

Sorafenib Inhibits the Imatinib-Resistant *KIT*^{T670I} Gatekeeper Mutation in Gastrointestinal Stromal Tumor

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Abstract Purpose: Resistance is commonly acquired in patients with metastatic gastrointestinal stromal tumor who are treated with imatinib mesylate, often due to the development of secondary mutations in the KIT kinase domain. We sought to investigate the efficacy of second-line tyrosine kinase inhibitors, such as sorafenib, dasatinib, and nilotinib, against the commonly observed imatinib-resistant *KIT* mutations (*KIT*^{V654A}, *KIT*^{T670I}, *KIT*^{D820Y}, and *KIT*^{N822K}) expressed in the Ba/F3 cellular system.

Experimental Design: *In vitro* drug screening of stable Ba/F3 *KIT* mutants recapitulating the genotype of imatinib-resistant patients harboring primary and secondary *KIT* mutations was investigated. Comparison was made to imatinib-sensitive Ba/F3 *KIT* mutant cells as well as Ba/F3 cells expressing only secondary *KIT* mutations. The efficacy of drug treatment was evaluated by proliferation and apoptosis assays, in addition to biochemical inhibition of KIT activation.

Results: Sorafenib was potent against all imatinib-resistant Ba/F3 *KIT* double mutants tested, including the gatekeeper secondary mutation *KIT*^{WK557-8del/T670I}, which was resistant to other kinase inhibitors. Although all three drugs tested decreased cell proliferation and inhibited KIT activation against exon 13 (*KIT*^{V560del/V654A}) and exon 17 (*KIT*^{V559D/D820Y}) double mutants, nilotinib did so at lower concentrations.

Conclusions: Our results emphasize the need for tailored salvage therapy in imatinib-refractory gastrointestinal stromal tumors according to individual molecular mechanisms of resistance. The Ba/F3 *KIT*^{WK557-8del/T670I} cells were sensitive only to sorafenib inhibition, whereas nilotinib was more potent on imatinib-resistant *KIT*^{V560del/V654A} and *KIT*^{V559D/D820Y} mutant cells than dasatinib and sorafenib.

Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor of the gastrointestinal tract. Constitutive activation through oncogenic mutations of the KIT or platelet-derived growth factor receptor A (PDGFRA) receptor tyrosine kinase plays an important role in the pathogenesis of GISTs (1, 2). Imatinib mesylate (Gleevec, Novartis Pharmaceuticals) potently inhibits BCR-ABL, PDGFRA, PDGFRB, and KIT and has been the frontline therapy in chronic myelogenous leukemia and advanced GISTs since its regulatory approval.

Imatinib mesylate achieves a clinical response in ~80% of GIST patients (3, 4), which is directly dependent on the presence and genomic location of *KIT*/*PDGFRA* mutations. Patients with *KIT* exon 11 mutations have a partial response rate of 84%, whereas tumors with an exon 9 mutation or wild-type *KIT* are less likely to respond (5, 6). Although imatinib has improved survival in GIST, complete response is rare. Furthermore, it is now clear that the majority of patients who initially benefit from tyrosine kinase inhibitors eventually become resistant, with a median time to progression on imatinib mesylate of 2 years (7). After an initial response, 46% to 67% of patients will develop imatinib resistance through acquisition of a secondary mutation in the KIT kinase domain (8–10). These secondary mutations tend to be single amino acid substitutions in exon 17 most often, but they also occur in exons 13 and 14 (8). The mechanism for the development of second-site *KIT* mutations remains unclear, but resistant patients with identifiable secondary mutations have been treated with imatinib longer than resistant patients lacking secondary mutations (median, 27 versus 14.5 months; ref. 8). These findings suggest that clonal selection of existing mutations before imatinib therapy is unlikely to explain acquired resistance. The frequency of secondary mutations is also determined by the location of the primary *KIT* mutations, with GISTs harboring *KIT* exon 11 mutations more commonly becoming imatinib resistant due to

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acquisition of secondary mutations compared with *KIT* exon 9-mutated GIST. One possible explanation for this difference is that *KIT* exon 9 mutant GISTs are able to use alternative mechanisms, whereas *KIT* exon 11 GISTs are more dependent on KIT signaling (10). Polyclonal acquired resistance as a result of multiple secondary *KIT* mutations in geographically separate metastases has been reported in up to 18% (10–12). Consistent with secondary clonal evolution, the primary mutation is always detectable in each tumor and there is never more than one new mutation in the same sample. These findings suggest that long-term imatinib therapy leads to clonal selection of resistant tumor subclones. RNA interference *KIT* knockdown experiments revealed that imatinib-resistant cell lines carrying secondary *KIT* mutations remain dependent on KIT signaling for growth and survival (10). Alternative mechanisms, such as low-level genomic *KIT* amplifications, play only a minor role in imatinib resistance in GIST (8, 11).

The initial success of imatinib as a targeted therapeutic drug and the better understanding of drug-kinase interaction based on crystallographic structure have led to the development of several novel small-molecule compounds that have a variable spectrum of kinase inhibition. As the median survival is only 15 months once imatinib resistance develops (13), it is important to validate novel therapeutic strategies within the specific *KIT/PDGFR* genotype that confer imatinib resistance. Some of the alternative agents to imatinib being tested in clinical trials include nilotinib, sorafenib, and dasatinib. The initial results of these trials are still being analyzed, but the efficacy of these drugs might not be clear due to the heterogeneity of imatinib-resistant patients enrolled. Thus, a more systematic *in vitro* approach of validating individual drug efficacy vis-à-vis specific resistant genotypes might better guide therapy and clinical trial design. These small molecules function as KIT kinase inhibitors, and some in addition inhibit other downstream targets. Nilotinib (AMN107) was designed based on the crystal structures of imatinib and the ABL kinase complex. This multitargeted kinase inhibitor features higher affinity to the ATP-binding site of ABL kinase to override imatinib resistance caused by BCR-ABL mutation (14). In addition, nilotinib selectively inhibits the KIT and PDGFR tyrosine kinases (14). Another small molecule, which showed great promise in the chronic myelogenous leukemia patients that are resistant or intolerant to imatinib, is dasatinib (BMS-354825). Dasatinib is an ATP competitor, with dual SRC/ABL activity, which inhibits most BCR-ABL mutants by binding to the active form of the kinase (15). Furthermore, dasatinib potently inhibits KIT, both wild-type and juxtamembrane domain mutant KIT (16).

Sorafenib (BAY 43-9006, Nexavar) is a novel biaryl urea compound that was initially developed as a specific inhibitor of serine-threonine kinase RAF. In addition, sorafenib has activity with few receptor tyrosine kinases, including vascular endothelial growth factor receptor-2, vascular endothelial growth factor receptor-3, PDGFRB, and KIT (17). Sorafenib has shown broad-spectrum, dose-dependent antitumor activity against xenograft models of human colon, lung, breast, ovarian, and pancreatic cancers and melanoma (18).

Based on our hypothesis that individual KIT mutant oncoproteins will affect on drug sensitivity, we investigated the efficacy of sorafenib, nilotinib, and dasatinib on Ba/F3 cells expressing various KIT mutants, which recapitulate imatinib-resistant GIST genotypes.

Materials and Methods

Ba/F3 KIT mutant transformants. *KIT* mutations recapitulating the genotype found in four imatinib-resistant GIST patients were introduced by site-directed mutagenesis PCR, using QuikChange II XL Site-Directed Mutagenesis kit (Qiagen, Inc.), on a retroviral expression vector containing wild-type human *KIT* cDNA (GNNK isoform), pMSCV-WTKIT-IRES-GFP (a generous gift from Dr. Gary Gilliland, Harvard Medical School, Boston, MA). *KIT* double mutant isoforms were generated hosting a primary *KIT* exon 11 mutation and a secondary mutation in *KIT* exon 13, 14, or 17 as follows: *KIT*^{V560del/V654A}, *KIT*^{WK557-8del/T670I}, *KIT*^{V559D/D820Y}, and *KIT*^{V560del/N822K}. Single *KIT* mutants carrying the secondary mutation alone were also generated for comparison, including *KIT*^{T670I}, *KIT*^{D820Y}, and *KIT*^{N822K}. The imatinib-sensitive exon 11 single mutants *KIT*^{V559D} and *KIT*^{WK557-8del} as well as the imatinib less-sensitive exon 9 *KIT*^{AY502-3ins} were used as controls.

The parental cell line Ba/F3 (German Collection of Microorganisms and Cell Cultures, Human and Animal Cell Cultures Collection) is an interleukin-3 (IL-3)-dependent murine pro-B-cell line lacking intrinsic KIT expression. Retroviral vector plasmids containing mutant *KIT* cDNA isoforms (25 µg of each construct) and the hygromycin selection marker were cotransfected into Ba/F3 cells at the ratio of 25:1 via electroporation using GenePulser (Bio-Rad). Stable transfectants were established by triple selection. The electroporated cells were first selected with hygromycin for 7 to 10 days, and the remaining cells were sorted according to green fluorescent protein fluorescence. The selected green fluorescent protein-positive cells were grown for 2 weeks in the presence of hygromycin, and finally, cells were grown in the absence of IL-3. The cell surface KIT expression was confirmed by flow cytometry using PE-conjugated anti-CD117 (BD Biosciences) antibody. Total KIT expression was confirmed by Western blotting using anti-KIT (Onco-gene) antibody.

Cell proliferation assays. To determine the growth inhibition drug effects, Ba/F3 *KIT* mutant cells were starved from growth factor for 4 h. Fifteen minutes before drug administration, growth factors were added: 10 ng/mL IL-3 for the IL-3-dependent Ba/F3 *KIT* mutants (*KIT*^{D820Y} and *KIT*^{V560del/N822K}) and 20 ng/mL KL for the remaining KL-dependent Ba/F3 *KIT* mutant isoforms. Cells were incubated with 10 to 10,000 nmol/L of sorafenib, dasatinib, nilotinib, and imatinib at 37°C for 48 h. The concentrations of drug used in these studies are in line with the concentrations achievable in human plasma based on pharmacokinetic studies of the kinase inhibitors. Dasatinib and nilotinib were synthesized and kindly provided by Dr. Bayard Clarkson (Sloan-Kettering Institute, New York, NY). Imatinib and sorafenib were purchased commercially. Bromodeoxyuridine was then added and incubated for 6 h before harvesting. Cells were fixed and stained using the standard protocol of the BrdU-APC kit (PharMingen). Bromodeoxyuridine incorporation was determined by flow cytometry. All experiments were done in triplicate. A minimum of 2×10^5 events was acquired and the data were analyzed using FlowJo software (version 5.7.2). Cell growth inhibition curves and IC₅₀ values were plotted and calculated using GraphPad Prism software, version 4.03.

Apoptosis assays. Induction of apoptosis on Ba/F3 *KIT* transformants was evaluated by flow cytometry using the Annexin V-PE Apoptosis Detection kit (PharMingen) in duplicate experiments. Cells at a density of 1×10^6 , activated with KL, were cultured in 24-well plates and starved from growth factors for 4 h before drug delivery. Fifteen minutes before adding the drug, growth factors were added. After drug incubation for 48 h using the concentrations indicated above, cells were harvested and stained with anti-Annexin V-PE antibody using the provider's protocol. A minimum of 10,000 events was analyzed by FACScan (Becton Dickinson) within an hour after staining. The data and results were analyzed by FlowJo.

Immunoprecipitation and Western blotting. The Ba/F3 cells expressing single and double *KIT* mutants were treated with the indicated doses of each drug. Cells were starved from serum and growth factors for 2 h,

Table 1. Comparison of *in vitro* cell proliferation inhibition of imatinib-resistant KIT single and double mutants expressed in the Ba/F3 cell system

Mutations	Imatinib	Dasatinib	Sorafenib	Nilotinib
560Vdel/V654A	3,927	585	1,074	192
557-8WKdel/T670I	>10,000	>10,000	1,063	>10,000
V559D/D820Y	3,202	432	944	297
V560del/N822K*	NA	NA	NA	NA
V559D [†]	63	27	66	44
557-8WKdel [†]	460	58	211	83
502-503AY [†]	509	74	400	671
T670I	>10,000	7,543	918	>10,000
D820Y*	NA	NA	NA	NA
N822K	>10,000	868	3,550	3,083

NOTE: Values are given in IC₅₀ (nmol/L) using imatinib, dasatinib, sorafenib, and nilotinib treatment. Data are representative of two or more experiments done in triplicate (average values are shown).

*The indicated mutants did not transform Ba/F3 cells to factor independence and therefore unable to test the efficacy of kinase inhibitors to inhibit proliferation in the absence of IL-3.

[†] Imatinib-sensitive KIT mutants used as control.

and only growth factors were added back to medium 15 min before drug administration. Drugs were incubated at 37°C in the absence of serum for 90 min. After treatment, cells were harvested and subjected to protein extraction. Whole-cell lysate (200 µg) was incubated with 2 µg of anti-KIT antibody (Assay Designs, Inc.) for an hour, and then the mixture was incubated with Magna beads (Pierce Biotechnology) overnight at 4°C. The beads were washed and isolated using a magnetic column and resuspended in LDS sample buffer (Invitrogen). Electrophoresis and immunoblotting were done on the protein extracts using the standard protocol. Phosphorylated KIT was detected with anti-phosphotyrosine antibodies PY20 and PY99 (Santa Cruz Biotechnology), and total KIT was detected by mouse monoclonal anti-c-KIT (Santa Cruz Biotechnology). The secondary antibodies used were donkey anti-mouse secondary (Santa Cruz Biotechnology).

Results

To test existing second-line inhibitors on imatinib-resistant KIT oncoproteins, we developed stable Ba/F3 transfectants expressing single and double mutant isoforms and selected by IL-3-independent growth. Eight of the 10 KIT mutant forms induced transformation by conferring factor-independent growth to Ba/F3 cells. *KIT*^{D820Y} and *KIT*^{V560del/N822K} Ba/F3 mutants did not achieve IL-3 independence. Ba/F3 transfectants expressing double KIT mutants, *KIT*^{V560del/V654A}, *KIT*^{WK557-8del/T670I}, *KIT*^{V559D/D820Y}, and *KIT*^{V560del/N822K}, and single KIT mutant isoforms, *KIT*^{T670I} and *KIT*^{N822K}, were tested with sorafenib, dasatinib, nilotinib, and imatinib. The efficacy of each drug was compared with imatinib-sensitive Ba/F3 *KIT*^{V559D}, *KIT*^{WK557-8del}, and *KIT*^{AY502-3ins}.

Dasatinib is more potent than other kinase inhibitors on imatinib-sensitive Ba/F3 KIT mutants. The *in vitro* cell proliferation assay showed that dasatinib inhibited cell growth of imatinib-sensitive Ba/F3 *KIT*^{V559D} with an IC₅₀ of 27 nmol/L, whereas imatinib, nilotinib, and sorafenib had an IC₅₀ of 63, 44, and 66 nmol/L, respectively. Dasatinib results are summa-

rized in Table 1. Dasatinib induced apoptosis in >20% of cells at 10 nmol/L, whereas the other drugs induced programmed cell death at 100 nmol/L. The biochemical assays showed consistent *KIT*^{V559D} phosphorylation inhibition by all four inhibitors tested, with dasatinib being the most potent. KIT kinase activity was distinctly inhibited using <10 nmol/L dasatinib, whereas imatinib, nilotinib, and sorafenib showed the same results at 100 nmol/L.

Similarly, the growth of *KIT*^{WK557-8del} cells was inhibited by dasatinib with an IC₅₀ of 58 nmol/L, whereas imatinib, nilotinib, and sorafenib had an IC₅₀ of 460, 83, and 211 nmol/L, respectively (Fig. 1). Furthermore, significant induction of apoptosis was noted at 100 nmol/L of dasatinib and nilotinib.

Dasatinib inhibited the imatinib less-sensitive Ba/F3 *KIT*^{AY502-3ins} with an IC₅₀ of 74 nmol/L, whereas imatinib, nilotinib, and sorafenib had an IC₅₀ of 509, 671, and 400 nmol/L, respectively. Dasatinib induced significant (>90%) programmed cell death of *KIT*^{AY502-3ins} cells at 100 nmol/L, whereas nilotinib induced ~65% and sorafenib induced 35% of programmed cell death at the same concentration. Imatinib did not induce overt apoptosis until 1,000 nmol/L. In keeping with the cell proliferation inhibition and apoptosis results, imatinib did not inhibit KIT phosphorylation at 100 nmol/L but showed distinct inhibition on KIT activity at 1,000 nmol/L.

Imatinib-resistant gatekeeper single *KIT*^{T670I} and double *KIT*^{WK557-8del/T670I} mutants are responsive to sorafenib but resistant to dasatinib and nilotinib. Sorafenib was the

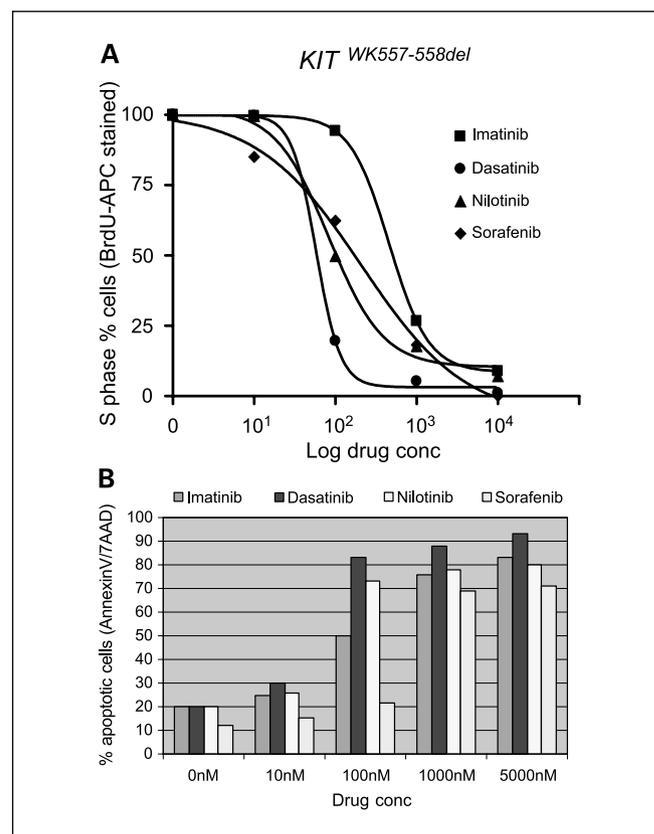


Fig. 1. Imatinib-sensitive juxtamembrane mutation *KIT*^{WK557-8del} showing sensitivity for all drugs tested; however, dasatinib and nilotinib inhibited cell growth (A) at an IC₅₀ of 58 and 83 nmol/L, respectively, and induced significant apoptosis at 100 nmol/L (B). 7-AAD, 7-aminoactinomycin D.

only drug that inhibited the cell proliferation of the Ba/F3 $KIT^{WK557-8del/T670I}$ transfectant with an IC_{50} of 1,063 nmol/L and induction of significant programmed cell death between 1,000 and 5,000 nmol/L (Fig. 2). Sorafenib also showed distinct inhibition of the kinase activity of the KL-dependent double mutant $KIT^{WK557-8del/T670I}$ isoform at ~1,000 nmol/L. In contrast, imatinib, dasatinib, and nilotinib did not inhibit its phosphorylation even up to 10,000 nmol/L. Similar experiments were done on the Ba/F3 cells expressing the gatekeeper single mutant KIT^{T670I} , which showed a comparable drug sensitivity profile. Sorafenib significantly inhibited the kinase activity of this single mutant at 1,000 nmol/L, inhibited cell growth with an IC_{50} of 918 nmol/L, and induced significant apoptosis between 1,000 and 5,000 nmol/L, whereas imatinib, nilotinib, and dasatinib did not show effects on both biochemical and biological assays (Fig. 2).

Nilotinib inhibited cell growth and induced apoptosis of imatinib-resistant $KIT^{V560del/V654A}$ mutant cells at lower doses than dasatinib and sorafenib. Nilotinib potently inhibited the proliferation of Ba/F3 $KIT^{V560del/V654A}$ cells with an IC_{50} of 192 nmol/L. This double mutant was also sensitive, but at higher concentrations, to dasatinib and sorafenib inhibition at

an IC_{50} of 585 and 1,074 nmol/L, respectively (Fig. 3A). The apoptosis assays showed similar results for these three inhibitors. Interestingly, imatinib showed induction of apoptosis in 50% of cells at 1,000 nmol/L, whereas at 5,000 nmol/L 90% of cells entered programmed cell death (Fig. 3B). Biochemically, nilotinib, dasatinib, and sorafenib inhibited KIT phosphorylation at 1,000 nmol/L. Imatinib partially inhibited the phosphorylation of $KIT^{V560del/V654A}$ oncoprotein at 1,000 nmol/L, which was completely inhibited at 5,000 nmol/L (Fig. 3C).

Imatinib-resistant $KIT^{V559D/D820Y}$ was sensitive to nilotinib, dasatinib, and sorafenib inhibition. All three inhibitors tested decreased cell proliferation of the imatinib-resistant Ba/F3 $KIT^{V559D/D820Y}$ cells (see Table 1). Nilotinib inhibited cell growth at an IC_{50} of 297 nmol/L, whereas sorafenib and dasatinib had similar effects at a slightly higher dose, IC_{50} of 944 and 432 nmol/L, respectively. Dasatinib induced significant apoptosis at 1,000 nmol/L, whereas nilotinib and sorafenib showed moderate effect on induction of apoptosis between 1,000 and 5,000 nmol/L. Biochemically, all three drugs showed distinct inhibition of KIT phosphorylation at 1,000 nmol/L.

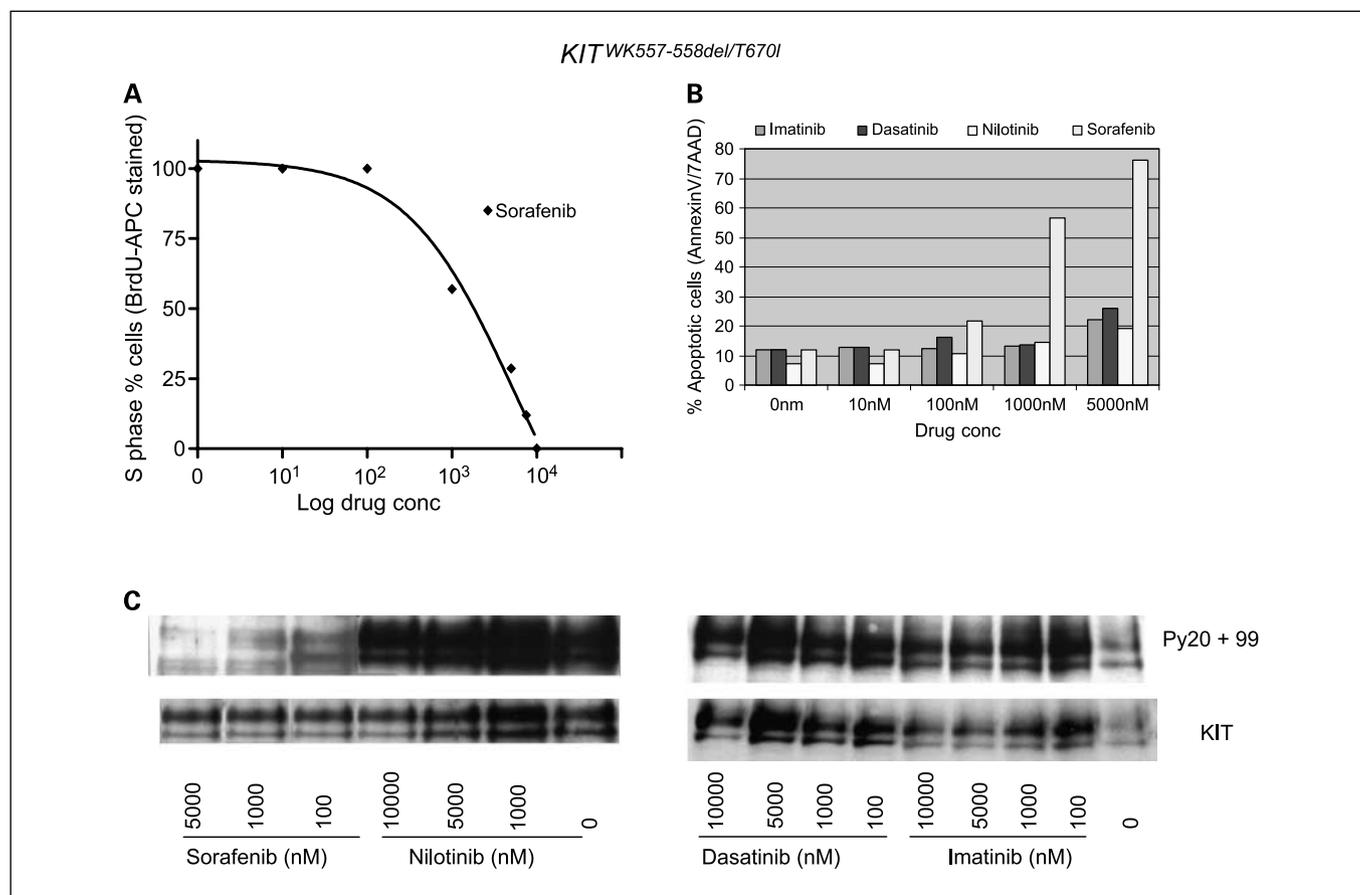


Fig. 2. Sorafenib potently inhibited cell proliferation and kinase activity and induced overt apoptosis of the Ba/F3 transfectant expressing $KIT^{WK557-8del/T670I}$ mutant. Imatinib, dasatinib, and nilotinib showed no effect on cell growth or KIT kinase activity. **A**, Ba/F3 cells stably expressing $KIT^{WK557-8del/T670I}$ were treated with escalating doses of sorafenib in the presence of 20 ng/mL KL for 48 h. Cellular proliferation as determined by bromodeoxyuridine (*BrdU*) incorporation assays was inhibited by sorafenib at an IC_{50} of 1,063 nmol/L. Other drugs did not show growth inhibition (data not shown) up to 10,000 nmol/L. **B**, apoptosis assays, measured by Annexin V and 7-aminoactinomycin D staining, showed that sorafenib induced significant apoptosis at 1,000 nmol/L. Imatinib, nilotinib, and dasatinib did not induce overt apoptosis up to 5,000 nmol/L. **C**, immunoblotting for phosphorylated (PY20 + PY99) and total forms of KIT was done to evaluate the inhibitory effect of the drugs on KIT activation. Sorafenib showed marked KIT activation inhibition at 1,000 nmol/L, whereas the other three drugs did not show an inhibitory effect up to 10,000 nmol/L.

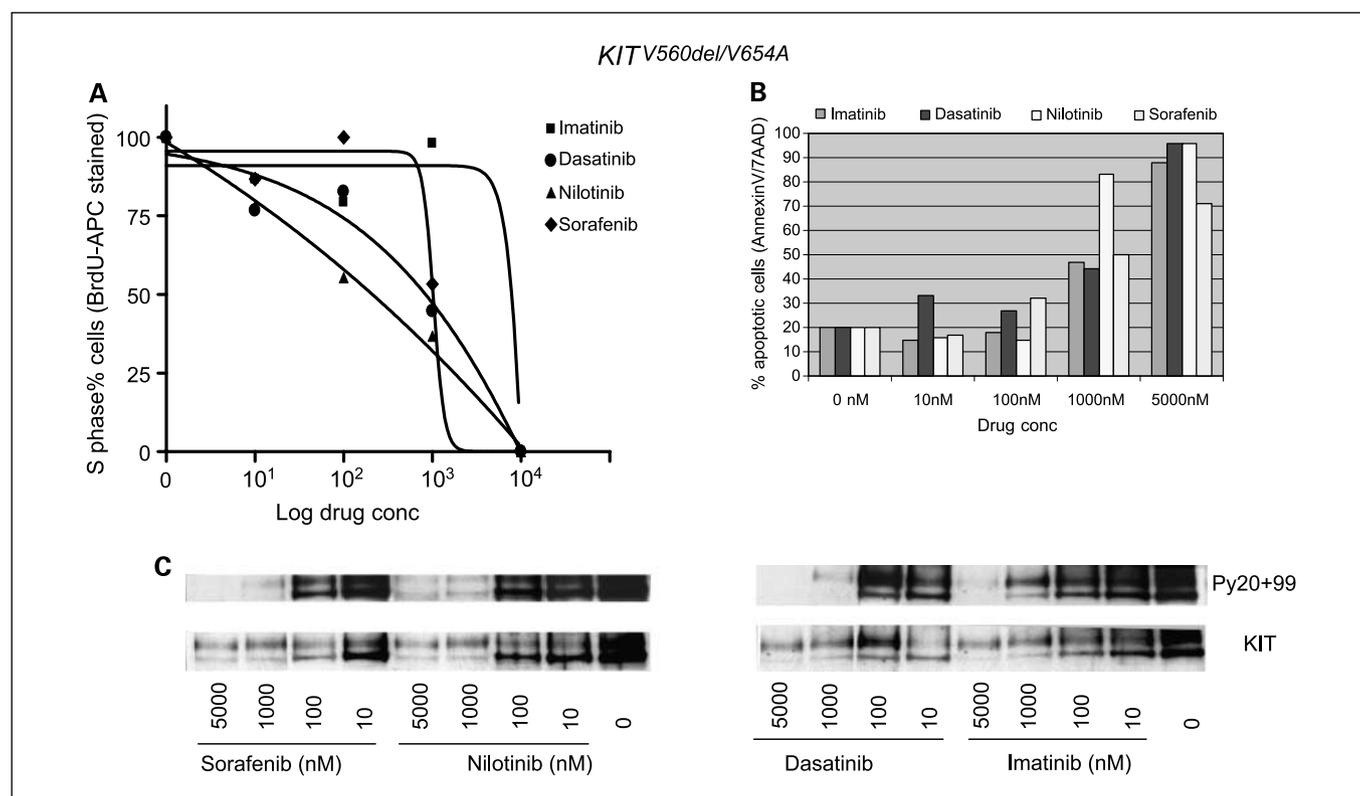


Fig. 3. A, sorafenib, dasatinib, and nilotinib showed significant inhibition on cell growth of Ba/F3 cells expressing *KIT^{V560del/V654A}* at 1,000 nmol/L or lower. Imatinib showed moderate effects between 1,000 and 5,000 nmol/L. B, nilotinib induced significant apoptosis between 100 and 1,000 nmol/L, whereas dasatinib, sorafenib, and imatinib did between 1,000 and 5,000 nmol/L. C, nilotinib, dasatinib, and sorafenib showed significant inhibition on KIT activation at 1,000 nmol/L, whereas imatinib at 5,000 nmol/L.

Phosphorylation of imatinib-resistant *KIT^{V560del/N822K}* is inhibited by nilotinib at lower concentrations than dasatinib and sorafenib. The Ba/F3 *KIT^{V560del/N822K}* cells did not achieve IL-3 independence. Biochemically, nilotinib completely inhibited KIT phosphorylation at 100 nmol/L concentration, whereas dasatinib and sorafenib at 1,000 nmol/L (data not shown). Proliferation and apoptosis studies done on these IL-3-dependent cells did not show an effect with any of the drugs tested. This result might be related to other IL-3-dependent signaling pathways not targeted by the drugs tested.

Discussion

Although most GIST patients respond to imatinib initially, they often develop progressing lesions at multiple metastatic sites within 2 to 5 years after starting therapy. In most imatinib-resistant GISTs, KIT is reactivated and the downstream signaling pathways remain KIT dependent (8, 11). In about half of imatinib-resistant patients, the development of a second-site *KIT* mutation is the cause for KIT reactivation (8, 11, 12), whereas in the remaining cases it is largely unknown. The second-site mutations occur without exception on the same allele as the primary mutation, affect either the first or second kinase domains (exons 13, 14, and 17), and are mostly single amino acid substitutions (8, 10). Crystallographic studies showed that imatinib binds only to the inactive form of KIT (19). Secondary *KIT* mutations in the kinase domains stabilize the active form of KIT and thus weaken or prevent its drug

binding, leading to clinical resistance. As the imatinib alternatives presently tested in clinical trials show a limited survival benefit (13), there is an urgent need to validate novel therapeutic strategies, tailored to the mechanism of resistance in individual patients.

As shown by several groups, the location of the primary *KIT* or *PDGFR α* mutation directly predicts the clinical response to imatinib in GIST patients. Tumors with an underlying primary *KIT* mutation in exon 11 represent the subgroup with better response rates than other mutational subtypes (5, 6). Furthermore, this group is also at higher risk to develop secondary mutations compared with *KIT* exon 9-mutated GISTs (8, 11, 12). This observation further supports that the probability of developing a secondary mutation increases with duration of imatinib treatment, which often is longer in GISTs harboring exon 11 mutation than in those with exon 9 mutation or wild-type GISTs. However, the presence of secondary *KIT* mutations per se within the acquired imatinib resistance setting is not an indicator of a worse prognosis compared with imatinib-refractory tumors without secondary mutations. The latter group escapes the inhibitory efficacy of imatinib through yet unresolved molecular mechanisms (12).

Another level of complexity relies on the fact that long-term imatinib therapy leads to clonal selection of distinct resistant tumor subclones, the so-called "polyclonal acquired resistance" (10–12). Thus, each tumor nodule under progression may undergo individual clonal evolution, resulting in multiple secondary mutations developed at different metastatic sites

within the same patient. As such, designing salvage strategies in these imatinib-resistant settings should address inhibition of all the known genomic activating mutations in the oncoprotein.

Most of the prior *in vitro* drug screening studies on imatinib-resistant KIT mutants investigate only one drug at a time (9, 20; see Table 2). Thus, comparing results between different laboratories, using different cell systems (primary GIST cells, Ba/F3, HEK, etc.), is quite difficult. Our study undertakes a detailed investigation of three imatinib alternative small molecules on a large panel of Ba/F3 KIT single and double mutant cells using biological and biochemical methods of assessing drug response.

Previous reports (12) suggested that GISTs with secondary KIT exon 14 gatekeeper mutations (T670I) show a more aggressive behavior with earlier metastasis and shorter progression-free survival. Of interest is that KIT^{T670I} mutation has not been identified as a primary mutation in GIST and only occurs under the selective pressure of imatinib therapy. The gatekeeper amino acid T670 of KIT was identified as one of the key hydrogen bonds for imatinib binding (19, 21); thus, mutations at this position have a profound effect on drug binding. The bulky isoleucine substituting the threonine residue abolishes the affinity not only for imatinib but also for dasatinib (22, 23). Similarly, the gatekeeper mutation in BCR-ABL, T315I, is resistant to nilotinib at up to 10,000 nmol/L (14). In keeping with these findings, the Ba/F3 KIT cells expressing a T670I single or double mutant were insensitive to all drugs except sorafenib. Sorafenib was initially designed to inhibit cell proliferation by targeting the RAF/mitogen-activated protein kinase/extracellular signal-regulated kinase/extracellular signal-regulated kinase pathway and tumor angiogenesis by targeting vascular endothelial growth factor receptor-2/PDGFRB (17). It was recently approved by the Food and Drug Administration for the treatment of advanced renal cell carcinoma. Sorafenib was also shown to interact with KIT, inhibiting its KIT kinase activity in *in vitro* kinase assays (18). These data support the ongoing phase II National Cancer Institute-sponsored multicenter investigation of sorafenib in imatinib- and sunitinib-resistant GIST (Clinicaltrials.gov identifier NCT00265798). However, the biochemical or biological consequences of sorafenib inhibition in the context of KIT mutant cells have not been yet investigated. Importantly, our results show that sorafenib inhibits the KIT^{T670I} gatekeeper mutation either expressed as a single or double mutant

oncoprotein in the Ba/F3 cellular system. Thus, sorafenib inhibited the KIT kinase activity and cell growth, as well as induced apoptosis at 1,000 nmol/L concentration, in both Ba/F3 KIT^{WK557-8del/T670I} double mutant and Ba/F3 KIT^{T670I} single mutant cells. In fact, sorafenib was the only drug potent against all different secondary mutations tested, including KIT exons 13 and 17.

Dasatinib is a potent, orally bioavailable inhibitor of several kinases, including BCR-ABL and SRC family of kinases (24). It was recently approved by the Food and Drug Administration for the treatment of chronic myelogenous leukemia and Philadelphia chromosome-positive ALL with resistance or intolerance to previous therapy, including imatinib (25). Dasatinib was previously shown to be more potent than imatinib in inhibiting KIT^{V559D} (23), KIT^{V560G} (16), and wild-type KIT (15). Our results also indicate that dasatinib is more effective than other kinase inhibitors on imatinib-sensitive exon 11 mutant Ba/F3 KIT^{V559D} and KIT^{WK557-8del} cells.

Schittenhelm et al. (16) reported that dasatinib inhibits the kinase activity of KIT^{V560G/D816V} double mutant cells in a dose-dependent manner. Screening the sensitivity of different activating tyrosine kinase oncoproteins carrying the gatekeeper mutation, Carter et al. (23) showed that dasatinib has no effect on KIT^{V559D/T670I} double mutant. Their results also indicate that similar gatekeeper mutations in BCR-ABL (T315I) and EGFR (T670M) oncoproteins are resistant to dasatinib therapy. Our data support that KIT^{T670I} expressed either as a single or double mutant oncoprotein in the Ba/F3 cells confers resistance to dasatinib. In contrast, Ba/F3 cells expressing KIT exon 13 or exon 17 single or double mutants were sensitive to dasatinib inhibition. Dasatinib was recently examined in a phase I study in which GIST patients were enrolled (Clinicaltrials.gov identifier NCT00099606); final data are not yet published.

Nilotinib was specifically designed as a more potent inhibitor of mutant and wild-type BCR-ABL compared with imatinib (14). In addition, Weisberg et al. (14) showed that nilotinib is as effective as imatinib in inhibiting exon 11 KIT^{V560del} and exon 13 KIT^{K642E} mutant Ba/F3 cells. In a different study, nilotinib showed potent inhibition against KIT^{V560G} mutant cells (26). Not previously reported, our results indicate nilotinib efficacy on the KIT double mutants involving exon 11 and exons 13 or 17. Specifically, nilotinib induced cell growth inhibition in imatinib-resistant KIT^{V560del/V654A} and KIT^{V559D/D820Y} mutant cells at lower concentrations than

Table 2. Imatinib, dasatinib, and nilotinib drug sensitivity in previously reported imatinib-sensitive and imatinib-resistant KIT mutant oncoproteins

KIT mutation	Cell line	Drug	IC ₅₀ (nmol/L)	Author/Reference
V560 del	Ba/F3	Nilotinib	27	Weisberg et al. (14)
V560G	HMC-1 ⁵⁶⁰	Nilotinib	108	Verstovsek et al. (26)
V560G	HMC-1.1	Dasatinib	5-10	Schittenhelm et al. (16)
V559D	HEK-293	Imatinib	90	Carter et al. (23)
V559D	HEK-293	Dasatinib	8	Carter et al. (23)
V559D	HEK-293	Sunitinib	100	Carter et al. (23)
V559D/T670I	HEK-293	Imatinib	>10,000	Carter et al. (23)
V559D/T670I	HEK-293	Dasatinib	>10,000	Carter et al. (23)
V559D/T670I	HEK-293	Sunitinib	70	Carter et al. (23)
WKdel/V654A	Ba/F3	Sunitinib	50-100	Prenen et al. (20)
WKdel/T670I	Ba/F3	Sunitinib	50-100	Prenen et al. (20)

dasatinib and sorafenib. In contrast, nilotinib was not effective against the gatekeeper KIT exon 14 single (KIT^{T670I}) or double ($KIT^{WK557-8del/T670I}$) mutant. Similarly, Weisberg et al. (14) showed that, although nilotinib inhibited all the other secondary mutations of BCR-ABL, it had no effect in inhibiting the T315I gatekeeper mutation, which is analogous to the T670I mutation in KIT. Nilotinib was investigated in combination with imatinib in patients who are imatinib resistant (Clinicaltrials.gov identifier NCT00135005); final results of the study are pending.

Previous preclinical data using the Ba/F3 cellular system showed that imatinib-resistant KIT V654A and T670I mutants are sensitive to sunitinib (SU11248; ref. 20). The growth of Ba/F3 cells expressing $KIT^{WK557-8del/V654A}$ or $KIT^{WK557-8del/T670I}$ was completely inhibited by sunitinib at concentrations below 500 nmol/L (IC_{50} , 50-100 nmol/L; Table 2). Although other imatinib-resistant KIT mutants, such as the more common second kinase domain KIT mutations, were not investigated, Ba/F3 cells expressing PDGFRA-D842V were less sensitive to sunitinib, and their growth proliferation was inhibited at 5 μ mol/L.

Anecdotal evidence based mainly on molecular modeling of mutated KIT receptors suggests that some secondary mutations acquired in the imatinib resistance setting might be weaker than others (27). Although most second-site KIT mutations affect the imatinib-binding site, the extent of structural changes conferred varies with the individual amino acid substitution; thus, T670I substantially modifies the binding pocket, whereas V654A induces only relatively confined changes (8, 27). When compared with the biochemical activity of KIT kinase, Tamborini et al. (27) showed that, in both substitutions, the phosphorylation of the receptor persisted in the presence of 3 mmol/L imatinib, in keeping with an activated status of the KIT receptors. However, the single and double mutant KIT^{V654A} oncoprotein expressed in COS cells seemed to be unphosphorylated at 6 mmol/L imatinib, whereas the single and double mutant KIT^{T670I} did not. These results suggest that the effects of V654A mutation might be subverted by a dose escalation of the imatinib and it would be expected that a clinical response would be restored. Our results are in support of these findings, showing that imatinib partially inhibited the

phosphorylation of $KIT^{V560del/V654A}$ oncoprotein at 1,000 nmol/L, which was completely inhibited at 5,000 nmol/L. Similarly, induction of apoptosis with imatinib was evident at 1,000 nmol/L in 50% of cells, whereas at 5,000 nmol/L most cells entered programmed cell death.

Alternative salvage options other than inhibiting KIT signaling pathway are presently being explored. These strategies promise a therapeutic solution to the challenge of heterogeneous imatinib resistance. Bauer et al. (28) investigated the activity of heat shock protein 90 inhibitor 17-allylamino-18-demethoxy-geldanamycin (17-AAG) in enhancing cellular degradation of constitutively activated KIT oncoprotein. Thus, 17-AAG was effective in inhibiting KIT-dependent imatinib-sensitive and imatinib-resistant GIST cell lines but not KIT-independent GIST cells, suggesting that its effects result mainly from inactivation of KIT oncoprotein. Taking a different approach, Sambol et al. (29) showed the efficacy of flavopiridol, a cyclin-dependent kinase inhibitor, on a primary GIST cell line carrying an imatinib-sensitive KIT^{K642E} exon 13 mutation, by induction of apoptosis and transcriptional down-regulation of *KIT*. A possible combination of targeted kinase inhibitors with drugs such as flavopiridol or 17-AAG may be more effective in the setting of polyclonal acquired resistance.

In conclusion, second-line tyrosine kinase inhibitors are selectively potent against imatinib-resistant *KIT* mutations and might provide an effective alternative to imatinib in the management of these patients. Specifically, sorafenib was found to be effective against all the *KIT* double mutants tested, including the secondary mutation in the "gatekeeper" residue (KIT^{T670I}), which is resistant to the other kinase inhibitors. In contrast, imatinib-resistant $KIT^{V560del/V654A}$ and $KIT^{V559D/D820Y}$ mutant cells were inhibited by all three inhibitors tested, with nilotinib showing a lower IC_{50} than dasatinib and sorafenib. These *in vitro* data set the stage for designing future clinical trials, where selection of the second-generation kinase inhibitor will be tailored based on the individual mechanism of imatinib resistance. The presence of a KIT^{T670I} gatekeeper mutation for example in the resistant disease may indicate sorafenib or sunitinib as the next therapeutic option, whereas other secondary mutations may be more susceptible to nilotinib.

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