Sorafenib Inhibits Imatinib-Resistant KIT and Platelet-Derived Growth Factor Receptor β Gatekeeper Mutants

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Abstract

Purpose: Targeting of KIT and platelet-derived growth factor receptor (PDGFR) tyrosine kinases by imatinib is an effective anticancer strategy. However, mutations of the gatekeeper residue (T670 in KIT and T681 in PDGFRβ) render the two kinases resistant to imatinib. The aim of this study was to evaluate whether sorafenib (BAY 43-9006), a multitargeted ATP-competitive inhibitor of KIT and PDGFR, was active against imatinib-resistant KIT and PDGFRβ kinases.

Experimental Design: We used *in vitro* kinase assays and immunoblot with phosphospecific antibodies to determine the activity of sorafenib on KIT and PDGFRβ kinases. We also exploited reporter luciferase assays to measure the effects of sorafenib on KIT and PDGFRβ downstream signaling events. The activity of sorafenib on interleukin-3–independent proliferation of Ba/F3 cells expressing oncogenic KIT or its imatinib-resistant T670I mutant was also tested.

Results: Sorafenib efficiently inhibited gatekeeper mutants of KIT and PDGFRβ (IC$_{50}$ for KIT T670I, 60 nmol/L; IC$_{50}$ for PDGFRβ T681I, 110 nmol/L). Instead, it was less active against activation loop mutants of the two receptors (IC$_{50}$ for KIT D816V, 3.8 μmol/L; IC$_{50}$ for PDGFRβ D850V, 1.17 μmol/L) that are also imatinib-resistant. Sorafenib blocked receptor autophosphorylation and signaling of KIT and PDGFRβ gatekeeper mutants in intact cells as well as activation of AP1-responsive and cyclin D1 gene promoters, respectively. Finally, the compound inhibited KIT-dependent proliferation of Ba/F3 cells expressing the oncogenic KIT mutant carrying the T670I mutation.

Conclusions: Sorafenib might be a promising anticancer agent for patients carrying KIT and PDGFRβ gatekeeper mutations.

The KIT and platelet-derived growth factor receptors (PDGFR) are members of the type III subclass of receptor tyrosine kinases. KIT is the receptor for stem cell factors (SCF), whereas PDGFRα and PDGFRβ are the receptors for platelet-derived growth factors (PDGF; ref. 1). The structure of these receptors includes an extracellular domain with five immunoglobulin-like motifs, a single membrane-spanning domain, and a cytoplasmic tyrosine kinase domain. The kinase domain is split by a kinase insert sequence into an ATP-binding region and a phosphotransferase region (1).

KIT, PDGFRα, and PDGFRβ are frequently activated in neoplastic diseases. More than 30 gain-of-function mutations in KIT, either single amino acid changes or small deletions/insertions, have been identified in such highly malignant human neoplastic diseases as gastrointestinal stromal tumors (GIST) and mastocytosis. GISTs are the most common type of sarcoma arising in the digestive tract and are generally distinguished from other abdominal sarcomas by the expression of KIT. Approximately 80% of these tumors show activating mutations in KIT (2). GIST mutations cluster in the KIT juxtamembrane region, whereas most mutations associated with mastocytosis target a specific aspartate residue (D816) in the kinase activation loop (3). Fusion of PDGFRα with different genes has been found in chronic myeloid leukemia and in hypereosinophilic syndrome (4). Moreover, GIST cases that are negative for KIT mutations almost invariably display activating point mutations in the juxtamembrane domain or in the activation loop of PDGFRα (5). The closely related PDGFRβ receptor is often activated by rearrangements in chronic myelomonocytic leukemia (6).

Imatinib (imatinib mesylate, STI571; Gleevec or Glivec) is an ATP-competitive inhibitor that has revolutionized drug therapy of chronic myeloid leukemia. Imatinib is very effective [*in vitro* half-maximal inhibitory concentration (IC$_{50}$) of 25 nmol/L] against the chronic myeloid leukemia–causing kinase BCR-ABL (7). It also efficiently inhibits KIT (*in vitro* IC$_{50}$, 410 nmol/L) and PDGFR (*in vitro* IC$_{50}$, 380 nmol/L). Consequently, it has

been successful in the treatment of cancer patients carrying activating KIT or PDGFR mutations (2, 5, 7). In clinical studies, 75% to 90% of patients with advanced GISTs treated with imatinib experienced a clinical benefit (7).

However, KIT and PDGFRα variants carrying mutations in the kinase activation loop (D816 in KIT; refs. 8, 9; and D842 in PDGFRα; ref. 5, which corresponds to D850 in PDGFRβ) are refractory to imatinib. Therefore, mastocytosis (KIT) and GIST (KIT or PDGFRα) patients with these mutations respond poorly to imatinib (2, 5). X-ray analysis has shown that imatinib binds preferentially to the inactive form of the kinase. It is conceivable that these mutations disrupt the kinase structure and so disable interaction with the drug (10). Moreover, some patients who initially respond to imatinib subsequently relapse. This secondary resistance usually results from the emergence of tumor clones with mutations in the kinase domain that prevent drug binding. In particular, some mutations in KIT (T670I; refs. 11–14) or PDGFR (T674I in PDGFRα and T681I in PDGFRβ) cause imatinib resistance (15–17). These mutations correspond to the Thr[315]-to-isoleucine substitution (T315I) in BCR-ABL that frequently causes resistance in patients with chronic myeloid leukemia. Given its role in controlling the susceptibility of kinases to drug inhibition, this particular residue has been designated as a "gatekeeper." The gatekeeper threonine interacts with imatinib via a hydrogen bond. It seems that its replacement with a large bulky amino acid (isoleucine) prevents the drug-kinase interaction (10). Other mutations in KIT and PDGFR have been reported to cause secondary resistance and these include mutations of D816 in KIT and D842 in PDGFRα (2).

Sorafenib, also known as BAY43-9006, is a multikinase inhibitor of the bi-aryl-urea chemical class (18). It targets the RAF family of serine/threonine kinases and the tyrosine kinase receptors VEGFR-2 (KDR), VEGFR-3 (Flt-4), Flt-3, PDGFR, and KIT (19). Sorafenib is undergoing advanced clinical trials and has been recently Food and Drug Administration–approved under the name of Nexavar for the treatment of advanced renal cell carcinoma (18). We recently showed that sorafenib is a potent inhibitor of the wild-type and mutant RET kinase. Importantly, sorafenib also inhibited RET gatekeeper mutants (V804M/L; ref. 20). Here, we have investigated the activity of sorafenib against imatinib-resistant KIT and PDGFRβ mutants.

Materials and Methods

Compounds. Sorafenib [BAY 43-9006, *N*-(3-trifluoromethyl-4-chlorophenyl)-*N*′-(4-[2-methylcarbamoyl pyridin-4-yl]oxyphenyl) urea], was provided by Bayer HealthCare Pharmaceuticals. The compound was dissolved in DMSO.

Plasmids. pCMV-KIT, encoding wild-type mouse KIT, and pcDNA 3.1-PDGFRβ, encoding wild-type human PDGFRβ (21), were kindly donated by C. Sette (Dept. Sanita' Pubblica e Biologie Cellulare, Universita' di Roma, Rome, Italy) and C.H. Heldin (Ludwig Institute for Cancer Research, Uppsala University, Uppsala, Sweden), respectively. PDGFRβ T681I and D850V mutants and KIT T670I and KIT D814V mutants were generated by site-directed mutagenesis. All the mutations were confirmed by double-strand DNA sequencing. Because the mouse KIT D814V mutation corresponds to the human KIT D816V, for the sake of clarity, this mutant is called KIT D816V throughout this article.

Antibodies. Antibodies to KIT and PDGFRβ were as follows: KIT (Cell Signaling Technologies), phospho-KIT (pY721; Biosource, Invitrogen Corporation), PDGFRβ (Santa Cruz Biotechnology) and phospho-

PDGFRβ (pY1021; Santa Cruz Biotechnology). Antibodies to SHC were from Upstate Biotechnology, Inc., antibodies to phospho-SHC (which recognize phosphorylated SHC at Y317) were from Santa Cruz Biotechnology, antibodies to mitogen-activated protein kinases (MAPK) were from Cell Signaling Technologies, and antibodies to phospho-p44/42 MAPK (pMAPK), specific for MAPK (ERK1/2) phosphorylated at Thr[202]/Tyr[204], were from Cell Signaling Technologies. Monoclonal anti–α-tubulin was from Sigma Chemical, Co. Secondary antibodies coupled to horseradish peroxidase were from Santa Cruz Biotechnology.

Cell cultures and luciferase assays. HEK293 cells were from the American Type Culture Collection and were grown in DMEM supplemented with 10% FCS, 2 mmol/L of L-glutamine, and 100 units/mL of penicillin-streptomycin (Life Technologies). NIH3T3 fibroblasts were cultured in DMEM supplemented with 10% calf serum, 2 mmol/L of L-glutamine, and 100 units/mL of penicillin-streptomycin. HeLa cells were grown in DMEM supplemented with 10% FCS, 2 mmol/L of L-glutamine, and 100 units/mL of penicillin-streptomycin.

Transient transfections were carried out with the LipofectAMINE reagent according to the manufacturer's instructions (Life Technologies). HeLa cells (1×10^6) were transiently transfected with vectors expressing KIT wt, KIT T670I and KIT D816V, and the AP1-luciferase vector (Stratagene) containing six AP1-binding sites upstream from the Firefly luciferase cDNA. Twenty-four hours after transfection, cells were serum-starved and 100 ng/mL of SCF (Prepotech) was added to the KIT wt and KIT T670I–transfected cells. NIH3T3 mouse fibroblasts (1×10^6) were transiently transfected with vectors expressing PDGFRβ wt, PDGFRβ T681I, or PDGFRβ D850V, and with the cyclin D1-luciferase vector (22) containing -1,745 bp of the human cyclin D1 promoter upstream from the Firefly luciferase cDNA. This vector was kindly provided by S.J. Gutkind (NIH, Bethesda, MD). Twenty-four hours after transfection, cells were serum-starved and 100 ng/mL of PDGF BB (Prepotech) were added to PDGFRβ wt and PDGFRβ T681I–transfected cells. Ten nanograms of pRL-null (a plasmid expressing the enzyme Renilla luciferase from *Renilla reniformis*) was used as an internal control. Firefly and Renilla luciferase activities were assayed using the Dual-Luciferase Reporter System (Promega Corporation). Light emission was quantitated using a Berthold Technologies luminometer (Centro LB 960) and expressed as a percentage of residual activity compared with untreated cells. Average results of three independent assays ± SD are indicated. Student's *t* test was used to assess statistical significance.

Stable pools of Ba/F3 cells expressing KIT Δ557-558 or the imatinib-resistant Δ557-558/T670I double mutant were selected for interleukin-3 (IL-3)–independent and G418-resistant growth.[3] Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum with or without IL-3 culture supplement (BD Biosciences). For cell proliferation assays, Ba/F3 cells were plated in complete medium with or without IL-3 and different doses of sorafenib. After 72 h, cell growth was evaluated by measuring luminescence with a CellTiter-Glo kit (Promega Corporation). Average IC$_{50}$ (nmol/L) of the compound for Ba/F3 cellular proliferation was calculated by using linear regression methods (GraphPad Software Inc.) from at least three experiments (minus IL-3: *n* > 3; plus IL-3: *n* = 3) and was reported means ± SE.

Protein studies. Immunoblotting experiments were done according to standard procedures. Briefly, cells were harvested in lysis buffer [50 mmol/L Hepes (pH 7.5), 150 mmol/L NaCl, 10% glycerol, 1% Triton X-100, 1 mmol/L EGTA, 1.5 mmol/L MgCl$_2$, 10 mmol/L NaF, 10 mmol/L sodium PPi, 1 mmol/L Na$_3$VO$_4$, 10 μg of aprotinin/mL, 10 μg of leupeptin/mL] and clarified by centrifugation at 10,000 × *g*. Protein concentrations were estimated with a modified Bradford assay (Bio-Rad). Antigens were revealed by an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech). Signal intensity was evaluated with the PhosphorImager (Typhoon 8600, Amersham Pharmacia Biotech) interfaced with the ImageQuant software.

For the *in vitro* KIT (23) and PDGFRβ (21) kinase assays, proteins (500 μg) were immunoprecipitated with the required antibodies.

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[3] Gedrich R. and Sullivan E., unpublished data.

Immunocomplexes were recovered with protein A Sepharose beads, washed five times with kinase buffer, and subjected to *in vitro* autophosphorylation by incubating (20 min at room temperature) the immunocomplex with kinase buffer, 2.5 μCi [γ-^{32}P]ATP, unlabeled ATP (20 μmol/L), and the indicated concentrations of the compound. Samples were separated by SDS-PAGE; gels were dried and exposed to autoradiography. Signal intensity was analyzed with the PhosphorImager (Typhoon 8600) interfaced with the ImageQuant software. The average results of three experiments done in duplicate $\pm$SD are reported. Kinase activity curves were plotted with the curve-fitting PRISM software (GraphPad InStat Software).

Statistical analysis. Two-tailed unpaired Student's *t* test (normal distributions and equal variances) were used for statistical analysis. Differences were significant at $P < 0.02$. Statistical analyses were done using the GraphPad InStat Software program (version 3.06.3).

Results

Sorafenib activity on KIT and PDGFRβ kinases. The IC$_{50}$ of sorafenib for KIT and PDGFRβ was 68 and 57 nmol/L, respectively (19). We tested if sorafenib inhibits imatinib-resistant KIT and PDGFRβ kinases that have mutations in the gatekeeper residue (KIT T670I and PDGFRβ T681I) or in the activation loop (KIT D816V and PDGFRβ D850V; Fig. 1A). In an immunocomplex kinase assay, we measured KIT T670I and KIT D816V proteins' autophosphorylation *in vitro* in the presence of different sorafenib concentrations. The drug strongly inhibited the T670I KIT gatekeeper mutant (IC$_{50}$, 60 nmol/L), but it was less active on the D816V activation loop mutant (IC$_{50}$, 3.8 μmol/L; Fig. 1B). An *in vitro* kinase assay with a GST-KIT (TK) recombinant protein carrying the D816V mutation confirmed these findings (data not shown). Also, the

PDGFRβ gatekeeper mutant (T681I) was potently inhibited by sorafenib *in vitro* (IC$_{50}$, 110 nmol/L). Similar to the D816V KIT mutant, the PDGFRβ activation loop mutant (D850V) was less efficiently inhibited (IC$_{50}$, 1.17 μmol/L; Fig. 1C).

Next, we tested the inhibitory effects of sorafenib on KIT and PDGFRβ autophosphorylation in intact cells. HEK293 cells were transiently transfected with either wild-type, gatekeeper, or activation loop mutant receptors. Transfected cells were treated with different doses (0.1, 0.5, and 1 μmol/L) of the compound for 2 h. Because they did not display detectable basal phosphorylation levels, cells expressing wild-type or gatekeeper mutant receptors were stimulated with 100 ng/mL of the specific ligand (SCF for KIT or PDGF BB for PDGFRβ) for 10 min before harvesting. Consistent with their oncogenic properties, activation loop mutants displayed constitutive kinase activity and did not require ligand stimulation (1). Receptor activation was monitored by Western blotting with specific phospho-KIT (pY721) and phospho-PDGFRβ (pY1021) antibodies. Relative phosphorylation levels in compound-treated compared with vehicle-treated cells were calculated with PhosphorImager. Sorafenib was very effective in blocking wild-type KIT and PDFGRβ phosphorylation (Fig. 2). At the 100 nmol/L dose, the compound blocked receptor phosphorylation by >90%. Moreover, gatekeeper mutants were very sensitive to sorafenib (100 nmol/L sorafenib blocked KIT T670I and PDGFRβ T681I receptor phosphorylation in intact cells by 80%; Fig. 2). Instead, the drug was clearly less active against the activation loop mutants: 1 μmol/L of sorafenib inhibited KIT D816V and PDGFRβ D850V by ~70%, whereas the inhibitory effect at 100 nmol/L was barely detectable.

Fig. 1. Effect of sorafenib on the *in vitro* kinase activity of KIT and PDGFRβ gatekeeper and activation loop mutants. *A,* the KIT and PDGFRβ mutants studied. Black bars, the transmembrane domain. *B,* HEK293 cells were transiently transfected with CMV-6 vectors expressing KIT T670I and KIT D816V mutants. KIT proteins were immunoprecipitated (500 μg) and subjected to *in vitro* autophosphorylation. Signal intensity was analyzed with PhosphorImager. The average results of three experiments done in duplicate $\pm$ SD are plotted with the curve-fitting PRISM software. The IC$_{50}$ for each protein is indicated. *C,* HEK293 cells were transiently transfected with pcDNA 3.1 vectors encoding PDGFRβ T681I and D850V mutants. Proteins were immunoprecipitated (500 μg) and subjected to *in vitro* autophosphorylation assay. Reactions were processed as described above.
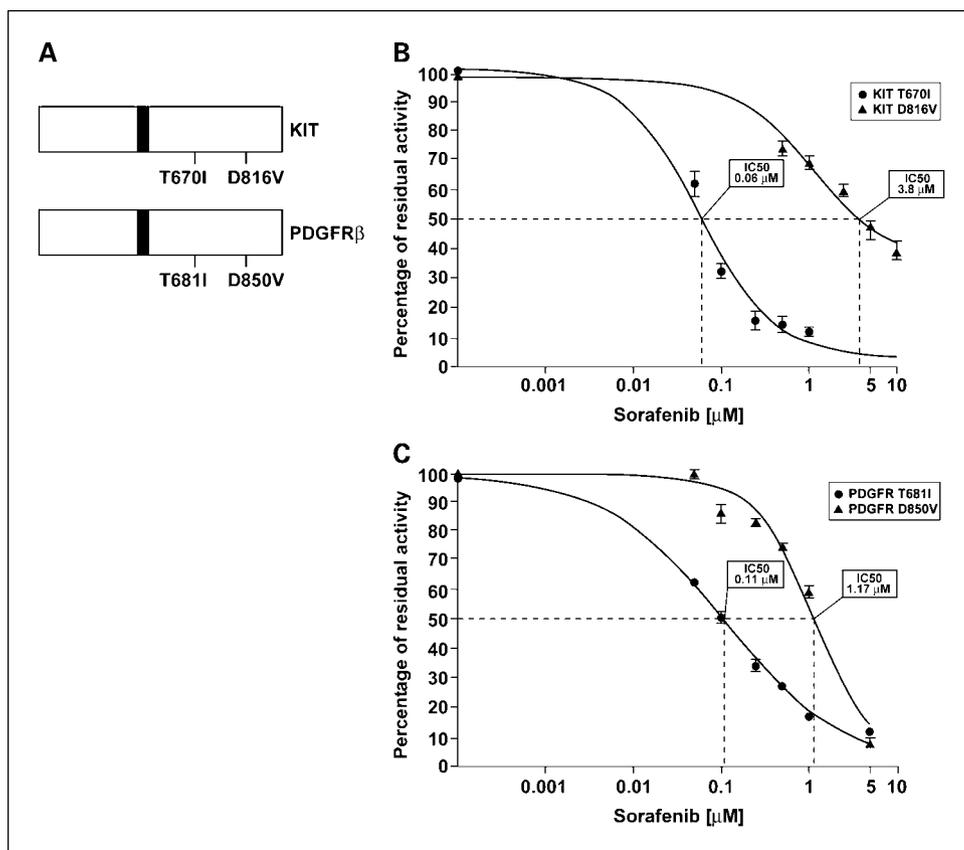
Sorafenib activity on KIT and PDGFRβ signaling. Then, we investigated whether sorafenib intercepts KIT and PDGFRβ downstream signaling. Ligand-stimulation of HEK293 cells adoptively expressing wild-type KIT and PDGFRβ or their gatekeeper mutant–induced SHC adaptor protein and MAPK phosphorylation (ref. 24; Fig. 3). The activation loop mutants of KIT and PDGFRβ also induced SHC and MAPK phosphorylation and did not require ligand triggering because of their constitutive kinase activity (Fig. 3). Treatment of cells with different doses of sorafenib strongly inhibited wild-type and gatekeeper mutant–dependent SHC and MAPK phosphorylation. In contrast, stimulation of SHC and MAPK phosphorylation by the activation loop mutants was less efficiently blocked by sorafenib; in this case, marked inhibition was seen only at a concentration of 1 μmol/L (Fig. 3).

The effects of sorafenib on KIT and PDGFRβ transcriptional activity. In a first set of experiments, we noted that KIT activation triggered the transcription of a luciferase reporter downstream from an AP1-responsive promoter in NIH3T3 murine fibroblasts and that PDFGRβ was a potent activator of a cyclin D1 promoter in HeLa cells. For PDFGRβ, we could not use NIH3T3 cells because fibroblasts express endogenously high levels of the receptor (data not shown). Thus, we evaluated whether sorafenib blocked receptor activity on these promoters. The AP1-luciferase reporter activity was hindered when KIT and KIT T670I cells were treated for 24 h with 0.1, 0.5, and 1 μmol/L of sorafenib (~10-fold reduction at 1 μmol/L; P < 0.02; Fig. 3C). Similarly, cyclin D1-luciferase promoter activity was inhibited when PDGFRβ and PDGFRβ

T681I cells were treated with 0.1, 0.5, and 1 μmol/L of sorafenib (~40-fold reduction at 1 μmol/L; P < 0.02; Fig. 3D). Although sorafenib was less active on the activation loop mutants of the two receptors, it still exerted a significant inhibitory activity at 1 μmol/L (~2-fold reduction of KIT D816V and ~7-fold reduction of PDGFRβ D850V) and some activity at 0.5 μmol/L (1.5-fold reduction for KIT D816V and 4-fold reduction for PDGFRβ D850V; P < 0.02; Fig. 3C and D).

The effects of sorafenib on KIT mitogenic activity. The murine pro–B cell line Ba/F3 requires the cytokine IL-3 for proliferation and survival. Expression of constitutively active KIT mutants confers IL-3–independent growth to Ba/F3 cells (25). Thus, Ba/F3 cells were transfected with plasmids expressing either the imatinib-sensitive oncogenic KIT mutant containing a deletion of residues 557 to 558 (Δ557-558) in the juxtamembrane domain or the imatinib-insensitive KIT double mutant containing both the Δ557-558 and the T670I mutations (3). In the absence of IL-3, sorafenib potently inhibited the growth of both the Δ557-558 and Δ557-558/T670I KIT–expressing cells (Table 1). The compound had virtually no effect on the proliferation of parental or Ba/F3-transfected cells grown in the presence of IL-3, indicating that the antiproliferative effects of the compound were selective and dependent on activated KIT signaling (Table 1).

Discussion

Here we show that sorafenib targets a number of mutant KIT and PDGFRβ kinases. Thus, based on its advanced clinical
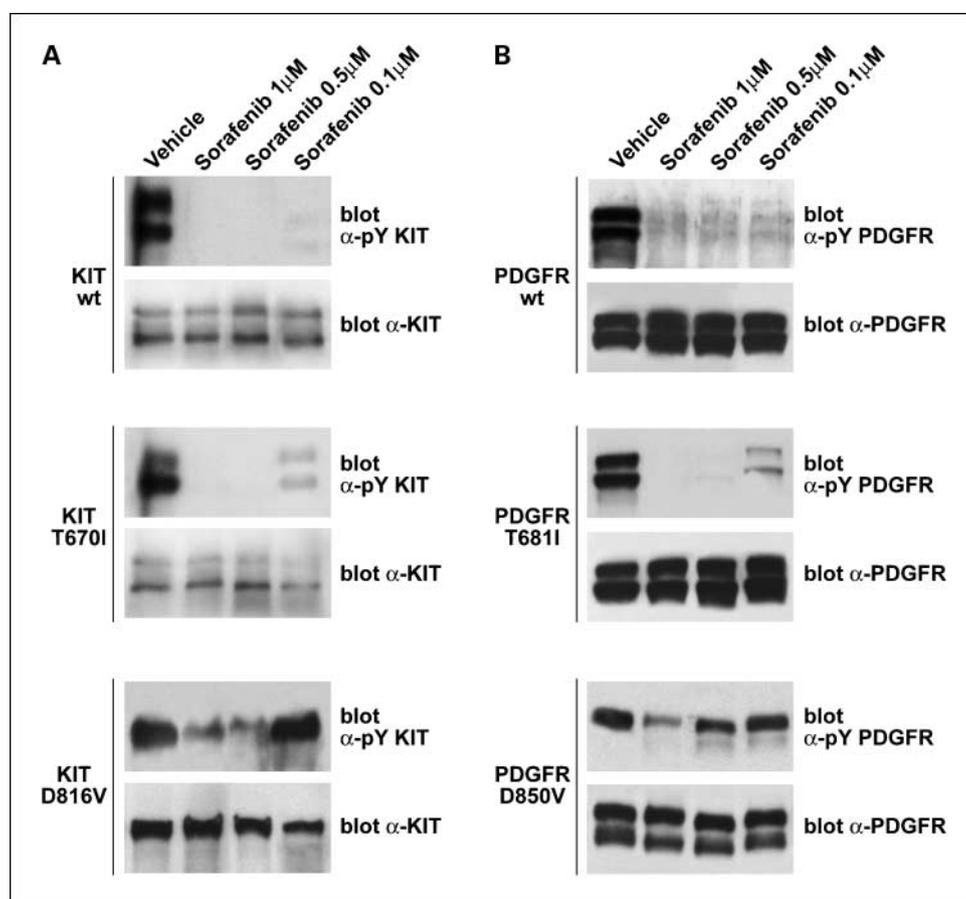

Fig. 2. Effect of sorafenib on KIT and PDGFRβ gatekeeper and activation loop mutants in intact cells. HEK293 cells were transfected with vectors expressing KIT, KIT T670I, KIT D816V (*A*), or PDGFRβ, PDGFRβ T681I, and PDGFRβ D850V (*B*). Twenty-four hours after transfection, cells were serum-starved. Two hours before being harvested, cells were treated with the indicated concentration of sorafenib. KIT wt and KIT T670I–transfected cells were stimulated with 100 ng/mL of SCF for 10 min, whereas PDGFRβ wt and PDGFRβ T681I–transfected cells were stimulated with 100 ng/mL of PDGF BB for 10 min. Cell lysates were analyzed by immunoblot with the antibodies to KIT, phospho-KIT (*pY KIT*), PDGFRβ (*PDGFRβ*) and phospho-PDGFRβ (*pY PDGFRβ*). *A*, proteins (0.5 mg) were immunoprecipitated with anti-KIT before being subjected to immunoblot (*top* and *middle*). Each experiment is representative of at least three independent experiments. Signal intensity was analyzed with PhosphorImager. Relative phosphorylation levels compared with vehicle-treated cells were calculated.
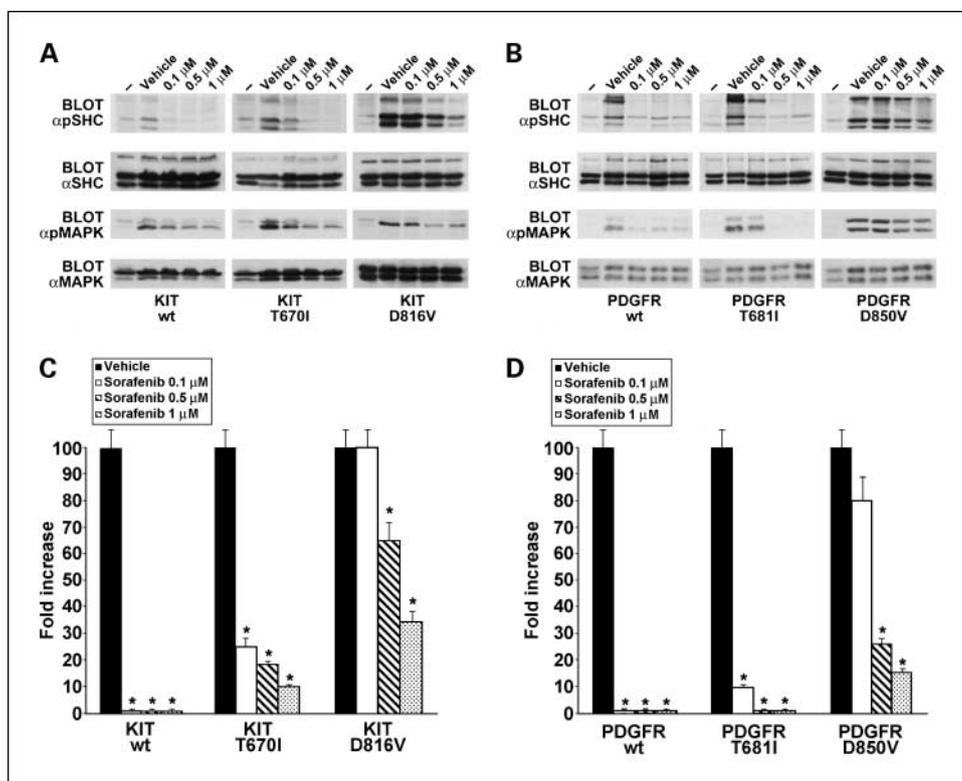
Fig. 3. Effect of sorafenib on KIT and PDGFRβ intracellular signaling. HEK293 cells were transfected with vectors expressing KIT wt, KIT T670I, KIT D816V (*A*), PDGFRβ wt, PDGFRβ T681I, and PDGFRβ D850V (*B*). Twenty-four hours after transfection, cells were serum-starved. Two hours before being harvested, cells were treated with the indicated concentration of sorafenib, and KIT wt and KIT T670I – transfected cells were then stimulated with 100 ng/mL of SCF for 10 min, whereas PDGFRβ wt and PDGFRβ T681I – transfected cells were stimulated with 100 ng/mL of PDGF BB for 10 min. Cell lysates were analyzed by immunoblot with antibodies to SHC, phospho-SHC (*pSHC*), MAPK, and phospho-p44/42 MAPK (*pMAPK*). Each experiment is representative of at least three experiments. *C*, NIH3T3 cells (1 ×10^6) were transiently transfected with vectors expressing KIT wt, KIT T670I, and KIT D816V, and the AP1-luciferase vector. Twenty-four hours after transfection, cells were serum-starved and 100 ng/mL of SCF was added to the KIT wt and KIT T670I – transfected cells. *D*, HeLa cells (1 ×10^6) were transiently transfected with vectors expressing PDGFRβ wt, and PDGFRβ T681I- and PDGFRβ D850V, and with the cyclin D1-luciferase vector. Twenty-four hours after transfection, cells were serum-starved and 100 ng/mL of PDGF BB were added to PDGFRβ wt and PDGFRβ T681I – transfected cells. The drug was added 24 h before harvesting at the indicated concentrations. Luciferase activity is expressed as a percentage of residual activity compared with cells that had not been treated with sorafenib. Columns, average results of three independent assays; bars, SD; *, $P < 0.02$, Student's *t* test was used to assess statistical significance (vehicle versus treatment).

development, sorafenib may be effective in the treatment of cancer driven by KIT or PDGFR mutations. We also show that sorafenib is less active against the activation loop mutants of KIT and PDGFRβ (D816V in KIT and D850V in PDGFRβ) than against wild-type and gatekeeper mutants (T670 in KIT and T681 in PDGFRβ) of the two receptors. Nevertheless, in intact cells, we observed the inhibition of activation loop mutants at concentrations between 0.5 and 1.0 μmol/L. Whatever the mechanism, such an increase in potency in cellular assays with

respect to *in vitro* kinase activity warrants further investigation to verify whether sorafenib could also be used to inhibit these mutants in a clinical setting.

Noteworthy, other compounds proved their efficacy against KIT or PDGFR activation loop mutants. These included dasatinib (BMS-354825), a small molecule inhibitor of SRC and ABL tyrosine kinases (26); PKC412, a staurosporine-derived tyrosine kinase inhibitor that targets protein kinase C; VEGFR-2, FLT-3, PDGFR and KIT (15, 27), and EXEL-0862,

Table 1. Sorafenib inhibits growth of Ba/F3 cell expressing the imatinib-resistant Δ557-558/T670I KIT gatekeeper double mutant

Compound	Ba/F3 cells*				
	Δ557-558		Δ557-558/T670I		Parental
	-IL-3	+IL-3	-IL-3	+IL-3	+IL-3
Sorafenib (IC$_{50}$)	6 ± 1	4,920 ± 1,070	14 ± 2	5,940 ± 1,920	6,400 ± 1,360

*Parental Ba/F3 cells or cells expressing KIT D557-558 or the imatinib-resistant D557-558/T670I double mutant were plated in complete medium with or without IL-3 and different doses of sorafenib. After 72 h, cell growth was evaluated by measuring luminescence with a CellTiter-Glo kit. Average IC$_{50}$ (nmol/L) for Ba/F3 cellular proliferation was calculated by using linear regression method (GraphPad Software, Inc.) from at least three experiments (minus IL-3: $n > 3$; plus IL-3: $n = 3$) and is reported as means ± SE.

Table 2. Inhibitory concentration of various TKIs against KIT and PDGFR imatinib-resistant mutants

Mutants	Receptor	Sorafenib (nmol/L)	Dasatinib (nmol/L)	PKC412 (nmol/L)	EXEL-0862 (nmol/L)	SU-11248 (nmol/L)
Gatekeeper	KIT	60	10,000 (26)	100 (35)	Not reported	70 (33)
	PDGFR	110	Not reported	50 (15)	Not reported	Not reported
Activation loop	KIT	3,800	1-100 (26)	44 (27)	42 (28)	Not reported
	PDGFR	1,170	Not reported	50 (39)	Not reported	>1,000 (34)

a novel kinase inhibitor active against fibroblast growth factor receptors, VEGFRs, PDGFR, FLT3, and KIT (Table 2; ref. 28).

On the other hand, gatekeeper mutation induces resistance to many inhibitors, rendering the identification of second-line treatments quite difficult (29–31). Although other strategies have been envisaged to counteract the activity of these mutants, such as the use of the heat shock protein 90 inhibitor 17-allylamino-18-demethoxy-geldanamycin (32), compounds able to inhibit gatekeeper mutants are urgently needed to provide a solution to the challenge of molecular resistance due to secondary mutations at this site. Thus far, only a few compounds have been reported to be active on these mutants. SU-11248 was found to inhibit the KIT T670I kinase (33, 34) and PKC412 inhibited both KIT and PDGFR gatekeeper mutants (Table 2; refs. 15, 35). Our data indicate that sorafenib is active against KIT and PDGFR gatekeeper mutations. Indeed, these mutants were efficiently inhibited at a drug concentration (60 nmol/L for KIT T670I and 110 nmol/L for PDGFRβ T681I) that is well below the average plasma concentration of the drug. Pharmacokinetic studies have shown that on multiple oral doses of 400 mg b.i.d., the C_{max} value of sorafenib was 9.35 mg/L, which corresponds to a concentration of ~ 20 μmol/L. Up to 99% of the drug is bound to serum proteins leading to an unbound concentration of the drug of ~ 200 nmol/L (36).

Consistent with our findings, Lierman and coworkers have recently shown that FIPL1-PDGFRα, the oncogenic rearrangement of PDGFRα, another PDGFR family member, found in HES (patients with idiopathic hypereosinophilic syndrome), and its T674I gatekeeper mutant are efficiently inhibited by sorafenib (37).

In conclusion, our study suggests that sorafenib might be a useful therapeutic agent to treat tumors harboring the imatinib-resistant KIT T670I or PDGFR T681I mutants. In the chronic myeloid leukemia model, it has been reported that the combination of imatinib with an inhibitor of imatinib-resistant BCR-ABL mutants can be used to prevent the emergence of resistance (38). Similarly, the combination of sorafenib with imatinib might be envisaged to reduce the risk of the emergence of treatment-resistant neoplastic clones harboring KIT or PDGFR mutants.

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