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ORIGINAL ARTICLE

The oral HDAC inhibitor pracinostat (SB939) is efficacious and synergistic with the JAK2 inhibitor pacritinib (SB1518) in preclinical models of AML

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Acute myeloid leukemia (AML) is currently treated with aggressive chemotherapy that is not well tolerated in many elderly patients, hence the unmet medical need for effective therapies with less toxicity and better tolerability. Inhibitors of FMS-like tyrosine kinase 3 (FLT3), JAK2 and histone deacetylase inhibitors (HDACi) have been tested in clinical studies, but showed only moderate single-agent activity. High efficacy of the HDACi pracinostat treating AML and synergy with the JAK2/FLT3 inhibitor pacritinib is demonstrated. Both compounds inhibit JAK-signal transducer and activator of transcription (STAT) signaling in AML cells with $JAK2^{V617F}$ mutations, but also diminish FLT3 signaling, particularly in FLT3-ITD (internal tandem duplication) cell lines. *In vitro*, this combination led to decreased cell proliferation and increased apoptosis. The synergy translated *in vivo* in two different AML models, the SET-2 megakaryoblastic AML mouse model carrying a $JAK2^{V617F}$ mutation, and the MOLM-13 model of FLT3-ITD-driven AML. Pracinostat and pacritinib in combination showed synergy on tumor growth, reduction of metastases and synergistically decreased JAK2 or FLT signaling, depending on the cellular context. In addition, several plasma cytokines/growth factors/chemokines triggered by the tumor growth were normalized, providing a rationale for combination therapy with an HDACi and a JAK2/FLT3 inhibitor for the treatment of AML patients, particularly those with FLT3 or JAK2 mutations.

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INTRODUCTION

Acute myeloid leukemia (AML) is a myeloid malignancy characterized by deregulated proliferation, increased self-renewal and limited differentiation of myeloid blasts. AML is typically diagnosed in elderly patients and the standard of care treatment is mainly chemotherapy. Most patients relapse and perish from the disease or the associated complications. Aggressive chemotherapeutic treatment can only be used in a minority of patients; hence, there is a great medical unmet need, for effective targeted therapy with less toxicity and better tolerability.[1]

Histone deacetylase inhibitors (HDACi) are a class of drugs that alter the acetylation status of both histone and non-histone proteins, thereby affecting a range of cellular functions of neoplastic cells, such as transcriptional activation, cell proliferation, immune responses, cell differentiation, survival and angiogenesis.[2,3] HDACis, including pracinostat (SB939), have shown clinical activity in AML and myelodysplastic syndrome, as well as myeloproliferative neoplasms (MPNs); however, it seems that efficacy as a single agent is only moderate.[4–6]

JAK2 mutations or fusion proteins leading to constitutive activation of JAK2 have long been known to have a role in MPNs and leukemia.[7,8] JAK2 inhibitors, such as pacritinib (SB1518),[9] an oral inhibitor currently in Phase II clinical studies, as well as other JAK2 inhibitors show significant efficacy in treating MPNs,[10–12] reducing the JAK-STAT (signal transducer and activator of transcription) signaling, spleen size, $JAK2^{V617F}$ mutation burden, as well as levels of certain cytokines/growth factors relevant in MPNs. Nuclear JAK2 has been reported to have a second, epigenetic function that might contribute to leukemogenesis.[13,14] The JAK-family kinases were shown to cause phosphorylation of Y41 on histone H3, displacing heterochromatin protein 1α from its position bound to histone H3. Sustained displacement of the heterochromatin protein 1α triggers increased expression of oncogenic transcription factors, such as LMO2, enhanced mitotic recombination, chromosomal disjunction and aneuploidy. All these changes promote oncogenesis and are consistent with the phenotypic consequences observed after constitutive JAK2 activation in hematological malignancies.[13,15]

A mutation in the FMS-like tyrosine kinase 3 (FLT3), the FLT3 internal tandem duplication (ITD), causes constitutive active FLT3 signaling, leading to activation of the downstream STAT5. The FLT3–ITDs are described in up to 35% of all AML patients,[16,17] and a single FLT3–ITD is sufficient to induce a myeloproliferative phenotype, as shown in genetic mouse models,[18,19] demonstrating the importance of mutated FLT3 in the pathogenesis of acute leukemia.

The HDACi givinostat (ITF2357) has been reported to reduce levels of total JAK2 as well as STAT5 in the $JAK2^{V617F}$ mutant cells.[20] In addition, the HDACi panobinostat and the JAK2/FLT3/RET inhibitor TG101209 are reported to exert synergistic cytotoxic effects against cell lines carrying the $JAK2^{V617F}$ mutation.[21] Another interesting recent observation is that HDACi selectively target FLT3–ITD for degradation in AML cells.[22] In addition, superior activity on AML cell apoptosis has been reported for a combination of an HDACi and a FLT3 inhibitor.[23,24] On the basis of these encouraging observations, we explored at multiple levels

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the *in vitro* and *in vivo* synergy between the HDACi pracinostat and the JAK2/FLT3 inhibitor pacritinib.

Pracinostat is an oral pan-HDACi with favorable pharmacokinetics[25] and good tolerability in patients,[26,27] which is currently explored as a single agent in multiple Phase II clinical studies, for solid tumors as well as myelodysplastic syndrome, AML and myelofibrosis. Pacritinib[9] is an oral JAK2/FLT3 kinase inhibitor, also with favorable pharmacokinetics and good tolerability, which is currently in Phase II clinical studies for myelofibrosis and lymphoma.[12] The studies described in this manuscript provide a rationale for the combination of these two drugs as a treatment for AML patients, especially those with either mutated FLT3 or JAK2.

MATERIALS AND METHODS

Compounds

Pracinostat (SB939) as hydrochloride salt and pacritinib (SB1518) as citrate salt were synthesized by SAI Advantium Pharma Ltd (Hyderabad, India). For *in vitro* studies, drugs were dissolved in dimethyl sulfoxide (10 mM stock); for *in vivo* studies, the dosing solutions for oral gavage were prepared in 0.5% methylcellulose (w/v) and 0.1% Tween-80 in H_2O (MC/Tween), stored at 4 °C, and prepared freshly at least every week. All *in vivo* doses described for pacritinib refer to the free base.

Cells

Cell lines used were obtained from either the American Type Culture Collection (Manassas, VA, USA) or the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). SET-2, KG-1, F36-P, HEL92.1.7, THP-1, MV4-11, MOLM-13, ML-2, ME-1, SH-2, HL-60, MOLM-16, 32D, K562, KARPAS-1106P and RS4;11 cells were all cultivated according to the vendor's instructions, tested for mycoplasma contamination (Mycoplasma *Plus* PCR Primer Set, Stratagene; Agilene Technologies Inc., Santa Clara, CA, USA) and verified by STR profiling (John Hopkins University, Baltimore, MD, USA). Granulocyte macrophage colony-stimulating factor to supplement F-36P cell growth medium was obtained from i-DNA Biotechnology (Singapore), fetal bovine serum was obtained from PAA Laboratories GmbH (Pasching, Austria).

Primary AML cells

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) from a total of 16 patients. Cultivation, expansion and analysis were carried out as described previously.[28]

Cell proliferation assay and determination of in vitro synergy

Cells were seeded in 96-well plates at a predetermined optimal density (in the log phase and rested for 2 h) before drug treatment and then treated for 48 h with drug concentrations from 10 μM to 1.5 nM in 9 serial-dilution steps with 0.1% solvent, and using the CellTiter-Glo Assay (Promega, Madison, WI, USA), according to the manufacturer's instructions, in a total volume of 100 μl. At least three independent experiments were performed in triplicates. IC_{50} were determined using XLfit (IDBS, Guildford, UK). To calculate the *in vitro* synergy of two drugs, they were combined at a constant ratio, based on the individual drug's IC_{50} concentrations, with the highest doses used being $8\times$ IC_{50} concentrations.[29] For sequential treatments, cells were treated with drug 1 for 24 h followed by a 24 h treatment with drug 2; simultaneous treatments were performed for 48 h. Synergy was determined using the CompuSyn software (v2007; ComboSyn Inc., Paramus, NJ, USA). Combinatorial indices (CIs) were calculated[29] $CI<1$ (synergy), $CI>1$ (antagonism) and $CI=1$ (additive) for *in vitro* combinations.

Lysis and western blots

Cell lysis, protein quantification and western blots were performed as described previously.[20] Antibodies against pFLT3 Y591 (no. 3461), pSTAT3 Y705 (no. 9145), pJAK2 Y1007/1008 (no. 3776), STAT5 (no. 9310), and cleaved poly-adenosine di-phosphate ribose polymerase N214 (no. 9541) as well as horse-radish peroxidase linked secondary antibodies were from Cell Signaling Technologies (Danvers, MA, USA). The antibodies against pSTAT5 Y694 (no. 611965) and STAT3 (no. 610190) were obtained from BD Biosciences (San Jose, CA, USA). The β-actin antibody (no. 2066) and the FLT3 antibody were from Sigma Aldrich (St Louis, MO, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. The LMO2 antibody (no. 91652) was obtained from Abcam (Cambridge, MA, USA).

Subcutaneous animal models

Female BALB/c nude mice were obtained from the Biological Resource Centre (Biopolis, Singapore), female SCID mice were purchased from BioLASCO (Taipei, Taiwan) and female SCID/Beige mice were obtained from Charles River Laboratories (Willmington, MA, USA). BALB/c nude mice (BALB/cOlaHsd-*Foxn1*^{nu}) were 8–10 weeks of age; SCID mice (C.B-17/IcrHan™Hsd-*Prkdc*^{scid}) 5–11 weeks of age, and SCID-Beige mice (C.B-17.Cg-*Prkdc*^{scid}*Lyst*^{bg}/Crl) were 9 weeks old. Standard protocols were followed, in compliance with the National Institutes of Health and National Advisory Committee for Laboratory Animal Research guidelines (IACUC approval no. 0800371).

For subcutaneous (s.c.) AML models, mice were implanted with 5×10^6 cells (or 1×10^7 for MV4–11 cells) in the right flank. Cells were re-suspended in 50 μl serum-free growth medium, mixed 1:1 with Matrigel (Cat no. 354248; BD Bioscience) and injected in a total volume of 100 μl. Tumor volumes were monitored using caliper measurements and volumes calculated using the formula: tumor volume (mm^3) $=(w^2\times l)/2$ (w = width and l = length in mm of the tumor xenograft). Pracinostat and pacritinib were administered by per oral gavage in a volume of 10 ml/kg if not otherwise stated. Pracinostat was dosed at 25–125 mg/kg either daily or every other day. Pacritinib was dosed 50–150 mg/kg daily or twice daily. Tumors were excised on the last day of treatment, 3 h post-dose. *In vivo* synergy was determined using the Clarke's CI (CCI) Equation:[30] $CCI=A/B-(C/B\times D/B)$, and must be <0 to be synergistic, where A is the average tumor measurement from the combination group; B, the average measurement from the vehicle control; and C and D, the average measurements from monotherapy 1 and 2, respectively. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

Orthotopic AML model

SCID mice (from Biological Resource Centre) were injected intravenously with 1×10^7 HL-60 cells in 100 μl serum-free medium. Mice were monitored three times per week for signs of paralysis. A separate take-rate control group was analyzed on day (d) 29 as previously described,[31] indicating a good take-rate. Treatment of the experimental group started 1 day later, on d30 after inoculation (before the first mouse in this group showed symptoms of paralysis). Blood counts were performed from tail blood, using a ScilVet abc hematology analyzer (Scil Animal Care Company, Gurnee, IL, USA) according to the manufacturer's instructions. Statistical analyses were performed using GraphPad Prism 5.

Pharmacokinetic analysis of pracinostat and pacritinib

The bioanalysis to detect levels of either pracinostat or pacritinib from 50 μl murine plasma was performed as described previously.[9,20] Two independent experiments were performed, either in 16 weeks old female BALB/c nude mice (BALB/cOlaHsd-*Foxn1*^{nu}) from Biological Resource Centre ($n=3$–5 per time point), or in 8–12 weeks old female BALB/c mice (BALB/cOlaHsd) from Biological Resource Centre ($n=4$ per time point). Pharmacokinetic parameters were calculated by a non-compartmental method using WinNonlin 5.2 software (Pharsight Corp., Sunnyvale, CA, USA).

Cytokine/growth factor analysis

Murine or human plasma samples were analyzed by Millipore Corp. (Billerica, MA, USA) for cytokine/chemokine/growth factor measurements using the Luminex xMAP technology. The Milliplex MAP Mouse Cytokine/Chemokine panel (premixed 32-plex, MPXMCYTO-LIX) was used for mouse samples.

RESULTS

Pracinostat inhibits JAK-STAT as well as FLT3-STAT5 signaling

Cells, expressing wild-type (wt) JAK2 (32D murine myeloid cells) or mutant JAK2^{V617F} (HEL92.1.7 and SET-2) were treated with pracinostat in concentrations ranging from 125–500 nM for 24 h, and levels of pJAK2, JAK2, pSTAT5 and STAT5 were measured.

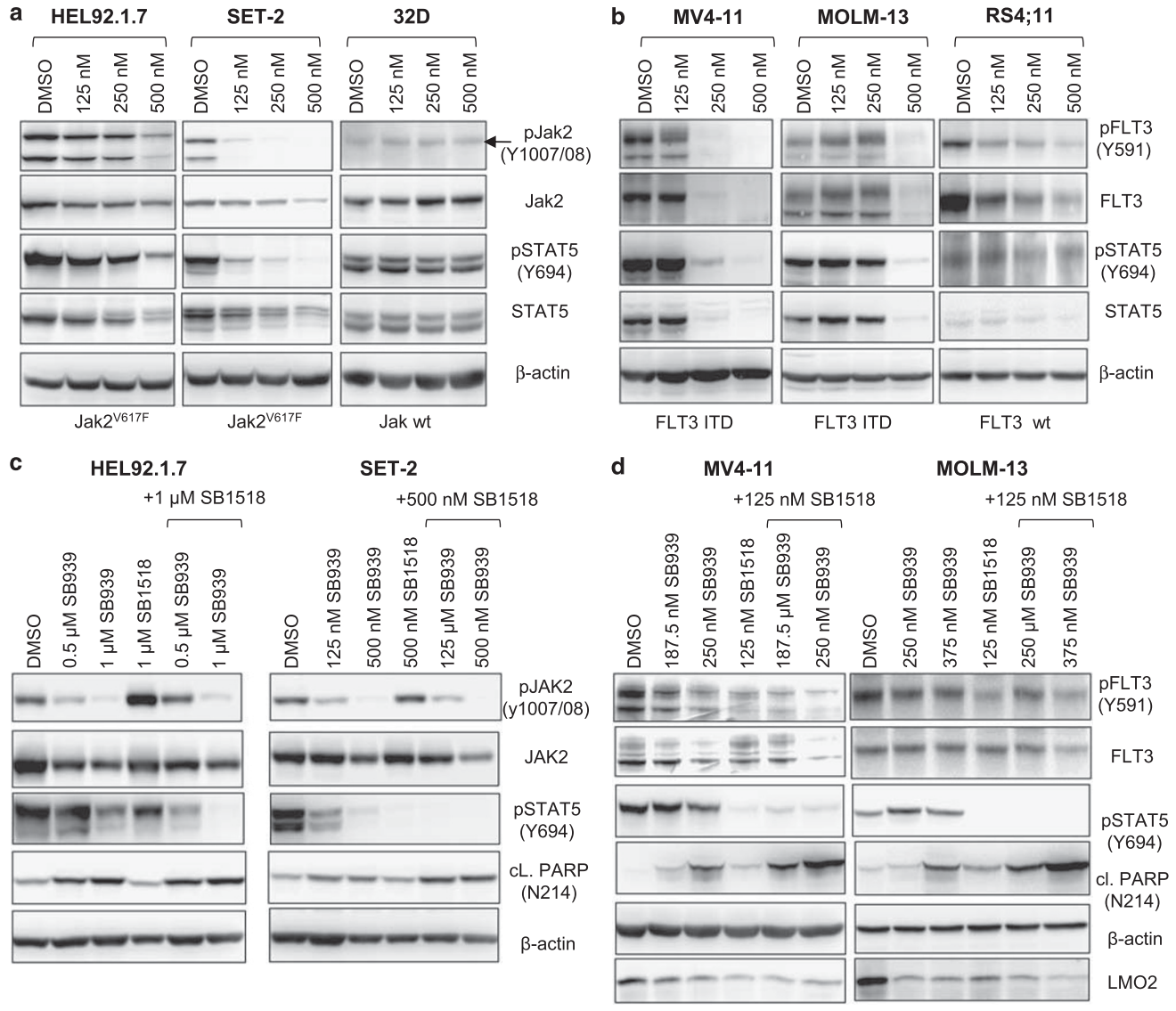


Figure 1. Pracinostat downregulates JAK and FLT3 signaling in JAK2^{V617F} and FLT-ITD cell lines, and shows synergy in combination with pacritinib. (**a**–**d**) Western blot analyses from 25 or 50 μg (pFLT3 and FLT3 blots only) of total cell lysate of the indicated cell lines with JAK2^{V617F}, JAK2, or FLT3-ITD or FLT3 wt are shown. (**a**, **b**) Cells were treated with the indicated concentrations of pracinostat (SB939) for 24 h. (**c**, **d**) Cells were treated for 24 h with pracinostat with pacritinib (SB1518) added in the indicated concentrations for the last 2 h of incubation before lysis.

Pracinostat treatment decreased both pJAK2 (Y1007/08) and pSTAT5 (Y694) levels, and also total JAK2 and STAT5 protein in JAK2^{V617F} mutant cells (see Figure 1a). These proteins remained unaffected in the JAK2 wt cells 32D and KARPAS 1106-P (Figure 1a right hand panel and data not shown). To determine the effects of pracinostat on cells with either mutated or wt FLT3, MV4-11 cells or MOLM-13 cells carrying FLT3 ITDs were compared with RS4;11 cells with wt FLT3. In FLT3-ITD cell lines, pracinostat led to a near-total ablation of pFLT3 (Y591) at 500 nM in MV4-11 and MOLM-13 cells, respectively, with very steep dose response (Figure 1b). There was a concomitant decrease in total FLT3 as well as pSTAT5, described to be a direct downstream substrate of FLT3 in FLT3-ITD cell lines.[32] In RS4;11 cells, which express wt FLT3, levels of pFLT3 and FLT3, and pSTAT5 were also reduced, but to a lesser extent and more gradually with dose than in the FLT3-ITD cell lines. In contrast to the FLT3-ITD cell lines, there was no decrease in total STAT5; however, the levels of total STAT5 are very low.

Pracinostat and pacritinib show in vitro synergy on STAT signaling and apoptosis

To determine whether pacritinib, a JAK2/FLT3 kinase inhibitor, combined with pracinostat would lead to even more pronounced inhibition of STAT signaling and cause increased cell death in JAK2- or FLT3-driven disease models, JAK2^{V617F} mutant cell lines were treated simultaneously with pracinostat and pacritinib. As described previously, pacritinib treatment alone increased pJAK2 levels in the JAK2^{V617F} cell lines,[9] whereas downstream pSTAT5 was decreased. The combination of pacritinib and pracinostat completely abrogated the JAK2-autophosphorylation in Set-2 cells (Figure 1c). The same effect, although to a lesser extent, was observed in HEL92.1.7 cells. Concurrently with the decrease in pSTAT5, there was an increase in cleaved poly-adenosine di-phosphate ribose polymerase levels, indicating enhanced cell death after the combination of pracinostat and pacritinib. This was also more pronounced in SET-2 cells than HEL92.1.7 cells (Figure 1c). The combination also increased efficacy in reducing levels of pFLT3 and total FLT3, as well as increasing cleaved

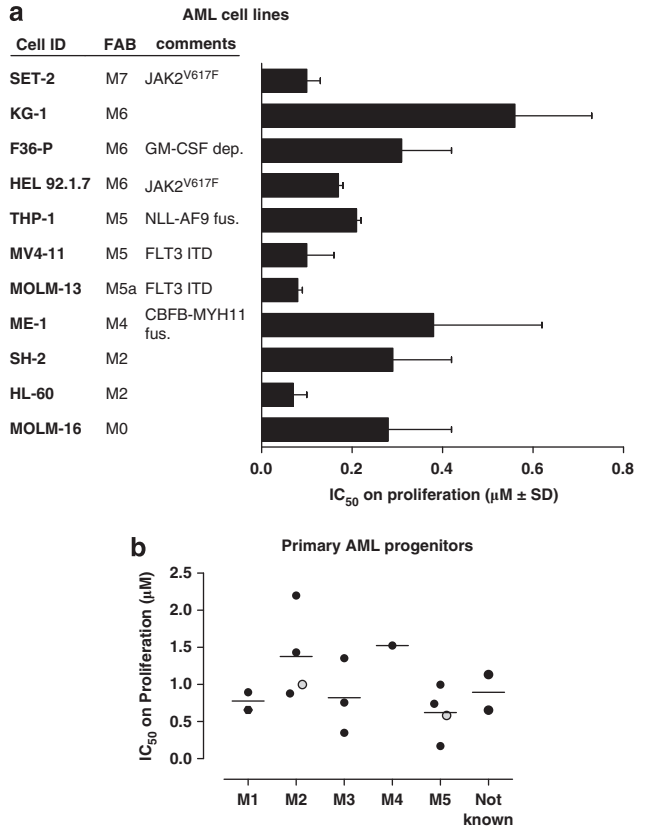


Figure 2. Pracinostat potently inhibits cell proliferation in AML cell lines and primary AML cells. (**a**) IC_{50} of cell lines tested in 48 h cell proliferation assays (CellTiterGlo); FAB: French–American–British classification of AML cells. Results show mean ± s.d. from at least three rounds of experiments, each performed in triplicates. (**b**) Primary AML blasts from 16 patients were expanded and then treated on d12–d13 with dimethyl sulfoxide or pracinostat serially diluted in nine steps from 10 μM to 1.5 nM for 48 h. Results show means from two rounds of blast expansion/proliferation assays. Gray-shaded icons depict blast cells carrying the FLT3-ITD.

poly-adenosine di-phosphate ribose polymerase in the FLT3-ITD cell lines MV4-11 and MOLM-13 (Figure 1d). There was also a greater reduction in the oncogenic transcription factor LMO2, suggesting that pracinostat and pacritinib also might synergize on the epigenetic level (bottom panel of Figure 1d) in FLT3-ITD cell lines.

Pracinostat potently inhibits proliferation of different AML subtypes as a single agent and is synergistic with pacritinib in $JAK2^{V617F}$ or FLT3-ITD AML cell lines

AML cells are among the most sensitive cancer cells to HDAC inhibition.[20] The IC_{50} on cell proliferation in a panel of 11 AML cell lines ranged from 70–560 nM, with the panel covering most subtypes of AML according to the French–American–British classification, with the exception of M1 and M3 (see Figure 2a). Primary AML cells, expanded from peripheral or bone marrow blasts in the presence of FLT3 ligand, stem cell factor, interleukin (IL)-3 and IL-6 were on average less potently inhibited, with mean IC_{50} values for the different French-American-British (FAB) classification types ranging between 622 nM and 1.5 μM. Individual blasts had IC_{50} as low as 169 nM and others as high as 2.2 μM (Figure 2b, Supplementary Figures 1 and 2). Within the small number of cells and cell lines tested, there was no clear trend for any AML subtype to be more sensitive to HDAC inhibition than others. The two FLT3-ITD primary AML blasts (Figure 2b, shaded gray) had similar sensitivity towards pracinostat as the FLT3 wt blasts. For pacritinib, cell lines dependent on either JAK2 or FLT3 signaling (such as SET-2, MV4-11 or MOLM-13) were the most sensitive to JAK2/FLT3 inhibition, both on cell proliferation as well as on the target biomarker levels (described elsewhere[9]). Similarly, primary AML blasts containing the FLT-3 ITD were amongst the most pacritinib-sensitive cells in cell proliferation assays.[33] *In vitro* synergy was observed when combining pracinostat and pacritinib (i.e., CIs < 1) in both FLT3-ITD cell lines and $JAK2^{V617F}$ cell lines, but not in HL-60 or KG-1 cells, which are wt for both genes. However, synergy was observed for F-36P cells, which are wt for both JAK2 and FLT3, but are fully dependent on JAK2 signaling. The best effects were observed in FLT3-ITD cell lines when both drugs were administered simultaneously, with CIs of 0.77 and 0.41 at a combination of doses that effectively blocked 90% of cell proliferation (ED_{90}) for MOLM-13 and MV4-11, respectively (Table 1). Weaker synergy was noted in $JAK2^{V617F}$ cell lines when pracinostat was administered 24 h before pacritinib, with CIs of 0.95 and 0.81 at ED_{90}, respectively, for SET-2 and HEL92.1.7 cells. The addition of pacritinib before pracinostat was antagonistic in all cell lines tested at ED_{90} (Table 1).

Table 1. *In vitro* combination of pracinostat (SB939) and pacritinib (SB1518)

Cell line	*Sequence*	*CI at* ED_{50} ± *s.d.*	*CI at* ED_{90} ± *s.d.*	n
MOLM-13 (FLT3 ITD)	Simultaneously	0.89 ± 0.1	0.77 ± 0.1	2
	SB939 first	1.49 ± 0.3	1.48 ± 0.4	
	SB1518 first	1.50 ± 0.1	1.32 ± 0.0	
MV4-11 (FLT3 ITD)	Simultaneously	0.55 ± 0.1	0.41 ± 0.2	2
	SB939 first	0.72 ± 0.1	0.55 ± 0.3	
	SB1518 first	1.69 ± 0.5	2.49 ± 1.3	
HL-60	Simultaneously	1.4 ± 0.2	1.35 ± 0.2	4
	SB939 first	1.35 ± 0.5	1.57 ± 0.3	
	SB1518 first	1.67 ± 0.2	2.17 ± 1.1	
KG1	Simultaneously	1.6 ± 0.2	1.7 ± 0.1	2
	SB939 first	1.3 ± 0.5	1.0 ± 0.2	
	SB1518 first	1.8 ± 0.1	2.7 ± 0.6	
SET-2 (JAK2mt)	Simultaneously	0.91	1.27	1
	SB939 first	1.13	0.95	
	SB1518 first	0.98	1.41	
HEL92.1.7 (JAK2 mt)	Simultaneously	2.07 ± 0.9	2.40 ± 1.4	2
	SB939 first	0.99 ± 0.0	0.81 ± 0.1	
	SB1518 first	2.12 ± 1.0	2.30 ± 0.7	
F36P (GM-CSFdep, JAK2 wt)	Simultaneously	0.74 ± 0.2	0.6 ± 0.2	3
	SB939 first	0.98 ± 0.3	0.8 ± 0.3	
	SB1518 first	1.81 ± 0.8	1.9 ± 1.0	

Abbreviations: CI, combinatorial indice; GM-CSF, granulocyte macrophage colony-stimulating factor; mt, mutant; wt, wild type.

Pracinostat is efficacious in various animal models of human AML

Pracinostat as a single therapy was tested in different mouse models of human AML. Treatment of mice bearing MV4-11 xenografts with pracinostat (25 or 50 mg/kg per day for 21 days) induced significant ($P < 0.001$) inhibition of tumor growth (TGI), by 59 and 116%, respectively. Complete tumor regression was observed in 6 out of 10 animals at the end of the treatment after the 50-mg dose (Figure 3a). To determine the anti-tumor efficacy in a model that is less sensitive to pracinostat, based on

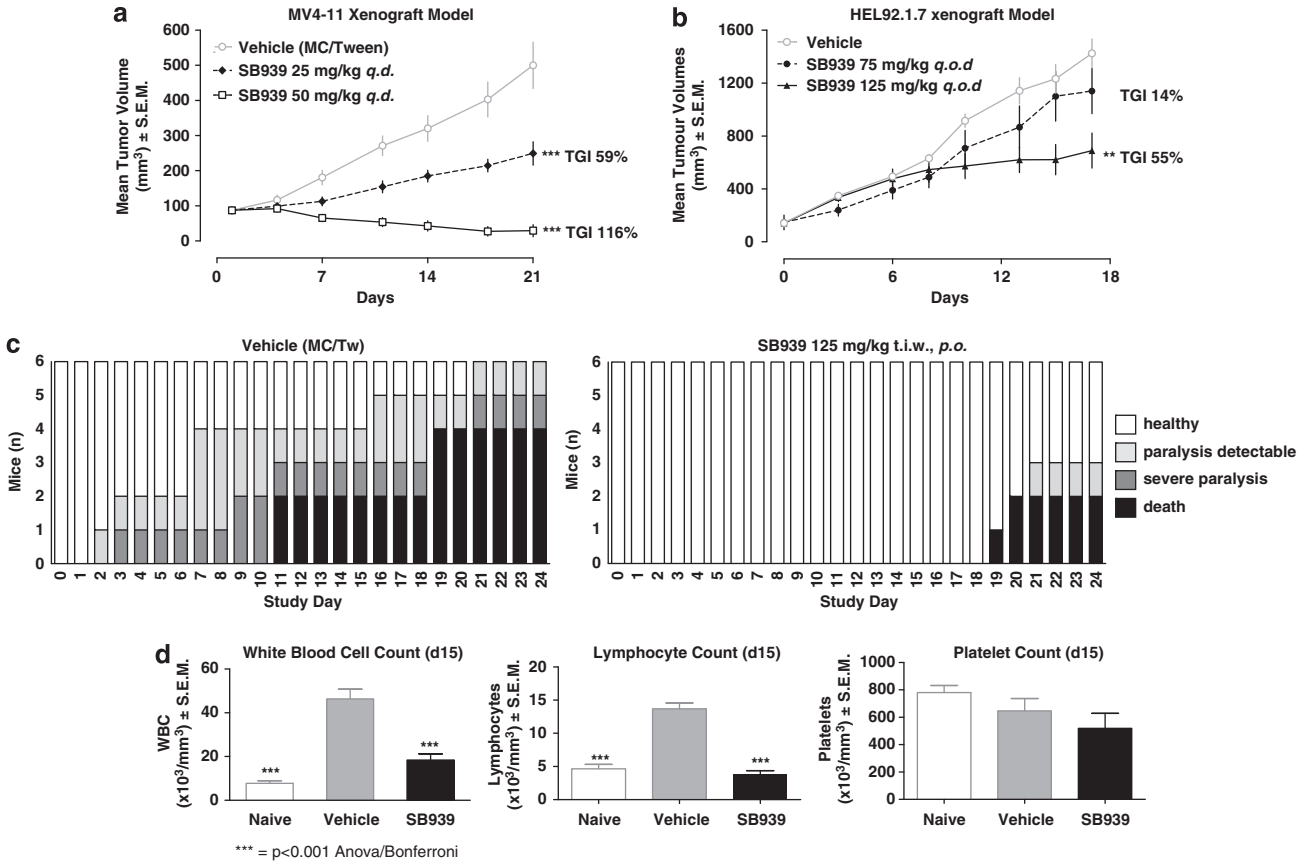


Figure 3. Pracinostat is efficacious in two s.c. and an orthotopic model of AML. (**a**) Female BALB/c nude mice ($n = 10$ per group) were inoculated with 1×10^7 MV4-11 cells s.c. into the right flank, and treatment as indicated was started on study d9. (**b**) Female BALB/c nude mice ($n = 5$ per group) were inoculated s.c. with 5×10^6 HEL92.1.7 cells, treatment was started on d18. (**c**) Female SCID mice ($n = 6$ per group) were injected with 1×10^7 HL-60 cells intravenously and scored daily for symptoms of paralysis. Treatment with pracinostat (SB939) 125 mg/kg three times per week was initiated on d30, before the first animal showed symptoms of paralysis. Daily disease scores are shown in (**c**); blood counts of naive mice, vehicle- or pracinostat-treated mice are shown in (**d**). 'Naive mice' refers to age-matched SCID mice that were not injected with HL-60 cells. Statistical significance was determined using analysis of variance/Bonferroni, ***$P < 0.001$ and **$P < 0.01$ significance compared with vehicle-treated animals.

cell proliferation *in vitro*, mice bearing HEL92.1.7 xenografts were treated with pracinostat at either 75 or 125 mg/kg every other day for a total of 17 days. Dose-dependent TGI (55%) was observed, which was statistically significant ($P < 0.01$) for the higher dose. Pracinostat was very well tolerated in both studies (maximum body weight losses of 7 and 4.7%, respectively, in the MV4-11 and HEL92.1.7 model). See Supplementary Table 1 for an overview of the efficacy in different models. To assess the efficacy of pracinostat in a more physiological relevant model, HL-60 cells were engrafted orthotopically (via tail vein injection). Treatment with 125 mg/kg pracinostat in a thrice weekly schedule was started once the disease was established on d30, (as measured by fluorescence-activated cell sorting analysis of human cell-surface markers on HL-60 cells in the murine bone marrow and peripheral blood, data not shown), but before the onset of the first symptoms, such as hind-leg paralysis. Pracinostat treatment led to a 17-day delay of the disease onset and a 50% reduction of death caused by progressive AML on study d24 (Figure 3c, upper panel). White blood counts on d15 of the study were six-fold elevated and lymphocyte counts three-fold elevated in vehicle-treated animals compared with disease-free SCID mice (i.e., mice that were not inoculated with HL-60 cells). Pracinostat treatment led to a significant ($P < 0.001$) decrease in blood counts, effectively normalizing blood counts to the levels of disease-free mice, without significantly reducing the numbers of platelets at the same time. These data demonstrated the efficacy and good tolerability of pracinostat in an orthotopic model of AML (Figure 3d).

Pracinostat and pacritinib are synergistic in two different AML disease models

The SET-2 model of megakaryoblastic leukemia was chosen to test the *in vivo* combination of pacritinib and pracinostat, as the growth of this cell line has been shown to be dependent on the JAK2-STAT5 signaling.[34] Pacritinib as a monotherapy, dosed at 150 mg/kg twice daily (i.e., the maximum tolerated dose) led to a 61% TGI, whereas pracinostat dosed at 75 mg/kg q.o.d (less than half the maximum tolerated dose) inhibited tumor growth by 56%. On the basis of tumor volume, the TGI of the drug combination was 86% (Figure 4a). On the basis of tumor weight, monotherapy led to 47 and 42.5% TGI for pacritinib or pracinostat, respectively (Figure 4b), whereas the combination TGI by 75%, giving rise to a CCI value of -0.06 (indicating synergy). Interestingly, several infrequent, but obvious metastases, were found in the lymph nodes of mice in each group, except in mice dosed with pacritinib alone or the animals treated with the combination of both drugs (data not shown). When analyzing tumors at the end of the study after chronic treatment with either monotherapy or the drug combination, pSTAT5 levels were decreased by about 50% by pacritinib alone and by 75% by the combination treatment. Pracinostat alone led to increased pSTAT5 levels compared with

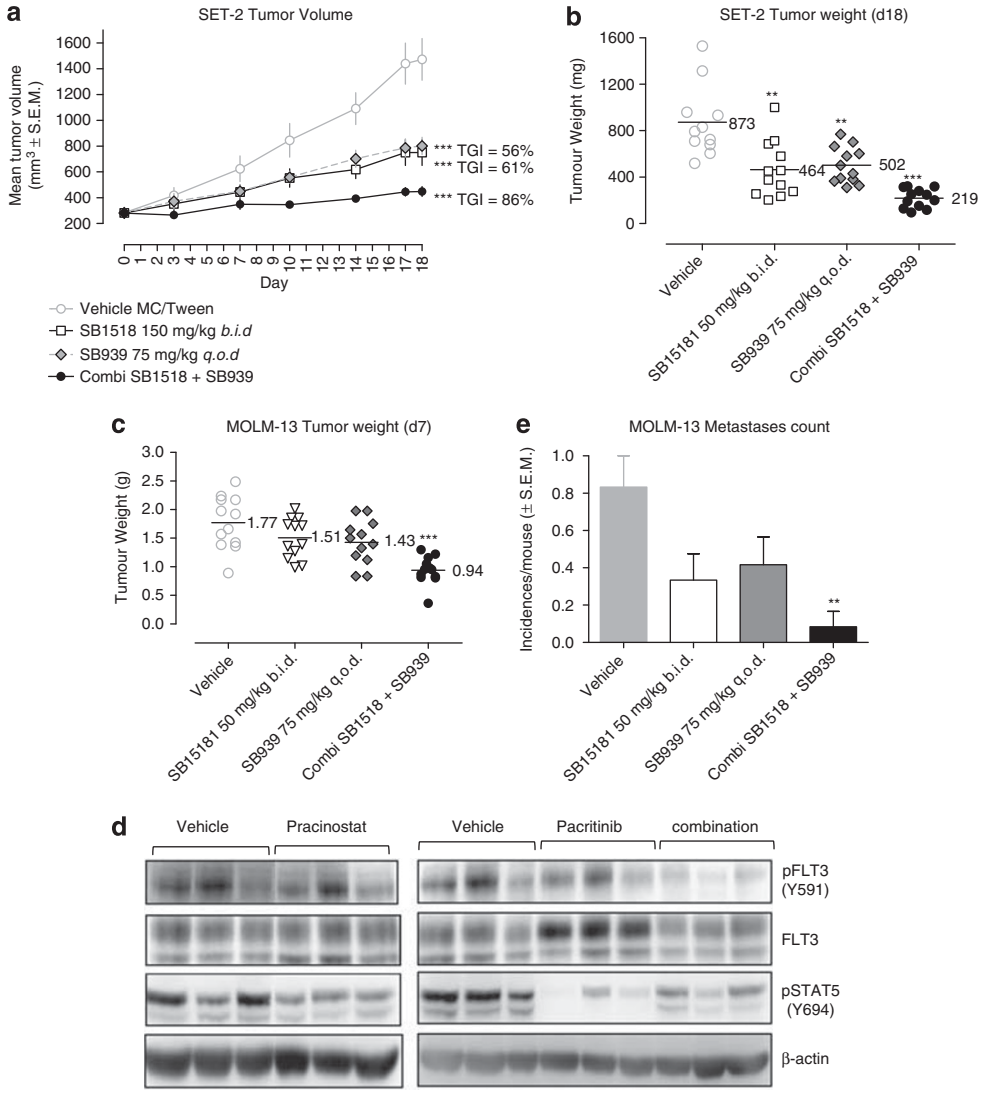
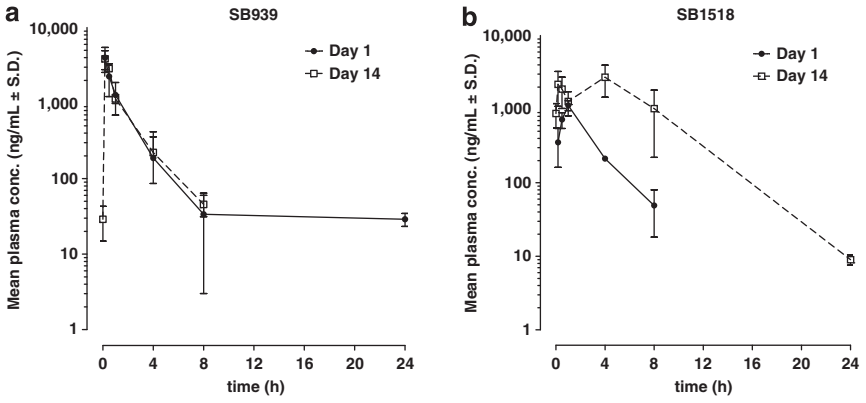


Figure 4. Pracinostat combined with pacritinib is efficacious and synergistic *in vivo* in two different models of human AML. Female SCID-beige mice ($n = 12$ per group) were inoculated with 5×10^6 SET-2 cells and treatment was started on d33 post-implantation (**a**, **b**). For the combination group, both pacritinib (SB1518) and pracinostat (SB939) were administered in half the volume (as a 20 mg/kg solution) every other day; when both compounds were given simultaneously, all other doses were given at 10 mg/kg, at least 8 h apart for 19 days (d0–d18). There was one non-treatment-related death in the vehicle group on study d11 (gavaging error). Female SCID mice ($n = 12$ per group) were inoculated with 5×10^6 Molm-13 cells (**c**, **d**). Treatment was started on d11, animals were dosed every other day for 8 days, in contrast to (**a**), animals were dosed with pacritinib only daily (in the evenings), pracinostat dosing remained unchanged, all dosing was done in a 10 mg/kg solution. On the last day, mice were killed and excised tumors were weighed. The doses showing significant TGI versus vehicle (on the last day of the study) using analysis of variance/Dunnet's post test, are indicated with $^{**}P < 0.01$ or $^{***}P < 0.001$. (**e**) Tumors from the MOLM-13 efficacy study (**c**, **d**) were harvested on the last day 3 h post pacritinib dosing and immediately lysed. Western blot analyses for pFLT3, FLT3, pSTAT5 and β-actin are shown from the lysates of three randomly selected tumors from each treatment group of (**c**).

vehicle treated animals (Supplementary Figure S3). Subsequently, the combination of pracinostat and pacritinib was also tested in mice bearing FLT3-ITD-driven MOLM-13 s.c. xenografts. *In vitro* data showed that the MOLM-13 cells were the most sensitive to both pracinostat and pacritinib,[9] and that the synergy of combination treatment was greater than in SET-2 cells (Table 1, Figure 2a). Therefore, a lower dose of pacritinib was used (50 mg/kg daily) in combination with pracinostat (75 mg/kg q.o.d) for the *in vivo* study with MOLM-13. This MOLM-13 xenograft model is extremely aggressive, within 10 days after cell injection when treatment was initiated, with mean tumor volumes being 553 mm^3. Tumors were excised on d7 of the study (after 8 days of treatment) when the mean tumor volume in the vehicle group was 2952 mm^3 (and the average tumor weight was 1770 mg). Treatment with pracinostat or pacritinib as a monotherapy led to a 19.2 and 14.7% reduction in tumor weight, respectively, whereas the combination treatment reduced the average tumor weight by 46.9%, to 940 mg ($P < 0.001$) (Figure 4c), giving rise to a synergistic CCI of -0.16. Western blot analysis of the tumors (harvested 3 h post pacritinib treatment) on d7 showed that both pacritinib and, to a lesser extent, also pracinostat decreased levels of pSTAT5. Levels of pFLT3 were only decreased marginally, but chronic treatment with pacritinib alone increased expression total levels of FLT3 significantly. In combination, pacritinib and pracinostat not only normalized FLT3 levels, but also reduced pSTAT5 levels and nearly abolished all pFLT3 (Figure 4d).

Many of the s.c. implanted tumors in this model metastasized (to the lungs, the nearest breast fat-pad and/or the lymph nodes). In the vehicle group, 9/12 mice had 1 or 2 metastases, some of



Parameter	75 mg/kg (Combination)		75 mg/kg (Single)*
	Day 1	Day 14	Day 1
$t_{1/2}$ (h)	1.3	1.5	2.4
T_{max} (h)	0.17	0.17	0.17
C_{max} (ng/mL)	4006	3857	3948
$AUC_{0-\infty}$ (h*ng/mL)	4342	4600	2761

Parameter	150 mg/kg (Combination)		150 mg/kg (Single)*
	Day 1	Day 14	Day 1
$t_{1/2}$ (h)	1.6	2.4	2.2
T_{max} (h)	1	4	1.3
C_{max} (ng/mL)	1129	2717	3324
$AUC_{0-\infty}$ (h*ng/mL)	3769	18063	7317

Figure 5. Pharmacokinetic analysis of pracinostat and pacritinib administered in combination. Female BALB/c nude mice were dosed with 150 mg/kg pacritinib (SB1518) plus 75 mg/kg pracinostat (SB939) simultaneously either once ('day 1') or for a total of 14 days (i.e., 7 doses of SB939, administered every other day in the morning, plus SB1518 for 14 times, every day morning and evening, 'day 14'). Plasma was collected at 10, 30 and 60 min post-dose on d1/d14 ($n = 5$) as well as 4, 8 and 24 h post-dose ($n = 3$). Pharmacokinetic parameters were calculated by a non-compartmental method, using WinNonlin software, and compared with values obtained in previous experiments in the same mouse strain. * Indicates that values were extrapolated from BALB/c nude mice dosed with 50 mg/kg pacritinib or pracinostat. Results for pracinostat are shown in (**a**), and for pacritinib in (**b**).

them weighing as much as 208 mg. Metastatic incidences (i.e., obvious, individual metastases per mouse) were reduced by 60 and 50%, respectively, after single-agent treatment with pacritinib or pracinostat, respectively (Figure 4e). In combination, metastatic incidents were reduced by 90%, leading to a CCI of -0.1, indicating that the combination was able to inhibit metastatic spread synergistically. The pharmacokinetic parameters of pracinostat were similar after 14 days of co-administration with pacritinib, with the concentration–time curve being nearly identical on d1 and d14 (Figure 5a). The C_{max} and $AUC_{0-\infty}$ of pacritinib were increased approximately two-fold in C_{max} and approximately four-fold in AUC after chronic dosing in combination (Figure 5b). Pacritinib also exhibited a two-fold increase in exposure following repeated dosing in toxicokinetic studies in mice as single agent (data not shown). Therefore, the observed *in vivo* synergy is unlikely because of a moderate increase in pacritinib exposure in the combination study design.

Effects of pracinostat, pacritinib or the combination on tumor-induced growth factors and cytokines/chemokines

HDACi as well as JAK2 inhibitors have been described to affect the production of various growth factors, chemokines and cytokines,[35–38] thereby influencing tumor growth. Plasma cytokine/growth factor/chemokine levels in naive mice (without SET-2 xenograft), or xenografted mice treated with vehicle, pracinostat or pacritinib alone, or a combination thereof, were analyzed using multiplex analysis/enzyme-linked immunosorbent assay. SET-2 tumors caused the plasma levels of IL-6, IP-10, KC, MCP-1 and MIP-1β to increase (Figure 6) compared with levels present in naive mice (i.e., mice that were not inoculated with tumor cells). Single-agent treatment with pacritinib or pracinostat led to the normalization of IL-6, IP-10, KC and MIP-1α. A synergistic effect for the combination of pacritinib or pracinostat was observed for the chemokine MCP-1. Plasma levels of MCP-1 were elevated from 44 (in naive mice) to 168 pg/ml in tumor-bearing mice. Pracinostat or pacritinib as single agents reduced levels by 40 and 10%, respectively. In combination, MCP-1 levels were reduced by 69% (to 53 pg/ml).

DISCUSSION

In this study, we show the efficacy and tolerability of the pan HDACi pracinostat in various *in vitro* and *in vivo* models of AML, and show synergistic effects at multiple levels in combination with the JAK2/FLT-3 inhibitor pacritinib in both the *in vitro* and *in vivo* setting. We also explored the possible mechanisms for these synergistic anti-tumor effects. Previously reported synergistic effects of an HDACi combined with a JAK2 inhibitor was attributed to impaired chaperone function of heat shock protein 90 by the HDACi, promoting proteasomal degradation and depleting total JAK2 levels.[21] FLT3 is another heat shock protein 90 client protein, where mutant forms such as FLT3-ITD are more dependent on the chaperone association than their wt counterpart.[39] In addition, earlier studies have shown that HDAC inhibition depleted mRNA levels of $JAK2^{V617F}$.[21] Therefore, not surprisingly, we have demonstrated that pracinostat not only reduced JAK2/STAT5 protein levels in cells bearing a JAK2 mutation, but also FLT3/STAT5 levels in cells with a FLT-3 mutations (as seen in Figure 1). Recent studies with the HDACi trichostatin A demonstrate that JAK2/STAT3 signaling was decreased by upregulation of the expression of *suppressors of cytokine signaling (SOCS) 1* and *3* genes.[40] A possible direct effect of the HDAC inhibition on the phosphorylation of JAK2, STAT5 and FLT3 was not investigated in this study.

SB939 showed potent inhibition predominantly in JAK2V617F- or FLT3-ITD-harboring cell lines (Figure 2a). The cell line with the lowest IC_{50} of 70 nM was HL-60, which carries an *N-RAS* mutation. HDACi have been shown to block Ras-dependent signaling and growth transformation.[41] Surprisingly, in HEL92.1.7 and MOLM-13 cells, the pracinostat IC_{50} on proliferation is lower than the IC_{50} on inhibition of JAK2 or FLT3-ITD protein levels, respectively.

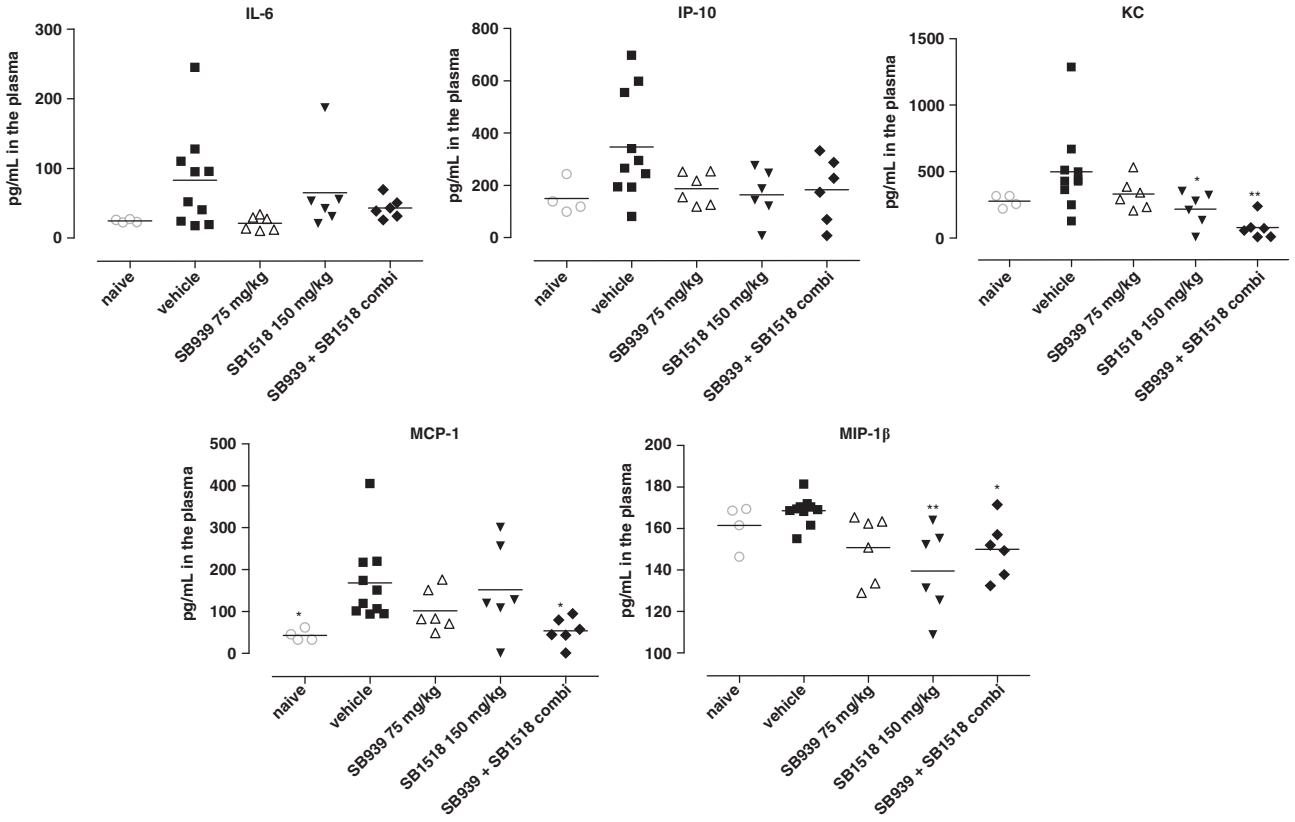


Figure 6. Pracinostat and pacritinib have synergistic effects on AML-induced plasma cytokines/growth factors/chemokines. Plasma levels of a panel of 32 cytokines/growth factors/chemokines were analyzed using the MAP Mouse Cytokine/Chemokine panel from Millipore. Plasma was collected from SCID-beige mice bearing s.c. SET-2 tumors on d18, 3 h post-dose, after chronic dosing with pacritinib and pracinostat from the mice used in the efficacy study described in Figure 4.

This discrepancy might be a result of modulation of other genes besides $JAK2^{V617F}$ and FLT3-ITD by HDAC inhibition.

Pacritinib is an equipotent inhibitor of JAK2 and FLT3, which is effective in reducing JAK2/STAT5 and FLT-3 JAK2 signaling in JAK2 and FLT3 mutant cells, respectively.[33] The combination of pracinostat and pacritinib led to synergistic effects with a complete inhibition of downstream STAT5 signaling, an increased efficacy on cell proliferation and the induction of apoptosis. *In vitro* combination studies in different cell lines with either wt or mutant JAK2 or FLT3 also demonstrated synergy, mostly in cells that carried the mutant protein. One exception was the F36-P cell line. The growth of this cell line depends on exogenously added granulocyte macrophage colony-stimulating factor,[42] which signals exclusively via JAK2, making it a JAK2 wt-dependent cell line. This indicates that synergy between a JAK2 inhibitor and an HDACi might also work in cells that are fully dependent on JAK2 (wt) signaling. Consistent with this, similar *in vitro* synergy was observed in the JAK2 wt SET-2 cells and F36-P cells but not in FLT3 mutant cell lines with the specific pan-JAK inhibitor ruxolitinib in combination with pracinostat (data not shown).

LMO2 is a transcription factor involved in normal hematopoiesis, but also leukemogenesis that is overexpressed in many AML cells.[43] Interestingly, LMO2 levels were downregulated synergistically in MOLM-13 cells with pacritinib and pracinostat, and may be a result of another synergistic interaction between JAK2 and HDAC. Dawson *et al.*[43] demonstrate that JAK2 inhibition leads to lower levels of histone H3 Y41 phosphorylation on the promoter of LMO2, whereas increasing the binding of heterochromatin protein 1α at the same site, resulting in lower expression of LMO2. JAK2 may have an epigenetic role in the nucleus to influence the status of H3 acetylation. It has been demonstrated previously that phosphorylation of H3 (on S10) leads to increased efficiency of a subsequent H3 acetylation, resulting in synergistic modifications of gene expression.[44]

Pacritinib, as well as targeting JAK2, is a potent FLT3 inhibitor. Our group has recently discovered that treatment of FLT3-ITD cells with FLT3 inhibitors lacking JAK2 activity (e.g., ABT-869, VX-680 or sunitinib), leads to an upregulation of JAK2 activity, causing secondary resistance.[33] Therefore, although combinations of FLT3 inhibitors and HDACi have been described to show synergy in *vitro*,[23,24] this combination without the additional JAK2 inhibition could lead to resistance after chronic dosing and not show enhanced efficacy in the *in vivo* setting. This may explain why none of the studies showing *in vitro* synergy reported any *in vivo* synergy data. Pacritinib as a dual JAK2/FLT-3 inhibitor is therefore ideally suited for a combination with an HDACi and superior to an inhibitor that only affects FLT3 kinase without targeting other JAK family kinases.

Although the combination of pracinostat and pacritinib showed synergy *in vitro*, the synergy was greater in the *in vivo* setting in both AML models tested. This indicates that there are additional synergistic mechanisms that are only working in the whole animal setting. One example is the synergistic effects observed on metastases. In AML patients, leukemia cutis and extramedullary involvement of organs such as the lungs are common. Respiratory distress syndrome secondary to lung involvement causes a significant percentage of the morbidity/mortality associated with AML.[45] Therefore, the synergy observed in reducing metastatic sites in the animal model is certainly of great relevance for AML patients. Interestingly, significantly higher plasma levels of MCP-1 were measured in untreated AML patients with extramedullary sites involved than in those with complete remission.[46]

This highlights the potential therapeutic benefit with our observation that pracinostat and pacritinib synergistically decrease MCP-1 plasma levels as well as metastatic occurrences. In both models tested, chronic treatment with one drug alone led to the increase of a signaling pathway. Pacritinib in the Molm-13 model or pracinostat (Set-2 model) led to increased FLT3 or pSTAT5 levels, respectively, whereas the combination treatment in both studies was most efficiently suppressing the signaling, indicating that a combination treatment can overcome treatment-induced resistance.

Effects of tumor-induced elevations of cytokine and chemokine levels may be another mechanism for the synergy observed with pracinostat and pacritinib. HDACi as well as JAK2 inhibitors have been described to affect the production of various growth factors and cytokines,[35–37] thereby influencing tumor growth. Manshouri *et al.*[38] recently showed that resistance to JAK2-inhibitor treatment of MPNs is mediated by cytokines produced by the bone marrow stroma. Distinctly high levels of IL-6, FGF4 and CXCL10/IP-10 were detected in co-cultures of stromal cells and SET-2 cells, mediating resistance to the JAK2 inhibitor antiprimod. Although SCID beige mice that lack B-cells, T-cells, as well as natural killer cells, were used for the SET-2 AML model in our studies, high circulating levels IL-6, IP-10, KC and MCP-1 were measured in the tumor-bearing mice without drug treatment. Treatment with pacritinib or pracinostat as single agents led to the normalization of IL-6, IP-10, KC and MIP-1α, and a synergistic normalization of MCP-1 levels was observed with the combination treatment.

Taken together, our studies demonstrated the synergistic efficacy of a combination of pracinostat and pacritinib in *in vitro* and *in vivo* models of AML and offer mechanistic insights for this synergy. These data provide a scientific rationale for the combination of pracinostat and pacritinib for advanced acute leukemia, which warrants further exploration in a clinical trial.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Blood Cancer Journal website (http://www.nature.com/bcj)