Published in final edited form as:

Gastroenterology. 2006 December ; 131(6): 1734–1742. doi:10.1053/j.gastro.2006.09.017.

Effects of PKC412, nilotinib and imatinib against GIST-associated PDGFRA mutants with differential imatinib sensitivity:

Mutant PDGFRA: PKC412, nilotinib, imatinib

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Abstract

Background & Aims—Activating mutations in platelet-derived growth factor receptor alpha (PDGFRA) have been reported in a subset of gastrointestinal stromal tumor (GIST) patients who do not express 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 three tyrosine kinase inhibitors, PKC412 and nilotinib, and imatinib, on two 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-PDGFRA mutants. The effects of nilotinib and PKC412, alone and combined, were investigated *in vivo*.

Results—PKC412 potently inhibited the V561D-PDGFRA mutant *in vitro* and the D842V-PDGFRA mutant *in vitro* and *in vivo*. Both imatinib and nilotinib displayed potent activity *in vitro* against the V561D-PDGFRA mutant, but were significantly less efficacious against D842V-PDGFRA. However, when combined with either imatinib or PKC412, nilotinib showed no evidence for antagonism and acted in a cooperative fashion against D842V-PDGFRA.

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-PDGFRA-associated GIST, although the reduced sensitivity of D842V-PDGFRA probably limits the potential of nilotinib monotherapy for D842V-PDGFRA-associated GIST.

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Grant Support: J.D.G. is supported by NIH grant CA66996, and a Specialized Center of Research Award from the Leukemia and Lymphoma Society. J.D.G. is also supported by NIH grants CA36167 and DK50654. P.W.M. and D.F. are employees of Novartis Pharma AG, Basel, Switzerland. J.D.G. has a financial interest with Novartis Pharma AG.

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Introduction

GIST is the most common mesenchymal tumor of the GI tract. Approximately 90% of GISTs have been shown to have gain-of-function mutations in the receptor for steel factor, c-Kit¹. Imatinib (Gleevec™, STI571; Novartis Pharma AG) is a small molecule kinase inhibitor that selectively targets KIT and PDGFR tyrosine kinases, as well as BCR-ABL². Imatinib is an effective therapy for GIST and is currently used to treat KIT-positive metastatic or unresectable tumors^{3,4}. 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^{3,4}. These responses are in accordance with *in vitro* studies which suggest that GISTs harboring regulatory-region c-Kit mutations are more sensitive to imatinib than GISTs harboring enzymatic-region mutations⁵.

Of GISTs in which only wild-type KIT can be detected, approximately 16% harbor PDGFRA mutations, which result in up-regulation of the same downstream signaling components as KIT^{6,4}. As with 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-PDGFR mutation, is the most common PDGFRA mutation and has been reported to be resistant to imatinib *in vitro*^{3,6,7}. GIST patients possessing exon 18 mutations in PDGFRA typically do not respond well to imatinib therapy. However, the D842V-PDGFR mutation does respond to other tyrosine kinase inhibitors, such as the staurosporine derivative PKC412⁸. The rarer V561D mutation is located at the juxtamembrane domain of exon 12 in the PDGFRA gene⁹.

In light of the development of primary and secondary resistance of GIST towards 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 non-selective inhibitor of protein kinases^{10,11}, and nilotinib (AMN107), a novel inhibitor of Bcr-Abl, c-Kit and PDGFR¹². In addition to inhibiting FLT3, which is mutated in 35% of AML patients, PKC412 inhibits KIT and PDGFR^{10,11}; PKC412 is currently in clinical trials in patients with acute myeloblastic leukemia (AML) 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 target¹²; 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 sensitivity. We also explored the effects of combinations of the three inhibitors against these mutants *in vitro*, and investigated the antiproliferative activity of PKC412, alone and in combination with nilotinib, against the D842V-PDGFR mutant *in vivo*.

Materials and Methods

Cell lines and cell culture

Constructs of D842V-, V561D-, and wild-type (wt)-PDGFRA 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 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-PDGFR α -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 1mg/mL G418.

Chemical compounds, antibodies, and immunoblotting

PKC412, nilotinib and imatinib were synthesized at Novartis Pharma AG, Basel, Switzerland, and were dissolved in DMSO to make 10 mM stock solutions. Serial dilutions were then made, also in DMSO, to obtain final dilutions for cellular assays. Anti p-Tyr (clone 4G10, Upstate Biotechnology, NY) and anti p-Tyr (pY99, Sc-7020, Santa Cruz, CA) were each used at 1:1000 for immunoblotting. PDGFR α antibody (C-20, Santa Cruz Biotechnology, CA) 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 described¹¹.

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-PDGFR α -Ba/F3 cells. Dose-response curves were generated and combination indices were calculated as previously described by isobologram analysis¹².

Mouse studies and in vivo imaging

D842V-PDGFR α -Ba/F3 cells were transduced with a retrovirus encoding firefly luciferase (MSCV-Luc), and selected with puromycin at a concentration of 0.5-1 μ g/mL to generate the D842V-PDGFR α -Ba/F3-luciferase (luc+) cell line. Cells free of *Mycoplasma* and viral contamination were resuspended in Hank's Balanced Salt Solution (HBSS; Mediatech, Inc., VA) prior to IV administration to mice. Solutions of nilotinib were prepared by dissolving 200 mg in 1.0mL of NMP to give a clear solution, and diluted daily prior to administration with 9.0 mL PEG300. 6% w/w PKC412 in Gelucire® 44/14 (Gattefosse, France) was diluted with 1X 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^{nu}) (5-6 weeks of age; Taconic, NY) were administered a total of 600,000 D842V-PDGFR α -Ba/F3-luc+ cells by tail vein injection. Mice were imaged and total body luminescence quantified as previously described¹³. Baseline imaging one day after tumor cell inoculation was used to establish treatment cohorts with matched tumor burden. Cohorts of mice were treated with oral administration of vehicle, 50-100mg/kg/day PKC412 (formulated as above; 4-6 days total treatment with 100mg/kg, 6 days total treatment with 50mg/kg), 150mg/kg/day nilotinib (formulated as above; 6 days total treatment), or a combination of PKC412 and nilotinib (6 days total treatment). Repeat imaging was performed at various intervals. At the planned end of the PKC412 and nilotinib combination study (approximately one week following initial IV injection of D842V-PDGFR α -Ba/F3-luc+ cells), mice were sacrificed, body and spleen weights were recorded, and tissues were preserved in 10% formalin for histopathological analysis.

Results

Effects of PKC412, nilotinib and imatinib on proliferation of D842V-PDGFR α -Ba/F3 cells and tyrosine phosphorylation in D842V-PDGFR α -Ba/F3 cells

D842V-PDGFR α -Ba/F3 cells treated with a range of concentrations of PKC412, nilotinib or imatinib for 72 h. PKC412 exhibited an IC₅₀ in the 0.05-0.1 μ M range against D842V-PDGFR α -Ba/F3 cells (Figure 1A), whereas the IC₅₀ values for nilotinib and imatinib were in the 0.1-0.5 μ M range (Figure 1B and 1C). Addition of IL-3, the normal growth factor for Ba/F3 cells, resulted in near complete rescue, suggesting selective inhibition of D842V-PDGFR α with minimal non-specific toxicity of each of these agents at the concentrations tested.

The effects of PKC412, imatinib and nilotinib on cellular tyrosine kinase activity in D842V-PDGFR α -Ba/F3 cells were studied. The robust signal observed for the pattern of phosphorylated bands in the D842V-PDGFR α -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-PDGFR α -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-PDGFR α 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-PDGFR α -Ba/F3

Combinations of PKC412, nilotinib and imatinib were tested against D842V-PDGFR α -Ba/F3 cells. Overall, positive combination effects were observed between nilotinib plus imatinib, nilotinib plus PKC412, and imatinib plus PKC412 (Figure 2). Calcsyn analysis of the combined effects of nilotinib plus imatinib suggests synergistic to nearly additive effects across a range of doses (ED₂₅-ED₇₅), with antagonism at ED₉₀ (Table I). The nilotinib plus PKC412 combination resulted in nearly additive to synergistic effects across a range of doses (ED₂₅-ED₉₀) (Table I), 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 I).

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 to vehicle control-treated cells) (Figure 3). Cellular tyrosine phosphorylation was inhibited to a greater extent when combinations of each of the three agents were used, as compared to single agent- and vehicle control-treated cells (Figure 3). The results suggest that inhibition of cellular tyrosine phosphorylation by combined treatment of D842V-PDGFR α -expressing cells correlates with enhanced inhibition of cellular proliferation via combined treatment.

Effects of PKC412, nilotinib, and imatinib on proliferation of V561D-PDGFR α -Ba/F3 cells and tyrosine phosphorylation in V561D-PDGFR α -Ba/F3 cells

V561D-PDGFR α -Ba/F3 cells were treated with a range of concentrations of PKC412, nilotinib, or imatinib. PKC412 displayed an IC₅₀ of approximately 0.025 μ M against V561D-PDGFR α -Ba/F3 cells (Figure 4A). The IC₅₀ of nilotinib or imatinib against V561D-PDGFR α

-Ba/F3 cells was approximately 0.01 μ M (Figure 4B and C). IL-3 rescue was observed with all three 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 h 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 three 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 to 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, and dose-response curves were generated (Figure 5).

Generally, both combinations led to varying degrees of antagonism across a range of doses in the V561D-PDGFRA-Ba/F3 cell line (Table I).

In vivo analysis of nilotinib and PKC412 against D842V-PDGFRA-Ba/F3 cells

To directly assess the *in vivo* anti-tumor efficacy of PKC412 alone, as well as in combination with nilotinib, we utilized a mouse model in which tumor burden was quantified by non-invasive imaging of luminescent D842V-PDGFRA-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. Non-invasive imaging was used to serially assess tumor burden, and mice with established disease were divided into cohorts with similar tumor burden.

In two independent studies, mice were treated for a total of 4 or 6 days, respectively, with either vehicle (n=3) or PKC412 at 100mg/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 to vehicle-treated mice (Figure 6A-D). In another study, PKC412 (50mg/kg) or nilotinib (150mg/kg/day) were administered via oral gavage alone or in combination, as was vehicle for a total of six 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 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 malignancy 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 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^{10,11}. 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^{14,15}. PKC412 has also been shown to be effective against the

FIP1L1-PDGFR α kinase associated with hypereosinophilic syndrome and chronic eosinophilic leukemia¹⁶, and can override imatinib resistance occurring in point mutated FIP1L1-PDGFR α -induced myeloproliferative disease¹⁷. In addition, GIST patient samples positive for the imatinib resistant mutant c-KIT or PDGFR α were found to respond well to PKC412⁸.

Because most GISTs express gain-of-function KIT or PDGFR α , it was of interest to investigate the effectiveness of PKC412 as a single agent *in vitro* against mutant PDGFR α 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 PDGFR α -associated GIST. Additionally, we studied the activity of nilotinib alone, and nilotinib in combination with imatinib, against differentially imatinib-sensitive PDGFR α mutants. Finally, we present here a novel *in vivo* imaging approach to studying the effects of tyrosine kinase inhibitors against the D842V-PDGFR α mutant.

Cooperative effects have been observed *in vitro* with combinations of nilotinib and imatinib in KIT and Bcr-Abl-expressing cells, with differences in the cellular influx and efflux of the two agents being implicated as contributing to the underlying mechanism of the additive/synergistic effects^{18,19,20}. 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 chemoresistance²¹, or Oct-1, an organic cation transporter found to be important for the influx of imatinib, but not for nilotinib uptake²⁰. 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-PDGFR α expression (and not associated with V561D-PDGFR α expression) that are targeted by one or the other of the agents whose combination has been investigated in this study.

The potency of imatinib against D842V-PDGFR α -Ba/F3 proliferation and cellular tyrosine phosphorylation was in accordance with previous observations^{22,8}. The proliferation of D842V-PDGFR α -Ba/F3 cells was inhibited by nilotinib at concentrations similar to those required for imatinib. Generally, *in vitro* D842V-PDGFR α -Ba/F3 cells were significantly less responsive than V561D-PDGFR α -Ba/F3 cells towards imatinib and nilotinib; the differential sensitivity of the D842V and V561D mutants to these inhibitors is consistent with what has been reported in the literature⁷. 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-PDGFR α -Ba/F3-luc+ cells with a well-tolerated dose of PKC412 reduced lower tumor burden in comparison to vehicle-treated mice, suggesting that PKC412 is efficacious against the D842V-PDGFR α mutant *in vivo*.

V561D-PDGFR α -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-PDGFR α mutant in terms of inhibition of cellular proliferation and total cellular tyrosine phosphorylation. The activity of nilotinib as a single agent (IC₅₀ approximately 0.00125 μ M) was similar to that of imatinib as a single agent (IC₅₀ approximately 0.00125 μ M); imatinib has previously been reported to be a potent inhibitor of the V561D mutant⁷. Despite the impressive activity of each single agent against the V561D-PDGFR α mutant, though, 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-PDGFR α mutant. These results suggest that nilotinib, imatinib, or PKC412 could potentially be used as single agents against GISTs harboring the V561D-PDGFR α

mutant. However, the results do not support the combined use of nilotinib plus imatinib, or nilotinib plus PKC412, against this mutation.

Due to 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-PDGFR α 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-PDGFR α -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 two 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 in order to achieve clinical responsiveness. Therefore, the benefits of using nilotinib in combination with another tyrosine kinase inhibitor in D842V-PDGFR α -associated GIST patients would have to be weighed against the increased risk of side-effects due to 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.

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Figure 1.

Effects of PKC412, nilotinib and imatinib on proliferation of D842V-PDGFR α -Ba/F3 cells. Cell viability is reported as percentage of control (untreated) cells. Error bars represent the standard error of the mean for each data point. A. Three-day treatment of D842V-PDGFR α -Ba/F3 cells (+/- IL-3) with PKC412 (n=2). B. Three-day treatment of D842V-PDGFR α -Ba/F3 (+/-IL-3) and PDGFR α -Ba/F3 cells with nilotinib (n=2). C. Three-day treatment of D842V-PDGFR α -Ba/F3 cells (+/-IL-3) with imatinib (n=2). D. Immunoblot: Treatment of D842V-PDGFR α -Ba/F3 cells for 1 hour with nilotinib, imatinib, and PKC412, respectively. Densitometry for this immunoblot yielded the following values, reported as area (pixels): D842V control#1 pTyr/PDGFR α =9112/7680; AMN107 pTyr/PDGFR α =8146/9632; imatinib pTyr/PDGFR α =6859/10382; D842V control #2 pTyr/PDGFR α =8431/6960; PKC412, 0.1 μ M pTyr/PDGFR α =5541/9609; PKC412, 1 μ M pTyr/PDGFR α =5759/13461.

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.

Figure 3.

Effects of combinations of PKC412, nilotinib, and imatinib on tyrosine phosphorylation in D842V-Ba/F3 cells. PTYR immunoblot showing effects of nilotinib, imatinib, and PKC412-alone and combined- against D842V-Ba/F3 cells. Lane 1, control; Lane 2, nilotinib alone; Lane 3, PKC412 alone; Lane 4, imatinib alone; Lane 5, nilotinib+PKC412; Lane 6, nilotinib+imatinib; Lane 7, imatinib+PKC412. D842V-Ba/F3 cells were treated for 4 hours with PKC412 (0.1 μ M), imatinib (0.5 μ M), or nilotinib (0.5 μ M), alone or combined. The pTyr (pY99) antibody was used for the pTyr immunoblot, and alpha-tubulin was used as a loading control. Densitometry for this immunoblot yielded the following values, reported as area (pixels): D842V control pTyr/tubulin=21983/19993; nilotinib alone pTyr/tubulin=18897/20791; PKC412 alone pTyr/tubulin=19030/24114; imatinib alone pTyr/tubulin=19116/24576; nilotinib+PKC412 pTyr/tubulin=13103/25584; nilotinib+imatinib pTyr/tubulin=14208/25865; imatinib+PKC412 pTyr/tubulin=13571/27210.

Figure 4.
Effects of PKC412, nilotinib and imatinib on proliferation of V561D-PDGFRα-Ba/F3 cells. Cell viability is reported as percentage of control (untreated) cells. Error bars represent the standard error of the mean for each data point. A. Three-day treatment of V561D-PDGFRα-Ba/F3 cells (+/- IL-3), PDGFRα-Ba/F3 cells and parental Ba/F3 cells with PKC412 (n=2). B. Three-day treatment of PDGFRα-V561D-Ba/F3 (+/-IL-3) and PDGFRα-Ba/F3 cells with nilotinib (n=2). C. Three-day treatment of V561D-PDGFRα-Ba/F3 cells (+/-IL-3) and PDGFRα-Ba/F3 cells with imatinib (n=2). D. Immunoblot: Treatment of V561D-PDGFRα-Ba/F3 cells for 1 hour with nilotinib, imatinib and PKC412, respectively.

Figure 5. Proliferation studies showing combined effects of nilotinib with imatinib and PKC412, respectively, against V561D-PDGFRα-Ba/F3 cells. A. Three-day treatments of V561D-PDGFRα-Ba/F3 cells with nilotinib, imatinib or a combination of nilotinib plus imatinib. B. Three-day treatments of V561D-PDGFRα-Ba/F3 with nilotinib, PKC412, or a combination of nilotinib plus PKC412.

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 (100mg/kg). Nomenclature (A0, B0, C30, C0, 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 percent baseline luminescence). Vehicle (n=3) and PKC412 (100mg/kg) (n=3). C. Imaging data showing NCr nude mice treated for 6 days with vehicle vs PKC412 (100mg/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 percent baseline). Vehicle (n=3) and PKC412 (100mg/kg) (n=3). E. Bioluminescence as a measure of tumor burden in vehicle- or PKC412 or nilotinib-treated mice. Vehicle (n=3), PKC412 (50mg/kg) (n=3), nilotinib (150mg/kg) (n=3), nilotinib +PKC412 (n=3).

Table I

Combination indices calculated for dose-response curves shown in Figures 2 and 5.

Cell Lines (Treatments)	Combination Indices:			
	ED25	ED50	ED75	ED90
D842V-Ba/F3 (AMN107+imatinib)	0.48812	0.73221	1.09836	1.64762
D842V-Ba/F3 (AMN107+PKC412)	1.00967	0.80135	0.68007	0.61817
D842V-Ba/F3 (imatinib+PKC412)	1.38372	1.01669	0.77981	0.62462
V561D-Ba/F3 (AMN107+imatinib)	0.80100	1.00712	1.81556	5.23012
V561D-Ba/F3 (AMN107+PKC412)	1.12460	1.42547	1.81579	2.32421