

The ability of sorafenib to inhibit oncogenic PDGFR β and FLT3 mutants and overcome resistance to other small molecule inhibitors

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ABSTRACT

Background and Objectives

Activated tyrosine kinases are implicated in the pathogenesis of chronic and acute leukemia, and represent attractive targets for therapy. Sorafenib (BAY43-9006, Nexavar[®]) is a small molecule B-RAF inhibitor that is used for the treatment of renal cell carcinoma, and has been shown to have activity against receptor tyrosine kinases from the platelet-derived growth factor receptor (PDGFR) and vascular endothelial growth factor receptor (VEGFR) families. We investigated the efficacy of sorafenib at inhibiting mutants of the receptor tyrosine kinases PDGFR β , KIT, and FLT3, which are implicated in the pathogenesis of myeloid malignancies.

Design and Methods

We tested the effect of sorafenib on the proliferation of hematopoietic cells transformed by ETV6-PDGFR β , FLT3 with an internal tandem duplication or D835Y point mutation, and the KIT(D816V) mutant. The direct effect of sorafenib on the activity of these kinases and their downstream signaling was tested using phospho-specific antibodies.

Results

We show that sorafenib is a potent inhibitor of ETV6-PDGFR β and FLT3 mutants, including some of the mutants that confer resistance to PKC412 and other FLT3 inhibitors. Sorafenib induced a cell cycle block and apoptosis in the acute myeloid leukemia cell lines MV4-11 and MOLM-13, both expressing FLT3 with an internal tandem duplication, whereas no effect was observed on four other acute myeloid leukemia cell lines. The imatinib-resistant KIT(D816V) mutant, associated with systemic mastocytosis, was found to be resistant to sorafenib.

Interpretation and Conclusions

These results warrant further clinical studies of sorafenib for the treatment of myeloid malignancies expressing activated forms of PDGFR β and FLT3.

Key words: oncogene, tyrosine kinase, resistance.

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Myeloproliferative diseases are frequently characterized by the expression of activated tyrosine kinases such as BCR-ABL, JAK2(V617F), KIT(D816V), FIP1L1-PDGFR α , and ETV6-PDGFR β .^{1,2} The importance of these oncogenic kinases for the proliferation and survival of the leukemic cells has been demonstrated by the use of mouse models, as well as by the clinical application of selective small molecule kinase inhibitors.³ Imatinib, a potent inhibitor of ABL, KIT, PDGFR α and PDGFR β , is now used as a standard treatment for BCR-ABL-positive chronic myeloid leukemia (CML), FIP1L1-PDGFR α -positive chronic eosinophilic leukemia (CEL), and ETV6-PDGFR β -positive chronic myelomonocytic leukemia (CMML).⁴⁻⁶ Treatment of these myeloid malignancies with imatinib results in rapid and durable responses, but does not eradicate the leukemic stem cells. In addition to imatinib, a number of other inhibitors targeting ABL, KIT, PDGFR α and PDGFR β have been identified and tested in pre-clinical studies.^{7,8}

A mutation of FLT3, a member of the class III receptor tyrosine kinase family, is the most common tyrosine kinase mutation in acute myeloid leukemia (AML). FLT3 is highly expressed in most patients with AML and approximately 30% of these patients have activating mutations in FLT3. The most frequent (~25%) is an internal tandem duplication (ITD) within the cytoplasmic juxtamembrane (JM) region. Less frequent (~7%) are point mutations in the activation loop of the tyrosine kinase domain, such as the D835Y mutation.⁹ Both types of mutations result in constitutive activation of FLT3 and its downstream signaling pathways, providing the cells with a proliferative and survival advantage. A number of structurally different FLT3 inhibitors have been developed, including CEP701, MLN518, and PKC412, and are currently being tested in clinical trials.¹⁰⁻¹² In clinical practice, however, the development of resistance to these small molecule inhibitors due to acquired mutations in the target kinase is an important limitation to the application of these targeted drugs for cancer therapy. Acquired resistance to imatinib has been described in leukemia as well as in gastro-intestinal stromal tumors (GIST).^{4,13,14} Alternatively, primary resistance to imatinib has also been observed in systemic mastocytosis associated with the KIT(D816V) mutant, and in patients with PDGFR α (D842V).^{15,16} Not unexpectedly, in a clinical trial to test the efficacy of PKC412 for the treatment of FLT3 mutation-positive AML, acquired resistance due to a point mutation in the FLT3 kinase domain was already observed, indicating that also in AML the development of resistance may complicate the clinical use of FLT3 inhibitors.¹⁷

Several studies have shown that a mutant form of a kinase that is resistant to a specific inhibitor may still be sensitive to small molecule inhibitors with different chemical structures.^{7,8,18} In order to deal with the problem of resistance it will be necessary to have several inhibitors available that target the same kinase and that can be used

consecutively or in combination for cancer therapy. To further expand our list of available tyrosine kinase inhibitors for the treatment of leukemia, we tested the efficacy of sorafenib at inhibiting PDGFR family tyrosine kinases. Sorafenib is a small molecule kinase inhibitor that was originally developed as an inhibitor of B-RAF¹⁹ but was found to have additional activity against several tyrosine kinases such as VEGFR, PDGFR α , KIT, wild-type FLT3 and oncogenic RET mutants.^{20,21} We previously showed that sorafenib is a potent inhibitor of FIP1L1-PDGFR α and its imatinib-resistant T674I mutant.²² In this study we tested the efficacy of the kinase inhibitor sorafenib at inhibiting ETV6-PDGFR β , imatinib-resistant KIT(D816V), activated FLT3, as well as FLT3 mutants that are resistant to PKC412.

Design and Methods

Vector construction

Cloning of the MSCV-FLT3-ITD and MSCV-FLT3-D835Y constructs and additional point mutants that confer resistance to PKC412 was described previously.²³ The open reading frames of ETV6-PDGFR β and KIT(D816V) were amplified by polymerase chain reaction (PCR) and cloned in the MSCV-puro vector (Clontech, Mountain View, CA, USA). All constructs were verified by sequencing.

Cell culture

293T cells were grown in DMEM medium supplemented with 10% fetal bovine serum (FBS). Retroviral vectors were generated by co-transfection of 293T cells with the MSCV constructs and an ecotropic packaging vector. Retroviral vectors were harvested and used to transduce Ba/F3 cells. Ba/F3 cells were grown in RPMI-1640 medium supplemented with 10% FBS and mouse interleukin (IL)-3 (1 ng/mL). Ba/F3 cells were transformed to IL3-independent growth by expression of the tyrosine kinase constructs and were then grown in the absence of IL3. The human cell lines MV4-11, MOLM-13, HL-60, K562, KG-1 and THP-1 were grown in RPMI-1640 medium supplemented with 10% FBS (MV4-11 and MOLM-13) or 20% FBS. To construct dose-response curves, cells were seeded at 3×10^5 viable cells/mL, and viable cell numbers were determined at the beginning and after 24 hours (Ba/F3 cells) or 48 hours (human cell lines) using the Vi-Cell™ XR cell counter (Beckman Coulter, Fullerton, CA, USA), based on trypan blue exclusion.

Western blotting

Cells were treated with the kinase inhibitor for 90 minutes and then lysed in cold lysis buffer containing 1% Triton X-100 and phosphatase inhibitors. MV4-11 cells were lysed, using cell lysis buffer from Cell Signalling technologies (Beverly, MA, USA), and the FLT3 protein

was first immunoprecipitated with an anti-FLT3 antibody. Samples were reduced and gel electrophoresis was performed using NuPage Bis-Tris 4-12% gels (Invitrogen, Carlsbad, CA, USA). Standard western blotting procedures were used with the polyclonal anti-FLT3, monoclonal anti-ERK2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), monoclonal anti-phospho-FLT3, monoclonal anti-phospho-ERK1/2 (Cell Signalling), antiphosphotyrosine (4G10; Upstate Biotechnology, Lake Placid, NY, USA) and anti-mouse/anti-rabbit peroxidase-labeled antibodies (Amersham Biosciences, Munich, Germany). Sorafenib was custom synthesized and a 10 mM stock was stored in dimethyl sulfoxide (DMSO) at -20°C. Dilutions were made in RPMI-1640 medium immediately before use.

Apoptosis and cell cycle assay

Apoptotic cells were detected by flow cytometric analysis, using annexin-V and propidium iodide staining (Roche, Penzberg, Germany). Cell cycle analysis was performed using the CycleTEST™ PLUS DNA Reagent Kit (Becton Dickinson, San José, CA, USA). Cells were analyzed on a FACSCanto cytometer (Becton Dickinson) and the data were analyzed with FACSDiva software (Becton Dickinson).

Results

Activity of sorafenib against ETV6-PDGFR β , KIT(D816V), FLT3-ITD and FLT3(D835Y)

Sorafenib is a small molecule kinase inhibitor with potent activity against B-RAF, but it is also known to inhibit a subset of tyrosine kinases, including PDGFR and VEGFR family tyrosine kinases.²⁰ We have recently shown that sorafenib is a potent inhibitor of FIP1L1-PDGFR α and its imatinib-resistant T674I mutant form.²² To determine the potency of sorafenib to inhibit other common oncogenic mutants of the PDGFR family tyrosine kinases PDGFR β , KIT and FLT3, we tested the effect of sorafenib on the proliferation of Ba/F3 cells transformed by ETV6-PDGFR β , KIT(D816V), FLT3-ITD and FLT3(D835Y).

Treatment of Ba/F3 cells expressing ETV6-PDGFR β , FLT3-ITD and FLT3(D835Y) with sorafenib resulted in a dose-dependent inhibition of their proliferation (Figure 1A). In contrast, treatment of Ba/F3 cells expressing KIT(D816V) with sorafenib had no effect, and even at 1 μ M sorafenib no significant decrease in proliferation or survival was observed (Figure 1A). For Ba/F3 cells transformed by ETV6-PDGFR β , FLT3-ITD and FLT3(D835Y), a 50% inhibition of cell proliferation was observed at 50 nM, 2 nM and 500 nM, respectively.

Western blot analysis confirmed that the effect on proliferation was due to a direct inhibition of the activated kinases ETV6-PDGFR β , FLT3-ITD and FLT3(D835Y), while there was no visible effect on the phosphorylation of KIT(D816V) (Figure 1B). Treatment of the correspon-

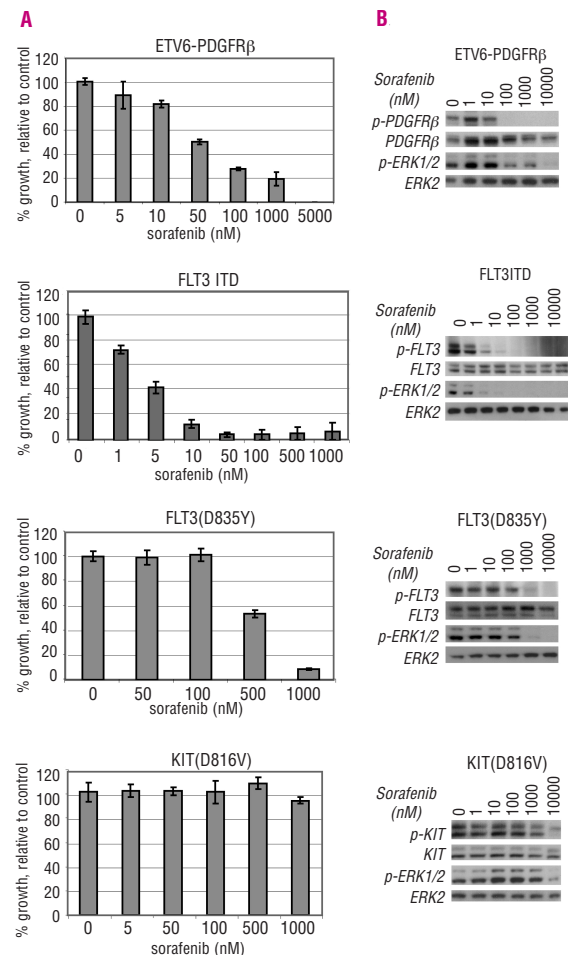


Figure 1. Sorafenib activity against ETV6-PDGFR β , KIT(D816V), FLT3-ITD and FLT3(D835Y). (A) Graphs showing the effect of sorafenib on proliferation of Ba/F3 cells expressing ETV6-PDGFR β , FLT3-ITD, FLT3(D835Y) or KIT(D816V). (B) Western blot analysis confirmed a direct inhibitory effect of sorafenib on ETV6-PDGFR β , FLT3-ITD and FLT3(D835Y). Phosphorylation of ERK1/2 decreased in parallel. Sorafenib had no visible effect on phosphorylation of KIT(D816V).

ding Ba/F3 cells with an increasing dose of sorafenib also resulted in a dose-dependent decrease in phosphorylation of ERK1/2, downstream effectors of ETV6-PDGFR β and FLT3-mediated transformation. In Ba/F3 cells transformed by KIT(D816V) there was no effect of sorafenib on ERK1/2 phosphorylation, indicating that the inhibitor has no direct effect on KIT(D816V) or MAPK signaling in these cells. In all transformed Ba/F3 cells studied, the effect on phosphorylation of the target tyrosine kinases or ERK1/2 correlated with the inhibition of cell proliferation.

Sorafenib induces apoptosis of MV4-11 cells and MOLM-13 cells

To further document the effect of sorafenib on FLT3-ITD, we performed studies using two human AML cell lines, MV4-11 and MOLM-13, known to harbor mutations in FLT3.²⁴ The presence of an internal tandem

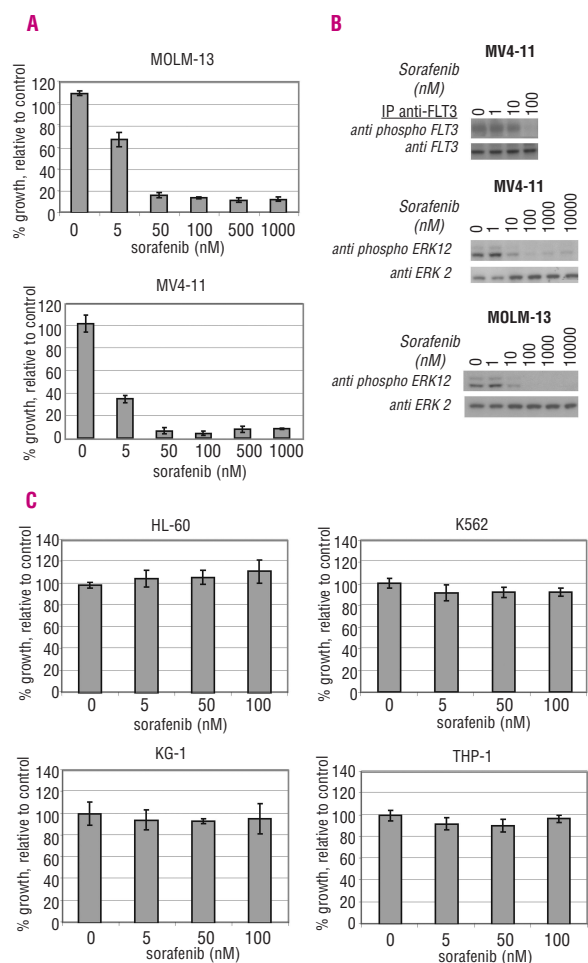


Figure 2. Sorafenib potently inhibits cell growth of MV4-11 and MOLM-13 cells. (A) Graphs illustrating the inhibition of growth of MV4-11 and MOLM-13 cells by sorafenib. (B) A direct inhibitory effect of sorafenib on FLT3-ITD in MV4-11 cells was confirmed after immunoprecipitation of FLT3 and western blot analysis using anti-phosphotyrosine antibody. Western blot analysis showing a decrease in ERK1/2 phosphorylation in MV4-11 and MOLM-13 cells upon sorafenib treatment. (C) Sorafenib had no inhibitory effect on the proliferation of HL-60, K562, KG-1 or THP-1 cells.

duplication in the juxtamembrane domain of FLT3 was confirmed in both cell lines by PCR (*data not shown*). Proliferation of the cell lines was significantly decreased by treatment with increasing doses of sorafenib, and 50% inhibition of proliferation was obtained at 3 nM and 10 nM for MV4-11 and MOLM-13, respectively (Figure 2A). Sorafenib doses up to 100 nM had no effect on the proliferation of four other human myeloid cell lines that do not express mutant FLT3 (Figure 2C).

Due to the low level of expression of FLT3 in the MOLM-13 and MV4-11 cell lines, we could only detect FLT3 after immunoprecipitation in MV4-11 cells. Analysis of the phosphorylation status of FLT3-ITD in the MV4-11 cells confirmed a direct effect of sorafenib on FLT3 phosphorylation in these cells (Figure 2B). In both cell lines, the expected reduction of phosphoryla-

tion of ERK1/2 downstream of FLT3 was observed (Figure 2B). Sorafenib not only inhibited the proliferation of these cells, but also induced apoptosis and block of their cell cycle. After 48 hours' treatment with 100 nM sorafenib, the total population of dead cells of MV4-11 and MOLM-13 had increased from 19.2% to 66.9% and from 22.0% to 61.1%, respectively, with a clear population of apoptotic cells present (Figure 3). In contrast, no significant increase in the numbers of apoptotic cells could be detected for HL-60, K562, KG-1 and THP-1 under these conditions (Figure 3).

In addition, treatment of MV4-11 and MOLM-13 cells for 48 hours with 100 nM sorafenib resulted in an increase of cells in the G₀/G₁ phase of the cell cycle, documenting a cell cycle arrest in the sorafenib-treated cells (Figure 4).

Different mutations in the ATP-binding domain of FLT3 confer various levels of resistance to sorafenib

Using an *in vitro* mutagenesis approach we have recently identified mutations in the ATP-binding domain of FLT3 that confer resistance to the small molecule inhibitor PKC412.²³ We next tested whether these mutations also confer resistance to sorafenib. The different point mutations that confer resistance to PKC412 were cloned in the FLT3-ITD background and expressed in Ba/F3 cells. At a concentration of 100 nM sorafenib, at least 50% inhibition of cell growth was observed for four of the mutants: A627T, N676K, N676Y, and N676D. Mutations at positions A627 and N676 thus only cause a mild shift in the sensitivity of FLT3-ITD to sorafenib. For the G697R point mutation ~200 nM sorafenib was required to obtain a 50% inhibition of proliferation. FLT3-ITD with a point mutation at position 691 (F691L and F691I) was less sensitive to sorafenib, and treatment with 1000 nM of inhibitor reduced the proliferation by less than 40% (Figure 5).

Western blot analysis of the phosphorylation status of FLT3 yielded comparable results, indicating that the F691I and F691L point mutations confer a high level of resistance to sorafenib (IC₅₀ >1000 nM), with no complete inhibition of phosphorylation of FLT3 even at 10 μM. The other tested mutants remain sensitive to sorafenib at concentrations below 500 nM (IC₅₀ values between 10 and 200 nM) (Figure 5). The G697R mutant, known to be resistant to a variety of FLT3 inhibitors, appears to be completely dephosphorylated after treatment with 1000 nM of sorafenib, confirming that this mutant remains sensitive to sorafenib, although at slightly higher concentrations than FLT3-ITD without additional mutations in the ATP-binding domain (Figure 5).

We also tested whether the N676D mutation would confer resistance to sorafenib in the context of FLT3 (D835Y). Ba/F3 cells transformed by FLT3(D835Y) N676D were highly resistant to sorafenib, with a cellu-

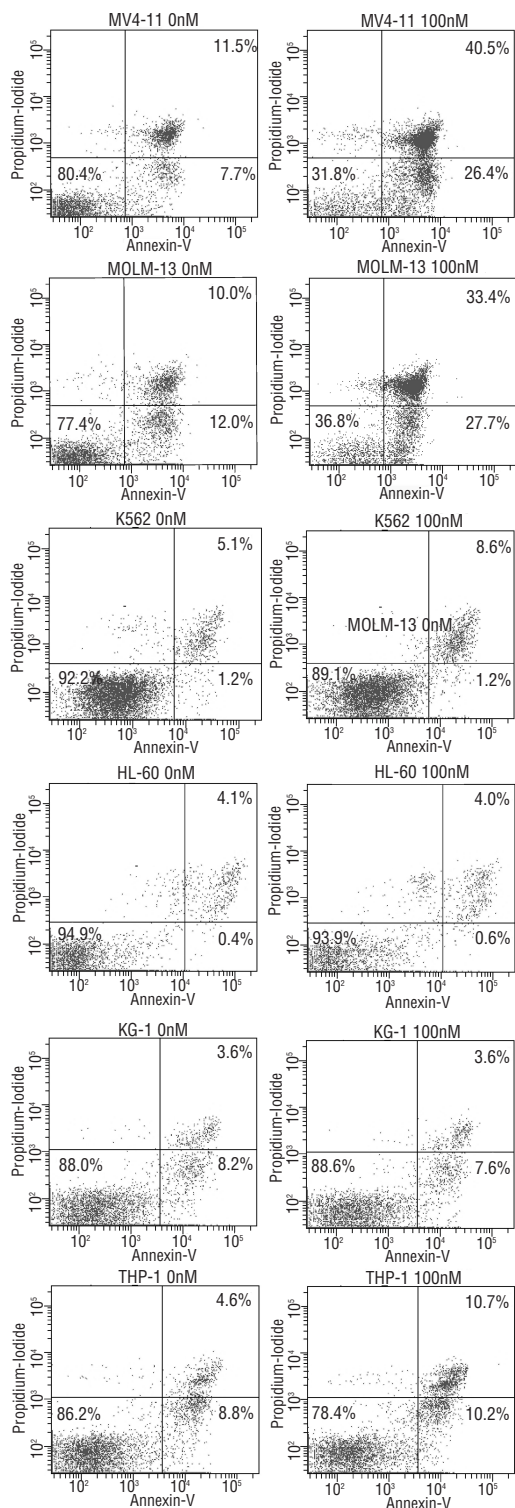


Figure 3. Induction of apoptosis in human AML cell lines and not in other myeloid cell lines. Apoptosis assay of human AML cell lines after 48 hours treatment with 100 nM sorafenib. In MV4-11 and MOLM-13 cells there was a clear induction of apoptosis, whereas no significant influence on the apoptotic populations of HL-60, K562, KG-1 or THP-1 cells was observed after 48 hours of treatment with sorafenib.

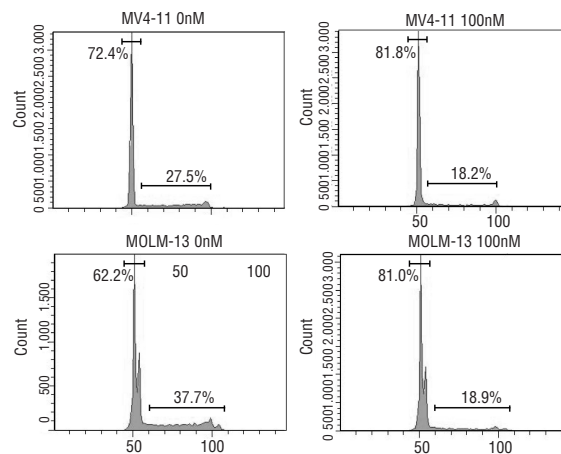


Figure 4. Sorafenib induces cell cycle arrest in MV4-11 and MOLM-13 cell lines. Cell cycle analysis showed an increase in cell cycle block when the cell lines were treated with sorafenib for 48 hours.

lar IC₅₀ value of >1000 nM. Analysis of the phosphorylation status of FLT3(D835Y)N676D after treatment with sorafenib confirmed the resistance of FLT3(D835Y)N676D to more than 1 μM of sorafenib (Figure 6).

Discussion

Molecularly targeted therapy for the treatment of cancer has been introduced with various levels of success. Treatment of BCR-ABL-positive CML, FIP1L1-PDGFRα-positive CEL and ETV6-PDGFRβ-positive CMML with the tyrosine kinase inhibitor imatinib has been quite successful, and most patients achieve a durable complete hematologic response.⁴⁻⁶ Treatment of BCR-ABL-positive CML patients in blast crisis, however, has been more difficult. Despite the fact that most of these patients initially show a response to imatinib therapy, many of them relapse due to the development of resistance to imatinib through acquisition of additional mutations in the kinase domain.¹³ Interestingly, some of the mutants that are resistant to imatinib remain sensitive to small molecule inhibitors with different structural properties.^{7,8,22} These observations indicate that patients who become resistant to one inhibitor could still be treated successfully with other inhibitors, and that it is important to test a variety of inhibitors for their activity against oncogenic kinases and their respectively known resistant mutants. This strategy is currently being tested in the context of BCR-ABL-positive CML in which imatinib-resistant patients are being treated with novel inhibitors such as AMN107 and BMS354825.^{7,25}

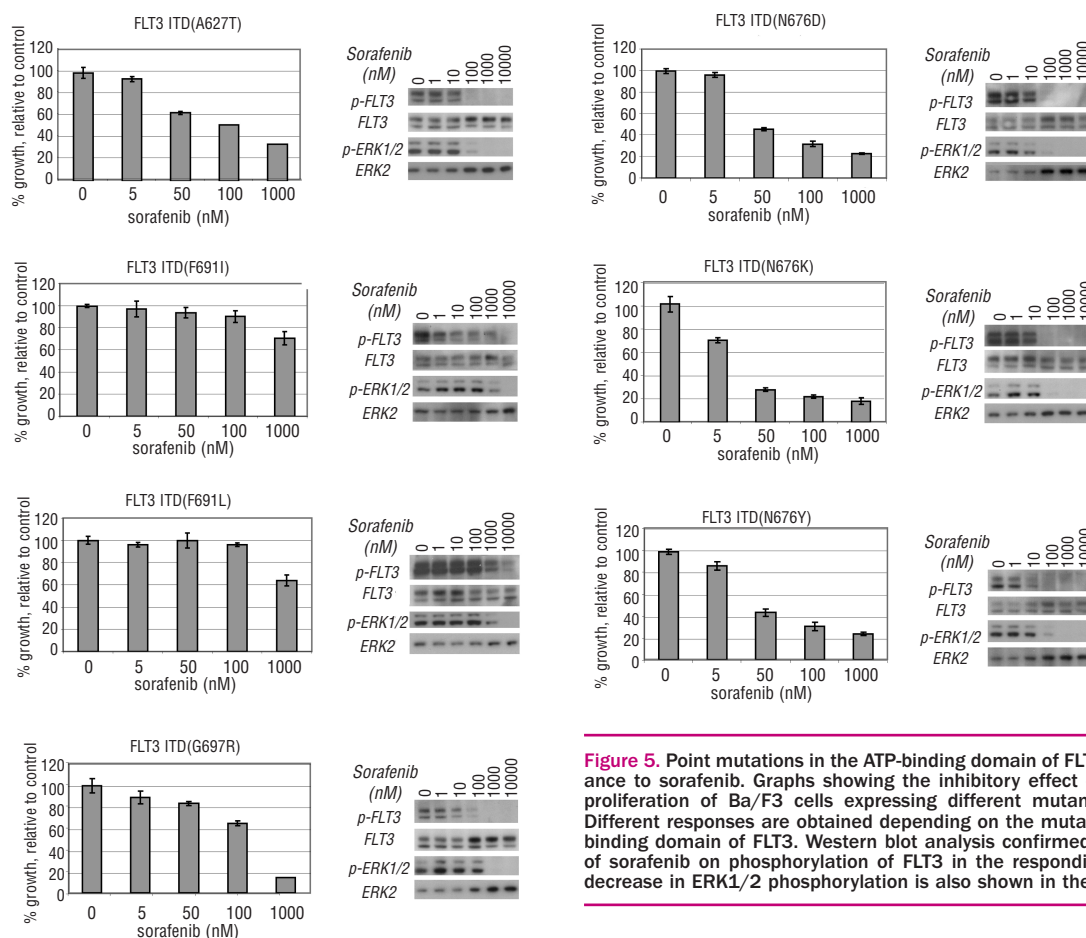


Figure 5. Point mutations in the ATP-binding domain of FLT3 confer resistance to sorafenib. Graphs showing the inhibitory effect of sorafenib on proliferation of Ba/F3 cells expressing different mutants of FLT3-ITD. Different responses are obtained depending on the mutation in the ATP-binding domain of FLT3. Western blot analysis confirmed a direct effect of sorafenib on phosphorylation of FLT3 in the responding cell lines. A decrease in ERK1/2 phosphorylation is also shown in these cell lines.

Given the central role of FLT3 mutations in the pathogenesis of AML, FLT3 inhibitors have been developed and are currently being tested in clinical trials. However, based on the experience with imatinib resistance in CML in blast crisis, the development of resistance to FLT3 inhibitors is also likely to occur in AML, and, indeed, has already been observed in one AML patient in a clinical trial of PKC412.¹⁷ At the time of relapse, this patient had acquired a N676K mutation in the ATP-binding domain of FLT3, which was shown to confer resistance to PKC412. Using an *in vitro* mutagenesis screen, we have recently identified four different positions in the ATP-binding site of FLT3 that, when mutated, confer resistance to PKC412, further documenting that resistance to PKC412 can develop due to mutations in the kinase domain of FLT3.²³

In this study we tested the efficacy of sorafenib at inhibiting the activated tyrosine kinases ETV6-PDGFR β , FLT3-ITD, FLT3(D835Y), and KIT(D816V). We demonstrated that sorafenib is a potent inhibitor of oncogenic mutants of PDGFR family tyrosine kinases PDGFR β and FLT3. ETV6-PDGFR β and FLT3-ITD are inhibited with cellular IC₅₀ values below 50 nM, while FLT3 with the D835Y activating mutation is slightly less sensitive to sorafenib with a cellular IC₅₀ value of approximately 500

nM. Steady-state serum concentrations of sorafenib up to 4 μ M could be safely achieved in patients with a dose of 100 mg/day.^{26,27} Since ETV6-PDGFR β and FLT3-ITD are completely inhibited at sorafenib concentrations below 1000 nM, an efficacious dose may be 100 mg/day or lower for ETV6-PDGFR β and FLT3-ITD positive patients. FLT3(D835Y) was found to be less sensitive to sorafenib, with a cellular IC₅₀ value of ~500 nM. Since this is still eight times below the achievable serum concentration, it can be expected that sorafenib could be used to inhibit FLT3(D835Y).

In contrast, our results illustrate that the corresponding D816V mutation in KIT confers a high level of resistance to sorafenib, with a cellular IC₅₀ value >1 μ M. These data indicate that related tyrosine kinases that are sensitive to a specific small molecule inhibitor in their wild type configuration do not necessarily have a similar resistance mutation profile.

In addition, we analyzed the potency of sorafenib to override resistance to PKC412 and other FLT3 inhibitors caused by mutations in the ATP-binding domain of FLT3. Our data indicate that mutations of the amino acids alanine 626 (A626), asparagine 676 (N676) and glycine 697 (G697) cause a shift in the sensitivity of FLT3-ITD to sorafenib, but do not confer a high level of

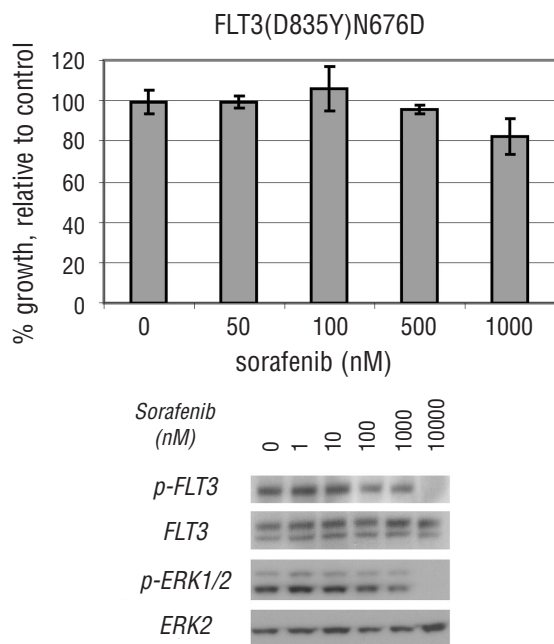


Figure 6. The N676D mutation causes resistance to sorafenib in the FLT3(D835Y) context. Graphs showing lack of inhibitory effect of sorafenib on proliferation of Ba/F3 cells expressing FLT3(D835Y)-N676D. Western blot analysis confirmed the resistance of FLT3(D835Y)-N676D to sorafenib. Decreases in FLT3 and ERK1/2 phosphorylation were only observed for sorafenib concentrations >1000 nM.

resistance. In contrast, a mutation of phenylalanine 691 (F691) to either leucine or isoleucine resulted in a large shift of the IC_{50} value for sorafenib to more than $1 \mu\text{M}$. These results suggest that a number of the mutations that confer resistance to PKC412 remain sensitive to sorafenib, including the G697R mutation, previously shown to confer high level resistance to PKC412, SU5614, and K-252a.²³ Finally, the N676D mutation was

also analyzed in the context of FLT3(D835Y). Given the fact that FLT3(D835Y) is already less sensitive than FLT3-ITD to sorafenib, it could be expected that this extra mutation would confer additional resistance to sorafenib. We confirmed that FLT3(D835Y)-N676D was indeed highly resistant to sorafenib. These data indicate that resistance in the context of FLT3(D835Y) could occur with a broader range of mutations than in the context of FLT3-ITD. These observations also suggest that it could be important to consider higher doses of sorafenib for the treatment of FLT3(D835Y)-positive AML than for FLT3-ITD-positive AML.

Sorafenib has recently been approved by the FDA for the treatment of renal cell carcinoma, which opens possibilities for the introduction of this compound in the treatment of patients with leukemias with activated PDGFR β or FLT3. Unfortunately, our data do not support a role for sorafenib in the treatment of imatinib-resistant mastocytosis associated with the common KIT(D816V) mutation. Sorafenib may remain a valuable inhibitor for the treatment of leukemias and solid tumors with other types of activating KIT mutations, which remain to be investigated. It is unlikely that sorafenib will be useful as a single agent for the treatment of AML but, given the fact that sorafenib is well tolerated and that FLT3-ITD is inhibited by sorafenib at low nanomolar concentrations, combinations of sorafenib with chemotherapy may open new possibilities to improve the treatment of AML patients, as has been described for other FLT3 inhibitors. Further studies addressing the potential of such combinations are warranted.

Author Contributions

EL performed the research, analyzed the data and wrote the paper; IL, HVM and NM performed the research; PM and JC supervised the study, analyzed the data and wrote the paper.

Conflict of Interest

The authors reported no potential conflicts of interest.

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