PAK Kinase Inhibition Has Therapeutic Activity in Novel Preclinical Models of Adult T-Cell Leukemia/Lymphoma

Elaine Y. Chung¹, Yun Mai¹, Urvi A. Shah², Yongqiang Wei¹,³, Elise Ishida¹, Keisuke Kataoka⁴, Xiaoxin Ren⁵, Kith Pradhan⁶, Boris Bartholdy¹, Xiaolei Wei¹,³, Yiyu Zou², Jinghang Zhang⁵, Seishi Ogawa⁷, Ulrich Steidl¹, Xingxing Zang⁵, Amit Verma²,⁵, Murali Janakiram², and B. Hilda Ye¹

Abstract

Purpose: To evaluate therapeutic activity of PAK inhibition in ATLL and to characterize the role of PAK isoforms in cell proliferation, survival, and adhesion of ATLL cells in preclinical models.

Experimental Design: Frequency and prognostic impact of PAK2 amplification were evaluated in an ATLL cohort of 370 cases. Novel long-term cultures and in vivo xenograft models were developed using primary ATLL cells from North American patients. Two PAK inhibitors were used to block PAK kinase activity pharmacologically. siRNA-based gene silencing approach was used to genetically knockdown (KD) PAK1 and PAK2 in ATLL cell lines.

Results: PAK1/2/4 are the three most abundantly expressed PAK family members in ATLL. PAK2 amplifications are seen in 24% of ATLLs and are associated with worse prognosis in a large patient cohort. The pan-PAK inhibitor PF-3758309 (PF) has strong in vitro and in vivo activity in a variety of ATLL preclinical models. These activities of PF are likely attributed to its ability to target several PAK isoforms simultaneously because genetic silencing of either PAK1 or PAK2 produced more modest effects. PAK2 plays a major role in CADM1-mediated stromal interaction, which is an important step in systemic dissemination of the disease. This finding is consistent with the observation that PAK2 amplification is more frequent in aggressive ATLLs and correlates with inferior outcome.

Conclusions: PAK2, a gene frequently amplified in ATLL, facilitates CADM1-mediated stromal interaction and promotes survival of ATLL cells. Taken together, PAK inhibition may hold significant promise as a targeted therapy for aggressive ATLLs.

Introduction

Adult T-cell Leukemia/Lymphoma (ATLL) is a highly aggressive T-cell neoplasm caused by human T-cell lymphotropic virus (HTLV)-1 (1, 2). Patients diagnosed with the aggressive subtypes carry some of the worst prognosis of any of the non-Hodgkin lymphomas. The 5-year overall survival (OS) rate of patients with ATLL is reported to be only 14% (3). In accordance with the observation that PAK2 amplification is more frequent in aggressive ATLLs and correlates with inferior outcome.

Purpose: To evaluate therapeutic activity of PAK inhibition in ATLL and to characterize the role of PAK isoforms in cell proliferation, survival, and adhesion of ATLL cells in preclinical models.

Experimental Design: Frequency and prognostic impact of PAK2 amplification were evaluated in an ATLL cohort of 370 cases. Novel long-term cultures and in vivo xenograft models were developed using primary ATLL cells from North American patients. Two PAK inhibitors were used to block PAK kinase activity pharmacologically. siRNA-based gene silencing approach was used to genetically knockdown (KD) PAK1 and PAK2 in ATLL cell lines.

Results: PAK1/2/4 are the three most abundantly expressed PAK family members in ATLL. PAK2 amplifications are seen in 24% of ATLLs and are associated with worse prognosis in a large patient cohort. The pan-PAK inhibitor PF-3758309 (PF) has strong in vitro and in vivo activity in a variety of ATLL preclinical models. These activities of PF are likely attributed to its ability to target several PAK isoforms simultaneously because genetic silencing of either PAK1 or PAK2 produced more modest effects. PAK2 plays a major role in CADM1-mediated stromal interaction, which is an important step in systemic dissemination of the disease. This finding is consistent with the observation that PAK2 amplification is more frequent in aggressive ATLLs and correlates with inferior outcome.

Conclusions: PAK2, a gene frequently amplified in ATLL, facilitates CADM1-mediated stromal interaction and promotes survival of ATLL cells. Taken together, PAK inhibition may hold significant promise as a targeted therapy for aggressive ATLLs.

ATLL diagnosed in North American (NA-ATLL) are of Caribbean descent. Compared with the Japanese patients (J-ATLL), NA-ATLLs tend to have a more aggressive clinical course, higher rates of chemo-refractory disease, and worse prognosis (4–6). Such differences in clinical behavior may be accounted for by distinct somatic mutation patterns between these two patient populations (7). Although there is no standard therapy for ATLL, the initial treatment often employs combination chemotherapy followed by allogeneic transplantation (1). Yet, the high rate of chemo-resistance disease often leads to treatment failure and dismal survival outcomes. Hence, an attractive strategy is to design mechanism-based treatment that could target novel pathways critical in maintaining ATLL phenotype in vivo.

The current model for ATLL pathogenesis depicts the initial infection of CD4 T cells by HTLV-1, followed by downregulation of the HTLV-1 Tax protein, and progressive accumulation of genetic and epigenetic changes in the infected T cells that ultimately lead to oncogenic transformation and disease progression (2). In an integrated genomics and transcriptomics study of a large cohort of 426 J-ATLL patients, many frequently altered genes encoding candidate driver mutations in ATLL transformation and progression were reported (8). These genes are involved in the TCR/NF-κB signaling, T-cell trafficking, and immune surveillance. Among the 35 genes frequently targeted by both somatic alterations and the HTLV-1 onco-protein Tax, p21-activated kinase (PAK) 2 is amplified in 24% of the cases (8). PAKs are a family of serine/threonine kinases composed of six isoforms (PAK1-6).
Translational Relevance

Adult T-cell leukemia/lymphoma (ATLL) is a rare but extremely aggressive cancer, associated with a dismal outcome and lack of effective therapies. Analysis of a large cohort of ATLL samples shows 24% of patients with ATLL carry amplification of the p21-activated kinase (PAK) 2 gene and the amplification is associated with a worse prognosis. Functional studies show that the PAK kinase inhibitor PF-3758309 has profound anti-ATLL activity in 

In vitro cytotoxicity assay

PF-3758309 (PF, Selleckchem; Catalog No. S7094) is an ATP-competitive inhibitor that targets PAK1, PAK2, PAK3, and PAK4. IPA-3 (Tocris; Catalog No. 3622) is a selective non-ATP competitive PAK1 inhibitor that also binds PAK2 and PAK3. The IC50 of PF-3758309 and IPA3 were determined using resazurin (R&D Systems; Catalog No. AR002)-based viability assays in which cells were exposed to various concentrations of the reagents for 48 hours. To evaluate the response to IPA-3, a structurally related inactive compound, PIR3.5 (Tocris; Catalog No. 4212), was also tested.

Adhesion assay

A total of 7 × 10⁶ HK cells/well were seeded in a 96-well plate and allowed to attach overnight before the assay. Parental ATLL cells or ATLL cells that were transfected with siRNA oligos 48 hours earlier were fluorescently labeled with the Calcein AM dye according to the manufacturer’s instructions (Invitrogen; Catalog No. C3100MP). Labeled parental ATLL cells were then treated with either DMSO or PF at 2.5 μmol/L for 1 hour prior to exposure to HK. Subsequently, ATLL cells were added to the HK feeder-coated 96-well plate in triplicate at 7 × 10⁶ cells/well and allowed to adhere for 180, 150, 120, 90, 30, and 15 minutes. Non-attached cells were washed away four times with 1x PBS. Absorbance reading was performed at 480 to 520 nm with the Fluostar plate reader. Adherence of ATLL cells to HK feeder was calculated as the fraction A/B. A is the absorbance values after washing away unattached cells minus average absorbance values of HK cells alone after washing. B is the absorbance values of cells before washing minus average absorbance values of HK cells alone before washing.

Xenograft studies of in vitro PF-3758309 activity

A patient-derived xenograft (PDX) model was established from patient PBMCs (ATL18) in NSG (NOD/SCID/γc−/−) mice by serially passaging subcutaneous tumor mass in female NSG mice. In this model, distal dissemination is a constant feature that
always involves lung, liver, and spleen whereas kidney and bone marrow are less frequently infiltrated. A cell line-derived xenograft (CDX) model was similarly developed using ATLA43Tb(−) cells but metastasis in this model is largely restricted to the lung. PF treatment was initiated when tumors reached ~100 mm (3). Animals were randomly assigned to one of the three groups to be treated by vehicle, 6 or 12 mg/kg/day of PF. PF was formulated in 2% carboxymethyl cellulose (24) and administered intraperitoneally. Throughout the experiment, overall health of the animals was closely monitored, body weight and tumor growth were frequently recorded. Tumor sizes were measured using an electronic caliper. Tumor volume (mm$^3$) was calculated using the formula of \( V = \frac{4}{3} \pi (r^2 \cdot h) \), where \( r \) and \( h \) are the tumor length and width in mm, respectively. Tumor growth inhibition (TGI) was calculated as TGI (%) = \( \frac{(V_c - V_t)}{V_c} \times 100 \), where \( V_c \) and \( V_t \) are the median tumor volume of control and treated groups at the end of the study and \( V_0 \) is the median tumor volume at the start of the study. All mice were maintained and treated in compliance with IACUC approved protocols at Albert Einstein College of Medicine.

**Results**

**Amplification of PAK2 is a frequent event in ATLL and correlates with inferior survival**

Copy number variation in a cohort of 426 Japanese ATLL samples was studied using SNP array karyotyping (8). Among the 26 focally amplified region is a band on 3q29, encoding PAK2 (Supplementary Fig. S2A). In all, PAK2 amplification was detected in 24.4% of all ATLL cases with a preferential enrichment in the aggressive subtypes (acute/lymphomatous vs. chronic/smoldering: 27.5% vs. 16.0%; Benjamini–Hochberg correction, \( q = 0.09 \)). There is a trend for increased PAK2 mRNA expression in 3q29 amplified cases (Supplementary Fig. S2B). Most importantly, PAK2 amplification was significantly associated with a trend for poor overall survival \( (P = 0.029, \text{Fig. 1A}) \).

**Expression patterns of PAK family members in ATLL**

Given PAK2 amplification in ATLL, we wanted to examine expression pattern of this kinase in primary samples. We first established mRNA expression profiles for all six PAK family members in this disease. In acute ATLL, a significant fraction of peripheral blood mononuclear cells (PBMCs) are leukemia T cells. We therefore performed qRT-PCR assays for PAK1, PAK2, PAK4, PAK3, and PAK6 on 6 PBMC samples from patients with ATLL and compared them to four healthy controls (Fig. 1B). PAK2 is expressed at the highest levels among all samples followed by PAK3 and PAK6. PAK5 mRNA is undetectable in most patient samples. The same pattern is observed from the RNA-seq dataset of the Japanese ATLL cohort (Supplementary Fig. S2C).

To measure PAK mRNA expression in pure populations of ATLL cells, we then analyzed eight ATLL cell lines and compared the results to normal resting and activated CD4$^+$ T cells, a pooled normal Treg sample, and four cell lines representing other T-cell non-Hodgkin lymphomas (Jurkat and CEM, acute lymphoblastic leukemia; HuT78, cutaneous T-cell lymphoma; OCLv13.2, peripheral T-cell lymphoma; Fig. 1C). The pattern for PAK isoforms among ATLL cell lines largely resembles that seen with patient PBMCs, although PAK3 and PAK6 mRNAs exhibit much more intragroup heterogeneity. Compared with normal resting CD4 T cells, ATLL cell lines under-express several PAK isoforms including PAK1, PAK2, PAK4, and PAK6. The same trend exists in another ATLL gene expression dataset (Supplementary Fig. S2D; ref. 25). Of note, when compared with activated CD4 T cells and other types of T-cell malignancies, PAK2 mRNA is also reduced in ATLL cell lines (Fig. 1C).

In general, Tregs have the lowest levels of all PAKs among all tested samples except for PAK2, which is expressed at comparable levels in Tregs and ATLL cell lines. Although the cause of this apparent ATLL-associated PAK2 down-regulation is currently unknown, the observation does suggest that the level of PAK2 may have become rate-limiting in this disease and thus gene amplification events are positively selected in aggressive ATLLs. This interpretation is in line with observations made on PAK1/2 proteins. Western blot analysis shows significant variability of PAK1 protein across all diagnostic categories of T-cell malignancies, whereas PAK2 protein exhibits less variability across the panel (Fig. 1D). It should be noted that the characteristic ATLL-associated reduction of PAK2 mRNA is not reflected at the protein level. We interpreted this result as a suggestion that posttranscriptional mechanisms such as protein stability may operate to compensate for the reduced PAK2 mRNA expression. In summary, our result indicates that PAK1/2 are the most abundantly expressed PAK isoforms in ATLL and that PAK2 is regulated differently from the other PAKs during ATLL development.

**PAK inhibitors have *in vitro* activity against primary ATLL samples and ATLL cell lines**

Given the PAK family expression patterns in ATLL, we selected two PAK inhibitors for pharmacologic PAK inhibition. PF-3758309 (PF) is the first PAK inhibitor to advance to clinical trial. It is an ATP-competitive inhibitor with documented activity against all PAK family members to various degree (26, 27). IPA-3 is an allosteric inhibitor that has specific activity against group I PAKs but spares group II PAKs (28). In all, seven J-ATLL cell lines, seven North American patient samples, and three NA-ATLL cell lines were tested (Fig. 2A–C, Supplementary Fig. S3). For PF, the 48 hours IC$_{50}$ values range from 1.8 to 13.4 μmol/L for the seven Japanese cell lines tested. IL2 requirement culture condition did not appear to impact PF sensitivity. For NA-ATLLs, the IC$_{50}$ values range from 1.2 to 14.4 μmol/L for the seven primary patient samples and 2.9 to 8.2 μmol/L for the three cell lines. Of note, ATL6a/6b/6c and ATL19a/19b are serial samples collected from chemo-refractory patients treated with high-dose chemotherapy in combination with antiviral agents. We also tested IPA-3 responsiveness in six Japanese cell lines and two North American ATLL samples. Relatively low IC$_{50}$ were observed (6.6–16.9 μmol/L), demonstrating in vitro efficacy of both pharmacologic inhibitors in both Japanese and North American ATLLs (Fig. 2C; Supplementary Fig. S3C and S3D). Interestingly, activated normal CD4 T cells are highly sensitive to PF (IC$_{50}$ = 2.5 μmol/L), suggesting that PAK kinases play an extremely important role in their proliferation/survival.

To explore the role of individual PAK isoforms in the observed PF activity, we examined changes in total PAK1/2 proteins as well as phospho PAK1/2/4 following 1 hour of drug treatment at 2.0 to 2.5 μmol/L, which are below the majority of 48 hours IC$_{50}$ values for the selected samples including six J-ATLL cell lines and four NA-ATLL samples (Fig. 2D). Both PAK1 and PAK2 were readily detected in all samples examined except ED40515(−), which has the lowest levels of both PAKs. Lower levels of total PAK1 was also
noted for ATL55T(+) and Su9T01. We examined all four types of phosphorylated PAK1/PAK2 and found that all of them exhibited cell line to cell line variability; yet, there is a close correlation between phospho-Ser20-PAK2 and the ability of a cell line to form tight aggregates in suspension culture (not shown). Interestingly, PF treatment reduced PAK2 phosphorylation on two residues (phospho-Ser20 and phospho-Ser141) while enhancing the signal on the third (phospho-Thr402; Fig. 2D). Because binding of
PAK Inhibition Has Preclinical Activity in ATLL

**Figure 2.** In vitro response to PAK inhibitors in Japanese and North America ATLL cells. Representative PF response curves of established ATLL cell lines (A) and primary patient samples (B). Additional PF and IPA-3 response curves are shown in Supplementary Fig. S2. All cultures were treated for 48 hours prior to cell viability measurement with the resazurin assay. C, Summary of IC50 doses for PF and IPA-3 in all tested samples. PF IC50 doses for normal pooled PBMC and activated CD4 T cells are also listed. D, Western blot analysis of total and phosphorylated PAK1/2 as well as phosphorylated PAK4 protein in response to 1-hour treatment with PF. All J-ATLL cell lines were treated at 2.5 µmol/L, while the NA-ATLL cell line ATL18 was treated at 2.0 µmol/L. Vertical line has been inserted to indicate a repositioned gel lane.
PF to the primed ATP-binding site in PK is expected to lock the enzyme in this partially activated state, the observed changes in PK2 is consistent with the model for its activation (9). In comparison, under the PK treatment conditions, phosphorylated PK1 was either not altered (phospho-Ser199–PK1) or often enhanced (phospho-Thr423–PK1) but never reduced. In most samples tested, strong phospho-PK4 signals were detected. Yet, PK only reduced phospho-PK4 in two cell line, ED40515(−) and Su9T01, which are the most sensitive samples to PF (Fig. 2C). Two conclusions can be made from these observations. First, at 2.0 to 2.5 μmol/L PF could effectively inhibit PK2 kinase activity in all samples containing activated PK2; whereas all seven tested samples contained activated PK4, it was only reduced by PF in the two sensitive cell lines. Signals representing activated PK1 were very weak and not reduced in these experiments. Second, when it comes to correlation with in vitro PK response, the best predictor of sensitivity appears to be low level expression of both PK1 and PK2 as well as phospho-PK4 (Fig. 2D).

Pharmacologic PK inhibition induced time- and dose-dependent apoptosis in ATLL cell lines

Because the resazurin-based in vitro viability test measures both cell proliferation and survival effects, we next evaluated these two cellular responses separately. Six J-ATLL and three NA-ATLL cell lines were treated with the indicated doses of PF and apoptosis was measured using Annexin V/PI staining (Fig. 3A; Supplementary Fig. S4). In all samples tested, most apoptotic cells were detected at 48 hours compared with 24 hours, and 10 μmol/L of PF triggered more pronounced apoptotic cell death than 2.0 μmol/L at 24 hours (ATL18 and ATL21; Supplementary Fig. S4). The two most sensitive Japanese cell lines [ED40515(+) and Su9T01] also exhibited the largest apoptotic fractions (40%–50%). Next, we examined cell cycle changes in PF-treated samples (Fig. 3B; Supplementary Figs. S5 and S6). The most significant phenotype was a prominent G1–S transition block detected in all samples, which was accompanied by corresponding S phase reductions. Variable extent of G2–M arrest was also observed. In three cell lines showing notable G2–M accumulation [ATL55T(+) and ED40515(+) and ATL43Tb(−) at 40 hours only], CYCLIN B1 and phospho-Ser10 H3 signals were increased thus confirming the activation of G2 checkpoint. However, this is not the case in ED40515(−) or Su9T01 (Supplementary Fig. S5B).

To determine the molecular mechanisms of PF response, we analyzed three cell signaling molecules (STAT5 and ERK1/2) known to be regulated by PAKs and important for ATLL biology (9, 29, 30). In addition, c-MYC and three apoptosis regulators (MCL1, BAD, and BAX) were also examined. Of note, c-MYC and BAD have previously been shown to be the kinase substrates of PK2 and PKA1/2, respectively (10). ATLL cells were treated with DMSO or PF for 2, 4, and 8 hours, and whole cell lysates were analyzed by Western blotting (Fig. 3C). The IL-2-independent cell line, ATL43Tb(−), did not contain activated/phosphorylated STAT5. In all three IL-2-dependent cell lines, PK inhibition notably reduced PY-STAT5 as early as 2 hours into the treatment. Oncogenic c-MYC levels were reduced after PK inhibition in all samples. Anti-apoptotic MCL1 was also markedly reduced whereas the level of pro-apoptotic BAX was elevated at early stage of the treatment. Because PK1 can promote ERK1/2 activation independent of its kinase activity (31), we also examined phospho-ERK1/2. All three J-ATLL cell lines showed no or very little phospho-ERK1/2 signals at the baseline, but the two IL-2-dependent lines [ATL55T(+) and ATL43Tb(−)] upregulated this signal following PK inhibition. The cell line ATL18 contained strong phospho-ERK1/2 at the baseline, which was markedly inhibited by PF at 4 and 8 hours, implying that ERK1/2 activation is controlled by different mechanisms in this sample. In the three Japanese cell lines, neither total nor phospho-Ser112-BAD was altered by PF treatment; yet, both were consistently reduced by PF in ATL18 starting from the 2-hour time point. Most of these early changes could still be detectable at 48 hours (Supplementary Fig. S7). The unexpected increase in phospho-BAD may reflect other kinases that can also act on the Ser112 residue (10). Overall, despite the variability in ERK1/2 and BAD, the observed molecular changes were consistent with and likely contributed to the apoptosis and cell-cycle inhibition phenotypes shown in Fig. 3A and B.

Pharmacologic PK inhibition impaired CADM1-mediated stromal adhesion by ATLL cells

In monotypic suspension culture, primary ATLL cells often form large aggregates, which will dissociate when plated on a feeder layer of HK cells (a human follicular dendritic cell line; ref. 23), mimicking in vivo microenvironment (Fig. 4A). Adherence to HK also markedly improved cell viability with unfractionated patient PBMCs and freshly sorted CD4+ leukemic cells (Fig. 4B), suggesting that adhesion-induced cell signaling events regulate both cytoskeletal remodeling and cell death control programs. Because PAKs regulate cancer cell migration and lamellipodia formation by modulating RacGTP activity, and the CADM1–Tiam1–Rac complex has been shown to promote dissemination of ATLL cells in vivo (11, 15), we examined the impact of pharmacologic PK inhibition on the ability of ATLL cells to adhere to HK (Fig. 4C). Using a Calcein AM-based cell adherence assay, we monitored HK adherence of seven ATLL cell lines over a period of 3 hours, comparing the results obtained with and without PF pretreatment. Among the seven cell lines tested, two were CADM1-negative [Supplementary Fig. S8A, Su9T01, ED40515(−)] and neither showed significant HK adherence in 3 hours (Fig. 4C). All five CADM1-expressing cell lines including the ATL18, demonstrated time-dependent HK adherence with variable kinetics. Most importantly, PF pretreatment significantly reduced HK adherence in all five CADM1-expressing cell lines but did not alter the low, basal HK binding activity of the CADM1-negative line, Su9T01. Flow cytometry analysis revealed that defective binding to HK was not due to PF-induced CADM1 downregulation (Supplementary Fig. S8B). These observations support the notion that PAKs positively regulate stromal adherence by CADM1-expressing ATLL cells (Supplementary Fig. S8C).

PK2 but not PK1 plays an important role in ATLL stromal interaction

To examine involvement of PK isoform(s) in the HK adherence process, we analyzed PK1 and PK2 protein during the first 150 minutes of ATLL-HK contact in three adhesion-competent cell lines (Fig. 4D). Activated/phospho-Ser144-PK1 was only detected in ATL43T(+)-cells, and was modestly reduced by PF pretreatment. Phospho-Ser141-PK2 was detected in both ATL43T(+) and ATL55T(+) cells and this signal was nearly
completely inhibited by PF pretreatment. All cell lines expressed phospho-Ser20-PAK2. This signal as well as total PAK2 were reduced after at 30 and 150 minutes following HK exposure in ATL55T(+) and ED40515(-) cells. In ATL43T(+), however, these PAK2 signals appear to be slightly increased after 30 minutes of HK interaction. In all three cell lines, PF pretreatment decreased phospho-Ser20-PAK2 at the baseline (0 minute) and late stage of HK exposure (150 minutes). In summary, changes in total and/or phospho-Ser20-PAK2 but not any of the PAK1 signals correlated with PF-induced adhesion defect.

Figure 3.
PAK inhibition induces time- and dose-dependent cytotoxicity in ATLL cell lines. ATLL cell lines exposed to PF for 24 or 48 hours at the indicated concentrations were analyzed by Annexin V (AV)/propidium iodide (PI) staining for apoptosis (A) and EdU/PI for cell-cycle changes (B). A, Viable fraction was defined as AV-negative/PI-negative cells; apoptotic fraction was defined as all AV-positive cells. Results shown are mean two independent experiments. Two-tailed Student t test was used for pair-wise comparison as indicated. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Original flow cytometry plots are in Supplementary Fig. S4. B, Stacked bar graphs indicate percentage of cells in G1, S, and G2-M phases. Original flow cytometry plots for all time points are in Supplementary Figs. S5 and S6. C, Western blot analysis of selected proliferation and survival regulators in response to PF treatment for the indicated amount of time. All J-ATLL samples were treated with PF at 2.5 μmol/L, while the doses for the NA-ATLL cell lines are marked in the graph. DMSO, vehicle control.
Figure 4.
PAK inhibition impairs stromal adhesion by CADM1-positive ATLL cells. A, Interaction with a lymph node stromal cell line, HK, disrupted homophilic cell-cell interaction among primary ATLL cells. B, Exposure to HK feeder for 2 days significantly enhanced viability of primary ATLL cells. Viability was assessed based on the Trypan Blue exclusion method. Results shown are mean ± SD. *, P < 0.01. C, Binding of ATLL cells to the HK feeder was measured in a 3-hour time course using the Calcein-AM-based adherence assay. To measure the impact of PAK inhibition, ATLL cells were pretreated with PF at 2.5 μmol/L for 1 hour prior to exposure to the HK feeder. Results shown are representative of at least two independent experiments. D, Impact of PF pretreatment on PAK1 and PAK2 activation following adherence to the HK feeder. Before exposure to the HK feeder, ATLL cells were pretreated with PF at 2.5 μmol/L for 1 hour. Whole cell extracts were prepared at the indicated time points and analyzed by Western blotting for the indicated markers. E, Effect of siRNA-mediated PAK1 and PAK2 knockdown in ATL55T(+) cells was demonstrated by Western blot analysis 48 hours after siRNA transfection. F, HK binding capability of PAK1 and PAK2 KD cells in E was evaluated as described in C. Relative adhesion measured at 3-hour after HK exposure is shown. Percentage reduction by PF is calculated using the adhesion of DMSO-treated PAK1/2 KD cells as 100% (numbers in blue).
To directly evaluate the function of individual PAK isoforms, we turned to siRNA-based knockdown (KD) approach. Both PAK1 and PAK2 were substantially and specifically reduced at 48 hours following siRNA transfection in ATL55T(+) (Fig. 4E). Interestingly, we observed cross-talk between PAK1 and PAK2 because PAK1 protein was increased in PAK2 KD cells. We then subjected these cells to 1 hour pretreatment by PF before analyzing their capability to adhere to the HK feeder. As shown in Fig. 4F, PAK1 KD by itself reduced HK binding capability by 15.3% and 32.6% of the remainder can be further suppressed by PF. In comparison, PAK2 KD lead to 33.3% loss of HK binding capability and the remainder activity was largely resistant to PF (PF-associated binding loss was only 18.1%). Because PAK1 level increased in PAK2 KD cells, the result indicates that the loss of PAK2 function in cell adhesion could not be compensated by PAK1. Combined with the observations in Fig. 4D, these data suggest that PAK2 is the major target of PF activity in the HK adhesion assay whereas PAK1 has a minor contribution. This conclusion is further supported by time course analysis of PAK1 and PAK2 KD cells in adhesion test (Supplementary Fig. S8D).

PAK2 but not PAK1 promotes cell autonomous ATLL cell survival

Using siRNA-mediated KD approach, we also examined the role of PAK1 and PAK2 in cell proliferation and survival in ATL55T(+) and ATL43Tb(-) cells. ATL55T(+) cells underwent moderate and substantial apoptosis following PAK1 and PAK2 KD, respectively (Fig. 5B; Supplementary Fig. S9A); yet, PAK1 or PAK2 KD did not alter survival of ATL43Tb(-) cells (Fig. 5B; Supplementary Fig. S9B). We did not observe notable cell cycle changes in either cell lines (Supplementary Fig. S9C). As to the molecular changes that may account for the apoptosis phenotype following PAK1 and PAK2 KD, we observed a reduction in total- and PY-STAT5, p-ERK1/2, and MCL1 proteins 72 hours following siRNA transfection (Fig. 5C). The pro-apoptosis protein BAD was elevated at 48 hours only. Interestingly, similar changes were also

![Figure 5](https://www.aacrjournals.org)
PF treatment potently inhibited tumor growth and distal dissemination in an ATLL PDX model. A, PF has strong and dose-dependent activity in preventing tumor growth at the subcutaneous site. NSG mice bearing ATL18-derived tumor mass were randomly assigned to three treatment arms: vehicle control, 6 or 12 mg/kg i.p. doses daily. Results presented are mean normalized tumor volumes with error bars indicating standard error of the mean. P values from group comparisons on day 16 are marked on the right of the graph (two-tailed Student t test). B, Histologic appearance of subcutaneous tumor mass and major organs from vehicle and 12 mg/kg PF treated mice. Animals were euthanized on day 19 posttreatment. C, Enumeration of ATLL cells disseminated to spleen, liver, and lung in the vehicle and 12 mg/kg PF treatment groups. Animals were euthanized after 29 or 36 days of treatment. The number of leukemic cells was quantified using hCD45 staining followed by flow cytometry. D, Expression of CADM1 is nearly ubiquitous in disseminated ATLL cells but highly variable in the subcutaneous tumor mass. Results shown are percentage of CADM1-positive cells within the hCD45+ gate. V#1, V#2, and V#3, 3 animals from the vehicle group. PF#1 and PF#2, two mice from the 12 mg/kg PF treatment group. Tumor, subcutaneous tumor mass; organs, averaged percentage of CADM1+ cells in spleen, liver, and lung.
detected in the apoptosis-resistant ATL43Tb(−) cell line except PY-STAT5, which is absent at baseline in this IL-2-independent cell line (Supplementary Fig. S10). This raises an interesting question as to whether PY-STAT5 is a critical mediator of PAK1/2 function in cell survival.

**PAK inhibition has potent activity in xenograft-based ATLL animal models**

In addition to the NA-ATLL cell lines, we have also established and characterized PDX models using primary samples from our patients. In these models, distal tissue/organ dissemination from subcutaneous engraf site is a prominent feature and the pattern is similar to human ATLL (Fig. 6B). Using a PDX models derived from ATL18 and a CDX model derived from ATL43Tb(−), we evaluated in vivo anti-ATLL activity of PF. In the initial experiment using the CDX model, PF administered at 12 mg/kg twice a week achieved significant TGI in 19 days (TGI = 54.9%; Supplementary Fig. S11A). We then initiated a follow up study using the PDX model, where PF was administered daily over a period of 3 weeks (Fig. 6A). At the 12 mg/kg daily dose, PF nearly completely inhibited the subcutaneous tumor growth (TGI = 86.8% on day 16), whereas a substantial albeit more moderate inhibitory effect was achieved with 6 mg/kg (TGI = 59.0% on day 16). Ki67 staining revealed reduced cell proliferation in the subcutaneous tumor mass in PF-treated animal; however, TUNEL-based apoptosis assay did not detect notable differences due to PF treatment (Supplementary Fig. S11D). Most importantly, PF completely prevented distal dissemination of leukemic cells from the subcutaneous site. This is evident from the histology of spleen, liver, lung, kidney, and bone marrow (Fig. 6B) as well as human CD45-based flow cytometry quantification of infiltrating leukemic cells (Fig. 6C). We also examined CADM1 expression pattern at different sites of disease presentation. In keeping with a requirement for CADM1 in tissue/organ infiltration by ATLL cells, nearly all leukemic cells located in internal organs expressed this adhesion molecule (83%–100%) whereas in the subcutaneous site, CADM1 expression was much more variable (30.3%–88.3%) (Fig. 6D). These results demonstrate that PF has potent anti-ATLL activity in xenograft ATLL models where it can strongly inhibit not only in situ tumor growth but also distal tissue/organ dissemination. Based on the overall health status, body weight change, and heart histology, the PF treatment protocol did not cause measurable, off-site toxicity in the test animals (Supplementary Fig. S11B and S11C).

**Discussion**

ATLL is a very aggressive T-cell malignancy with no effective therapy (1, 3, 5). Motivated by the observation that PAK2 is amplified in 24% of Japanese patients with ATLL (8), we examined therapeutic activity of PAK inhibition and characterized roles of PAK family kinases in this disease. We report here that PAK1/2/4 are the three most abundantly expressed PAK isoforms in ATLL. Functional studies demonstrate that the pan-PAK inhibitor PF has strong in vitro and in vivo anti-ATLL activity in a number of preclinical models. The observed activity of PF is likely attributed to its ability to target several PAK isoforms simultaneously because genetic silencing of either PAK1 or PAK2 only produced variable and moderate cytotoxicity. PAK2, but likely not PAK1, is the major mediator of CADM1-mediated stromal interaction, which is an important step in systemic dissemination of the disease. This is consistent with the observation that PAK2 is more frequently amplified in the aggressive subtypes of ATLL.

Among the six PAK isoforms, PAK2 and PAK4 are ubiquitously expressed, whereas the other four are restricted to specific tissue/organ (9, 32). In all ATLL samples tested, PAK2 mRNA is the most abundantly expressed followed by PAK1 and PAK4 (Fig. 1B and C). The protein products of all three of these genes can be readily detected in ATLL samples tested (Fig. 1D). Although PAK1 and PAK2 have virtually identical substrate specificities, they play different and nonredundant roles in development and cancer (32). For example, PAK1 but not PAK2 have been implicated as a direct regulator of nuclear gene expression program by phosphorylating transcription factors CBP and STAT5 (29, 33, 34). For cytoskeletal remodeling, rapid turnover of focal contacts at the leading edge of migrating breast cancer cells requires PAK2 (35, 36), whereas modulation of RhoA activity during master cell degranulation is controlled by PAK2 but not PAK1 (37, 38). In this study, we have found that ATLLs under-express PAK2 mRNA relative to normal CD4 T cells and other T-cell malignancies (Fig. 1C, Supplementary Fig. S2D); yet, PAK2 protein levels appear to be comparable to those found in other T-cell malignancies (Fig. 1D) implying additional posttranscriptional regulations. We also found that PAK2 has a more important contribution than PAK1 to stromal adhesion and cell survival in ATLL cells (Figs. 4F and 5B). It is therefore possible that as a disease category, ATLLs tend to under-express PAK2 mRNA, and this may have rendered PAK2 levels limiting for in vitro disease progression, and hence PAK2-amplification events are positively selected in aggressive ATLLs.

We selected PAK1 and PAK2 for gene-specific functional studies since they are the most abundantly expressed PAKs in ATLL. Knockdown experiments showed that the contribution of these two genes to cell autonomous proliferation and survival is not universal. In ATL43Tb(−) cells grown in suspension, they play negligible roles; in ATL55T(+) cells, PAK2 KD caused significant apoptosis while PAK1 KD-induced cell death was more modest. Given the fact that the PF IC50 dose for ATL55T(+) is nearly twice that for ATL43Tb(−) (Fig. 2A) and yet ATL55T(+) was more sensitive to PAK1/2 KD, it is possible that targets other than PAK1 and PAK2 also mediated PF toxicity in the in vitro drug sensitivity tests. One such possibility is PAK4. Among the six Japanese cell lines studied by western blotting, the two most sensitive ones have very low phospho-Ser474-PAK4 signals [Su9T01 and ATL43Tb(−)] (Fig. 2B). Therefore, when all of our findings are considered as a whole, there is a formal possibility that both PAK2 and PAK4 mediated PF-induced toxicity in the in vitro toxicity assays. Non-PAK kinases that could be targeted by PF under our experimental conditions could also be involved (24).

Multi-organ involvement is the cause of mortality in ATLL. Therefore, ATLL treatment must effectively block systemic dissemination of leukemic cells. Two experiments in this study addressed this issue. In the adherence assays, PF significantly inhibited CADM1-dependent interaction with HK largely through PAK2 (Fig. 4). Although the importance of CADM1 in ATLL progression and systemic dissemination has been documented in the literature (11, 13, 14), this is the first time that a specific member of PAK is shown to operate downstream of CADM1 in this disease. Because stromal interaction significantly enhanced survival of primary ATLL cells (Fig. 4A and B), and this process is largely PAK2-dependent, PAK2 is expected to function as a major survival factor in ATLL microenvironment as well. It is worth...
noticing here that, in our xenograft study, PF dosed daily at 12 mg/kg potently inhibited subcutaneous tumor growth and completely prevented distal dissemination of leukemic cells (Fig. 6). Combined with the results from our biochemical analysis and PAK1/2 knockdown experiments, such PF activities should be largely attributed to PAK2 with minor contributions from other PAK isoforms, such as PAK1 and PAK4.

In summary, our findings in this study showed that PAK2, a gene frequently amplified in ATLL, facilitates CADM1-mediated stromal interaction and promotes ATLL cell survival. Furthermore, PAK inhibition through an inhibitor such as PF may hold great promise as a targeted therapy for aggressive ATLLs. Although PF was withdrawn from further clinical testing due to inadequate oral availability, additional PAK inhibitors have since been designed (24) and some of them may possess a favorable therapeutic window for a disease associated with frequent PAK2 amplification.

Disclosure of Potential Conflicts of Interest
U. Stein reports receiving other commercial research support from GlaxoSmithKline. Bayer Healthcare, and Aileron Therapeutics; holds ownership interest (including patents) in Slexis Therapeutics; and is a consultant/advisory board member for Pieris Pharmaceuticals, Aileron Therapeutics, Slexis Therapeutics, and Bayer Healthcare. M. Janakiram is a consultant/advisory board member for Miragen and Seattle Genetics. No potential conflicts of interest were disclosed by the other authors.

References
PAK Inhibition Has Preclinical Activity in ATLL


PAK Kinase Inhibition Has Therapeutic Activity in Novel Preclinical Models of Adult T-Cell Leukemia/Lymphoma

Elaine Y. Chung, Yun Mai, Urvi A. Shah, et al.


Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-18-3033

Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2019/03/12/1078-0432.CCR-18-3033.DC1

This article cites 38 articles, 16 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/25/12/3589.full#ref-list-1

Sign up to receive free email-alerts related to this article or journal.

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

To request permission to re-use all or part of this article, use this link:
http://clincancerres.aacrjournals.org/content/25/12/3589
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.