ORIGINAL RESEARCH

Pilot study of the antifibrotic effects of the multikinase inhibitor pacritinib in a mouse model of liver fibrosis

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Background: Fibrotic diseases result from an exuberant response to chronic inflammation. Myelofibrosis is the end result of inflammation in bone, caused by an inflammatory process triggered by production of abnormal myeloid cells driven by mutations affecting the JAK–STAT pathway. Inflammatory cytokine overproduction leads to increased mesenchymal cell proliferation, culminating in fibrosis. Although JAK2 inhibitors, such as the JAK1/2 inhibitor ruxolitinib and the JAK2/FLT3/CSF1R/IRAK1 inhibitor pacritinib suppress abnormal clone expansion in myelofibrosis, ruxolitinib does not appear to prevent or reverse bone-marrow fibrosis in most patients. In two Phase III clinical trials, pacritinib, however, demonstrated improvements in platelet counts and hemoglobin and reductions in transfusion burden in some patients with baseline cytopenias, suggesting it may improve bone-marrow function. Unlike ruxolitinib, pacritinib suppresses signaling through IRAK1, a key control point for inflammatory and fibrotic signaling.

Purpose: To investigate potential antifibrotic effects of pacritinib in an animal model of liver fibrosis relevant to the observed course of human disease.

Methods: Pacritinib, negative control (vehicle), and positive control (the angiotensin 2-receptor antagonist and PPARγ partial agonist telmisartan) were assessed in the murine Stelic animal model, which mimics the clinically observed progression from hepatic steatosis to nonalcoholic steatohepatitis, liver fibrosis, and hepatocellular carcinoma. Histopathological analysis used hematoxylin and eosin staining. Body and liver weight changes, nonalcoholic fatty-liver disease activity scores, and plasma cytokeratin 18 fragment levels (a biomarker of hepatic necrosis) were measured.

Results: Pacritinib-treated mice had significantly ($P<0.01$) reduced fibrotic areas in liver compared to vehicle control and significantly ($P<0.05$) lower levels of CK18. The antifibrotic effect of pacritinib was comparable to that of telmisartan, but without significant effects on fat accumulation.

Conclusion: These results, the first to demonstrate hepatic antifibrotic effects for pacritinib in an animal model of liver disease, provide preliminary support for potential clinical applications of pacritinib in fibrotic diseases other than myelofibrosis.

Keywords: Janus kinase 2, interleukin 1 receptor-associated kinase 1, colony-stimulating factor 1-receptor kinase, steatosis, myelofibrosis, liver fibrosis

Introduction

Fibrosis is the end product of chronic inflammation, and has aptly been called "a wound-healing response that has gone out of control."[1] Common to all fibrotic diseases are activation and proliferation of endothelial cells and fibroblasts in response to inflammation induced by Toll-like receptors that activate IRAK1 causing downstream inflammatory and profibrotic cytokines, including TGFβ. Fibroblasts subsequently

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differentiate into myofibroblasts and secrete increasing levels of extracellular matrix proteins including collagens.[2] This ultimately results in replacement of normal tissues with fibrotic tissue, along with attendant disruption in function that may be limited to single organs (eg, liver fibrosis, pulmonary fibrosis) or involve a systemic process, such as scleroderma.[3]

Myelofibrosis (MF) is a clonal hematopoietic neoplasm originating at the level of multipotential hematopoietic stem cells characterized by an inflammatory response in the bone marrow, leading to progressive fibrosis that impairs normal marrow function, resulting in cytopenias and extramedullary hematopoiesis.[4] A hallmark of bone-marrow response to MF cells is vascular proliferation[5] and increasing reticulin and collagen fibrosis in the bone marrow associated, with marked elevation in inflammatory cytokines, including TGFβ.[6] A central role for JAK–STAT signaling in the pathogenesis of myeloproliferative neoplasms, such as MF, was suggested by the discovery that a gain-of-function mutation in the *JAK2* gene (*JAK2*[V617F]) was found in 50%–60% of patients with MF.[7–9] In light of the unmet need for the treatment of patients with MF, inhibitors of JAK2 have been extensively studied in the clinic.

Ruxolitinib is an oral JAK1/2-kinase inhibitor that has been approved by the US Food and Drug Administration for patients with intermediate- or high-risk MF and those with polycythemia vera who have had inadequate response to or are intolerant of hydroxyurea.[10] Ruxolitinib has been shown to result in potent reductions in inflammatory cytokine-expression levels in patients with MF,[11] and its approval in MF was based on data from studies that demonstrated only modest reductions in bone-marrow fibrosis in a fraction of treated patients (16%), with fibrosis unchanged (32%) or progressing (19%) in a higher percentage of patients.[12–15] Therefore, effective antifibrotic strategies in MF remain an unmet need upon which translational research continues to focus.

Pacritinib is an oral inhibitor of JAK2, FLT3, CSF1R, and IRAK1[16–18] that has demonstrated activity in clinical studies of patients with MF and other myeloproliferative neoplasms.[19–22] In Phase II studies, pacritinib was associated with reduction in spleen volume and improvement in symptoms in patients with MF[20] and myeloid malignancies[22] without substantial limiting myelosuppression. In the randomized Phase III PERSIST-1[23] and PERSIST-2[20] studies of pacritinib versus best available therapy in patients with MF, improved hematopoietic function (eg, increased platelet count, increased hemoglobin, reduced transfusion burden) was noted in patients with baseline cytopenias, suggesting

restoration in marrow function possibly due to an effect in part on bone-marrow fibrosis.

The kinase profile of pacritinib was evaluated in a kinome-wide screening study and demonstrated – in addition to its effect on JAK2 – that it potently inhibits phosphorylation of IRAK1 at an IC_{50} <20 nM.[18] IRAK1 is a kinase situated at a critical juncture of inflammatory signaling from Toll-like receptors and cellular signaling by IL1 leading to downstream activation of both p38 MAPK and p-ERK. The biomarker profile of pacritinib was determined in a panel of human primary cell-based systems designed to model various disease states, including inflammation and fibrosis.[24] At physiologically relevant levels in a system modeling T-cell-dependent B-cell activation, pacritinib robustly reduced levels of the proinflammatory cytokines soluble IL6 (sIL6), TNFα, sIL17A, and sIL17F. Along with TGFβ, IL6 is crucial to promoting T_H17-cell differentiation.[25] These cells in turn secrete IL17A and IL17F, which promote neutrophil recruitment and infiltration, leading to inflammation and fibrosis.[26,27] IL17 also stimulates macrophages to produce proinflammatory cytokines.[28] In addition to its effects on biomarkers, pacritinib has been found to have an antiproliferative effect on endothelial cells and fibroblasts.[24] Inhibition of several key kinases involved in the elaboration of proinflammatory cytokines, particularly IL17A, and the noted antiproliferative effects provide a strong rationale for examining whether pacritinib is able to modify the fibrotic process in MF. Since clinical assessment of this hypothesis requires invasive procedures in clinical trials of extended duration, this was tested in a standard, well-characterized preclinical model of a fibrotic disease.

Liver fibrosis is a response to chronic hepatocyte injury[29] that may progress to end-stage cirrhosis.[30,31] Although it is potentially reversible,[32] there are currently no approved therapies that have been shown to modify the course of liver fibrosis. Numerous rodent models have been developed in which liver injury is induced by trauma, toxins, or chronic infections.[33–36] None of these, however, recapitulate the natural history commonly seen in patients with nonalcoholic (NA) liver disease. Recently, the Stelic animal model (STAM) was introduced.[37] In this model, designed to replicate the clinically observed, insulin-resistance-driven progression from hepatic steatosis to NA steatohepatitis (NASH), liver fibrosis, and hepatocellular carcinoma, neonatal male mice are treated with a carefully titrated dose of streptozotocin, followed by feeding with a high-fat diet. Fatty liver (FL) is induced by 5 weeks and NASH by 6 weeks in 100% of the animals. Here, we report the effects of pacritinib in the STAM mouse model, compare these with effects of telmisartan, an

angiotensin 2-receptor antagonist and PPARγ partial agonist with known antifibrotic effects in rodent models, and present hypotheses on the mechanisms potentially involved.

Methods
Animal model
The STAM mouse model has been described previously.[37] Two-day old male C57BL/6J mice (Japan SLC, Tokyo, Japan) were given a single 200 µg subcutaneous dose of streptozotocin. At 4 weeks of age, they were fed a sterilized high-fat diet (HFD32; CLEA, Tokyo, Japan). The sterilized high-fat diet and water were provided ad libitum. Animals were housed and cared for in accordance with the Japanese Pharmacology Society Guidelines for Animal Use,[38] and the study was approved by the SMC Laboratories institutional animal care and use committee. They were maintained in a specific-pathogen-free facility under controlled temperature, humidity, lighting, and air exchange. Pacritinib (CTI Bio-Pharma, Seattle, WA, USA) was suspended in vehicle consisting of 0.5% weight/volume methylcellulose (4,000 cP) and 0.1% volume/volume Tween 80. Telmisartan (Boehringer Ingelheim, Ingelheim am Rhein, Germany), the positive control, was dissolved in pure water. Groups consisted of eight mice each. The treatment schedule for the vehicle (negative control), pacritinib, and telmisartan is shown in Table 1. Body weight was recorded prior to treatment and then daily. Mice were observed for signs of toxicity, moribundity, and mortality for approximately 60 minutes after each administration. Animals were killed by exsanguination through direct cardiac puncture under ether anesthesia at 9 weeks of age.

Histopathological analysis
For hematoxylin and eosin (H&E) staining, sections were cut from paraffin blocks of liver tissue prefixed in Bouin solution and stained with Mayer's hematoxylin (Lillie's modification; Muto Pure Chemicals, Tokyo, Japan) and eosin (Wako Pure Chemicals Industries, Osaka, Japan) solution. NA fatty-liver disease (NAFLD) activity score was calculated according to the criteria of Kleiner et al.[39]

Plasma cytokeratin 18 fragment levels
Plasma CK18 (M30) level was quantified using the Mouse Cytokeratin 18-M30 enzyme-linked immunosorbent-assay kit (Cusabio, College Park, MD, USA) as per label instructions. Briefly, plasma (1:500 in sample diluent) was added to the wells and incubated at 37°C for 2 hours. After removal of liquid, biotin antibody was added and wells incubated at 37°C for 1 hour, then washed with buffer three times and incubated with 100 µL of HRP–avidin for 1 hour. Enzymatic activity was detected using tetramethylbenzidine substrate and absorbance measured at 450 nm after a 30-minute incubation.

Statistical tests
Statistical analyses were performed using Bonferroni multiple-comparison tests on GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). Results are expressed as the means ± SD.

Results
Deaths
Deaths prior to day 21 in the treatment groups were none in the vehicle group, one in the telmisartan group (day 10) and three in the pacritinib group (days 5, 7, and 10). For animals in the pacritinib group, severe weight loss was noted prior to death, but pathology results did not reveal any unusual findings (eg, vascular occlusion or cerebral bleeding). Based on these results, the 200 mg/kg pacritinib dose was deemed intolerable and mice treated with 150 mg/kg thereafter (Table 1). No further deaths occurred.

Weight changes
There was no significant difference in mean body weight between the vehicle and pacritinib groups on any day. The mean body weight of the positive control (telmisartan) group was significantly lower than that of the vehicle group on day 21, as assessed by the two-way analysis of variance (ANOVA) Bonferroni test (Figure 1). On the necropsy day (day 0 of week 9), there were no significant differences in mean body weight among any of the treatment groups, as assessed by the one-way ANOVA Bonferroni test (Figure 2A).

Table 1 Treatment schedule for all groups during weeks 6–9

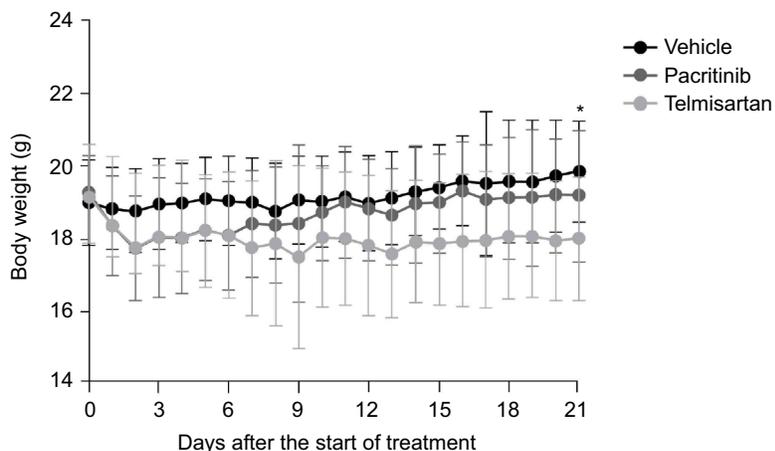| Test substance	Dose (mg/kg)	Volume (mg/kg)	Regimens	Death (week)
Vehicle	NA	10	Oral, twice daily, 6-9 weeks	9
Pacritinib	200	10	Oral, twice daily (day 0 to morning of day 10)	9
	150	10	Oral, twice daily (afternoon of day 10 to day 21)	
Telmisartan	10	10	Oral, once daily, 6–9 weeks	9

Abbreviation: NA, not applicable.

Figure 1 Body-weight changes in all treatment groups (n=8 per group). *P<0.05, telmisartan vs vehicle.
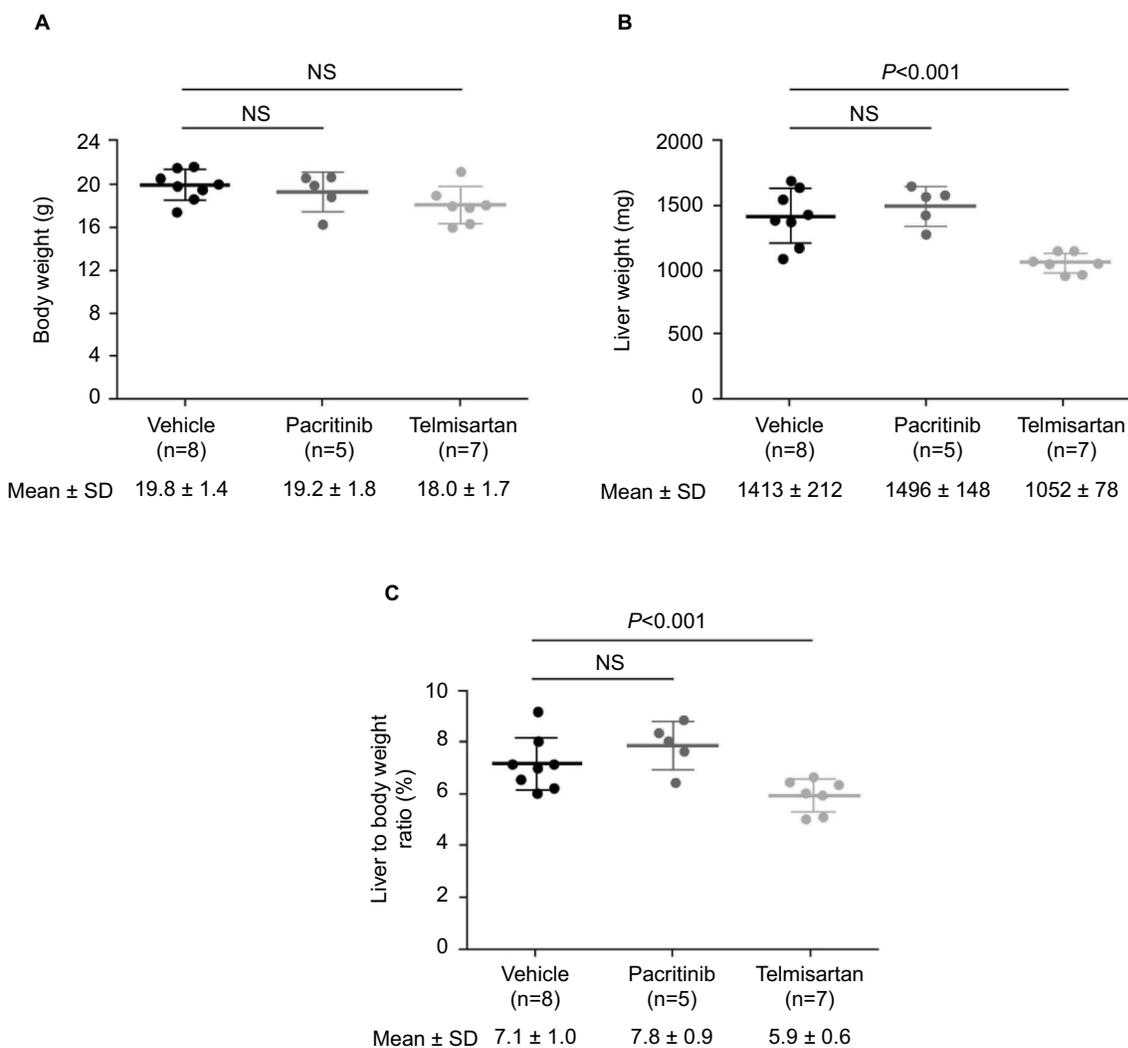

Figure 2 (**A**) Body weight, (**B**) liver weight, and (**C**) liver:body weight ratio on day of death.
Abbreviation: NS, not significant.

The telmisartan group had significantly decreased mean liver weight ($P<0.001$) and mean liver:body weight ratio ($P<0.05$) compared with the vehicle group (Figure 2, B and C), and there were no significant differences between the pacritinib and vehicle groups.

Histological analysis

Representative micrography of the H&E-stained liver sections is shown in Figure 3. Liver sections from the vehicle group exhibited severe micro- and macrovesicular fat deposition, hepatocellular ballooning, and inflammatory cell infiltration. The telmisartan group showed a significant ($P<0.01$) reduction in NAFLD-activity score compared with the vehicle group (Table 2, Figure 4). Although a trend toward lower steatosis and hepatocyte-ballooning scores were noted in the pacritinib group relative to the vehicle group, the difference in NAFLD score between the two groups was not significant. Representative micrography of Sirius red-stained

sections of liver is shown in Figure 5. Liver sections from the vehicle group exhibited collagen deposition in the pericentral region of liver lobules. Both the pacritinib and the telmisartan groups showed significant ($P<0.01$ for pacritinib, $P<0.001$ for telmisartan) decreases in the mean percentage of fibrosis area compared with the vehicle control group ($0.74\%\pm0.17\%$, $0.73\%\pm0.12\%$, and $1.08\%\pm0.16\%$, respectively).

Plasma CK18 levels

On the day of death, mean plasma CK18 M30 levels were 282.9 ± 24.2, 237.6 ± 29.5, and 357.7 ± 39.7 mIU/mL for vehicle, pacritinib, and telmisartan, respectively (Figure 6). Relative to vehicle (negative control) CK18 M30 levels were significantly lower ($P<0.05$) in the pacritinib group and significantly higher ($P<0.001$) in the telmisartan group.

Discussion

Pharmacological inhibition of JAK2 has previously been reported to attenuate liver fibrosis in rodent models, although the mechanism has not been fully elucidated.[40] The observation that pacritinib reduces levels of sIL6, sIL17A, and sIL17F in a system of peripheral blood mononuclear cells, fibroblasts, and endothelial cells that models T-cell-dependent B-cell activation[24] suggests a number of potential intervention points in addition to JAK2 signaling through which it could exert an anti-inflammatory and/or antifibrotic effect (Figure 7).

IRAK1 is a serine/threonine kinase that plays a key role in the IL1–IL6 axis[41] and has a major role in steatosis associated liver cirrhosis and progression to hepatocellular carcinoma.[42] Knockdown of *IRAK1* in human hepatic stellate cells (HSCs) results in reductions in the release of inflammatory cytokines associated with local inflammation and promotion of fibrosis.[43] IRAK1 is critical to signaling by Toll-like receptors activated by fatty acids and other lipid derivatives, and appears to be central to lipid-mediated inflammation.[44,45] In mouse models of acute and chronic inflammation, IRAK1 deletion dampens inflammatory responses by disfavoring naïve T-cell differentiation into T_H17 cells, thereby decreasing levels of IL17, the proinflammatory cytokine that plays a pivotal role
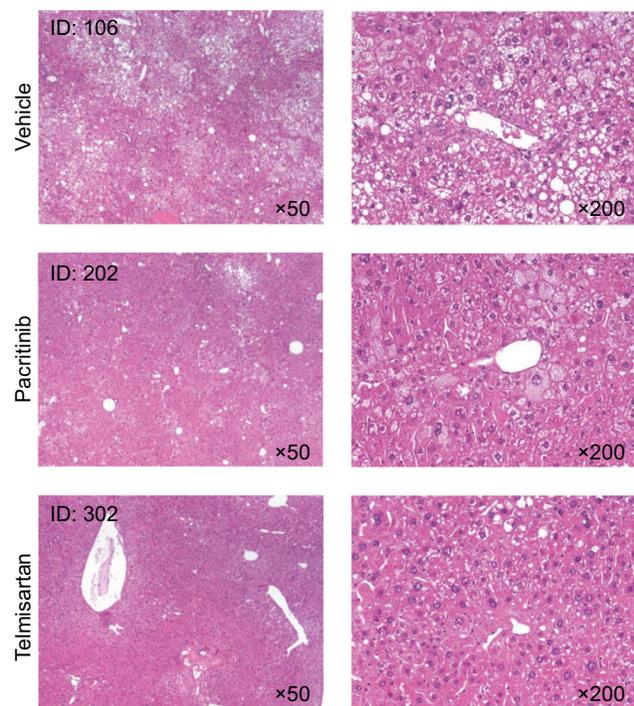
Figure 3 Representative micrography of H&E-stained liver sections on day of death.
**Abbreviation:** H&E, hematoxylin and eosin.

Table 2 Nonalcoholic fatty-liver disease-activity score[39]

| Group	n	Steatosis				Lobular inflammation				Hepatocyte ballooning			
		0	1	2	3	0	1	2	3	0	1	2	
Vehicle	8	–	6	2	–	–	1	6	1	–	1	7	5.1±0.6
Pacritinib	5	3	2	–	–	–	2	1	2	1	2	2	3.6±1.5
Telmisartan	7	3	4	–	–	1	5	1	–	2	1	4	2.9±1.6
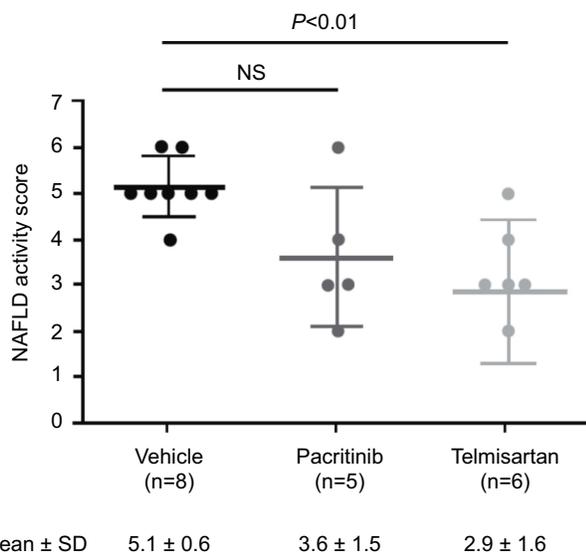
Figure 4 NAFLD-activity scores for all groups on day of death.
Abbreviations: NAFLD, nonalcoholic fatty-liver disease; NS, not significant.

| Mean ± SD | 5.1 ± 0.6 | 3.6 ± 1.5 | 2.9 ± 1.6 |
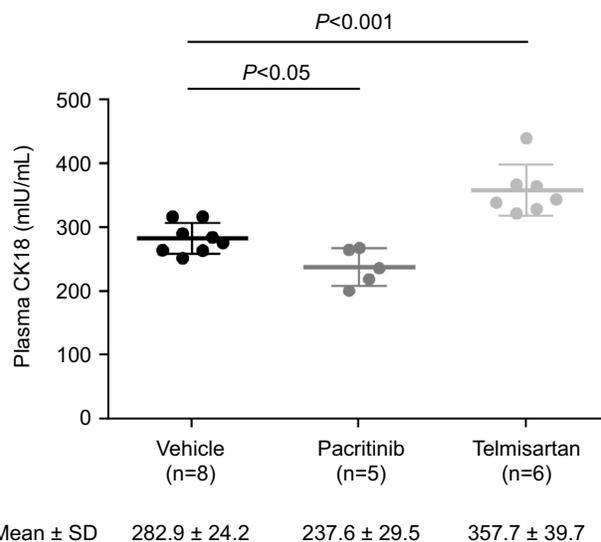
Figure 6 Plasma levels of CK18 fragment M30 on day of death.

| Mean ± SD | 282.9 ± 24.2 | 237.6 ± 29.5 | 357.7 ± 39.7 |
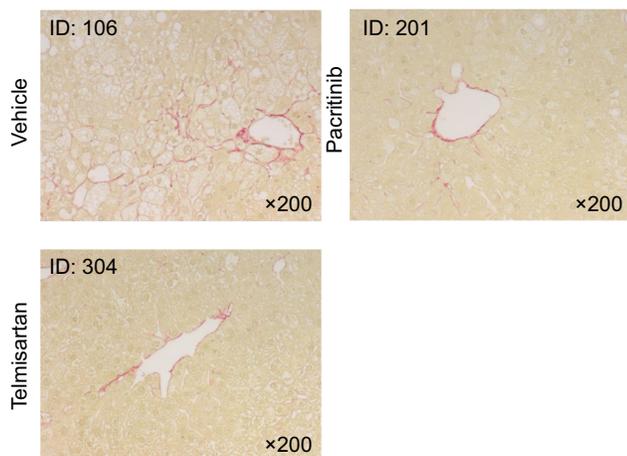
Figure 5 Representative photomicrographs of Sirius red-stained liver sections on day of death.

in HSC activation, which gives rise to ~90% of myofibroblasts in liver-fibrosis models.[46–48] Therefore, inhibition of IRAK1 by pacritinib may underlie the observed reduction in IL6 levels, causing a consequent depletion of $T_H 17$ cells, IL17A, and IL17F.

Pacritinib may also inhibit $T_H 17$-cell differentiation through effects on the transcription factor RORC (RORγt in mice), induction of which depends on full activation of STAT3 in processes dependent upon IRAK1 and JAK2, both of which pacritinib inhibits. Following differentiation, JAK2 associated with the IL23/IL12Rβ1 receptor plays a role in increased IL17A transcription, thus representing another possible intervention point for pacritinib. Finally, pacritinib

also inhibits CSF1R kinase, thereby disfavoring the differentiation of monocytes to macrophages,[49] which promote myofibroblast survival and contribute to the development of liver fibrosis.[50,51] Notably, controlling macrophage differentiation as an antifibrotic strategy in MF via a different pathway (using recombinant human pentraxin 2) is the subject of ongoing clinical investigation.[52]

The present study investigated whether pacritinib, acting through one or more of these mechanisms, could exert antifibrotic effects in a mouse model that recapitulated the clinical progression commonly seen in human liver disease. In the STAM mouse model, pacritinib had no significant effect on body weight, liver weight, liver:body weight ratio, or NAFLD score relative to vehicle. As such, it did not significantly affect fat accumulation, the inflammatory trigger for liver fibrosis. Nonetheless, it significantly reduced fibrotic area, suggesting inhibition of the inflammatory and subsequent fibrotic response to steatosis. In the same assay, telmisartan, an angiotensin 2 receptor antagonist and PPARγ partial agonist that has demonstrated antifibrotic[53] and hepatoprotective[54] activity in rodent models, most likely through downregulation of TGFβ and suppression of HSC activation,[55,56] was used as a positive control. In contrast to pacritinib, telmisartan had significant effects on liver weight, liver:body weight ratio, and NAFLD score, in addition to fibrosis area. These results are consistent with a clinical study that reported significantly improved NAFLD and fibrosis scores for telmisartan plus lifestyle modifications relative to lifestyle modifications alone in human patients with NASH.[57] Differential effects of pacritinib and telmisartan in the STAM
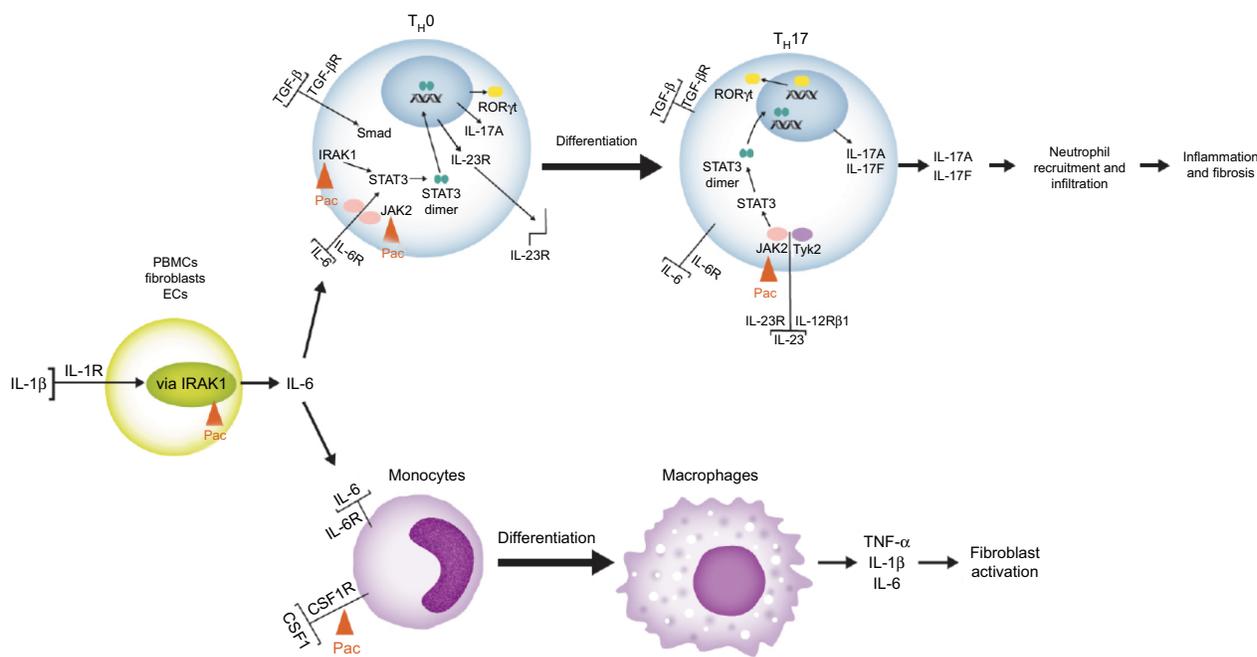
Figure 7 Inflammation pathways and potential pacritinib intervention points in fibrosis.
Notes: In peripheral blood monocytes, fibroblasts and endothelial cells, binding of IL1β induces IL6 secretion in a process involving IRAK1. Observed phenotypic reductions in IL6 levels induced by pacritinib may reflect IRAK1 inhibition, with downstream effects on inflammation, fibroblast activation, and fibrosis. Upper schema: in naïve T cells, binding of IL6 (together with TGFβ) ultimately induces differentiation into T_H17 cells. Both JAK2 and IRAK1 (both of which pacritinib inhibits) are necessary for STAT3 activation; activated STAT3 dimerizes and translocates to the nucleus, where it binds to DNA, leading to expression of the transcription factor RORγt, which is critical for T_H17-cell differentiation, and export of IL23R. In T_H17 cells, IL23 binds to the IL23R/IL12Rβ1 receptor to activate STAT3 in another JAK2-dependent process; binding of the STAT3 dimer promotes expression of IL17A, while binding of RORγt promotes the expression of IL17A and IL17F, both of which are secreted by T_H17 cells, leading to neutrophil recruitment and infiltration and downstream inflammation and fibrosis. Lower schema: IL6 acts as a switch to induce differentiation of monocytes to macrophages rather than dendritic cells; a second critical factor in monocyte differentiation is CSF1R, the kinase activity of which pacritinib also inhibits; macrophages secrete the proinflammatory cytokines TNFα, IL1β, and IL6, which are involved in fibroblast activation.
Abbreviations: Pac, pacritinib; PBMCs, peripheral blood mononuclear cells; ECs, endothelial cells.

model likely reflect the additional mechanism of action, PPARγ partial agonism, associated with telmisartan. This has effects on hepatic fatty oxidation, hepatic lipogenesis, and peripheral as well as hepatic insulin sensitivity.[58] Finally, the present study examined levels of circulating CK18 fragment in all three groups of animals. Plasma CK18 fragment levels represent a biomarker of the extent of hepatocyte apoptosis, with increased levels predicting clinically observed liver fibrosis,[59] NASH occurrence, and NASH severity.[60] CK18 levels were significantly reduced relative to vehicle control in animals treated with pacritinib, a finding in line with the significantly reduced extent of liver fibrosis observed by histopathology in this group.

The present pilot translational study has several limitations. A relatively small number of animals was tested, and biomarkers that could link the observed activity of pacritinib to the proposed mechanisms of action were not examined. Further studies are needed to elucidate the pharmacological basis for the effects of pacritinib in liver fibrosis. Bearing these caveats in mind, this is the first study to demonstrate

hepatic antifibrotic effects for pacritinib in a nonclinical model of liver disease.

The results of this study lend support to longitudinal assessment of the effect of pacritinib on marrow fibrosis in patients with MF enrolled in upcoming clinical trials, and moreover provide preliminary support to pilot clinical development in liver cirrhosis, along with other fibrotic conditions, such as pulmonary fibrosis and scleroderma.

Acknowledgments

Disclosure

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