Sorafenib Induces Apoptosis Specifically in Cells Expressing BCR/ABL by Inhibiting Its Kinase Activity to Activate the Intrinsic Mitochondrial Pathway

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Abstract
Although the BCR/ABL tyrosine kinase inhibitor imatinib is highly effective for treatment of chronic myelogenous leukemia and Philadelphia chromosome–positive (Ph+) acute lymphoblastic leukemia, relapse with emerging imatinib resistance mutations in the BCR/ABL kinase domain poses a significant problem. Here, we show that the multikinase inhibitor sorafenib inhibits proliferation and induces apoptosis at much lower concentrations in Ton.B210 cells when driven by inducibly expressed BCR/ABL than when driven by interleukin-3. The increased sensitivity to sorafenib was also observed in cells inducibly expressing BCR/ABL with the imatinib-resistant E255K or T315I mutation. Sorafenib-induced apoptosis in these cells and Ph+ leukemic cells was synergistically enhanced by bortezomib or ABT-737 and inhibited by pan-caspase inhibitor BOC-d-fmk or the overexpression of Bcl-XL. It was further revealed that sorafenib activates Bax and caspase-3 and reduces mitochondrial membrane potential specifically in BCR/ABL–driven cells. Sorafenib also inhibited BCR/ABL–induced tyrosine phosphorylation of its cellular substrates and its autophosphorylation in Ton.B210. It was finally shown that sorafenib inhibits the kinase activity of BCR/ABL as well as its E255K and T315I mutants in in vitro kinase assays. These results indicate that sorafenib induces apoptosis of BCR/ABL–expressing cells, at least partly, by inhibiting BCR/ABL to activate the mitochondria-mediated apoptotic pathway. Thus, sorafenib may provide an effective therapeutic measure to treat Ph+ leukemias, particularly those expressing the T315I mutant, which is totally resistant to imatinib and the second generation BCR/ABL inhibitors. [Cancer Res 2009;69(9):OF1–10]

Introduction
The BCR/ABL fusion gene, generated by a reciprocal t(9;22) chromosomal translocation causing the Philadelphia chromosome, is the molecular signature of chronic myelogenous leukemia (CML) and is also observed in 30–40% of acute lymphoblastic leukemia (ALL; refs. 1, 2). Imatinib is a specific inhibitor of the BCR/ABL tyrosine kinase and has shown unprecedented efficacy for the treatment of these leukemias (1–4). However, the resistance to imatinib may develop in significant portions of patients under treatment, especially in those with CML in advanced stages or with Philadelphia chromosome–positive (Ph+) ALL, mostly due to the emergence of mutations in the BCR/ABL kinase domain that may inhibit binding of imatinib to the kinase domain, including the most frequent E255K and T315I mutations (1–4). We previously showed that BCR/ABL with either E255K or T315I also exhibited the increase in activity to induce autophosphorylation and tyrosine phosphorylation of various cellular proteins, including signal transducers and activators of transcription 5 (STAT5; refs. 5, 6). Thus, it is possible that these mutations may confer the growth advantage on leukemic cells to expand in the absence of selective pressure from imatinib treatment. Therefore, studies are urgently needed to develop new therapies to prevent or counteract the imatinib resistance, particularly that with T315I mutation, which confers complete resistance not only to imatinib but also to the second generation BCR/ABL inhibitors nilotinib and dasatinib (7).

Sorafenib (BAY43-9006) was originally developed as an orally bioavailable small-molecule Raf kinase inhibitor (8). Sorafenib has been approved by Food and Drug Administration for treatment of renal cell carcinoma after a phase III clinical study in 2005. BCR/ABL activates various intracellular signaling pathways, such as those involving Ras, Raf-1, extracellular signal-regulated kinase (Erk), phosphatidylinositol 3-kinase, STAT5, and nuclear factor-κB, which normally play roles in regulation of hematopoiesis by hematopoietic cytokines and other extracellular stimuli (1, 2). We have recently shown that BCR/ABL also activates signaling pathways involving Rap1 and B-Raf, which play important roles in induction of cell proliferation and inhibition of apoptosis (9). Thus, inhibitors for the Raf-1 and B-Raf kinases should be attractive candidates for the development of novel therapies against the imatinib resistance. Sorafenib also inhibits multiple tyrosine kinases, including VEGFR-2, VEGFR-3, PDGFR-α, Fli3, and c-Kit (8). In accordance with this, recent studies have shown a significant effect of sorafenib on acute myelogenous leukemia cells harboring the constitutively activated Fli3 internal tandem duplication mutations (10, 11). In addition, sorafenib has been reported to induce apoptosis of a variety of hematopoietic tumor cells, including those expressing BCR/ABL (12–15). Although the various mechanisms have been reported, it has remained to be known whether and how sorafenib specifically inhibits the BCR/ABL–activated signaling events to affect proliferation and survival of leukemic cells.

In this study, we show that sorafenib induces apoptosis of BCR/ABL–driven cells, at least partly, through inhibition of the BCR/ABL kinase activity. The present study further suggests that sorafenib
alone or in combination with other reagents, including rottlerin, ABT-737, and bortezomib, may provide effective therapies for Ph+ leukemias, particularly those expressing imatinib-resistant mutations including T315I.

Materials and Methods

Cells and reagents. Ton.B210 cells were kindly provided by Dr. George Q. Daley (16). Ton.B210/E255K or Ton.B210/T315I cells, which inducibly express BCR/ABL with E255K or T315I mutation, respectively, were described previously (6). Ton.32D210 cells were established by sequentially transfecting an interleukin 3 (IL-3)–dependent cell line, 32Dcl3, with pTet-On (Clontech) and pcDNA-BCR/ABL, as described previously (16). CML cell lines KU812 and K562 were obtained from the Riken cell bank (Ibaraki), and MOLM-1 cells were kindly provided by Dr. Yoshinobu Matsuo (Fujisaki Cell Center). TMD-5 cells, a double Ph+ ALL-derived cell line expressing the p190 form of BCR/ABL, were kindly provided by Dr. Shuji Tohda (17). PLAT-E, an ecotropic virus packaging cell line, was kindly provided by Dr. Toshio Kitamura (18). Retrovirus vectors pSFFV-neo-Bcl-XL (Addgene plasmid 8749; ref. 19) and pMXs-puro (20) were obtained from Addgene and Dr. Toshio Kitamura, respectively. Sorafenib and ABT-737 were synthesized based on the published structures. Imatinib was kindly provided by Novartis, and bortezomib was from Millennium Pharmaceuticals. Rottlerin and doxycycline were purchased from Calbiochem and Sigma, respectively. BOC-d-fmk and DiOC6 were purchased from BioVision and Invitrogen, respectively. Antibodies against Abl, STAT5, Cbl, Dok-1, and CrkL were purchased from Santa Cruz Biotechnology. Antiphosphotyrosine monoclonal antibody (4G10) and anti–Erk antibody were from Upstate Biotechnology, and anti–Bax monoclonal antibody (YTH-6A7) was from Trevigen. Phosphospecific antibodies against STAT5 (Tyr694), Erk, eIF4E, and eIF2α and an anti–cleaved caspase-3 antibody were from Cell Signaling. Anti–β-actin antibody was purchased from Sigma.

Analyses of cell proliferation, viability, and apoptosis. Cell proliferation and viability were assessed by counting viable and nonviable cell numbers by the trypan blue dye exclusion method. Flow cytometric analysis of cell cycle and apoptosis was performed as described previously (6). Flow cytometric analysis of apoptosis was also performed by staining cells with Annexin V-FITC and PI and analyzed by flow cytometry in D. Bottom, percentages of apoptotic cells with sub-G1 DNA content (C) or percentages of cells that have become positive for Annexin V after treatment with indicated concentrations of sorafenib (D).

Figure 1. Sorafenib induces apoptosis selectively in Ton.B210 cells expressing BCR/ABL. A and B, Ton.B210 cells cultured with 1 μg/mL doxycycline to induce BCR/ABL expression in the absence of IL-3 or cultured without doxycycline in the presence of IL-3 were cultured with indicated concentrations of sorafenib for 48 h. Numbers of viable cells and nonviable cells were counted after trypan blue staining. Relative viable cell numbers compared with those of control cells cultured without sorafenib (A) and percentages of viable cells (B). C and D, Ton.B210 cells expressing BCR/ABL or cultured with IL-3 were treated for 24 h with indicated concentrations of sorafenib. DNA content was analyzed by flow cytometry in C. Cells were stained with Annexin V-FITC and PI and analyzed by flow cytometry in D. Bottom, percentages of apoptotic cells with sub-G1 DNA content (C) or percentages of cells that have become positive for Annexin V after treatment with indicated concentrations of sorafenib (D).
conformational change and caspase-3 cleavage, cells were fixed and permeabilized using Cytofix/Cytoperm kit (Becton Dickinson) according to manufacturer’s instructions. Fixed cells were incubated with either anti-Bax or anti–cleaved caspase-3 on ice for 40 min and then with FITC-conjugated goat anti-mouse IgG1 (Southern Biotech) or FITC-conjugated goat anti-rabbit IgG (Beckman Coulter), respectively, for 30 min in the dark. After washing, the samples were analyzed by flow cytometry.

Immunoprecipitation, immunoblotting, and in vitro kinase assays. Immunoprecipitation, immunoblotting, and in vitro kinase assays were performed essentially as described previously (5, 9). In brief, BCR/ABL and its mutants were immunoprecipitated from cell lysates with anti-Abl and reacted for 10 min at room temperature with 1 μg of the glutathione S-transferase (GST)–CrkL-F fusion protein (21) in the presence or absence of 1 μmol/L cold ATP. To examine the inhibitory effects of sorafenib and imatinib on BCR/ABL kinase activity, the inhibitor or 0.025% DMSO as vehicle control was added to the reaction mixture on ice 15 min before adding ATP and transferring to room temperature. The reaction products were resolved by SDS–PAGE and subjected to Western blot analysis with antiphosphotyrosine, followed by reprobing with anti-CrkL and anti-Abl.

Overexpression of Bcl-XL in Ton.B210 cells. A retroviral expression plasmid for Bcl-XL, pMXs-puro-Bcl-XL, was constructed by subcloning the EcoRI fragment coding for human Bcl-XL from pSFFV-neo-Bcl-XL into pMXs-puro. PLAT-E cells were transfected with pMXs-puro-Bcl-XL or pMXs-puro using the Lipofectamine reagent (Life Technologies-Bethesda Research Laboratories) according to the manufacturer’s instruction. The recombinant retroviruses were harvested 48 h after transfection and used to infect Ton.B210 cells. Infected cells were then selected in medium containing 2 μg/mL puromycin, and pools of infected cells were used for subsequent experiments.

Statistical analysis. Values shown are means ± SD of experiments performed in triplicate and representative of at least three repeated independent experiments. The statistical analysis was done using the two-tailed Student’s t test and indicated as follows: *, P < 0.05; **, P < 0.01; ***, P < 0.001; NS, not significant (P > 0.05).

Results

Sorafenib induces apoptosis much more effectively in BCR/ABL–driven cells than in IL-3–driven cells. We first examined the effects of sorafenib on Ton.B210 cells, an IL-3–dependent cell line that inducibly expresses BCR/ABL when cultured with doxycycline (16). As shown in Fig. 1A, sorafenib at as low as 2.5 μmol/L significantly inhibited proliferation of BCR/ABL–driven cells, whereas sorafenib at 5 μmol/L only moderately inhibited

![Figure 2. Sorafenib-induced apoptosis in cells expressing the imatinib-resistant BCR/ABL E255K or T315I mutant. Ton.B210/E255K cells (A) or Ton.B210/T315I cells (B) cultured with 10 ng/mL doxycycline to induce the BCR/ABL E255K or T315I mutant expression, respectively, in the absence of IL-3 or those cultured without doxycycline in the presence of IL-3 were treated with indicated concentrations of sorafenib for 24 h and analyzed for cellular DNA content.](http://www.aacrjournals.org)
Figure 3. Sorafenib synergistically enhances rottlerin-induced, bortezomib-induced, or ABT-737–induced apoptosis of cells expressing BCR/ABL or its T315I mutant. 

Ton.B210 cells expressing BCR/ABL (A) or its T315I mutant (B) were treated with or without 2.5 μmol/L (A) or 5 μmol/L (B) sorafenib in the presence of 1 μmol/L rottlerin, 2.5 ng/mL bortezomib, or 1 μmol/L ABT-737, as indicated, for 24 h and analyzed for apoptosis. Unless indicated otherwise, differences in values induced by sorafenib, rottlerin, bortezomib, or ABT-737 in the presence or absence of other reagents were statistically significant (P < 0.001). C, TMD-5 cells were treated with indicated concentrations of sorafenib for 48 h and analyzed for the cellular DNA content. D, K562 cells were treated with or without 5 μmol/L sorafenib in the presence of 1 μmol/L rottlerin or 1 μmol/L ABT-737, as indicated, for 24 h and analyzed for cellular DNA content. Unless indicated otherwise, differences in values induced by sorafenib, rottlerin, or ABT-737 in the presence or absence of other reagents were statistically significant (P < 0.001).
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IL-3–dependent proliferation. Moreover, sorafenib at 2.5 or 7.5 μmol/L reduced viability of BCR/ABL–driven cells moderately or drastically, respectively, whereas sorafenib at 7.5 μmol/L only modestly reduced viability of IL-3–driven cells (Fig. 1B). In accordance with this, treatment with sorafenib at as low as 2.5 μmol/L for 24 h induced apoptosis in Ton.B210 cells expressing BCR/ABL as assessed by the fraction of cells with sub-G1 cellular DNA content (Fig. 1C). Apoptosis was induced drastically in these cells treated with 10 μmol/L sorafenib. On the other hand, sorafenib at 10 μmol/L induced accumulation of IL-3–driven cells in the G0-G1 phase but only modest apoptosis in these cells. Flow cytometric analyses of cells stained with Annexin V-PI also showed that apoptosis was induced significantly by 2.5 to 7.5 μmol/L sorafenib specifically in BCR/ABL–driven Ton.B210 cells but not in IL-3–driven cells (Fig. 1D).

Cells expressing the imatinib-resistant E255K or T315I BCR/ABL mutant are also sensitive to sorafenib. We next examined whether sorafenib can also induce apoptosis in Ton.B210/E255K cells inductively expressing BCR/ABL with the imatinib-resistant E255K or T315I mutation in the kinase domain. As shown in Fig. 2, sorafenib at 5.0 μmol/L or higher concentrations induced more significant apoptosis in Ton.B210/E255K or Ton.B210/T315I cells driven by the BCR/ABL mutant than in those driven by IL-3. These cells driven by the E255K or T315I mutant were less sensitive than Ton.B210 cells driven by BCR/ABL without any kinase domain mutation, because sorafenib at 5 μmol/L induced apoptosis only marginally in these cells. Nevertheless, these cells were sensitive to sorafenib, much more significantly when driven by the BCR/ABL mutants than by IL-3. These data indicate that, although more resistant than cells expressing imatinib-sensitive BCR/ABL cells expressing the imatinib-resistant BCR/ABL mutants, including the T315I mutant, are also sensitive to sorafenib.

Sorafenib synergistically enhances apoptosis induced by rottlerin, bortezomib, or ABT-737 in BCR/ABL–expressing cells. We next explored whether sorafenib may synergistically act with other therapeutic agents to induce apoptosis in BCR/ABL–expressing cells, because cells expressing the imatinib-resistant BCR/ABL mutants were also relatively resistant to sorafenib at 5 μmol/L, which corresponds to the steady-state plasma concentration attainable in most patients continuously taking the standard dose (400 mg bid) of sorafenib (22). As shown in Fig. 3A, rottlerin, bortezomib, and ABT-737 at concentrations inducing apoptosis in <10% of BCR/ABL–driven Ton.B210 cells when used alone induced apoptosis in more than a third of cells in combination with sorafenib at 2.5 μmol/L, which alone also failed to induce apoptosis in >10% of cells. Sorafenib did not show any enhancing effect with these agents in IL-3–driven Ton.B210 cells (data not shown). As shown in Fig. 3B, apoptosis induced by rottlerin, bortezomib, or ABT-737 in Ton.B210/T315I cells was also drastically enhanced by sorafenib at 5 μmol/L, which failed to induce significant apoptosis in these cells. Very similar effects were observed in Ton.B210/E255K cells (data not shown).

We next examined the effects of sorafenib on several Ph–leukemic cell lines. Sorafenib at 5 μmol/L induced significant apoptosis in TMD-5 Ph– ALL cells expressing the p190 form of BCR/ABL, as well as in KU812 CML cells (Fig. 3C; data not shown). On the other hand, sorafenib at 5 μmol/L barely induced apoptosis in K562 or MOLM-1 cells (Fig. 3D; data not shown). However, rottlerin and ABT-737, but not bortezomib, significantly enhanced sorafenib-induced apoptosis in these cells (Fig. 3D; data not shown). Thus, in various cell lines expressing BCR/ABL we examined, apoptosis was significantly induced by sorafenib at 5 μmol/L alone or in combination with other agents, including ABT-737 and rottlerin, in synergistic manners.

Sorafenib activates the mitochondrial apoptotic pathway leading to caspase activation specifically in BCR/ABL–expressing cells. To explore the molecular mechanisms involved in induction of apoptosis, we first examined whether activation of caspases is involved. As shown in Fig. 4A, the pan-caspase inhibitor BOC-d-fmk at least partly inhibited sorafenib-induced apoptosis in a dose-dependent manner. Next, we examined the effect of overexpression of the antiapoptotic Bcl2 family member Bcl-XL, which has been shown to play an important role in inhibition of the mitochondria-mediated induction of apoptosis in hematopoietic cells (23). As shown in Fig. 4B, BCR/ABL–driven Ton.B210 cells overexpressing Bcl-XL were remarkably more resistant to sorafenib-induced apoptosis compared with control cells cultured under the same conditions. These data suggest that sorafenib may activate the intrinsic mitochondria-mediated apoptotic pathway leading to caspase activation to induce apoptosis in BCR/ABL–expressing cells.

To confirm this, we next examined the effects of sorafenib on activation of Bax and caspase-3 as well as on Δψm in Ton.B210 cells driven by BCR/ABL or IL-3. Treatment of BCR/ABL–driven Ton.B210 cells with 7.5 μmol/L sorafenib induced the activation-specific Bax conformational change, cleavage of caspase-3, and loss of Δψm, which were observed significantly as early as 8 h after treatment and increased thereafter in time-course–dependent manners (Fig. 5A; data not shown). BOC-d-fmk inhibited the sorafenib-induced cleavage of caspase-3, the executioner caspase-activated downstream in the caspase activation cascades (Fig. 5A). However, BOC-d-fmk did not show any inhibitory effect on Bax activation and loss of Δψm induced by sorafenib, thus indicating that the activation of caspases takes place downstream of these events. On the other hand, treatment of IL-3–driven Ton.B210 cells with sorafenib under the same conditions did not show any significant effect on activation of Bax or caspase-3 and only marginally affected Δψm (Fig. 5A). It was further observed that overexpression of Bcl-XL significantly inhibited the effects of sorafenib on activation of Bax and caspase-3 as well as on depolarization of Δψm in BCR/ABL–driven Ton.B210 cells (Fig. 5B). These data suggest that sorafenib activates caspases specifically in BCR/ABL–driven cells through the mitochondrial intrinsic pathway, which is mediated by Bax activation and negatively regulated by Bcl-XL, to induce apoptosis.

Sorafenib inhibits BCR/ABL, irrespective of imatinib-resistant mutations and tyrosine phosphorylation of its cellular substrates. We next investigated the effects of sorafenib on signal transduction events regulating proliferation and apoptosis of cells expressing BCR/ABL. In accordance with previous studies, sorafenib decreased phosphorylation of eIF4-E and induced phosphorylation of eIF2α in BCR/ABL–expressing Ton.B210 cells (Fig. 6A). However, these effects were observed in similar manners also in IL-3–driven Ton.B210 cells. Sorafenib also inhibited the activation-specific phosphorylation of Erk in similar manners in both BCR/ABL–driven and IL-3–driven Ton.B210 cells (Fig. 6A). These results suggest that these previously reported effects of sorafenib may not play decisive roles in specific induction of apoptosis in BCR/ABL–expressing cells.

We next examined the effects of sorafenib on tyrosine phosphorylation of BCR/ABL and its downstream effector molecules in Ton.B210 cells. As shown in Fig. 6A, sorafenib
inhibited the activation-specific tyrosine phosphorylation of STAT5 in BCR/ABL–driven, but not in IL-3–driven, Ton.B210 cells. Because the STAT5 phosphorylation is mediated by BCR/ABL or Janus-activated kinase 2 (Jak2) in BCR/ABL–driven or IL-3–driven cells, respectively, we speculated that sorafenib may inhibit BCR/ABL but not Jak2. In accordance with this speculation, treatment of cells with sorafenib and imatinib significantly reduced the tyrosine phosphorylation of BCR/ABL and abrogated the activation-specific tyrosine phosphorylation of STAT5 (Fig. 6B). Sorafenib and imatinib also abrogated tyrosine phosphorylation of Dok-1 and significantly inhibited that of CrkL (Fig. 6B). Thus, both inhibitors exhibited similar patterns of inhibition on tyrosine phosphorylation of BCR/ABL and its downstream signaling events, although the inhibitory effects of 10 μmol/L sorafenib were slightly less significant than those of 1 μmol/L imatinib. Essentially, the same results were obtained with a different cell line, 32Dcl3, expressing BCR/ABL (Fig. 6B). These results suggest that sorafenib, but not imatinib, also inhibits BCR/ABL with T315I and its downstream signaling events.

Finally, we examined the direct effect of sorafenib on BCR/ABL by the in vitro kinase assays. As shown in Fig. 6C, BCR/ABL phosphorylated CrkL on tyrosine only in the presence of ATP. Sorafenib added to the reaction mixture inhibited the CrkL phosphorylation in a dose-dependent manner, with 5 μmol/L of sorafenib showing an inhibitory effect comparable with that of 1 μmol/L imatinib. Sorafenib at 5 μmol/L also remarkably inhibited the kinase activity of E255K mutant, which was inhibited to a lesser degree by 1 μmol/L imatinib compared with naive BCR/ABL without mutation (Fig. 6D). Sorafenib at 5 μmol/L strikingly inhibited the kinase activity of T315I mutant, which was not at all inhibited by 1 μmol/L imatinib, as expected (Fig. 6D). Together, these results suggest that sorafenib directly inhibits the kinase activity of BCR/ABL, which should contribute significantly to its antiproliferative and proapoptotic effects specifically observed in BCR/ABL–expressing cells.

Discussion

Sorafenib has been reported to inhibit various tyrosine kinases in addition to Raf kinases (8). However, its effect on the ABL or BCR/
ABL kinase has not been documented previously. Although we have not performed detailed kinetic studies, sorafenib at 5 μmol/L significantly inhibited the kinase activity of BCR/ABL in our in vitro kinase assays, which was comparable with the effect of imatinib at 1 μmol/L (Fig. 6C). The specific inhibition of BCR/ABL–mediated, but not IL-3–induced and Jak2-mediated, tyrosine phosphorylation of STAT5 in sorafenib-treated cells (Fig. 6A) further supports the notion that sorafenib directly inhibits the BCR/ABL kinase activity. However, the inhibition of BCR/ABL–mediated tyrosine phosphorylation of various cellular substrates in Ton.B210 induced by 10 μmol/L sorafenib was slightly less than that induced by 1 μmol/L imatinib (Fig. 6B; data not shown), which

Figure 5. Sorafenib induces activation of Bax and caspase-3 and loss of ∆ψm specifically in BCR/ABL–expressing cells. A, Ton.B210 cells expressing BCR/ABL or cultured without doxycycline in the presence of IL-3 were treated with or without 7.5 μmol/L sorafenib and 100 μmol/L BOC-d-fmk, as indicated, for 12 h. Cells were then analyzed for activation of Bax or caspase-3 and loss of ∆ψm, as indicated, by flow cytometry, as described in Materials and Methods. Bottom, percentages of affected cells. B, Ton.B210 cells infected with control vector (puro) or overexpressing Bcl-XL were treated with or without 7.5 μmol/L sorafenib for 18 h. Cells were then analyzed as described above.
suggests that sorafenib may not penetrate into cells as efficiently as imatinib. Nevertheless, the present study strongly suggests that sorafenib at \( V_{10} \) mol/L partially, but directly, inhibits BCR/ABL kinase activity in cells.

Previous studies have shown that sorafenib inhibited proliferation and survival of a variety of cell types, including leukemic cells, through various mechanisms, such as inhibition of the Raf/MEK/Erk signaling pathway, down-regulation of Mcl-1 expression, and induction of the endoplasmic reticulum stress (8, 12–15, 24). In accordance with these reports (12, 14), we observed that sorafenib not only inhibited activation-specific phosphorylation of Erk but also induced phosphorylation of eIF2\( \alpha \) and dephosphorylation of eIF4E, which are involved in endoplasmic reticulum stress signaling and Mcl-1 down-regulation, respectively (Fig. 6A).

However, these effects of sorafenib were observed in similar manners in both BCR/ABL–driven and IL-3–driven cells and, thus, should not play decisive roles in activation of the mitochondrial apoptotic pathway, which was observed specifically in BCR/ABL–driven cells. On the other hand, sorafenib specifically inhibited BCR/ABL–dependent phosphorylation of various cellular substrates and directly inhibited the kinase activity of BCR/ABL in \textit{in vitro} kinase assays (Fig. 6). Therefore, the present study strongly suggests that inhibition of the BCR/ABL kinase activity by sorafenib should play an essential role in the induction of apoptosis in BCR/ABL–driven cells. As compared with imatinib, however, sorafenib less efficiently and only moderately inhibited the BCR/ABL kinase activity while inducing significant apoptosis in these cells. It is thus speculated that the inhibition of BCR/ABL by sorafenib may act cooperatively with other BCR/ABL–independent effects of sorafenib, including those discussed above, to induce apoptosis effectively in BCR/ABL–driven cells.

Very recently, Rahmani and colleagues (15) reported that sorafenib inhibits tyrosine phosphorylation of STAT5 in BCR/ABL–expressing cells, including BaF3 cells expressing BCR/ABL.
with or without E255K and T315I mutations. The authors, however, concluded that it is not through inhibition of BCR/ABL, because the tyrosine phosphorylation of CrkL, as examined by the phosphorylated CrkL-specific antibody, was not affected by sorafenib treatment in K562 cells. We also observed that the tyrosine phosphorylation of STAT5 was very efficiently inhibited by sorafenib (Fig. 6). However, sorafenib did not show any significant inhibitory effect on the STAT5 phosphorylation mediated by Jak2 in IL-3–driven cells (Fig. 6A). Furthermore, although the inhibitory effect of sorafenib on the tyrosine phosphorylation of STAT5 was more remarkable than that of BCR/ABL in the two cell lines we examined, imatinib similarly inhibited STAT5 phosphorylation more efficiently than that of BCR/ABL (Fig. 6B). Tyrosine phosphorylation of other substrates of BCR/ABL was also affected to different extents by sorafenib with that of CrkL inhibited much less significantly than that of Dok-1 (Fig. 6B). Nevertheless, the inhibitory effects of sorafenib on tyrosine phosphorylation of these substrates correlated with those of imatinib in the two cell lines we examined. Thus, it is speculated that the differences in sensitivity of inhibition of tyrosine phosphorylation may reflect the differences in threshold of the BCR/ABL kinase activity required for phosphorylation or the differences in sensitivity to tyrosine phosphatases. It should also be noted that the differences in experimental methods might have been partially responsible for the apparent discordance with the results reported by Rahmani and colleagues (22), because the anti–phosphorylated CrkL antibody has not worked reliably in our hands given an apparently high background signal (data not shown).

In accordance with previous studies, induction of significant apoptosis in some of the BCR/ABL–expressing cells required up to 10 μmol/L of sorafenib. Although the exact mechanisms causing relative resistance remain to be known, the strength of BCR/ABL signaling and antiapoptotic potentials of cells may be, at least in part, responsible for the differences in sensitivity to sorafenib. It is notable in this regard that, compared with Ton.B210 cells, Ton.B210/E255K or Ton.B210/T315I cells exhibited an increase in tyrosine phosphorylation of various BCR/ABL substrates, including STAT5, in accordance with our previous reports (5, 6), and in expression level of Bcl-XL, which is dependent on STAT5 activation in BCR/ABL–expressing cells (ref. 25; Fig. 6B; data not shown). In spite of the findings that some of the BCR/ABL–expressing cells were resistant to sorafenib at the trough plasma concentration expected from the clinical studies (22), the present study has shown that lower concentrations of sorafenib could induce significant apoptosis in synergistic manners in combination with subtoxic concentrations of bortezomib, ABT-737, and rotterlin (Fig. 3). These results also agree with previously reported results in various types of tumor cells, including leukemic cells, showing the synergistic effects of sorafenib with these reagents (24, 26, 27). In addition, sorafenib has been reported to exhibit synergistic effects with various other therapeutic agents, including AraC, adaphostin, and vorinostat, which act through a variety of different molecular mechanisms (13, 24, 28). Nevertheless, the mechanisms for the synergistic effects have remained to be defined. The Bcl2 family antagonist ABT-737 most likely enhances the effect of sorafenib by inhibiting Bcl-XL, which is supported by the observation that ABT-737 sensitized Ton.B210/T315I cells highly expressing Bcl-XL to sorafenib at 5 μmol/L, which alone is insufficient to induce significant apoptosis (Figs. 2B and 3B). Previously, Jane and colleagues (26) speculated that rotterlin enhanced the effect of sorafenib through inhibition of PKCδ and its downstream signaling pathways in glioma cell lines. However, our previous study revealed that rotterlin failed to inhibit PKCδ in Ton.B210 cells but uncoupled mitochondrial oxidative phosphorylation to enhance imatinib-induced apoptosis (6). Because sorafenib also inhibits BCR/ABL in common with imatinib, it is speculated that rotterlin synergistically enhances sorafenib-induced apoptosis also through the mitochondrial uncoupling effect. It was previously suggested that bortezomib synergistically enhanced sorafenib-induced apoptosis, at least partly, through synergistic activation of c-Jun NH2-terminal kinase (JNK) in various tumor cells (27). In Ton.B210 cells, however, sorafenib did not enhance activation of JNK but inhibited that of p38, most likely by directly inhibiting its kinase activity (8). Because inhibition of p38 reportedly augments bortezomib-induced apoptosis, it is possible that sorafenib synergistically enhances the effect of bortezomib through this mechanisms (29). Future studies are warranted to explore the possible synergistic combination of sorafenib with various therapeutic agents and to define the molecular mechanisms involved in synergy.

Importantly, the present study has shown that sorafenib induces apoptosis of cells expressing BCR/ABL harboring the T315I mutation in the “gatekeeper region” (7). This result is in fact in agreement with the very recent report by Rahmani and colleagues (15), in which the authors showed that sorafenib was effective against cells expressing BCR/ABL with E255K, M351T, and T315I mutation. We have further shown that sorafenib directly and similarly inhibited the kinase activity of BCR/ABL with or without the gatekeeper mutation T315I. In this regard, it is noteworthy that previous reports have shown that sorafenib inhibited various oncogenic tyrosine kinases with imatinib-resistant gatekeeper mutants similar to the BCR/ABL T315I mutant (30–32). Together with its proven clinical safety and its synergistic effects with various therapeutic agents, sorafenib, with its efficacy against the most troublesome T315I mutation, may prove to be very valuable for development of the effective molecular targeted therapy against Ph1+ leukemia with the T315I mutant resistant to imatinib as well as to the second generation BCR/ABL inhibitors, which needs to be tested in future clinical trials.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
References

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