Inhibition of mutant FLT3 receptors in leukemia cells by the small molecule tyrosine kinase inhibitor PKC412

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Summary

Constitutively activating FLT3 receptor mutations have been found in 35% of patients with acute myeloblastic leukemia (AML). Here we report the identification of a small molecule FLT3 tyrosine kinase inhibitor PKC412, which selectively induced G1 arrest and apoptosis of Ba/F3 cell lines expressing mutant FLT3 ($IC_{50} < 10$ nM) by directly inhibiting the tyrosine kinase. Ba/F3-FLT3 cell lines made resistant to PKC412 demonstrated overexpression of mutant FLT3, confirming that FLT3 is the target of this drug. Finally, progressive leukemia was prevented in PKC412-treated Balb/c mice transplanted with marrow transduced with a FLT3-ITD-expressing retrovirus. PKC412 is a potent inhibitor of mutant FLT3 and is a candidate for testing as an antileukemia agent in AML patients with mutant FLT3 receptors.

Introduction

Acute myelogenous leukemia (AML) is a malignant disorder of hematopoietic cells characterized by an accumulation of highly proliferative blasts blocked in differentiation at various stages. The disease is maintained by transplantable leukemic stem cells capable of substantial self-renewal (Bonnet and Dick, 1997). Cytogenetics, age, and history of a preleukemic syndrome are major prognostic determinants. Allogeneic bone marrow transplant (BMT) has had a significant impact on the disease and long-term survival. However, although the number of young patients treated with either BMT or aggressive chemotherapy has increased steadily over the last 3 decades, such that currently, patients under age 60 can anticipate 25%–30% ten-year survival, patients over the age of 60, as well as those with secondary AML or prior myelodysplastic syndrome, continue to do extremely poorly. Remarkably, the major chemotherapy drugs used during induction therapy, cytosine arabinoside and an anthracycline such as daunorubicin, have not changed in more than 25 years, although a number of useful new agents have value in patients with relapsed disease.

The majority of patients with AML have genetic mutations that result in precursor cells exhibiting either blocked differentiation or accelerated proliferation, or both (Pabst et al., 2001; Tanaka et al., 1997; Olsson et al., 1996). Many of these mutant genes have been cloned and shown to cause leukemias in mice, or to cooperate with other oncogenes to cause leukemia in various models. Identification of these mutant genes, and elucidation of their function, is of critical importance because they represent potentially ideal therapeutic targets for small molecule drugs, antibodies, or other agents.

The class III receptor tyrosine kinase, FLT3 (*F*ms-*L*ike *T*yrosine kinase-3; FLK-2, *F*etal *L*iver *K*inase-2; or STK-1, human *S*tem Cell *T*yrosine *K*inase-1) (Rosnet and Birnbaum, 1993), has a number of features that suggest that it is a particularly good therapeutic target. FLT3 is activated by mutations that have now been detected in about 30% of all patients with AML. Two types of activating mutations have been described. Internal tandem duplications within the juxtamembrane (JM) domain of FLT3 were first reported in AML in 1996 (Nakao et al., 1996). Five of 30 patients were found to have tandem, in-frame, duplications primarily within exon 11, resulting in duplication of a small segment of the JM domain. Two patients had small insertions in addition to duplications. No abnormalities of FLT3 were detected in remission samples. The high frequency of JM domain mutations has been confirmed in several large studies, with an overall incidence of about 20%–25% of AMLs, and <5% of patients with myelodysplastic syndrome (MDS) (Nakao et al.,

SIGNIFICANCE

Acute myelogenous leukemia (AML) is a malignant disorder of hematopoietic cells with an incidence of approximately 5 cases per 100,000 population per year. Although standard chemotherapy or stem cell transplantation can induce remission in most patients, the disease recurs frequently, and the majority of patients will ultimately die of their disease. The discovery of the oncogenes that cause AML has led to increasing efforts to define novel, oncogene-targeted therapy. The FLT3 tyrosine kinase inhibitor is mutated in about 30% of patients. We identify a small molecule inhibitor of FLT3, PKC412, and demonstrate that it is selectively toxic to leukemic cells expressing mutant FLT3 in cell lines and in murine models of leukemia. Inhibition of FLT3 represents a promising approach to the treatment of AML.

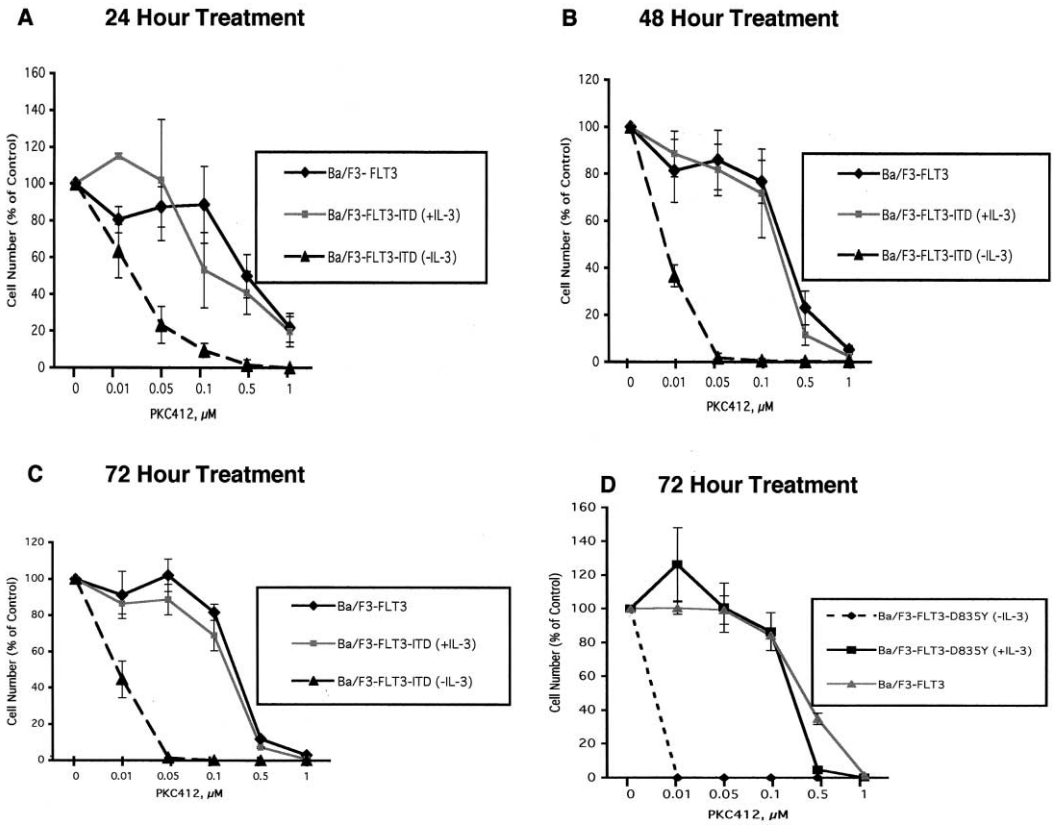

Figure 1. PKC412 inhibition of cellular proliferation and viability in Ba/F3-FLT3-ITD cells

A–C: Growth of Ba/F3-FLT3-ITD cells cultured for 24 (**A**), 48 (**B**), and 72 (**C**) hr, respectively, in the presence of increasing concentrations of PKC412, and either in the presence or absence of IL-3. Parental Ba/F3 cells were similarly treated at the indicated time points for comparison. Data points represent the average of three independent experiments.

D: Growth of parental Ba/F3-FLT3 cells and Ba/F3-FLT3-D835Y cells cultured for 72 hr in the presence of increasing concentrations of PKC412, the latter cultured in the presence and absence of IL-3. Data points represent the average of two independent experiments.

1996; Horiike et al., 1997; Rombouts et al., 2000; Kondo et al., 1999; Kiyoi et al., 1999, 1998), but have not been seen in acute lymphoblastic leukemias (ALLs) unless they are biphenotypic (Xu et al., 1999). Expression of the mutant FLT3 gene has generally been found to be a poor prognostic feature (Kondo et al., 1999; Kiyoi et al., 1999; Iwai et al., 1999). Interestingly, deletion of the wild-type allele or mutation of both FLT3 genes has been reported in a minority of patients, but may be associated with even poorer prognosis. Mutations in FLT3 have not been seen in nonhematopoietic cancers, perhaps because expression of FLT3 is limited to only a few tissues outside of the blood and immune system.

The second type of mutation in FLT3 involves point mutations within the "activation loop" of the kinase (Yamamoto et al., 2001). These point mutations have been reported in an additional 7% of AML cases, most often involving an asparagine (Asp) residue at position 835. This residue is homologous to Asp 816 in c-KIT, also known to confer an activated phenotype when mutated in systemic mastocytosis. In both KIT and FLT3, mutations within the activation loop are believed to change the conformation of this domain, causing it to adopt an "activated" configuration, resulting in kinase activation. D835 mutations generally occur independently of FLT-3-ITD (Yamamoto et al., 2001). FLT3-ITD and D835 mutations worsen disease-free survival (Yamamoto et al., 2001).

Both types of mutations of FLT3 (FLT3-ITD and FLT3-D835) result in constitutive activation of FLT3 kinase activity. Expression of either of these mutants in factor-dependent cell lines is associated with rapid conversion to factor-independent proliferation, enhanced viability, and increased tyrosine phosphorylation of FLT3 itself and other signaling proteins (Kiyoi et al., 1998; Hayakawa et al., 2000). Further, transplantation of murine bone marrow cells infected with a retrovirus expressing a FLT3-ITD mutant results in a rapidly lethal myeloproliferative disease (Kelly et al., 2002).

Overall, these data strongly support the notion that inhibition of FLT3 could have important therapeutic utility in AML. FLT3 is commonly mutated, and enhances proliferation and viability through constitutive activation of the tyrosine kinase. Thus, tyrosine kinase inhibitors that selectively target FLT3 would be anticipated to have potential therapeutic utility.

We report here the identification and characterization of PKC412, an inhibitor of FLT3, and demonstrate that this agent induces cell cycle arrest and apoptosis of leukemic cells expressing mutant FLT3, but not cells expressing wild-type FLT3. Furthermore, PKC412 was found to have significant ability to

extend survival of mice with FLT3-ITD-induced leukemia. The results confirm that FLT3 is potentially an excellent therapeutic target for AML and suggest that PKC412 and similar agents are worthy of evaluation in clinical trials.

Results

Selective inhibition of proliferation of Ba/F3-FLT3-ITD cells by PKC412

A diverse set of small molecule tyrosine kinase inhibitors were screened for their ability to selectively kill growth factor-independent Ba/F3-FLT3-ITD cells, without inducing apoptosis in parental, growth factor-dependent Ba/F3 cells. These compounds were previously identified in other drug screens at Novartis Pharma AG as inhibitors of kinases related to FLT3, such as PDGFR−β, c-KIT, c-FMS, and others. One compound, PKC412, inhibited the proliferation of Ba/F3-FLT3-ITD cells with an IC_{50} (inhibitory concentration, 50%) of less than 10 nM within 24–72 hr, and was nontoxic toward parental Ba/F3 cells at concentrations up to 100 nM (Figures 1A–1C). Reduced cell growth was due to both the induction of apoptosis (Annexin V staining, Figure 2A) and cell cycle arrest (Figure 2B). Ba/F3-FLT3-ITD cells could be rescued from the inhibitory effects of PKC412 by IL-3 (Figures 1A–1C and 2A). After 72 hr of treatment, the IC_{50} for Ba/F3-FLT3-ITD treated with PKC412 was several orders of magnitude lower than the IC_{50} for the same cells cultured in the presence of IL-3 (Figures 1A–1C). This suggests that PKC412 does not significantly inhibit any kinases in the signaling pathways used by the IL-3 receptor to support growth or proliferation of Ba/F3 cells.

PKC412 was also found to inhibit proliferation and viability of Ba/F3-FLT3-D835Y cells (Figure 1D). As with Ba/F3-FLT3-ITD cells, the inhibitory effects of PKC412 could be reversed by the addition of IL-3.

The effects of PKC412 and STI571 were compared on Ba/F3 cell lines expressing either FLT3-ITD or p210BCR/ABL (Figure 3). PKC412 was only toxic to Ba/F3.p210BCR/ABL cells at a concentration of 1 μM, a dose significantly higher than that required for the inhibition of the growth of FLT3-ITD or FLT3-D835Y-expressing Ba/F3 cells. Further, the effect of 1 μM PKC412 on BCR/ABL-expressing cells was not specific to BCR/ABL, as parental Ba/F3 cells were also killed at this concentration of drug. Approximately 100× more PKC412 was required to inhibit BCR/ABL-expressing cells than STI571, and PKC412 was toxic to the cells both in the presence and in the absence of IL-3, whereas STI571 was selectively toxic to BCR/ABL-expressing cells cultured only in the absence of IL-3. These results are consistent with unpublished in vitro kinase assays indicating that this compound is not an effective inhibitor of c-ABL kinase. Similarly, the c-ABL-kinase inhibitor STI571 was not toxic to Ba/F3-FLT3-ITD cells except at a very high dose (10 μM), consistent with the previous observation that FLT3 is not a kinase target of this drug. These results suggest that the use of paired Ba/F3 cell lines expressing different oncogenic kinases can be useful in demonstrating the specificity of kinase inhibitors.

Inhibition of wild-type FLT3 tyrosine kinase activity and mutant FLT3 tyrosine kinase activity by PKC412

The above studies demonstrated that PKC412 was an effective inhibitor of proliferation and viability induced by expression of a mutant FLT3 receptor in Ba/F3 cells. A different approach was used to determine if PKC412 also inhibited ligand-dependent activation of wild-type FLT3 receptors, since the addition of FLT3 ligand (FLT3L) did not support proliferation of Ba/F3 cells in the absence of IL-3 (data not shown), consistent with previous observations (Hayakawa et al., 2000). Serum-deprived, Ba/F3-FLT3-WT cells were stimulated for 2 or 10 min, respectively, with FLT3L in the presence or absence of PKC412. FLT3L stimulated tyrosine phosphorylation in Ba/F3 cells expressing the wild-type FLT3 receptor as expected, namely of substrates in the 100 kDa range. This increase in tyrosine phosphorylation was abolished by 0.01–1 μM PKC412 (Figure 4), indicating that kinase activity of wild-type FLT3 was also likely to be inhibited by PKC412.

PKC412 inhibits autophosphorylation of mutant FLT3 receptors

The results in Figure 1 suggested that FLT3 might directly inhibit the tyrosine kinase activity of mutant, and wild-type, FLT3 receptors. This was examined directly by determining if PKC412 could inhibit FLT3 receptor tyrosine autophosphorylation. Like other class III receptor tyrosine kinases, FLT3 is known to undergo autophosphorylation in response to ligand activation, and both ITD and D835 mutants have high levels of ligand-independent tyrosine phosphorylation. Potent inhibition of FLT3 tyrosine phosphorylation was seen in Ba/F3-FLT3-ITD and Ba/F3-FLT3-D835Y cells treated with 0.01–1 μM of PKC412 within 15 min, with no apparent effect on levels of FLT3-ITD or FLT3-D835Y expression (Figure 5). These results suggest that PKC412 works either by direct inhibition of FLT3 tyrosine kinase activity, or by inhibiting a receptor-associated kinase involved in receptor tyrosine phosphorylation.

PKC412 directly inhibits FLT3 kinase activity in vitro

The ability of PKC412 to inhibit FLT3-catalysed phosphorylation was assessed using a tyrosine kinase inhibition assay using the cytoplasmic kinase domain of FLT3. In this assay, PKC412 demonstrated an IC_{50} value of 528 ± 161 nM ($n = 15$), confirming that PKC412 is a direct inhibitor of the FLT3 kinase. The major human metabolite of PKC412, CGP052421 (metabolite M [34]) had an IC_{50} value of 643 ± 79 nM ($n = 3$), whereas STI571 did not have significant activity at concentrations >10,000 nM.

Effect of PKC412 on Ba/F3-FLT3-ITD cells overexpressing FLT3

The results above indicate that PKC412 can directly inhibit the kinase activity of both wild-type and mutant FLT3. Further, the cell culture experiments shown in Figure 1 also suggest that inhibition of cell growth is highly correlated with expression of mutant FLT3. Since PKC412 is known to inhibit other kinases as well as FLT3, additional studies were done to determine if FLT3 could be confirmed as the most important target of this compound in intact cells. Ba/F3-FLT3-ITD cells were cultured in the presence of increasing concentrations of PKC412 (up to 0.04 μM) over a span of two months to generate a polyclonal subline of Ba/F3-FLT3-ITD cells less sensitive to PKC412 (Figure 6A). In these less sensitive cells, but not in Ba/F3-FLT3-ITD cells similarly cultured in the absence of PKC412, significant overexpression of FLT3-ITD protein was observed (Figure 6B).

Amplification of the gene encoding a drug target and overexpression of the target protein are commonly observed mechanisms of drug desensitization. For example, we have previously reported that chronic exposure of Ba/F3-p210BCR/ABL cells

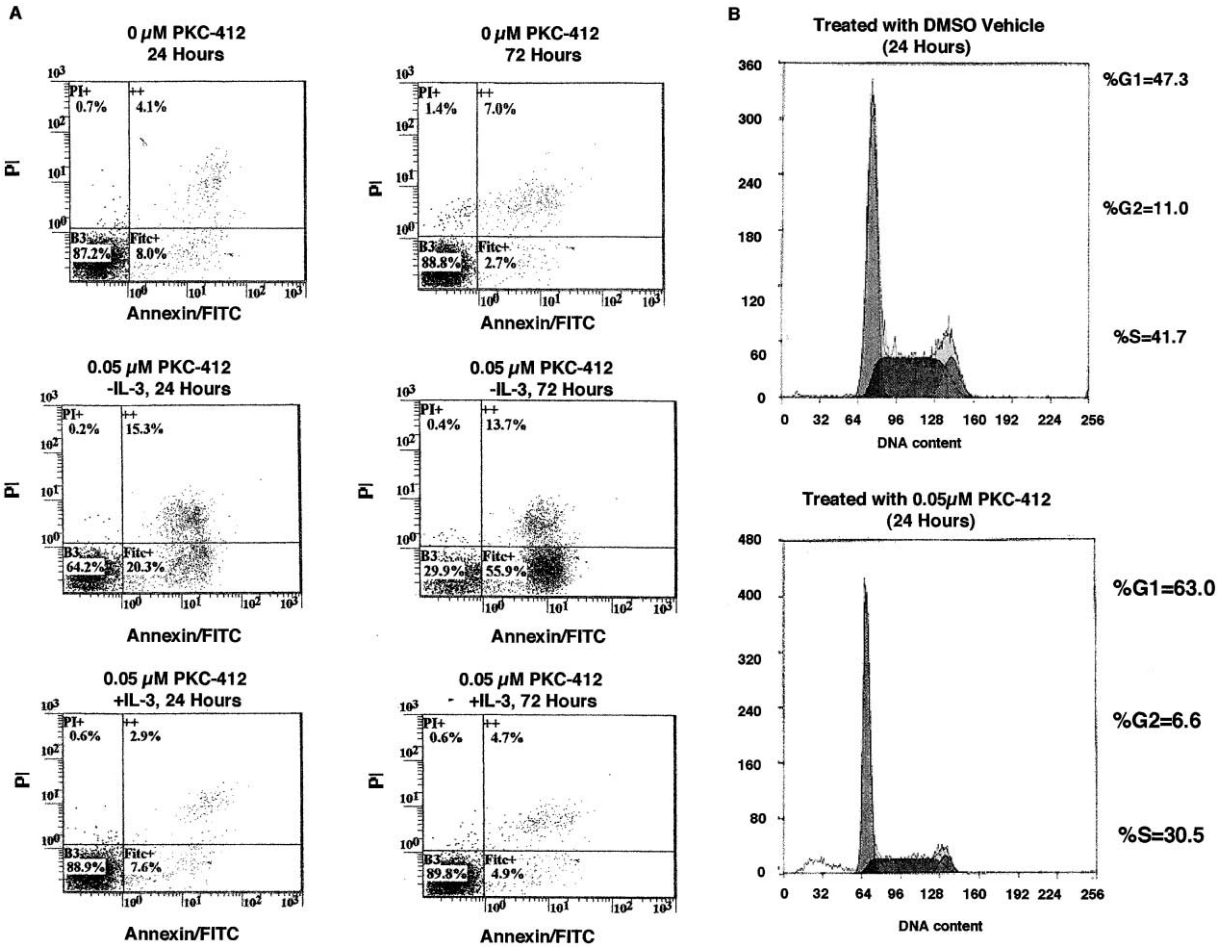

Figure 2. PKC412 induction of apoptosis and cell cycle arrest in Ba/F3-FLT3-ITD cells

A: PKC412 induction of apoptosis in Ba/F3-FLT3-ITD in 24 hr (left vertical panel) and 72 hr (middle vertical panel). Drug effects were reversed with IL-3 at both time points.

B: PKC412 inhibition of cell cycle progression in Ba/F3-FLT3-ITD cells. Ba/F3-FLT3-ITD cells were treated with DMSO vehicle for 24 hr or PKC412 for 24 hr (right vertical panel).

to STI571 results in amplification of the BCR/ABL transgene (Weisberg and Griffin, 2000). Here, these results provide strong support for the notion that FLT3 is the relevant target for PKC412 in these experiments.

In vivo inhibition of FLT3-ITD-mediated transformation by PKC412

Two independent trials were performed using a BMT assay to test the efficacy of PKC412 on the FLT3/ITD-induced myeloproliferative disorder (MPD) in vivo. We assessed efficacy of PKC412 in established FLT3-ITD disease to most closely mimic therapy of established disease in humans. Our previous observations with the FLT3/ITD BMT assay indicated that disease was well established at approximately 30 days posttransplant, and that >90% of animals succumbed to disease by day 90 (Kelly et al., 2002). In trial 1, the mice were treated with drug from day 30 on. In trial 2, drug was administered from day 25 on. Assuming that 20% of drug-treated animals and 80% of untreated animals would develop MPD, we designed each trial to have 80% power to detect this difference at the 0.05% significance level. Pharmacokinetic analysis in Balb/c mice indicated that q24 hr dosing by oral gavage at 100 mg/kg maintained plasma concentrations of PKC412 at or above IC_{50} levels. Throughout each trial, any animals with massive splenomegaly by physical exam (spleen boundary at the dorsal midline) or that were moribund were sacrificed and analyzed. Data collected included total and differential white blood cell counts, gross morphological features, and spleen weight (Table 1). Organs from all animals were fixed and analyzed for histological features of disease. The immunophenotype of spleen cells of 4 animals from each group was also analyzed.

Kaplan-Meier analyses for survival (Figures 7A and 7B) of placebo- and drug-treated mice for each of the two trials demonstrated that PKC412 prolonged survival, with p values of 0.0005 and 0.009 for trials 1 and 2, respectively, using a log rank analysis. A Kaplan-Meier plot with the combined data from trials 1 and 2 is shown in Figure 7C. Assessment of plasma PKC412 concentrations was performed at the trial 1 study endpoint. Plasma samples were obtained from six mice sacrificed 8–12 hr after dosing, and showed plasma concentrations of

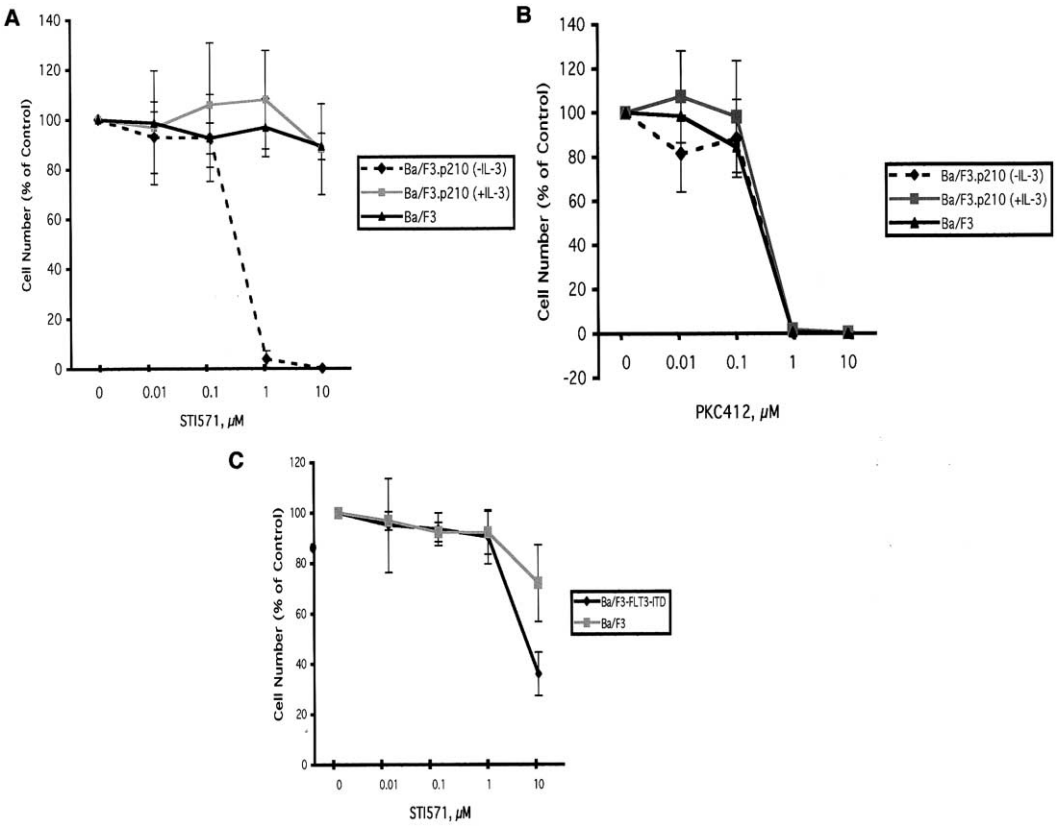

Figure 3. Selectivity of PKC412 for FLT3-expressing cells

A and B: Ba/F3.p210 cells were cultured for 72 hr in the presence and absence of IL-3 with increasing concentrations of STI-571 (**A**) or PKC412 (**B**). Ba/F3 cells were similarly treated in each experiment in the presence of IL-3. For each, data points represent the average of two independent experiments. Parental Ba/F3 cells (cultured in the presence of IL-3) and Ba/F3-FLT3-ITD cells (cultured in the absence of IL-3) were treated for 72 hr with STI-571
C: Data points represent the average of three independent experiments.

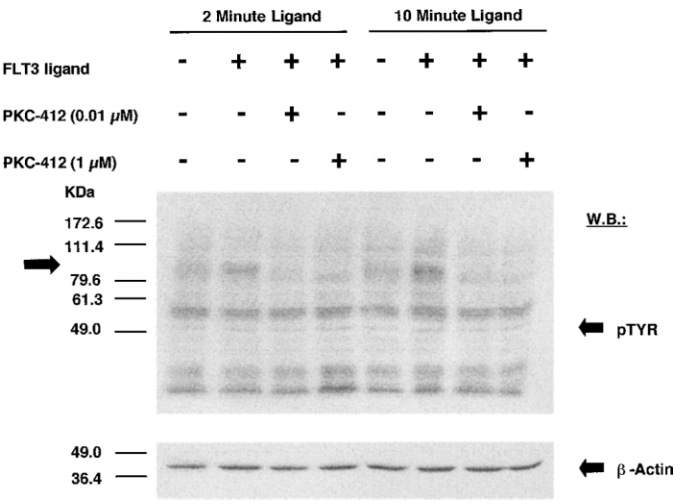

Figure 4. Inhibition of wild-type FLT3-mediated cellular tyrosine phosphorylation by PKC412

Ba/F3-FLT3 cells were serum-starved overnight and treated for 1 hr with 0.01 μM or 1 μM PKC412, prior to 2 min or 10 min of FLT3 ligand stimulation, respectively. Cellular tyrosine phosphorylation was analyzed by immunoblotting with a pTYR antibody. A β-actin antibody was used as a loading control. Elevation of tyrosine phosphorylation of bands at approximately 100 kDa is shown (left hand arrow).

545 ± 237 ng/ml (range: 333–379 ng/ml; 545 ng/ml = 954 nM), which were well above the PKC412 IC_{50} for inhibition of Ba/F3-FLT3-ITD cells in tissue culture.

Analysis of the data from each trial (Table 1) showed that the majority of placebo animals developed characteristic features of FLT3/ITD MPD, while none of those treated with PKC412 did. The mean spleen weight was 401 mg for placebo animals, corresponding to a 4-fold increase in size compared to irradiated transplanted controls (100 mg). In contrast, PKC412-treated mice displayed only a slight increase in mean spleen weight (80 mg). Similarly, the mean white blood cell count for placebo animals was 25.8×10^6 /ml compared to 3.6×10^6 /ml for drug-treated animals.

Histopathologic examination of the spleen from representative animals (Figure 8) further supported the interpretation of a dramatic reduction in FLT3-ITD-induced disease in the drug-treated mice compared to placebo controls. Splenic architecture was effaced in the placebo mice by a marked expansion of red pulp comprised of maturing myeloid cells and scattered admixed megakaryocytes. The spleen displayed marked hypercellularity and myeloid hyperplasia consisting predominantly of mature granulocytic elements. In contrast, the drug-treated animals showed a partial recovery of splenic architecture, a reduction in myeloid hyperplasia, and a corresponding increase in the proportion of other hematopoietic lineages.

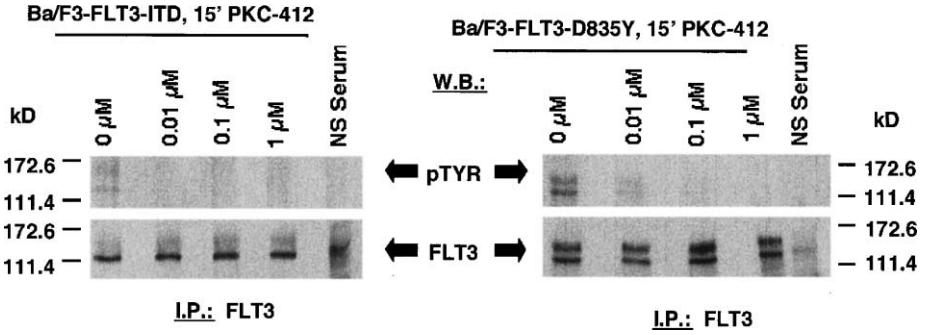

Figure 5. Inhibition of FLT3-ITD and FLT3-D835Y autophosphorylation by PKC412

FLT3-ITD was immunoprecipitated from Ba/F3-FLT3-ITD cells treated for 15 min with varying concentrations of PKC412. Immunoblotting was performed on all samples with a pTYR antibody (upper panel) and a FLT3 antibody (lower panel). Rabbit serum was used as a control for nonspecific binding of the FLT3 antibody. FLT3-D835Y was immunoprecipitated from Ba/F3-FLT3-D835Y cells and treated as for FLT3-ITD.

The immunophenotype of single cell suspensions from the spleens of 4 placebo and 4 PKC412-treated animals from trial 1 was examined using myeloid markers Gr-1 and Mac-1 to evaluate the proportion of myeloid cells present. These data showed that the placebo mice had a profile consistent with classic FLT3-induced myeloproliferative disease (Kelly et al., 2002). In contrast, mice treated with PKC412 had an average of 5% myeloid cells, which is comparable to a disease-free Balb/c mouse (4%) (data not shown).

Discussion

The FLT3 receptor tyrosine kinase gene is mutated in approximately one-third of patients with AML. The mutations have been detected in all FAB subtypes of AML in both adult and pediatric patients, but are rarely detected in either ALL or MDS. The most common mutations result in tandem duplications of a variable number of amino acids within the juxtamembrane domain, and have been reported in approximately 20%–25% of patients (Na-

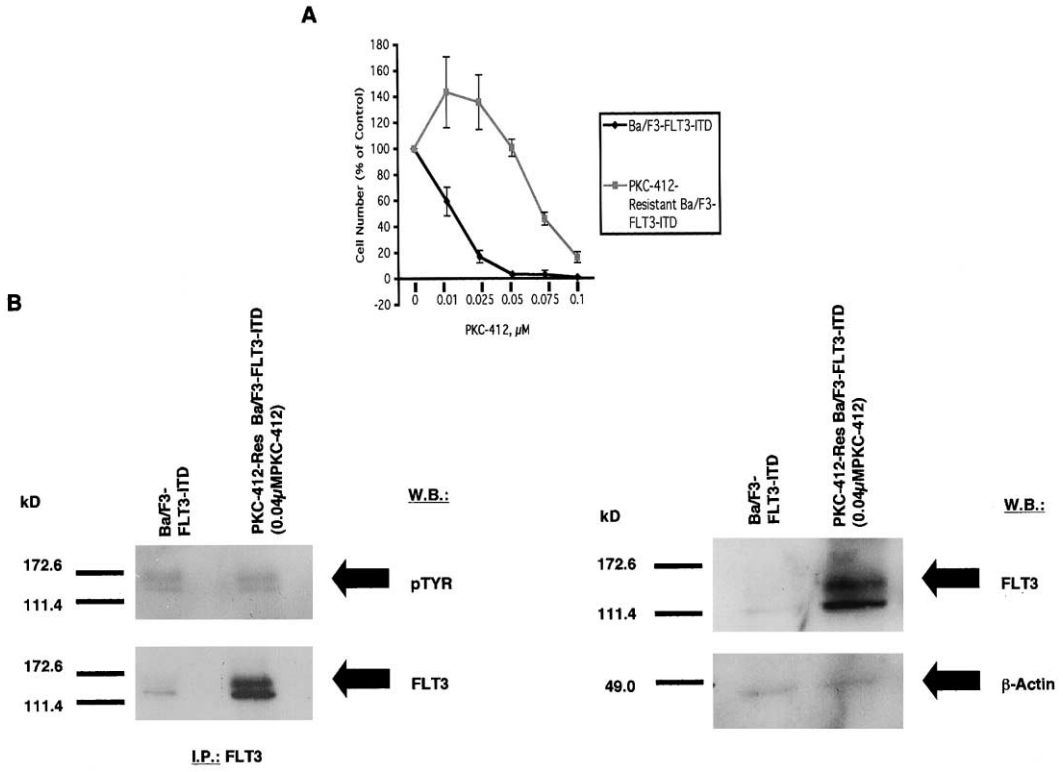

Figure 6. PKC412 Resistance in Ba/F3-FLT3-ITD cells

A: Proliferation of PKC412-resistant Ba/F3-FLT3-ITD cells in the presence of PKC412. Ba/F3-FLT3-ITD cells cultured for several months in the presence of gradually increasing concentrations of PKC412 (up to 0.04 μM) were treated with PKC412 and compared to Ba/F3-FLT3-ITD cells that had not been previously cultured in the presence of drug. Data points represent the average of two independent experiments.

B: Overexpression of FLT3-ITD in PKC412-resistant Ba/F3-FLT3-ITD cells. FLT3-ITD was immunoprecipitated from nonresistant and PKC412-resistant Ba/F3-FLT3-ITD cells and analyzed by immunoblotting with a pTYR antibody and a FLT3 antibody, respectively (left panel). Whole cell protein lysates of nonresistant and PKC412-resistant Ba/F3-FLT3-ITD cells were analyzed by immunoblotting with a FLT3 antibody (right panel). A β-actin antibody was used as a loading control.

Table 1. Phenotypic analysis of placebo and PKC412-treated animals

	Spleen weight (mg)		WBC ($\times 10^6$/ml)	
	Placebo	PKC412	Placebo	PKC412
Mean	401	80	25.8	3.6
Median	387	83	12	3.2
Range	105–801	61–98	4.0–160.0	0.4–12.0
n	22	13	22	22
p value	3×10^{-6}	3×10^{-6}	6×10^{-5}	6×10^{-5}

Data are presented for placebo animals and PKC412-treated animals at trial endpoint. The white blood cell counts and spleen weights are shown per milliliter (10^6/ml) and in milligrams (mg), respectively. In each case, the mean vlaue, the median value, the range, and the number of animals (n) are listed.

kao et al., 1996; Rombouts et al., 2000; Kondo et al., 1999; Kiyoi et al., 1997). An additional 7% of patients have point mutations within the activation loop of the kinase domain, most often involving asparagine 835 (Yamamoto et al., 2001). The presence of a mutation has been generally reported to be independently associated with a poor prognosis (Yamamoto et al., 2001).

The mutations result in constitutive activation of the tyrosine kinase activity of FLT3, autophosphorylation of the receptor, and tyrosine phosphorylation of a number of signaling molecules such as STAT5 and SHP2 (Hayakawa et al., 2000; Fenski et al., 2000). Expression of a mutant receptor in an IL-3-dependent hematopoietic cell line, such as Ba/F3, induces IL-3-independent proliferation. In contrast, cells expressing the wild-type receptor do not proliferate in the absence of growth factors without the addition of FLT3 ligand (Dehmel et al., 1996). Transplantation of murine marrow infected with a retrovirus expressing FLT3 juxtamembrane domain mutants into irradiated mice results in a lethal myeloproliferative syndrome, without evidence of any significant block in differentiation of granulocyte lineage cells (Kelly et al., 2002). Thus, as is the case with a number of other tyrosine kinase oncogenes, the major biological effect of mutant FLT3 receptors may be primarily to induce proliferation and enhance viability of immature myeloid cells. These mutant receptors would therefore be likely to cooperate with other AML oncogenes such as AML1/ETO or PML/RAR α that function primarily to block differentiation, resulting in the production of an aggressive leukemia.

The high incidence of activating mutations of FLT3 has suggested that inhibitors of mutant FLT3 might have therapeutic activity in AML. Two antagonists of the FLT3 receptor (AG1296 and AG1295) have been identified that inhibit FLT3-mediated transformation and induce apoptosis in cells expressing constitutively activated FLT3 or primary leukemic blasts from AML patients harboring FLT3/ITD mutations, respectively (Levis et al., 2001a; Tse et al., 2001). These compounds were effective at micromolar concentrations (Levis et al., 2001a, 2001b). Herbimycin A was also found to be effective at killing cells expressing mutant FLT3 (Zhao et al., 2000). The cytotoxicity of herbimycin A was found to be due to the targeting of Hsp90 (Minami et al., 2001). Both Herbimycin A and the Hsp90 inhibitor Radicicol caused mutant FLT3-transformed 32D cells to selectively undergo apoptosis (Minami et al., 2001). The SUGEN compound SU11248, which inhibits KDR and PDGF-R, was demonstrated to inhibit wild-type and ITD-FLT3 receptors (IC_{50} = 10 nM)

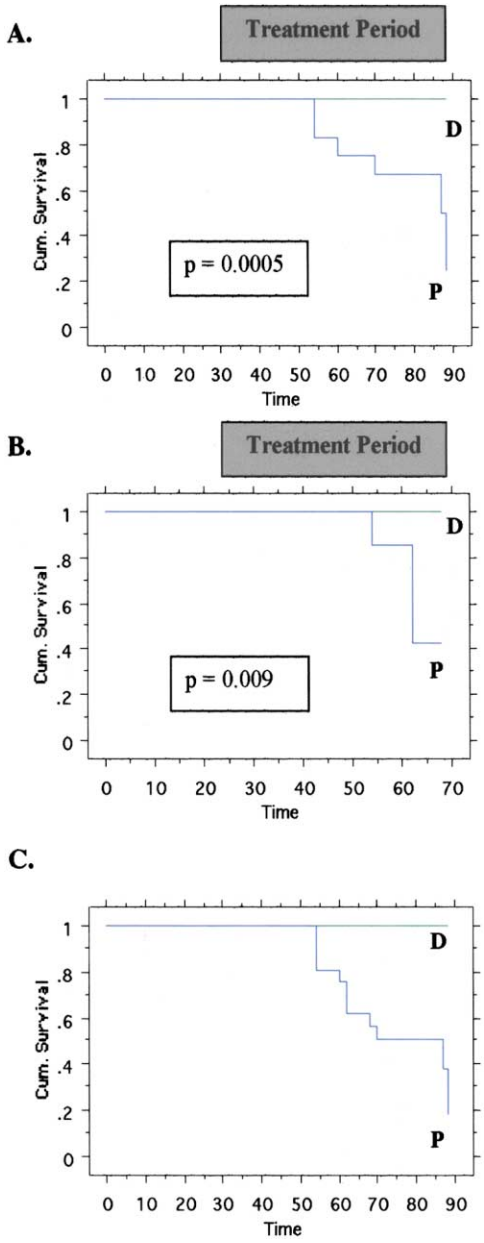

Figure 7. In vivo inhibition of FLT3-ITD-mediated transformation by PKC412

Kaplan-Meier plots of survival.

A and B: Mice transplanted with bone marrow transduced with FLT3-ITD treated with placebo (n = 22) or with PKC412 at 100 mg/kg/day (n = 22). The percentage of surviving mice (*y*-axis) is plotted with respect to time in days (*x*-axis). The p values were obtained using the log rank test. The period of drug administration is indicated by the shaded boxes, day 30–88 in Trial 1 (**A**) and day 25–68 in Trial 2 (**B**).

C: Combined data from trials 1 and 2.

(O'Farrell et al., 2001). The KDR receptor inhibitors SU5416 and SU5614 inhibited growth of ITD-FLT3-expressing cells less potently (IC_{50} = 250 nM and 100 nM, respectively) (Yee et al., 2001). Two novel FLT3 inhibitors, CEP-701 and CEP-5214, have been identified, which inhibit FLT3 tyrosine kinase activity in vitro (IC_{50} = 1–2 nM) and in vivo, and are antileukemic in an ITD-FLT3 mouse leukemia model (Levis et al., 2001b; Allebach

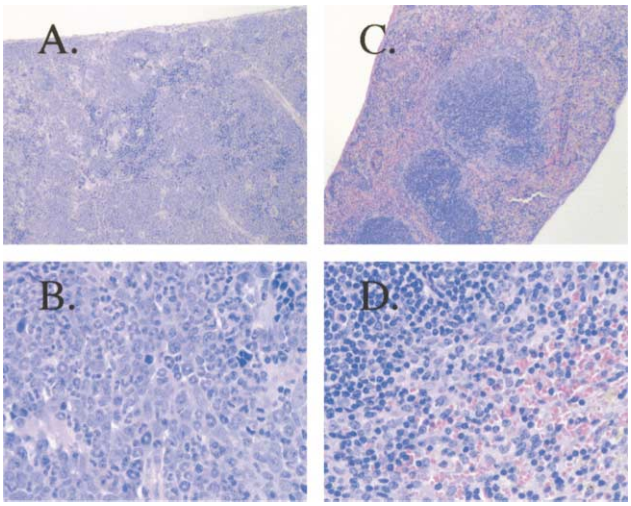

Figure 8. Histopathological analysis of animals treated with PKC412 or placebo at study endpoint

A (10×) and **B** (50×): In placebo-treated animals, splenic architecture was effaced by a marked expansion of red pulp comprised of maturing myeloid cells and scattered admixed megakaryocytes. The spleen displayed characteristic features of a MPD with marked hypercellularity and myeloid hyperplasia consisting predominantly of mature granulocytic elements.
C (low power) and **D** (high power): In contrast, the drug-treated animals showed a partial recovery of splenic architecture, a reduction in myeloid hyperplasia, and a corresponding increase in the proportion of other hematopoietic lineages.

et al., 2001). These preliminary studies suggested that inhibitors of FLT3 could have a substantial cytotoxic effect on leukemic cells expressing mutant receptors. It seems likely that several of these inhibitors will be tested in clinical trials in AML, and it will be of interest to compare their efficacy and toxicities. It is quite possible that the non-FLT3 targets of each drug will influence both their efficacy and toxicity; these targets are likely to differ significantly.

In this study, we report the identification of PKC412 (N-benzoyl staurosporine) as a highly active inhibitor of both mutant and wild-type FLT3 tyrosine kinases, and show that the drug is cytotoxic to both cell lines and primary cells expressing mutant FLT3 receptors. PKC412 was selected in a screen to identify compounds that were cytotoxic to Ba/F3 cells expressing FLT3-ITD, and was found to inhibit proliferation of both Ba/F3-ITD and Ba/F3-D835Y cells with an IC_{50} of less than 10 nM, while the IC_{50} against Ba/F3 cells expressing p210BCR/ABL or Ba/F3 cells growing in IL-3 was >500 nM. PKC412 induced cell cycle arrest of Ba/F3-ITD cells in less than 24 hr, and apoptosis within 24–48 hr. The effects of PKC412 on both proliferation and viability are consistent with the hypothesis that the major biological effects of this activated tyrosine kinase receptor oncogene are to induce, or amplify, ligand-independent proliferation and to prolong viability of myeloid progenitor cells.

In keeping with the cellular findings, we have also shown that PKC412 is a potent inhibitor of FLT3 tyrosine kinase activity with an IC_{50} value of 530 nM. PKC412 is a synthetic derivative of the naturally occurring alkaloid staurosporine, and was previously shown to be an inhibitor of protein kinase C (the major transducer of lipid second messengers and phorbol esters), KDR, a tyrosine kinase associated with a vascular endothelial growth factor (VEGF) receptor, the platelet derived growth factor receptor β (PDGFRβ), and KIT, the receptor for Steel Factor (35). Thus, the kinases inhibited by PKC412 differ substantially from those targeted by STI571 (Gleevec, Novartis Pharma), which inhibits ABL, PDGFRβ, and KIT, but does not significantly inhibit FLT3, PKC, or KDR at concentrations below 10 μM. In other preclinical studies, PKC412 inhibited VEGF-dependent angiogenesis in a growth factor implant model (Fabbro et al., 2000). Orally administered PKC412 also strongly inhibited retinal neovascularization and laser-induced choroidal neovascularization in murine models (Fabbro et al., 2000). It was also synergistic with ionizing irradiation in animal tumor models, again thought to be because of its antiangiogenic activities (Fabbro et al., 2000). In a Phase I trial of patients with solid tumors, significant nausea, vomiting, and diarrhea developed at the highest dose tested, 300 mg/kg/day (Propper et al., 2001). Only two of 32 patients developed myelosuppression. The estimated median elimination half-life was 1.6 days (range, 0.9 to 4.0 days). Steady-state PKC412 plasma levels at the top three dose cohorts (150 to 300 mg) were 0.2 to 0.7 μmol/L, 20 to 70 times the IC_{50} for Ba/F3-FLT3-ITD cells in culture. Thus, PKC412 can be safely administered to patients at doses that achieve plasma levels sufficient to inhibit mutant FLT3 in AML cells.

Since PKC412 has a number of potential targets, as is the case for most other tyrosine kinase inhibitors, several approaches were taken to determine if FLT3 could be confirmed as the actual target of this inhibitor. First, it was shown that PKC412 inhibited proliferation of Ba/F3 cells transformed by mutant FLT3, but not Ba/F3 cells transformed by p210BCR/ABL (except at concentrations 50×–100× higher than those required to inhibit FLT3). Second, the toxicity of PKC412 for Ba/F3-ITD cells was reversed completely by the addition of IL-3, indicating that kinases such as Jak-2 that are known to be required for IL-3 receptor signaling are not targets of PKC412. Third, PKC412 inhibited tyrosine phosphorylation of FLT3 itself and also inhibited in vitro kinase activity of a purified FLT3 kinase domain peptide. Fourth, and perhaps most significant, prolonged exposure of cells generated in tissue culture to sublethal concentrations of PKC412 resulted in a dramatic overexpression of FLT3. This overexpression of FLT3 resulted in the cells becoming less sensitive to PKC412. Overexpression of a drug target is a well-known mechanism of drug desensitization, and we have previously shown, for example, that similar treatment of Ba/F3p210BCR/ABL cells with STI571 resulted in a similar overexpression of BCR/ABL (Weisberg and Griffin, 2000). For PKC412-resistant cells, a number of mechanisms for the increased protein level must be considered and evaluated, such as gene amplification, increased transcription, and altered translation or protein stability. Preliminary studies suggest that both the FLT3 RNA and DNA are increased several fold in PKC412-resistant cells (data not shown). Taken together, these results suggest that FLT3 is directly inhibited by PKC412, and that this is the most critical target of the drug in the cell line Ba/F3-FLT3-ITD.

To determine if the significant inhibitory effects of PKC412 against FLT3 observed in tissue culture could be translated to an animal model of leukemia, mice transplanted with marrow cells transduced with a FLT3-ITD retrovirus were treated daily with PKC412 or vehicle control in two independent trials, starting 32 or 25 days after the transplant, respectively. This model is well suited to analysis of efficacy of FLT3 inhibitors in vivo because it utilizes primary bone marrow cells, and disease is

mediated solely by the FLT3-ITD (Kelly et al., 2002). In previous studies, mice receiving these transplants were found to develop a myeloproliferative syndrome that was lethal in 30 to 90 days in 100% of recipients (Kelly et al., 2002). Administration of PKC412 to the mice was not started until 25–32 days after the transplant, in order to allow time for the mice to develop an aggressive, potentially lethal, leukemia before starting treatment, thereby providing a better model for therapy of human disease. Remarkably, at the time the study was stopped, all 22 mice in the control arms had developed fatal MPD, while none of 22 mice treated with PKC412 had either developed visible signs of leukemia or expired. Further, spleen weights and blood leukocyte counts were significantly lower in mice treated with PKC412 at the end of the studies, compared to mice receiving control. Serum samples taken at the termination of the first study from six representative animals had levels of PKC412 (or the predominant metabolite CGP052421) of 545 $\pm$ 237 ng/ml (range 333–879 ng/ml; 545 ng/ml = 954 nM), about 100-fold higher than the IC_{50} for Ba/F3-FLT3-ITD cells in tissue culture. As the mice tolerated these high serum levels of PKC412 for 60 days without overt signs of toxicity, these results suggest there is a substantial therapeutic window in vivo.

Overall, the results presented here strongly support the notion that FLT3 is potentially a good drug target in AML. FLT3 is frequently mutated, and both types of known mutations are inhibited by PKC412 at nanomolar concentrations in culture. Further, PKC412 causes extensive and rapid apoptosis of FLT3-expressing cells, a desirable feature for a potential antileukemic drug. Finally, the significant effects in an animal model of leukemia suggest that the in vitro activity can also be observed in vivo. It will be of considerable interest to determine whether FLT3 can be successfully inhibited in leukemic cells upon treatment of AML patients in vivo, and if this has a beneficial therapeutic effect.

Experimental procedures

Cell lines and cell culture

The IL-3-dependent murine hematopoietic cell line Ba/F3 was transduced with either wild-type FLT3-, FLT3-ITD-, or FLT3-D835Y-containing MSCV retroviruses harboring a neomycin selectable marker, and selected for resistance to neomycin (Kelly et al., 2002). FLT3-ITD transduced cells were selected for growth in G418 (1 mg/ml). Expression of FLT3 or mutant FLT3 was documented by flow cytometry and Western blot with anti-FLT3 antibody. Polyclonal Ba/F3 cell lines expressing wild-type, FLT3-ITD, or FLT3-D835Y are termed Ba/F3-FLT3-WT, -FLT3-ITD, and -FLT3-D835Y, respectively. Ba/F3-FLT3-ITD and Ba/F3-FLT3-D835Y could proliferate independently of IL-3, while parental cells and Ba/F3-FLT3-WT remained factor dependent. All cell lines were maintained in the presence of 10% WEHI-conditioned medium as a source of IL-3 to reduce the likelihood of any line acquiring additional mutations. IL-3 was removed from media prior to experimentation. Ba/F3 cells expressing p210BCR/ABL have been previously described (Daley and Baltimore, 1988; Sattler et al., 1996; Okuda et al., 1996). All cell lines were cultured at 37°C with 5% CO_2 at a concentration of 2×10^5 to 5×10^5 in RPMI (Mediatech, Inc, Herndon, VA) with 10% fetal calf serum and supplemented with 1% glutamine.

Chemical compounds and biologic reagents

PKC412 and STI571 (Gleevec, Glivec) were obtained from Novartis Pharma AG, Basel, Switzerland, and dissolved in DMSO to make an initial stock solution. Serial dilutions were then made, also in DMSO, to obtain final dilutions for cellular assays. FLT3 ligand (PeproTech, Inc., Rocky Hill, NJ) was added to some cell cultures at a concentration of 100 ng/ml for up to 10 min.

Cell viability, cell cycle, and apoptosis measurements

Following staining with trypan blue (Sigma, St. Louis, MO), the number of viable cells was determined by counting unstained cells with a hemacytometer. Cell viability is reported as percentage of control (untreated) cells, and data are presented as the average of 2–3 independent experiments, as indicated in the figure legends. Error bars represent the standard error of the mean for each data point. Apoptosis of drug-treated cells was measured using the Annexin-V-Fluos Staining Kit (Boehringer Mannheim, Indianapolis, IN). Briefly, 500,000–1 $\times$ 10^6 cells cultured in the presence or absence of drug were washed 1$\times$ with phosphate-buffered saline (PBS) and centrifuged for 5 min at 1500 rpm. Washed cell pellets were resuspended in 100 μl of 20% Annexin-V-fluorescein labeling reagent and 20% propidium iodide (PI) in HEPES buffer. Cells were incubated for 15 min at room temperature, followed by dilution with 0.8 ml of HEPES buffer. Samples were then analyzed by flow cytometry. As controls, cells were incubated for 15 min with PI alone, Annexin-V-fluorescein labeling reagent alone, or HEPES buffer, and then diluted with HEPES buffer and analyzed by flow cytometry.

Cell cycle analysis was performed using approximately 500,000 cells, which were centrifuged at 1500 rpm for 5 min, washed in PBS, and the pellet resuspended in 500 μl of propidium iodide solution (50 μg/ml propidium iodide, 0.1% NP-40, 0.1% sodium citrate). The mixture was stored in the dark at 4°C for a minimum of 15 min, and then analyzed by flow cytometry.

Antibodies

Anti-pTyr, monoclonal antibody (mAb) #4G10, was a gift from Dr. Brian Druker, University of Oregon Health Sciences Center (Portland, OR) and was diluted 1:2500 for use in immunoblotting. FLT3/Flk-2 (C-20) was purchased from Santa Cruz Biotechnology, Inc., and used at 1:200 for immunoblotting. Monoclonal anti-β-actin (Clone AC-15) was purchased from Sigma and diluted 1:1000 for immunoblot.

Immunoprecipitation and immunoblotting

Cells were lysed in lysis buffer (0.02 M Tris [pH 8.0], 0.15 M NaCl, 10% glycerol, 1% NP-40 (wt/vol), 0.1 M NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 40 μg/ml leupeptin, and 20 μg/ml aprotinin). Protein lysates were incubated for 25 min on ice, with vortexing at 5 min intervals, and then centrifuged for 15 min at 12,000 $\times$ g. Supernatants were saved, and the Bio-Rad Protein Assay was used to determine protein yields (Bio-Rad Laboratories, Hercules, CA). Equivalent amounts of protein were subsequently loaded directly onto a gel for immunoblotting experiments. For immunoprecipitation, cell lysates were incubated with FLT3/Flk-2 (C-20) antibody and protein A Sepharose overnight with rocking at 4°C. As a control, cell lysates were also incubated with protein A Sepharose beads alone. Following incubation, immune complexes were washed 2$\times$ with lysis buffer, 2$\times$ with 1$\times$ PBS, and were dissolved in Laemmeli's sample buffer by boiling for 5 min. For immunoblotting and immunoprecipitation, whole cell lysates and immune complexes, respectively, were resolved on a sodium dodecyl sulfate (SDS)-7.5% polyacrylamide gel. Following this, protein was electrophoretically transferred to a Protran nitrocellulose transfer and immobilization membrane (Schleicher and Schuell, Dassel, Germany). The membrane was then blocked overnight at 4°C with 5% nonfat dry milk in 1$\times$ TBS (10 mM Tris-HCl [pH 8.0], 150 mM NaCl) and then probed for 2 hr at 25°C with pTYR antibody or overnight at 4°C with FLT3/Flk-2 (C-20) antibody in 1$\times$ TBST buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween20). Following 3 washes with 1$\times$ TBST, membranes were incubated for 1 hr at 25°C with anti-mouse immunoglobulin (horseradish peroxidase-linked whole antibody from sheep) or anti-rabbit immunoglobulin (horseradish peroxidase linked whole antibody from donkey) (Amersham Life Science, Inc., Arlington Heights, IL). The membrane was washed 5$\times$ in 1$\times$ TBST buffer, with 5 min intervals between buffer changes, and bound antibodies were detected with enhanced luminol and oxidizing reagent as specified by the manufacturer (NEN Life Science Products, Boston, MA). Bound antibodies were removed with stripping buffer (2% SDS, 0.0625 mol/L Tris [pH 6.8], and 0.7% 2-mercaptoethanol) 50°C for 30 min. The filter was then probed with additional antibodies.

Development of PKC412 resistant Ba/F3-FLT3-ITD cell lines

Ba/F3-FLT3-ITD cells were cultured in the presence of increasing concentrations of PKC412 (0.01 μM to 0.04 μM) over a period of approximately two months. Cells were initially cultured in the presence of 0.01 μM PKC412,

with culture medium changes every 6–7 days, for a total of 28 days. Cells were then cultured in 0.02 μM PKC412 for an additional 25 days, and finally cells were transferred to media containing 0.04 μM PKC412. Surviving cells continued to be passaged thereafter in the presence of 0.04 μM PKC412, and were analyzed for resistance to the drug.

In vitro kinase assay

The coding sequence of the cytoplasmic kinase domain of human FLT3 (aa 563–993) was amplified by PCR from human cDNA libraries (Clontech) and cloned into pFbacG01 vector made compatible for ligation by digestion with BamH1 and HindIII resulting in a cDNA carrying N-terminal GST fused in frame to the cytoplasmic kinase domain of FLT3 (pFbacG01-GST-FLT3). To generate viral DNA, the pFbacG01-GST-FLT3 was transfected into DHC10Bac (GIBCO). DNA from positive colonies was transfected into either Sf9 or Sf21 cells (American Type Culture Collection) using Cellfectin (GIBCO) reagent (Homann et al., 2001).

Recombinant baculovirus was collected from the transfected cell culture and used for infection. After two rounds of infection, the media was used for large-scale protein expression as described (Homann et al., 2001). Sf9 cell pellets from 5-liter cultures were resuspended in 50 ml of ice-cold lysis buffer (25 mM Tris-HCl [pH 7.5], 2 mM EDTA, 1% NP-40, 1 mM DTT, 1 mM PMSF), stirred on ice for 15 min, and then centrifuged at 5000 rpm for 20 min. The centrifuged cell lysate was then loaded onto a 2 ml glutathione-sepharose column (Pharmacia) and washed three times with 10 ml of 25 mM Tris-HCl [pH 7.5], 2 mM EDTA, 1 mM DTT, and 200 mM NaCl. The GST-tagged protein was eluted by 10 applications (1 ml each) of 25 mM Tris-HCl [pH 7.5], 10 mM reduced-glutathione, 100 mM NaCl, 1 mM DTT, and 10% Glycerol, and stored at −70°C. Protein tyrosine kinase assays using the purified GST-FLT3 were carried out in a final volume of 30 μl containing 200–1800 ng of enzyme protein (depending upon the specific activity), 20 mM Tris-HCl [pH 7.6], 3 mM $MnCl_2$, 3 mM $MgCl_2$, 1 mM DTT, 10 μM Na_3VO_4, 3 μg/ml poly (Glu, Tyr) 4:1, 1% DMSO, 8.0 μM ATP, and 0.1 μCi [γ^{33}P] ATP. The activity was assayed in the presence or absence of PKC412 by measuring the incorporation of ^{33}P from [γ^{33}P] ATP into the poly (Glu, Tyr) substrate as described (Homann et al., 2001). Assay (30 μl) were carried out in 96-well plates at RT for 20 min, and terminated by the addition of 20 μl of 125 mM EDTA. 40 μl of the reaction mixture was transferred onto Immobilon-PVDF membrane (Millipore, Bedford, MA) previously soaked for 5 min with MeOH, rinsed with water, soaked for 5 min with 0.5% H_3PO_4, and mounted on a vacuum manifold with a disconnected vacuum source (Homann et al., 2001). Membranes were removed and washed on a shaker 4× with 1.0% H_3PO_4, then once with EtOH. Following addition of 10 μl/well of Microscint TM (Packard), samples were counted in a Packard TopCount. The IC_{50} value was calculated by linear regression analysis of the percentage inhibition curve. One unit of protein kinase activity is defined as 1 nmole of ^{33}P ATP transferred from [γ^{33}P] ATP to the substrate protein per minute per mg of protein at 37°C.

Mouse studies

Mouse strains and bone marrow transplantation

BALB/c mice were purchased from Taconic (Germantown, NY). Bone marrow transplantation assays were carried out as described previously (Schwaller et al., 1998; Liu et al., 2000; Kelly et al., 2002). Briefly, 4- to 6-week-old male donor mice were primed with intraperitoneal injection of 5′ fluorouracil (150 mg/kg, Sigma) and subsequently sacrificed after 6 days by CO_2 asphyxiation. Bone marrow was flushed from femurs and tibias, and red blood cells were lysed (Red Blood Cell Lysis, RBCL buffer, Sigma). Cells were cultured overnight with IL-3 (6 ng/ml, R&D systems), IL-6 (10 ng/ml, R&D systems), and Stem Cell Factor (10 ng/ml, PeproTech) in RPMI with 10% FCS (transplant medium). Cells were transduced by 2 rounds of spin-infection, at 24 hr and 48 hr postharvesting. Centrifugation of 1 ml of viral supernatant and 4×10^6 cells in 3 ml of transplant media containing 5 μg/ml polybrene and 7.5 mM HEPES buffer was carried out for 90 min at 1800 g. Cells were washed in PBS, resuspended in Hanks balanced salt solution (Life Technologies), and injected (1–3 × 10^6 cells/0.5 ml) into the lateral tail vein of lethally irradiated (2 × 450 cGy) female recipient mice. Mice were housed in microisolator cages with autoclaved chow and acidified water.

Drug preparation and administration

6% w/w PKC412 in Gelucire® 44/14 (Gattefosse, France) was stored at 4°C as a waxy-solid formulation. Prior to administration, the Gelucire/PKC412 waxy solid mixture was warmed in a 44°C water bath until liquid. The liquid mixture was then diluted with sterilized deionized water to produce a final PKC412 concentration of 12.0 mg/ml. The animals were weighed prior to treatment, and every 7 days thereafter, to ensure that a consistent dose (100 mg/kg/day) of drug was administered. Dosing was performed every 24 hr by gavage of a maximum volume of 150 μl per animal using 22 gauge gavage needles (Hornbecks). Placebo animals received the same volume of a Gelucire® 44/14 and water solution prepared in the same way as described above for the PKC412/Gelucire mixture.

Statistical analysis and study design

In each trial, mice were dosed with either drug or placebo. In comparing the survival time of the mice, all times were measured from the day of BMT, and the log rank test was used to attach a significance level to the difference in the survival curves. Mice who remained alive at the end of the study or who were sacrificed in an apparently healthy condition at the end of the study were considered censored in this analysis.

Gross morphological analysis

Mice were examined for splenomegaly every 3–4 days by palpation of the abdomen. Animals with spleens that crossed the dorsal midline were sacrificed and analyzed. Peripheral blood was collected from the retro-orbital cavity using a heparinized glass capillary. A blood smear was prepared and stained with Wright and Giemsa (Sigma). Manual white blood cell counts were performed and a sample of blood was analyzed using the ADIVA 120 Hematology system (Bayer) for total and differential blood counts. Anesthesia was achieved using Methoxyfluroane (Australia) and then animals were sacrificed by cervical dislocation. The spleen, liver, heart, lungs, intestine, hind limb bones, and kidneys were examined and collected. The spleen weight and any other unusual observations were recorded. Samples of all organs were stored in 10% Formalin/PBS solution (Sigma). A single cell suspension from the spleen and bone marrow was prepared and stored in 10% dimethylsulphoxide/90% Fetal Calf Serum.

Histopathology

Murine tissues were fixed for at least 72 hr in 10% neutral buffered formalin (Sigma), dehydrated in alcohol, cleared in xylene, and infiltrated with paraffin on an automated processor (Leica). 4 micron-thick tissue sections from paraffin embedded tissue blocks were placed on charged slides, and deparaffinized in xylene, rehydrated through graded alcohol solutions, and stained with hematoxylin and eosin.

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