

Comparison of antitumor effects of multitargeted tyrosine kinase inhibitors in acute myelogenous leukemia

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Abstract

We compared the antitumor activities of the multitargeted tyrosine kinase inhibitors imatinib, sorafenib, and sunitinib to determine which inhibitor is best suited to be used for the treatment of acute myelogenous leukemia (AML). In nine human AML cell lines, sorafenib and sunitinib were more potent inhibitors of cellular proliferation than imatinib (IC₅₀, 0.27 to >40, 0.002-9.1, and 0.007-13 μmol/L for imatinib, sorafenib, and sunitinib, respectively). Sorafenib and sunitinib were potent inhibitors of cells with *fms*-like tyrosine kinase 3 internal tandem duplication (IC₅₀, 2 and 7 nmol/L) and *c-KIT* N822K mutations (IC₅₀, 23 and 40 nmol/L). In four cell lines (MV4-11, Kasumi-1, KG-1, and U937) that spanned a range of drug sensitivities, sorafenib and sunitinib had similar activity in apoptosis and cell cycle assays, except that sunitinib did not promote apoptosis in U937 cells. Both drugs inhibited mitogen-activated protein kinase signaling but had no effect on AKT signaling in most of the cell lines tested. Sorafenib was substantially more bound than sunitinib in human plasma (unbound fraction, 0.59% versus 8.4%) and cell culture medium (unbound fraction, 1.3% versus 39%), indicating that sorafenib was more potent than sunitinib and that unbound sorafenib concentrations with activity against most AML cell lines are achievable *in vivo*. There was more intracellular accumulation of sorafenib than

of sunitinib and imatinib in AML cells. Between 1 and 10 μmol/L, sorafenib inhibited the proliferation of six of nine primary AML blast samples by ≥50%. Our results highlight the pharmacologic features of sorafenib that may provide it an advantage in the treatment of AML. [Mol Cancer Ther 2008;7(5):1110-20]

Introduction

Receptor tyrosine kinases such as *c-KIT*, *fms*-like tyrosine kinase 3 (FLT3), platelet-derived growth factor receptor (PDGFR), and vascular endothelial growth factor receptor play important roles in regulating cell proliferation, differentiation, and survival by activating downstream effectors such as signal transducers and activators of transcription (STAT), protein kinase B/AKT, and extracellular signal-regulated kinase 1/2 (ERK1/2; refs. 1-4). Disruption of cell growth and survival because of aberrant activation of receptor tyrosine kinases may promote leukemogenesis in acute myelogenous leukemia (AML; refs. 1, 5-7). FLT3 and *c-KIT* are expressed in ~90% of people with AML, and activating mutations causing ligand-independent receptor dimerization and constitutive phosphorylation and activation of the receptor have been observed for both receptor tyrosine kinases (5, 6). FLT3 mutations occur in about one third of patients with AML and can be (a) internal tandem duplication of 3 to 400 bp in the juxtamembrane domain (in 23% of patients) and (b) point mutations mainly involving aspartic acid 835 in the second tyrosine kinase domain (in 7% of patients; ref. 6). Activating mutations in *c-KIT* involving the extracellular or second tyrosine kinase domains have been reported in AML, predominantly in patients with t(8;21) and inv(16) (in 12-48% of patients; refs. 5, 8, 9). The presence of FLT3 internal tandem duplication and *c-KIT* mutations is associated with poor prognosis and survival in adults and children with AML (10, 11). Multitargeted tyrosine kinase inhibitors that have activity against various aberrant receptor tyrosine kinases are a new and promising class of therapeutic agents for the treatment of AML.

Several small-molecule multitargeted tyrosine kinase inhibitors have been registered for the treatment of cancer. Imatinib mesylate, an inhibitor of Bcr-Abl, *c-KIT*, and PDGFR, is approved for the treatment of chronic myelogenous leukemia, gastrointestinal stromal tumors, and Philadelphia chromosome-positive chronic myelogenous leukemia in children (12, 13). Sorafenib tosylate, a diphenylurea derivative, has shown potent efficacy in several tumors and is approved for the treatment of advanced renal cell carcinoma and hepatocellular carcinoma (14). Sorafenib, initially developed as a potent inhibitor of C-RAF and B-RAF, was later found to also inhibit other

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receptor tyrosine kinases, including c-KIT, FLT3, PDGFR, and vascular endothelial growth factor receptor (14). Sunitinib malate, an inhibitor of c-KIT, FLT3, PDGFR, and vascular endothelial growth factor receptor, is approved for the treatment of advanced renal cell carcinoma and imatinib-resistant gastrointestinal stromal tumors (15).

In this study, we compared the activities of the tyrosine kinase inhibitors imatinib, sorafenib, and sunitinib in a panel of adult and pediatric human AML cell lines, which represent different FAB types, chromosomal abnormalities, and genetic variants in tyrosine kinases, to determine which inhibitor is best suited to be used for the treatment of AML.

Materials and Methods

Reagents

Imatinib methanesulfonate, sunitinib malate, and sorafenib were purchased from Toronto Research Chemicals. Cell culture reagents, including RPMI 1640 and fetal bovine serum, were purchased from Invitrogen. DMSO and interleukin-3 were purchased from Sigma. [³H]imatinib (specific activity, 1 Ci/mmol; concentration, 1.0 mCi/mL), [³H]sorafenib (specific activity, 2 Ci/mmol; concentration, 1.0 mCi/mL), and [³H]sunitinib (specific activity, 1 Ci/mmol; concentration, 1.0 mCi/mL) were purchased from Moravex Biochemicals.

Cell Lines

The human AML cell lines HL-60, Kasumi-1, NB4, ML-2, MV4-11, KG-1, THP-1, U937, and M-07e were purchased from the American Tissue Culture Collection. All cell lines were cultured in RPMI 1640 containing 10% or 20% fetal bovine serum and incubated at 37°C in a humidified atmosphere with 5% CO₂. M-07e cells were supplemented with 10 ng/mL interleukin-3. The nine cell lines represent different FAB types and unique leukemogenic translocation/fusion genes (Supplementary Table S1).⁴ Cytogenetic profiles were confirmed by the Department of Pathology, St. Jude Children's Research Hospital.

Primary AML Blasts

Leukemia cells from bone marrow or peripheral blood were obtained from nine patients with newly diagnosed AML following informed consent according to St. Jude Children's Research Hospital and Stanford University guidelines. Enrichment of blasts was carried out using indirect microbeads based on the magnetic-activated cell sorting (Miltenyi Biotec) data sheet protocol. Briefly, cells were resuspended in magnetic-activated cell sorting buffer (1× PBS) containing 0.5% fetal bovine serum and 0.4% EDTA. Cell suspensions were then incubated with antibodies against CD3-PE (Miltenyi Biotec), CD20-PE (Miltenyi Biotec), CD235a-PE (BD Biosciences), CD16-PE (Miltenyi Biotec), or CD14-PE (Miltenyi Biotec) for 10 min

in the dark at 4°C to deplete contamination from T cells, B cells, erythrocytes, neutrophils, or monocytes. Cells were washed once with 10 mL magnetic-activated cell sorting buffer to remove unbound antibodies before 20 μL anti-PE microbeads (Miltenyi Biotec) per 1 × 10⁷ cells were added. The antibody selection was determined by the percentage of contaminating cells from the bone marrow differential. The PE-labeled cell suspension was applied to a LS column (Miltenyi Biotec), the column was further washed twice with 3 mL ice-cold magnetic-activated cell sorting buffer, and the flow through effluent was collected and processed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described below. This process resulted in enrichment to >90% blasts.

MTT Assay

A MTT assay was carried out with the MTT Cell Proliferation Kit I (Roche). Briefly, 1.5 × 10⁴ MV4-11, 4 × 10⁴ Kasumi-1, 2 × 10⁴ KG-1, and 5 × 10³ U937 cells were resuspended in 100 μL culture medium and seeded in each well of a 96-well microplate. After 24 h, cells were treated with imatinib, sorafenib, or sunitinib over the concentration range of 0.001 to 40 μmol/L for 72 h. Drugs were individually diluted in the culture medium to the desired concentration and 20 μL were added to each well. Cells were exposed to a final DMSO concentration of <0.1%, and DMSO was added in culture as a solvent control. At the end of treatment, 12 μL MTT labeling reagent (5 mg/mL MTT in PBS) was added to each well and the microplates were incubated at 37°C with 5% CO₂ for 4 h. Then, 100 μL of a solubilization solution were added to each well and the plates were left overnight in the incubator. Once the purple formazan crystals were completely solubilized, the absorbance of the samples was checked at 562 nm by a uQuant Universal Microplate Spectrophotometer (Bio-Tek). The concentration inhibiting 50% of cellular proliferation (IC₅₀) was estimated by the maximum likelihood estimation, as implemented in the software program Adapt II (University of Southern California), by fitting the viability versus concentration data in the following equation:

$$V = (Y_{\max} * K_m^n + Y_{\min} * C^n) / (K_m^n + C^n)$$

Here, V is the viability, Y_{\max} is the maximum response, Y_{\min} is the minimum response, K_m^n is the Michaelis-Menten constant, and C_n is the concentration. For each drug and cell line, three independent experiments were done with eight replicates each.

For primary AML blast samples, cells (1.6 × 10⁵/90 μL RPMI 1640) isolated from the enrichment procedure described above were seeded in sterile 96-well plates and immediately cultured in the presence of sorafenib 0.1 to 10 μmol/L in duplicate wells for 96 h at 37°C, 5% CO₂. Control wells (six replicates) contained cells and culture medium. MTT labeling reagent was added to each well, the microplates were incubated at 37°C with 5% CO₂ for 6 h, and the solubilization solution (isopropanol with 0.04 N HCl) was added to each well and absorbance was measured immediately.

⁴Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

Apoptosis and Cell Cycle Assays

Cells at concentrations similar to those described for the MTT assay were seeded in six-well plates. After incubation for 24 h, cells were treated with varying concentrations of sorafenib or sunitinib that spanned a concentration range that inhibited proliferation in the MTT assay for 24 and 72 h. At the end of the treatment, cells were collected and counted. Cells were assessed for apoptosis and cell cycle distribution with Annexin V and propidium iodide in the Flow Cytometry and Cell Sorting Shared Resource at St. Jude Children's Research Hospital. For each drug and cell line, two independent experiments were done.

Western Blot Analysis

Logarithmically growing cells at a concentration of 3×10^6 were seeded in six-well plates with a low-serum-containing medium (1% fetal bovine serum) and incubated for 24 h. Cells were then treated with varying concentrations of sorafenib or sunitinib that spanned a concentration range that inhibited proliferation in the MTT assay for 1 h. After treatment, cells were collected and washed with ice-cold PBS and lysed in a cell lysis buffer (Cell Signaling) by adding a protease inhibitor cocktail (Roche Diagnostic) plus 1 mmol/L phenylmethylsulfonyl fluoride. Protein concentration was determined by a BCA protein assay kit (Pierce) and 20 to 30 μ g protein in equal amounts were loaded on 4% to 12% NuPAGE Bis-Tris gels (Invitrogen) and transferred onto a polyvinylidene difluoride membrane (Invitrogen). The membrane was first probed with antibodies specific to the phosphoprotein and visualized by the ECL Plus Kit (Amersham Pharmacia Biotech), and the same membrane was then stripped and probed with corresponding antibodies for total protein. The following primary antibodies were used: antibodies to human ERK, phospho-ERK1/2 (Thr²⁰²/Tyr²⁰⁴), p70S6 kinase, phospho-p70S6 kinase (Thr³⁸⁹), STAT5, and phospho-STAT5 (Tyr⁶⁹⁴) were purchased from Cell Signaling; antibodies to human AKT, phospho-AKT (Thr⁴⁷³ and Ser³⁰⁸), and Glyceraldehyde-3-phosphate dehydrogenase were purchased from Santa Cruz Biotechnology.

Mutational Analysis

To determine whether tyrosine kinase mutations were present in the nine AML cell lines, exons with known hotspots in various cancers were sequenced for c-KIT, FLT3, PDGFR α , NRAS, KRAS, BRAF, and CRAF. The following exons were sequenced: c-KIT, exons 8, 9, 11, 13, and 17; FLT3, exons 11 to 14, 16, and 20; PDGFR α , exons 12, 14, and 17 to 19; NRAS and KRAS, exons 1 and 2; BRAF, exons 11 and 15; and CRAF, exon 12. Genomic DNA or cDNA was used as a template to amplify DNA. Genomic DNA was isolated by the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. Total RNA was extracted from cells with TRIzol (Invitrogen), and cDNA was synthesized by the SuperScript First-Strand Synthesis Kit (Invitrogen). Primers were designed by using Primer 5.0 version, and PCR reactions were done by using the HotStart Taq Master Mix (Qiagen) following recommended conditions. The efficiency and

quality of PCR for all the genes were confirmed by running the PCR products on a 2.0% agarose gel. The PCR products were subsequently subjected to direct sequencing after cleanup by using ExoSAP-IT (USB). Sequence analysis was done at the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children's Research Hospital. Supplementary Table S2⁴ lists the mutations identified.

Unbound Fraction in Plasma and Cell Culture

Unbound fractions of imatinib, sorafenib, and sunitinib in human plasma and cell culture medium containing 10% fetal bovine serum were determined by microequilibrium dialysis as described previously (16). Briefly, equilibrium dialysis was done on a 96-well Equilibrium DIALYZER with a regenerated cellulose membrane with a 5-kDa cutoff (Harvard Apparatus). Experiments were carried out with 200 μ L plasma containing clinically relevant steady-state concentrations of imatinib and sorafenib (1 μ mol/L) or sunitinib (0.1 μ mol/L) or 200 μ L cell culture medium with drug concentrations ranging from 0.01 to 10 μ mol/L and a tracer amount of ³H-labeled drug against an equal volume of PBS (pH 7.4).

Equilibrium dialysis was done on a rotator (Harvard Apparatus) at 37°C in a humidified atmosphere of 5% CO₂. The optimal time to equilibrium was assessed in quadruplicate in human plasma and cell culture 2, 4, 6, 16, 20, and 24 h after the start of the experiment and was found to be 6 h. At equilibrium, 75 μ L aliquots (in quadruplicate) of both compartments were transferred to scintillation vials and mixed with 5 mL Emulsifier-Safe high flash-point economy LSC cocktail for aqueous samples (Perkin-Elmer). Radioactivity in the buffer (Cu) and plasma (Cp) or cell culture (Cc) compartments was measured by liquid scintillation counting for 1 min on a Beckman LS 6500 multi-purpose scintillation counter (Beckman Instruments). Unbound drug fraction (fu) was calculated as $fu = Cu / Cp$ or $fu = Cu / Cc$. To express fu as a percentage, fu was multiplied by 100.

The IC₅₀ was corrected for the unbound fraction in cell culture medium by multiplying the mean IC₅₀ by the unbound fraction in cell culture. To compare *in vitro* unbound IC₅₀ to unbound exposures achieved in humans, C_{ss,ave,unbound} was estimated by multiplying C_{ss,ave,total} by the unbound fraction in human plasma. C_{ss,ave,total} was estimated from pharmacokinetic data in the literature by the following equation: C_{ss,ave,total} = area under the concentration-time curve at steady-state/dosing interval (17–19). C_{ss,ave,total} values were estimated to be 3 μ mol/L, 10 μ mol/L, and 100 nmol/L for imatinib, sorafenib, and sunitinib, respectively.

Intracellular Drug Accumulation

Logarithmically growing cells at a concentration of 3×10^6 were washed and seeded in six-well plates containing serum-free medium with 0.2 μ mol/L ³H-labeled imatinib or sorafenib or 0.1 μ mol/L ³H-labeled sunitinib. Cells were incubated for 2 h at 37°C with 5% CO₂ and then washed twice with ice-cold PBS, lysed with 0.5 mL of 1 N (1 mol/L) NaOH, and agitated for 10 min, and 0.25 mL of a 2 N (2 mol/L) HCl was added. An aliquot (25 μ L) of the

lysate was used to estimate protein concentration by the BCA protein assay kit. Radioactivity was measured in the cell lysate by liquid scintillation counting. Cellular accumulation was normalized to milligram protein as follows: cellular accumulation per milligram protein = reading (dpm) / [(0.6 × protein concentration µg/mL) / 1,000], and then normalized to initial radioactivity. Independent experiments were done twice in triplicate.

Results

Sorafenib and Sunitinib Are More Potent Inhibitors of Cellular Proliferation Than Imatinib

A comparison of the antiproliferative activity of imatinib, sorafenib, and sunitinib was done in nine human AML cell lines. Sorafenib and sunitinib were more potent than imatinib against Kasumi-1 cells with a c-KIT N822K mutation (heterozygous): with IC₅₀ of 40 and 23 nmol/L for sorafenib and sunitinib, respectively, versus 270 nmol/L for imatinib (Table 1). Sorafenib and sunitinib were potent inhibitors of the proliferation of MV4-11 cells with a FLT3 internal tandem duplication (homozygous), with IC₅₀ of 2 and 7 nmol/L, respectively (Table 1). Sorafenib and sunitinib also inhibited proliferation in the other seven cell lines, with IC₅₀ of 2.7 to 9.1 µmol/L for sorafenib and 1.1 to 13 µmol/L for sunitinib; M-07e cells were the least sensitive to sorafenib (IC₅₀, 9.1 µmol/L) and sunitinib (IC₅₀, 9.8 µmol/L) and NB4 cells to sunitinib (IC₅₀, 13 µmol/L). Other than Kasumi-1, the other eight AML cell lines were insensitive to imatinib, with IC₅₀ exceeding 20 µmol/L in most cell lines.

Sorafenib Is a More Potent Inhibitor of Cellular Proliferation Than Sunitinib Based on Unbound Fraction in Cell Culture Medium

The tyrosine kinase inhibitors we studied are highly bound to plasma proteins, and we expected a certain

degree of binding in cell culture medium. Therefore, to relate *in vitro* IC₅₀ for inhibiting cellular proliferation to exposures that are achievable *in vivo*, the unbound fractions of imatinib, sorafenib, and sunitinib in cell culture medium and human plasma were determined by equilibrium dialysis. Over the concentration range of 0.01 to 10 µmol/L, the binding of imatinib, sorafenib, and sunitinib in cell culture medium was independent of concentration. The unbound fractions in cell culture medium were similar for imatinib and sunitinib, with mean ± SD of 34 ± 1.7% and 39 ± 3.1%, respectively, but the binding in cell culture medium was substantially higher for sorafenib, with a mean ± SD of 1.3 ± 0.17%. Table 1 lists IC₅₀ corrected for unbound fractions in cell culture medium. Based on unbound IC₅₀, sorafenib is a more potent inhibitor of proliferation than imatinib and sunitinib are in all AML cell lines.

Unbound fractions in human plasma were 9.5 ± 0.5%, 0.59 ± 0.07%, and 8.4 ± 0.7% for imatinib, sorafenib, and sunitinib, respectively, indicating that sorafenib was much more highly bound than imatinib or sunitinib in human plasma. Based on the unbound fraction in human plasma, C_{ss,ave,unbound} values were estimated to be 0.29, 0.059, and 0.0084 µmol/L at the approved doses of 400, 400, and 50 mg for imatinib, sorafenib, and sunitinib, respectively. The unbound concentrations for sorafenib that inhibited the cellular proliferation of most cell lines *in vitro* can be achieved *in vivo* (Table 1). In contrast, only unbound concentrations of sunitinib and imatinib that can inhibit proliferation of cell lines with a c-KIT (Kasumi-1) or FLT3 (MV4-11) mutation can be achieved *in vivo*.

Sorafenib Has Higher Intracellular Accumulation Than Sunitinib and Imatinib

To determine whether differences in activity against cellular proliferation were due in part to differences in

Table 1. Total and unbound concentrations of tyrosine kinase inhibitors that inhibit the proliferation of AML cell lines

Cell line	IC ₅₀ (µmol/L)					
	Imatinib (C _{ss,ave,unbound} = 0.29 µmol/L)		Sunitinib (C _{ss,ave,unbound} = 0.0084 µmol/L)		Sorafenib (C _{ss,ave,unbound} = 0.059 µmol/L)	
	Total	Unbound	Total	Unbound	Total	Unbound
HL-60	29 ± 12	9.9	2.2 ± 0.60	0.86	3.3 ± 0.43	0.043
Kasumi-1	0.27 ± 0.065	0.092	0.023 ± 0.0050	0.009	0.040 ± 0.011	0.001
KG-1	17 ± 0.80	5.8	1.1 ± 0.16	0.43	2.7 ± 0.93	0.035
NB4	>40	>14	13 ± 3.7	5.1	6.6 ± 0.78	0.086
ML-2	16 ± 7.8	5.4	3.3 ± 0.46	1.3	3.7 ± 0.70	0.048
MV4-11	>20	>6.8	0.007 ± 0.0003	0.003	0.002 ± 0.0007	0.00003
THP-1	>20	>6.8	2.9 ± 0.65	1.1	6.2 ± 1.3	0.081
U937	18 ± 2.9	6.1	6.2 ± 2.3	2.4	6.9 ± 0.54	0.090
M-07e	>20	>6.8	9.8 ± 2.2	3.8	9.1 ± 2.8	0.12

NOTE: Total IC₅₀ are the mean ± SD of three independent experiments done in eight replicates. The unbound IC₅₀ for inhibiting proliferation was estimated for each drug and cell line by multiplying the mean total IC₅₀ by the unbound fraction in cell culture (34%, 39%, and 1.3%, for imatinib, sunitinib, and sorafenib, respectively). To relate unbound concentrations achieved clinically to unbound IC₅₀ *in vitro*, C_{ss,ave,unbound} in human plasma was estimated by multiplying C_{ss,ave,total} (3 µmol/L, 100 nmol/L, and 10 µmol/L for imatinib, sunitinib, and sorafenib, respectively, at the approved adult doses of 400, 50, and 400 mg, respectively) by the % unbound fraction in plasma (9.5%, 8.4%, and 0.59% for imatinib, sunitinib, and sorafenib, respectively).

intracellular drug exposure, the accumulation of sorafenib, sunitinib, and imatinib was determined in Kasumi-1, KG-1, NB4, ML-2, and MV4-11. Sorafenib exhibited the highest intracellular accumulation followed by sunitinib and then by imatinib (Fig. 1). Intracellular accumulation of all tyrosine kinase inhibitors was highest in MV4-11 cells, with intracellular accumulation relative to initial radioactivity being ~60% for sorafenib (Fig. 1A), 40% for sunitinib (Fig. 1B), and 20% for imatinib (Fig. 1C). For all three drugs, there was higher intracellular accumulation in the more sensitive cells lines than in the less sensitive ones (e.g., MV4-11 versus KG-1).

Sorafenib and Sunitinib Induce Apoptosis and Arrest in G₀-G₁ Phase of the Cell Cycle

Because sorafenib and sunitinib were more potent inhibitors of cellular proliferation than imatinib was in all the AML cell lines evaluated, we compared the ability of sorafenib and sunitinib to induce apoptosis and alter cell cycle distribution in MV4-11, Kasumi-1, KG-1, and U937, which exhibit a range of sensitivities to these agents. Sorafenib and sunitinib induced apoptosis in a time- and concentration-dependent manner (Fig. 2). After 72 h of drug exposure, sorafenib and sunitinib induced apoptosis in >50% of MV4-11 and Kasumi-1 cells at concentrations greater than or equal to the IC₅₀ for inhibiting cellular proliferation, with maximum apoptosis observed in 60% to 80% of cells at 0.1 and 1.0 μmol/L, respectively (Fig. 2A and B). In the less sensitive cell line KG-1, 10 μmol/L sorafenib or sunitinib was required to induce apoptosis in ~50% of cells (Fig. 2C). In another less sensitive cell line U937, sorafenib induced apoptosis at ≥10 μmol/L; sunitinib did not induce apoptosis at concentrations of up to 15 μmol/L (Fig. 2D). Sorafenib and sunitinib induced a concentration-dependent accumulation of cells in the G₀-G₁ phase of the cell cycle in MV4-11, Kasumi-1, and KG-1 cells, with a corresponding decrease in the percentage of cells in the S and G₂-M phases (Fig. 3). After 72 h of treatment, the extent of cell cycle block was associated with antiproliferative effects, with MV4-11 > Kasumi-1 > KG-1. A cell cycle block was not observed in U937 cells. Similar effects were observed with sunitinib, except in U937 cells, wherein an accumulation of cells in G₂-M at 10 μmol/L (Fig. 3D) coincided with the lack of induction of apoptosis.

Sorafenib Inhibits Proliferation of Primary AML Blasts

To examine the effect of sorafenib in leukemia cells, freshly isolated primary blasts from nine consecutive patients with AML were cultured in the presence of 0.1 to 10 μmol/L sorafenib for 4 days. Patient demographics, FAB type, karyotype, and FLT3 mutational status are summarized in Supplementary Table S3.⁴ Between 1 and 10 μmol/L, sorafenib inhibited the proliferation of blasts by ≥50% in 6 of 9 (67%) samples (Fig. 4). Six of nine samples were from patients with AML. FLT3 status was confirmed in four of six AML blast samples, and all were FLT3 wild-type; of these, inhibition of proliferation by sorafenib was observed in three (patients 3, 5, and 9; Fig. 4). Sorafenib also inhibited the proliferation of primary blasts from a patient with biphenotypic leukemia

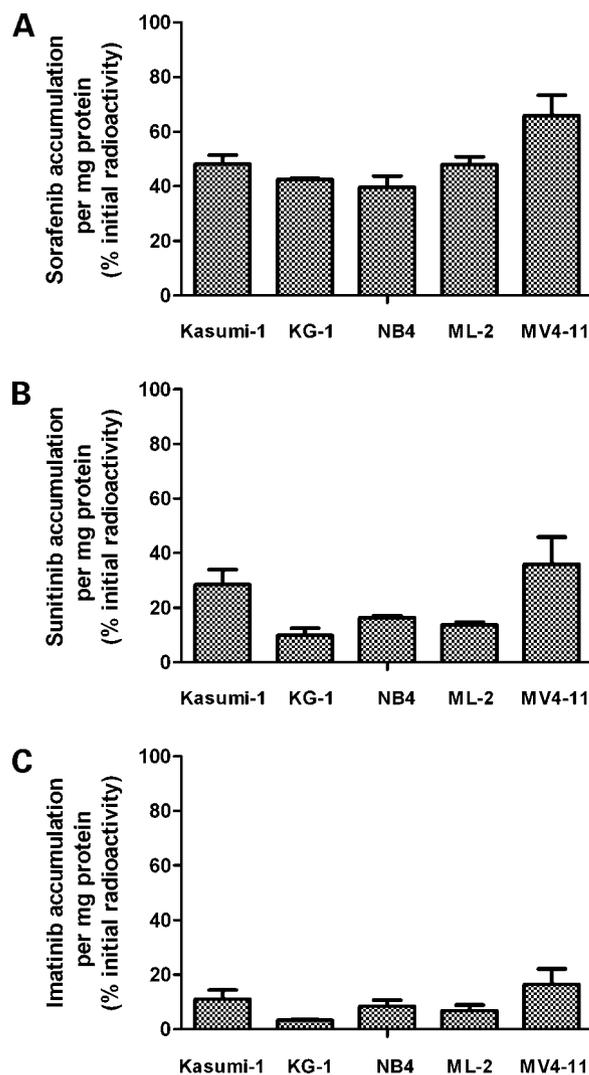


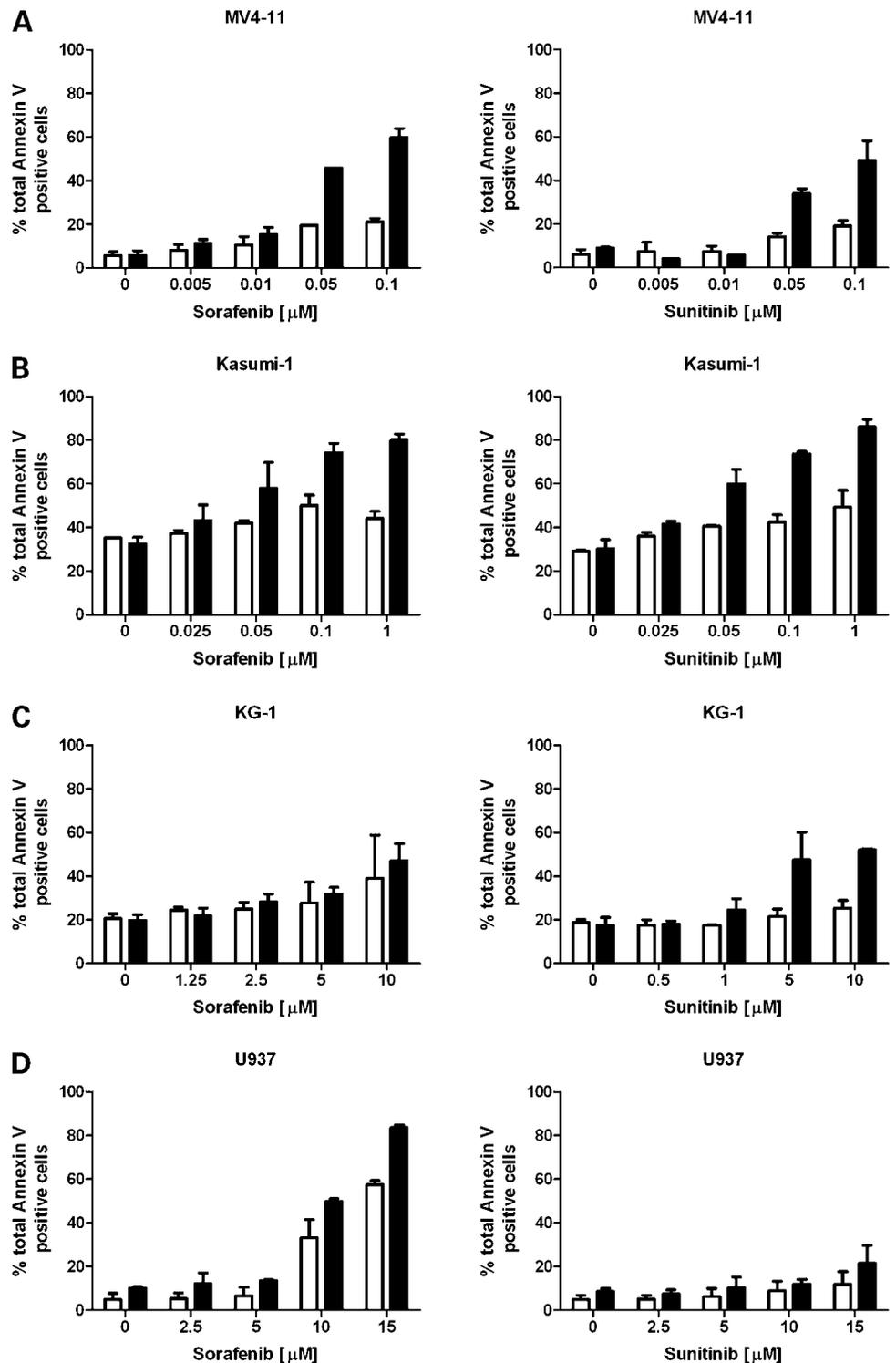
Figure 1. Intracellular accumulation of sorafenib, sunitinib, and imatinib in AML cell lines. Cells were incubated with ³H-labeled sorafenib (A), sunitinib (B), and imatinib (C) for 2 h. Radioactivity was measured in cell lysates, and cellular accumulation was normalized to milligram protein and then expressed as percent of initial radioactivity. Data represent two independent experiments done in triplicate.

and who was also positive for the Bcr-Abl gene translocation (patient 8; Fig. 4).

Treatment with Sorafenib and Sunitinib Is Associated with a Decrease of Phospho-ERK, Phospho-p70S6 Kinase, and Phospho-STAT5

The effect of sorafenib and sunitinib on receptor tyrosine kinase downstream signaling pathways was investigated in MV4-11, Kasumi-1, KG-1, and U937. After 1 h of drug treatment, sorafenib inhibited ERK phosphorylation in all four cell lines at concentrations comparable with the IC₅₀ for inhibiting cellular proliferation and in the range of concentrations inducing apoptosis (Fig. 5). Sorafenib had no effect on AKT phosphorylation, except in U937 cells, wherein phospho-AKT was inhibited at 5 and 10 μmol/L. Sorafenib inhibited p70S6 kinase phosphorylation in MV4-11 cells and

Figure 2. Induction of apoptosis by sorafenib and sunitinib in AML cell lines. MV4-11 (A), Kasumi-1 (B), KG-1 (C), and U937 (D) cells were treated with increasing doses of sorafenib or sunitinib for 24 h (open columns) or 72 h (closed columns), and Annexin V binding and propidium iodide staining were analyzed by flow cytometry. Data represent two independent experiments.



STAT5 in MV4-11 and KG-1 cells. Similar effects were observed with sunitinib, except, in U937 cells, wherein there was no inhibition of phospho-ERK or phospho-AKT at concentrations up to 10 $\mu\text{mol/L}$, which coincided with the resistance of U937 cells to sunitinib-induced apoptosis.

Discussion

Multitargeted tyrosine kinase inhibitors are a promising class of therapeutic agents for the treatment of AML. We did a comprehensive comparison of the activity of sorafenib and sunitinib in a panel of adult and pediatric

human AML cell lines representing different FAB types, chromosomal abnormalities, and genetic variants in tyrosine kinases. In general, sorafenib and sunitinib had similar activities in cell proliferation, apoptosis and cell cycle

assays, and inhibition of mitogen-activated protein kinase and STAT signaling. However, with consideration of the unbound fraction in cell culture medium, sorafenib was substantially more potent than sunitinib. The unbound

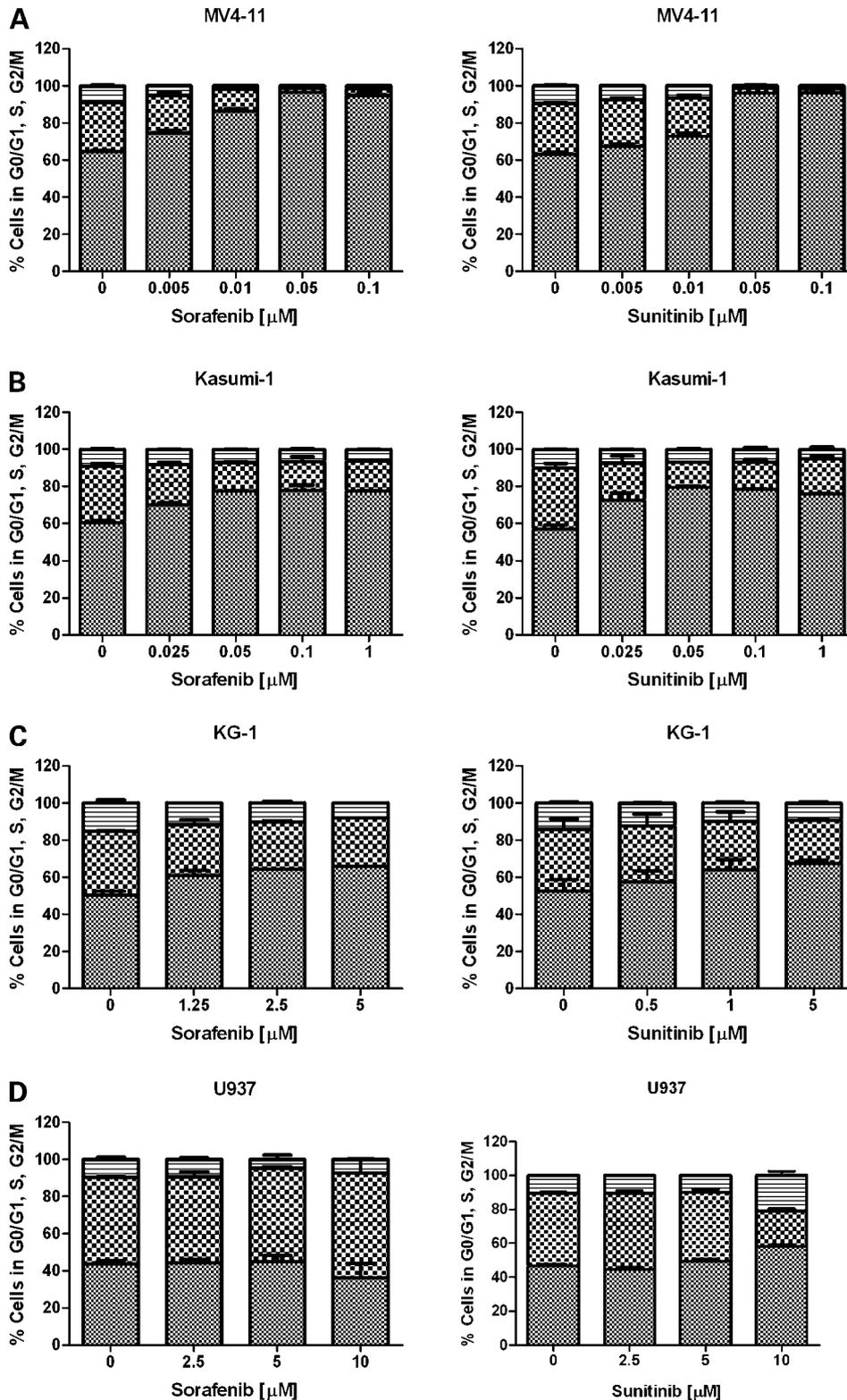


Figure 3. Induction of cell cycle arrest by sorafenib and sunitinib in AML cell lines. MV4-11 (A), Kasumi-1 (B), KG-1 (C), and U937 (D) cells were treated with increasing concentrations of sorafenib or sunitinib for 72 h, and propidium iodide staining was analyzed by flow cytometry. Cross-hatched columns, percentage of cells in G₀-G₁ phase; checkered columns, percentage of cells in S phase; horizontally hatched columns, percentage of cells in G₂-M phase. Data represent two independent experiments.

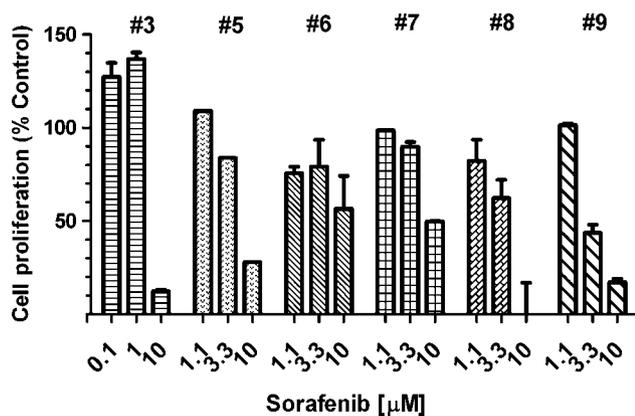


Figure 4. Sorafenib inhibits the proliferation of primary leukemic blast cells. Cells were cultured in the presence of sorafenib 0.1 to 10 $\mu\text{mol/L}$ for 96 h and cellular proliferation was determined using a MTT assay. Data represent one experiment done in duplicate and are expressed as the percentage of cell proliferation compared to DMSO-treated control cells.

concentrations of sorafenib that inhibited proliferation and promoted apoptosis in most AML cell lines can be achieved *in vivo*, whereas, for sunitinib, only unbound concentrations capable of inhibiting the proliferation of cell lines with a c-KIT or FLT3 mutation can be achieved *in vivo*. There was also higher intracellular accumulation of sorafenib than sunitinib in AML cells. Taken together, the results highlight the pharmacologic features of sorafenib that may provide it an advantage over sunitinib for the treatment of AML. Current studies are evaluating sorafenib for the treatment of relapsed/refractory AML as well as in combination with chemotherapy in patients with newly diagnosed AML.

In nine AML cell lines evaluated, all were less sensitive to imatinib than sorafenib and sunitinib in a proliferation assay. One reason imatinib may not be as effective as sorafenib and sunitinib is because it exhibits lower intracellular accumulation in AML cells. Another reason imatinib may not be as effective as these two agents is because it inhibits fewer kinases involved in aberrant signaling pathways in AML. For example, imatinib does not have activity against vascular endothelial growth factor receptor, identified as having a role in regulating cell proliferation, differentiation, and survival in leukemia (1). In a panel of 119 protein kinases, imatinib inhibited far fewer proteins than sorafenib and sunitinib (20). Due to overlap in the function of receptor tyrosine kinases and their downstream effectors, it is conceivable that agents that affect multiple targets in signaling pathways may be more efficacious than those affecting fewer proteins. However, the potential benefit of inhibiting multiple kinases simultaneously in AML requires further investigation.

During the *in vitro* evaluation of sorafenib, sunitinib, and imatinib in AML cell lines, we showed that the extracellular (e.g., free fraction in cell culture medium) and intracellular (e.g., accumulation) pharmacokinetics were different.

Knowledge of these drug properties may provide important information that can be used during further preclinical and clinical development. For example, we can use extracellular unbound concentrations that produce desired biological and antitumor effects in cell-based models to establish a starting target exposure in mice and humans during evaluation of pharmacokinetic-pharmacodynamic-efficacy relationships. Furthermore, we can evaluate the extent of intracellular accumulation as an indicator of drug sensitivity/resistance. For instance, there was higher intracellular accumulation of all drugs in the more sensitive cells lines than in the less sensitive ones (e.g., MV4-11 versus KG-1), and sunitinib had lower intracellular accumulation than sorafenib in all cell lines. This suggests that transporter expression may be an important determinant of activity for sunitinib and sorafenib as has been shown previously for imatinib (21–26); sunitinib may be more predisposed to transporter-related cellular resistance compared with sorafenib. After identifying which transporters are involved in cellular accumulation, a decision could be made to select one drug over the other based on transporter expression in the primary tumor of interest (e.g., AML blasts cells).

We evaluated the *in vitro* activity of sorafenib against leukemic cells from newly diagnosed children using the MTT assay. Sorafenib showed activity against 6 of 9 (67%) leukemic blast cells in the concentration range of 1 to 10 $\mu\text{mol/L}$, which we showed, based on assessment of unbound fraction in cell culture medium, are clinically relevant concentrations. Of four blast samples where FLT3 status was known, all were wild-type and sorafenib inhibited the proliferation of three of these. Sunitinib at a concentration of 1 $\mu\text{mol/L}$ has been shown to inhibit the proliferation of freshly isolated AML cells by >50% in 6 of 10 (60%) samples without mutations in FLT3 (27). However, based on unbound fraction in cell culture (39%), this equates to a concentration of 39 nmol/L, which is above the unbound average steady-state concentration ($C_{ss,ave,unbound}$) of 8 nmol/L achieved clinically. Sorafenib also inhibited the proliferation of primary blasts from a patient with biphenotypic leukemia and who was also positive for the Bcr-Abl gene translocation. Recently, sorafenib has been shown induce apoptosis in imatinib-resistant human leukemia cells expressing high levels of Bcr-Abl at clinically relevant concentrations of 10 to 15 $\mu\text{mol/L}$ and cells expressing mutations resistant to imatinib, dasatinib, and nilotinib (28). Previous studies have shown correlations between *in vitro* drug sensitivity of primary blasts cells and short-term clinical response in AML, although clear relationships with long-term clinical outcomes such as event-free and overall survival have not been observed (29–31). The *in vitro* testing of sorafenib does not necessarily indicate it will have activity in AML but provides an additional model system to evaluate drug activity at clinically relevant concentrations.

Several recent investigations have shown that inhibition of FLT3 does not prevent mitogen-activated protein kinase

phosphorylation or AKT signaling in some primary AML cells possibly because of activation of parallel downstream signaling pathways (32, 33). In our study, sorafenib and sunitinib inhibited mitogen-activated protein kinase

signaling (phospho-ERK1/2) in four AML cell lines evaluated but had no effect on phospho-AKT under the *in vitro* conditions employed. However, U937 cells were an exception, wherein sorafenib inhibited phospho-AKT but

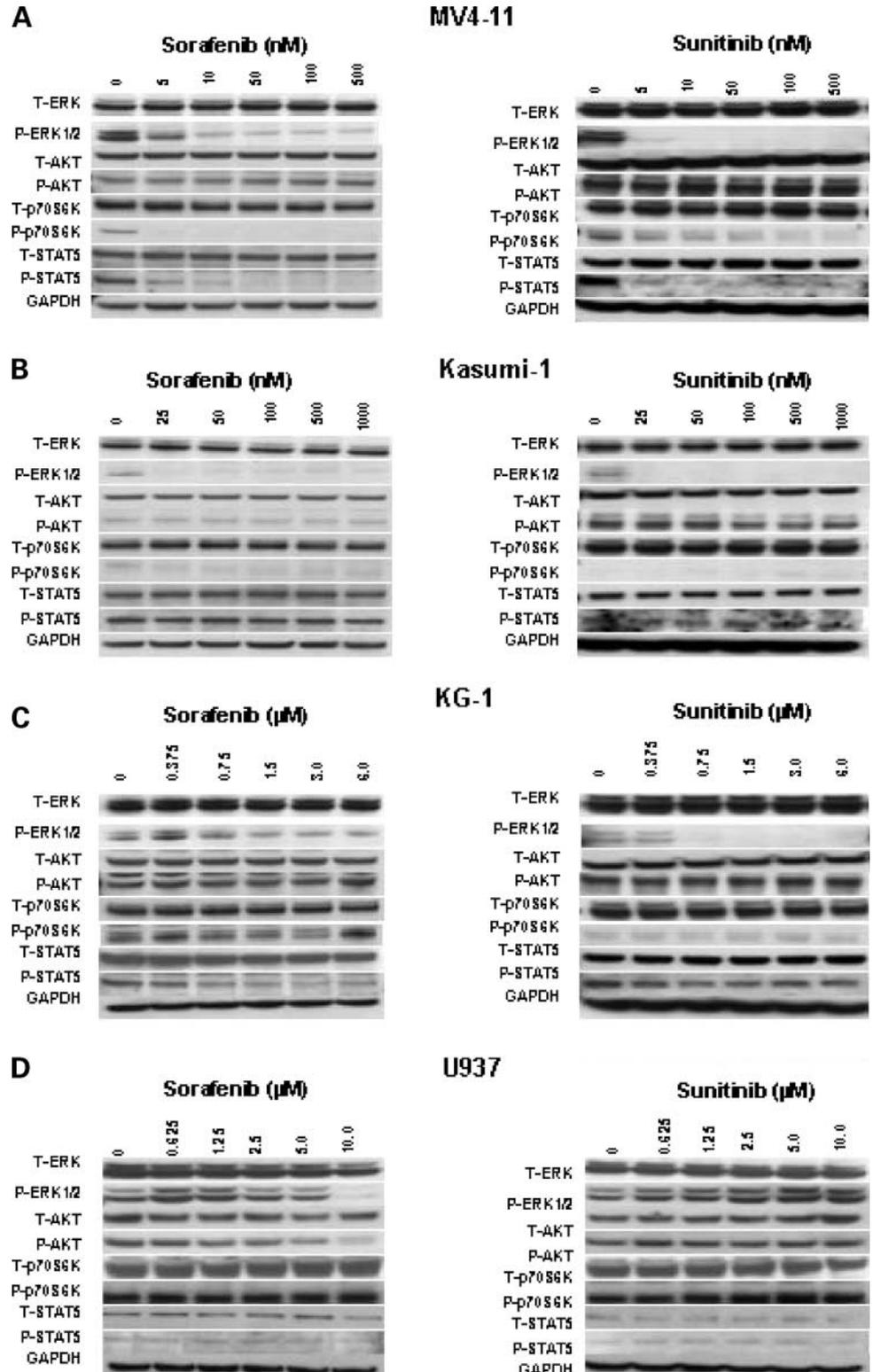


Figure 5. Effect of sorafenib and sunitinib on mitogen-activated protein kinase, AKT, and STAT signaling in AML cell lines. MV4-11 (A), Kasumi-1 (B), KG-1 (C), and U937 (D) cells were serum starved overnight and then treated with increasing concentrations of sorafenib or sunitinib for 1 h. Protein lysates were prepared and analyzed by Western blot. Equal amount of protein was loaded in each lane and probed sequentially with antibodies for the phosphorylated and total forms of ERK1/2, AKT, p70S6 kinase, and STAT5. Glyceraldehyde-3-phosphate dehydrogenase served as a loading control. Data represent one of two independent experiments.

sunitinib inhibited neither phospho-ERK1/2 nor phospho-AKT. U937 cells have a frameshift mutation (insertion of four bases) at position 1,423 in exon 5 of PTEN, which results in premature termination of the coding sequence and may contribute to activation of PI3K/AKT signaling and phospho-AKT inhibition by sorafenib in this cell line (34). The results of our study and those of previous investigations in AML cell lines and primary blasts suggest that optimal therapy of AML with receptor tyrosine kinase inhibitors involves combination treatment with specific inhibitors of downstream signaling pathways. There has been recent interest in combining sunitinib with an inhibitor of AKT/mTOR signaling (27) as well as evaluating a dual FLT3/AKT inhibitor in AML cell lines (35).

Multitargeted tyrosine kinase inhibitors such as sorafenib and sunitinib represent a promising class of therapeutic agents for the treatment of AML. Although sorafenib and sunitinib were similar in most pharmacologic assays, they were different in some ways, giving sorafenib a potential advantage over sunitinib in the clinic. As multiple signal transduction pathways are activated in AML, the evaluation of tyrosine kinase inhibitors in combination with inhibitors of downstream signaling pathways as well as with chemotherapy will likely improve the efficacy of these promising novel agents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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