Journal of Experimental Pharmacology

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ORIGINAL RESEARCH

Comprehensive kinase profile of pacritinib, a nonmyelosuppressive Janus kinase 2 inhibitor

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This article was published in the following Dove Press journal:
Journal of Experimental Pharmacology
16 August 2016
Number of times this article has been viewed

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Abstract: Pacritinib, potent inhibitor of Janus kinase 2 (JAK2), JAK2V617F, and fms-like receptor tyrosine kinase 3, is in Phase III development in myelofibrosis. Among type 1 inhibitors, pacritinib shows a lack of myelosuppression at doses that both inhibit JAK2/signal transducer and activator of transcription 3 pathway and demonstrate clinical efficacy. To elucidate these mechanisms and identify other disease targets, a kinome analysis screened 439 recombinant kinases at 100 nM pacritinib concentration. For kinases with >50% inhibition, pacritinib was titrated from 1 to 100 nM. JAK2, JAK2V617F, FLT3, colony-stimulating factor 1 receptor, and interleukin-1 receptor-associated kinase 1 achieved half-maximal inhibitory concentrations <50 nM. Pacritinib did not inhibit JAK1 (82% control at 100 nM). Lack of myelosuppression may stem from inhibiting JAK2 without affecting JAK1 and reducing hematopoietic inhibitory cytokines by suppressing interleukin-1 receptor-associated kinase 1 or colony-stimulating factor 1 receptor. The pacritinib kinome suggests therapeutic utility in acute myeloid leukemia, myelodysplastic syndrome, chronic myelomonocytic leukemia, solid tumors, and inflammatory conditions.

Keywords: kinase analysis, myelofibrosis, hematologic malignancies, Janus kinase 2, JAK2V617F, fms-like receptor tyrosine kinase 3

Introduction

Janus kinase 2 (JAK2) is involved in the signaling cascades critical for maintaining normal hematopoiesis. JAK2 is activated by cytokines that control granulopoiesis (granulocyte-colony stimulating factor, interleukin [IL]-3, granulocyte macrophage-colony stimulating factor), erythropoiesis (erythropoietin), thrombopoiesis (thrombopoietin), eosinopoiesis (IL-5), and T-cell differentiation signaling (IL-12).[1] Inhibition of JAK1 and JAK2, such as by ruxolitinib, decreases the activation of signal transducer and activator of transcription (STAT)3/5, and while helping patients with myelofibrosis by improving splenomegaly and quality of life, it suppresses erythropoiesis, myelopoiesis, and thrombopoiesis, resulting in dose-related anemia, neutropenia, and thrombocytopenia in clinical studies.[2,3]

Pacritinib, a novel inhibitor of both JAK2 and fms-like receptor tyrosine kinase 3 (FLT3), was developed as a selective JAK2/FLT3 inhibitor with minimal suppression of JAK1.[4] It has demonstrated promising antitumor activity in lymphoid and myeloproliferative neoplasms in both preclinical studies[5,6] and clinical trials.[7–11] Evaluation of pacritinib in preclinical models of advanced myeloid malignancies and myelofibrosis demonstrated pharmacological activity. Two Phase I–II studies in patients with primary or secondary myelofibrosis showed that pacritinib reduced splenomegaly

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Dovepress
http://dx.doi.org/10.2147/JEP.S110702

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and symptom scores and could be used safely in patients regardless of baseline platelet counts. Interestingly, neither evidence of treatment-related decline in platelet counts[12,13] nor subsequent increase in anemia was reported. These data were recently confirmed in a Phase III trial.[14] Clinical trials of a majority of other JAK inhibitors that are less selective for JAK2 report dose-related anemia and/or thrombocytopenia.

The JAK2 mutation JAK2V617F is frequently found in patients with myeloproliferative neoplasms, occurring in almost all patients with polycythemia vera and in approximately half of the patients with essential thrombocythemia and idiopathic myelofibrosis.[1,15] This gain-of-function mutation results in the expression of a constitutively activated JAK2.[16] In most of the patients with germ-line JAK2, other mutations that activate this pathway have been recently discovered, including mutations in calreticulin and the thrombopoietin receptor gene (MPL).[17]

As an inhibitor of FLT3, pacritinib may have utility in the treatment of leukemia. A family of class III receptor tyrosine kinases, including c-fms, c-Kit, FLT3, and platelet-derived growth factor receptors α and β, are important in the maintenance, growth, and development of hematopoietic and nonhematopoietic cells.[18] In acute myeloid leukemia (AML), FLT3 mutations are the most frequent genetic mutations and are involved in the signaling pathway of autonomous proliferation and differentiation block in leukemia cells.[19] In addition, several clinical studies have confirmed that FLT3 internal tandem duplications are strongly associated with a poor prognosis.[19] Because high-dose chemotherapy and stem cell transplantation cannot overcome the adverse effects of FLT3 mutations,[19] the development of FLT3 inhibitors is a promising therapeutic strategy. Although JAK2V617F mutation rarely occurs in de novo AML, STAT3 activation is common.[20] Since STAT proteins are phosphorylated and activated by JAKs, the frequent pSTAT activation in AML suggests the involvement of JAK2 extrinsic regulators and other proteins in the JAK–STAT pathway. In addition, JAK–STAT represents one alternate pathway by which leukemic cells circumvent FLT3 inhibition. In vitro studies show that FLT3 inhibitors upregulate the JAK–STAT pathway and that JAK2 inhibition may overcome resistance to FLT3 inhibition, suggesting that dual inhibition may improve outcomes in AML.[5]

To help elucidate the mechanisms underlying pacritinib's lack of hematopoietic suppression despite its low nanomolar inhibition of JAK2/STAT3 and to identify other target kinases, we performed a kinome-wide screen to evaluate its spectrum of kinase inhibition.

Methods

Materials

Pacritinib was provided by CTI BioPharma, Corp. (Seattle, WA, USA). Other kinase inhibitors outlined in Table S1 were obtained either from Selleckchem (Houston, TX, USA) or Sigma-Aldrich Co. (St Louis, MO, USA), with an average purity of >98%. Kinases were purchased from Thermo Fisher Scientific (Waltham, MA, USA), SignalChem (Richmond, BC, Canada), ProQinase GmbH (Freiburg, Germany), or Carna Biosciences Inc. (Kobe, Japan).

Kinase assay methods

In vitro profiling of the 439-member kinase panel was performed at Reaction Biology Corporation (Malvern, PA, USA) using the HotSpot assay platform.[21] Pacritinib was tested through the full kinase panel to confirm both its activities in relation to its primary targets (JAK2, JAK2V617F, and FLT3) and its activities in relation to kinases not in our original conceptualization. The gold standard for kinase profiling, HotSpot technology, is a miniaturized assay platform for radioisotope-based filter binding.

Briefly, kinase–substrate pairs along with the required cofactors were prepared in a reaction buffer containing 20 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (pH 7.5), 10 mM magnesium chloride ($MgCl_2$), 1 mM ethylene glycol tetraacetic acid, 0.02% Brij 35® (G-Biosciences, St. Louis, MO, USA), 0.02 mg/mL bovine serum albumin, 0.1 mM sodium orthovanadate (Na_3VO_4), 2 mM dithiothreitol, and 1% dimethyl sulfoxide. Small test molecules were then added into the reaction mixture, and approximately 20 minutes later, 10 μM of a mixture of nonradiolabeled adenosine triphosphate (ATP) and ^{33}P-labeled ATP was combined to start the reaction. Reactions were carried out at 25°C for 120 minutes. The reaction mixtures were then spotted onto P81 ion-exchange filter paper. The dry filters were then washed in 0.75% phosphoric acid to remove any unbound phosphate. After subtracting the background from the control reactions, which contained the inactive enzyme, substrate, and radiolabeled ATP, kinase activity was expressed as the percent of kinase activity remaining in the test samples compared to the vehicle (dimethyl sulfoxide) reactions.

For the screening assays, pacritinib was tested at 100 nM. For all kinases that were inhibited by >50% at 100 nM, graduated dosing from 1 to 100 nM was performed. The half maximal inhibitory concentration (IC_{50}) values and curve fits were obtained using Prism® (GraphPad Software, Inc., La Jolla, CA, USA). Kinome tree representations were prepared

using Kinome Activity Mapper (Reaction Biology Corporation, Malvern, PA, USA). For reference, mean peak plasma concentrations of free pacritinib at steady state were calculated after administering the standard daily dose of 400 mg.

Statistical methods

Outlier detection

Raw data were measured as the percentage of compound activity for each kinase–substrate pair in duplicate. Negative values were truncated to zero, and any pairs with missing observations or duplicate values were removed from the analysis. For each pair, the coefficient of variation was calculated, and the difference (D) from duplicate observations used kernel density estimation and quantile–quantile plots to be double exponentially distributed.[21] Using maximum likelihood methods,[22] location and scale parameters were estimated, as were mean and standard deviation. To account for the noise inherent in assay measurements, any observation within 1 standard deviation of the mean distribution of D was further analyzed for compound activity.[21]

Based on the resulting data set, the method to detect distribution-based outliers was then applied to the coefficient of variation.[23] To detect outliers, data (except for the highest and lowest 1%) were positioned onto the quantile–quantile plot for log-normal distribution[21] and the data parameters were robustly estimated.[22] To determine whether extreme data points were outliers, we computed the threshold to set the expected range of observations. Any result beyond the threshold of the coefficient of variation was considered an outlier and excluded from further analysis.

Hierarchical clustering

For data on kinase activity, hierarchical clustering was conducted as described previously.[21]

Results

Pacritinib inhibited kinases in several unrelated families in the <50 nM range (Table 1). Pacritinib suppressed all members of the JAK/FLT pathways at low nanomolar concentrations, except for JAK1, on which it was inactive at 100 nM (82% control). For reference, after administering the daily dose of 400 mg, the mean peak plasma concentrations of pacritinib free drug at steady state equaled approximately 200 nM, while the total steady-state levels of pacritinib were in the 5–7 μM range (Figure 1). Hence, the 100 nM concentration of pacritinib evaluated in this study is readily achievable clinically.

Along with the anticipated inhibition of JAK2, FLT3, and their respective common mutations, pacritinib also inhibited several kinases in other families identified as potential therapeutic targets in oncology, including IL-1 receptor-associated kinase 1 (IRAK1) and c-fms (colony-stimulating factor 1

Table 1 Kinase inhibition profile for pacritinib at 100 nM concentration[a]

Kinase	IC_{50} (nM)	Ligand
JAK-associated kinases		
JAK1	Indeterminate	IL-2, IFN
JAK2	6.0	EPO-R, MPL
JAK2 (V617F)	9.4	
FLT3	14.8	FLT3 ligand
FLT3 (ITD)	13.4	
FLT3 (D835Y)	4.7	
TYK2	27.0	IL-12, IFN-α and -β, IL-10, IL-2
JAK3	18.3	Type I cytokine family receptors in lymphoid and NK cells; IL-8 in neutrophils
Non-JAK tyrosine kinases		
TRKC	18.4	NT-growth factor (neuronal tumors)
TNK1	15.0	Nonreceptor TK (ras/raf/MAPK pathway)
ROS1	18.4	Insulin receptor family; proto-oncogene
c-kit	43.8	c-kit ligand (Steele factor)
c-src	46.7	Proto-oncogene
CSF1R (c-fms)	39.5	CSF1
Nontyrosine kinases of interest		
HIPK4	14.5	P53 serine 9
IRAK1	13.6	IL-1 (serine/threonine-protein kinase)

Note: [a]Kinases with IC_{50} <50 nM are shown.

Abbreviations: CSF1R, colony-stimulating factor 1 receptor; EPO-R, erythropoietin receptor; FLT3, fms-like receptor tyrosine kinase 3; HIPK4, homeodomain-interacting protein kinase 4; IC_{50}, half maximal inhibitory concentration; IFN, interferon; IL, interleukin; IRAK1, interleukin-1 receptor-associated kinase 1; ITD, internal tandem duplication; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; MPL, thrombopoietin receptor gene; NK, natural killer; NT, neurotrophin; TK, tyrosine kinase; TYK2, tyrosine kinase 2; TNK1, tyrosine kinase nonreceptor 1; TRKC, Tropomyosin receptor kinase C.

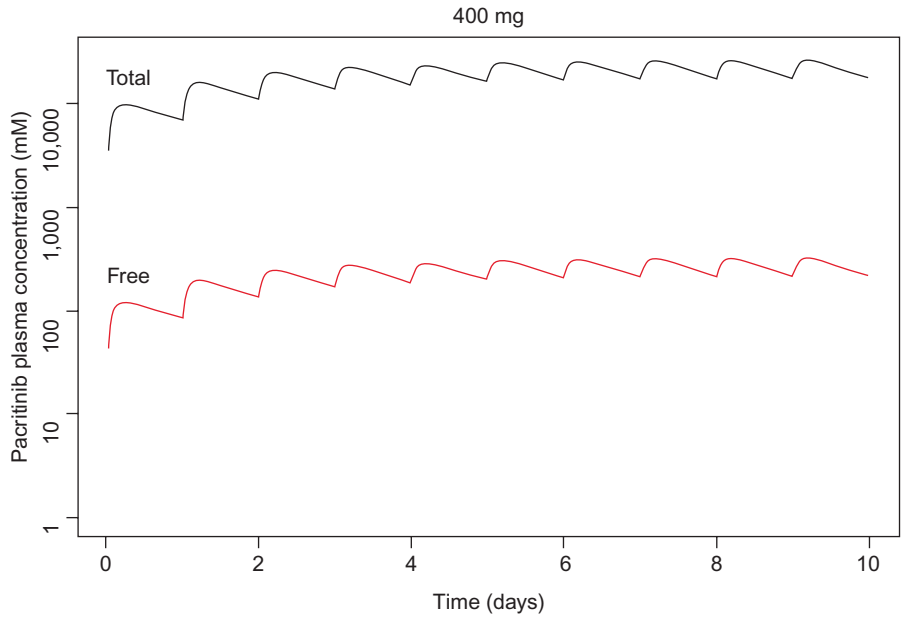

Figure 1 Simulated steady-state peak plasma concentration (C_{max}) for plasma protein-bound and free pacritinib after administration of 400 mg daily dosage of pacritinib in humans.

receptor [CSF1R]),[24] ROS1,[25] and tyrosine kinase nonreceptor 1.[26] The kinome map (Figure 2) shows relative pacritinib inhibition, demonstrating a high level of inhibitory activity against specific kinases in disparate families.

Phosphorylated CSF1R at concentrations <1 μM was inhibited in studies of normal human macrophages and macrophages from patients with chronic lymphocytic leukemia.[27] In addition, phosphorylated IRAK1 was inhibited in myeloid leukemia cell lines and fresh samples from patients with acute myelogenous leukemia at concentrations <500 nM.[28]

Discussion

Since 2001, more than 20 kinase inhibitors have received approval from the US Food and Drug Administration for the treatment of patients with cancers and inflammatory diseases. Most of them are type 1 inhibitors that target the ATP binding site. With the identification of the common JAK2 mutation (V617F), a number of type 1 kinase inhibitors entered development. Ruxolitinib, a JAK1 and JAK2 inhibitor, was the first to receive regulatory approval for the treatment of intermediate- or high-risk myelofibrosis and hydroxyurea-resistant polycythemia vera.[29] Treatment with ruxolitinib improves disease-related symptoms, reduces spleen size in both diseases, and appears to be associated with a survival advantage. However, ruxolitinib is associated with dose-related anemia and thrombocytopenia and its use is not recommended in patients with severe thrombocytopenia.[2,30] Pacritinib is also a type 1 ATP-binding site inhibitor, but unlike ruxolitinib and most other JAK inhibitors in development (Table 2),[31] pacritinib shows specificity for JAK2 and does not affect JAK1. In clinical studies using oral, once-daily dosing at 400 mg, pacritinib achieves steady-state concentrations of approximately 200 nM of free drug (Figure 1) – substantially above the IC_{50} values of the other recombinant kinases shown – and in most patients, pacritinib does not appear to cause either thrombocytopenia or anemia.

The published results of kinase inhibition profiles of other JAK2 inhibitors in development indicate that except for pacritinib, all inhibit JAK1, JAK2, and JAK3.[32–34] Unfortunately, kinome-wide screening has not been published for JAK inhibitors, except for momelotinib. Our kinome analysis discovered important inhibitory effects on these formerly overlooked kinases, which adds dimension to the mechanisms of action and potential targets with therapeutic benefit of pacritinib.

Of the type 1 inhibitors for myeloproliferative disorders, ruxolitinib shows low nanomolar inhibition of JAK1, the kinase triggered by the activation of interferon, IL-6, IL-2, and other lymphokines, which is important in signaling by innate and acquired immune responses.[32,35] Suppression of JAK1 phosphorylation is associated with a decreased ability to clear viral infections.[32,35] Moreover, suppression of JAK1 signaling decreases natural killer and dendritic cell function, further impairing the immune response.[36–38] Suppression of these signaling pathways may be associated with the opportunistic infections that have been associated with

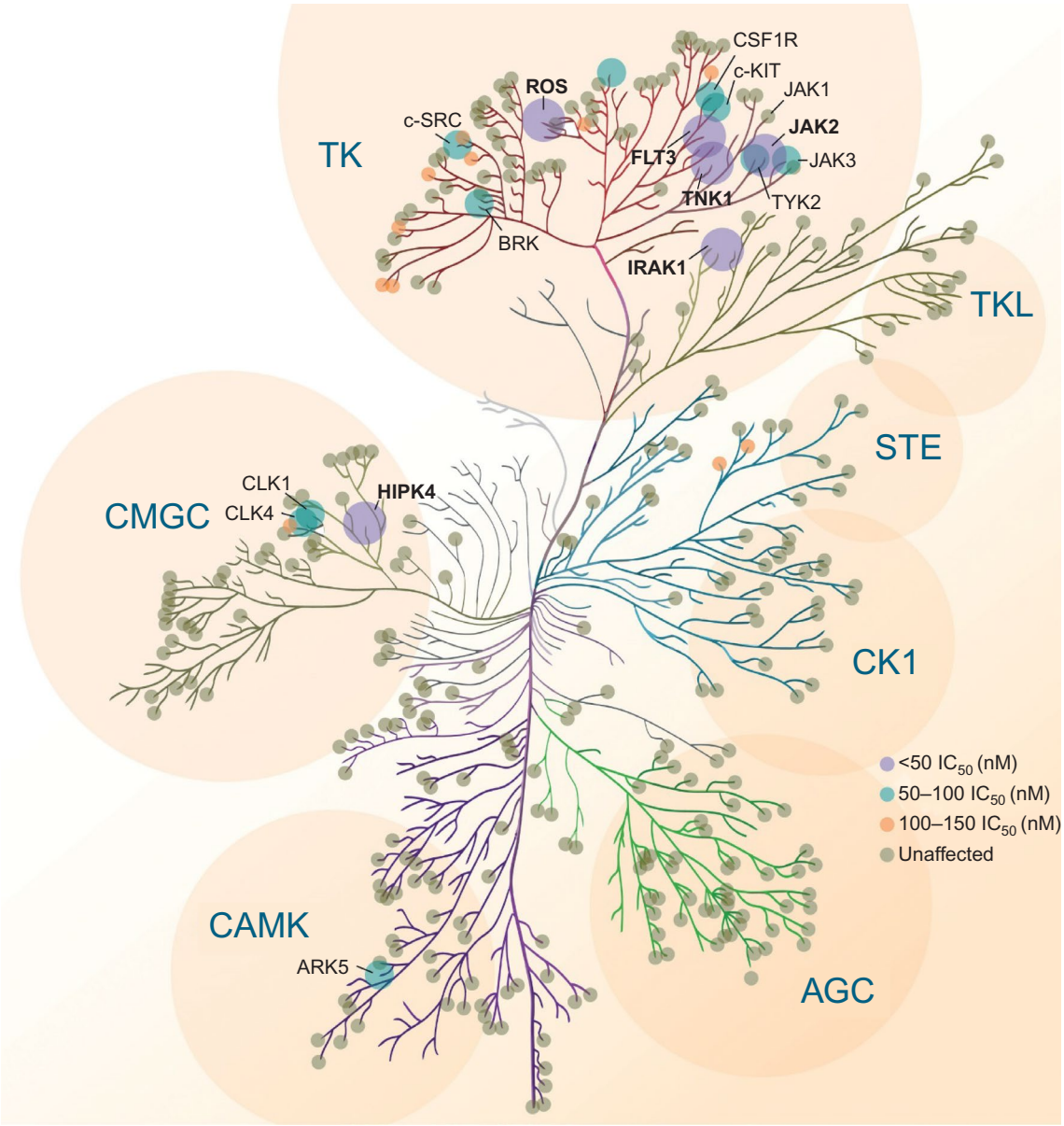

Figure 2 Pacritinib activity kinome map.
Abbreviations: AGC, protein kinase A, G, C group; ARK, Beta-adrenergic receptor kinase; BRK, breast tumor kinase; CK1, casein kinase; CLK, CDC Like Kinase 1; CSF1R, colony-stimulating factor 1 receptor; FLT3, fms-like receptor tyrosine kinase 3; HIPK4, homeodomain-interacting protein kinase 4; IC_{50}, half maximal inhibitory concentration; IRAK1, interleukin-1 receptor-associated kinase 1; JAK, Janus kinase; STE, homolog of sterile; TK, tyrosine kinase; TKL, tyrosine kinase-like group of kinases; TNK1, tyrosine kinase nonreceptor 1; TYK2, tyrosine kinase 2.

ruxolitinib therapy.[2,3,38,39] The lack of suppression of signaling mediated by JAK1 may have a role in the lack of myelosuppression associated with pacritinib, although the mechanism remains to be elucidated.

The suppression of inflammatory pathways has been associated with the production of hematopoietic inhibitory cytokines such as members of the tumor necrosis factor family, transforming growth factor beta, and macrophage inflammatory protein 1 alpha.[24] Although pacritinib does not suppress signaling through JAK1 but does produce early-onset reduction in symptoms associated with myelofibrosis (data on file), our current results suggest that this may occur due to its inhibition of inflammatory pathways mediated by IRAK1 and CSF1R. Serine–threonine kinase IRAK1 is a critical effector of the myeloproliferative diseases associated with the loss of microRNA-146a and the dysregulation of NF-κB.[40,41] IRAK1 is activated by Toll-like receptors through a complex with MYD88,[42] the main system for recognizing foreign stimuli such as lipopolysaccharide for the innate immune system, and has a critical role in the inflammatory response.[36] IRAK1 may prove to be a critical signal in the interaction between oncogenesis and the inflammatory response. Moreover, activating mutations in MYD88 are associated with a number of neoplastic diseases, including

Table 2 Relative kinase profiles comparing pacritinib, momelotinib, ruxolitinib, and fedratinib

Kinase	IC_{50} (nM)		IC_{50} at 1 μM[34]	
	Pacritinib	Momelotinib[32,33]	Ruxolitinib	Fedratinib
JAK-associated kinases				
JAK1	+	++++	++	++
JAK2	++++	++++	++	++
JAK2 (V617F)	++++	+	NR	NR
FLT3	++++	+++	NR	NR
FLT3 (ITD)	++++	NR	NR	++
FLT3 (D835Y)	++++	NR	NR	++
TYK2	+++	NR	++	++
JAK3	++++	+	++	++
Non-JAK tyrosine kinases				
TRKC	++++	NR	NR	NR
TNK1	++++	NR	NR	NR
ROS1	++++	++	NR	NR
c-kit	+++	NR	NR	NR
c-src	+++	+	NR	NR
CSF1R (c-fms)	+++	++	NR	NR
Nontyrosine kinases of interest				
HIPK4	++++	++	NR	NR
IRAK1	++++	NR	NR	NR

Notes: Pacritinib scale: ++++, <25 nM; +++, 25 to <50 nM; +, >100 nM. Momelotinib scale: ++++, <25 nM[32]; +++, <100 nM; ++, <1 μM; +, >1 μM.[33] Ruxolitinib and fedratinib scales: ++, IC_{50} at 1 μM.

Abbreviations: CSF1R, colony-stimulating factor 1 receptor; FLT3, fms-like receptor tyrosine kinase 3; HIPK4, homeodomain-interacting protein kinase 4; IC_{50}, half maximal inhibitory concentration; IRAK1, interleukin-1 receptor-associated kinase 1; ITD, internal tandem duplication; JAK, Janus kinase; NR, not reported; TNK1, tyrosine kinase nonreceptor 1; TYK2, tyrosine kinase 2.

Waldenström macroglobulinemia.[42] IRAK1 has also been recently identified as a therapeutic target in myelodysplastic syndromes (MDS)[24] and AML.[43] In AML, IRAK1 may have a role in both stromal–tumor interactions and stromal protection from leukemic cells from AML-directed therapy.[24] In primary MDS, inhibiting IRAK1 in marrow or cell lines delayed proliferation and compromised MDS progenitor function.[24] Recent results demonstrated that pacritinib inhibits the phosphorylation of IRAK1 in AML cells and reduces both the viability and survival of AML cell lines harboring various mutations.[44] IRAK1 has also been shown to be upregulated in chronic myelomonocytic leukemia (CMML); pacritinib was found to have activity in primary CMML cells and synergistic activity when combined with 5-azacitidine.[45]

Activation of CSF1R, which is also inhibited by pacritinib, may also have an anti-inflammatory effect.[46] In addition, CSF1R activation has been shown to have an important role in interactions between nurse-like cells in protecting low-grade lymphomas and chronic lymphocytic leukemia cells from therapeutic interventions and supporting proliferation.[47] Recently, a CSF1R inhibitor has shown clinical activity in the treatment of tenosynovial giant cell tumors.[48]

Along with its lack of myelosuppression, the potential for pacritinib to interfere with microenvironmental tumor interactions through the suppression of IRAK1 and CSF1R suggests its potential for use in combination with established therapies in a variety of liquid and solid tumors. Pacritinib also inhibits the phosphorylation of CSF1R (c-fms), a relevant target in lymphoid and myeloproliferative neoplasms and potentially inflammatory autoimmune diseases.[49]

Other targets identified in the pacritinib kinome (Table 1) underlie other cancers and may prove important for their treatment. For non-JAK/FLT3 tyrosine kinases, ROS1 showed promising response to pacritinib and its ligand is a proto-oncogene that activates fusions in non-small-cell lung cancer and cholangiocarcinoma. Among non-JAK tyrosine kinases, CSF1R ligand accounts for mutations in CMML and AML and interacts with nurse-like cells in chronic lymphocytic leukemia and mantle cell lymphoma. For nontyrosine kinases of interest, IRAK1 is a proven target in MDS and CMML. These potential indications are under exploration in ongoing clinical trials.[50–53] Preliminary results from an ongoing clinical trial of pacritnib monotherapy in relapsed/primary refractory FLT3-mutated AML demonstrated an overall response rate of 17% (4/23), including three complete remissions with incomplete blood count recovery and one partial remission at the 28-day assessment period.[54] Most toxicities were low grade (grade 1–2) and the most frequent

were nausea/vomiting (53%), diarrhea (33%), and increased alanine aminotransferase (30%).[54]

The major limitations of this study include the acellular nature of the screening assays and the need to validate that the kinase inhibition identified has similar effects in neoplastic cells. This has been done for two of the kinases of most interest, CSF1R in human monocytes and IRAK1 in myeloid leukemic cell lines and fresh cells from patients with AML,[28] both of which will be fully described in other manuscripts. These findings will need to be corroborated through ongoing in vivo models of disease and in clinical trials. In addition, the effect of pacritinib on common mutations involving these kinases would be of interest to validate that the effects demonstrated in the kinase assays are relevant to common activating mutations and gene fusions associated with malignant transformation.

In conclusion, pacritinib inhibits a variety of tyrosine kinases (JAK2, JAK2V617F, FLT3) and nontyrosine kinases (CSF1R, IRAK1, tyrosine kinase nonreceptor 1, and ROS1) at low nanomolar levels. Pacritinib demonstrated combined ability to suppress signaling by kinases involved in microenvironmental tumor interactions as well as by kinases thought to be involved in tumor progression. Pacritinib suppresses known driver mutations in JAK2, FLT3, IRAK1, and CSF1R, and shows clinical tolerability and efficacy in both non-Hodgkin lymphoma and chronic myeloproliferative diseases. Combined with its lack of myelosuppression and potentially less immunosuppressive properties than most other JAK2 inhibitors that also suppress signaling through JAK1, pacritinib has the potential to be used in combination with established therapies for the treatment of patients with a broad variety of neoplastic and inflammatory diseases.

Acknowledgments

The data in this manuscript were initially presented at the 56th American Society of Hematology Annual Meeting, December 6, 2014 in San Francisco, CA, and the meeting abstract was published online as Singer J, et al *Blood*. 2014;124(21):1874 http://www.bloodjournal.org/content/124/21/1874. The authors of this manuscript received no grant funding for this work.

Disclosure

The authors have the following competing interests: JWS, SA: CTI BioPharma, Corp. (ctibiopharma.com): employment, equity ownership; RSK: Incyte Corporation (incyte.com), Celgene Corp. (celgene.com): research funding, advisory role; RM: Incyte Corporation (incyte.com), CTI BioPharma, Corp., NS Pharma, Inc. (nippon-shinyaku.co.jp), Gilead Science, Inc. (gilead.com), Celgene Corp. (celgene.com): research funding; and SV: CTI BioPharma, Corp.: research funding. Medical writing and editorial assistance was provided, under the direction of the authors, by Hope J Lafferty, AM, ELS. The authors report no other conflicts of interest in this work.

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