Sorafenib induces apoptosis in HL60 cells by inhibiting Src kinase-mediated STAT3 phosphorylation

Wei Zhao[a], Tao Zhang[a], Bingqian Qu[a], Xingxin Wu[a], Xu Zhu[a], Fanyu Meng[a], Yanhong Gu[a,b], Yongqian Shu[b], Yan Shen[a], Yang Sun[a] and Qiang Xu[a]

Signal transducer and activator of transcription 3 (STAT3) is constitutively active in approximately 50% of acute myeloid leukemia (AML) cases and mediates multiple cellular processes including cell resistance to apoptosis. Inhibition of constitutively active STAT3 has been shown to induce AML cell apoptosis. Our aim was to ascertain if sorafenib, a multikinase inhibitor, may also inhibit STAT3 signaling and, therefore, be efficacious for AML. We found that sorafenib inhibited proliferation and induced apoptosis in human AML cell line (HL60) cells. In addition, sorafenib exposure reduced constitutive STAT3 phosphorylation in HL60 cells and repressed STAT3 DNA-binding activity and Mcl-1 and Bcl-2 expression. Similar results were obtained with the Src kinase inhibitor I, suggesting that sorafenib suppresses STAT3 phosphorylation by inhibiting Src-kinase activity. Furthermore, significant inhibition of Src kinase activity by sorafenib was observed in the kinase assay. In addition, Src could be coimmunoprecipitated with STAT3, and the phosphorylation of STAT3 was significantly inhibited by sorafenib only in cell lines in which phosphorylated Src is highly expressed. Taken together, our study indicates that sorafenib blocks Src kinase-mediated STAT3 phosphorylation and decreases the expression of apoptosis regulatory proteins Mcl-1 and Bcl-2, which are associated with increased apoptosis in HL60 cells. These findings provide a rationale for the treatment of human AML. *Anti-Cancer Drugs* 22:79–88 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2011, **22**:79–88

Keywords: acute myeloid leukemia, human acute myeloid leukemia cell line, signal transducer and activator of transcription 3, sorafenib, Src

[a]State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University and [b]Department of Clinical Oncology, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China

Correspondence to Qiang Xu, PhD, or Yang Sun, PhD, State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing 210093, China
Tel/fax: +86 25 8359 7620; e-mail: molpharm@163.com.

Wei Zhao and Tao Zhang contributed equally to this study.

Received 24 May 2010 **Revised form accepted** 11 August 2010

Introduction

The signal transducer and activator of transcription (STAT) family plays an important role in numerous cellular events such as differentiation, proliferation, and apoptosis [1,2]. Normally, the activation of STAT is highly regulated and transient. However, several STAT family members, including STAT3, are constitutively activated in diverse human tumors [3,4]. The constitutively active STAT3 induces oncogenic processes, such as dysregulated growth, survival, angiogenesis, and immune modulation, and thereby contributes to malignant transformation and progression [5]. STAT3 is activated upon phosphorylation. Approximately 50% of acute myeloid leukemia (AML) patients are found to have constitutive STAT3 phosphorylation [6], and their disease-free survival is significantly shorter as compared with those patients without constitutive STAT3 phosphorylation [7].

Constitutive STAT3 phosphorylation may be induced by hematopoietic growth factors that stimulate the Janus kinase (Jak)/STAT pathway [8], and it may be related, at least partially, to the activation of protein tyrosine kinases (PTKs) [9]. Src family nonreceptor tyrosine kinases are now recognized as important signaling intermediates involved in the initiation and progression of various human malignancies and cytokine signaling [10]. Phosphorylation of tyrosine 416 increases the activity of Src kinase and constantly leads to the activation of downstream signaling pathway [11]. Earlier studies indicated that Src kinase could activate STAT3 in various tumors [12,13]. However, until now the relationship between Src and STAT3 in AML has not been evaluated in detail.

Sorafenib is a potent PTKs inhibitor that has shown efficacy against a wide variety of tumors. It has been shown to block tumor cell proliferation and angiogenesis by inhibiting Raf, vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), Fms-like tyrosine kinase-3 (FLT-3), and c-Kit kinases. Recent results showed that sorafenib induced apoptosis in a variety of leukemia cells including U937, human lymphoma cell line (Jurkat), and K562 cells, suggesting potential activity of sorafenib as a novel mechanism-based therapeutic agent in AML [14,15]. Some studies have shown that sorafenib directly targets mutant FLT3 kinase, thereby inhibiting the growth and

This study was carried out at Nanjing University.
Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Website (www.anti-cancerdrugs.com).

0959-4973 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0b013e32833f44fd

survival of AML cells [16]. Moreover, downregulation of Mcl-1 has been shown to be involved in the apoptosis of leukemia cells induced by sorafenib [14,17]. In addition, sorafenib induces apoptosis in AML cells through Bim-mediated activation of the intrinsic apoptotic pathway [18]. These apoptosis regulatory proteins are known to be the downstream targets of the STAT3 pathway [5], indicating that sorafenib might have some influence on STAT3 activity in leukemia.

In this study, the effects of sorafenib on Src and STAT3, which have oncogenic activities in various human tumors, [10,19] were investigated. Our data show that sorafenib inhibits both Src tyrosine kinase and downstream STAT3 phosphorylation, and induces growth inhibition and apoptosis in HL60 cells.

Materials and methods

Cells and reagents

Human AML cell line (HL60), human chronic myeloid leukemia cell line (K562), and Jurkat were obtained from the Institute of Biochemistry and Cell Biology (Shanghai, China). The cells were incubated in RPMI 1640 medium (Gibco, Maryland, USA) supplemented with 10% newborn calf serum, in a humidified atmosphere of 5% CO_2 at 37°C. Sorafenib (Bayer, Germany) was dissolved in dimethyl sulfoxide to a concentration of 20 mmol/l as a stock solution. STAT3 inhibitor peptide, PpLYKTK-mts, (Calbiochem, California, USA) was dissolved in water and stored at −20°C. AG490 (Calbiochem) was dissolved in ethanol and stored at −20°C until used. Src kinase inhibitor I and SU6656 (Calbiochem) were dissolved in dimethyl sulfoxide and stored at −20°C. Fluorescein FragEL TM DNA Fragmentation Detection Kit was purchased from Calbiochem.

Anti-STAT3, anti-phospho-STAT3 (Tyr705), anti-Jak2, anti-phospho-Jak2 (Tyr1007/1008), anti-phospho-Src (Tyr416), anti-phospho-Src (Tyr527), anti-Src, anti-Mcl-1, anti-Bcl-2, anti-Bcl-xL, anti-cleaved caspase 3, and anti-cleaved caspase 9 were purchased from Cell Signaling Technology (Beverly, Massachusetts, USA). Protein A/G, anti-glyceraldehyde 3-phosphate dehydrogenase, anti-β actin, and anti-α tubulin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA). All other chemicals were purchased from Sigma Chemical Co. (St Louis, Missouri, USA).

Cell proliferation and viability assays

Cell viability was analyzed by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay according to the manufacturer's protocol (Promega MTS assay kit, California, USA). In brief, HL60 cells (3×10^4 cells/well) were plated in 96-well plates (Corning, New York, USA) and were treated with 0–10 μmol/l sorafenib. At each timepoint, the cells were pulsed with the MTS/phenazine methosulfate solution (Promega, Madison, Wisconsin, USA). Ninety-six-well plates were incubated at 37°C for 4 h, and then absorbance at a wavelength of 492 nm was measured on a spectrophotometer (Molecular Devices Corp, California, USA).

Cell proliferation was analyzed by tritiated-thymidine uptake (^{3}H-TdR) (Perkin Elmer, Boston, Massachusetts, USA), as described earlier [20]. In brief, HL60 cells (3×10^4 cells/well) were incubated in 96-well plates in the medium alone or with different concentrations of sorafenib for 60 h. The cells were pulsed with 1 μC$_i$ ^{3}H-TdR during the last 12 h of the 72-h cultures, then harvested on glass fiber filters using a Combi cell harvester (Molecular Device), and finally the incorporated radioactivity was determined using a Beckman LS6000SC scintillation counter (Beckman Coulter Inc., Fullerton, California, USA).

Cell cycle analysis

The cell cycle was measured as described earlier [20]. Approximately 1×10^5 cells were collected at the specified timepoints after culture. Next, the cells were washed twice with ice-cold phosphate buffered saline, fixed in ice-cold 70% ethanol for 30 min, and pretreated with RNase (Sigma) for 1 h. The cells were stained with 10 μg/ml propidium iodide, and the cell cycle profile was determined on a FACScan flow cytometer and analyzed using the CellQuest software (Becton Dickinson, San Jose, California, USA).

Annexin V assay

The annexin V assay was performed in accordance with the manufacturer's protocol (Jingmei Biotech, Shenzhen, China). In brief, HL60 cells (2×10^5) were collected and resuspended in 100 μl of binding buffer containing 1 μl of annexin V–fluorescein isothiocyanate and 1 μl of propidium iodide, and then incubated for 15 min in the dark at room temperature. Fluorescence was measured on a FACScan flow cytometer and analyzed using the CellQuest software.

Terminal deoxynucleotidyl transferase 2′-deoxyuridine, 5′-triphosphate nick end labeling assay

HL60 cells (2×10^6) were exposed to different concentrations of sorafenib. Next, the cells were harvested for the terminal deoxynucleotidyl transferase 2′-deoxyuridine, 5′-triphosphate nick end labeling assay according to the manufacturer's protocol (Calbiochem). In brief, the cells were fixed with 4% formaldehyde at room temperature for 10 min, resuspended in 80% ethanol, and incubated in Tris-buffered saline buffer. Thereafter, the samples were resuspended with proteinase K (20 μg/ml) for 5 min and then incubated in 60 μl of solution containing TdT and Fluorescein-FragEL TdT Labeling Reaction Mix (3:57) for 80 min at 37°C in the dark. Next, the cells were washed with Tris-buffered saline buffer, measured on a FACScan flow cytometer and analyzed using the CellQuest software.

Western blot analysis

Western blot analysis was performed as described earlier [21]. Proteins were extracted in a lysis buffer (30 mmol/l Tris, pH 7.5, 150 mmol/l sodium chloride, 1 mmol/l phenylmethylsulfonylfluoride (PMSF), 1 mmol/l sodium orthovanadate, 1% Nonidet P-40, 10% glycerol, and phosphatase and protease inhibitors). The protein sample was electrophoresed by SDS-PAGE and transferred onto nitrocellulose membranes. After blocking with 5% nonfat milk, the membrane was incubated with a primary antibody followed by a horseradish peroxidase-conjugated secondary antibody. Detection was carried out using a LumiGLO chemiluminescent substrate system (KPL, Guildford, UK).

Subcellular fractionation

HL60 cells (4×10^6) were lysed using the digitonin buffer [22]. Thereafter, cytosolic and membrane fractions were separated by centrifugation, solubilized in Laemmli buffer, and boiled for 5 min. Proteins were analyzed by western blot analysis to evaluate cytochrome c release into the cytosol.

Electrophoretic mobility shift assay

Nuclear isolation and the electrophoretic mobility shift assay were performed using a Gel Shift Assay System (Pierce, Rockford, Illinois, USA) according to the manufacturer's protocol. The consensus oligonucleotides probe hSIE (high-affinity sis-inducible element 5′-AGCTTCAT TTCCCGTAAATCCCTA-3′) was used. Gel shift-binding buffer (1 ×) was incubated with nuclear extracts, which were prepared from the HL60 cells treated with various concentrations of sorafenib for 30 min using the Nuclear Extract kit (Active Motif, Carlsbad, California, USA). A biotin-labeled probe was added and the reaction was incubated for 20 min at room temperature. DNA–protein complexes were resolved by 5% nondenaturing polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane followed by the detection of the biotin-labeled probe by the Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific, Rockford, Illinois, USA) according to the manufacturer's instructions. The membranes were exposed to X-ray films to visualize the bands.

Chromatin immunoprecipitation assay

HL60 cells (2×10^7) were harvested and chemically crosslinked with 1% formaldehyde for 10 min at 37°C. Fixation was quenched by the addition of a 1/20 volume of 2.5 mol/l glycine for 5 min at room temperature. The cells were pelleted, washed with ice-cold 1 × phosphate buffered saline and lysed with the nuclear lysis buffer [1% SDS, 10 mmol/l EDTA, 50 mmol/l Tris–HCl (pH 8.1), 0.5 mmol/l PMSF, and protease inhibitors]. Chromatin solution was sonicated for 15 s pulses at the maximum power for 5 min and diluted 10-fold with ChIP dilution buffer [0.01% SDS, 1.1% Triton X-100, 1.2 mmol/l EDTA, 16.7 mmol/l Tris–HCl (pH 8.1), 167 mmol/l NaCl, 0.5 mmol/l PMSF, and protease inhibitors]. Samples were precleared with protein A agarose beads for 1 h at 4°C. Next, the cell extracts were incubated with anti-STAT3 antibody (1 : 50) overnight at 4°C. Chromatin-antibody complexes were isolated with protein A agarose beads and washed five times with ChIP dilution buffer. Protein/DNA complexes were eluted from the beads in 250 µl elution buffer (1% SDS, 100 mmol/l sodium bicarbonate) for 15 min at room temperature. The eluates (500 µl total) were combined, treated with 5 mol/l NaCl and warmed at 65°C for 4 h to reverse the crosslinks. The extracts were then treated with 10 mg/ml RNase A and 10 mg/ml proteinase K at 45°C for 1 h, and DNA was purified by phenol/chloroform extraction and ethanol precipitation. STAT3-binding Mcl-1 and Bcl-2 expression were analyzed using a standard two-step real-time PCR. Primers were designed according to the STAT3-binding site in the human Mcl-1 and Bcl-2 promoter, respectively.

Mcl-1:

forward: 5′-ACTCAGAGCCTCCGAAGACC-3′;

reverse: 5′-GCGAGCAGCTCCTTTATCAC-3′.

Bcl-2:

forward: 5′-CTGGAGAGTGCTGAAGATTG-3′;

reverse: 5′-CACACTACAAGTAACACGGC-3′.

In-vitro kinase activity assay

The HTScan Kinase Assay Kit (Cell Signaling Technology) was used to determine the direct effects of sorafenib on Jak2 and Src kinase activity. In this assay, serial 10-fold dilutions of sorafenib, starting with 100 µmol/l, were prepared in 96-well plates. The reaction conditions were as follows: purified kinase 50 ng, ATP 20 µmol/l, and substrate peptide 1.5 µmol/l in kinase reaction buffer.

Statistics

All experiments were repeated three to five times with the similar outcome. The *P* values between two experimental groups were tested using a two-tailed Student's *t* test. The level of significance was set at a *P* value of 0.05. Where applicable, data were reported as the mean ± standard deviation.

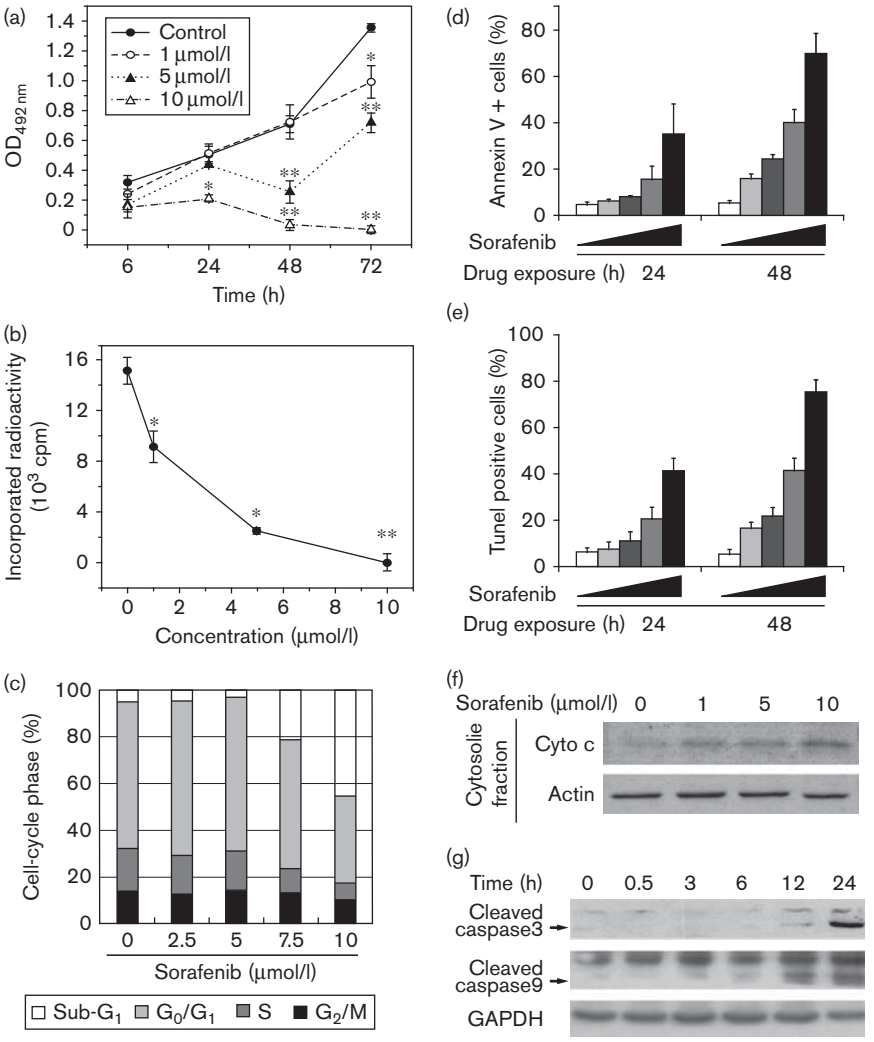
Results

Sorafenib inhibits proliferation and induces mitochondrial depolarization and apoptosis in HL60 cells

To characterize the effects of sorafenib on HL60 cells, the proliferation, and viability of HL60 cells were examined. The ^{3}H-TdR incorporation assay and MTS assay showed inhibition of cellular proliferation and loss of cellular viability in a dose-dependent manner (Fig. 1a and b). Flow cytometric analyses were carried out to further investigate the inhibition of leukemic cell growth

induced by sorafenib. As shown in Fig. 1c, an increased sub-G1 peak and a decreased G1/G2 fraction were found in the sorafenib-treated HL60 cells, suggesting DNA fragmentation and apoptosis. Furthermore, exposure of HL60 cells to sorafenib for 24 h showed a moderate induction of apoptosis at a concentration as low as 5 μmol/l as indicated by annexin V analysis (Fig. 1d). Higher concentrations and longer exposure intervals resulted in a marked increase in cell death (e.g. 8.7 and 35.6% at 5 and 10 μmol/l, respectively; 36.6 and 68.6% at 24 and 48 h, respectively). Essentially equivalent findings were observed in the terminal deoxynucleotidyl transferase 2′-deoxyuridine, 5′-triphosphate nick end labeling assay (Fig. 1e). In addition, exposure to sorafenib resulted in the release of cytochrome *c* into the cytosol (Fig. 1f), accompanied by the cleavage of caspase 3 and caspase 9 (Fig. 1g). Taken together, these findings indicate that exposure to sorafenib results in inhibition of proliferation and a striking induction of caspase activation, mitochondrial depolarization, and apoptosis in HL60 cells.

Fig. 1



Effects of sorafenib on the proliferation and apoptosis of human acute myeloid leukemia cell line (HL60) cells. HL60 cells were treated with or without different concentrations of sorafenib. (a) HL60 cells were counted by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay from 6 to 72 h. *$P<0.05$, **$P<0.01$ versus control group. (b) Sixty hours after sorafenib treatment, the medium was added with 1 μCi tritiated-thymidine, then the HL60 cells were cultured for another 12 h and counted using the tritiated-thymidine incorporation assay. *$P<0.05$, **$P<0.01$ versus drug-untreated group. (c) HL60 cells were treated with or without increasing concentrations of sorafenib for 24 h. Next, they were processed by propidium iodide staining for cell cycle analysis. (d and e) HL60 cells were treated with 0, 2.5, 5, 7.5, and 10 μmol/l sorafenib for 24 and 48 h. Next, they were processed by the annexin V assay (d) or terminal deoxynucleotidyl transferase 2-deoxyuridine, 5-triphosphate nick end labeling assay (e) for cell apoptosis. (f) HL60 cells were exposed to different concentrations of sorafenib for 24 h, then the cell mitochondria-free cytosolic fractions were obtained and subjected to western blot analysis to monitor the release of cytochrome *c*. (g) HL60 cells were treated with or without 10 μmol/l sorafenib for the indicated time, and then the cell extract was detected by western blot analysis for cleaved caspase 3/9. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; OD, optical density.

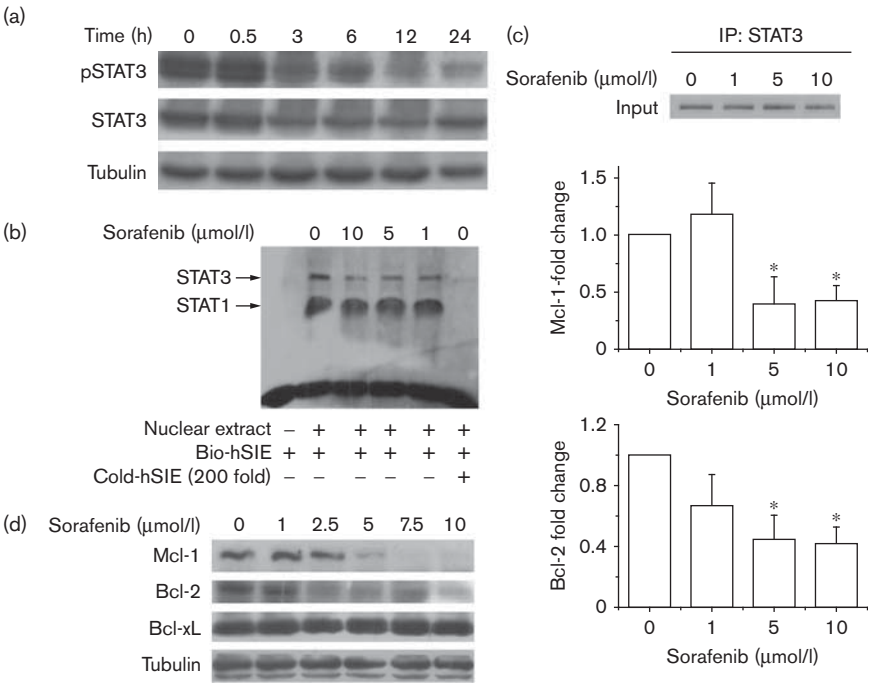
Sorafenib inhibits STAT3 phosphorylation and DNA-binding activity

Next, we investigated the effect of sorafenib on STAT3. HL60 cells were exposed to 10 μmol/l sorafenib for 0.5–24 h. Western blot assay showed that sorafenib decreased the pTyr705 STAT3 level in a time-dependent manner (Fig. 2a). Furthermore, electrophoretic mobility shift assay analysis of the nuclear extracts showed reduced STAT3 DNA-binding activity in the sorafenib-treated HL60 cells (Fig. 2b). Mcl-1 and Bcl-2 proteins are apoptosis regulatory proteins known to be downstream of the STAT3 pathway. To directly determine if sorafenib inhibits STAT3 DNA-binding activity in the promoter of Mcl-1 and Bcl-2, ChIP assays were performed. As determined by real-time PCR, copy numbers of the PCR products from the cells treated with sorafenib were significantly lower than those of the untreated cells (Fig. 2c). These data indicated sorafenib repressed STAT3 DNA-binding activity associate with Mcl-1 and Bcl-2 expression. In addition, the level of antiapoptotic proteins Mcl-1 and Bcl-2 declined in a dose-dependent manner after treatment with sorafenib for 24 h. However, no obvious change was observed at the protein level in Bcl-xL (Fig. 2d).

Sorafenib directly inhibits Src kinase activity

Jak2/STAT3 signaling is the predominant pathway for STAT3 phosphorylation [23]. However, little change was detected in Jak2 kinase phosphorylation in peripheral blood mononuclear cells treated with 10 μmol/l sorafenib (Supplemental Fig. 1a; Supplementary digital content, *http: //bbs.nju.edu.cn/file/S/shuishou/Supplemental_Fig.pdf*). Moreover, Jak2 kinase activity was not affected by sorafenib (0.001–100 μmol/l) in the in-vitro kinase assay (Supplemental Fig. 1b). These results suggest that sorafenib does not inhibit the activity of Jak2 kinase. As Src kinase is also associated with STAT3 phosphorylation, we then examined the potential role of sorafenib on Src kinase activity. As shown in Fig. 3a, Src shares more homology than Jak2 in the ATP-binding domain with Raf, VEGFR, and PDGFR, implying Src kinase to be a potential target of sorafenib. Thus, we investigated the effects of sorafenib on the phosphorylation of Src kinase in HL60 cells. After treatment with sorafenib for 24 h, the expression of pTyr416 Src was significantly decreased. In contrast, no apparent difference was found in Tyr527 Src phosphorylation (Fig. 3b). Furthermore, the in-vitro kinase assay showed that sorafenib inhibited Src kinase activity in a

Fig. 2



Downregulation of signal transducer and activator of transcription 3 (STAT3) activity by sorafenib. (a) Human acute myeloid leukemia cell line (HL60) cells were incubated with 10 μmol/l sorafenib up to 24 h. The levels of pTyr705 STAT3 and STAT3 were detected by western blot analysis. (b) HL60 cells were treated with various concentrations of sorafenib for 30 min. Next, nuclear extracts were isolated, and DNA binding of STAT3 was determined by electrophoretic mobility shift assay. (c) HL60 cells were incubated with 0, 1, 5, and 10 μmol/l sorafenib for 6 h and crosslinked for the chromatin immunoprecipitation assay using STAT3 antibody. Input lanes represent 2% total chromatin used in the chromatin immunoprecipitation assay. Primers for real-time PCR were designed according to the STAT3-binding site of each promoter. *$P<0.05$ versus drug-untreated group. (d) HL60 cells were incubated with increasing concentrations of sorafenib for 24 h. The levels of Mcl-1, Bcl-2, Bcl-xL were detected by western blot analysis. hSIE, high-affinity sis-inducible element 5′-AGCTTCATTTCCCGTAAATCCCTA-3′.

dose-dependent manner (Fig. 3c). These data indicate that sorafenib directly inhibits Src kinase activity by inhibition of Tyr416 Src phosphorylation.

Sorafenib suppresses Src-signal transducer and activator of transcription 3 interaction

STAT3 has been reported to be one of the downstream effectors of Src in tumor cells. In this study, we found that activated Src could be immunoprecipitated with STAT3 and the interaction could be remarkably reduced by sorafenib (Fig. 4a). To further confirm the involvement of Src kinase in the regulation of STAT3 phosphorylation, three leukemia cells lines with different levels of pTyr416 Src (Fig. 4b) were treated with increasing concentrations of sorafenib for 4 h. In HL60 and K562 cells in which pTyr416 Src is highly expressed, the expression of pSTAT3 was downregulated in a dose-dependent manner by sorafenib. In contrast, in Jurkat cells, which have a low Src activity, the expression of pSTAT3 was not affected (Fig. 4c). These data suggest that Src kinase is required for the sorafenib-induced downregulation of STAT3 activity.

Inhibition of Src/STAT3 signaling leads to cell apoptosis and decreased expression of Mcl-1

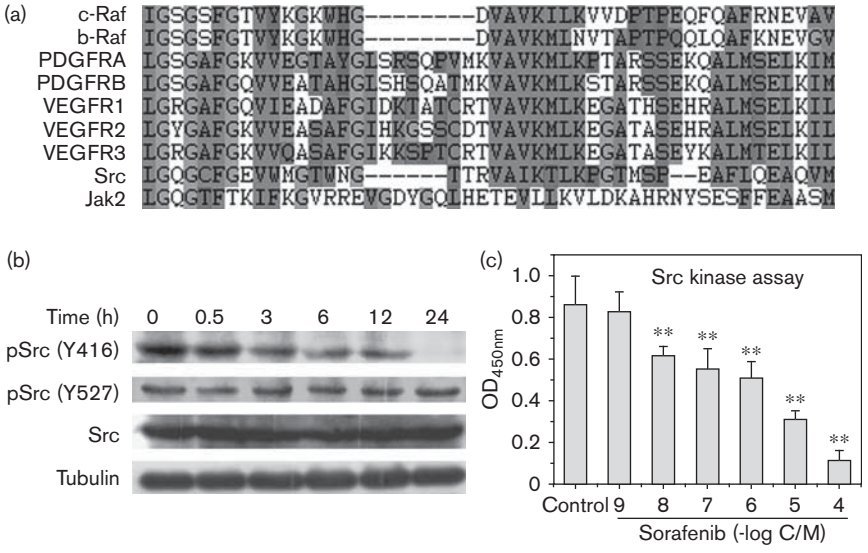
To further confirm the involvement of Src in sorafenib-induced apoptosis, HL60 cells were treated with the specific inhibitor of Src, Src kinase inhibitor I. As shown in Fig. 5a, the cells treated with Src inhibitor I displayed a significant decrease in the levels of phosphorylation of STAT3, Mcl-1, and Bcl-2 compared with the drug-untreated cells. In addition, the HL60 cells treated with the Src inhibitor also showed increased apoptosis (Fig. 5b) and loss of viability (Fig. 5c).

To evaluate the role of STAT3 in sorafenib-induced apoptosis in HL60 cells, the cells were exposed to the Jak2/STAT3 inhibitor, AG490, and STAT3 peptide inhibitor, PpLYKTK-mts. Exposure to STAT3 inhibitor resulted in decreased expression of the antiapoptotic proteins Mcl-1 and Bcl-2 in the HL60 cells, whereas the Bcl-xL protein was not influenced (Fig. 6a). Meanwhile, although the phosphorylation of STAT3 was only partially inhibited, the percentage of apoptotic HL60 cells increased compared with the control (Fig. 6b). These data support the notion that Src/STAT3 signaling is involved in the sorafenib-induced Mcl-1 downregulation and apoptosis in HL60 cells.

Discussion

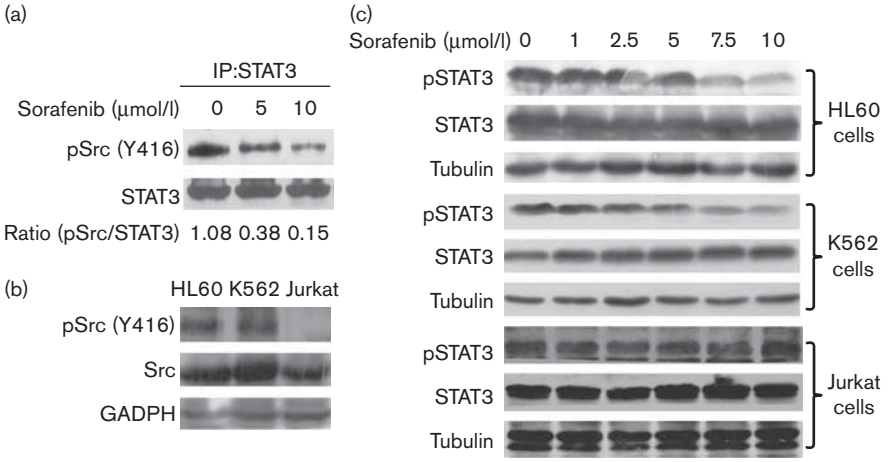
Sorafenib is a multityrosine kinase inhibitor that has been approved for the treatment of advanced renal cell cancer and hepatocellular carcinoma [24,25]. Recent studies show that sorafenib induces apoptosis in AML cells [14,16]. A commonly observed theme is that sorafenib induces apoptosis by inhibition of PTK phosphorylation and loss of the antiapoptotic protein Mcl-1 in AML cells. However, there remains a temporal disconnect between the inhibition of PTK phosphorylation and the loss of Mcl-1. Our study indicates that sorafenib directly inhibits the Src

Fig. 3



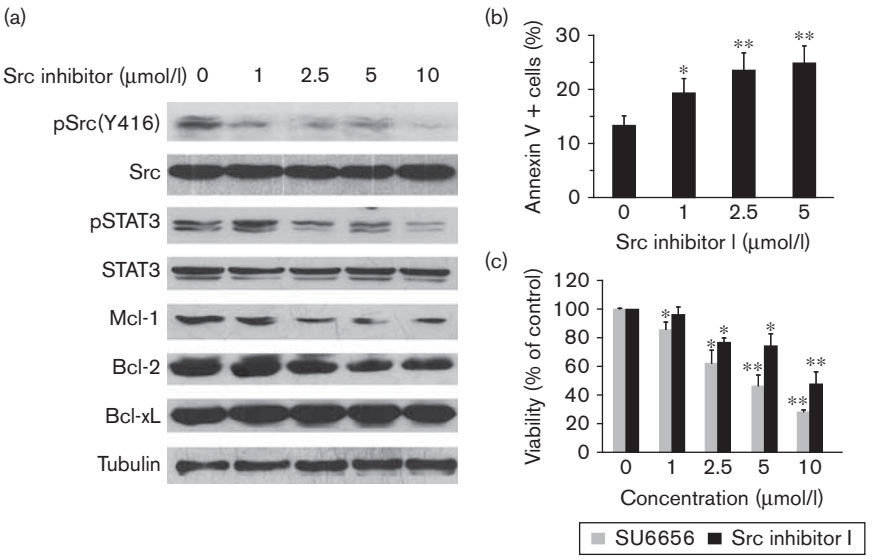
Effects of sorafenib on Src kinase activity. (a) The protein sequences of the ATP-binding pockets of the known sorafenib-sensitive protein tyrosine kinase were shown. The sequences of Src and Jak2 were added for comparison. (b) Human acute myeloid leukemia cell line cells were incubated with 10 μmol/l sorafenib for 24 h. pTyr416 Src, pTyr527 Src, and total Src were detected by western blot analysis. (c) Src in-vitro tyrosine kinase assay was performed with serial 10-fold dilutions of sorafenib from 0.001 μmol/l to 100 μmol/l. $^{**}P<0.01$ versus control group. Jak, Janus kinase; OD, optical density; PDGFR, platelet-derived growth factor receptor; VEGFR, vascular endothelial growth factor receptor.

Fig. 4



Effects of sorafenib on Src-signal transducer and activator of transcription 3 (STAT3) signaling. (a) Human acute myeloid leukemia cell line (HL60) cells were incubated with 0–10 μmol/l sorafenib for 4 h, and the levels of pTyr416 Src and STAT3 were detected by immunoprecipitation and western blot analysis. The relative decrease in pTyr416 Src was quantified by densitometry of three separate experiments. (b) The whole cell lysates of HL60, K562, and Jurkat cells were analyzed by western blotting for pTyr416 Src and Src. (c) HL60, K562, and Jurkat cells were incubated with increasing concentrations of sorafenib for 4 h, and then the levels of pTyr705 STAT3 and STAT3 were detected by Western blot analysis. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Fig. 5

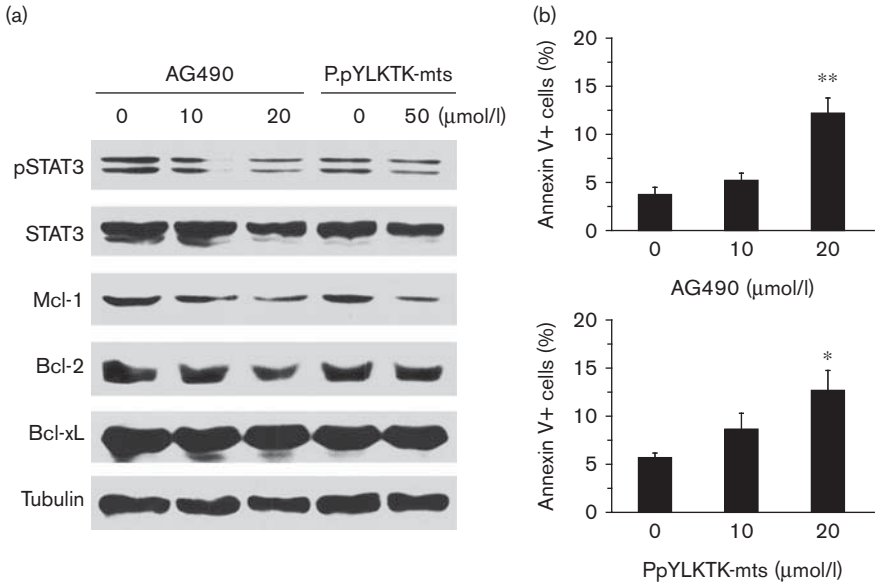


Inhibition of Src leads to cell apoptosis and decreased expression of Mcl-1. (a) Human acute myeloid leukemia cell line (HL60) cells were incubated with Src inhibitor I for 24 h. Levels of pTyr416 Src, Src, pTyr705 signal transducer and activator of transcription 3, STAT3, Mcl-1, Bcl-2, and Bcl-xL were detected by western blot analysis. (b) HL60 cells were treated with increasing concentrations of Src inhibitor I for 48 h, and cell apoptosis was detected by flow cytometric analysis. *$P<0.05$ versus drug-untreated group. (c) HL60 cells were treated with 1 to 10 μmol/l Src inhibitor SU6656 and Src inhibitor I for 48 h, and then the percentage of cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. *$P<0.05$, **$P<0.01$ vs. drug-untreated group.

kinase activity and thereby represses STAT3 phosphorylation. Furthermore, sorafenib decreases expression of the anti-apoptotic proteins Mcl-1 and Bcl-2, which is associated with increased apoptosis in AML cells (Fig. 7).

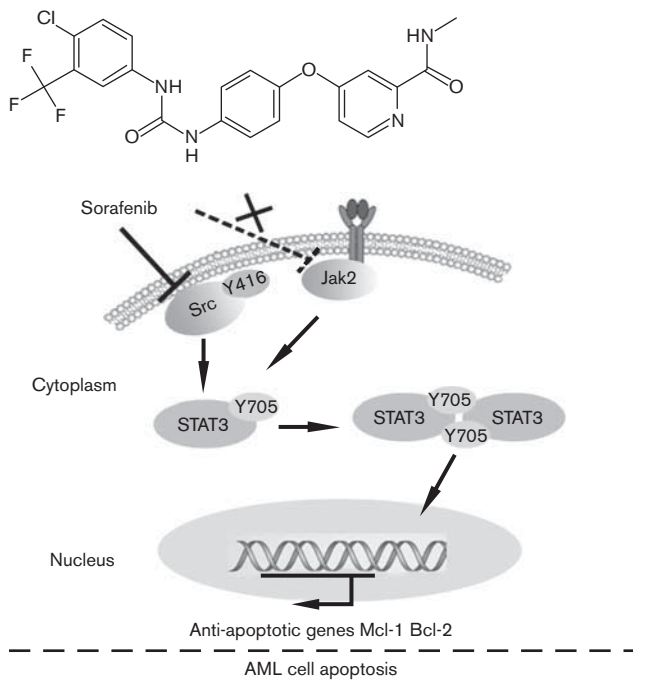
Constitutive phosphorylation of STAT3 has been shown to participate in oncogenesis through the upregulation of genes encoding the antiapoptotic proteins and cell cycle regulators such as Bcl-xL, Mcl-1, and cyclins D1/D2.

Fig. 6



Inhibition of signal transducer and activator of transcription 3 (STAT3) phosphorylation leads to cell apoptosis and decreased expression of Mcl-1. Human acute myeloid leukemia cell line (HL60) cells were exposed to various concentrations of Janus kinase 2/STAT3 inhibitor AG490 and STAT3 peptide inhibitor PpYLKTK-mts for 24 h. (a) Levels of STAT3, pSTAT3, Mcl-1, Bcl-2, and Bcl-xL were detected by western blot analysis. (b) Cell apoptosis was detected by flow cytometric analysis. *$P<0.05$ and **$P<0.01$ versus control group.

Fig. 7



Scheme of sorafenib-induced apoptosis of acute myeloid leukemia (AML) cells. In this model, sorafenib directly inhibits Src kinase rather than Janus kinase 2 (Jak2), resulting in the inhibition of signal transducer and activator of transcription 3 (STAT3) phosphorylation. As a consequence, the expressions of antiapoptotic genes such as Mcl-1 and Bcl-2 are also downregulated, closely associated with the inhibition of cell proliferation and induction of apoptosis in AML cells.

Inhibition of constitutive STAT3 phosphorylation has been shown to inhibit tumor cell growth *in vitro* and *in vivo* and provides a novel strategy for cancer therapy [5,26]. Four recently published studies reported the effect of sorafenib in reducing the cellular levels of Tyr705 phosphoSTAT3 in pancreatic cancer, esophageal cancer, medulloblastoma cells, and cholangiocarcinoma cells [27–30]. Our data confirmed the effect of sorafenib on STAT3 phosphorylation in AML cells (Fig. 1). Furthermore, we identified that Src kinase, the upstream of STAT3, was required for the sorafenib-induced down-regulation of STAT3 phosphorylation.

Several mechanisms have been proposed to explain constitutive STAT3 phosphorylation in AML. Schuringa *et al.* [8] showed that constitutive STAT3 phosphorylation in AML blasts resulted from autocrine secretion of interleukin-6. Other growth factors, thrombopoietin, and colony-stimulating factors are also known to activate STAT3 [31]. In addition, cellular transformation triggered by various tyrosine kinase oncoproteins, such as v-Src and Lck, is also associated with the constitutive phosphorylation of STAT3 [32]. However, the involvement of these oncoproteins has not been shown in AML. Using a homology comparison with a consensus sequence derived from the ATP-binding pockets of known sorafenib targets, we observed that the ATP-binding pocket of Src and Lck [33] closely resembled that of Raf, VEGFR, and PDGFR (Fig. 3a). Results of immunoblot and in-vitro Src kinase assay (Fig. 3b and c) indicated that sorafenib targeted Src kinase in HL60 cells. The IP assay and Src inhibitor assay

indicated that sorafenib inhibited the interaction of Src and STAT3 (Figs 4a and 5a). Besides, in HL60 and K562 leukemia cells in which pSrc was highly expressed, STAT3 phosphorylation was significantly inhibited by sorafenib. In contrast, in Jurkat cells with low Src activity, STAT3 phosphorylation was not affected by sorafenib (Fig. 4b). Thus, we conclude that the effects of sorafenib on STAT3 signaling in HL60 may be mainly mediated through the inhibition of Src kinase.

Mcl-1, one of the antiapoptotic Bcl-2 family members, has been reported to play a key role in sorafenib-mediated lethality, and its downregulation contributes significantly to cell apoptosis [27,34]. In our study, we observed a dose-dependent downregulation of Mcl-1 in the sorafenib-treated HL60 cells. Furthermore, exposure to sorafenib resulted in the release of cytochrome c into the cytosol. These findings are consistent with the studies performed by Rahmani *et al.* [14], who showed that overexpression of Mcl-1 largely inhibited caspase activation and release of cytochrome *c* into the cytosol in U937 cells. Thus, it is very probable that Mcl-1 is involved in the sorafenib-induced apoptosis of HL60 cells. Several mechanisms of the sorafenib-induced downregulation of Mcl-1 have been reported including dephosphorylation of the eIF4E translation initiation factor, inhibition of the transcriptional factor STAT3, and accelerated proteosomal degradation [14,27,30,34]. Earlier studies reported that the level of Mcl-1 mRNA in leukemia cells, such as Jurkat cells, was not affected by sorafenib [34]. Our data, however, suggest that the reduced Mcl-1 mRNA level is mediated by decreased STAT3 DNA-binding activity by sorafenib (Fig. 2b and c), which is consistent with recent publications [27,30]. Moreover, in our study, we observed that sorafenib did not inhibit STAT3 phosphorylation in Jurkat cells. This observation reinforces our hypothesis that STAT3 signaling plays an important role in the transcriptional regulation of Mcl-1 in sorafenib-treated cells. The difference between leukemia cell lines with regard to Mcl-1 regulation is likely because of the upstream signaling pathway of STAT3, such as Src kinase.

Earlier studies have shown that Src is highly expressed and activated in the clinical samples of human AML [35]. Dual Src and Abl kinase inhibitors, such as BMS-354825 (dasatinib), have been reported to exert remarkable in-vivo activity in imatinib-resistant Philadelphia chromosome-positive leukemia [36]. Our findings indicate a high prevalence of constitutive STAT3 phosphorylation in AML linked to Src tyrosine kinase and suggest that the Src family tyrosine kinase inhibitors merit clinical investigation in human AML.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (NSFC) (Nos. 30730107, 30913023, 30973920), the Provincial Science Foundation of Jiangsu (No. BK2008022), and the National Science & Technology Major Project (2009ZX09102-129, 2009 ZX09303-001). There is no conflict of interest to declare.

References

1 Yu H, Jove R. The STATs of cancer – new molecular targets come of age. *Nat Rev Cancer* 2004; **4**:97–105.
2 Bromberg J, Darnell JE Jr. The role of STATs in transcriptional control and their impact on cellular function. *Oncogene* 2000; **19**:2468–2473.
3 Germain D, Frank DA. Targeting the cytoplasmic and nuclear functions of signal transducers and activators of transcription 3 for cancer therapy. *Clin Cancer Res.* 2007; **13**:5665–5669.
4 Al Zaid Siddiquee K, Turkson J. STAT3 as a target for inducing apoptosis in solid and hematological tumors. *Cell Res* 2008; **18**:254–267.
5 Buettner R, Mora LB, Jove R. Activated STAT signaling in human tumors provides novel molecular targets for therapeutic intervention. *Clin Cancer Res* 2002; **8**:945–954.
6 Xia Z, Baer MR, Block AW, Baumann H, Wetzler M. Expression of signal transducers and activators of transcription proteins in acute myeloid leukemia blasts. *Cancer Res* 1998; **58**:3173–3180.
7 Benekli M, Xia Z, Donohue KA, Ford LA, Pixley LA, Baer MR, *et al.* Constitutive activity of signal transducer and activator of transcription 3 protein in acute myeloid leukemia blasts is associated with short disease-free survival. *Blood* 2002; **99**:252–257.
8 Schuringa JJ, Wierenga AT, Kruijer W, Vellenga E. Constitutive Stat3, Tyr705, and Ser727 phosphorylation in acute myeloid leukemia cells caused by the autocrine secretion of interleukin-6. *Blood* 2000; **95**:3765–3770.
9 Wetzler M, Brady MT, Tracy E, Li ZR, Donohue KA, O'Loughlin KL, *et al.* Arsenic trioxide affects signal transducer and activator of transcription proteins through alteration of protein tyrosine kinase phosphorylation. *Clin Cancer Res* 2006; **12**:6817–6825.
10 Ozawa Y, Williams AH, Estes ML, Matsushita N, Boschelli F, Jove R, *et al.* Src family kinases promote AML cell survival through activation of signal transducers and activators of transcription (STAT). *Leuk Res* 2008; **32**:893–903.
11 Thomas SM, Brugge JS. Cellular functions regulated by Src family kinases. *Annu Rev Cell Dev Biol* 1997; **13**:513–609.
12 Zhang Y, Turkson J, Carter-Su C, Smithgall T, Levitzki A, Kraker A, *et al.* Activation of Stat3 in v-Src-transformed fibroblasts requires cooperation of Jak1 kinase activity. *J Biol Chem* 2000; **275**:24935–24944.
13 Garcia R, Bowman TL, Niu G, Yu H, Minton S, Muro-Cacho CA, *et al.* Constitutive activation of Stat3 by the Src and JAK tyrosine kinases participates in growth regulation of human breast carcinoma cells. *Oncogene* 2001; **20**:2499–2513.
14 Rahmani M, Davis EM, Bauer C, Dent P, Grant S. Apoptosis induced by the kinase inhibitor BAY 43-9006 in human leukemia cells involves down-regulation of Mcl-1 through inhibition of translation. *J Biol Chem* 2005; **280**:35217–35227.
15 Lierman E, Folens C, Stover EH, Mentens N, Van Miegroet H, Scheers W, *et al.* Sorafenib is a potent inhibitor of FIP1L1-PDGFRalpha and the imatinib-resistant FIP1L1-PDGFRalpha T674I mutant. *Blood* 2006; **108**:1374–1376.
16 Zhang W, Konopleva M, Shi YX, McQueen T, Harris D, Ling X, *et al.* Mutant FLT3: a direct target of sorafenib in acute myelogenous leukemia. *J Natl Cancer Inst* 2008; **100**:184–198.
17 Rahmani M, Nguyen TK, Dent P, Grant S. The multikinase inhibitor sorafenib induces apoptosis in highly imatinib mesylate-resistant bcr/abl+ human leukemia cells in association with signal transducer and activator of transcription 5 inhibition and myeloid cell leukemia-1 down-regulation. *Mol Pharmacol* 2007; **72**:788–795.
18 Zhang W, Konopleva M, Ruvolo VR, McQueen T, Evans RL, Bornmann WG, *et al.* Sorafenib induces apoptosis of AML cells via Bim-mediated activation of the intrinsic apoptotic pathway. *Leukemia* 2008; **22**:808–818.
19 Homsi J, Cubitt C, Daud A. The Src signaling pathway: a potential target in melanoma and other malignancies. *Expert Opin Ther Targets* 2007; **11**:91–100.
20 Raje N, Kumar S, Hideshima T, Ishitsuka K, Chauhan D, Mitsiades C, *et al.* Combination of the mTOR inhibitor rapamycin and CC-5013 has synergistic activity in multiple myeloma. *Blood* 2004; **104**:4188–4193.
21 Zhang B, Shi ZL, Liu B, Yan XB, Feng J, Tao HM. Enhanced anticancer effect of gemcitabine by genistein in osteosarcoma: the role of Akt and nuclear factor-kappaB. *Anticancer Drugs* 2010; **21**:288–296.
22 Dai Y, Grant S. The histone deacetylase inhibitor sodium butyrate interacts synergistically with phorbol myristate acetate (PMA) to induce

mitochondrial damage and apoptosis in human myeloid leukemia cells through a tumor necrosis factor-alpha-mediated process. *Exp Cell Res* 2002; **277**:31–47.
23 Aggarwal BB, Sethi G, Ahn KS, Sandur SK, Pandey MK, Kunnumakkara AB, *et al.* Targeting signal-transducer-and-activator-of-transcription-3 for prevention and therapy of cancer: modern target but ancient solution. *Ann N Y Acad Sci* 2006; **1091**:151–169.
24 Flaherty KT. Sorafenib in renal cell carcinoma. *Clin Cancer Res* 2007; **13**:747s–752s.
25 Spinzi G, Paggi S. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008; **359**:2497–2498.
26 Dowlati A, Kluge A, Nethery D, Halmos B, Kern JA. SCH66336, inhibitor of protein farnesylation, blocks signal transducer and activators of transcription 3 signaling in lung cancer and interacts with a small molecule inhibitor of epidermal growth factor receptor/human epidermal growth factor receptor 2. *Anticancer Drugs* 2008; **19**:9–16.
27 Ulivi P, Arienti C, Amadori D, Fabbri F, Carloni S, Tesei A, *et al.* Role of RAF/MEK/ERK pathway, p-STAT-3 and Mcl-1 in sorafenib activity in human pancreatic cancer cell lines. *J Cell Physiol* 2009; **220**:214–221.
28 Delgado JS, Mustafi R, Yee J, Cerda S, Chumsangsri A, Dougherty U, *et al.* Sorafenib triggers antiproliferative and pro-apoptotic signals in human esophageal adenocarcinoma cells. *Dig Dis Sci* 2008; **53**: 3055–3064.
29 Yang F, Van Meter TE, Buettner R, Hedvat M, Liang W, Kowolik CM, *et al.* Sorafenib inhibits signal transducer and activator of transcription 3 signaling associated with growth arrest and apoptosis of medulloblastomas. *Mol Cancer Ther* 2008; **7**:3519–3526.
30 Blechacz BR, Smoot RL, Bronk SF, Werneburg NW, Sirica AE, Gores GJ. Sorafenib inhibits signal transducer and activator of transcription-3 signaling in cholangiocarcinoma cells by activating the phosphatase shatterproof 2. *Hepatology* 2009; **50**:1861–1870.
31 Drexler HG, Zaborski M, Quentmeier H. Thrombopoietin supports the continuous growth of cytokine-dependent human leukemia cell lines. *Leukemia* 1997; **11**:541–551.
32 Turkson J, Bowman T, Garcia R, Caldenhoven E, De Groot RP, Jove R. Stat3 activation by Src induces specific gene regulation and is required for cell transformation. *Mol Cell Biol* 1998; **18**: 2545–2552.
33 Zhao W, Gu YH, Song R, Qu BQ, Xu Q. Sorafenib inhibits activation of human peripheral blood T cells by targeting LCK phosphorylation. *Leukemia* 2008; **22**:1226–1233.
34 Yu C, Bruzek LM, Meng XW, Gores GJ, Carter CA, Kaufmann SH, *et al.* The role of Mcl-1 downregulation in the proapoptotic activity of the multikinase inhibitor BAY 43-9006. *Oncogene* 2005; **24**:6861–6869.
35 Lund TC, Coleman C, Horvath E, Sefton BM, Jove R, Medveczky MM, *et al.* The Src-family kinase Lck can induce STAT3 phosphorylation and DNA binding activity. *Cell Signal* 1999; **11**:789–796.
36 Talpaz M, Shah NP, Kantarjian H, Donato N, Nicoll J, Paquette R, *et al.* Dasatinib in imatinib-resistant Philadelphia chromosome-positive leukemias. *N Engl J Med* 2006; **354**:2531–2541.