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ORIGINAL ARTICLE

Pacritinib (SB1518), a JAK2/FLT3 inhibitor for the treatment of acute myeloid leukemia

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FMS-like tyrosine kinase 3 (*FLT3*) is the most commonly mutated gene found in acute myeloid leukemia (AML) patients and its activating mutations have been proven to be a negative prognostic marker for clinical outcome. Pacritinib (SB1518) is a tyrosine kinase inhibitor (TKI) with equipotent activity against FLT3 (IC_{50} = 22 nM) and Janus kinase 2 (JAK2, IC_{50} = 23 nM). Pacritinib inhibits FLT3 phosphorylation and downstream STAT, MAPK and PI3 K signaling in FLT3-internal-tandem duplication (ITD), FLT3-wt cells and primary AML blast cells. Oral administration of pacritinib in murine models of FLT3-ITD-driven AML led to significant inhibition of primary tumor growth and lung metastasis. Upregulation of JAK2 in FLT3-TKI-resistant AML cells was identified as a potential mechanism of resistance to selective FLT3 inhibition. This resistance could be overcome by the combined FLT3 and JAK2 activities of pacritinib in this cellular model. Our findings provide a rationale for the clinical evaluation of pacritinib in AML including patients resistant to FLT3-TKI therapy.
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Introduction

Acute myeloid leukemia (AML) is characterized by aberrant proliferation of myeloid progenitor cells that have lost the ability to differentiate into mature cells. There are more than 12 000 new cases in the United States every year.[1] Up to 35% of AML patients harbor a mutation in the FMS-like tyrosine kinase 3 (*FLT3*) gene, a member of the class III receptor tyrosine kinase family.[2] Constitutively activated mutants of FLT3 have been shown to be transforming in cultured cell lines and leukemogenic in mice.[3] Two major classes of activating mutations have been identified: internal-tandem duplications (ITDs) of 3 to 400 bp within the juxta-membrane domain or point mutations in the tyrosine kinase domain.[2] These genetic alterations give rise to constitutive signaling of FLT3 and activation of downstream oncogenic pathways, leading to dysregulated cell cycle control and apoptosis.[4,5] Clinically, FLT3-ITD is a negative prognostic marker that is associated with increased relapse rate, increased blast count and poor overall survival.[3,6,7] Overexpression of wild-type FLT3 in AML patients has been also shown to increase FLT3 auto-phosphorylation and was an unfavorable prognostic factor for overall survival.[8] Therefore, aberrantly activated FLT3 kinase is a validated molecular target for the treatment of AML.

Several small-molecule FLT3 inhibitors have been evaluated in clinical trials, either as single agents or in combination with chemotherapy.[2,9] To date, these candidates either did not generate sufficient initial response or failed to sustain therapeutic benefit, primarily due to development of secondary resistance.[10] Clinical data demonstrates that peripheral blood blasts decline, but bone marrow responses are very rare.[11,12] Among the possible mechanisms for these failures is the existence of independent alternative survival pathways that leukemic cells can tap into, either through further genetic lesions or metabolic adaptation.[2] These pathways may include components of the mTOR-PI3K-Akt, JAK-STAT or Ras-MAPK axes.[2] We envisaged that simultaneous targeting of additional independent pathways will render leukemic cells less likely to escape FLT3 mono-inhibition. In this respect, targeting JAK2 provides an interesting opportunity due to several pertinent observations: (a) JAK2 mutations have been reported in rare cases of AML, (b) phospho-JAK2 has been found to be elevated in AML primary samples and (c) the suppressor of cytokine signaling 1/2/3, negative regulators of JAK signaling, have been found to be downregulated in FLT3-TKI-resistant FLT3-ITD harboring AML cells.[13,14]

Pacritinib is a novel low molecular-weight compound with potent inhibitory activities against FLT3 and JAK2.[15] We have previously shown that pacritinib inhibits JAK2-mediated effects on cellular signaling, functional responses and disease symptoms in models of myeloid disease generated by activation of JAK2 signaling.[16] Pacritinib has also shown promising clinical activity in phase 1/2 trials in advanced myeloid and lymphoid malignancies.[17,18] Herein, we present new data indicating that blockade of FLT3 in conjunction with JAK2 signaling could enhance clinical benefit for AML patients harboring a FLT3-ITD mutation. This preclinical data provides a rationale for a clinical evaluation of pacritinib in AML including patients resistant to FLT3-TKI therapy.

Materials and methods

Compounds and reagents

Pacritinib (SB1518) was discovered and synthesized by S*BIO Pte Ltd. (Singapore, Singapore).[15,16] Sunitinib was obtained from Sequoia Research Products Ltd. (Pangbourne, UK). JAK inhibitor 1 (abbreviated as JAKi-1), a pan-JAKi (cat#420099) was purchased from Calbiochem (Gibbstown, NJ, USA). ABT-869 (linifanib, cat#1638) and VX-680 (cat#1540) were purchased from Axon Medchem BV (Groningen, Netherlands). INCB018424 (ruxolitinib) was purchased from Active Biochem (Maplewood, NJ, USA, cat#: A-1134). Stock solutions were

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prepared in dimethyl sulfoxide, with final dimethyl sulfoxide concentration of 0.1% in cell-based assays. For *in vivo* studies, dosing solutions were prepared in 0.5% methylcellulose (w/v) and 0.1% Tween-80 in H_2O (MC/Tween). Doses shown are free-base equivalents of pacritinib.

Cell culture and proliferation assay

SET-2, KG-1, ME-1, SH-2, F36-P, HEL92.7.1, MOLM-13 and MOLM-16 cells were obtained from DSMZ (Braunschweig, Germany). MV4-11, THP-1 and HL-60 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). MV4-11-P and MV4-11-R have been described previously.[13] All cell lines were cultivated according to the vendor's instructions using fetal bovine serum from PAA Laboratories (Pasching, Austria).

For proliferation assays in 96-well plates, 2000–6000 cells/well were seeded and treated the same day with compounds (in triplicates) at concentrations up to 10 μM for 48 h. Cell viability was monitored using the CellTiter-Glo assay (Promega, Madison, WI, USA). Dose response curves were plotted to determine IC_{50} values for the compounds using the XL-fit software (IDBS Ltd., Alameda, CA, USA).

To determine the *in vitro* synergy of two drugs they were combined at a constant ratio (based on the individual drug's IC_{50}), with nine concentration steps, threefold dilutions and the highest dose used being 8 × IC_{50} concentrations.[19] Synergy was determined using the CompuSyn software (v2007; ComboSyn Inc., Paramus, NJ, USA).

Primary cells, either peripheral blood mononuclear cells or bone marrow mononuclear cells from AML patients were obtained from AllCells (Emeryville, CA, USA) and ProteoGenex (Culver City, CA, USA). Cells were thawed and expanded as described earlier.[20] Between day 10 and 13, the expanded blasts were counted on a Z1 Coulter Particle Counter (Beckman Coulter Inc., Brea, CA, USA) and aliquoted as follows: $\sim 1 \times 10^5$ cells for FLT3 genotyping (as described earlier),[20] $\sim 5 \times 10^5$ cells for FACS analysis and $\sim 3 \times 10^6$ cells for a proliferation assay.

Caspase-3/7 assay

MV4-11 cells (100 μl) or AML blast cells (300 000 cells/ml) were treated with pacritinib in a concentration range between 10 μM and 0.5 nM for 16 h. Caspase-3/7 activity was measured using the Promega Caspase-Glo 3/7 assay (#TB323, Promega).

Flow cytometry

For cell cycle analysis, 5×10^5 cells/ml MV4-11, MOLM-13 and RS4;11 cells were treated for 24 h at the IC_{50} for viability of pacritinib. After treatment, cells were fixed using 70% ice-cold ethanol and stained with 20 ng/ml propidium iodide (BD Biosciences, Franklin Lakes, NJ, USA). For apoptosis analysis, MV4-11 cells were treated with 0.03 and 0.15 μM pacritinib for 48 and 72 h and stained using the AnnexinV-FITC apoptosis detection kit from BD Biosciences, according to the manufacturer's instructions. To characterize expanded AML blasts, cells were labeled with monoclonal antibodies against CD123 (#558663, BD Biosciences) and analyzed on a FACSCalibur equipped with the CellQuest Pro software (BD Systems, San Jose, CA, USA).

Western blot analyses

Cell lysis, protein quantification and western blots were performed as described previously.[21] Following SDS-polyacrylamide gel electrophoresis, proteins were transferred to polyvinylidene difluoride membranes. Western blots were performed according to standard methods. pFLT3 (Y591) (cat#3461), pSTAT3 (Y705) (cat#9135), pAkt (T308) (cat#4056), pAKT(T473) (cat# 9271), pp44/42 (T202/Y204) (cat#9101), anti-mouse IgG (cat#7074), and anti-rabbit IgG, HRP-linked (cat#7076) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). pSTAT5 (Y694, cat#611965) was obtained from BD Biosciences (San Jose, CA, USA) and â-actin (cat#2066) from Sigma (St Louis, MO, USA).

Animal models

Female athymic BALB/c nude mice (BALB/cOlaHsd-*Foxn1*^{nu}) were obtained from the Biological Resource Centre (Biopolis, Singapore) and were 9-16 weeks of age at the time of tumor implantation. Standard protocols were followed, in compliance with the National Institutes of Health and National Advisory Committee for Laboratory Animal Research guidelines (IACUC approval 0800371). Female BALB/c nude mice were implanted subcutaneously in the right flank with 1×10^7 MV4-11 human AML cells. Cells were re-suspended in 50 ìl serum-free growth medium, mixed 1:1 with Matrigel (cat. No: 354248; BD Bioscience) and injected in a total volume of 100 μl, using a 27-gauge needle. Tumor growth was measured twice per week using a caliper. For the efficacy study animals were randomized on day 11 after inoculation (study day 0) into 4 treatment groups, with a mean tumor volume of 146–150 mm^3. Statistical analyses on tumor growth inhibition or tumor weight (one-way analysis of variance, followed by Dunnett's post-test) were performed using Prism 5 (GraphPad Software, La Jolla, CA, USA). For the PK-PD study the average starting tumor volume was 328 mm^3 (range 144–550 mm^3). For the MOLM-13 *s.c.* model, 5×10^6 MOLM-13 cells were injected in 0.2 ml serum-free medium into the right flank of female mice severe combined immunodeficiency (C.B-17/IcrHan, Hsd-Prkdcscid) (Biolasco, Taipeh, Taiwan). Tumor volumes were determined by caliper measurements. After 15 days when tumors had reached a mean volume between 536 and 596 mm^3, mice were randomized into three groups of 12 animals each and drug treatment was initiated. Treatment was orally administered *b.i.d.* for 8 consecutive days. All animals were killed 3 h post-dose on day 7 and the tumors harvested. Tumor growth inhibition was calculated as described previously.[21] All statistical analyses were performed with GraphPadPrism 5.

Results

Pacritinib modulates FLT3 signaling pathways

Pacritinib is a small-molecule inhibitor of JAK2 ($IC_{50} = 23$ nM) with selectivity for JAK2 within the JAK family (JAK1 $IC_{50} = 1280$ nM, JAK3 $IC_{50} = 520$ nM, TYK2 $IC_{50} = 50$ nM) and targets FLT3 (FLT3-wt $IC_{50} = 22$ nM, FLT3-D835Y $IC_{50} = 6$ nM) at the same concentration range as JAK2.[16]

To investigate whether its enzyme inhibitory properties translate into modulation of FLT3 signaling pathways in the cellular context, the effects of pacritinib on FLT3 autophosphorylation (pFLT3 Y591) and downstream STAT5 phosphorylation (pSTAT5 Y694), pERK1/2 (T202,Y204) and pAkt (T308) were investigated in two FLT3-ITD-harboring cell lines (MV4-11 and MOLM-13) and one FLT3-wt-bearing cell line (RS4;11).

FLT3-ITD harboring MV4-11 cells were treated for 3 h with different concentrations of pacritinib and pFLT3, pSTAT5 and

pERK1/2 levels were quantified. Pacritinib led to a dose-dependent decrease of pFLT3, pSTAT5, pERK1/2 and pAkt with IC_{50} of 80, 40, 33 and 29 nM, respectively (Figure 1a). Similar potencies in inhibition of FLT3 signaling were achieved in the second FLT3-ITD cell line, MOLM-13, with IC_{50} of 180 and 20 nM on pFLT3 and pSTAT5, respectively (Figure 1b). To ascertain that the inhibition of FLT3 signaling is independent of the JAK2 activity of pacritinib, we treated the cells with the JAKi-1 (a selective pan JAKi) and analyzed the cells for pFLT3. Concentrations up to 1000 nM of this potent pan-JAKi did not reduce FLT3 phosphorylation (Figure 1a). Treatment of the cells with sunitinib, a multi-kinase inhibitor with FLT3 ($IC_{50} = 3$ nM) but no JAK2 activity, resulted in a potent inhibition of FLT3 signaling. To extend the evaluation of the effects of pacritinib to FLT3-wt signaling, RS4;11 cells were treated with various concentrations of pacritinib. The IC_{50} on auto-phosphorylation of FLT3-wt in RS4;11 was fourfold higher ($IC_{50} = 600$ nM) compared with FLT3-ITD in MV4-11 and MOLM-13 cells. However, STAT5 inhibition was detected at much lower concentrations of pacritinib ($IC_{50} = 8$ nM) (Figure 1c).

Overall, these data demonstrate that pacritinib effectively permeates leukemia cells to modulate FLT3 signaling pathways in cell lines driven by constitutively activated or ligand-activated FLT3.

Pacritinib induced apoptosis, cell cycle arrest and anti-proliferative effects in FLT3-mutant and FLT3-wt cells

As FLT3 signaling has a key role in important functional responses such as cell proliferation and survival, the effects of pacritinib on cell cycle and apoptosis were investigated. MV4-11 cells were treated with pacritinib (0.03 and 0.15 μM) for 48 or 72 h and analyzed for induction of apoptosis using Annexin V staining.

Pacritinib dose dependently increased cell populations in early and late stage apoptosis, without inducing necrosis (Figure 2a). The ability of pacritinib to induce apoptosis is also evident in Figure 2b where caspase-3/7 was dose dependently activated. To determine whether pacritinib treatment leads to a cell cycle arrest, FLT3-ITD and FLT3-wt cell lines were treated with pacritinib for 24 h. Figure 2c showed that 24 h of exposure to pacritinib arrested both FLT3-ITD- and FLT3-wt-expressing cells at G_1 phase and decreased the S-phase population in a dose-dependent manner. The IC_{50} of pacritinib on cell proliferation in RS4;11 (FLT3-wt) cells was 15-20-fold higher ($IC_{50} = 930$ nM) compared with FLT3-ITD-harboring cells MV4-11 ($IC_{50} = 47$ nM) and MOLM13 ($IC_{50} = 67$ nM) (Figure 3).[16]

These results indicate that inhibition of FLT3 signaling by pacritinib in cancer cells can lead to G1 arrest and caspase-dependent apoptotsis, with FLT3-ITD harboring cells being the most sensitive.

Pacritinib blocks proliferation in FLT3-ITD- or JAK2V617F-driven AML cell lines

The anti-proliferative effect of pacritinib was tested on 11 AML-derived cell lines with different French-American-British classification. Interestingly, pacritinib showed the highest potency in French-American-British classification M5 subtypes (THP-1, MV4-11 and MOLM-13), with the FLT3-ITD status further differentiating the last two cell lines from THP-1. Furthermore, the $JAK2^{V617F}$-harboring cell line, SET-2, was also very sensitive to pacritinib ($IC_{50} = 0.22$ μM, Figure 3). The data reflects the on-target specificity of pacritinib across diverse AML cell lines.

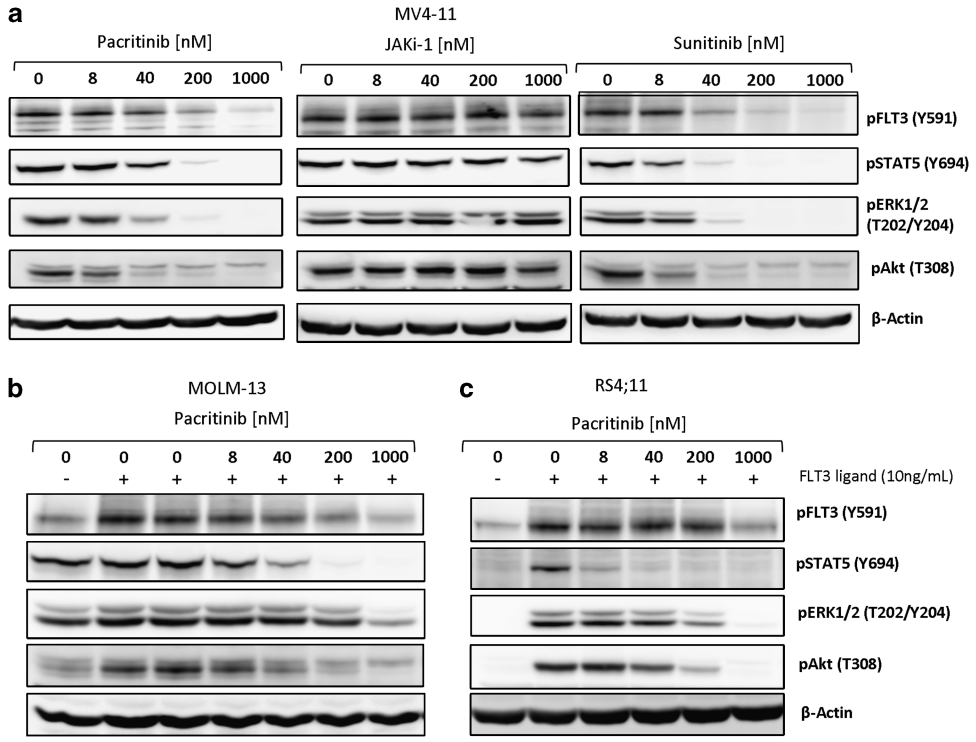

Figure 1 Pacritinib effectively blocks FLT3 signaling in FLT3-ITD (MV4-11, MOLM-13) or FLT3-wt (RS;4-11) cells. (**a**) MV4-11 cells were treated with pacritinib, JAKi-1 and sunitinib for 3 h as indicated. After lysis, phosphorylation status of FLT3, STAT5, ERK1/2 and Akt were detected by immunoblotting (IB). As a loading control, the same membranes were re-probed with anti-actin antibody. (**b**) MOLM-13 and (**c**) RS4;11 cells were pre-treated with pacritinib for 3 h and stimulated for 3 min with 10 ng/ml FLT3 ligand as indicated. After lysis, the phosphorylation status of FLT3 and total actin were detected by IB.

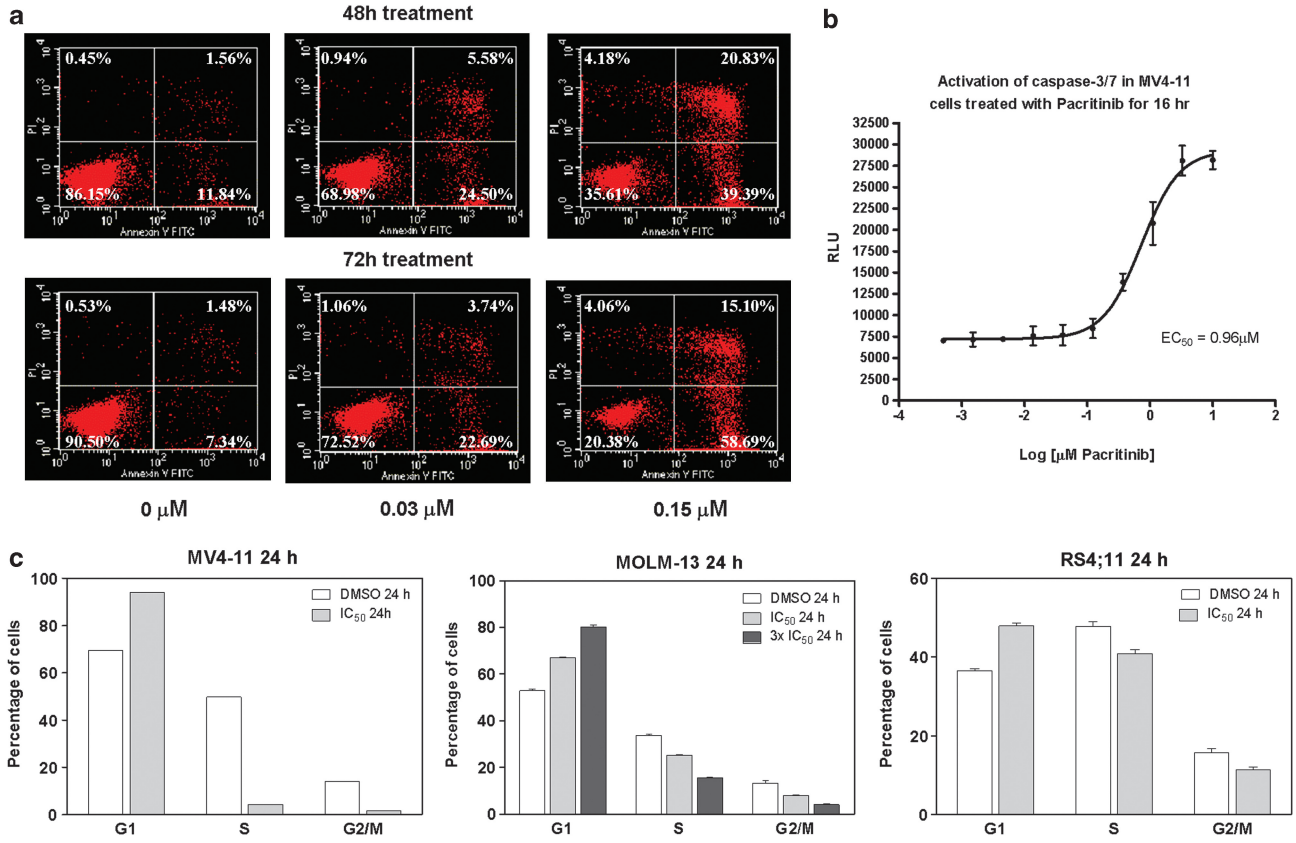

Figure 2 Pacritinib induces cell cycle arrest and apoptosis in FLT3-ITD- (MV4-11, MOLM-13) and FLT3-wt- (RS;4-11) harboring cancer cells. (**a**) MV4-11 cells were treated for 48 or 72 h with pacritinib followed by AnnexinV and propidium iodide co-staining. The left lower quadrant shows live cells, the left upper quadrant necrotic cells, the right lower and the right upper quadrant early and late apoptotic cells, respectively. The percentage of cells in each quadrant are indicated. (**b**) MV4-11 cells were treated with pacritinib for 16 h and caspase-3/7 activity was determined. An EC_{50} of 0.96 μM was calculated using the XL-fit software. (**c**) MV4-11, MOLM-13 and RS4;11 cells were treated for 24 h with pacritinib as indicated and cell cycle analysis was performed using propidium-iodide staining followed by flow cytometric measurement.

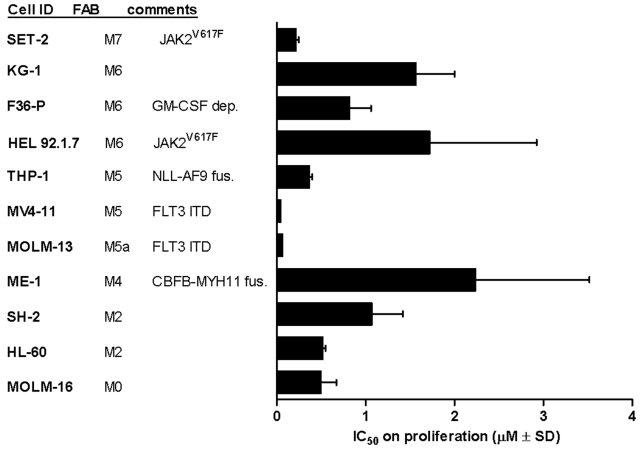

Figure 3 Pacritinib inhibits proliferation of AML cells with highest potency in FLT3-ITD harboring cells. Cells were treated for 48 h with pacritinib followed by the CellTiterGlo assay.

Pacritinib blocks signaling and induces cell cycle arrest and apoptosis in ex-vivo *expanded primary AML blast cells*

Having demonstrated that FLT3 inhibition leads to cell cycle arrest and apoptosis in established AML cell lines, it was pertinent to investigate whether pacritinib treatment could also compromise the viability of primary AML blast cells. The expanded AML blasts were analyzed using FACS and more than 90% of cell population from each sample were found to express the IL-3 receptor-α chain (CD123), a distinctive marker for human AML stem cells (Supplementary Figure 1).[22] This confirmed that the expanded cells were the intended population. Patient characteristics for the 14 AML samples are shown in Supplementary Table 1. Treatment of the AML blast cells with pacritinib for 3 h led to a dose-dependent decrease of pFLT3, pSTAT3 and pSTAT5 with an IC_{50} below 0.5 μM (Figure 4a).

The most sensitive sample to the anti-proliferative effect of pacritinib had an IC_{50} of 190 nM and the most resistant sample had an IC_{50} of 1300 nM (Figure 4b). The two samples harboring the FLT3-ITD mutation were among the most sensitive to pacritinib treatment. The relatively high sensitivity of the FLT3-wt blasts may be because the expansion medium contained FLT3-L, which would have activated FLT3 signaling in these cells. The inhibition of FLT3 signaling in the AML blast cells resulted in G1 cell cycle arrest and induction of caspase-dependent apoptosis (Figures 4c and d).

These data demonstrate that pacritinib induces FLT3 pathway inhibition and concomitant G1 cell cycle arrest and apoptosis in primary AML primary blasts as well as cell lines.

Pacritinib is efficacious in FLT3-ITD-bearing MV4-11 and MOLM-13 xenograft models

For evaluation of the *in vivo* efficacy of pacritinib on FLT3-ITD driven tumors, MV4-11 and MOLM-13 xenografts were

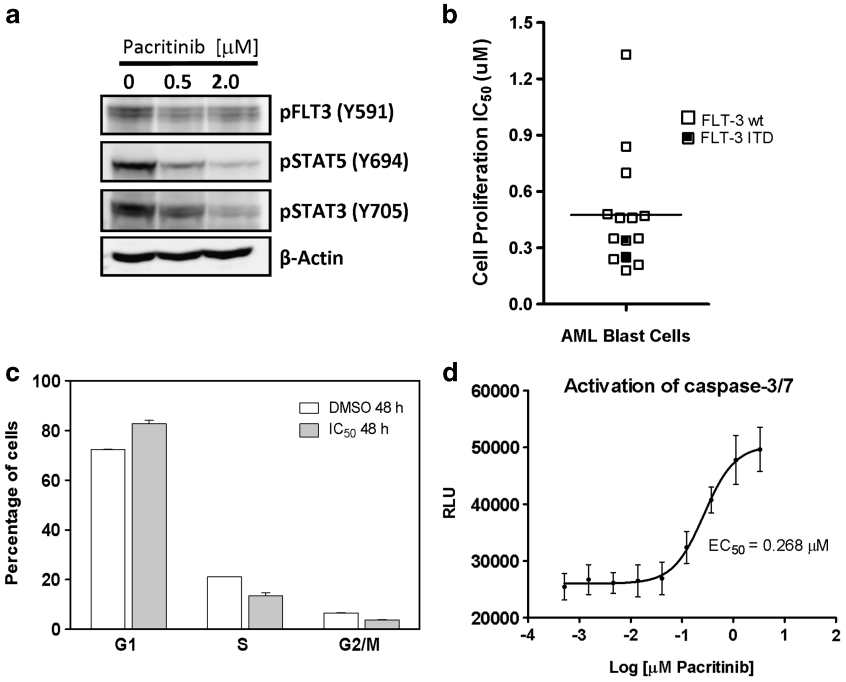

Figure 4 Pacritinib blocks proliferation and induces apoptosis in *ex vivo* expanded primary AML blast cells. (**a**) On day 12 of the *ex vivo* expansion protocol of AML blast cells, cells were treated with pacritinib 0.5 and 2 μM for 3 h. After lysis, the phosphorylation status of FLT3, STAT3 and STAT5 were detected by immunoblotting. (**b**) AML blasts were treated with pacritinib for 48 h and the IC_{50} for proliferation was evaluated using the CellTiter-Glo assay. (**c**) AML blasts were treated with 0.47 μM pacritinib for 48 h and cell cycle analysis was performed using propidium-iodide staining followed by flow cytometric measurement. (**d**) AML blasts were treated with pacritinib for 16 h and the EC_{50} on induction of apoptosis was determined by measuring caspase-3/7 activity.

established in nude or severe combined immunodeficiency mice. To demonstrate target engagement by pacritinib in the tumor tissues, tumor-bearing mice were first given a single dose of 150 mg/kg pacritinib and tumor samples taken at 2 and 4 h (MV4-11) or 3 h (MOLM-13) and the tumor lysates were analyzed for FLT3 signaling. In both xenograft models, acute pacritinib treatment was able to block FLT3 and downstream signaling (Figures 5a and b) in the tumors. To identify a possible effect on tumor growth, MV4-11 tumor-bearing mice (average starting tumor size of 130 mm^3) were treated once daily for 21 consecutive days. Pacritinib treatment induced dose-dependent inhibition of tumor growth (38% for 25 mg/kg, 92% for 50 mg/kg and 121% for 100 mg/kg, Figure 5c). Complete regression was observed in 3/10 and 8/8 mice for the 50 and 100 mg/kg/day groups, respectively. All doses were well tolerated with no significant body weight loss (data not shown).

In contrast to the MV4-11 efficacy study, the average tumor volume was much higher (543 mm^3) when treatment commenced in the MOLM-13 model. Treatment with 150 mg/kg *b.i.d.* for 7 consecutive days resulted in a tumor growth inhibition of 83% (Figure 5d). Analysis of the FLT3 signaling pathway in the tumor lysates 3 h after the last dose on day 7 showed a full inhibition of STAT5 phosphorylation (Figure 5e). In addition, post-mortem analysis showed that metastatic incidence (mostly in the lungs) was significantly reduced from 0.83 to 0.33 in the high-dose pacritinib group (Figure 5f).

These results demonstrate that treatment of FLT3-dependent tumors by pacritinib not only reduces the growth of the primary tumor, but also the formation of metastasis.

Selective FLT3-TKI up-regulates JAK2 signaling in FLT3-ITD-harboring AML cells

It has been proposed that one of the mechanisms of secondary resistance to FLT3-TKI in AML patients arises from enhanced STAT signaling.[13] Therefore we investigated, whether MV4-11 cells resistant to the FLT3-TKI linifanib/ABT-869 (MV4-11-R) shows higher JAK/STAT signaling compared with the parental MV4-11 (MV4-11-P) cells. Western blot analysis clearly shows, that both pJAK2(Y1007/8) and total JAK2 are significantly increased in MV4-11-R compared with MV4-11-P (Figure 6a). This result prompted us to explore whether acute treatment of MV4-11 cells with FLT3-TKI enhances JAK2 signaling in any way. Linifanib, sunitinib and VX-680 are FLT3-TKI without any significant activity against JAK2. MV4-11 cells were treated with linifanib, sunitinib and VX-680 for 24 h at the IC_{50} of cell proliferation and JAK2 signaling determined by western blot analysis. All three compounds increased pJAK2 signaling in the MV4-11 cells without changing total JAK2 protein levels (Figure 6b).

Having shown that JAK2 signaling is upregulated in MV4-11-R cells, we wondered if combined inhibition of JAK2 and FLT3 can nullify the resistance to FLT3i in MV4-11-R cells. Linifanib, a FLT3-TKI, is 127 times more potent against MV4-11-P compared with MV4-11-R (Figure 6c). Sunitinib, a multi-kinase inhibitor with FLT3 but not JAK2 activity, showed a 14-fold difference in potency of MV4-11-P compared with MV4-11-R. Pacritinib, a dual inhibitor of FLT3 and JAK2, showed only a 2.9-fold difference, with a high sensitivity of both cell lines ($IC_{50} = 153$ and 434 nM for MV4-11-P and MV4-11-R, respectively) indicating that JAK2 inhibition may overcome the resistance to FLT3 inhibition. Consistent with this, the JAK family inhibitor ruxolitinib, which has no FLT3 activity and is only active on the MV4-11-P at extremely high concentrations ($IC_{50} = 19.5$ μM), showed an opposite trend, being sevenfold more potent against MV4-11-R cells ($IC_{50} = 2.7$ μM). Taken together these data demonstrate the enhanced JAK2 dependency in MV4-11-R compared with MV4-11-P cells.

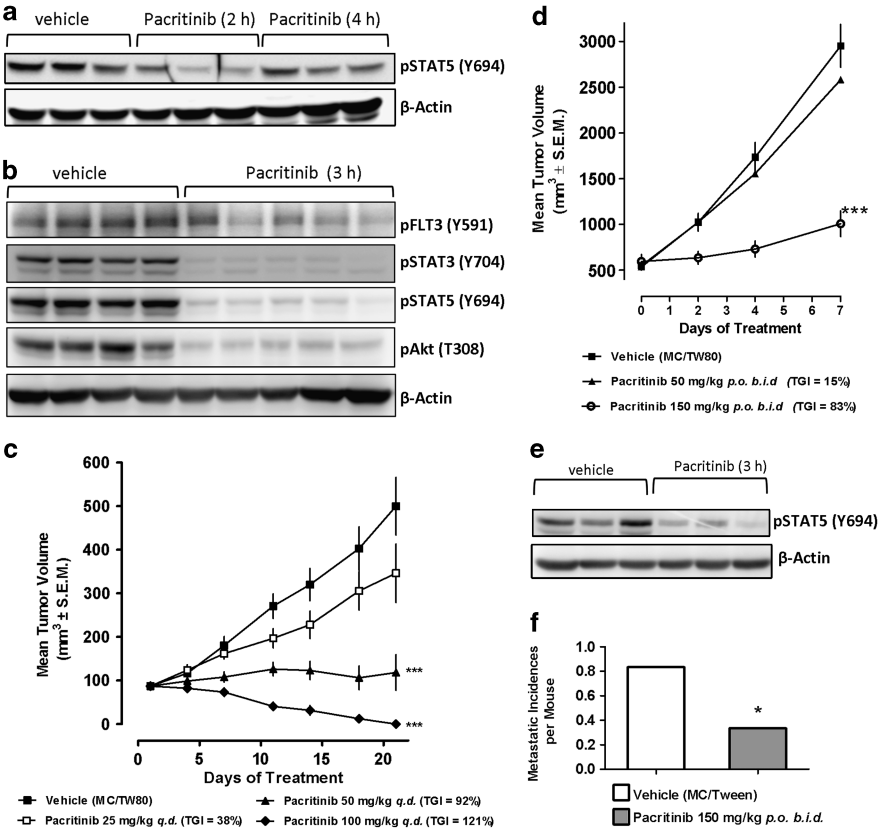

Figure 5 Pacritinib is efficacious in xenografts derived from cell lines harboring FLT3-ITD. (**a**) MV4-11 tumor-bearing mice received an acute dose of 150 mg pacritinib. At 2 and 4 h after dosing, mice were killed and tumors harvested. Phosphorylation status of STAT5 and total actin was determined by immunoblotting. (**b**) MOLM-13 tumor-bearing mice received an acute dose of 150 mg pacritinib. At 3 h after dosing, mice were killed and tumors harvested. Phosphorylation status of FLT3, STAT3, STAT5, Akt and total actin were determined by immunoblotting. (**c**) MV4-11 tumor-bearing nude mice ($n=10$/group) were treated daily for 21 days with the indicated doses of pacritinib hydrochloride salt or vehicle (MC/Tween). Doses shown are free base equivalents of pacritinib. The tumor growth inhibition (TGI) is indicated. Analysis of variance (ANOVA) with Dunnett's post-test was performed, $^{***}P<0.001$. (**d**) MOLM-13 tumor-bearing nude mice ($n=12$/group) with an average tumor volume between 548 and 596 mm^3 were treated daily for 8 days with the 150 mg/kg *b.i.d.* pacritinib citrate salt or vehicle (MC/Tween). Doses shown are free base equivalents of pacritinib. The TGI is indicated. ANOVA with Dunnett's post-test was performed, $^{***}P<0.001$. (**e**) At 3 h after the last treatment on day 7, MOLM-13 tumor bearing-mice were killed and tumors harvested. Phosphorylation status of STAT5 and total actin were determined by immunoblotting. (**f**) On day 7, MOLM-13 tumor-bearing mice were killed and analyzed for tumor metastasis ($n=12$/group). Unpaired *t*-test was performed, $^{*}P<0.05$.

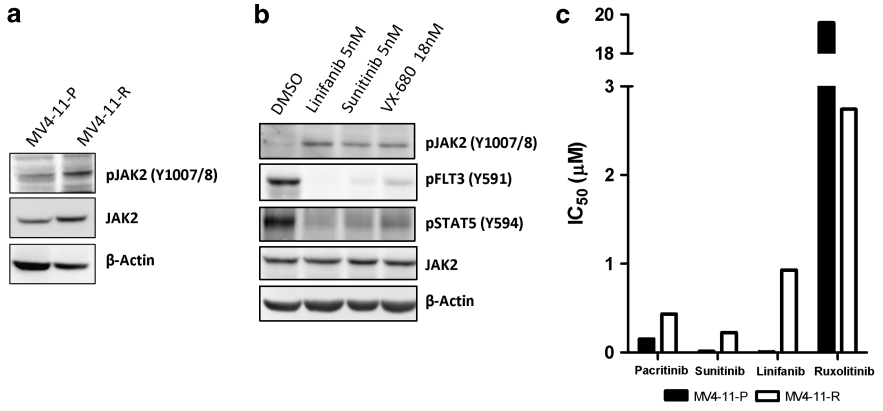

Figure 6 Activated JAK2 signaling in MV4-11 cells after selective inhibition of FLT3 induces FLT3-TKI resistance. **(a)** Parental MV4-11 cells (MV4-11-P) and linifanib-resistant MV4-11 cells (MV4-11-R) were lysed and pJAK2 and total JAK2 were detected by immunoblotting. (**b**) MV4-11 cells were treated with linifanib, sunitinib and VX-680 for 24 h. After lysis, pJAK and pFLT3 were detected by immunoblotting (IB). (**c**) Cells were treated for 48 h with various concentrations of pacritinib, ruxolitinib, sunitinib and linifanib followed by the CellTiterGlo assay.

Having demonstrated that JAK2 signaling is upregulated in MV4-11 cells within 24 h following acute treatment with FLT3 inhibitors and to further demonstrate that this is a resistance mechanism, we investigated whether combining a JAK2 inhibitor without significant FLT3 activity with a FLT3 inhibitor without significant JAK2 activity, might be synergistic. Indeed,

treatment of MV4-11 cells simultaneously with linifanib and JAKi-1 resulted in a confidence interval value of 0.73 and 0.8 for ED_{50} and ED_{90} respectively, reflecting the synergy of the two compounds (Supplementary Table 2). Similar data were obtained with the combination of linifanib and ruxolitinib (data not shown).

In summary, acute and chronic treatment of MV4-11 cells with FLT3-TKI leads to increased JAK2 signaling as a resistance mechanism. FLT3-TKI resistance can be reduced by additional JAK2 inhibition. Pacratinib provides these properties as a monotherapy and is highly effective in the parental and FLT3-TKI-resistant MV4-11 lines.

Discussion

FLT3 kinase, which is genetically altered in up to 35% of AML patients, is considered an attractive therapeutic target for this indication.[7,23] Various FLT3-TKIs, like linifanib, sunitinib, CEP-701, PKC412, AC-220 and MLN518 have been studied in clinical trials in AML patients, either as a single agent or in combination with standard chemotherapy.[24–27] These studies have shown initial clinical responses, which were not sustained over the long term as patients developed resistance to the drug.[28,29]

Pacritinib is a novel JAK2 inhibitor selective for JAK2 within the JAK family and equipotent against FLT3.[15,16] It is currently in phase II clinical trials for myelofibrosis and lymphoma where it is showing promising clinical activity and a favorable safety profile. We have previously reported its pharmacological profile and efficacy in preclinical models of JAK2-driven myeloid and lymphoid malignancies.[16] Herein we describe its efficacy in preclinical models of AML and provide a rationale for clinical trials in this indication.

Our present data demonstrate that pacritinib potently inhibits FLT3 auto-phosphorylation and downstream STAT5, MAPK and PI3K signaling pathways in AML cell lines with highest potency against cells harboring FLT3-ITD mutations. Blockade of FLT3 signaling was also demonstrated in primary AML blasts treated *ex-vivo* with pacritinib. In both cell lines and primary blasts, pacritinib treatment led to the induction of G1 arrest, inhibition of cell proliferation, as well as caspase-dependent apoptosis. The anti-proliferative effects of pacritinib on the FLT3-ITD harboring cell lines MV4-11 ($IC_{50} = 47$ nM) and MOLM-13 ($IC_{50} = 67$ nM), which have been reported previously,[16] are in the same range as the inhibition of intracellular FLT3 signaling. Pacritinib was highly efficacious in blocking tumor growth in mouse subcutaneous xenograft models generated with the FLT3-ITD-harboring cell lines, MV4-11 and MOLM-13. In the MV4-11 model, pacritinib dose-dependently blocked tumor growth and the highest dose led to complete tumor regression in all mice. Similarly, in mice with well established and aggressive MOLM-13 tumors, (average tumor size of 536–596 mm^3), pacritinib decreased FLT3 phosphorylation and downstream STAT5 signaling in tumor tissue and led to 83% tumor growth inhibition after 7 days of dosing. In contrast, linifanib, a multi-targeted receptor tyrosine kinase inhibitor with 4 nM FLT3 activity but no activity on JAK2, is reported to show only a modest effect on the inhibition of growth of large MOLM-13 tumors.[30] Interestingly, intra-pulmonary leukemic deposits were observed in the vehicle group of the MOLM-13 xenograft model and pacritinib treatment significantly reduced these deposits (Figure 5f). AML patients have been reported to develop extramedullary granulomas in the lung or liver.[31] Our findings suggest that besides limiting the growth of the primary tumor, pacritinib may also have the potential to reduce extramedullary leukemic growth in patients with AML.

Increasingly, other targets besides FLT3 have been suggested as potential therapeutic targets for AML due to the development of secondary resistance to FLT3-TKI. Examples include casein kinase 2 alpha (CK2α), CD47, CD123, PIM, mTORC1, PI3K and JAK2.[32–36] Recent observations indicate that high levels of phospho-JAK2 are associated with adverse clinical outcomes in AML.[14] Furthermore, a selective JAK2 inhibitor with no FLT3 activity, namely AZ-960,[37] has been shown to inhibit clonogenic growth and induce apoptosis in freshly isolated AML cells.[14] The authors concluded that JAK2 is a *bona fide* target for AML therapy. Recent publications suggested that therapeutic outcomes can be significantly improved with co-inhibition of FLT3 and the JAK/STAT pathway.[13] Inhibition of STAT5 signaling by a small molecule inhibitor of JAK2 is reported to sensitize leukemia stem cells to FLT3 inhibitors.[38] In addition, FLT3 inhibitor-resistant MV4-11-R cells have been shown to arise from hyper-activated STAT pathways due to downregulation of suppressor of cytokine signaling proteins but not PI3K/AKT or the MAPK pathway.[13]

In the present studies, we have shown that JAK2 signaling is activated in MV4-11 parental cells after acute treatment with FLT3-TKI as well as in a FLT3-TKI-resistant MV4-11-R cell line. An acquired JAK dependency in the FLT3-TKI-resistant cells was demonstrated by a sevenfold increased sensitivity of these cells to a JAKi devoid of FLT3 activity and the high sensitivity of these resistant cells to pacritinib. Moreover, co-treatment of MV4-11 cells with a JAK2 inhibitor devoid of significant FLT3 activity, with a FLT3 inhibitor devoid of significant JAK2 activity, showed a synergistic effect in inhibiting cell proliferation. Our data strongly argues for the combined inhibition of FLT3 and JAK2 in FLT3-ITD-positive patients in two scenarios: as a first-line therapy to minimize the development of secondary resistance or as a second-line therapy to re-sensitize resistant cells to FLT3 inhibition. Lestaurtinib (CEP-701), a potent JAK2/FLT-3 inhibitor, has been recently tested in a phase II trial in AML patients with mutant FLT3 following chemotherapy. The study showed that FLT3 inhibition highly correlated with remission rate.[39] However, the drug failed to provide long-term benefits for the patients. The authors suggested that the pharmacokinetic properties of lestaurtinib, which include significant variations in steady-state plasma levels and decreasing plasma levels over the course of treatment, might explain the failure.[39]

Pacritinib, with its combined potent JAK2/FLT3 inhibition and a favorable pharmacokinetic and safety profile that is now established in patients, may have a better chance of success. The JAK2 activity of pacritinib provided the rationale for its current clinical evaluation in patients with myelofibrosis and lymphoma. Importantly, these trials have demonstrated not only durable clinical benefit, but also favorable pharmacokinetics properties and a safety profile that includes no overt myelosuppression.[18,40] Interestingly, seven AML patients were included in one of the phase 1 myeloid malignancy studies and three of these patients showed clinical benefits (two stable diseases and one clinical improvement).[41] Taken together, the promising preclinical profile as well as the emerging clinical data provide a compelling rationale for a more extensive clinical evaluation of pacritinib in AML, including patients resistant to FLT3-TKI therapy.

Conflict of interest

Except for J Zhou and WJ Chng, all the authors are current or past employees of S*BIO.

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