

Efficacy of the Kinase Inhibitor SU11248 against Gastrointestinal Stromal Tumor Mutants Refractory to Imatinib Mesylate

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Abstract Purpose: The majority of gastrointestinal stromal tumors harbor mutations in the receptor tyrosine kinases KIT or platelet-derived growth factor receptor A (PDGFRA), and respond to treatment with the tyrosine kinase inhibitor imatinib. Some tumors, however, show primary resistance to imatinib treatment, and most others become resistant during treatment. The most common mechanism of imatinib resistance involves specific mutations in the kinase domains of KIT or PDGFRA. We tested the activity of SU11248, an orally active small-molecule tyrosine kinase inhibitor, to inhibit important imatinib-resistant KIT and PDGFRA mutants.

Experimental Design: Primary imatinib-resistant tumor cells and cell lines expressing clinically identified imatinib-resistant KIT-V654A, KIT-T670I, or PDGFRA-D842V mutant isoforms were evaluated for sensitivity to SU11248 by Western immunoblotting and proliferation assays. Three patients with the KIT-V654A mutation were treated with SU11248.

Results: Based on *ex vivo* assays, SU11248 potently inhibits KIT kinase activity of V654A and T670I mutants and suppresses proliferation of the cells expressing these mutations. Sensitivity of KIT-V654A and KIT-T670I mutants to SU11248 was confirmed using cell lines expressing these mutants. In contrast, SU11248 did not potently inhibit the PDGFRA-D842V mutant. In agreement with these results, two of the three imatinib-resistant patients with the KIT-V654A mutation responded to SU11248 treatment.

Conclusions: These studies suggest that SU11248 may be a useful therapeutic agent to treat gastrointestinal stromal tumors harboring the imatinib-resistant KIT-V654A or KIT-T670I mutations, but it has no effect on the activity of the PDGFRA-D842V mutant. Specific kinase inhibitors should be designed to inhibit the constitutive activating PDGFRA mutation at codon 842.

Imatinib mesylate (Gleevec, STI571; Novartis, Basel, Switzerland), a tyrosine kinase inhibitor with activity against ABL1 (ABL), ABL2 (ARG), KIT, platelet-derived growth factor receptor α (PDGFR α), and PDGFR β , is effectively used for the treatment of patients with chronic myelogenous leukemia, chronic eosinophilic leukemia, and gastrointestinal stromal tumors (GIST; refs. 1–4). Despite good responses to imatinib in most patients, primary and acquired resistance to the drug does occur in these diseases and forms a new major problem for targeted therapies.

GISTs that show primary resistance to imatinib either carry no mutations in KIT or PDGFRA, or carry specific activation loop mutations in KIT or PDGFRA, including the PDGFRA-D842V mutation (5–7). We and others have shown that the

most important mechanism for acquired resistance to imatinib is the reactivation of KIT, which occurs via secondary gene mutations in the KIT kinase domain, including the mutations Val⁶⁵⁴Ala and Thr⁶⁷⁰Ile (8–11). An understanding of the mechanism of imatinib resistance has prompted the search for alternative KIT and PDGFRA inhibitors that are effective against imatinib-resistant mutants (10). SU11248 is an orally active inhibitor of multiple receptor tyrosine kinases, with selectivity for PDGFR, vascular endothelial growth factor receptor, FLT3, and KIT (12, 13). It therefore exhibits direct antitumor effects against tumors depending on signaling through PDGFR, FLT3, and KIT, in addition to antiangiogenic activity through its potent inhibition of vascular endothelial growth factor

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receptor. *In vitro*, SU11248 inhibits these targets with IC₅₀ values in the low nanomolar range (14).

A clinical phase III randomized trial of SU11248 in GIST patients who became resistant to imatinib showed clinical activity in resistant tumors and a statistically significant improvement in both time to progression and overall survival in patients treated with SU11248 versus placebo (12). Preliminary positron emission tomography analysis showed that SU-11248 shrank tumors in 8% of patients while stabilizing the disease in 60%, giving an overall response rate of 68%. The GIST genotype was determined in 47 patients, and a total of 79%, 33%, 50%, and 100% of patients with KIT exon 9 mutants, KIT exon 11 mutants, no detectable mutations, and PDGFRA mutants, respectively, showed clinical benefit. However, data regarding the ability of SU11248 to inhibit specific imatinib-resistant mutants are not available. Given that knowledge of the sensitivity of a particular mutant could assist in determining whether SU11248 would be appropriate for a particular patient or not, we tested the effect of SU11248 on the most common imatinib-resistant mutants, KIT-V654A, KIT-T670I, and PDGFRA-D842V.

Materials and Methods

Patients. Progressive tumors from three patients treated with imatinib were evaluated. All three tumors expressed the acquired imatinib-resistant mutation V654A. Patient characteristics and clinical data of two of these patients were described previously (cases 10 and 19; ref. 10). The third patient was a 57-year-old male who progressed after 30 months of imatinib treatment. His progressive tumor, which was partially removed, expressed the primary KIT(delWK557-558) and secondary KIT-V654A mutation. During the next 5 months, the remaining tumor started to grow again. These three patients were enrolled in a trial of SU11248 and were treated with SU11248 until progression (Response Evaluation Criteria in Solid Tumors; ref. 15). The study was reviewed and approved by the institutional review board.

Primary tumor cells. GIST cells were obtained by tumor biopsy from three patients refractory to imatinib treatment in the Department of Clinical Oncology at University Hospital Leuven. Patients' clinical characteristics, histopathologic findings, and tumor genomic primary and progressive features are detailed elsewhere (cases no. 11, 14, and 19; ref. 10). All three tumors expressed acquired imatinib-resistant KIT mutations, either V654A or T670I, in addition to the primary KIT exon 9 or 11 activating mutations, which were identified based on mutational analysis of imatinib-nontreated tumor specimens. Thus, tested tumor cells harbored KIT(del557-558)/T670I, KIT(Ins503AY)/V654A, or KIT(Ins574PT)/V654A mutant isoforms.

In addition, primary GIST cells from the sporadic, high-risk GIST (bearing KITdel557-558 mutation), which was obtained from the biopsy of imatinib-naïve patient, and imatinib-sensitive GIST882 cell line, which exhibits constitutive kinase activity due to hemizygous KIT K642E missense mutation (16) were tested as a control.

Primary GIST cells were obtained from collagenase-disaggregated tumor specimens, seeded in 25 mm cell culture dishes (Corning, Inc., Corning, NY) and grown in DMEM supplemented with 10% fetal bovine serum, 1 mmol/L nonessential amino acids, 1.0 mmol/L sodium pyruvate, and 0.5 mmol/L 3-isobutyl-1-methylxanthine for inhibition of fibroblast growth until 90% confluence. Control, imatinib-sensitive GIST882 cell line was grown using the same medium as for primary GIST cells but without 3-isobutyl-1-methylxanthine supplement.

Progressive and the control primary GIST cells and the control imatinib-sensitive GIST882 cell line were tested for their response to the inhibitor after short-term culture by Western immunoassay. Cells were treated with various concentrations of DMSO alone or with SU11248 in DMSO to a range of doses (0.1, 0.5, 1.0, and 5 μmol/L for primary GIST cells, and 0.001, 0.025 and 0.5 μmol/L for GIST882 cells) for 90 minutes at room temperature. After treatment, cells were washed with ice-cold PBS and scraped into lyses buffer (20 mmol/L Tris-HCl, 1% Triton X-100, 5 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L Na₃VO₄, and a protease inhibitor cocktail). Lysates were incubated for 30 minutes at 4°C and then centrifuged for 20 minutes. Supernatants were removed and used for SDS gel electrophoresis and immunoblotting as described previously (17). Membranes (Amersham Pharmacia Biotechnology, Cambridge, United Kingdom) were immunoblotted overnight using anti-phospho-KIT (Y703; Zymed, San Francisco, CA; dilution 1:250). Horseradish peroxidase-conjugated anti-rabbit immunoglobulin was used as secondary antibody (dilution 1:2,500) and visualized with enhanced chemiluminescence (Pierce, Rockford, IL). Membranes were then stripped and reblotted to determine total protein levels using total KIT-antibody (dilution 1:500; anti-CD117, A4506; DAKO, Glostrup, Denmark).

The effect of SU11248 on cell proliferation of primary GISTs cells and GIST882 cell line was evaluated by Ki-67 immunostaining assay. Cells (1 × 10⁴) were seeded in eight-well chamber slides and grown in DMEM (supplemented as above) for 72 hours in triplicate. Then, cells were incubated with various concentrations of SU11248 for 72 hours. The proliferation index was determined by immunostaining using human anti-Ki67 antibody (dilution 1:200; DAKO) in drug-treated slides relative to no-drug controls.

Inhibitors and constructs. SU11248 was provided by Pfizer (La Jolla, CA). A 10 mmol/L stock solution of SU11248 was prepared in DMSO and was stored at -20°C. For experiments, dilutions of the stock solution were made in cell culture medium with the final DMSO concentration maximum 0.1%. PDGFRA and KIT expression constructs were described previously (10).

Ba/F3 cell growth assay and Western blot analysis. Ba/F3 cells stably expressing the different constructs were grown in RPMI 1640 supplemented with 10% fetal bovine serum as described previously (10). For dose-response curves, Ba/F3 cells were grown in 24-well plates with different concentrations of inhibitor. The number of viable cells was determined at the start and after 24 hours, using the AqueousOne solution (Promega, Mannheim, Germany). The effect of SU11248 on KIT or PDGFRA autophosphorylation of Ba/F3 cells exposed to varying concentrations of SU11248 for 90 minutes was evaluated by Western blot analysis, as previously described (18). The antibodies used were anti-phospho-PDGFRA and anti-PDGFRA (Santa Cruz Biotechnology, Santa Cruz, CA).

Results

Ex vivo response of imatinib resistant GISTs mutants to SU11248. To determine the efficacy of SU11248 to inhibit the imatinib-resistant T670I and V654A mutants of KIT, we established *ex vivo* cultures of GIST cells obtained from tumors expressing KIT(delWK557-558)/T670I, KIT(Ins503AY)/V654A, and KIT(Ins574PT)/V654A mutants. The KIT protein was expressed and phosphorylated in all imatinib-resistant GIST cells propagated *ex vivo* as well as in the control imatinib-naïve GIST harboring KIT(del557-558) mutation and a control GIST882 cell line, which express an activating KIT(K642E) mutant (Fig. 1).

The effect of SU11248 on the autophosphorylation of the KIT Y703 residue in cultured imatinib-resistant GIST cells and imatinib-sensitive control GIST cells was determined by Western blot. Observations were standardized for total KIT expression using anti-KIT antibody based on densitometric

quantification of autoradiographic signals (Supplementary Table S1). KIT autophosphorylation was totally inhibited by SU11248 at concentrations ranging from 0.5 to 1.0 $\mu\text{mol/L}$ in imatinib-resistant GIST cells that carry V654A mutations, and significantly or totally inhibited at concentrations of 1.0 and 5.0 $\mu\text{mol/L}$ in cells harboring the T670I mutation, respectively. In imatinib-sensitive GIST882 cells, KIT activation was significantly reduced by SU11248 already at 10 nmol/L concentration and completely inhibited at 50 nmol/L concentration of the drug. In imatinib-naïve, control primary GIST cells, KIT activation was totally inhibited already at 10 nmol/L concentration of the drug.

The antiproliferative effect of SU11248 was determined by immunostaining of cultured imatinib-resistant GIST cells and GIST882 cells with anti-Ki-67 antibody. As shown in Fig. 1, the

fraction of proliferating cells in all tested GIST cultures decreased up to 3-fold and in a dose-dependent manner after treatment with SU11248 for 3 days. After treatment with 1 $\mu\text{mol/L}$ SU11248, the proliferation rate of imatinib-resistant KIT(Ins503AY)/V654A and KIT(Ins574PT)/V654A mutants decreased to 35% and 55%, and that of the KIT(delWK557-558)/T670I mutant to 64%, compared with nontreated cells. Treatment of imatinib-sensitive GIST882 cells with SU11248 inhibited the proliferation with 31% compared with nontreated cells already at 50 nmol/L SU11248. Treatment of imatinib-naïve GIST with SU11248 resulted in complete cell proliferation arrest already at 10 nmol/L concentration of the drug.

In vitro effect of SU11248 on KIT and PDGFRA mutants expressed in Ba/F3 cells. To complement the *ex vivo* studies done on primary GIST cells, we also expressed the

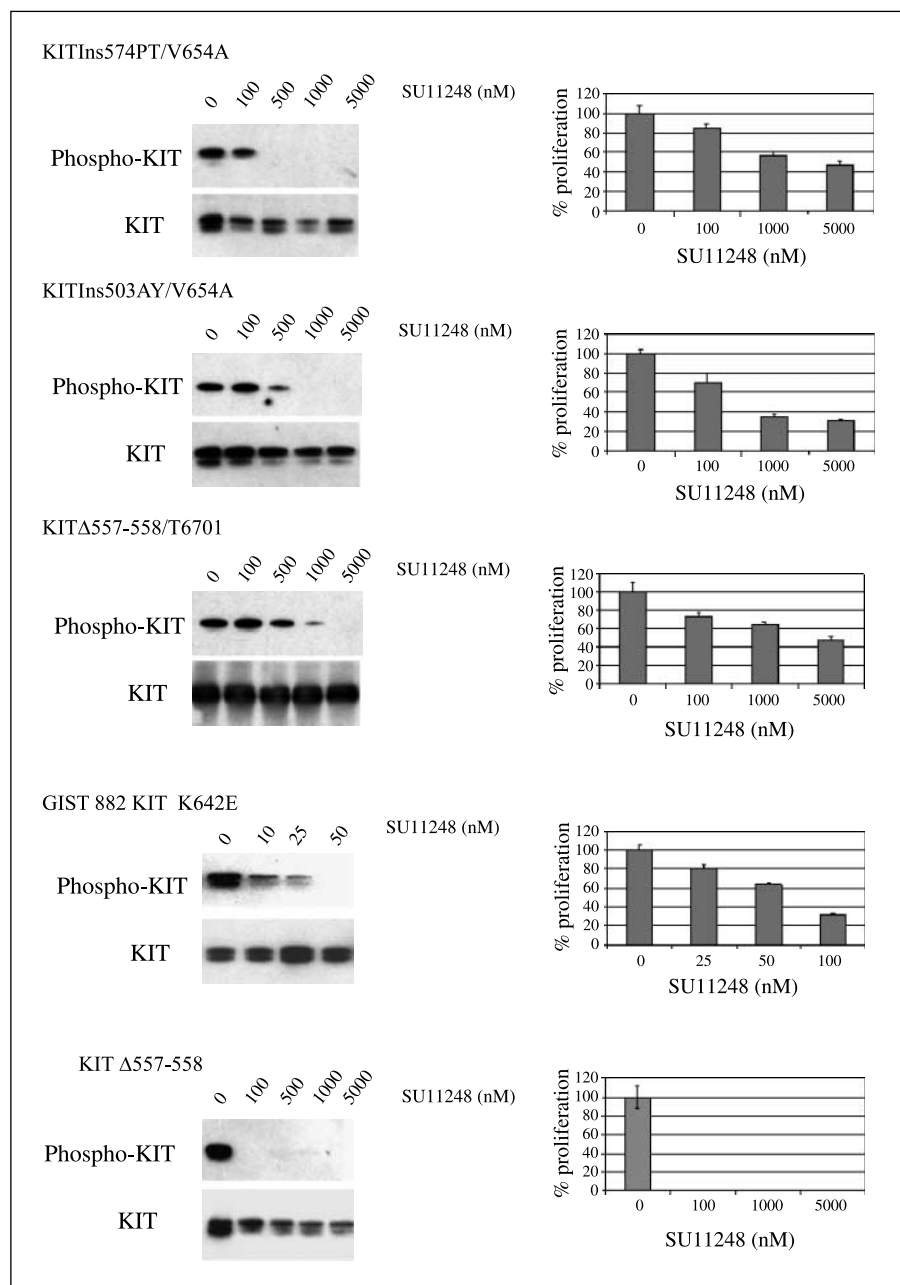
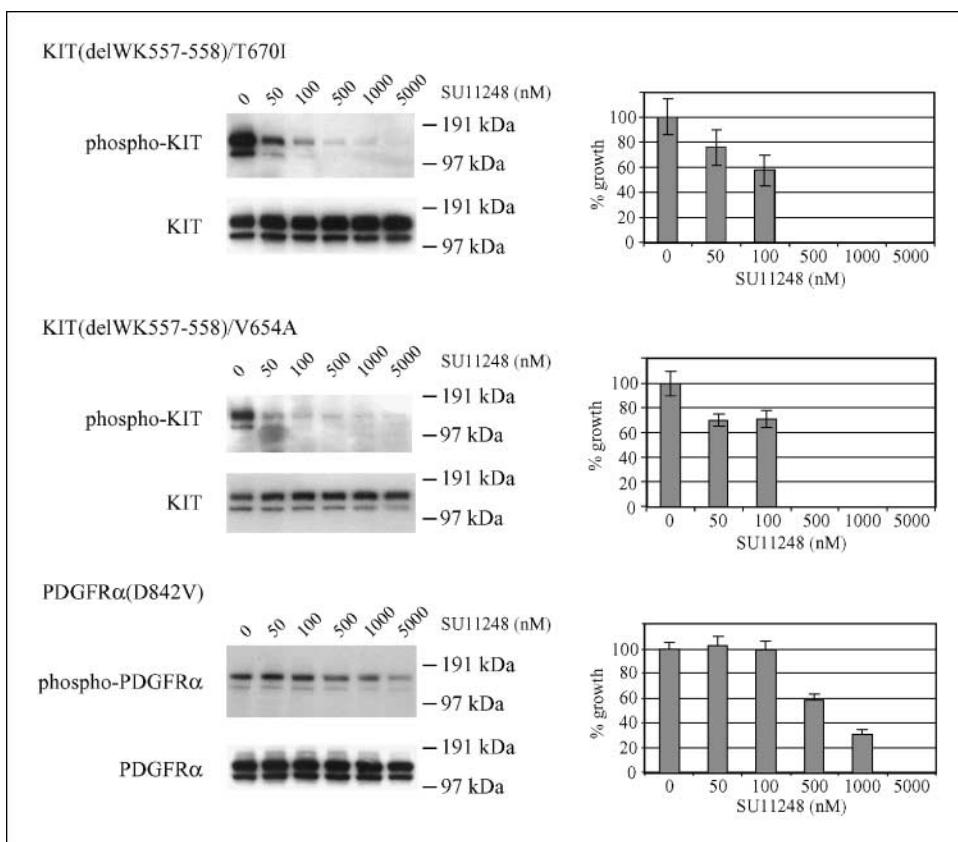


Fig. 1. *Ex vivo* sensitivity assay of GIST cells to SU11248. Primary imatinib-resistant GISTs that harbor KIT (del557-558)/T670I, KIT (Ins503AY)/V654A, or KIT (Ins574PT)/V654A mutations were cultured and treated with SU11248 *ex vivo*. As control, the imatinib-sensitive GIST882 cell line and imatinib-naïve primary GIST cells that express KIT (del557-558) mutation were used. *Left*, Western blot analysis of cells treated with SU11248 for 90 minutes. Whole cell lysates were analyzed using anti-KIT or anti-phospho-KIT (Y703) antibodies. *Right*, proliferation index (%) of cells after treatment with SU11248 for 72 hours as determined by Ki-67 immunostaining. Columns, mean of triplicate experiments; bars, SD.

Fig. 2. *In vitro* effect of SU11248 on KIT and PDGFRA mutants expressed in Ba/F3 cells. Ba/F3 cells stably expressing activating KIT(del557-558)/T670I, KIT(del557-558)/V654A, or PDGFRA-D842V mutants were treated with SU11248 *in vitro*. Left, Western blot analysis of Ba/F3 cells after treatment with SU11248 for 90 minutes indicates inhibition of autophosphorylation of the KIT mutants but not of the PDGFRA-D842V mutant. Total cell lysates were analyzed with anti-KIT, anti-phospho-KIT (Y703), anti-PDGFRα, or anti-phospho-PDGFRα (Y754). Right, dose-response curves of Ba/F3 cells expressing the KIT or PDGFRA mutants. Ba/F3 cells stably expressing the indicated constructs were treated with SU11248 and the number of viable cells was determined at start and after 24 hours. Columns, mean of triplicate samples; bars, SD.



KIT(delWK557-558)/T670I, KIT(delWK557-558)/V654A, and PDGFRA-D842V mutants in murine Ba/F3 cells. Ba/F3 cells expressing these mutants became growth factor independent and totally dependent on the kinase activity of the KIT or PDGFRA mutants for their growth and survival. We have previously shown that the Ba/F3 cells expressing these constructs are resistant to imatinib (10). In the current experiments, the growth of Ba/F3 cells expressing KIT(delWK557-558)/V654A or KIT(delWK557-558)/T670I was completely inhibited by SU11248 at concentrations below 500 nmol/L (cellular IC₅₀ 50-100 nmol/L). The direct effect of SU11248 on these kinases was confirmed by analysis of their phosphorylation state by Western blot analysis using a phosphospecific antibody (Fig. 2). The growth of Ba/F3 cells expressing the PDGFRA-D842V mutant was less sensitive to SU11248 and was completely inhibited at 5 μmol/L. Analysis of the phosphorylation state of PDGFRA-D842V mutant, however, showed resistance to

SU11248, with phosphorylation not completely inhibited even at a drug concentration up to 5.0 μmol/L (Fig. 2). These results indicate that the PDGFRA-D842V mutant is resistant to SU11248. The observed growth inhibition of the Ba/F3 cells is likely due to off-target effects of SU11248 in these cells as has also been observed for other small-molecule inhibitors (19).

Clinical response to SU11248. Based on our *ex vivo* and *in vitro* results, three GIST patients whose tumors expressed the secondary imatinib-resistant KIT mutation V654A were treated with SU11248 in the Department of Oncology, University Hospital Leuven, after progression on imatinib treatment. Two patients showed stable disease during SU11248 treatment for >11 months and are still on the drug. One GIST patient progressed after one cycle of SU11248, and the treatment was discontinued (Table 1). The treatment with high imatinib dose (800 mg daily) was restarted but the patient died 6 months later due to progressive disease.

Table 1. Clinical response to SU11248

Patient	KIT genotype		Response to SU11248 (RECIST criteria)
	Primary tumor	Tumor refractory to imatinib	
1 (case 19)*	Ins503AY	Ins503AY/V654A	Progression after 5-wk treatment, died of disease
2 (case 10)*	L576P	L576P/V654A	Stable disease for 11 mo, still on treatment
3	DeWK557-558	DeWK557-558/V654A	Stable disease for 11 mo, still on treatment

Abbreviation: RECIST, Response Evaluation Criteria in Solid Tumors.
*Data from reference 10.

Discussion

Although most GIST patients show good responses to imatinib, many of them develop resistance after a long duration of treatment. Acquired resistance to imatinib in GISTs commonly occurs via secondary gene mutation in the KIT kinase domain. This finding has implications for strategies to delay or prevent imatinib resistance and to apply newer targeted therapies.

The KIT-V654A and KIT-T670I mutations, located in the proximal kinase domain of the gene, have been reported as common, recurrent secondary mutations in GIST patients treated with imatinib, and have been proved to confer resistance to imatinib (10). In the current study, we have tested the new tyrosine kinase inhibitor SU11248 for the activity against three primary GISTs cells with these mutations, derived from imatinib-resistant patients. All three also harbored a primary mutation in the *KIT* gene, either within exon 9 or exon 11, which were disclosed in baseline pretreatment biopsies. SU11248 inhibited tyrosine phosphorylation of all these imatinib-refractory mutants at concentrations from 0.5 to 1.0 $\mu\text{mol/L}$. This inhibition was related to cell cycle arrest and cell growth inhibition after 72 hours of treatment with the drug. Moreover, the *in vitro* efficiency of SU11248 was equal for KIT(Ins503AY)/V654A and KIT(Ins574PT)/V654A mutants, indicating that primary KIT mutations in extracellular or juxtamembrane domains [in this case KIT(Ins503AY) or KIT(Ins574PT)] do not affect the efficiency of SU11248 in inhibiting growth of GISTs cells refractory to imatinib due to secondary mutations. The sensitivity to SU11248 on the KIT-T670I and KIT-V654A mutants was further validated *in vitro* using transformed Ba/F3 murine cells. Furthermore, SU11248 was also shown to be effective in inhibiting KIT-activating mutations of the imatinib-sensitive GIST882 cells and control, imatinib-naïve primary GIST cells at concentrations as low as 50 and 10 nmol/L, respectively. Interestingly, from 10- to 50-fold difference between the positive control imatinib-sensitive GIST and imatinib-resistant mutation-bearing GIST cells was observed, suggesting that the sensitivity to the drug of imatinib-resistant tumors may also depend on additional factors that may affect KIT kinase inhibition. One of the possibilities is the development of resistance mechanisms by the refractory cells that may reduce the active intracellular concentration of the applied inhibitors. Nevertheless, because

SU11248 can be safely administered at dose levels of 50 mg daily, yielding steady-state plasma levels above 2 $\mu\text{mol/L}$, our findings suggest that SU11248 can be an efficient second-line therapy for patients with GISTs that carry the acquired KIT-V654A or KIT-T670I mutations. This indication was supported by the clinical observations of three GISTs patients, whose tumors carried imatinib-resistant V654A mutation and who were treated with SU11248 in our hospital. In two of these patients, the disease has remained stable for the last 11 months, demonstrating the clinical benefit from the treatment. One patient progressed within 5 weeks of SU11248 treatment. Notably, tumor cells from this particular patient showed sensitivity to SU11248 in terms of KIT receptor autophosphorylation and cell proliferation inhibition by *ex vivo* assay. The reason for the discrepancy between *ex vivo* and *in vivo* data is unknown. Because the material from the post-SU11248 progressive tumor was not available for the further study, we were not able to search for possible additional genomic mutations or alternative functional alteration that could give tumor cells the selective advantage for growth in spite of SU11248 treatment. Further clinical studies on a larger cohort of patients are needed to confirm possible benefit from SU11248 treatment in patients with tumors showing KIT-T670I and KIT-V654A refractory mutants.

To further explore the sensitivity of other imatinib-resistant mutations to SU11248, we also tested Ba/F3 cells expressing the imatinib-resistant PDGFRA-D842V mutant. The D842V mutation is the most common activating PDGFR α mutation in GISTs and was also proven to be imatinib resistant (5, 10). We observed that, in contrast to tested imatinib-resistant KIT mutants harboring kinase proximal domain acquired mutations, SU11248 did not completely inhibit the PDGFRA-D842V mutant at 5 $\mu\text{mol/L}$ concentration of the drug, as assessed by Western blot analysis.

In conclusion, our *in vitro*, *ex vivo*, and clinical findings indicate that SU11248 has good inhibitory activity against the V654A and T670I mutations in KIT that confer resistance to imatinib. In contrast, The PDGFRA-D842V mutation that confers resistance to imatinib also confers resistance to SU11248. Our results show that the efficacy of SU11248 for the treatment of imatinib-resistant GISTs is dependent on the type of mutation, emphasizing again the need for customized, patient-tailored therapy of refractory GISTs according to molecular mechanisms of resistance.

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