Development of Antagonists of the Platelet-Derived Growth Factor Receptor Family

By Neill A. Giese, PhD, Nathalie A. Lokker, PhD, Jin-Chen Yu, PhD, Kenji Matsuno, PhD, Michio Ichimura, PhD, Shoji Oda, PhD, Robert Scarborough, PhD, and Anjali Pandey, PhD

ABSTRACT

*The platelet-derived growth factor receptor (PDGFR) family of tyrosine kinases are promising targets for the development of cancer therapeutics because their known aberrant signaling by autocrine activation or gain-of-function mutations in human tumors very likely play a role in the etiology of the disease. A cell-based PDGFR phosphorylation assay was utilized to screen natural product and synthetic compound libraries for the identification of small molecule kinase inhibitors. These efforts resulted in the identification of four classes of potent PDGFR inhibitors that include a macrocyclic lactone, aminopyrazoles, benzimidazoles, and quinazolines. The quinazoline series was further optimized for potency, specificity, oral bioavailability, and plasma half-life. One compound from this series, CT52923 (previously synthesized by Kyowa Hakko, Japan as KN734), was shown to exhibit remarkable specificity when tested against other kinases, including members of the closely related PDGFR family. The PDGFRs and stem cell factor receptor (*c-kit*) were inhibited with an IC_{50} of 100 to 200 nM, whereas 45- to >200-fold–higher concentrations of CT52923 were required to inhibit* fms*-like tyrosine kinase-3 (Flt-3) and colony-stimulating factor-1 receptor (CSF-1R), respectively. Other receptor tyrosine kinases, cytoplasmic tyrosine kinases, serine/threonine kinases, or members of the MAPK pathway were not significantly inhibited at 100- to 1000-fold–higher CT53923 concentrations. In addition, this compound also demonstrated specificity for inhibition of cellular responses. PDGF-induced smooth muscle cell migration or fibroblast proliferation were found to be blocked by CT52923 with IC_{50} values of 64 and 280 nM, respectively, whereas 50- to 100-fold–higher concentrations were required to inhibit these responses when induced with FGF. To further evaluate the therapeutic potential of CT52923, it was tested in a mouse model of chronic myelomonocytic leukemia (CMML) that involves the expression of a TEL/βPDGFR fusion protein in which the PDGFR kinase domain is constitutively activated. Oral administration of CT52923 to mice resulted in improved survival due to a significant delay in disease progression. This study illustrates a strategy for the development of kinase inhibitors for the treatment of cancer that includes target selection, compound identification, optimization of lead compound series, and "proof of principle" in a relevant preclinical cancer model.*

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INTRODUCTION

The platelet-derived growth factor receptor (*PDGFR*) family belongs to the larger family of receptor tyrosine kinases (RTK) that are characterized by an extracellular ligand-binding region, a single transmembrane spanning region, and an intracellular tyrosine kinase domain.[1] Currently, 58 human genes encoding RTKs, which are distributed into 20 subfamilies, have been identified.[2] The *PDGFR*s are classified as the RTK type III subfamily that is distinguished by five immunoglobulin-like repeats in the extracellular domain and a split kinase in the intracellular domain.[3] Signaling by RTKs requires ligand-induced

TALKING POINTS Physicians Pharmacy Formulary Cancer Nurses

Kinase inhibitors are anticipated to provide important new therapeutic modalities for the treatment of cancer.

Pharmaceutical development of kinase inhibitors requires a novel therapeutic approach.

The scientific rationale behind the development of kinase inhibitors for the potential treatment of various cancer is an important component to early pharmaceutical research

Kinase inhibitors may offer exciting and better tolerated therapeutic alternatives to current treatment approaches to various cancers.

Dr. Giese is a research fellow, Dr. Lokker is senior scientist, Dr. Yu is a scientist in the Biology Department, Dr. Scarborough is vice president and Dr. Pandey is associate director in the Medicinal Chemistry Department, all at COR Therapeutics, Inc. in South San Francisco, CA. Dr. Matsuno is leader in the Regulatory Affairs Department at Kyowa Hakko Kogyo Co., Ltd. in Tokyo, Japan, where Dr. Ichimura is senior researcher at the Pharmaceutical Research Institute and Dr. Oda is manager of the Drug Discovery Division.

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receptor oligomerization that results in tyrosine autophosphorylation.[4-6] Autophosphorylation serves a dual purpose in that it increases the receptors catalytic activity and creates the sites for physical interactions with a number of proteins that contain *src* homology region 2 (SH2) domains and protein tyrosine-binding (PTB) domains. These interactions result in the activation of signaling pathways that are mediated by small G proteins, lipid kinases, cytoplasmic kinases, and phospholipases.[6,7] These pathways are of particular importance for the genesis of proliferative diseases such as fibrosis and cancer because they induce cell proliferation, promote cell survival by inhibiting apoptosis, and alter normal cell differentiation—changes that are the hallmarks of oncogenic transformation. Deregulation of RTKs signaling leading to oncogenic activation has been shown to occur by a variety of mechanisms. The most common alteration leading to increased RTK signaling is receptor overexpression that has been identified in tumor tissues for approximately 30 RTKs. The importance of RTK overexpression in cancer has been best demonstrated recently for the epidermal growth factor receptor (EGFR) family. Treatment of breast cancer patients with a monoclonal antibody, Herceptin, directed against EGFR-2/erbB2 has proven to be an effective therapy when this receptor is overexpressed.[8,9] Aberrant RTK signaling leading to oncogenic transformation can also occur when the receptor and its ligand are expressed by the same cell, establishing an autocrine loop. Autocrine signaling by a number of RTKs including the EGFR and PDGFR, among others, has been shown to cause tumor formation in animal models and is a common event in human tumors.[10-15] More importantly, mutations or structural alterations that lead to constitutive receptor activation have been identified in human tumors for 13 RTK family members.[2,6] For example, kinase inhibitor therapy with imatinib (Gleevec) that targets mutant *c-kit* has been shown to be effective in the treatment of gastrointestinal stromal tumors (GISTs).[16-18] In this article, we describe methods for kinase inhibitor development that include target identification, lead discovery, lead optimization, in vitro characterization and demonstration of in vivo efficacy in a relevant tumor model.

METHODS FOR DEVELOPMENT OF KINASE INHIBITORS

Target Identification: Rationale for Selection of the PDGFR Family

RTK activation occurs in response to a specific extracellular stimulus and represents the most proximal step for initiating an intracellular signaling pathway, whereas nonreceptor kinases act downstream and participate in multiple pathways. Therefore, RTK inhibition at the proximal step should provide the greatest level of specificity and represents a novel mechanism-based therapeutic strategy to selectively block a signaling pathway that is known to mediate tumor formation. The success of this approach is dependent on the identification of kinase targets whose inappropriate signaling plays an important etiologic role in the cancer disease process. It is well established in animal model systems that RTKs can mediate oncogenic signaling as the result of receptor overexpression or the establishment of an autocrine loop without any direct genetic changes to the receptor itself. Based on these observations, kinase inhibitors are being actively investigated in clinical trials for tumors that overexpress EGFR family members. A much stronger rationale exists for selecting a kinase target that is known to have genetic alterations that cause its constitutive activation in primary human tumors. This rationale has been validated clinically recently with the demonstration that Gleevec, a *bcr/abl* kinase inhibitor, is an effective treatment for chronic myelogenous leukemia.[16,19,20] Based on this rationale, the members of the PDGFR family are especially attractive oncology targets for kinase inhibitor therapy because four of the five family members are known to contain activating mutations in human tumors.

The members of the PDGFR family include αPDGFR, βPDGFR, colony stimulating factor 1 receptor (CSF-1R), Flt-3, and *c-kit*.[2] Within this kinase family, *c-kit* gain-of-function mutations have been the most extensively studied. Mutated *c-kit* has been identified in mastocytosis, acute myelogenous leukemia (AML) and GISTs.[21-24] Early stage clinical trials using Gleevec to inhibit *c-kit* in GIST patients have shown positive results, which indicate that *c-kit* is a valid target for treating this disease.[17,18] Flt-3, the newest and least studied member of the PDGFR family, has been recently implicated in AML.[25] Greater than 30% of AML patients harbor a gain-of-function mutation in their Flt-3 gene that involves either an internal tandem duplication in the juxtamembrane region or a point mutation in the kinase domain activation loop.[26-28] Patients with Flt-3 mutations have higher white cell counts when compared to AML patients without Flt-3 mutations, show lower response rates to chemotherapy, and have shorter times to relapse, making Flt-3 kinase inhibition an attractive therapeutic strategy. Another member of the PDGFR family, CSF-1R has also been implicated in the development of AML. A point mutation in the C-terminal region has been described but the frequency of this mutation or its importance to tumor formation has not been well characterized.[29-32] The founding family member, βPDGFR, has been studied extensively for its role in cancer. The initial interest in the PDGFRs was due to the observation that the *v-sis* oncogene of simian sarcoma virus encoded the PDGF-B chain that, when expressed in fibroblasts or glial cells of an infected animal, gave rise to sarcomas and brain tumors, respectively.[15,33-35] This established for the first time that RTK autocrine signaling commonly observed in human tumors could play a key role in the oncogenic process. More recent studies have identified chromosomal translocations involving the βPDGFR in chronic myelomonocytic leukemia (CMML). A subset of CMML patients have a t(5;12) (q33;p13) or a t(5;7) (q33;q11.2) chromosomal translocation that results in the N-terminal ligand-binding domain of the PDGFR being replaced by the N-terminus of the transcription factor Tel

or the Huntingtin interacting protein 1.[36-38] Each of the fusion partners contains a self-association domain that mediates the formation of oligomers leading to constitutive activation of the βPDGFR kinase domain. Therefore, βPDGFR kinase inhibitor therapy would likely benefit these patients. Overall, there is strong rationale for targeting members of the PDGFR family for both hematological malignancies and certain solid tumors.

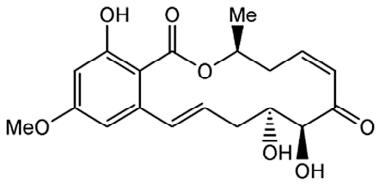
Lead Discovery

The most successful approach for the discovery of small molecule kinase inhibitors has been high-throughput screening of diverse natural product or synthetic compound libraries. Therefore, we developed a high-throughput cell-based two-site ELISA to screen compounds for inhibition of PDGFR tyrosine autophosphorylation.[39,40] The use of a whole cell assay has a distinct advantage over a purified kinase assay in that only compounds that inhibit the receptor in its native state will be detected. Greater than 100,000 natural product extracts of microorganisms from soil samples or synthetic compounds were tested in this screening assay resulting in the identification of four lead compounds with different chemical structures (Figure 1). These compounds included one natural product, CT56066, a macrocyclic lactone, and three synthetic compounds, CT50120, an aminopyrazole, CT38739, a benzimidazole, and CT56040 (previously synthesized by Kyowa Hakko, Japan as KN1022), a quinazoline.[41] All four compounds exhibited potent inhibitory activities against the βPDGFR with IC_{50} values of 20–500 nM. Because nearly all other protein kinase inhibitors described to date compete with the common substrate ATP for enzyme binding, it is surprising that the level of target specificity has been very high in some instances and almost completely lacking in others.[42] Therefore, determination of compound specificity is an essential component of a successful kinase inhibitor drug discovery program that is dependent on the development of a spectrum of secondary kinase assays. To evaluate compound specificity, we developed comparable cell-based assays for the other PDGFR family members and EGFR, whereas a purified enzyme was used for the nonreceptor kinase *src*. As shown in Table 1, when our four leads were tested against the closely related PDGFR family kinases, only CT56040 displayed some degree of specificity because 16-fold–higher concentrations were required to inhibit Flt-3 kinase as compared with other family members. No inhibition of EGFR was observed with any of the leads, and only CT56066 displayed significant inhibitory activity toward *src*. The reduced level of specificity of CT56066 as compared with the other synthetic leads was not unexpected because inhibitors derived from natural sources frequently lack specificity. For example, staurosporin was shown to inhibit all kinases with an IC_{50} of <1 μM (Table 1). Based on these observations, CT56040 was selected for lead optimization because it had sufficient potency, it had the best specificity for the PDGFR, and the quinazoline nucleus is an excellent pharmacophore.

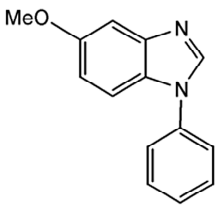
Lead Optimization

The primary goal of lead optimization is to increase potency and specificity as well as to achieve desirable in vivo properties including high oral bioavailability and a

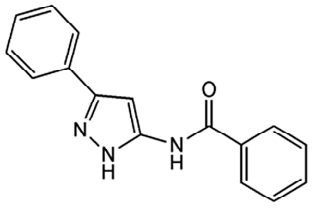
FIGURE 1. LEADS IDENTIFIED THROUGH HIGH-THROUGHPUT SCREENING



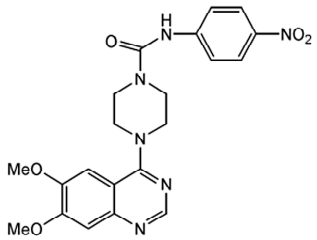
CT56066



CT38739



CT50120



CT56040

Presented are the four lead compounds that were identified as inhibitors of platelet-derived growth factor receptor autophosphorylation. These include a natural product CT56066, a macrocyclic lactone, and three synthetic compounds, CT50120, an aminopyrazole, CT38739, a benzimidazole, and CT56040, a quinazoline.

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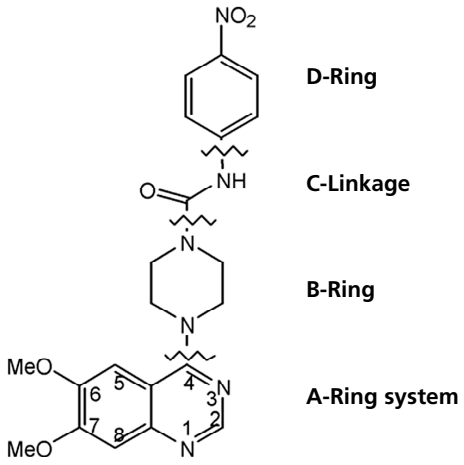
FIGURE 2. INITIAL LEAD PHARMACOPHORE DISSECTIONS



Presented is the pharmacophore of CT56040, the initial quinazoline platelet-derived growth factor receptor inhibitor lead dissected into four regions. Chemical modifications were performed in the various regions indicated for compound optimization.

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long plasma half-life. CT56040 is a very attractive candidate for lead optimization because the quinazoline template has been previously exploited to develop EGFR tyrosine kinase inhibitors that are currently in clinical trials (eg, ZD1839, CTI1033, CP358774/OSI774, and PKI166).[43-46] Based on the model shown in Figure 2, we initiated a systematic SAR exploration of the A bicyclic ring, the B piperazine, the C urea linkage, and the D-ring to extensively characterize this chemical series (and see patent issued: Matsuno K, Ichimura M, Nomoto Y, et al. Nitrogenous Heterocyclic Compounds. 1998. European Patent Office. WO 98/14431).[41] In the quinazoline A-ring, substitutions at the 6 & 7-position were favored. Replacement of the dimethoxyquinazoline with another heterocyclic ring system reduced activity in most cases. The substitution on the piperazine B-ring reduced inhibitory activity of these compounds against PDGFRs. The urea or the thiourea moiety at the C-linkage is critical for inhibitory potency. The hydrogen atom on the nitrogen of the urea/thiourea functionality appears to be absolutely necessary possibly due to hydrogen bonding with an amino acid residues in the ATP binding site. Within the D-ring, hydrophobic and aromatic substituents increase the potency, whereas polar substitution decreases the inhibitory activity. It is apparent that a wide range of functionality in the 4-position of the D-ring can be tolerated,

TABLE 1. INHIBITION OF KINASE ACTIVITIES BY LEAD COMPOUNDS

	IC_{50} (μM)					
Compound	αPDGFR	βPDGFR	c-kit	Flt-3	EGFR	Src-Kinase
CT56066	0.01	0.011	0.007	0.013	>30	0.3
CT50120	0.22	0.245	0.064	0.069	>30	8
CT56040	0.05	0.200	0.390	3.390	>30	>30
CT38739	0.07	0.195	0.065	0.210	NT	NT
Staurosporin	0.07	0.010	0.010	0.100	NT	0.07

Autophosphorylation of all RTKs were measured in intact cells using a two-site ELISA as described elsewhere.[39,40] For the src-kinase, substrate phosphorylation was measured in an in vitro assay with purified enzyme.[40]

PDGFR=platelet-derived growth factor receptor; EGFR=epidermal growth factor receptor; RTK=receptor tyrosine kinase.

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TABLE 2. SELECTIVE ANTAGONISM OF PDGFR FAMILY MEMBERS BY QUINAZOLINE ANALOGS

	IC_{50} (μM)			
Kinase	CT53481	CT56043	CT56046	CT52212
βPDGFR	0.45	>30	2.24	0.03
Flt-3	>30	1.09	3.97	0.03
c-kit	3.80	15.8	0.17	0.01
CSF-1R	>30	>30	>30	0.20

Various quinazoline derivatives based on the original CT56040 lead were tested for their level of enzyme specificity. Presented are examples of derivatives with various specificities for each member of the platelet-derived growth factor receptor (PDGFR) family including CT53481 for PDGFR, CT56043 for Flt-3, and CT56046 for c-kit. Another derivative CT52212 inhibited all four members of the PDGFR family. Autophosphorylation of all receptor tyrosine kinases were measured in intact cells using a two-site ELISA as described elsewhere.[39,40]

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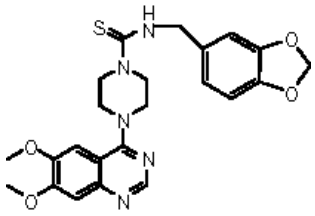
both with regard to steric bulk and the electronic nature of the substituents. Remarkably, after testing of over 700 quinazoline derivatives, compounds that are specific for individual PDGFR family members were obtained demonstrating the success of this strategy (Table 2). This SAR exploration also provided us with a urea-containing class of compounds that had similar potencies for inhibiting phosphorylation of all three RTKs including PDGFR, c-Kit, and Flt3 kinases with IC50 ranging from 10 to 200 nM as shown for CT52212 (Table 2). However, when the urea linkage is replaced by a thiourea the compounds are now highly specific for PDGFR and c-Kit with an IC50 value of 100–200 nM, whereas 30 to 300-fold higher concentrations were required to inhibit Flt3 and CSF-1 as shown for CT52923 in Table 3. In addition to having good specificity for PDGFR, CT52923 also displayed improved pharmaceutical properties that included potency in plasma, increased oral bioavailability and a long in vivo half-life (data not shown). Therefore, CT52923 was selected for further in vitro and in vivo characterization.

TABLE 3. TYROSINE KINASE SPECIFICITY OF PDGFR ANTAGONIST CT52923

IC_{50} (μM)



	CT52923	Staurosporin
PDGFR Family		
βPDGFR	0.14	0.09
αPDGFR	0.21	ND
c-kit	0.22	0.03
CSF-1R	>30	0.12
Flt-3	6.22	0.04
Other RTK		
VEGFR-2	17.3	0.02
EGFR	>100	ND
FGFR	160	0.42
Nonreceptor TK		
c-src	>30	0.07
c-abl	>30	<0.30
MAPK and MAPKK	>30	<1
Mek1	>30	<1
Mkk4	>30	<1
Mkk6	>30	<1
Erk2	>30	<1
Jnk1	>30	<1
p38	>30	<1

Autophosphorylation of all RTKs were measured in intact cells using a two-site ELISA as described elsewhere.[39,40] For all other kinases, substrate phosphorylation was measured in an in vitro assay with purified enzyme.[40]

PDGFR=platelet-derived growth factor receptor; RTK=receptor tyrosine kinase; VEGFR=vascular endothelial growth factor receptor; EGFR=epidermal growth factor receptor; FGFR=fibroblast growth factor receptor; TK=tyrosine kinase; MAPK=mitogen-activated protein kinase; MAPKK=mitogen-activated protein kinase kinase.

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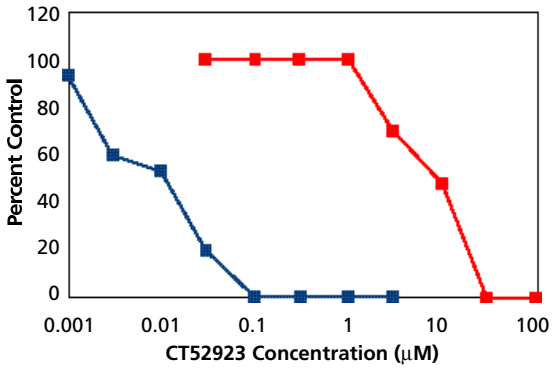
In Vitro Compound Characterization

As part of the continuing development of CT52923, more extensive target specificity testing was conducted. As shown in Table 3, CT52923 exhibited remarkable specificity when tested against other kinases including all members of the closely related PDGFR family. The PDGFR and *c-kit* were inhibited with an IC_{50} of 100–200 nM, whereas 120–>200-fold–higher concentrations were required to inhibit Flt-3 and CSF-1R kinases. Other receptor tyrosine kinases, cytoplasmic tyrosine kinases, serine/threonine kinases, or members of the MAPK pathway were not significantly inhibited at 100- to 1,000-fold–higher concentrations. To evaluate the biological specificity of CT52923, its ability to inhibit cell proliferation and migration in response to PDGF-BB or bFGF was measured because these two growth factors have been shown to induce very similar signaling pathways, changes in gene expression, and cellular responses in mesenchymal cells.[47] For cell proliferation, NIH3T3 cells were stimulated with PDGF-BB or βFGF in the presence of increasing concentrations of CT52923. CT52923 inhibited

FIGURE 3. CT52923 INHIBITION OF TEL/PDGFR-BAF3 CELL GROWTH



3 x 10^4 BaF3 cells expressing Tel/PDGFR fusion protein were plated in 1 mL of culture media with (red squares) or without (blue squares) 1 ng/mL IL3. Cell growth was measured in the presence of increasing concentrations (0.001–100 μM) of CT52923, and viable cells were counted after 3 days. Each point represents an average of triplicate data.

PDGFR=platelet-derived growth-factor receptor.

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PDGF-induced thymidine incorporation with an IC_{50} value of 280 nM, whereas the IC_{50} for bFGF was 46-fold higher at 13 µM ($P<.001$). Rat A10 smooth muscle cell migration was measured using modified Boyden chambers in which cells were induced by PDGF or bFGF in the presence of increasing concentrations of CT52923. PDGF-induced cell migration was inhibited by CT52923 with an IC_{50} value of 64 nM, whereas approximately 100-fold–higher concentration was required to inhibit migration stimulated by βFGF.[40] These results demonstrated that CT52923 was a very selective inhibitor of PDGF-mediated cell proliferation and migration.

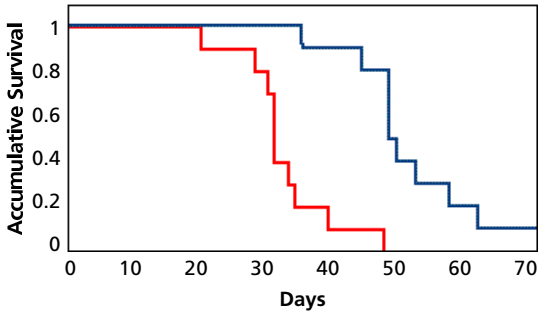
In Vivo Efficacy in a Preclinical Animal Model

To further explore the therapeutic potential of CT52923 it was tested in a mouse model of CMML. This was accomplished by first expressing the CMML Tel/PDGFR oncoprotein in BaF3 cells that causes oncogenic transformation and abrogates the IL3 requirement for in vitro cell growth.[48] To evaluate the ability of CT52923 to inhibit Tel/PDGFR-mediated BaF3 cell growth, cells were grown in culture media without IL3 and which contained various concentrations of inhibitor (0.001–100 µM). Cell growth was monitored over 3 days. As shown in Figure 3, CT52923 inhibited Tel-PDGFR mediated cell growth with an IC_{50} of 10 nM. This inhibition was specific for Tel/PDGFR because 1,000-fold were the concentrations of inhibitor required to inhibit cell growth mediated by IL3 (Figure 3). To extend these studies, we developed a mouse model of CMML. This was accomplished by injecting 10^5 BaF3 cells expressing Tel/PDGFR into the tail vein of nude mice to initiate the disease process. Beginning at 7 days postinjection, mice (n=10/group) were treated by oral gavage with vehicle or CT52923 at 60 mg/kg twice daily until day 42, when dosing was stopped. As shown in Figure 4, all vehicle-treated control mice died between day 20 and day 46, whereas CT52923 treated animals did not begin to die until day 40. Additional deaths occurred in the treated group as late as day 60, with one mouse surviving indefinitely. These studies demonstrate that CT52923 treatment caused a significant delay in the disease process and, in one case, completely protected the animal. To monitor the disease process, satellite animals were sacrificed 4 weeks following BaF3 cell inoculation and tumor cell infiltration into the spleen was examined. As shown in Figure 5, in control animals, massive splenomegaly was observed with the average spleen weight being 834 mg as compared with 150 mg for the CT52923 animals or 118 mg for unmanipulated mice. These studies provide the "proof of principle" that oral treatment with CT52923 can provide an effective treatment for CMML-like disease in animals and that advancement toward clinical trials is warranted.

CONCLUSIONS

The highly successful clinical trials employing imatinib for the treatment of CML have validated kinase inhibition as a therapeutic strategy in oncology.[19,20,49] Continued success with the development of new kinase inhibitors is dependent initially on the selection of appropriate kinase targets. The most valid kinase targets are those known to be activated constitutively in human tumors due to genetic alterations, as is the case for *bcr/abl* in CML. It is for this reason that we chose the PDGFR family for the development of kinase inhibitors. In a situation very analogous to *bcr/abl* in CML, the family members Flt-3 and *c-kit* are known to be activated by mutations in a significant percentage of AML cases, whereas βPDGFR is mutated in CML. Following target selection, the discovery and development of small molecule kinase inhibitors, for the most

FIGURE 4. KAPLAN-MEIER SURVIVAL CURVE OF CT52923-TREATED MICE IN A TEL/PDGFR LEUKEMIA MODEL



10^5 BaF3 cells expressing Tel/PDGFR were injected into mice via a tail vein. Mice (*n*=10) were dosed by oral gavage CT52923 at 60 mg/kg BID or vehicle alone (0.5% methyl cellulose) from day 7 to day 42. Shown are survival curves of CT52923-treated mice (blue line) and control mice (red line).

PDGFR=platelet-derived growth factor receptor.

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FIGURE 5. CT52923 INHIBITION OF SPLENOMEGALY IN A TEL/PDGFR LEUKEMIA MOUSE MODEL

Spleens were harvested at 4 weeks postBaF3-Tel/PDGFR cell injection. The average spleen weight in CT52923-treated mice was 150 mg; in vehicle-treated mice the average spleen weight was 834 mg. A representative spleen from each group is shown and compared with a normal unmanipulated mouse spleen that on average weighed 118 mg.

PDGFR=platelet-derived growth factor receptor.

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part, has followed traditional methods of high-throughput screening of synthetic compound libraries and directed analog profiling for compound optimization. The goal of compound optimization is to achieve high potency and specificity as well as acceptable in vivo properties including high bioavailability, a long plasma half-life, and a good safety profile. Once a clinical lead has been identified, its careful evaluation in a relevant preclinical cancer model is critical for the validation of the approach and the identification of variables that will facilitate the design of an optimal clinical trial. In summary, kinase inhibitor therapy has proven to be highly effective for the treatment of cancer and is likely to find additional applications especially for a PDGFR inhibitor in a variety of fibroproliferative diseases. OS

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