitors have been shown to inactivate STAT5 (Huang *et al.*, 2002;Fiskus *et al.*, 2006). Consequently, the effects of sorafenib on STAT5 activation were examined. As shown in Figure 3A, exposure of K562 cells to sorafenib induced a rapid (i.e., within 2 hr) inactivation of STAT5, which persisted throughout the entire exposure interval (16 hr, a period in which 40% of cells were apoptotic, data not shown). Levels of total STAT5 declined only slightly during this interval. In contrast, dephosphorylation of Crkl, a known target of Bcr/Abl (ten Hoeve *et al.*, 1994), was only modest, and was most prominent at later exposure intervals (e.g., 16 hr). Further analysis comparing the effects of sorafenib and the MEK1/2 inhibitors U0126 and PD184352 on phosphorylation of ERK1/2 and STAT5 in K562 cells was then performed (Figure 3B). While 5

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μM U0126 or PD184352 were more potent than sorafenib in diminishing ERK1/2 phosphorylation, neither U0126 nor PD184352 decreased STAT5 phosphorylation. Instead, these agents induced, if anything, a modest increase in STAT5 phosphorylation. Such findings are in accord with results of a very recent report suggesting that MEK1 negatively regulates JAK2 by enhancing phosphorylation at serine 523, an event associated with diminished STAT5 phosphorylation (Mazurkiewicz-Munoz *et al.*, 2006). Furthermore, STAT5 transcriptional activity monitored by luciferase reporter assays revealed that sorafenib (7.5 μM) blocked STAT5 activity to a similar extent as 1 μM IM (Figure 3C), whereas U0126 or PD184352 failed to diminish STAT5 activity. Together, these findings suggest that sorafenib triggers a rapid dephosphorylation and inactivation of STAT5, and that this phenomenon operates independently of the MEK1/2/ERK1/2 pathway.

STAT5 inactivation in Bcr/Abl$^+$ cells by sorafenib plays a functional role in lethality

To determine the functional significance of these findings, K562 cells were stably transfected with plasmids encoding a constitutively active form of STAT5 (pMX-STAT5A-N642H). In this construct, the asparagine residue at position 642 was mutated to histidine, a mutation known to be associated with high DNA binding and transactivation activities (Ariyoshi et al., 2000}. Two clones expressing the constitutively active STAT5, designated K562 cl4 and K562 cl18, were isolated (Figure 3D upper panel). Notably, these clones were significantly more resistant to sorafenib-mediated lethality when administered at either 7.5 or 10 μM ($P < 0.05$ or 0.02; Figure 3D lower panel). Together, these findings suggest that inactivation of STAT5 contributes functionally to sorafenib-mediated lethality in Bcr/Abl$^+$ cells. They also raise the possibility that STAT5 inactivation may represent a marker for sorafenib activity in these cells.

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Sorafenib induces Mcl-1 down-regulation, STAT5 inactivation, and eIF4E dephosphorylation in imatinib mesylate-resistant cells exhibiting Bcr/Abl kinase mutations

To determine whether these events also occurred in Bcr/Abl^{+} cells expressing various Bcr/Abl mutants, wild-type and mutant Ba/F3 cells were exposed to10 μM sorafenib for 2-24 hr, after which expression of phospho-STAT5 was monitored. Expression of the anti-apoptotic protein Mcl-1, a downstream target of STAT5 (Aichberger et al., 2005) which we have also shown to be down-regulated at the translational level by sorafenib (Rahmani *et al.*, 2005a), was monitored in parallel. As shown in Figures 4A-D, 10 μM sorafenib rapidly (e.g., within 2 hr) induced inactivation of STAT5 in each of the cell lines. While a slight rebound phenomenon was observed in E255K cells, activity in all cases was essentially abrogated after 24 hr. In addition, Mcl-1 expression was also rapidly down-regulated in cells expressing wild-type or each of the Bcr/Abl mutant proteins, and was largely complete after 8 hr of exposure. Furthermore, a rapid and pronouonced dephosphorylation of the translation initiation factor eIF4E was observed in Ba/F3 expressing wild type as well as mutant Bcr/Abl after sorafenib exposure. Thus, sorafenib rapidly and potently inactivated STAT5, dephosphorylated eIF4E, and down-regulated Mcl-1 expression in cells expressing mutant forms of Bcr/Abl which confer marked resistance to IM.

Sorafenib-mediated cell death involves BH3 domain only Bim.

Extensive evidences indicate that anti-apoptotic activity of Mcl-1 involves its interaction with and blockade of the proapoptotic BH3 domain only Bim (Gomez-Bougie et al., 2005). Given the finding that sorafenib downregulates Mcl-1, the possibility that Bim might play a functional role in sorafenib-mediated lethality appeared plausible. To test this hypothesis, K562 cells were

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stably transfected with constructs encoding for shRNA directed against Bim. As shown in Figure 5A, two clones exhibiting significant knockdown of Bim proteins were employed. Notably, cells in which Bim was knocked down were significantly more resistant to sorafenib mediated lethality than their control counterparts (Figure. 5B). These findings suggest that Bim plays a functional role in sorafenib-mediated lethality in K562 cells.

Sorafenib induces apoptosis in primary $CD34^+$ bone marrow cells obtained from patients who progressed on imatinib mesylate

Finally, the effects of sorafenib were examined with respect to apoptosis induction in $CD34^+$ bone marrow cells from 4 patients who experienced disease progression while receiving IM. As shown in Figure 6A, exposure to 10 or 15 μM sorafenib for 48 hr markedly increased apoptosis in three of four patients samples (e.g., 60-95%; $P < 0.01$ versus controls), whereas only the 15 μM concentration was effective in the fourth patient specimen. In separate studies, exposure of normal bone marrow $CD34^+$ to 10 or 15 μM sorafenib (48 hr) resulted in only a minor increase in apoptosis (Figure. 6B). These findings indicate that a clinically relevant concentration of sorafenib can induce apoptosis *in vitro* in primary $CD34^+$ cells from patients with CML, including those who have developed resistance to IM.

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DISCUSSION

Despite the success of IM in Bcr/Abl$^+$ leukemias, the development of drug resistance represents a significant barrier to cure (Walz and Sattler, 2006). Moreover, patients in accelerated or blast phase CML are relatively refractory to this agent. The development of second generation Bcr/Abl kinase inhibitors such as nilotinib and dasatinib represent a significant advance, because in addition to their enhanced activity, such agents remain active against leukemia cells bearing most Bcr/Abl mutations, including those residing in the activation loop and ATP-binding domain (Talpaz *et al.*, 2006;Walz and Sattler, 2006). However, these agents are unable to bind to Bcr/Abl exhibiting mutations in the gatekeeper region (e.g., T315I) (Talpaz *et al.*, 2006;O'Hare *et al.*, 2005;von Bubnoff *et al.*, 2006), raising the possibility that cells expressing this or related mutations will be selected for during therapy with second generation kinase inhibitors. Indeed, the appearance of this and related mutations has been observed in preliminary trials involving these agents (Talpaz *et al.*, 2006;von Bubnoff *et al.*, 2006). Therefore, a search for alternative strategies capable of eradicating cells bearing such mutations is clearly justified.

Previously, several groups, including our own, reported that sorafenib potently induced apoptosis in human leukemia cells, including Bcr/Abl$^+$ leukemias, through a mechanism involving down-regulation of Mcl-1 (Rahmani *et al.*, 2005a;Yu *et al.*, 2005). Furthermore, we demonstrated that this process stemmed from inhibition of Mcl-1 translation, a process associated with diminished phosphorylation of the eIF4E translation initiation factor. Recent studies suggest that Mcl-1 cooperates with Bcl-x_L to tether the proapoptotic proteins Bak and

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Bim, a multi-domain and BH3 only-domain protein respectively, and prevent their activation (Willis *et al.*, 2005; Gomez-Bougie et al., 2005). As Bcl-x_L is a well described downstream target of Bcr/Abl, it is tempting to speculate that interventions that downregulate both Mcl-1 and Bcl-x_L might act through this mechanism. However, in our earlier study, no major changes in Bcl-x_L protein levels were noted in sorafenib-treated cells, at least during the first 8 hr of treatment, nor did significant changes occur in the expression of anti-apoptotic proteins other than Mcl-1(Rahmani *et al.*, 2005a). Moreover, similar findings were observed in Bcr/Abl mutants (Rahmani and Grant, unpublished results), arguing against this possibility. The notion that sorafenib down-regulates Mcl-1 expression in Bcr/Abl$^+$ leukemia cells by multiple mechanisms cannot be excluded. For example, Mcl-1 expression is regulated at the transcriptional level by STAT5 (Aichberger et al., 2005), a downstream target of Bcr/Abl that has been implicated in Bcr/Abl-related leukemogenesis (de Groot *et al.*, 1999). In addition, previous studies demonstrated that sorafenib potently and rapidly diminishes expression of Mcl-1 in Bcr/Abl- leukemia cells primarily through translation inhibition (Rahmani *et al.*, 2005a). Thus, while sorafenib-mediated disruption of Bcr/Abl and STAT5 function cannot be excluded as contributing factors in Mcl-1 down-regulation, it is likely that interference with Mcl-1 translation represents the predominant mode of action. The finding that knockdown of Bim significantly diminished sorafenib lethality in K562 cells argues strongly for a functional role of this proapoptotic Bcl-2 family member in sorafenib-mediated lethality. In this regard, the bulk of evidence indicates that Mcl-1 physically interacts with Bim and blocks its proapoptotic activity (Gomez-Bougie et al., 2005). It is therefore conceivable that downregulation of Mcl-1 might lead to an increase in free Bim protein, thereby enhancing its proapoptotic activity. Additionally, other studies have shown that Mcl-1 down-regulation by itself may be sufficient to trigger

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apoptosis in certain transformed cells (Derenne *et al.*, 2002;Moulding *et al.*, 2000). In any case, to the extent that Mcl-1 down-regulation occurs independently of Bcr/Abl, such a mechanism would be operative in cells resistant to IM through multiple mechanisms, including increased expression of Bcr/Abl, or diverse mutations in the kinase domain. Indeed, each of the resistant cell types examined in this study displayed roughly equivalent sensitivity to sorafenib-induced lethality. Moreover, sorafenib was equally effective in blocking eIF4E phosphorylation in imatinib-sensitive and –resistant cells, including those expressing the T315I mutation.

The results of this study indicate, for the first time, that sorafenib induces a rapid and pronounced dephosphorylation of STAT5, a major survival transcription factor in myeloid leukemia cells (de Groot *et al.*, 1999). This was associated with diminished STAT5 activity as observed in cells exposed to IM, but in striking contrast to the actions of the MEK1/2 inhibitors U0126 and PD184352, which failed to diminish STAT5 phosphorylation or activity. This suggests that sorafenib inactivates STAT5 through a MEK1/2/ERK1/2-independent mechanism. Moreover, the capacity of constitutively active STAT5 to protect Bcr/Abl$^+$ cells from sorafenib lethality, argues that STAT5 inactivation plays a significant functional role in sorafenib-induced apoptosis. Notably, sorafenib down-regulated phospho-STAT5 levels in cells both sensitive and resistant to IM, including those bearing the T315I mutation. The finding that Sorafenib rapidly and profoundly diminished STAT5 phosphorylation while the Bcr/Abl downstream target Crkl (ten Hoeve *et al.*, 1994) was minimally affected suggests that sorafenib disrupts STAT5 signaling through a Bcr/Abl-independent mechanism. In this context, STAT5 is known to be phosphorylated by Janus tyrosine kinase (JAK2) as well as the non-receptor tyrosine kinase Src, members of kinase families that lie downstream of multiple tyrosine kinase receptors including

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PDGFR, VEGFR, which are recognized targets of sorafenib (Wilhelm et al., 2004). It is therefore possible that sorafenib inactivates STAT5 through a mechanism involving inhibition of PDGFR and VEGFR and their downstream kinases JAK2 and Src. However, the contribution of other mechanisms to this phenomenon cannot be excluded, and clearly additional studies will be required to resolve these issues.

The bulk of evidence from this and our earlier study (Rahmani *et al.*, 2005a) suggest that sorafenib acts independently of Bcr/Abl to induce apoptosis. Previously, we observed that sorafenib modestly diminished expression of total and phospho-Bcr/Abl in wild-type CML cells (Rahmani *et al.*, 2005a), effects which most likely reflect a reduction in Bcr/Abl translation. The finding that sorafenib diminished phosphorylation of Crkl only modestly, and at relatively late intervals, also argues against direct inhibition of Bcr/Abl as a primary mechanism of lethality. Thus, the actions of sorafenib stand in marked contrast to those of other tyrosine kinase inhibitors recently found to be active in IM-resistant leukemia cells. For example, the tyrphostin adaphostin (NSC680410) has been shown to inactivate/down-regulate Bcr/Abl in Bcr/Abl mutant cells, including those expressing T315I (Chandra *et al.*, 2006). On the other hand, the lethality of adaphostin in these cells stems from induction of oxidative damage (i.e., ROS generation), a phenomenon that may be independent of effects on Bcr/Abl (Chandra *et al.*, 2006). More recently, the aurora kinase inhibitor VX-680 has shown activity against patient-derived CML cells exhibiting the T315I mutation (Young *et al.*, 2006). This capacity is felt to stem from the ability of VX-680 to bind to the active form of the T315I variant Bcr/Abl, and to prevent phosphorylation of the activation loop (Young *et al.*, 2006). The present results also differ sharply from those of a very recent report demonstrating that the growth of hematopoietic cells

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bearing constitutively active FIP1L1-PDGFRα, the oncogenic kinase responsible for chronic eosinophilic leukemia (CEL) (Lierman *et al.*, 2006), was extremely sensitive to sorafenib. Notably, sorafenib was also highly active against IM-resistant cells expressing the FIP1L1-PDGFRα T647I mutation, which is similar to the T315I Bcr/Abl mutation (Lierman *et al.*, 2006). In this setting, sorafenib acts directly on the oncogenic kinase to inhibit cell survival. In striking contrast, the present results suggest that in cells bearing Bcr/Abl mutations rendering them resistant to IM, sorafenib acts downstream and/or independently of the Bcr/Abl kinase, rather than inhibiting it directly, and is therefore able to kill cells resistant to second generation Bcr/Abl kinase inhibitors such as dasatinib and nilotinib (e.g., those bearing the T315I mutation) (Talpaz *et al.*, 2006; von Bubnoff *et al.*, 2006).

In summary, the present findings suggest that as in the case of IM-resistant chronic eosinophilic leukemia (CEL) cells (Lierman *et al.*, 2006), CML cells resistant to IM may remain susceptible to sorafenib, albeit through a fundamentally different mechanism. While sorafenib kills IM-resistant CEL cells by inhibiting FIP1L1-PDGFRalpha bearing the T674I mutation that confers resistance, it appears to induce apoptosis in IM-resistant CML cells through a Bcr/Abl-independent mechanism. It is likely that activation of a distinct death pathway involving disruption of Mcl-1 translation and inhibition of STAT5 contributes significantly to this phenomenon. An important consideration is whether sorafenib will be able to eradicate CML stem cells postulated to account for disease recurrence following therapy. For example, imatinib mesylate has been shown to be relatively ineffective in eliminating such stem cells (Graham *et al.*, 2002), and recent studies suggest that newer generation kinase inhibitors (e.g., dasatinib) may also have limited activity against these cells (Copland *et al.*, 2006). In this context, the

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dependence of hematopoietic stem cells on Mcl-1 for survival (Opferman *et al.*, 2005) may be relevant. In any case, the present results suggest that investigation of sorafenib as an agent capable of eradicating IM-resistant CML cells, either alone, or perhaps in combination with other agents, deserves further consideration. Accordingly, studies addressing this issue are currently underway.

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FIGURE LEGENDS

Fig. 1. Sorafenib is active against Bcr/Abl-overexpressing K562 cells as well as K562 cells displaying a Bcr/Abl-independent form of IM resistance.

A) Western blot analysis comparing levels of Bcr/Abl in three cell lines: Parental K562 (K562P), imatinib mesylate-resistant K562 cells displaying a 4-5-fold increase in Bcr/Abl protein levels (K562-STI-R), and IM-resistant K562 displaying a decline in Bcr/Abl levels (K562-Bcr/abl^{-}). B) Each of the K562 cell lines was treated with the designated concentration of sorafenib for 48 hr, after which extent of apoptosis was determined using an annexin V staining/flow cytometric assay. Values represent the means ± S.D. for three separate experiments performed in triplicate.

Fig. 2. Sorafenib induces apoptosis in Ba/F3 cells expressing wild type and mutant Bcr/abl .

Ba/F3 cells expressing wild type Bcr/Abl (Bcr/Abl-wt) or mutant Bcr/Abl bearing one of three clinically relevant mutations conferring imatinib mesylate resistance (i.e., E255K, T315I, or M351T) were exposed to 1 μM imatinib mesylate (IM) for 24 or 48 hr (A) or to the designated concentration of sorafenib for 24 hr (B) or 48 hr (C), after which extent of apoptosis was determined by flow cytometry using the annexin V staining assay. Values represent the means ± S.D. for three separate experiments performed in triplicate. (D) Ba/F3 cells expressing wild type or mutant Bcr/Abl were exposed to the designated concentration of sorafenib for 24 hr after which protein lysates were prepared and subjected to Western blot analysis to monitor PARP cleavage. Each lane was loaded with 20 μg of protein; blots were subsequently reproped with antibodies to tubulin to document equivalent loading and transfer. Results of a representative study are shown; additional studies yielded equivalent findings.

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Fig. 3. Exposure to sorafenib results in STAT5 inactivation, while ectopic expression of a constitutively active STAT5 construct significantly reduces sorafenib mediated cell death. A) K562 cells were treated with 7.5 μM sorafenib for the designated intervals after which protein lysates were prepared and subjected to Western blot analysis. B) K562 cells were treated with 7.5 μM sorafenib, 5 μM U0126, or PD184352 for 2 hr after which protein lysates were prepared and subjected to Western blot analysis to monitor STAT5 and ERK1/2 phosphorylation. C) K562 cells were co-transfected with STAT5-luc or TA-luc and pRL-TK-luc plasmids, and treated with sorafenib (7.5 μM) or IM (1 μM) for 16 hr, after which firefly and Renilla luciferase activities were determined and analyzed as indicated in Methods. Values were expressed as a percentage relative to that obtained in untreated controls. D) upper panel: Proteins were extracted from 2 clones (cl4 and cl18) of K562 cells expressing constitutively active STAT5A (CA-STAT5A) or empty vector (pMX-neo), and subjected to Western blot analysis using anti-Flag M2 antibody. Each lane was loaded with 20 μg of protein; blots were subsequently reproped with antibodies to tubulin to document equivalent loading and transfer. Results of a representative study are shown; additional studies yielded equivalent findings. D) lower panel: Constitutively active STAT5A cells (CA-STAT5A-cl4 and CA-STAT5A-cl18) and empty vector cells pMX-neo were treated with 7.5 μM sorafenib for 24 hr after which extent of cell death was monitored using annexin V staining assay. Values represent the means ± S.D. for at least three separate experiments performed in triplicate. * and ** = significantly lower than values obtained for pMX-neo cells ($p < 0.05$ and $p < 0.02$ respectively)

Fig. 4. Exposure to sorafenib results in dephosphorylation of STAT5 and eIF4E, and downregulation of Mcl-1 in Ba/F3 expressing wild type as well as mutant Bcr/Abl.

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Ba/F3 cells expressing wild type Bcr/abl (Bcr/abl-wt) or mutated forms of Bcr/Abl (E255K, T315I, or M351T) were exposed to 10 μM sorafenib for the designated intervals after which cells were lysed and Western blot performed to monitor cleavage of PARP, phosphorylation of STAT5 and eIF4E, and levels of Mcl-1 protein. Each lane was loaded with 20 μg of protein; blots were subsequently reproped with antibodies to tubulin to document equivalent loading and transfer. The blots shown are representative of three separate experiments.

Fig. 5. Sorafenib-mediated lethality involves the BH3 only protein Bim.

(A) Protein lysates were prepared from two clones (Bim-shRNA4 Bim-shRNA10) of K562 cells stably transfected with shRNA construct against Bim and cells transfected with a shRNA construct directed against GFP and subjected to Western blot analysis to monitor Bim levels. (B) Bim-shRNA4, Bim-shRNA10, and GFP-shRNA cells were exposed to sorafenib (10 μM) for 24 h after which the extent of apoptosis was determined by annexin V staining assay. Values represent the means for 3 separate experiments ± S.D. * = significantly lower than values for GFP-shRNA cells ($p < 0.05$).

Fig. 6. Sorafenib induces apoptosis in primary CD34$^+$ cells isolated from patients with CML.

CD34$^+$ cells were isolated as described in Methods from the bone marrow of four patients with CML who had progressed following treatment with IM (A) or normal subject (B) and exposed to 10 and 15 μM sorafenib for 48 hr after which the extent of cell death was determined by flow cytometry using the annexin V staining assay. Values represent the means ± S.D. for each experiment performed in triplicate.

Figure 1

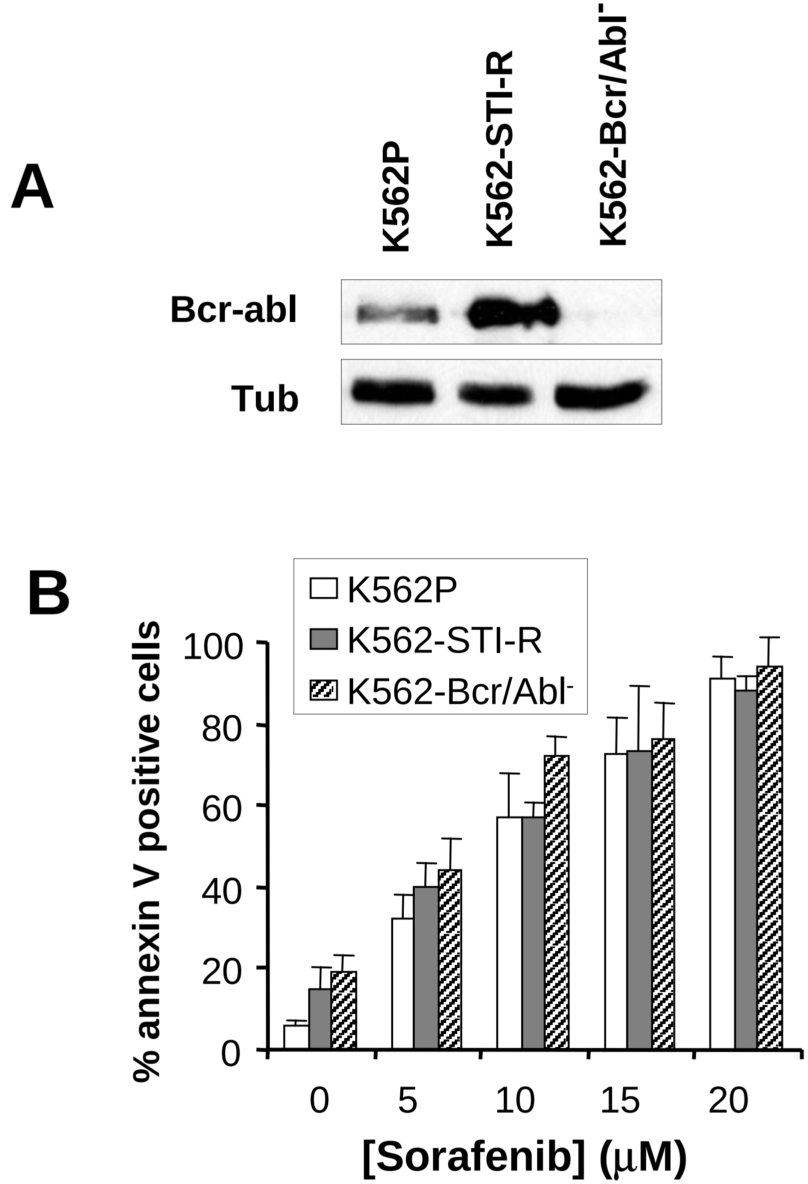

A
K562P
K562-STI-R
K562-Bcr/Abl⁻
Bcr-abl
Tub
B
K562P
K562-STI-R
K562-Bcr/Abl-
% annexin V positive cells
100
80
60
40
20
0
0
5
10
15
20
[Sorafenib] (μM)

Figure 2

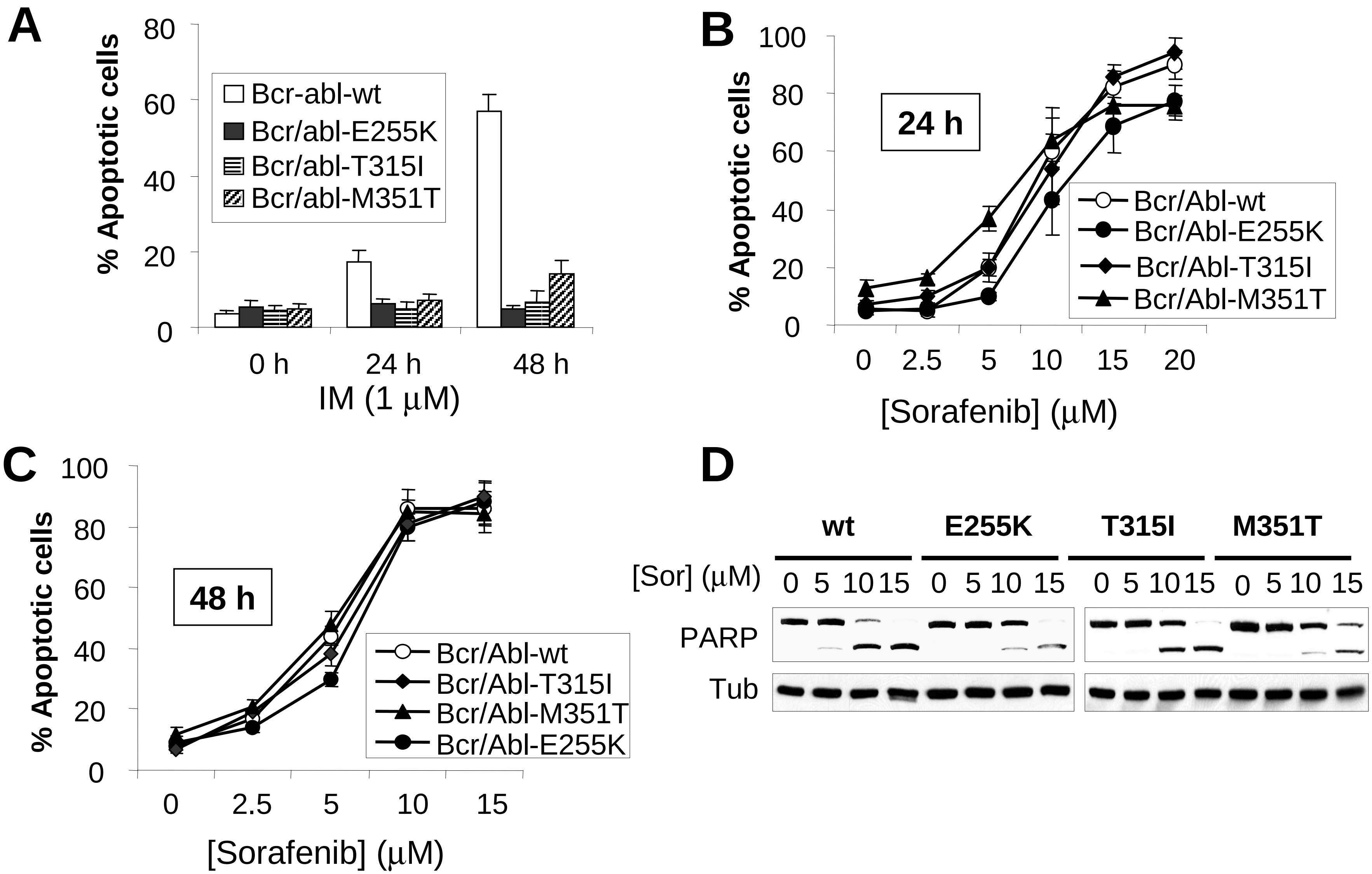

A
% Apoptotic cells
80
60
40
20
0
Bcr-abl-wt
Bcr/abl-E255K
Bcr/abl-T315I
Bcr/abl-M351T
0 h
24 h
48 h
IM (1 μM)
B
% Apoptotic cells
100
80
60
40
20
0
24 h
Bcr/Abl-wt
Bcr/Abl-E255K
Bcr/Abl-T315I
Bcr/Abl-M351T
0
2.5
5
10
15
20
[Sorafenib] (μM)
C
% Apoptotic cells
100
80
60
40
20
0
48 h
Bcr/Abl-wt
Bcr/Abl-T315I
Bcr/Abl-M351T
Bcr/Abl-E255K
0
2.5
5
10
15
[Sorafenib] (μM)
D
wt
E255K
T315I
M351T
[Sor] (μM)
0 5 10 15 0 5 10 15 0 5 10 15 0 5 10 15
PARP
Tub

Figure 3

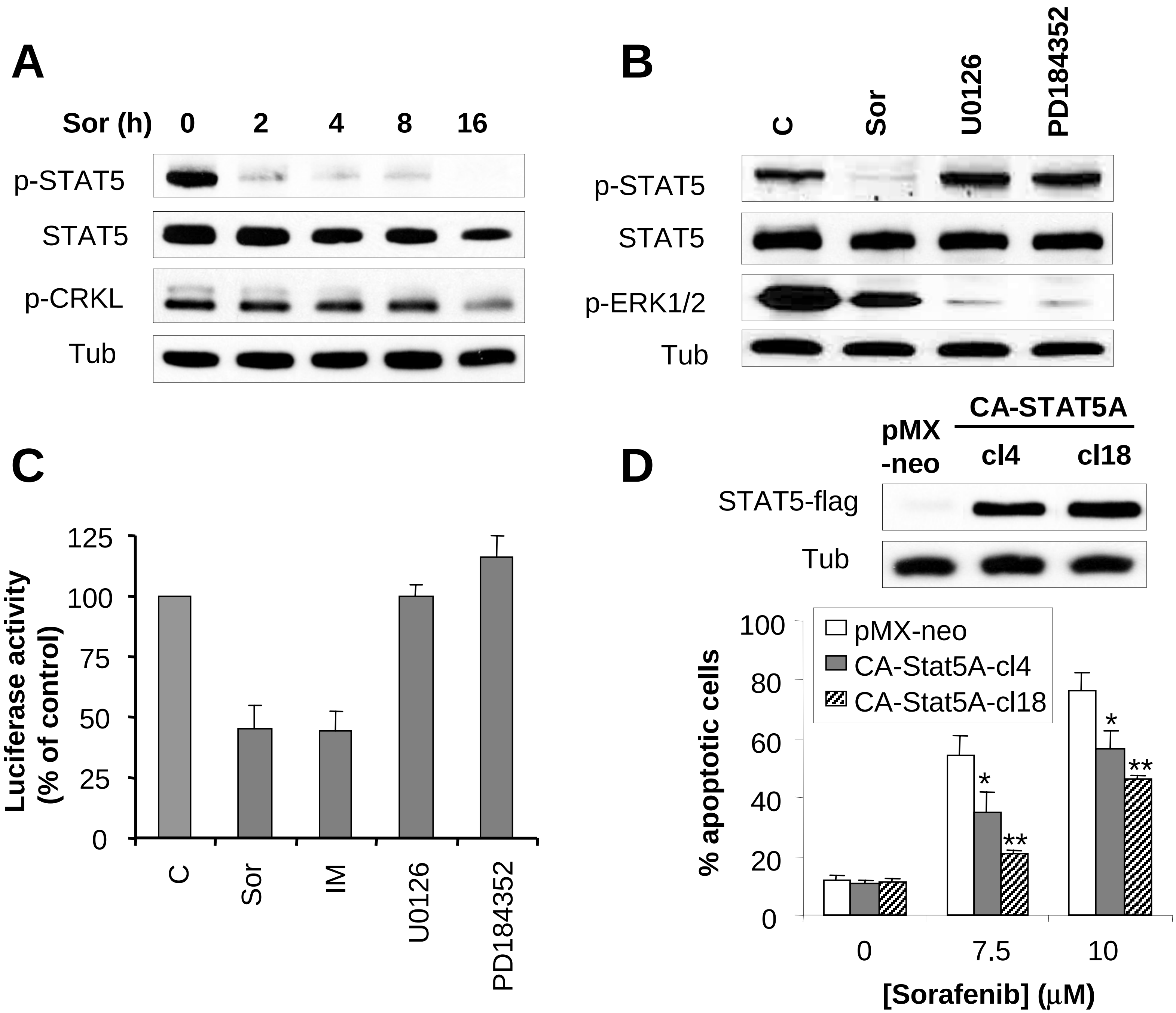

A
Sor (h) 0 2 4 8 16
p-STAT5
STAT5
p-CRKL
Tub
B
C
Sor
U0126
PD184352
p-STAT5
STAT5
p-ERK1/2
Tub
C
Luciferase activity (% of control)
125
100
75
50
25
0
C
Sor
IM
U0126
PD184352
D
pMX -neo
CA-STAT5A
cl4
cl18
STAT5-flag
Tub
% apoptotic cells
100
80
60
40
20
0
pMX-neo
CA-Stat5A-cl4
CA-Stat5A-cl18
*
**
0
7.5
10
[Sorafenib] (μM)

Figure 4

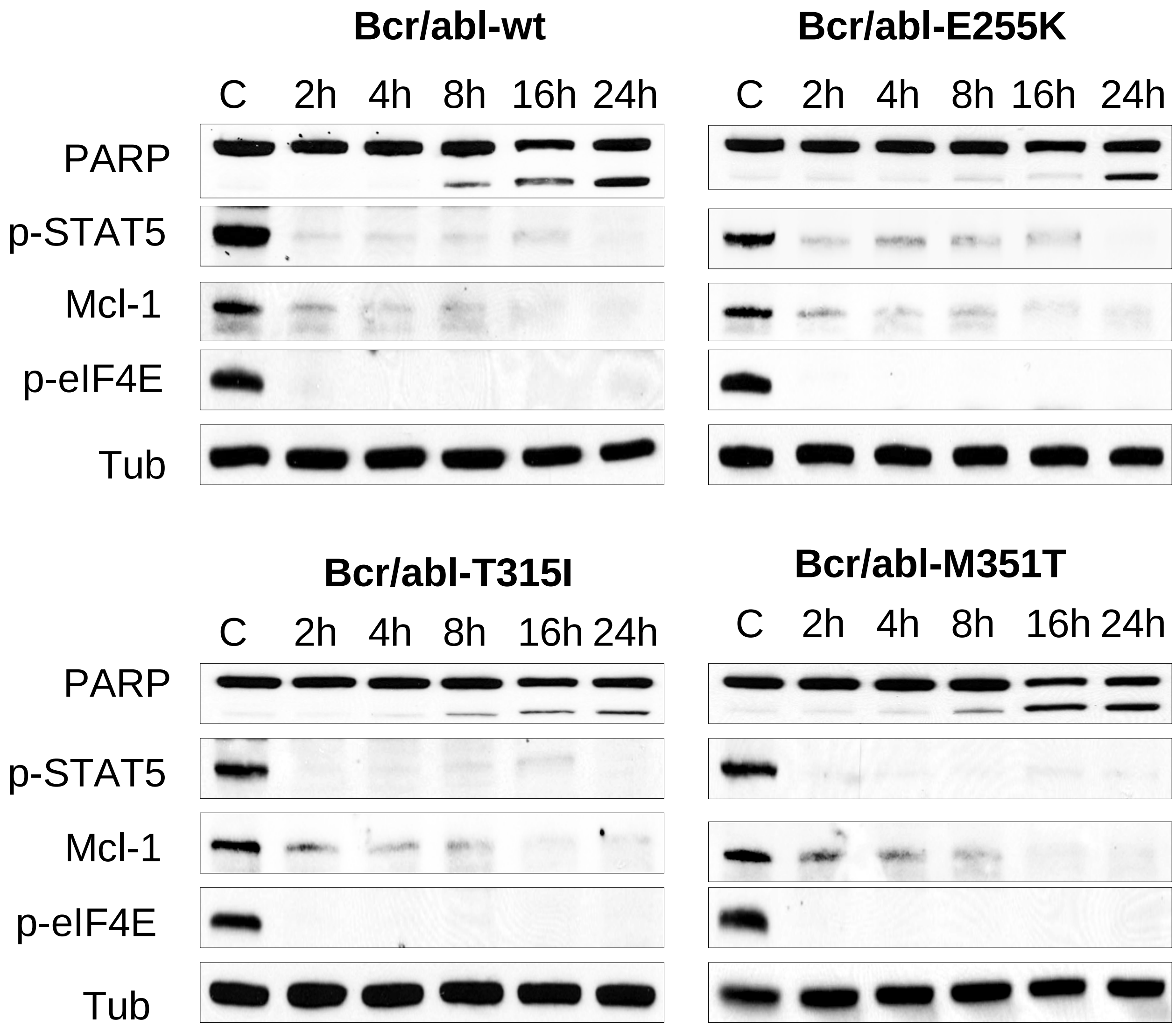
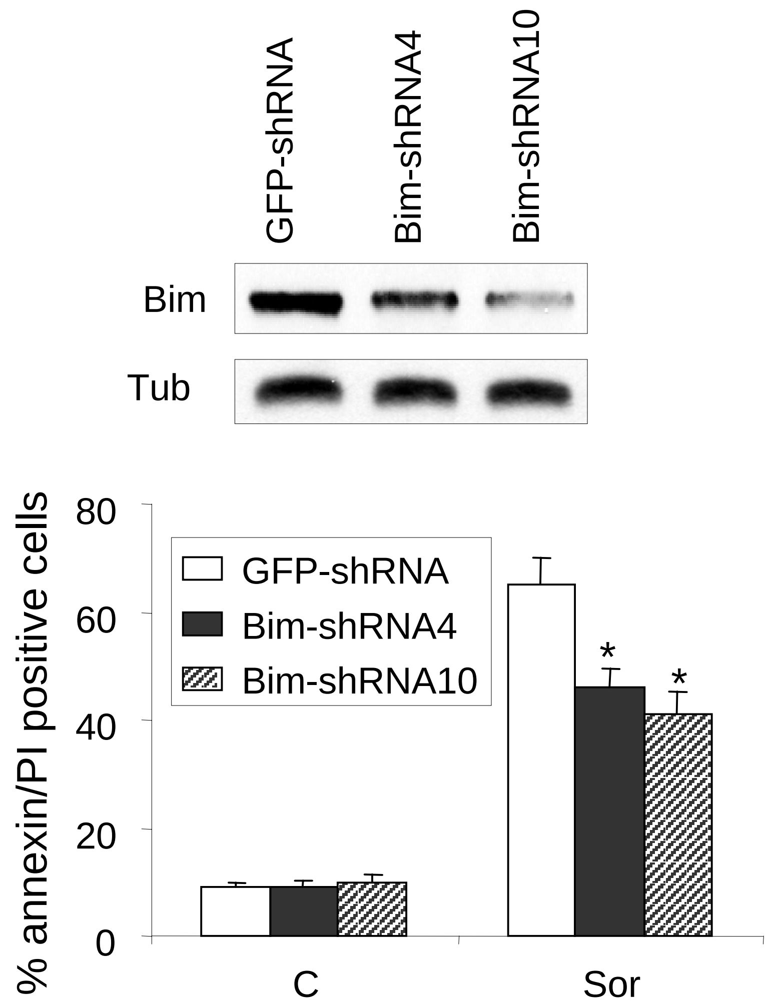
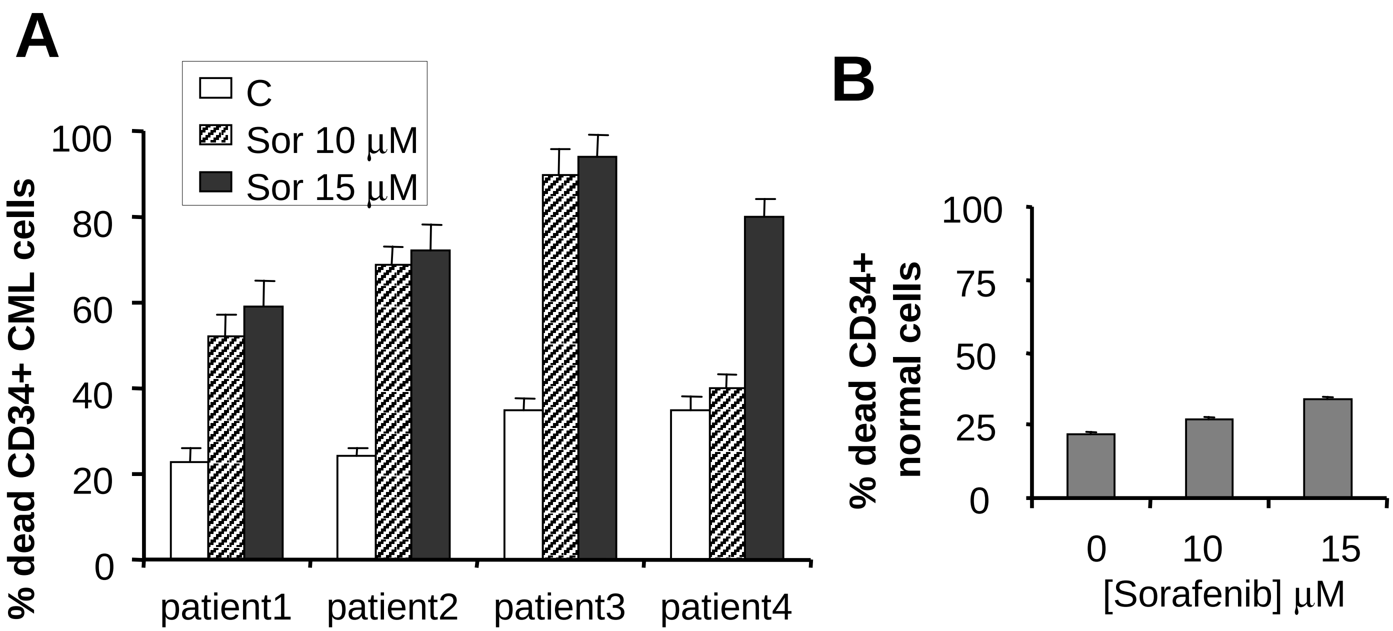

Bcr/abl-wt
C 2h 4h 8h 16h 24h
PARP
p-STAT5
Mcl-1
p-eIF4E
Tub
Bcr/abl-E255K
C 2h 4h 8h 16h 24h
Bcr/abl-T315I
C 2h 4h 8h 16h 24h
PARP
p-STAT5
Mcl-1
p-eIF4E
Tub
Bcr/abl-M351T
C 2h 4h 8h 16h 24h

Figure 5



GFP-shRNA
Bim-shRNA4
Bim-shRNA10
Bim
Tub
% annexin/PI positive cells
80
60
40
20
0
GFP-shRNA
Bim-shRNA4
Bim-shRNA10
*
*
C
Sor

Figure 6


A
C
Sor 10 µM
Sor 15 µM
100
80
60
40
20
0
% dead CD34+ CML cells
patient1
patient2
patient3
patient4
B
100
75
50
25
0
% dead CD34+ normal cells
0
10
15
[Sorafenib] µM