Alvocidib inhibits IRF4 expression via super-enhancer suppression and adult T-cell leukemia/lymphoma cell growth

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Abstract
Adult T-cell leukemia/lymphoma (ATL) is an intractable hematological malignancy with extremely poor prognosis. Recent studies have revealed that super-enhancers (SE) play important roles in controlling tumor-specific gene expression and are potential therapeutic targets for neoplastic diseases including ATL. Cyclin-dependent protein kinase (CDK) 9 is a component of a complex comprising transcription factors (TFs) that bind the SE region. Alvocidib is a CDK9 inhibitor that exerts antitumor activity by inhibiting RNA polymerase (Pol) II phosphorylation and suppressing SE-mediated, tumor-specific gene expression. The present study demonstrated that alvocidib inhibited the proliferation of ATL cell lines and tumor cells from patients with ATL. RNA sequencing (RNA-Seq) and chromatin immunoprecipitation sequencing (ChIP-Seq) disclosed that SE regulated IRF4 in the ATL cell lines. Previous studies showed that IRF4 suppression inhibited ATL cell proliferation. Hence, IRF4 is a putative alvocidib

Abbreviations: AKAP1, A-kinase anchor protein 1; AML, acute myeloid leukemia; ATL, adult T-cell leukemia/lymphoma; BATF3, basic leucine zipper ATF-like transcription factor 3; BCL-2, B-cell lymphoma 2; BRD4, bromodomain-containing protein 4; BrdU, bromodeoxyuridine; CCR4, CC chemokine receptor 4; CDK, cyclin-dependent protein kinase; ChIP-Seq, chromatin immunoprecipitation sequencing; CLL, chronic lymphocytic leukemia; c-MYC, MYC proto-oncogene; DEGs, differentially expressed genes; DMSO, dimethyl sulfoxide; DUSP22, dual specificity phosphatase 22; FBS, fetal bovine serum; FYN, proto-oncogene tyrosine-protein kinase fyn; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSEA, gene set enrichment analysis; HBZ, HTLV-1 bZIP factor; HDAC, histone deacetylase; IL2RA/CD25, interleukin 2 receptor, alpha chain; IRF4, interferon regulatory factor 4; KLHL8, kelch like family member 8; mAb, monoclonal antibody; MCL-1, MCL1 apoptosis regulator; BCL2 family member; MED1, mediator complex subunit 1; NOG, NOD.Cg-PrkdcscidIl2rg-/-ShiJic; PBMCs, peripheral blood mononuclear cells; PCA, principal component analysis; PI, propidium iodide; PIK3R1, phosphatidylinositol 3-kinase regulatory subunit alpha; Pol, polymerase; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; RIPA, radioimmunoprecipitation assay; RNA-Seq, RNA sequencing; RPKM, reads per kilobase of exon per million mapped reads; SE, super-enhancers; TAK/P-TEFB, Tat-associated kinase/positive transcription elongation factor b; TCR, T-cell receptor; TFs, transcription factors; TNFRSF8, tumor necrosis factor receptor superfamily member 8.

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1 | INTRODUCTION

Adult T-cell leukemia/lymphoma (ATL) is a T-cell lymphoma caused by human T-cell leukemia virus type I. The prognosis of the aggressive form of ATL is extremely poor. Dose-intensified, multiagent chemotherapy, mogamulizumab (monoclonal antibody [mAb] against CC chemokine receptor 4 [CCR4]), and lenalidomide have been developed and administered for ATL but have failed to sufficiently improve its prognosis. Allogeneic hematopoietic stem cell transplantation is the only curative treatment for ATL. However, most patients who benefit from this therapy are aged <70 years, which is only ~20%. Therefore, the etiology of ATL must be elucidated for the development of novel therapeutic agents against this disorder.

Progress is being made in the development of novel therapeutic agents against malignant tumors and hematological malignancies. New treatments include molecularly targeted drugs that focus on genetic abnormalities and epigenetic control. Bromodomain inhibitors control the expression of cancer-specific genes via super-enhancers (SEs). SEs are regions in which transcription factors (TFs), mediator complexes, chromatin regulators, and RNA polymerase (Pol II) form a cluster resembling a normal enhancer. They are typically ~9 kb in length, have extremely high protein densities, and are defined based on their chromatin immunoprecipitation (ChIP) sequences and other data. SEs promote the transcription of specific genes at considerably higher rates than typical enhancers and are known to regulate tumor-specific oncogenes.

SE regulation has become a therapeutic target for various cancers. In ATL, gene expression control via SE is vital. Studies on enhancer profiling have been conducted using samples from ATL patients. SEs regulate the expression of genes such as interleukin 2 receptor, alpha chain (IL2RA/CD25), tumor necrosis factor receptor superfamily member 8 (TNFRSF8/CD30), proto-oncogene tyrosine-protein kinase Fyn (FYN), CCR4, phosphatidylinositol 3-kinase regulatory subunit alpha (PIK3R1), and tumor protein 73 (TP73) in ATL and normal T cells. The cyclin-dependent protein kinase (CDK)7 inhibitor THZ1 suppresses cell proliferation, induces apoptosis, and blocks SE-dependent gene expression in ATL cells. Thus, treatments targeting SE may be efficacious in ATL therapy.

A recent study showed that CDK9 protein is part of a complex regulating SE. CDK9 is a component of the Tat-associated kinase/positive transcription elongation factor b (TAK/P-TEFb) multiprotein complex and a key cell cycle regulator. It regulates RNA Pol II-directed transcription by phosphorylating the C-terminal domain of the largest RNA Pol II subunit. It also binds SE by forming a complex with bromodomain-containing protein 4 (BRD4) and mediator complex subunit 1 (MED1). CDK9 inhibitors such as NVP-2, LDC067, and BAY1143752 have successfully treated acute myeloid leukemia (AML). BAY1143752 was purported to be effective against ATL in a mouse model study. The CDK9 inhibitor alvocidib (flavopiridol) has demonstrated efficacy against various hematological malignancies.

In a phase 1b/II study, alvocidib, cyclophosphamide, and rituximab were coadministered against refractory chronic lymphocytic leukemia (CLL). The therapeutic efficacy of the combination was demonstrated. The clinical efficacy of alvocidib against AML has been verified on >400 patients in sequential phase I and II trials. The clinical application of alvocidib is anticipated in the future. Here, we endeavored to verify the efficacy of alvocidib against ATL and determine whether it is potentially useful in clinical application. The objectives of this work were to clarify the mechanism by which alvocidib controls ATL, elucidate the molecular pathology of this disease, and develop novel therapeutic strategies against it.

2 | MATERIALS AND METHODS

2.1 | Cell lines

The ATL-derived cell lines ST-1, KOB, and KK-1 were established in the laboratory of the present research team. KOB and KK-1 were cultured in RPMI 1640 medium supplemented with 20% (v/v) fetal bovine serum (FBS) and 1 ng/ml interleukin-2 (IL-2; Cosmo Bio Co.). ST-1 was cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS alone.

2.2 | Primary ATL cells and normal CD4+ lymphocytes

Primary ATL cells were obtained from patients with ATL. ATL was diagnosed and its subtypes were classified according to previously defined criteria. Five patients presented with acute ATL, while the sixth (ATL-04) exhibited an unfavorable chronic type of ATL. The
proportion of abnormal lymphocytes in the peripheral blood was >80% in all patients (Table 1). Peripheral blood mononuclear cells (PBMCs) were isolated from ATL patients and normal healthy donors by Ficoll-Hypaque density gradient centrifugation (Sigma-Aldrich GmbH) and washed with phosphate-buffered saline (PBS). Normal human CD4+ T cells were purified from PBMCs with a Human CD4+ T-Cell Isolation Kit (Miltenyi Biotec GmbH). All cells were cultured in RPMI 1640 medium supplemented with 20% (v/v) FBS and IL-2 (1ng/ml).

2.3 | Cell proliferation assay

Cell viability was determined with Cell Titer-Glo Reagent v. 2.0 (Promega) according to the manufacturer’s recommended protocol. The 50% inhibitory value (IC_{50}) was calculated by nonlinear regression analysis with GraphPad Prism v. 4.0 (GraphPad Software).

2.4 | Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Both the ATL and primary ATL cells were incubated either with alvocidib (Selleck Chemicals) or dimethyl sulfoxide (DMSO) for 3h. The RNeasy Kit (Qiagen) was used to isolate total RNA. PrimeScript™ RT Master Mix (Perfect Real Time; Takara Biotechnology) was used to synthesize the cDNA. qRT-PCR was performed with QuantStudio™ 12K Flex (Applied Biosystems) and TaqMan™ Universal Master Mix II (Applied Biosystems). The data were analyzed by the 2^{-\Delta\Delta Ct} method and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. Interferon regulatory factor 4 (IRF4) (Hs00180031_m1) and GAPDH (Hs02786624_g1) were the PCR primers.

2.5 | Western blotting

Total protein was extracted from the cell pellets with radioimmunoprecipitation assay (RIPA) buffer and complete, EDTA-free protease inhibitor cocktail (Roche Diagnostics). The extracted samples were analyzed by automated quantitative Western blotting (Wes assay)23 in a Wes instrument (Protein Simple) and Compass software (https://www.proteinsimple.com/software_compas_simplewestern.html). Anti-IRF4 (EBH3S) XP(R) rabbit mAb (Cell Signaling Technology) and anti-β-actin (Cell Signaling Technology) were the used primary antibodies.

2.6 | Annexin V/propidium iodide (PI) staining

An annexin V-FITC apoptosis detection kit (BioVision) was used to evaluate cell death. The KOB, KK-1, and ST-1 ATL cell lines were assessed after 72h treatment with DMSO or alvocidib.

2.7 | Cell cycle analysis

A bromodeoxyuridine (BrdU) flow kit (BD Pharmingen GmbH) was used to analyze the cell cycle. The KOB, KK-1, and ST-1 ATL cell lines were assessed with a 40-min BrdU pulse after 48h treatment with DMSO or alvocidib.

2.8 | RNA sequencing (RNA-Seq) preparation and analysis

The ATL-related cell lines ST-1 and KOB were incubated either with 100nM alvocidib or DMSO for 4.5h. A Pure Link RNA mini kit

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**TABLE 1** Clinical data when the specimen was obtained from six ATL patients

| UPN   | Age | Sex | ATL subtype     | Time when the specimen was collected          | WBC (\mu l) | Abnormal lymphocyte (%) | LDH (U/L) | sIL-2R (U/L) | Corrected Ca (mg/dl) | Albumin (g/dl) |
|-------|-----|-----|----------------|-----------------------------------------------|------------|------------------------|-----------|--------------|----------------------|---------------|
| ATL-01| 31  | F   | Acute          | Before induction chemotherapy                 | 26,600     | 87                     | 770       | 71,088       | 9.2                  | 4.0           |
| ATL-02| 75  | M   | Acute          | Before induction chemotherapy                 | 72,200     | 89                     | 2357      | 27,150       | 9.3                  | 3.6           |
| ATL-03| 79  | F   | Acute          | Before induction chemotherapy                 | 39,600     | 89                     | 595       | 19,310       | 9.7                  | 3.6           |
| ATL-04| 46  | F   | Unfavorable    | Before induction chemotherapy                 | 38,600     | 86                     | 251       | 10,879       | 9.4                  | 3.3           |
| ATL-05| 65  | M   | Acute          | Before induction chemotherapy                 | 28,100     | 80                     | 281       | 15,629       | 10.9                 | 2.9           |
| ATL-06| 66  | M   | Acute          | Before second-line chemotherapy (first-line chemotherapy, CHOP-like regimen, was refractory) | 30,400     | 84                     | 405       | 44,511       | 11.7                 | 3.5           |

Abbreviations: ATL, adult T-cell leukemia/lymphoma; Ca, calcium; LDH, lactate dehydrogenase; UPN, unique patient number; WBC, white blood cell.
It is difficult to sustain an effective serum alvocidib concentration because the drug binds and is inactivated by serum proteins. \(^{27}\) Hence, we conducted a cell proliferation assay using short-duration alvocidib exposures. When the foregoing ATL cell lines were treated for 6h with alvocidib before rinsing, their proliferation was partially inhibited. The IC\(_{50}\) values for ST-1, KOB, and KK-1 were 7148.9, 832.8, and 461.9 nM respectively. Nevertheless, the 12-h alvocidib exposure inhibited ATL cell line proliferation as effectively as the 72-h alvocidib exposure (Figure 1C).

### 3.2 | Alvocidib induces apoptosis and cell cycle arrest

The ATL-related cell lines were subjected either to DMSO or 100nM alvocidib for 72h and examined by annexin V-PI to determine whether apoptosis induction explained the observed inhibition of proliferation. The alvocidib treatment significantly increased the number of apoptotic cells in all ATL-related cell lines. The rates of apoptosis in the ST-1, KOB, and KK-1 cells were 15.8±3.1%, 19.6±1.0%, and 16.5±0.3%, respectively (Figure 2A).

The ATL-related cell lines were then treated with DMSO or 100nM alvocidib for 48h and examined by BrdU assay with a 40-min BrdU pulse to investigate the influence of alvocidib on the cell cycle. The relative proportions of cells in the G\(_2\)-M phase had increased in all alvocidib-treated ATL-related cell lines.

### 3.3 | Effects of alvocidib on gene transcription profiles

RNA-Seq was performed and the effects of alvocidib concentration and exposure time on the ATL cell lines were observed to elucidate the molecular mechanisms by which alvocidib affects gene transcription. The 6-h treatment with 100nM alvocidib inhibited ATL cell proliferation. Gene profiling prior to 6h alvocidib exposure was studied to clarify the mechanism by which alvocidib inhibits cell proliferation.

RNA-Seq was performed on ST-1 and KOB treated with DMSO or 100nM alvocidib for 4.5h. A principal component analysis (PCA) was performed using reads per kilobase of exon per million mapped reads (RPKM) obtained from the triplicate RNA-Seq data. These results distinguished the DMSO- and alvocidib-treated ST-1 and KOB. (Figure 3A). The MA plots displayed the differentially expressed genes (DEGs) in the ST-1 and KOB cells after treatment with alvocidib (Figure 3B). Among these genes, the transcription of IRF4 and NOTCH1, \(^{28–31}\) which were reported as important genes in ATL pathogenesis, were significantly decreased.

The foregoing results indicated that there were heterogeneous gene expression backgrounds even within the same ATL cell lines. Therefore, certain overlapping DEGs could be important in ATL. A Venn diagram showed DEG overlap between the ST-1 and KOB cells.
Out of 365 genes, 249 were downregulated after alvocidib treatment (Figure 3C).

3.4 Alvocidib targets SE-associated genes

CDK9 regulates gene expression via RNA Pol II phosphorylation. Thus, alvocidib is expected to target a broad range of genes in addition to SEs. The present study focused on the regulation of gene expression by CDK9 via SE. Therefore, ChIP-Seq was performed using anti-BRD4 antibody on all three ATL cell lines to identify SE-associated genes. The genes controlled by SE were identified by SE ranking. There were 129 overlapping SE-associated genes among all three ATL cell lines (Figure 3C) including CD2, dual specificity phosphatase 22 (DUSP22), and IFR4 that were previously identified as SE-associated genes in ATL.
TNFRSF8/CD30 (Figure 4B) and CD28 were also detected in KK-1 and ST-1, respectively. Neither CCR4 (Figure 4C) nor IL-2RA/CD25 (Figure 4D) was identified as a type of SE. However, BRD4 signals were observed near the gene bodies as previously reported.11 The foregoing results and the RNA-Seq data disclosed that A-kinase anchor protein 1 (AKAP1), IRF4, and kelch like family member 8 (KLHL8) were included among the 249 transcriptionally down-regulated genes (Figure 3C). Earlier studies suggest that IRF4 plays important roles in ATL survival and progression.29-34 For this reason, the present work focused on IRF4 regulation by alvocidib. To this end, ChIP-Seq was performed using anti-BRD4 as well as ST-1 and KK-1 cells treated with DMSO or alvocidib. The alvocidib treatment substantially reduced relative BRD4 binding at the IRF4 locus (Figure 4E). The IRF4 mRNA and protein were significantly downregulated after alvocidib treatment (Figure 4F,G).

A previous study31 demonstrated that IRF4 activates T-cell receptor (TCR) signaling in ATL. A gene set enrichment analysis (GSEA) based on RNA-Seq demonstrated that TCR signaling downstream of IRF4 was suppressed in KOB and ST-1 cells subjected to alvocidib (Figure S1).

### 3.5 Alvocidib inhibited the growth of cell samples from patients with ATL

We investigated whether alvocidib has the same effect on the peripheral blood cells of all ATL patients. Over 90% of the cells in...
the peripheral blood of the patients with acute ATL were ATL cells (Table 1). The alvocidib concentrations required to inhibit ATL cell proliferation by 50% (IC50) in the ATL-01, ATL-02, ATL-03, ATL-04, ATL-05, and ATL-06 patient samples were 64.5, 62.5, 102.6, 36.3, 84.9, and 90.3 nM, respectively (Figure 5A). IRF4 suppression was identified as a mode of action of alvocidib in the ATL cell lines. We also found that IRF4 mRNA was significantly downregulated in all primary clinical ATL samples (Figure 5B).

3.6 | Alvocidib inhibited tumor growth in an in vivo mouse model

We verified the effects of alvocidib on an in vivo mouse model (Figure 6A) by subcutaneously injecting KOB cells into 10 NOG mice. The effects of alvocidib were evaluated based on the total volumes of the subcutaneous tumors. Engraftment was observed in 9/10 mice on day 12 or 13 after subcutaneous injection.
FIGURE 4 Regulation of super-enhancer (SE)-associated IRF4 by alvocidib. A, Enhancers ranked in increasing order of BRD4 signal in DMSO-treated, adult T-cell leukemia/lymphoma (ATL)-related cell lines. B-D, TNFRSF8/CD30 (B), CCR4 (C), and IL2RA/CD25 (D) loci in chromatin immunoprecipitation sequencing (ChIP-Seq) tracks displaying BRD4 signals. E, IRF4 loci in ChIP-Seq tracks displaying reduced BRD4 signals after alvocidib treatment. F, RT-PCR relative to GAPDH by 2−ΔΔCt method analyzed IRF4 mRNA expression levels in ATL-related cell lines treated either with DMSO or 100nM alvocidib. Data are means ± SD (error bars) of triplicate experiments. G, ATL-related cell lines were untreated, treated with DMSO, or treated with 250nM alvocidib for 72 h. Western blot analysis revealed that alvocidib downregulated IRF4 protein translation. Results were confirmed by triplicate analyses.
FIGURE 5 Effects of alvocidib on primary adult T-cell leukemia/lymphoma (ATL) samples. A. Inhibition of primary ATL cell sample survival by alvocidib. Cells were incubated in the presence of various alvocidib concentrations for 72 h. In vitro survival was determined with CellTiter Glo v. 2.0 (Promega). Relative viability of 100% was defined as total number of cells surviving in dimethyl sulfoxide (DMSO) after 72 h. Relative viability of cultured cells was determined using triplicate cultures and presented as means ± SD (error bars). B. RT-PCR relative to GAPDH by \(2^{\Delta\Delta Ct}\) method was used to analyze IRF4 mRNA expression levels in primary ATL cells treated either with DMSO or 100 nM alvocidib. Data are means ± SD (error bars) of triplicate experiments.
Engraftment was delayed until day 20 in the tenth mouse, and it was excluded from the subcutaneous tumor size analysis. On day 15, the mice were divided into alvocidib and control groups such that they had equal average total subcutaneous tumor volumes. Alvocidib (5 mg/kg) was intraperitoneally injected into five mice from days 15 to 19. The other four mice were injected with equal volumes of DMSO. The mean total subcutaneous tumor volumes ± standard deviations (SD) were 305.55 mm³ ± 282.53 and 314.21 mm³ ± 282.23 for the control and alvocidib groups, respectively, on the day of alvocidib initiation. The mean total volumes ± SD of the subcutaneous tumors were 800.03 mm³ ± 721.37 and 6596.88 mm³ ± 1898.70 for the control and alvocidib groups, respectively, on day 28 after injection (Figure 6B,C, and Table S1). Autopsies of the dead control mice revealed that the tumors consisted of CD4(+), CD8(−), and CD20(−) ATL cells and had infiltrated the livers, spleens, and kidneys (Figure S2).

4 | DISCUSSION

The present study demonstrated that practical concentrations of alvocidib inhibited ATL growth in cell lines, clinical samples, and an in vivo mouse model. IRF4 was regulated via SE and identified as candidate alvocidib target in ATL.

An assay of the inhibitory effect of alvocidib on ATL cell proliferation revealed that the IC₅₀ were 30.6, 60.1, and 55.8 nM for ST-1, KOB, and KK-1 cells, respectively (Figure 1A). The proliferation of normal CD4+ cells was not inhibited by alvocidib as high as 10 μM (Figure 1B). However, further studies are needed to test its safety including whether other hematopoietic cells, cycling and quiescent, such as hematopoietic stem cells, are affected or not under these conditions.

Considering the high protein-binding rate of alvocidib (82%-96%) in human serum, dosing schedules seem very important to reach and maintain the effective blood concentration in clinical trials. In a previous study, for example, a new dosing schedule was developed with a loading dose (30 min administration) followed by 4 hour continuous infusion. Our present study demonstrated that the exposure time to alvocidib was important to suppress the growth of ATL cells: 12 but not 6 h with IC₅₀ from 57.4 to 88.6 nM (Figure 1C). These results may be helpful to plan a dosing schedule of alvocidib, but pharmacological studies with ATL patients are needed for future clinical trials.

A previous study revealed an antitumor effect in mouse xenograft models bearing HL-60 tumors (a leukemia cell line) and...
SUDHL-4 (a lymphoma cell line) subjected to 7.5 mg/kg alvocidib. At least, alvocidib significantly suppressed ATL tumor growth in mouse models (Figure 6).

The present study showed that alvocidib decreased IRF4 transcription by suppressing SE. This mechanism inhibited downstream T-cell receptor (TCR) signaling (Figure S1). Hence, IRF4 may be a target of the anti-ATL effects of alvocidib. IRF4 and basic leucine zipper ATF-like transcription factor 3 (BATF3) activate TCR signaling and MYC proto-oncogene (c-MYC), thereby promoting ATL cell proliferation and survival. HTLV-1 bZIP factor (HBZ) is encoded by the minus-strand of the HTLV-1 provirus and induces BATF3. IRF4 overexpression and mutation indicate poor ATL prognosis. Mutations in IRF4 transcriptionally activate its downstream targets and are detected in ~10% of all ATL patients. Thus, IRF4 is recognized as an important molecular target in ATL. IRF4 downregulation by short hairpin RNA or antisense oligonucleotides suppresses ATL cell proliferation. Lenalidomide may also target IRF4 and is clinically approved for ATL therapy in Japan. However, comparatively high doses of it (1-20 μM) were required to inhibit IRF4 expression. The foregoing findings suggest that alvocidib may be effective for the treatment of ATL patients presenting with IRF4 mutations because it suppresses IRF4 transcription.

A previous report recognized c-MYC as an important target of the antitumor effects of alvocidib. RNA-Seq analysis disclosed that whereas 100nM alvocidib sufficed to suppress IRF4, it was inadequate for c-MYC suppression (data not shown). However, qRT-PCR revealed that 250nM alvocidib downregulated c-MYC, but only 100nM alvocidib was necessary to inhibit cell proliferation. Hence, repressing IRF4 may be more important than repressing c-Myc in terms of treating ATL.

The present study showed that a mechanism by which alvocidib inhibits ATL cell growth may be induced by regulating SE-mediated gene expression and suppressing IRF4. Hence, alvocidib may be a novel therapeutic agent for ATL. In our mouse model, a single course of standalone alvocidib treatment effectively inhibited ATL cell lines but was not curative (Figure 6). Therapeutic efficacy may be improved by optimizing the administration schedule of alvocidib alone. ATL is characterized by tumors, organ infiltration, and abnormal lymphocytes in the peripheral blood. In the present study, however, we only examined the effects of alvocidib on subcutaneous murine ATL tumors. Furthermore, we did not explore the tissue migration of alvocidib or its blood concentration. Therefore, the results of this study may not reflect the actual clinical efficacy of alvocidib. Future research should establish the optimal alvocidib administration schedule for ATL treatment in animal models. Subsequent human clinical safety and efficacy trials on alvocidib treatment for ATL should also be designed and conducted according to the results of the animal model assays.

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ETHICAL STATEMENT

The protocol for the research project has been approved by the constituted ethics committee of Nagasaki University, within which the work was undertaken, and that it conforms to the provisions of the Declaration of Helsinki. Approval of the research protocol by an Institutional Reviewer Board: Nagasaki University Ethical Review Board (approval No. 17112014-8).

INFORMED CONSENT

Informed consent was obtained from the patients.

ANIMAL STUDIES

The study was approved by the Nagasaki University Institutional Animal Care and Use Committee (approval No. 1801151432-8).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.