PAK1 is a Therapeutic Target in Acute Myeloid Leukemia and Myelodysplastic Syndrome

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- Targeting of PAK1 inhibits primary AML and MDS patients’ cells including leukemia stem cells, but spares healthy stem and progenitor cells
- Inhibition of PAK1 induces differentiation and apoptosis of AML cells through downregulation of MYC and a core network of MYC target genes

ABSTRACT
Poor clinical outcome of Acute Myeloid Leukemia (AML) and Myelodysplastic Syndrome (MDS) has been attributed to failure of current chemotherapeutic regimens to target leukemic stem cells. We recently identified p21-activated kinase (PAK1) as a downstream effector molecule of H2.0-like homeobox (HLX), a gene functionally relevant for AML pathogenesis. In this study, we find that inhibition of PAK1 activity by small molecule inhibitors or by RNA interference leads to profound leukemia-inhibitory effects both in vitro and in vivo. Inhibition of PAK1 induces differentiation and apoptosis of AML cells through downregulation of MYC oncogene and a core network of MYC target genes. Moreover, we find that PAK1 up-regulation occurs during disease progression and is relevant for patient survival in MDS. Importantly, we find that inhibition of PAK1 inhibits primary human leukemic cells including immature leukemic stem cell-enriched populations. Our studies highlight PAK1 as a novel target in AML and MDS, and support the use of PAK1 inhibitors as a therapeutic strategy in these diseases.

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INTRODUCTION

Acute Myeloid Leukemia (AML) and Myelodysplastic Syndrome (MDS) are heterogeneous neoplastic blood diseases with poor clinical outcome. AML has a 5-year overall survival of only 3-8% in patients aged 60 years and older (1, 2). While disease course and prognosis are highly variable in MDS, approximately 30-40% of patients with MDS will progress to AML (1, 2), and allogeneic stem cell transplantation remains the only curative option. Current chemotherapeutic approaches, which have remained unchanged for the past 40 years, target rapidly dividing cells, having limited effects on the pre-leukemic and leukemic stem cells (LSC) responsible for disease propagation and relapse. Targeting of (pre-)leukemic stem cells is vital to maintaining remission and improving survival in AML and MDS.

We recently identified p21-activated kinase (PAK1) as a downstream effector molecule of H2.0-like homeobox (HLX), a gene up-regulated in pre-leukemic stem cells and functionally relevant for AML pathogenesis (3). Utilizing data from a large cohort of AML patients, we found that higher levels of PAK1 in AML are associated with significantly inferior overall patient survival, implicating PAK1 as a potential clinical target in this disease. PAK1 is a member of the PAK family of serine/threonine kinases that act downstream of the Rho small GTPases Cdc42 and Rac1 in a variety of signaling pathways. PAKs in mammalian cells are classified into group I (PAK1, PAK2, and PAK3) and group II (PAK4, PAK5, and PAK6) on the basis of structural similarities, although group members exhibit significant functional differences (4). PAK family members have been shown to play important roles in cell proliferation and motility, as well as in cell transformation and tumor progression (5). PAK1 has been implicated in oncogenic transformation in several malignancies including breast, ovarian, melanoma, T-cell lymphoma, liver, and bladder cancers (6-14).

In this study, we assessed the importance of PAK1 in the myeloid malignancies AML and MDS. We find that inhibition of PAK1 both chemically and using short-hairpin RNA (shRNA) targeting has profound anti-leukemic effects both in vitro and in vivo. Inhibition or reduction of PAK1 leads to induction of monocytic differentiation and apoptosis via repression of MYC and a MYC transcriptional network. Moreover, we show that leukemia cells from human patients with AML are reliant on PAK1 function and that chemical inhibition of PAK1 preferentially inhibits leukemic cells over healthy cells, including in immature, LSC-enriched populations. Our studies highlight PAK1 as a novel target in AML and MDS and support the use of PAK1 inhibitors as a therapeutic strategy in these diseases.

METHODS

Cell culture
THP-1, MOLM-13, HL-60, and KG1a cells were purchased from ATCC and cultured according to ATCC recommendations. All cell lines were maintained in an incubator at 37°C and 5% CO2. Primary mononuclear samples were obtained from patients with AML, or from healthy donors. Patient characteristics are shown in Supplemental Figure 5A. The studies were approved by the IRB (#2008-942).

Knockdown of PAK1 and PAK2 by shRNA and chemical inhibition of PAK1
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To silence PAK1 and PAK2 by RNAi, we transduced cells with plko.1-based lentiviruses (Open Biosystems) by spinfection at 1800 rpm for 90 minutes at 37°C in 8 ug/mL polybrene. Stably transduced cell lines were selected with 1.2 ug/mL puromycin for 6 days. PAK1 specific sequences used were TRCN0000002225 (CGATGAGAAATACCAAGCATA) and TRCN0000002226 (GCGATCCTAAGAAGAATATA). PAK2 specific sequences used were TRCN0000002115 (CTCTAGGACCAAGATGATT) and TRCN0000002118 (TGGGAATGGAAGGATCGTTA). Small molecules IPA-3 (#3622, Tocris Bioscience), PIR3.5 (#4212, Tocris Bioscience), and FRAX-597 (Genentech, Inc.) were suspended in DMSO and assayed at the indicated concentrations.

**Proliferation, apoptosis, differentiation, and clonogenic assays**

Manual cell counts were performed by culturing 2 x 10^5 cells/mL in 1 mL culture medium in 24-well plates. Viable cells were counted daily using trypan blue exclusion. Cell differentiation was assessed by morphological observation after Diff Quik (IMEB) staining as well as FACS analysis using antibodies directed against human CD11b [CR3] (BD Biosciences) and CD15 [VIMC6] (Life Technologies) on a BD FACSARia II machine. Cell line and stem cell apoptosis was measured with Annexin-V FLUOS Staining Kit (Roche) and DAPI (1:1000) followed by flow cytometric analysis. Viable cells were defined as Annexin-V/DAPI-. For AML cell line colony assays, 3000 cells were plated in HSC002SF methylcellulose (R&D Systems, Inc.) with 5% FBS. Colonies were scored at day 10. For primary cell colony assays 1-3x10^5 mononuclear cells were plated in 1 mL HSC003 (R&D Systems, Inc.) methylcellulose media with 40 ug/mL LDL (Calbiochem), 10 ng/mL human IL-6 and 25 ng/mL human FLT3L. For serial re-plating assays, colonies were re-suspended and all cells were re-plated as above with indicated doses of IPA-3 and FRAX-597. Colonies were scored at day 10.

**Western Blotting**

Proteins were resolved on 10-12% polyacrylamide gels after lysis in denaturing buffer (R&D Systems, Inc.) and transferred to Nitrobind nitrocellulose membranes (Maine Manufacturing). Membranes were blocked for one hour in 2% milk in PBS containing 0.1% Tween-20 (TBS-T). For detection of protein phosphorylation, membranes were blocked in TBS-T with 2.5% BSA (Roche). Primary antibody incubations were carried out at 4°C overnight or at room temperature for one hour in TBS-T containing 2.5% BSA. The following primary antibodies were used: AKTS473 (1:1000, #9271S, Cell Signaling), AKT (1:1000, C67E7 #4691S, Cell Signaling), ACTIN (1:1000, C-11 #sc-1615, Santa Cruz Biotech), MYC (1:1000, D84C12 #5605, Cell Signaling) and PAK1 (1:1000, #2602, Cell Signaling). Secondary antibody incubations (LI-COR 926-32213 IRDye 800CW Donkey anti-Rabbit IgG (H+L) 1:5000 and LI-COR 926-68074 IRDye 680RD Donkey anti-Goat IgG (H+L) 1:5000) were performed at room temperature for one hour. Blots were imaged on a LI-COR Odyssey Imaging System.

**Microarray experiments, analysis and qRT-PCR**

Microarray data are available in the Gene Expression Omnibus, accession number GSE70791. For microarray, RNA was isolated from THP-1 cells after 5 hours of treatment with IPA-3 (6 ug/mL), FRAX-597 (2 ug/mL) or an equal volume of DMSO using Trizol Reagent (Invitrogen). After evaluation of the quality of RNA with an Agilent2100 Bioanalyzer, RNA was labeled with the GeneChip WT terminal labeling kit (Affymetrix), labeled cRNA of each individual sample was hybridized to Affymetrix Human Gene 2.0ST microarrays (Affymetrix), and scanned by GeneChip Scanner 3000 7G system (Affymetrix) according to standard protocols. For evaluation of individual genes, RT reaction was performed using iScript cDNA Synthesis Kit (Bio-Rad) and qPCR was performed using Power SYBR Green Master Mix (ThermoFisher) on a ViiA 7 Real Time PCR System. qRT-PCR primers: hPAK1 fwd: GATGCTGGAACCTAAACCA rev: GGCCGCTCTTTCTCTCTTT; hPAK2 fwd: ACAGAAGCACCCTCAGTATG rev:
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AAAGACTTTGGCAGCACCCTC; hDUSP7 fwd: GCGAGTTCACCTACAAGCAG rev: TCTGCATCAGATAGGCCACA; hMYC fwd: CCTGGTGCTCCATGAGGAGAC rev: CAGACTCTGACCTTTTGGCAGG; hAMD1 fwd: CTCACCAAGGGTACCCACAC rev: CGTCCCATACAATATGCTGCT;

In vivo leukemia models

All animal experiments were performed in compliance with institutional guidelines and approved by the Animal Institute Committee of the Albert Einstein College of Medicine (#20130102). NOD-SCID IL2Ry null (NSG) mice were sub-lethally irradiated (200 rad) and were intravenously injected (tail vein) with 3 x 10^5 THP-1 cells. At 46 days, tissues were fixed in Neutral Buffered Formalin fixative and embedded in paraffin for H&E staining. Tissues were imaged using EVOS Cell Imaging station (Life Technologies). 4 animals/construct/experiment, n=2. For xenografting of AML patient samples mononuclear cells were isolated and treated for 12 hours with either DMSO or IPA-3. 1 x 10^6 cells were transplanted into each animal of a cohort of 2-5 NSG mice/condition. Six weeks post-transplantation, murine bone marrows were assessed via flow cytometry for the presence of CD45^+CD33^+ human myeloid cells. The experiment was performed twice, with two independent primary AML patient samples.

Stem cell-enriched apoptosis assays

Viability of stem/progenitor cells was assessed based on a previously published assay (15). AML or healthy cells (AllCells) were cultured in Myelocult H5100 media (Stem Cell Technologies) supplemented with Primocin 100ug/mL, hIL3 10ng/mL, hSCF 100ng/mL, hTPO 50ng/mL, hIL6 10ng/mL, hFlt3L 20ng/mL, and hLDL 40ug/mL for 24 hours with IPA-3, FRAX-597 or DMSO. All drug treatments were performed in triplicate. After 24 hours in culture, cells were isolated, washed twice with PBS, and labeled with anti-CD38-PE-Cy7 (HIT2) and anti-CD34-APC for 20 mins.

Data sets and statistical analysis

For MDS analyses, we used the publicly available data set GSE19429 consisting of gene expression data of CD34^+ cells isolated from the bone marrow of 183 MDS patients. For survival analysis, patients were grouped into low- and high-expression categories by use of median expression of PAK1 or PAK2 and survival was assessed by log-rank test. Survival package in R was used to plot Kaplan–Meier curves. Correlation of PAK1 expression and survival in individual patients was assessed by correlation coefficient R and regression p-value. Multivariate analysis using Cox regression model was performed with SAS9.3 using the clinical information available for patients from the GSE19429 dataset (including gender, FAB classification, hemoglobin, bone marrow blasts, white blood cell, and PAK1 expression status (low/high), IPSS category). For AML analyses, we used the publicly available data set GSE14468 consisting of 598 patients with AML. Patients were grouped into low- and high-expression categories by the 33rd percentile of PAK1 expression. Fisher’s exact test of independence was used to assess correlations of patient characteristics (FAB subtype/karyotype/mutation) and PAK1 expression. Error bars represent mean plus/minus standard deviation unless specifically indicated. P values are by two-tailed student’s T-test unless specifically indicated.

RESULTS

Targeting PAK1 signaling slows growth and reduces leukemic colony formation capacity of human acute myeloid leukemia cells

To determine whether PAK1 is functionally important in AML, we first examined the effects of PAK1 inhibition on the growth of a panel of human AML cell lines (MOLM-13, HL-60, THP-1 and KG1a). We selected two small molecule inhibitors that target PAK1 directly: IPA-3, an allosteric inhibitor of PAK1-3 activation, and FRAX-597, an ATP-
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competitive PAK1-3 inhibitor currently under advanced pre-clinical investigation for solid tumor malignancies (16, 17). Treatment with IPA-3 or FRAX-597 inhibited the proliferation of all four cell lines in a dose-dependent manner with 24 hour IC50s in the range of 0.45 to 5.3 μg/mL (6 μM - 15 μM for IPA-3; 800 nM - 8 μM for FRAX-597) (Figure 1A,B; Supplemental Figure 1A,B). Leukemic colony formation capacity of THP-1 and MOLM-13 AML cells was also inhibited upon treatment with both PAK1 inhibitors (Figure 1C,D). Importantly, we observed only mild toxicity with PIR3.5, a structural isomer of IPA-3 that has no inhibitory activity toward PAK1 and serves as a negative control for PAK1 inhibition (17) (Supplemental Figure 1C). To confirm PAK1 inhibition by both IPA-3 and FRAX-597 in AML cell lines, we evaluated levels of phosphorylated AKT at Ser473, an established PAK1 target (16, 18, 19). Treatment with either drug resulted in decreased phosphorylation of AKT at Ser473 in a dose-dependent manner (Supplemental Figure 1D,E).

To confirm these findings were due to specific inhibition of PAK1, we employed RNA interference with two independent shRNA sequences targeting human PAK1. We achieved reductions of 89% (shPAK1#1) and 54% (shPAK1#2) in PAK1 transcript levels and a corresponding decrease in the amount of PAK1 protein (Supplemental Figure 1F,G). PAK1 knockdown was confirmed by significant reduction in AKT Ser473 phosphorylation (Supplemental Figure 1G). PAK1 knockdown inhibited both the proliferation and leukemic colony formation capacity of THP-1 cells (Figure 1E,F). Notably, PAK1 knockdown was specific for PAK1 and did not result in reducing levels of PAK2, nor did PAK1 knockdown result in a compensatory increase in PAK2 levels (Supplemental Figure 1F). PAK3 was not expressed in the AML cell lines. Furthermore, to assess the importance of PAK2 in AML, we used two different shRNAs to reduce PAK2 expression in THP-1 and MOLM-13 cells. We found that PAK2 inhibition had no effect on proliferation of these AML cell lines (Supplemental Figure 1H,I).

**PAK1 inhibition induces apoptosis of AML cells**

To investigate the cellular mechanism of PAK1 dependent inhibition of cell growth and clonogenic capacity, cell lines were treated with increasing concentrations of IPA-3 or FRAX-597 for 24 hours and assessed for apoptosis and cell cycle arrest. Treatment with either compound resulted in a dose-dependent induction of apoptotic cell death in all four of the malignant myeloid cell lines tested, with observable progression through an early apoptotic stage (Figure 2A,B; Supplemental Figure 2A). Additionally, we observed a significant increase in apoptosis of AML cells with PAK1 knockdown compared to the non-silencing control (Figure 2C). Treatment of AML cell lines with either IPA-3 or FRAX-597 for 24 hours resulted in no changes in the cell cycle, despite reported roles for PAK1 in the regulation of cell cycle progression in other cell types (20) (Supplemental Figure 2B).

**Inhibition of PAK1 induces myelomonocytic differentiation of AML cells**

A block in terminal myeloid differentiation is a hallmark of myeloid leukemic cells. To determine if modulating PAK1 activity could overcome the differentiation block in AML, we assessed the expression of myelomonocytic surface markers following incubation of AML cell lines with increasing concentrations of IPA-3 or FRAX-597 for 24 hours. Treatment with either drug induced up-regulation of surface expression of mature myelomonocytic markers CD11b and CD15, indicating PAK1 inhibition differentiates AML cells toward the monocytic lineage (Figure 3A; Supplemental Figure 3A). We confirmed a direct role for PAK1 in affecting myeloid differentiation, as shRNA knockdown of PAK1 similarly induced differentiation of AML cells toward the monocytic
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lineage (Figure 3B; Supplemental Figure 3B). The appearance of cytomorphological features of mature myeloid cells, including multi-lobed nuclei and an increase in the cytoplasm:nucleus ratio, correlated with the induction of myelomonocytic markers following drug treatment or PAK1 knockdown (Figure 3C,D; Supplemental Figure 3C).

PAK1 inhibition leads to leukemic cell death by downregulation of a MYC transcriptional network

To obtain insight into the molecular mechanism for the induction of apoptosis and differentiation resulting from PAK1 inhibition in AML, we performed gene expression microarrays following treatment with either IPA-3 or FRAX-597. Consistent with our observed phenotypes, Ingenuity Pathway Analysis confirmed considerable enrichment for networks that regulate cancer, hematological disease, cell growth and proliferation and cell death and survival (Supplemental Figure 4A). Gene Set Enrichment Analysis confirmed our results that PAK1 inhibition leads to an increase in differentiation and apoptosis (Supplemental Figure 4B). Furthermore, we observed that treatment with either drug led to transcriptional changes inversely correlated with a MYC_UP.V1_UP gene set, comprising genes that are up-regulated in primary breast cancer cells overexpressing MYC (NES IPA-3 -1.309, p=0.02; NES FRAX-597 -2.359, p<0.001) (Figure 4A) (21). Strikingly, both inhibitors of PAK1 led to a downregulation of MYC as well as a core set of genes directly targeted by MYC (Figure 4B). We validated reduction of MYC expression by western blot following treatment with both inhibitors (Figure 4C; Supplemental Figure 4C). Furthermore, we confirmed MYC network downregulation by either inhibitor or PAK1 shRNA by validating a reduction in expression of several downstream targets of MYC including DUSP7 and AMD1 by qPCR (Figure 4D,E; Supplemental Figure 4D) (22-24).

PAK1 reduction inhibits AML progression in vivo

Given the in vitro anti-leukemic effects of PAK1 inhibition, we examined the in vivo effects of loss of PAK1 using a murine xenotransplantation model of AML. THP-1 cells expressing a non-silencing control or either of two PAK1 targeting constructs were injected into sub-lethally irradiated NSG mice. Gross organ analysis and histopathology at six weeks following transplantation revealed dramatically reduced infiltration of leukemic cells and preservation of normal NSG tissue architecture in the bone marrow, liver, and spleen of mice transplanted with PAK1 shRNAs compared to the non-silencing control (Figure 5A,B). Mice injected with cells expressing either of the PAK1 shRNAs had a 91% reduction in engraftment of THP-1 cells in the liver compared to mice injected with the non-silencing control cells (Figure 5C). Consistent with our in vitro studies, the few THP-1 cells that could be isolated from PAK1 knockdown experimental animals had increased CD11b cell surface expression and displayed a more differentiated morphology in cytospin preparations compared to non-silencing control cells (Figure 5D,E).

Targeting of PAK1 inhibits primary AML patients’ cells including immature, leukemia stem cell-enriched populations, while sparing healthy stem and progenitor cells

Current chemotherapeutic approaches in AML and MDS target rapidly dividing cells, having minimal effects on the quiescent leukemic and pre-leukemic stem cells responsible for disease propagation and relapse. To investigate whether leukemic stem and progenitor cells are dependent on PAK1 function, we assessed the efficacy of PAK1 inhibition on leukemic colony formation of primary human mononuclear cells isolated from healthy controls or patients with active AML. PAK1 inhibition by either IPA-3 or FRAX-597 led to a dose-dependent reduction of leukemic colony formation capacity of
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AML patient cells, while colony formation capacity of healthy control cells was only modestly affected, indicating that primary leukemic cells exhibit significantly greater sensitivity to PAK1 inhibition than healthy cells (Figure 6A). Furthermore, we found that PAK1 inhibition with either IPA-3 or FRAX-597 inhibits the ability of AML primary patient cells to serially replate in a dose-dependent manner, in comparison to treatment with DMSO (Supplemental Figure 5B).

It has previously been shown that LSC most often reside in the CD34+CD38- cell compartment in patients with AML (25, 26). We sought to assess the effectiveness of PAK1 inhibition specifically on these LSC-enriched populations by culturing bone marrow mononuclear cells from human AML patients and healthy controls with increasing concentrations of IPA-3 or FRAX-597. FACS analysis revealed that PAK1 inhibition led to significantly decreased viability of CD34+CD38- cells from AML patients. In contrast, no significant decrease in cell viability was observed in healthy control CD34+CD38- cells with either PAK1 inhibitor (Figure 6B). Similarly, we found significant differential reduction in viability in the CD34+CD38- cell compartment in human leukemic patient samples compared to healthy donor samples (Figure 6C). In order to assess the in vivo leukemia-initiating capacity of human AML cells in the presence of PAK1 inhibition, we performed xenografting studies in NSG mice using primary samples from two patients with AML. We detected significantly reduced CD45+CD33+ human AML cell engraftment in the bone marrow after six weeks for both patient samples treated with IPA-3 compared to DMSO (Figure 6D; Supplemental Figure 5C).

In order to further assess the relevance of PAK1 in myeloid malignancies, we evaluated PAK1 expression in patients with MDS, a heterogeneous hematological stem cell disorder which frequently progresses to AML. We found that MDS patients with high PAK1 expression in bone marrow CD34+ cells have significantly worse survival compared to PAK1 low expressing patients (p<0.0001; Figure 6E; GSE19429) (27) and that there is a linear correlation between PAK1 mRNA levels and worse overall survival in individual patients (p<0.0001, R²=0.08607; Supplemental Figure 6A). The hazard ratio of PAK1 high versus PAK1 low expressers was HR=3.120 (95% CI: 1.865-5.413). PAK2 expression showed no correlation with overall survival (Supplemental Figure 6B). Of note, we found that elevation of PAK1 expression is significantly more frequent in patients with the MDS subtype RAEB, the subtype of MDS with the highest risk for progression to AML (Supplemental Figure 6C). To assess whether the impact of PAK1 expression on overall survival is independent of known prognostic factors for MDS, we performed multivariate analysis using a Cox regression model (taking into account sex, hemoglobin, FAB subtype, bone marrow blast percentage, white blood cell count). In this analysis, high PAK1 status remained an independent prognostic factor (p=0.0334; HR=1.884; Supplemental Figure 6D). In addition, we performed multivariate analysis considering IPSS score as a co-variable. In this analysis, PAK1 high status also remained an independent prognostic factor (p=0.0003) (Supplemental Figure 6E).

**DISCUSSION**

Our studies highlight the importance of PAK1 as a novel target in AML and MDS. We show that leukemia cells are reliant on PAK1 function and that PAK1 inhibition has significant effects on leukemic tumor cells in vitro and in vivo. Inhibition of PAK1 with small molecule inhibitors IPA-3 and FRAX-597, or PAK1 shRNA mediated knockdown, dramatically reduces the proliferation as well as the leukemic colony formation capacity of AML cell lines as well as primary patient samples. Furthermore, reduction of PAK1 in
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an \textit{in vivo} xenotransplantation AML model results in nearly complete loss of infiltration of leukemic cells, and preservation of normal tissue architecture in the bone marrow, liver, and spleen upon PAK1 knockdown.

MDS and AML are defined as clonal hematopoietic stem cell disorders arising \textit{de novo} or as a result of previous chemo- or radiotherapy. Current treatment regimens in AML and MDS target rapidly dividing cells, having minimal effects on the quiescent leukemic and pre-leukemic stem cells responsible for disease progression and relapse. Novel therapies that are able to target pre-leukemic and leukemic stem cells are urgently needed to maintain remission and improve survival. MDS is considered a pre-leukemic disease as MDS patients frequently progress to AML. We find that PAK1 up-regulation is more frequent in RAEB MDS, the subtype at highest risk of progression to AML, and that higher levels of \textit{PAK1} in MDS CD34\textsuperscript{+} cells correlate with inferior patient survival. \textit{PAK1} inhibition induces apoptosis of LSC-enriched cell populations and inhibits leukemic colony formation capacity and \textit{in vivo} engraftment of primary AML cells in xenotransplantation experiments. Importantly, PAK1 inhibition targets primary human leukemic cells while sparing healthy control stem cells. These findings provide a promising therapeutic window that can be exploited by targeting of PAK1 in leukemia.

Several potent and specific small-molecule inhibitors of group I PAKs are in the advanced stages of preclinical development, including a Phase I trial in solid tumors. Our own group initially identified PAK1 as a downstream effector molecule of HLX, a gene up-regulated in pre-leukemic stem cells and functionally relevant for AML pathogenesis (3). PAK1 was also recently shown to act downstream of FLT3-ITD and KITD816V mutations via FAK signaling in myeloproliferative disease (28). Interestingly, three of the four AML cell lines and several patient samples we tested in this study do not contain either FLT3-ITD or KITD816V mutations yet were still sensitive to PAK1 inhibition. Furthermore, the AML patient samples we studied included FAB subtypes M4 and M5, which more frequently have elevated \textit{PAK1} expression from our assessments of a large cohort of AML patients (GSE14468; Supplemental Figure 7A,B). Four patients from these experiments, however, had subtype M1 or M2 and also responded to PAK1 inhibition, despite these subtypes more frequently having low \textit{PAK1} expression (Supplemental Figure 7A,B). Together, these results indicate PAK1 could be a broadly applicable target to combat the heterogeneity of AML, and PAK1 signaling may be downstream of multiple pathways important for AML growth, and less dependent on mutation status (Supplemental Figure 7A-E).

Despite reported roles for PAK1 in the regulation of cell cycle progression in other cell types (20), we find that in AML, PAK1 inhibition limits the growth of cells by inducing apoptosis and monocytic differentiation without affecting cell cycling. Using microarray studies, we identified downregulation of a large MYC oncogene regulated transcriptional network following PAK1 inhibition, including MYC target genes previously implicated in AML pathogenesis such as DUSP7. MYC oncogene has roles in regulating cell proliferation, apoptosis and differentiation (29). Dysregulation of MYC is one of a series of oncogenic events implicated in myeloid leukemias (29, 30). Retroviral overexpression of MYC has been shown to induce AML (31, 32) and several studies have shown that fusion oncogenes in myeloid leukemias including AML1-ETO, PML-RAR\textalpha and PLZF-RAR\textalpha induce leukemogenesis through activation of MYC (31). Exactly how PAK1 activates transcription of MYC in AML is still under investigation. For instance, in gastric cells, PAK1 modification of beta-catenin was previously shown to lead to beta-catenin nuclear localization and subsequent activation of MYC transcription (33). However, we
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did not find this effect in AML cells (data not shown). In FLT3-ITD expressing
myeloproliferative disease, PAK1 inhibition reduced the presence of activated nuclear
STAT5 leading to reduced transcription of MYC (28). Additionally, other recent PAK1
studies have indicated PAK1 may itself activate transcription in the nucleus in addition to
its kinase activities in the cytoplasm (34).

In summary, our studies present PAK1 as a novel target in AML and MDS, and show
that inhibition of PAK1 has leukemia-inhibitory effects not only on bulk leukemia blast
cells but also in more immature, LSC-enriched populations. These conclusions support
the further development and clinical testing of PAK1 inhibitors as a therapeutic strategy
in AML and MDS.

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Authorship Contributions
A.P. and R.S. contributed equally to this work. A.P. and R.S. designed and performed
research, analyzed data, made figures and wrote the manuscript; Y.Y. performed
statistical analyses and made figures; B.B. performed statistical analyses and made
figures; G.P. assisted in sample collection; J.B. contributed vital clinical data; K.G., J.C.
and A.V. designed research and analyzed and interpreted data; U.S. designed and
analyzed research and contributed to writing of the manuscript.

Conflict of interest
The authors have declared no conflict of interest.

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homeobox regulates early hematopoiesis and promotes acute myeloid leukemia. Cancer

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Figure Legends

Figure 1. Targeting PAK1 inhibits growth and reduces clonogenicity of human acute myeloid leukemia cells. (A) MOLM-13, HL-60, THP-1 and KG1a cells were treated with increasing concentrations of IPA-3. Cell viability was measured by cell counts with trypan blue exclusion every 24 hours. Experiment performed in triplicate, (p-values are shown for last time point indicated). (B) MOLM-13, HL-60, THP-1 and KG1a cells were treated with increasing concentrations of FRAX-597. Statistics are indicated as in (A). (C) 3,000 THP-1 or MOLM-13 cells were seeded in methylcellulose containing indicated concentrations of IPA-3 or (D) FRAX-597. (E) Two unique shRNAs targeting human PAK1 were expressed in THP-1 cells. Cell growth was measured by cell counts with trypan blue exclusion. Average of three independent experiments. (F) Colony formation analysis of THP-1 cells treated with two independent PAK1 knockdown sequences. Average of two independent experiments. *p<0.05, **p<0.01, ***p<0.001 for all experiments.

Figure 2. PAK1 inhibition induces apoptosis of AML cells. (A) MOLM-13, HL-60, THP-1 and KG1a cells were treated with increasing concentrations of IPA-3. Apoptosis was measured at 24 hours by flow cytometry using Annexin V and DAPI staining. Average of two independent experiments. (B) MOLM-13, HL-60, THP-1 and KG1a cells were treated with increasing concentrations of FRAX-597. Average of two independent experiments. (C) Human THP-1 AML cells were infected with lentiviruses targeting PAK1 for knockdown or a non-targeting control. Average of two independent experiments (**p<0.001).

Figure 3. Inhibition of PAK1 induces myelomonocytic differentiation of AML cells. (A). THP-1, MOLM-13, and HL-60 cells were treated with indicated concentrations of IPA-3 or FRAX-597 for 24 hours and analyzed by FACS for expression of CD11b and CD15 cell surface markers for myelomonocytic differentiation. Y-axis plots fold change Mean Fluorescence Intensity (MFI). Average of two independent experiments, error bars represent standard error of the mean. (B) Expression of CD11b and CD15 cell surface markers on THP-1 infected with lentiviruses targeting PAK1 for knockdown. (C) HL-60 AML cells were treated with indicated concentrations of DMSO (0.2%), IPA-3 or FRAX-597 for 24 hours. Cells were then spun onto slides (cytospin) and stained with Wright-Giemsa stain. (D) THP-1 AML cells were infected with lentivirus targeting PAK1 for knockdown or a non-targeting control and selected with puromycin. Cells were spun onto slides and stained with Wright-Giemsa stain.

Figure 4. PAK1 inhibition leads to leukemic cell death by downregulation of MYC and a MYC transcriptional network. (A) Gene-Set Enrichment Analysis (GSEA) of genes expressed differently in IPA-3 (6ug/mL) or FRAX-597 (2ug/mL) treated THP-1 cells compared to DMSO. (B) Ingenuity Pathway Analysis (IPA) generated image showing the downregulation of genes from the MYC network following treatment with either IPA-3 (6ug/mL) or FRAX-597 (2ug/mL). Intensities indicate degree of up or downregulation. (C) Western blot shows reduction of MYC protein levels after 4 hours of treatment with IPA-3 or FRAX-597 in THP-1 cells. Concentrations shown are ug/mL. (D) qPCR validation of downregulated MYC network genes after treatment of THP-1 cells with either DMSO, IPA-3 (6ug/mL) or FRAX-597 (2ug/mL). (E) qPCR validation of downregulated MYC network genes after knock-down of PAK1 in THP-1 AML cells.
PAK1 is a therapeutic target in AML and MDS

Figure 5. PAK1 reduction inhibits AML progression in vivo. (A) Gross liver and spleen images 46 days after transplantation of 3x10^5 sh control, shPAK1#1 or shPAK1#2 THP-1 cells (4 animals/group per experiment; n=2). (B) H & E staining of femur, liver and spleen sections of transplanted animals. (C) Chimerism of human THP-1 cells in the liver quantified by FACS analysis for cells expressing human CD45. Data represent the mean +/- SD. (D) CD11b expression levels on the surface of human CD45 expressing THP-1 cells isolated from the livers of animals transplanted with THP-1 cells expressing the control shRNA lentivirus or one of two PAK1 targeting sequences. (E) Cytospin preparations and Wright-Giemsa staining of THP-1 cells FACS-sorted from the livers of transplanted animals. The few remaining cells that could be isolated from animals receiving PAK1 knockdown cells exhibit signs of myelomonocytic differentiation. *p<0.05 for all experiments.

Figure 6. Targeting of PAK1 inhibits AML patients’ cells including immature, leukemia stem cell-enriched populations, while sparing healthy stem and progenitor cells. (A) Colony formation assay of primary AML patient MNC samples or healthy control MNC samples treated with IPA-3 (healthy n=4, AML n=5) or FRAX-597 (healthy n=4, AML n=3). (B) Cell viability (DAPI-Annexin V-) of CD34^+CD38^- cells from AML patients and healthy controls after 24 hours in vitro culture with IPA-3 or FRAX-597 (n=3 for each drug). (C) Cell viability (DAPI-Annexin V-) of CD34^+CD38^- cells from AML patients and healthy controls after 24 hours in vitro culture with IPA-3 or FRAX-597 (n=3 for each drug). (D) Percentage bone marrow engraftment of human CD45^+CD33^- cells six weeks following xenografting with primary AML patient samples treated with IPA-3. Experiment performed in duplicate with two independent primary AML patient samples. (E) Kaplan-Meier curve of overall survival of patients with high versus low PAK1 expression in CD34^+ bone marrow cells isolated from MDS patients. Patients with “high” PAK1 expression are those with PAK1 expression levels higher than the median expression level of PAK1 in the dataset. The p-value (log-rank test) is indicated. *p<0.05, **p<0.01 for all experiments.
Figure 3

(A) Fold Change MFI for CD11b and CD15 in THP-1, MOLM-13, and HL-60 cells treated with DMSO, 2 μg/mL IPA-3, 4 μg/mL IPA-3, 2 μg/mL FRAX-597, and 4 μg/mL FRAX-597.

(B) Fold Change MFI for CD11b and CD15 in THP-1 and MOLM-13 cells treated with sh control, shPAK1 #1, and shPAK1 #2.

(C) Microscope images showing DMSO, 2 μg/mL IPA-3, 4 μg/mL IPA-3, 2 μg/mL FRAX-597, and 4 μg/mL FRAX-597 treatments at 20x magnification.

(D) Microscope images showing sh control, shPAK1 #1, and shPAK1 #2 treatments at 50x magnification.
Figure 4

A

Enrichment Score

IPA-3
DMSO
FRAX-597
DMSO

NES: -1.309 p = 0.02
NES: -2.359 p < 0.001

B

IPA-3
FRAX-597

Upregulated
Downregulated

C

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<tr>
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D

Fold Change (relative to DMSO)

MYC
DUSP7
AMD1

E

Fold Change (relative to shControl)

MYC
DUSP7
AMD1

shPAK1 #1
shPAK1 #2
Figure 6

A

Colonies (relative to DMSO)

IPA-3 concentration (ug/mL)

Healthy Controls

AML Patients

* p < 0.05

** p < 0.01

FRAX-597 concentration (ug/mL)

Colonies (relative to DMSO)

Healthy Controls

AML Patients

* p < 0.05

** p < 0.01

B

CD34⁺CD38⁻ viability (relative to DMSO)

DMSO 2ug/ml 4ug/ml 6ug/ml

Healthy Controls

AML Patients

* p < 0.05

** p < 0.01

IPA-3

FRAX-597

C

CD34⁺CD38⁻ viability (relative to DMSO)

DMSO 2ug/ml 4ug/ml 6ug/ml

Healthy Controls

AML Patients

* p < 0.05

** p < 0.01

IPA-3

FRAX-597

D

% Bone Marrow Engraftment

Human CD45⁺CD33⁺ Cells

DMSO IPA-3

* p < 0.05

E

MDS Patient Survival

Low PAK1

High PAK1

p < 0.0001

Years

0 2 4 6 8
PAK1 is a therapeutic target in acute myeloid leukemia and myelodysplastic syndrome

Ashley Pandolfi, Robert F. Stanley, Yiting Yu, Boris Bartholdy, Gopichand Pendurti, Kira Gritsman, Jacqueline Boultwood, Jonathan Chernoff, Amit Verma and Ulrich Steidl