Effects of PKC412, Nilotinib, and Imatinib Against GIST-Associated PDGFRA Mutants With Differential Imatinib Sensitivity

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Background & Aims: Activating mutations in platelet-derived growth factor receptor α (PDGFRA) have been reported in a subset of gastrointestinal stromal tumor (GIST) patients who do not express the mutant stem cell factor receptor c-kit. The responsiveness of mutant PDGFRA-positive GIST to imatinib depends on the location of the PDGFRA mutation; for example, the V561D juxtamembrane domain mutation is more sensitive to imatinib than the D842V kinase domain mutation. In this study, we compare the effects of 3 tyrosine kinase inhibitors, such as the staurosporine derivative PKC412 and nilotinib, and imatinib, on 2 GIST-related PDGFRA mutants, V561D and D842V, which possess differential sensitivity to imatinib. Methods: The effects of PKC412, nilotinib, and imatinib, alone and in combination, were evaluated via in vitro proliferation studies performed with V561D- or D842V-PDGFRα mutants. The effects of nilotinib and PKC412, alone and combined, were investigated in vivo. Results: PKC412 potently inhibited the V561D-PDGFRα mutant in vitro and the D842V-PDGFRα mutant in vitro and in vivo. Both imatinib and nilotinib displayed potent activity in vitro against the V561D-PDGFRα mutant but were significantly less efficacious against D842V-PDGFRα. However, when combined with either imatinib or PKC412, nilotinib showed no evidence for antagonism and acted in a cooperative fashion against D842V-PDGFRα. Conclusions: Our findings support the clinical testing of PKC412 for treatment of mutant PDGFRA-GIST. The data also support the use of nilotinib as a treatment option for V561D-PDGFRα-associated GIST, although the reduced sensitivity of D842V-PDGFRα probably limits the potential of nilotinib monotherapy for D842V-PDGFRα-associated GIST.

T he gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor of the gastrointestinal (GI) tract. Approximately 90% of GISTs have been shown to have gain-of-function mutations in the stem cell factor receptor c-kit. Imatinib (Gleevec, STI571; Novartis Pharma AG, Basel, Switzerland) is a small molecule kinase inhibitor that selectively targets c-kit and platelet-derived growth factor receptor (PDGFR) tyrosine kinases, as well as breakpoint cluster region-Abelson (Bcr-Abl). Imatinib is an effective therapy for GIST and is currently used to treat c-kit-positive metastatic or unresectable tumors. However, despite the success of imatinib against this malignancy, acquired drug resistance can develop.

The responsiveness of GISTs to imatinib varies significantly, depending on which exon the c-kit mutation is located. For instance, whereas GIST patients with c-kit exon 11 mutations are the most prevalent and these patients respond well to imatinib, patients whose tumors express the c-kit exon 9 mutation are less responsive. These responses are in accordance with in vitro studies that suggest that GISTs harboring regulatory-region c-kit mutations are more sensitive to imatinib than GISTs harboring enzymatic-region mutations. Of GISTs in which only wild-type c-kit can be detected, approximately 16% harbor PDGFRA mutations, which result in up-regulation of the same downstream signaling components as c-kit. As with c-kit–associated GISTs, the responsiveness of PDGFRA-associated GISTs to imatinib treatment varies depending on the exonic location of the PDGFRA mutation. The exon 18, D842V-PDGFRα, mutation is the most common PDGFRA mutation and has been reported to be resistant to imatinib in vitro. GIST patients possessing exon 18 mutations in PDGFRA typically do not respond well to imatinib therapy. However, the D842V-PDGFRα mutation does respond to other tyrosine kinase inhibitors, such as the staurosporine derivative PKC412. The rarer V561D mutation is located at the juxtamembrane domain of exon 12 in the PDGFRA gene.

In light of the development of primary and secondary resistance of GIST toward imatinib emerging as a significant clinical challenge in the treatment of this disease, there is a need to identify additional compounds that can serve as alternative strategies for the treatment of imatinib-resistant GIST patients. As such, we were interested in studying the in vitro and in vivo efficacy of other known inhibitors of PDGFRA against some of the mutants seen in patients with GIST. Here, we report findings with PKC412, a nonselective inhibitor of protein kinases, and nilotinib (AMN107), a novel inhibitor of Bcr-Abl, c-kit, and PDGFR. In addition to inhibiting FLT3, which is mutated in 35% of acute myeloblastic leukemia patients, PKC412 inhibits c-kit and PDGFR. PKC412 is currently in clinical trials in patients with acute myeloblastic leukemia with mutations in FLT3. Nilotinib is a potent inhibitor of Bcr-Abl, designed to override resistance to imatinib caused by point mutations in Bcr-Abl that disrupt the binding of imatinib to its target; nilotinib is currently undergoing clinical evaluation in myeloproliferative diseases and in GIST. In this study, we investigated the antiproliferative and kinase inhibitory effects of PKC412, nilotinib, and imatinib against GIST-related PDGFRA mutants showing varying degrees of imatinib resistance.
sensitivity. We also explored the effects of combinations of the 3 inhibitors against these mutants in vitro and investigated the antiproliferative activity of PKC412, alone and in combination with nilotinib, against the D842V-PDGFRA mutant in vivo.

Materials and Methods

Cell Lines and Cell Culture

Constructs of D842V-, V561D-, and wild-type (wt) PDG-FRA complementary DNA (cDNA) cloned into pcDNA3.1 (obtained from M.C. Heinrich, Department of Pathology, Division of Hematology and Oncology, Oregon Health & Science University Cancer Institute, Portland, OR) were stably transfected into Ba/F3 cells by electroporation, and cells were selected for neomycin resistance and interleukin (IL)-3-independent growth. All cells were cultured in the presence of 5% CO2 at 37°C, at a concentration of 5 × 10^5 cells/mL, in Cellgro RPMI 1640 medium (Mediatech, Inc., Herndon, VA), supplemented with 10% fetal calf serum (FCS; Harlan Bioproducts, Indianapolis, IN), 1% glutamine, and penicillin/streptomycin. Parental Ba/F3 cells or wt-PDGFRA-Ba/F3 cells were cultured with 15% WEHI-conditioned medium as a source of IL-3. All transfected cells were cultured in media supplemented with 1 mg/mL G418.

Chemical Compounds, Antibodies, and Immunoblotting

PKC412, nilotinib, and imatinib were synthesized at Novartis Pharma AG, Basel, Switzerland, and were dissolved in dimethyl sulfoxide (DMSO) to make 10 mmol/L stock solutions. Serial dilutions were then made, also in DMSO, to obtain final dilutions for cellular assays. Anti-p-Tyr (clone 4G10; Upstate Biotechnology, Lake Placid, NY) and anti-p-Tyr (pY99, Sc-7020; Santa Cruz Biotechnology, Santa Cruz, CA) were each used at 1:1000 for immunoblotting. PDGFRA antibody (C-20; Santa Cruz Biotechnology) was used at 1:200 for immunoblotting. The α-tubulin (clone DM1A) antibody was purchased from Sigma-Aldrich (St. Louis, MO) and was used at a 1:2000 dilution. Protein lysis preparation and immunoblotting were carried out as previously described.11

Proliferation Studies

The trypan blue exclusion assay has been previously described11 and was used for all cell proliferation studies. For drug combination studies, PKC412 and nilotinib, PKC412 and imatinib, or nilotinib and imatinib were added simultaneously at fixed ratios to either D842V- or V561D-PDGFRA-Ba/F3 cells. Dose-response curves were generated, and combination indices were calculated as previously described by isobologram analysis.12
Mouse Studies and In Vivo Imaging

D842V-PDGFRα-Ba/F3 cells were transduced with a retrovirus encoding firefly luciferase (MSCV-Luc) and selected with puromycin at a concentration of 0.5–1 μg/mL to generate the D842V-PDGFRα-Ba/F3-luciferase (luc/F3) cell line. Cells free of Mycoplasma and viral contamination were resuspended in Hank’s balanced salt solution (HBSS; Mediatech, Inc., VA) prior to intravenous (IV) administration to mice. Solutions of nilotinib were prepared by dissolving 200 mg in 1.0 mL of 1-methyl-2-pyrrolidone to give a clear solution and diluted daily prior to administration with 9.0 mL PEG300. Six percent wt/wt PKC412 in Geltucire 44/14 (Gattefosse, France) was diluted with 1X phosphate-buffered saline (PBS) and warmed in a 42°C water bath until liquid. Placebo mice received vehicles for both PKC412 and nilotinib, administered 30–45 minutes apart.

Male NCr athymic nude outbred mice (CrTac:NCr-Foxn1−/−) (5–6 weeks of age; Taconic, NY) were administered a total of 600,000 D842V-PDGFRα-Ba/F3-luc+ cells by tail vein injec-

![Figure 2. Proliferation studies showing combination effects of PKC412, nilotinib, and imatinib against D842V-PDGFRα-Ba/F3 cells. (A) Three-day treatments of D842V-PDGFRα-Ba/F3 cells with nilotinib, imatinib, or a combination of nilotinib plus imatinib. (B) Three-day treatments of D842V-PDGFRα-Ba/F3 cells with PKC412, nilotinib, or a combination of PKC412 plus nilotinib. (C) Three-day treatments of D842V-PDGFRα-Ba/F3 cells with PKC412, imatinib, or a combination of PKC412 plus imatinib.](image)

**Table 1.** Combination Indices Calculated for Dose-Response Curves Shown in Figures 2 and 5

<table>
<thead>
<tr>
<th>Cell lines (treatments)</th>
<th>Combination indices</th>
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<tbody>
<tr>
<td></td>
<td>ED25</td>
</tr>
<tr>
<td>D842V-Ba/F3 (nilotinib+Imatinib)</td>
<td>0.48812</td>
</tr>
<tr>
<td>D842V-Ba/F3 (nilotinib+PKC412)</td>
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</tr>
<tr>
<td>D842V-Ba/F3 (nilotinib+PKC412)</td>
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<tr>
<td>V561D-Ba/F3 (nilotinib+Imatinib)</td>
<td>0.80100</td>
</tr>
<tr>
<td>V561D-Ba/F3 (nilotinib+PKC412)</td>
<td>1.12460</td>
</tr>
</tbody>
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NOTE. ED25–ED75, where ED50 is the median effective dose.
against D842V-PDGFRA-Ba/F3 cells (Figure 1A), whereas the IC₅₀ values for nilotinib and imatinib were in the 0.1–0.5 μmol/L range (Figure 1B and C). Addition of IL-3, the normal growth factor for Ba/F3 cells, resulted in near complete rescue, suggesting selective inhibition of D842V-PDGFRA with minimal nonspecific toxicity of each of these agents at the concentrations tested.

The effects of PKC412, imatinib, and nilotinib on cellular tyrosine kinase activity in D842V-PDGFRA-Ba/F3 cells were studied. The robust signal observed for the pattern of phosphorylated bands in the D842V-PDGFRA-Ba/F3 cells was modestly inhibited by 1-hour treatments with concentrations of nilotinib and PKC412 that were effective in inhibiting proliferation of cells (Figure 1D). Treatment of D842V-PDGFRA-Ba/F3 cells with imatinib led to more pronounced, yet still partial, inhibition of total cellular tyrosine phosphorylation levels with no observed decrease in D842V-PDGFRA expression (Figure 1D). The high level of cellular tyrosine phosphorylation and partial inhibition of this signal in the presence of the tyrosine kinase inhibitors are consistent with the aggressive, resilient phenotype of the D842V mutant.

Combination Effects of PKC412, Nilotinib, Imatinib: D842V-PDGFRA-Ba/F3

Combinations of PKC412, nilotinib, and imatinib were tested against D842V-PDGFRA-Ba/F3 cells. Overall, positive combination effects were observed between nilotinib plus imatinib, nilotinib plus PKC412, and imatinib plus PKC412 (Figure 2). CalcuSyn analysis of the combined effects of nilotinib plus imatinib suggests synergistic to nearly additive effects across a range of doses (ED25–ED75, where ED50 is the median effective dose), with antagonism at ED90 (Table 1). The nilotinib plus PKC412 combination resulted in nearly additive to synergistic effects across a range of doses (ED25–ED90) (Table 1), and the combined effects of imatinib plus PKC412 were nearly additive to synergistic effects across a range of doses (ED50–ED90), with moderate antagonism observed at ED25 (Table 1).

Effects of Combinations of PKC412, Nilotinib, and Imatinib on Tyrosine Phosphorylation in D842V-Ba/F3 Cells

We investigated the effects of combinations of nilotinib and imatinib, nilotinib and PKC412, and imatinib and PKC412 on cellular tyrosine phosphorylation in D842V-Ba/F3 cells. We found that tyrosine phosphorylation, especially of high-molecular-weight substrates, was modestly inhibited by treatment for 4 hours with each drug as a single agent at their approximate IC₅₀ (as compared with vehicle control-treated cells) (Figure 3). Cellular tyrosine phosphorylation was inhibited to a greater extent when combinations of each of the 3 agents were used, as compared with single agent- and vehicle control-treated cells (Figure 3). The results suggest that inhibition of cellular tyrosine phosphorylation by combined treatment of D842V-PDGFRA-expressing cells correlates with enhanced inhibition of cellular proliferation via combined treatment.
Effects of PKC412, Nilotinib, and Imatinib on Proliferation of V561D-PDGFRA-Ba/F3 Cells and Tyrosine Phosphorylation in V561D-PDGFRA-Ba/F3 Cells

V561D-PDGFRA-Ba/F3 cells were treated with a range of concentrations of PKC412, nilotinib, or imatinib. PKC412 displayed an IC$_{50}$ of approximately 0.025 μmol/L against V561D-PDGFRA-Ba/F3 cells (Figure 4A). The IC$_{50}$ of nilotinib or imatinib against V561D-PDGFRA-Ba/F3 cells was approximately 0.01 μmol/L (Figure 4B and C). IL-3 rescue was observed with all 3 inhibitors, and wild-type PDGFRA-expressing cells were unaffected by either inhibitor at doses that inhibited mutant-PDGFRA-expressing cells. This suggests selective inhibition of mutant PDGFRA.

Treatment of V561D-PDGFRA-Ba/F3 cells for 1 hour with PKC412, nilotinib, or imatinib led to a decrease in levels of total cellular tyrosine phosphorylation with no observed decrease in PDGFRA expression (Figure 4D). The increased potency of the 3 inhibitors in decreasing levels of cellular tyrosine phosphorylation in V561D-PDGFRA-Ba/F3 cells is consistent with the increased potency of the inhibitors against proliferation of Ba/F3 cells expressing the V561D mutant, as compared with cells expressing the D842V mutant.

Combination Effects of Nilotinib With Imatinib and PKC412: V561D

The effects of combinations of nilotinib with either imatinib or PKC412 were evaluated against V561D-PDGFRA-Ba/F3 cells (Table 1). Generally, both combinations led to varying degrees of antagonism across a range of doses in the V561D-PDGFRA-Ba/F3 cell line (Figure 5).

In Vivo Analysis of Nilotinib and PKC412 Against D842V-PDGFRA-Ba/F3 Cells

To assess directly the in vivo antitumor efficacy of PKC412 alone, as well as in combination with nilotinib, we utilized a mouse model in which tumor burden was quantified by noninvasive imaging of luminescent D842V-PDGFRα-Ba/F3 cells (Figure 6). Murine Ba/F3 cells expressing the D842V-PDGFRA mutant were engineered to stably express firefly luciferase, and NCr nude mice were then inoculated with these cells. Noninvasive imaging was used to assess serially the tumor burden, and mice with established disease were divided into cohorts with similar tumor burden.

In 2 independent studies, mice were treated for a total of 4 or 6 days, respectively, with either vehicle (n = 3) or PKC412 at 100 mg/kg (n = 3). In these studies, tumor burden, as assessed by bioluminescence, was observed to be overall lower in mice treated with PKC412 as compared with vehicle-treated mice (Figure 6A–D). In another study, PKC412 (50 mg/kg) or nilotinib (150 mg/kg/day) was administered via oral gavage alone or in combination, as was vehicle for a total of 6 days of treatment. At the end of the treatment period, tumor burden was lower in PKC412-treated mice but did not appear to be decreased by nilotinib (Figure 6E). These results suggest efficacy of PKC412 as a single agent against the D842V-PDGFRA mutant, whereas nilotinib is a less effective inhibitor of the D842V-PDGFRA mutant in vivo.

Discussion

The vast majority of GISTs are characterized by activating mutations of c-kit or PDGFRA. The management of GISTs has been significantly changed by the development of imatinib, which has proven to be an effective treatment for this malig-
nancy by inducing clinical responses in patients and improving overall survival. However, imatinib resistance in GIST is becoming a significant challenge in the treatment of this disease. Reports of resistance to imatinib after initial clinical response are emerging, with secondary point mutations in either c-kit or PDGFRA being identified as common mechanisms for acquired resistance to imatinib.

The staurosporine derivative, PKC412, is a broad-spectrum inhibitor of Ser/Thr and Tyr protein kinases that has among its targets protein kinase C (PKC), vascular endothelial growth factor receptor (VEGF-R2), FLT3, PDGFR, and c-kit. PKC412 has previously been shown to be an effective inhibitor of the imatinib-resistant c-kit mutant D816V, which is associated with systemic mast cell disease. PKC412 has also been shown to be effective against the FIP1L1-PDGFRA kinase associated with hypereosinophilic syndrome and chronic eosinophilic leukemia and can override imatinib resistance occurring in point-mutated FIP1L1-PDGFRA-induced myeloproliferative disease. In addition, GIST patient samples positive for the imatinib-resistant mutant c-kit or PDGFRA were found to respond well to PKC412.

Because most GISTs express gain-of-function c-kit or PDGFRA, it was of interest to investigate the effectiveness of PKC412 as a single agent in vitro against mutant PDGFRA conferring differential sensitivity toward imatinib. We were also interested in investigating the in vitro effects of PKC412 in combination with the Bcr-Abl inhibitors imatinib and nilotinib, the latter of which has not yet been investigated with respect to mutant PDGFRA-associated GIST. Additionally, we studied the activity of nilotinib alone, and nilotinib in combination with imatinib, against differentially imatinib-sensitive PDGFRA mutants. Finally, we present here a novel in vivo imaging approach to studying the effects of tyrosine kinase inhibitors against the D842V-PDGFRA mutant.

Cooperative effects have been observed in vitro with combinations of nilotinib and imatinib in c-kit and Bcr-Abl-expressing cells, with differences in the cellular influx and efflux of the 2 agents being implicated as contributing to the underlying mechanism of the additive/synergistic effects. It has recently been shown that nilotinib and imatinib can interact with cell transporters, such as the multidrug efflux transporter ABCG2, which has been linked to chemoresistance, or Oct-1, an organic cation transporter found to be important for the influx of imatinib but not for nilotinib uptake. Alternatively, the synergy observed between nilotinib plus imatinib or PKC412, or imatinib plus PKC412, against D842V-Ba/F3 cells could be attributed to the presence of signaling components/cellular proteins unique to D842V-PDGFRA expression (and not associated with V561D-PDGFRA expression) that are targeted by one or the other of the agents whose combination has been investigated in this study.

Figure 5. Proliferation studies showing combined effects of nilotinib with imatinib and PKC412, respectively, against V561D-PDGFRA-Ba/F3 cells. (A) Three-day treatments of V561D-PDGFRA-Ba/F3 cells with nilotinib, imatinib, or a combination of nilotinib plus imatinib. (B) Three-day treatments of V561D-PDGFRA-Ba/F3 with nilotinib, PKC412, or a combination of nilotinib plus PKC412.
The potency of imatinib against D842V-PDGFRA-Ba/F3 proliferation and cellular tyrosine phosphorylation was in accordance with previous observations. The proliferation of D842V-PDGFRA-Ba/F3 cells was inhibited by nilotinib at concentrations similar to those required for imatinib. Generally, in vitro D842V-PDGFRA-Ba/F3 cells were significantly less responsive than V561D-PDGFRA-Ba/F3 cells toward imatinib and nilotinib; the differential sensitivity of the D842V and V561D mutants to these inhibitors is consistent with what has been reported in the literature. However, the potency of PKC412 in inhibiting proliferation of the D842V mutant was similar to the potency of PKC412 against the V561D mutant. In addition, treatment of mice injected with D842V-PDGFRA-Ba/F3-luc+ cells with a well-tolerated dose of PKC412 reduced lower tumor burden in comparison with vehicle-treated mice, suggesting that PKC412 is efficacious against the D842V-PDGFRA mutant in vivo.

V561D-PDGFRA-Ba/F3 cells responded well to PKC412 treatment in vitro, with high potency observed both in terms of inhibition of cellular proliferation and tyrosine phosphorylation. Nilotinib also displayed significant potency against the V561D-PDGFRA mutant in terms of inhibition of cellular proliferation and total cellular tyrosine phosphorylation. The activity of nilotinib as a single agent (IC_{50} approximately 0.00125 μmol/L) was similar to that of imatinib as a single agent (IC_{50} approximately 0.00125 μmol/L); imatinib has previously been reported to be a potent inhibitor of the V561D mutant. Despite the impressive activity of each single agent against the V561D-PDGFRA mutant, the combination of either nilotinib plus imatinib, or nilotinib plus PKC412, did not result in additive to synergistic effects across a range of doses. In contrast, varying degrees of antagonism were observed between the compounds against the V561D-PDGFRA mutant. These results suggest that nilotinib, imatinib, or PKC412 could potentially be

Figure 6. In vivo analysis of PKC412 alone and PKC412 combined with nilotinib against D842V-PDGFRA-Ba/F3 cells. (A) Imaging data showing NCr nude mice treated for 4 days with vehicle vs PKC412 (100 mg/kg). Nomenclature (A0, B0, C30, A3, and B1) corresponds to the cage (A, B, or C) and number (0, 1, 3, 30) of each mouse. (B) Bioluminescence plotted for (A) as a measure of tumor burden in vehicle or PKC412-treated mice (shown as percentage baseline luminescence). Vehicle (n = 3) and PKC412 (100 mg/kg) (n = 3). (C) Imaging data showing NCr nude mice treated for 6 days with vehicle vs PKC412 (100 mg/kg). Nomenclature (C1, D0, D33, D1, C0, C3) corresponds to the cage (C or D) and number (0, 1, 3, 33) of each mouse. (D) Bioluminescence plotted for (C) as a measure of tumor burden in vehicle or PKC412-treated mice (shown as percentage baseline). Vehicle (n = 3) and PKC412 (100 mg/kg) (n = 3). (E) Bioluminescence as a measure of tumor burden in vehicle- or PKC412- or nilotinib-treated mice. Vehicle (n = 3), PKC412 (50 mg/kg) (n = 3), nilotinib (150 mg/kg) (n = 3), nilotinib + PKC412 (n = 3).
used as single agents against GISTs harboring the V561D-PDGFRα mutant. However, the results do not support the combined use of nilotinib plus imatinib, or nilotinib plus PKC412, against this mutation.

Because of the emergence of primary and secondary resistance of GIST to imatinib, there is a growing need for the identification and development of additional inhibitors that can be used therapeutically for imatinib-resistant GIST or which can be safely and effectively used in combination with imatinib against GIST. In general, PKC412 appears to be an effective inhibitor of both the D842V- and V561D-PDGFRα mutants as a single agent, whereas each mutation is differentially sensitive to imatinib and nilotinib, respectively. In addition, the combination of PKC412 with either nilotinib or imatinib, respectively, in vitro resulted in greater inhibition of D842V-PDGFRα-Ba/F3 cellular proliferation and cellular tyrosine phosphorylation than any one agent alone, with varying degrees of additive-synergistic effects between the compounds observed across a wide range of doses. The combinations of nilotinib, imatinib, and PKC412 could result in a cooperative antiproliferative effect from effects on multiple targets, especially in the case of PKC412, which is a broad-spectrum inhibitor.

The strategy of administering 2 different inhibitors could in theory lead to improved patient responsiveness, as well as suppression of the emergence of drug-resistant mutations characteristic of development of some forms of acquired resistance. Our data suggest that high doses of nilotinib would need to be administered to patients harboring the D842V mutant to achieve clinical responsiveness. Therefore, the benefits of using nilotinib in combination with another tyrosine kinase inhibitor in D842V-PDGFRα-associated GIST patients would have to be weighed against the increased risk of adverse effects because of the high dose of nilotinib required to cause tumor regression. The demonstrated efficacy of PKC412 as a single agent, and the positive enhancement of effects of PKC412 against the D842V mutant in the presence of Abl inhibitors, lends conviction to the potential use of PKC412 alone or in combination therapy for GIST.

References

