PKC412 overcomes resistance to imatinib in a murine model of FIP1L1-PDGFRα-induced myeloproliferative disease

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Summary

FIP1L1-PDGFRα causes hypereosinophilic syndrome (HES) and is inhibited by the tyrosine kinase inhibitor imatinib (Gleevec). Imatinib is a potent inhibitor of ABL, ARG, PDGFRα, PDGFRβ, and KIT and induces durable hematologic responses in HES patients. However, we observed relapse with resistance to imatinib as consequence of a T674I mutation in FIP1L1-PDGFRα, analogous to the imatinib-resistant T315I mutation in BCR-ABL. We developed a murine bone marrow transplant model of FIP1L1-PDGFRα-induced myeloproliferative disease to evaluate the efficacy of PKC412, an alternative inhibitor of PDGFRα, for the treatment of HES. PKC412 is effective for treatment of FIP1L1-PDGFRα-induced disease and of imatinib-induced resistance due to the T674I mutation. Our data establish PKC412 as molecularly targeted therapy for HES and other diseases expressing activated PDGFRα and demonstrate the potential of alternative kinase inhibitors to overcome resistance in target tyrosine kinases.

Introduction

Small molecule inhibitors of tyrosine kinases have emerged as effective therapies for hematologic malignancies as well as solid tumors (Cohen, 2002; Fabbro et al., 2002). Perhaps the best-characterized example is the application of imatinib, a selective inhibitor of the ABL, ARG, PDGFRα, PDGFRβ, and KIT tyrosine kinases, in the treatment of BCR-ABL-positive CML and CML in blast crisis (Capdeville et al., 2002). Imatinib is safe and efficacious in this context (Druker et al., 2001a, 2001b), and it has been suggested that imatinib should now be considered as the first line therapy of choice for chronic phase CML (Druker, 2003).

An unexpected dividend of these pioneering efforts to demonstrate the value of molecular-targeted therapy of CML has been the observation that imatinib has efficacy in other challenging clinical cancer contexts. For example, the observation that the majority of gastrointestinal stromal cell tumors (GIST) harbor activating mutations in KIT led to clinical trials that demonstrated remarkable activity of imatinib in this context (Demetri et al., 2002). Similarly, chronic myelomonocytic leukemias (CMML) associated with the constitutively activated TEL-PDGFRβ fusion are also highly responsive to treatment with imatinib (Apperley et al., 2002).

SIGNIFICANCE

Imatinib is an effective, molecular-targeted therapy for BCR-ABL-positive CML. However, the development of clinical resistance to imatinib due to point mutations in the ABL kinase domain has emerged as an increasingly important problem. As a class of new therapeutic agents, a considerable variety of small molecule inhibitors of tyrosine kinases are currently under development as therapeutics for both hematological malignancies and solid tumors. Here we provide proof of principle that resistance to imatinib as consequence of point mutation in PDGFRα can be overcome by the use of an alternative PDGFRα inhibitor, PKC412. Our results indicate that the use of structurally diverse small molecule inhibitors that target the same tyrosine kinase, but employ different binding modes, can be an effective approach in circumventing or preventing development of resistance.

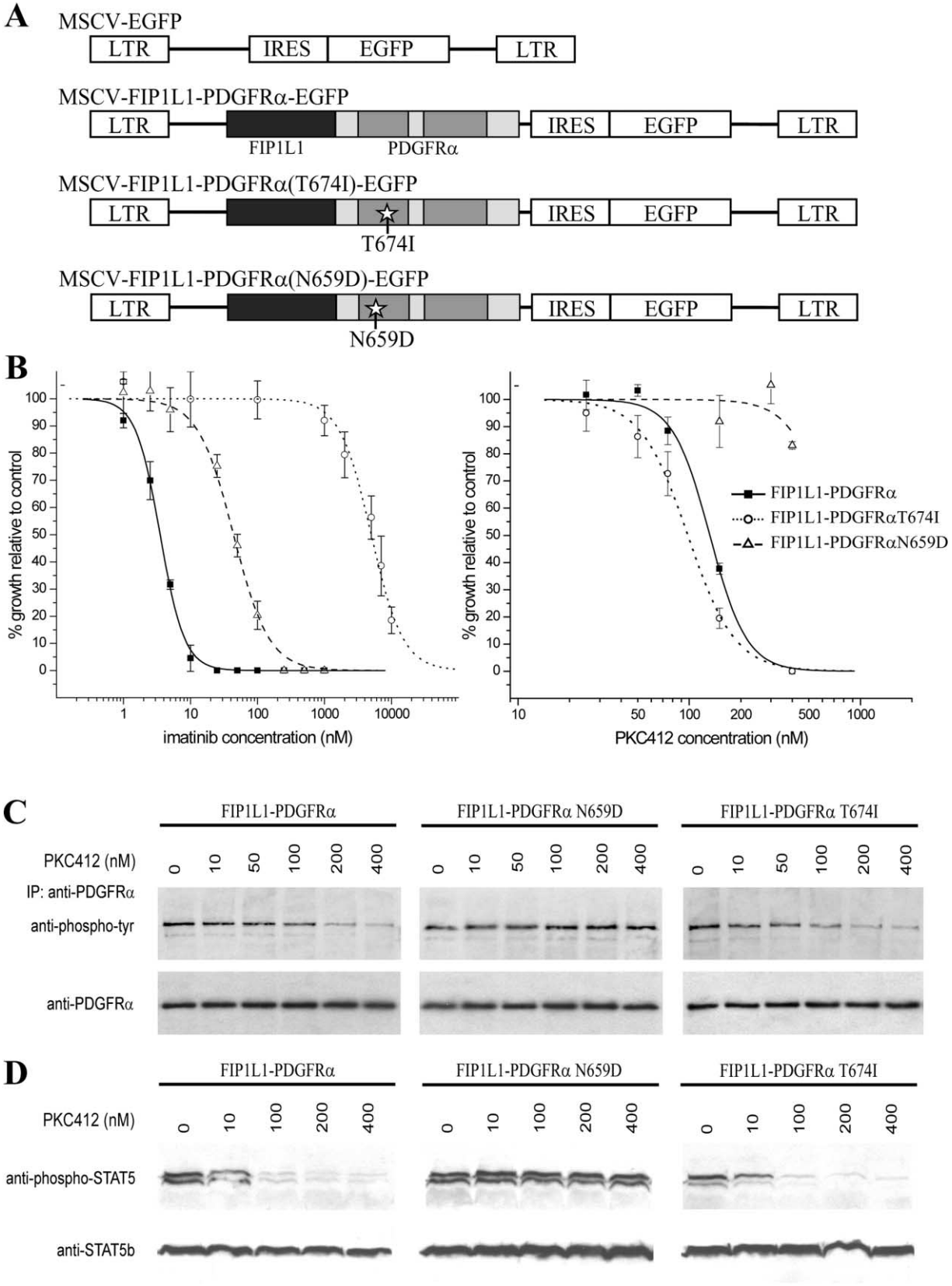

Figure 1. PKC412 directly inhibits FIP1L1-PDGFRα in vitro

A: Constructs used in this study. The stars mark the positions of the T674I and N659D mutations.
LTR, long terminal repeat. IRES, internal ribosomal entry site. EGFP, enhanced green fluorescent protein.
B: Dose response curves of Ba/F3 cells stably expressing wild-type, T674I, or N659D FIP1L1-PDGFRα. The percentage of growth, relative to the growth of cells

Imatinib therapy of CML, GIST, and CMML are examples of rational clinical trial design based on the presence of mutations in tyrosine kinases that are known targets of imatinib. However, imatinib has also been used to treat a spectrum of diseases for which the molecular basis of disease is not known. One example is the use of imatinib to treat hypereosinophilic syndrome (HES), a myeloproliferative disease that most prominently affects the eosinophil lineage (Gleich et al., 2002; Ault et al., 2002; Pardanani et al., 2002; Cortes et al., 2003). Based on the clinical observation that a majority of HES patients have dramatic clinical response to imatinib, we investigated the possibility that one or more known targets of imatinib was the cause of HES. We discovered a novel FIP1L1-PDGFRα fusion protein, expressed as consequence of interstitial chromosomal deletion on 4q12, in the majority of patients with HES (Cools et al., 2003). FIP1L1-PDGFRα-positive HES patients achieve rapid and durable clinical responses to imatinib, and FIP1L1-PDGFRα is potently inhibited by imatinib in vitro. Furthermore, definitive evidence for FIP1L1-PDGFRα as the therapeutic target of imatinib in HES came from the identification of a T674I imatinib resistance mutation in the kinase domain of FIP1L1-PDGFRα in a patient who relapsed while on therapy (Cools et al., 2003). The catalytic sites of tyrosine kinases are highly conserved, and the T674I mutation in PDGFRα corresponds to the T315I mutation in ABL, a common resistance mutation in BCR-ABL-positive CML patients that relapse while on imatinib therapy (Gorre et al., 2001; Shah et al., 2002). In summary, our previous results demonstrate that FIP1L1-PDGFRα is specifically inhibited by imatinib, and that the T674I mutation in the PDGFRα kinase domain confers resistance to imatinib as does the T315I mutation in the context of BCR-ABL.

PKC412 is a selective inhibitor of FLT3, PKC, KDR, KIT, PDGFRα, and PDGFRβ (Andrejauskas-Buchdunger and Regenass, 1992; Fabbro et al., 1999, 2000; Weisberg et al., 2002). The safety and pharmacokinetic profiles of PKC412 have been determined in Phase I clinical trials (Propper et al., 2001), and this inhibitor is currently being evaluated in a Phase II clinical trial as molecular-targeted therapy for AML with activating mutations in FLT3 (Stone et al., 2003). We investigated the potential use of PKC412 as an alternative option for the treatment of FIP1L1-PDGFRα-positive HES and as a strategy to overcome the clinical resistance to imatinib. PKC412 is a potent inhibitor of the kinase activity of the FIP1L1-PDGFRα fusion protein and inhibits FIP1L1-PDGFRα-mediated transformation of hematopoietic cells in vitro and in vivo. Of particular note, PKC412 retained full inhibitory activity against the imatinib-resistant T674I mutant form of FIP1L1-PDGFRα and may be of value to overcome or prevent resistance to imatinib.

Results

PKC412 specifically inhibits wild-type FIP1L1-PDGFRα

PKC412, a derivative of staurosporine (N-benzoyl-staurosporine), is a potent inhibitor of PKC, FLT3, KIT, KDR, PDGFRα, and PDGFRβ (Andrejauskas-Buchdunger and Regenass, 1992; Fabbro et al., 1999, 2000; Weisberg et al., 2002). To determine whether PKC412 inhibited FIP1L1-PDGFRα, we investigated the dose-response relationship for Ba/F3 cells stably expressing the fusion protein. Ba/F3 is a murine hematopoietic cell line that requires IL3 for growth, but activated kinases including BCR-ABL and FIP1L1-PDGFRα transform Ba/F3 cells to growth factor independence (Daley and Baltimore, 1988; Cools et al., 2003). We have previously reported that imatinib inhibits the growth of FIP1L1-PDGFRα-expressing Ba/F3 cells with a cellular IC_{50} of 3 nM (Cools et al., 2003). PKC412 also inhibited the growth of these cells with a cellular IC_{50} of approximately 130 nM (Figure 1). Addition of IL3 to the growth media restored cell growth in the presence of PKC412, indicating that PKC412 inhibition was not due to nonspecific toxicity (data not shown). We next introduced a point mutation into the ATP binding pocket of PDGFRα at asparagine residue 659 (FIP1L1-PDGFRα(N659D)) that was predicted to result in resistance to PKC412 (J.C. and D.G.G., unpublished data). Ba/F3 cells transformed with FIP1L1-PDGFRα(N659D) were not inhibited at concentrations of PKC412 as high as 400 nM, and a cellular IC_{50} could not be achieved (Figure 1). These data indicate that the N659D mutation in the context of FIP1L1-PDGFRα confers resistance to PKC412 and that the growth inhibitory effects of PKC412 for cells transformed by the wild-type FIP1L1-PDGFRα were specifically due to inhibition of the fusion protein. These observations correlated with the IC_{50} for inhibition of FIP1L1-PDGFRα tyrosine kinase activity as assessed by reduction in tyrosine phosphorylation content of the fusion protein as well as its downstream target, Stat5. The IC_{50} of PKC412 for FIP1L1-PDGFRα or Stat5 was approximately 100 nM, and $>$400 nM for the N659D mutant (Figure 1).

PKC412 inhibits the imatinib-resistant FIP1L1-PDGFRα(T674I) mutant

We next investigated whether PKC412 was able to inhibit FIP1L1-PDGFRα containing the T674I mutation conferring resistance to imatinib (Cools et al., 2003), and conversely, whether imatinib could inhibit FIP1L1-PDGFRα containing the N659D mutation conferring resistance to PKC412. We observed that PKC412 inhibited the FIP1L1-PDGFRα(T674I) imatinib-resistant mutant with a cellular IC_{50} of approximately 100 nM, whereas imatinib inhibited the FIP1L1-PDGFRα(N659D) PKC412-resistant mutant with a cellular IC_{50} of approximately 50 nM (Figure 1). These data correlated with IC_{50} for inhibition of tyrosine kinase activity by PKC412, as assessed by Western blot analysis of tyrosine phosphorylation of FIP1L1-PDGFRα or Stat5 (Figure 1). Interestingly, these data indicate that PKC412 is a more potent inhibitor of the FIP1L1-PDGFRα(T674I) mutant than of wild-type FIP1L1-PDGFRα.

in the absence of drug, is plotted for increasing concentrations of imatinib or PKC412, respectively.

C: Analysis of the phosphorylation status of FIP1L1-PDGFRα (wild-type, T674I mutant, or N659D mutant). FIP1L1-PDGFRα was immunoprecipitated from Ba/F3 cells stably expressing the different constructs and incubated with increasing concentrations of PKC412. Detection was performed with anti-phosphotyrosine or anti-PDGFRα (loading control).

D: Analysis of the phosphorylation status of the downstream effector STAT5. Western blotting was performed using whole cell lysates of Ba/F3 cells stably expressing the different constructs and incubated with increasing concentrations of PKC412. Detection was done with anti-phospho-STAT5 or anti-STAT5b (loading control).

A mouse model for FIP1L1-PDGFRα-induced myeloproliferative disease

These results demonstrated that PKC412 is an effective in vitro inhibitor of the imatinib-resistant FIP1L1-PDGFRα(T674I) mutant identified in a patient with clinical resistance to imatinib. To test in vivo efficacy of PKC412 inhibition of FIP1L1-PDGFRα, we developed a mouse model for FIP1L1-PDGFRα-induced myeloproliferative disease. Briefly, bone marrow cells from donor mice treated with 5-fluorouracil were transduced with retrovirus containing the *FIP1L1-PDGFRA* fusion gene or related mutants (constructs shown in Figure 1), followed by transplantation into lethally irradiated syngeneic recipients. All animals developed a myeloproliferative disease characterized by peripheral blood leukocytosis (mean white blood cell counts (WBC) = 654×10^3/ml) and splenomegaly (mean spleen weight 832 mg) with a latency of 14–21 days and 100% penetrance. Histopathologic analysis demonstrated maturing myeloid hyperplasia in the bone marrow dominated by an increase of all forms of the granulocyte lineage (neutrophils, eosinophils, and basophils), effacement of splenic architecture and expansion of the red pulp by a similar population, and extramedullary hematopoiesis with a predominance of granulocytic forms in the liver, lung, kidney, and Peyer's patches (Figures 2A–2D, data not shown). Although numerous eosinophils were observed in many mice (5% to 20% eosinophils in the peripheral blood), all cells within the granulocytic lineage were increased, with neutrophils demonstrating the greatest increase. Overall, the phenotype closely resembled myeloproliferative disease induced by other constitutively activated tyrosine kinase fusions, including BCR-ABL (Daley et al., 1990), TEL-PDGFRβ (Tomasson et al., 2000), TEL-JAK2 (Schwaller et al., 1998), and FLT3-ITD (Kelly et al., 2002a). Flow cytometry of single cell suspensions from spleen and bone marrow cells confirmed the histologic findings of chronic myeloproliferative disease, with dramatic increase in Gr-1^+/Mac-1^+ cells and few T or B cells (Figures 2E and 2F). As described in more detail below, the FIP1L1-PDGFRα(T674I) resistance mutation induced myeloproliferative disease that was indistinguishable from the wild-type fusion protein.

Validation of the mouse model for the study of PDGFRα inhibitors

Induction of myeloproliferative disease by FIP1L1-PDGFRα in the murine BMT assay, although not exclusively characterized by prominent eosinophilia, provided a model system to test the efficacy of PKC412 and imatinib for treatment of disease induced by wild-type and mutant fusion proteins. Imatinib is an effective therapy for hypereosinophilic syndrome associated with the wild-type FIP1L1-PDGFRα fusion, and relapse on imatinib has been associated with acquisition of a T674I mutation. Therefore, we first validated the mouse model by testing the ability of imatinib to prolong survival in animals with myeloproliferative disease induced either by the wild-type FIP1L1-PDGFRα or the FIPL1-PDGFRα(T674I) mutant.

Myeloproliferative disease was induced by retroviral vectors containing either the wild-type or T674I mutant form of FIP1L1-PDGFRα, and the recipient mice were treated by gavage with imatinib (125 mg/kg/day in two divided doses) or placebo. Recipient mice that received bone marrow expressing the wild-type FIP1L1-PDGFRα did not develop disease as assessed by spleen weight and WBC when treated with imatinib, whereas placebo-treated mice rapidly developed myeloproliferative disease characterized by marked leukocytosis and splenomegaly. In contrast, mice transplanted with cells expressing the T674I mutant did not respond to imatinib and developed disease with the same penetrance and latency as the placebo group (Table 1). These data correlate with the clinical responses to imatinib in human HES associated with the FIP1L1-PDGFRα fusion and the T674I resistance mutation (Cools et al., 2003) and validate the model for evaluation of efficacy of PKC412.

PKC412 is efficacious for the treatment of both FIP1L1-PDGFRα and imatinib-resistant FIP1L1-PDGFRα(T674I)-induced myeloproliferative disease

The in vitro results demonstrated the ability of PKC412 to inhibit both FIP1L1-PDGFRα and the imatinib-resistant FIP1L1-PDGFRα(T674I) mutant. We next investigated the in vivo efficacy of PKC412 for the treatment of myeloproliferative disease induced by FIP1L1-PDGFRα or the imatinib-resistant FIP1L1-PDGFRα(T674I) mutant. Myeloproliferative disease was again induced using retroviral vectors containing either the wild-type or the T674I mutant form of FIP1L1-PDGFRα. The recipient mice were divided into three groups that received imatinib, PKC412, or placebo, respectively. In this study design, animals were treated daily until one week after all the placebo-treated mice had been sacrificed due to progressive disease. Animals in the placebo groups developed massive splenomegaly and marked leukocytosis and were all sacrificed by day 22 (T674I mutant) or day 27 (wild-type FIP1L1-PDGFRα).

In control arms, mice treated with imatinib or placebo recapitulated the observations described above, namely that FIP1L1-PDGFRα disease was sensitive to treatment with imatinib whereas the FIP1L1-PDGFRα(T674I)-induced disease was imatinib resistant. In particular, all animals transplanted with bone marrow cells expressing FIP1L1-PDGFRα that were treated with imatinib were alive at the study endpoint (day 33) (Figure 3A) and had a marked reduction in spleen weight and leukocyte count (Table 2). In contrast, mice treated with imatinib, but transplanted with bone marrow cells expressing the FIP1L1-PDGFRα(T674I) imatinib-resistant mutation, developed disease with the same penetrance and latency as the placebo-treated animals (Figure 3B) and had increased spleen weights and leukocyte counts comparable to placebo-treated animals (Table 2).

In striking contrast to the effects observed with imatinib, PKC412 was effective in treating myeloproliferative disease induced either by the FIP1L1-PDGFRα or the FIP1L1-PDGFRα(T674I) mutant (Figures 3A and 3B). Most mice transplanted with bone marrow cells expressing FIP1L1-PDGFRα that were treated with PKC412 did not develop myeloproliferative disease, as assessed by spleen weight and WBC counts (Table 2). There was a significant prolongation of their survival (Figure 3A) and a clear reduction of their spleen weights and leukocyte counts compared to the placebo group (Table 2). PKC412 was also effective for treatment of myeloproliferative disease induced by the FIP1L1-PDGFRα(T674I) imatinib resistance mutation, producing a significant prolongation of survival (Figure 3B) and reduction in spleen weight and leukocyte count compared to placebo-treated animals (Table 2). Similar results with imatinib and PKC412 treatment were obtained in a third, independent trial, confirming the efficacy of PKC412 for the treatment of myeloproliferative disease caused by FIP1L1-PDGFRα and FIP1L1-PDGFRα(T674I) (data not shown).

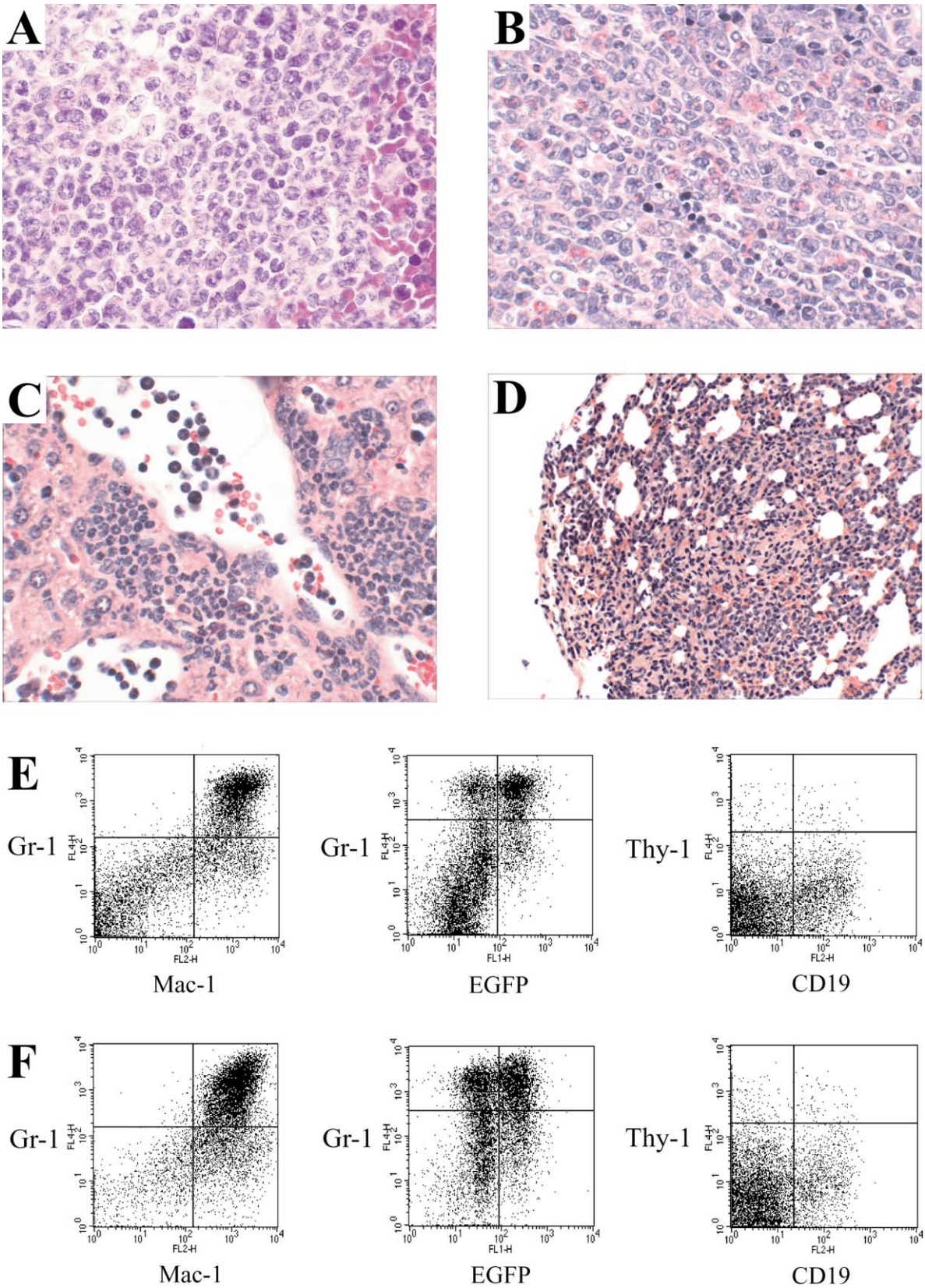

Figure 2. Mice transplanted with bone marrow cells expressing FIP1L1-PDGFRα develop a myeloproliferative disease

A–D: Histopathology of the bone marrow (**A**, 100×), spleen (**B**, 40×), liver (**C**, 40×), and lung (**D**, 20×) of a mouse that developed a FIP1L1-PDGFRα-induced myeloproliferative disease, documenting myeloid infiltration.

E and **F:** Immunophenotype of cells from spleen (**E**) and bone marrow (**F**) of the same mouse, illustrating a high percentage of mature myeloid cells in spleen and bone marrow.

Table 1. Efficacy of imatinib for the treatment of FIP1L1-PDGFRα-induced myeloproliferative disease, and resistance to FIP1L1-PDGFRα(T674I)-induced disease (trial 1)

	FIP1L1-PDGFRα wild-type		FIP1L1-PDGFRα T674I
	Placebo	Imatinib	Imatinib
Spleen weight (mg)			
Mean	832	111	801
Median	852	106	780
Range	667–922	93–140	700–1,007
n	8	8	6
WBC (× 10^6/ml)			
Mean	654.4	6.0	496.5
Median	620.2	5.2	507.7
Range	593.8–773.0	4.6–9.4	434.6–535.8
n	5	7	4

Spleen weights and white blood cell counts (WBC) of mice in the different groups of trial 1, determined at time of death or at trial endpoint. n: number of mice analyzed.

Table 2. Efficacy of PKC412 for the treatment of both FIP1L1-PDGFRα- and FIP1L1-PDGFRα(T674I)-induced myeloproliferative disease (trial 2)

	FIP1L1-PDGFRα		
	Placebo	Imatinib	PKC412
Spleen weight (mg)			
Mean	729	101	241
Median	690	99	199
Range	588–922	82–132	104–586
n	9	9	8
WBC (×10^6/ml)			
Mean	534.1	4.8	12.7
Median	554.3	4.8	12.4
Range	388.9–639.0	4.4–5.3	5.8–20.0
n	4	4	3
	FIP1L1-PDGFRα T674I		
	Placebo	Imatinib	PKC412
Spleen weight (mg)			
Mean	743	649	157
Median	778	645	157
Range	556–803	543–785	90–217
n	7	8	9
WBC (×10^6/ml)			
Mean	493.2	548.2	3.8
Median	460.6	591.5	3.1
Range	28.7–879.8	364.9–657.6	1.9–7.2
n	5	7	6

Spleen weights and white blood cell counts (WBC) of mice in the different groups of trial 2, determined at time of death or at trial endpoint. n: number of mice analyzed.

The effect of imatinib and PKC412 treatment was also confirmed by flow cytometry of single cell suspensions from spleen (Figure 4) and histopathologic analysis of bone marrow and spleen (Figure 5). We observed, consistent with in vitro data, that imatinib appeared to be somewhat more effective than PKC412 for treatment of the disease induced by wild-type FIP1L1-PDGFRα (Figures 3 and 4; Table 2).

Taken together, our in vitro and in vivo data indicate that PKC412 is an effective inhibitor of FIP1L1-PDGFRα and the clinically derived imatinib-resistant mutant, FIP1L1-PDGFRα(T674I).

Discussion

Small molecule inhibitors of tyrosine kinases are effective therapeutic agents for both hematologic malignancies and solid tumors, and it is likely that this paradigm for treatment of cancer will be rapidly extended (Cohen, 2002; Fabbro et al., 2002). However, to the extent that tyrosine kinase inhibitors are effective in treatment of disease, we can anticipate the development of resistance to single agents as has previously been observed for virtually all antibiological agents including antimicrobial and antiviral agents, and chemotherapy. A validated approach to preventing or circumventing resistance is the use of combina-

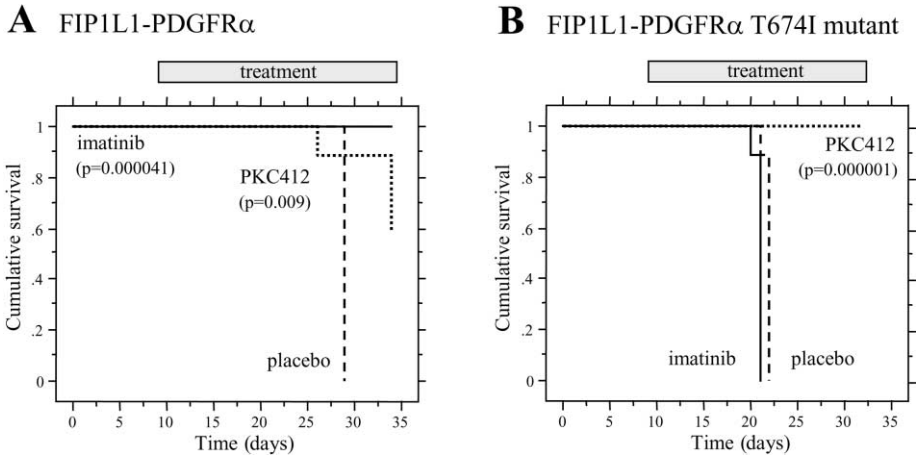

Figure 3. PKC412 increases survival in a murine BMT model for disease induced by either FIP1L1-PDGFRα or the imatinib-resistant FIP1L1-PDGFRα(T674I) mutant

A: Kaplan Meier plot showing the disease-free survival (*y* axis) of mice transplanted with bone marrow cells expressing FIP1L1-PDGFRα (wild-type) and treated with placebo, imatinib, or PKC412. There is a significant difference in survival between the drug-treated groups (imatinib or PKC412) versus the placebo group. The drop in the curve on day 33 reflects animals that appeared healthy at sacrifice, but were found to have moderately enlarged spleens at necropsy.

B: Kaplan Meier plot showing the disease-free survival (*y* axis) of mice transplanted with bone marrow cells expressing the imatinib-resistant FIP1L1-PDGFRα(T674I) mutant and treated with placebo, imatinib, or PKC412. There is no difference in survival between imatinib treatment and placebo, confirming in vivo resistance of the T674I mutant to imatinib. There was, however, a significant difference in survival between PKC412 treatment and placebo. The treatment period and p values are indicated on the plots.

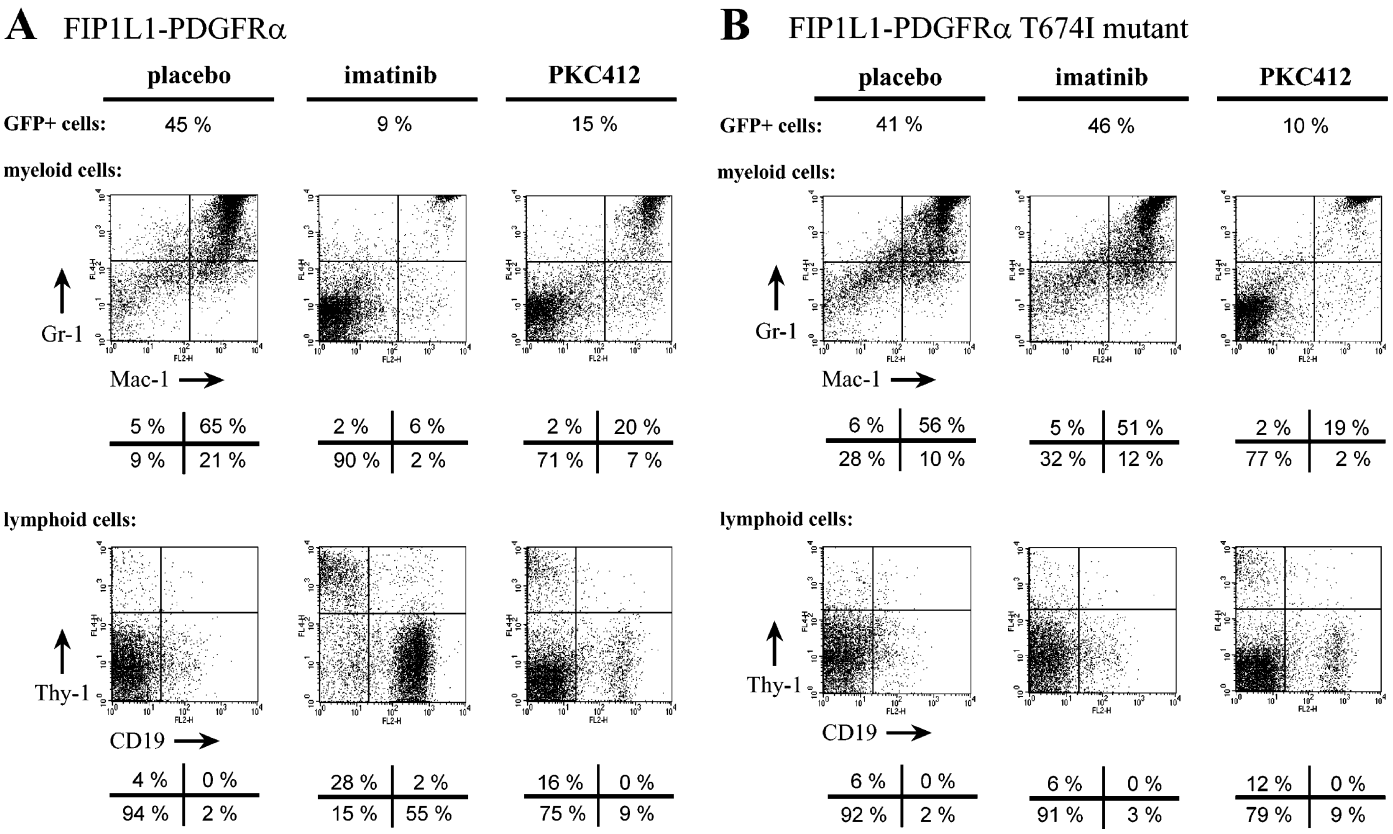

Figure 4. Immunophenotype of spleen cells of placebo-, imatinib-, and PKC412-treated mice

A and **B:** FACS analysis of spleen cells taken at death or trial endpoint from mice with disease caused by FIP1L1-PDGFRα (**A**) or the imatinib-resistant FIP1L1-PDGFRα(T674I) mutant (**B**). Shown is the percentage of GFP-positive (transformed) cells, myeloid cells, and B and T cells in spleens with or without drug treatment. All viable cells analyzed were included in the statistical analysis including those located at the outer boundaries of the quadrants. Effective treatment is illustrated by a decrease in GFP-positive cells, a reduction of Gr-1/Mac-1-positive cells, and an increase in B and T cells in the spleen.

tions of non-cross-resistant drugs that also have nonoverlapping or minimal toxicities. In the case of tyrosine kinase inhibitors, such combinations would ideally include small molecule inhibitors which recognize different structural elements of the target protein.

In this report, we investigate this strategy in the context of treatment of hypereosinophilic syndrome associated with the *FIP1L1-PDGFRA* fusion (Cools et al., 2003). The cloning of the fusion gene was fueled by the clinical observation that the majority of HES patients have dramatic responses to imatinib (Gleich et al., 2002). Furthermore, the acquisition of an imatinib resistance mutation of FIP1L1-PDGFRα in a patient while on imatinib therapy provides convincing evidence that the fusion is the therapeutic target of imatinib in HES (Cools et al., 2003). Together, these data provide the foundation for development of alternative small molecule inhibitors that target the imatinib resistance mutation. The T674I mutation is precisely analogous to the T315I imatinib resistance mutation that occurs in the context of BCR-ABL in CML and CML blast crisis (Gorre et al., 2001; Shah et al., 2002). Consequently, it is highly probably that imatinib binds to an inactive conformation of PDGFRa, analogous to that through which it binds c-ABL. Inhibition of the BCR-ABL(T315I) has proven to be a difficult problem. Significant progress has been made in development of compounds that circumvent other imatinib resistance mutations in BCR-ABL, but none thus far have a pharmacokinetic and toxicity profile that would allow for clinical application, and there are none that are effective for the BCR-ABL(T315I) mutant (La Rosee et al., 2002).

Here we report data from in vitro and in vivo models of myeloproliferative disease induced by FIP1L1-PDGFRα indicating that the imatinib-resistant BCR-ABL(T315I) equivalent in the context of FIP1L1-PDGFRα(T674I) can be effectively inhibited with PKC412. As a staurosporin derivative, in contrast to imatinib, this molecule is expected to inhibit PDGFRa by binding within the ATP binding pocket of the active conformation of the kinase, as observed, for example, in the case of CSK and CDK2 (Lamers et al., 1999; Toledo et al., 1997). PKC412 is also an attractive candidate for analysis in this context because it is currently in a Phase II clinical trial as a FLT3 inhibitor and has an acceptable toxicity and safety profile (Propper et al., 2001; Stone et al., 2003).

We first demonstrated that FIP1L1-PDGFRα and two related mutants confer factor-independent growth to Ba/F3 cells. As expected, imatinib inhibits the FIP1L1-PDGFRα wild-type, but not the clinically derived imatinib resistance mutation FIP1L1-PDGFRα(T674I). PKC412, in contrast, inhibits both the wild-type and the imatinib-resistant mutant T674I. The inhibitory effect of

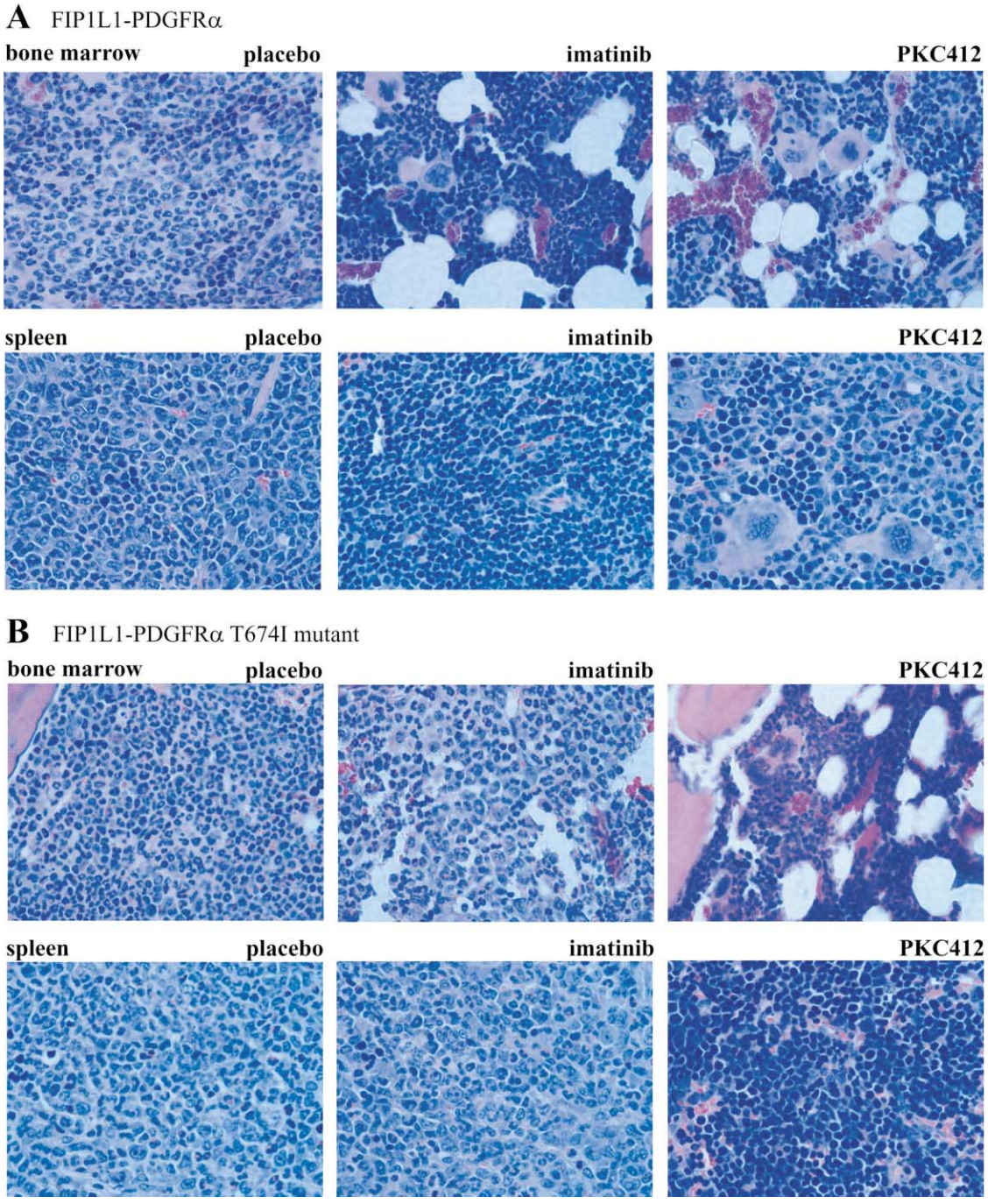

Figure 5. Histopathology of bone marrow and spleen of placebo-, imatinib-, and PKC412-treated mice

A and **B:** Histopathology of bone marrow (60×) and spleen (60×) from mice at death or trial endpoint with FIP1L1-PDGFRα-induced disease (**A**) or FIP1L1-PDGFRα(T674I)-induced disease (**B**), after treatment with placebo, imatinib, or PKC412. Effective treatment is illustrated by reappearance of a variety of cell types and fat in the bone marrow and reappearance of lymphoid cells in the spleen.

PKC412 is not observed in the presence of IL3, indicating that inhibition is not due to nonspecific toxicity. However, PKC412, like all tyrosine kinase inhibitors that target the highly conserved, active conformation of the enzymes, is selective but not completely specific and inhibits PKC, KDR, KIT, and FLT3 in addition to PDGFRα and PDGFRβ (Fabbro et al., 1999, 2000; Weisberg et al., 2002). To formally test the specificity of PKC412 for FIP1L1-PDGFRα in this context, we generated a FIP1L1-PDGFRα(N659D) mutation that we predicted would be PKC412 resistant based on unpublished observations in the context of other tyrosine kinases. FIP1L1-PDGFRα(N659D) was PKC412 resistant, indicating that inhibition of Ba/F3 cells transformed by FIP1L1-PDGFRα could be attributed solely to inhibition of the fusion gene. Of considerable interest, as discussed below, the

PKC412-resistant mutant retained full sensitivity in vitro to imatinib.

We next sought to develop a murine model of FIP1L1-PDGFRα-induced myeloproliferative disease to further test these observations in vivo. When we transduced unselected bone marrow cells with retrovirus containing the *FIP1L1-PDGFRA* gene, we observed a myeloproliferative phenotype similar to that observed with other constitutively activated tyrosine kinases such as BCR-ABL (Daley et al., 1990), FLT3-ITD (Kelly et al., 2002a), or TEL-PDGFRβ (Tomasson et al., 2000), with leukocytosis and splenomegaly attributable to a dramatic expansion of Gr-1/Mac-1-positive cells. Histopathologically, all members of the granulocyte lineage (neutrophils, eosinophils, and basophils) were markedly expanded. In general, the greatest overall increase was noted in the neutrophilic forms, but most animals had absolute eosinophil counts over 1500/μl. Human hypereosinophilic syndrome has a variable phenotype, with diagnostic criteria including a prolonged elevation of eosinophils above 1500/μl, though most patients have a more dramatic elevation of their eosinophil count (Chusid et al., 1975; Weller and Bubley, 1994). The bone marrow of HES patients is typically comprised of 30%–60% eosinophilic precursors. Although the total leukocyte may be normal, most patients also have an expansion of neutrophil lineage cells in both bone marrow and peripheral blood (Weller and Bubley, 1994).

We first validated the model using imatinib therapy of disease induced either by the FIP1L1-PDGFRα fusion or the imatinib-resistant T674I mutant. We observed clinical outcome similar to that in humans with HES, namely that disease induced by the wild-type FIP1L1-PDGFRα was effectively treated with imatinib, whereas disease induced by the imatinib resistance mutation was not.

These data set the stage for the pivotal experiment to test the effect of PKC412 on the imatinib resistance mutant. PKC412 was effective in treating disease induced by the imatinib-resistant mutant FIP1L1-PDGFRαT674I, although it appears to be somewhat less effective than imatinib both in vitro and in vivo in inhibition of the wild-type FIP1L1-PDGFRα. One animal treated with PKC412 developed a marked myeloproliferative disease. The reason for breakthrough of disease is not certain, but could include the development of resistance mutations analogous to the N659D, inadequate dosing, or increased clearance of the drug in this animal.

These observations provide proof-of-principle that acquired resistance to one tyrosine kinase inhibitor can be overcome by use of an alternative, structurally unrelated inhibitor that binds to the kinase domain in a different manner. As imatinib therapy has been FDA approved and PKC412 is currently in Phase II trials for other indications, it is plausible that this strategy can be clinically tested soon. However, it is not clear whether it would be best to treat with a single agent until resistance develops or to treat initially with combination therapy. A recent report that imatinib-resistant mutations in BCR-ABL may exist prior to the initiation of therapy suggests that the latter approach may be more effective (Shah et al., 2002). The finding that some imatinib resistance mutations are PKC412 sensitive (T674I) while other mutations result in resistance to PKC412 but retain imatinib sensitivity (N659D) also supports the use of concurrent combination therapy.

The feasibility of combination therapy will depend in part on safety profiles for these selective, but not specific inhibitors. Although imatinib and PKC412 inhibit a similar spectrum of tyrosine kinases that includes PDGFRα, PDGFRβ, and KIT, imatinib also inhibits ABL and ARG, while PKC412 inhibits PKC and FLT3 (Fabbro et al., 1999, 2000; Capdeville et al., 2002; Weisberg et al., 2002). Thus, there may be unacceptable toxicities from using these agents in combination. To the extent that these agents have nonoverlapping or minimal toxicities, it may be most prudent to use these agents in combination at initiation of therapy.

Finally, our data indicate that PKC412 may be useful in treating any disease in humans associated with activating mutations in PDGFRα or PDGFRβ. These would include, but not be limited to, gastrointestinal stromal cell tumors associated with activating mutations in PDGFRα (that are clinically resistant to imatinib) (Heinrich et al., 2003), myeloproliferative disease associated with the BCR-PDGFRα fusion gene (Baxter et al., 2002), or myeloproliferative disease associated with *PDGFRB* fusion genes, including TEL-PDGFRβ (Golub et al., 1994), HIP1-PDGFRβ (Ross et al., 1998), H4-PDGFRβ (Schwaller et al., 2001), RABPT5-PDGFRβ (Magnusson et al., 2001), or a spectrum of other translocations associated with *PDGFRB* rearrangements (Baxter et al., 2003). In addition, although most of these are imatinib sensitive, PKC412 may have use in these contexts, as in HES, in either preventing or treating imatinib resistance mutations. In addition, it is plausible that PKC412 could be useful in treating or overcoming resistance in cancers or noncancerous diseases associated with overexpression of *PDGFRA*, including central nervous system tumors (MacDonald et al., 2001). Lastly, *FIP1L1-PDGFRA* itself may be capable of contributing to the pathogenesis of cancer in other tissues, as has been observed with activating KIT mutations in both systemic mastocystosis and gastrointestinal stromal cell tumors (Nagata et al., 1995; Longley et al., 1996; Hirota et al., 1998). Since FIP1L1 is ubiquitously expressed, it would be reasonable to screen for the *FIP1L1-PDGFRA* fusion in other tumors, especially since effective therapy is currently available.

Experimental procedures

Constructs

The retroviral construct MSCV-FIP1L1-PDGFRα-ires-EGFP and the corresponding T674I mutant were described previously (Cools et al., 2003). The N659D mutation was introduced by PCR. Constructs are shown in Figure 1.

Cell culture and retroviral transduction

293T cells were grown in DMEM supplemented with 10% FBS. Ba/F3 cells were grown in RPMI supplemented with 10% FBS and 1 ng/ml mouse IL-3. Production of retroviral vectors and transduction was described (Schwaller et al., 1998). Transformed Ba/F3 cells were grown in the absence of IL3. The kinase inhibitors imatinib and PKC412 were stored as 10 mM stock solutions in water (imatinib) or DMSO (PKC412). These inhibitors were diluted in RPMI medium for use. For Western blotting, Ba/F3 cells were incubated in the presence of imatinib for 90 min before lysis. For dose response curves, Ba/F3 cells were incubated for 24 hr in the presence of imatinib, and the number of viable cells at the start and end point was determined by use of the Celltiter96AQ$_{ueous}$one solution proliferation assay (Promega). Dose response curves were fitted using the OriginPro 6.1 software (OriginLab).

Bone marrow transplantation and treatment of the animals

Balb/c mice were purchased from Taconic. Bone marrow transplant assays (injecting 1×10^6 cells per recipient mouse) and drug treatment of the mice were performed as described previously (Schwaller et al., 1998; Kelly et al., 2002b; Weisberg et al., 2002). Imatinib (stored as powder at 4°C) was resuspended in a 0.5% methylcellulose (MC) solution in water prior to use. PKC412 (6% w/w in Gelucire® 44/14 (GC) (Gattefosse, France)) was stored

at 4°C as a waxy solid formulation. Prior to administration, the GC/PKC412 waxy solid mixture was melted in a 44°C water bath and diluted with sterilized deionized water. The animals were weighed on a regular basis to ensure that a consistent dose (125 mg/kg/day for imatinib and 100 mg/kg/day for PKC412) of drug was administered. Dosing was performed every 12 hr for imatinib and every 24 hr for PKC412 by oral gavage of a maximum volume of 150 μl per animal using 22 gauge gavage needles (Hornbecks). Placebo animals received the same volume of an MC or GC solution. Any animals with splenomegaly (spleen boundary detectable at the dorsal midline) or that were moribund were sacrificed and analyzed for signs of hematological disease.

Peripheral blood was collected from the retroorbital cavity using a heparinized glass capillary. Blood smears were stained with Wright and Giemsa. Manual and automated (ADIVA 120 Hematology system, Bayer) total and differential blood cell counts were performed. Histopathologic exam of relevant organs (spleen, liver, heart, lungs, intestine, hindlimb bones, and kidneys) and preparation of single cell suspensions from spleen and bone marrow for flow cytometry were performed as described (Schwaller et al., 1998).

Statistical analysis

In comparing the survival time of the mice, all times were measured from the day of bone marrow transplant, estimated by the method of Kaplan and Meier, and assessed using the log rank test.

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). The tissue sections (4 μm) 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.

Immunoprecipitation and Western blotting

Immunoprecipitation was performed using the anti-Myc antibody (Cell Signaling) and Protein G agarose (Roche). Each precipitation was started from 6×10^6 Ba/F3 cells stably expressing myc-tagged FIP1L1-PDGFRα wild-type or T674I mutant. Cells were lysed in lysis buffer (Cell Signaling) containing 1 mM Na_3VO_4, 20 μM phenylarsine oxide (Calbiochem), and complete tablets (Roche). For Western blotting, Ba/F3 cells were collected by centrifugation and directly lysed in 1 × loading buffer containing 2% SDS and 40 μM DTT (Cell Signaling), separated using 10%–12% SDS-PAGE, and transferred to membranes. The antibodies used were: anti-phospho-STAT5 and anti-phospho-tyrosine (P-Tyr-100/102) (Cell Signaling), anti-PDGFRα (Upstate), anti-STAT5b (Santa-Cruz), anti-mouse-PO, and anti-rabbit-PO (Amersham Pharmacia Biotech). Detection was performed using the Western Lightning system (Perkin Elmer).

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