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High expression of NAMPT in adult T-cell leukemia/lymphoma and anti-tumor activity of a NAMPT inhibitor

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ABSTRACT

Adult T-cell leukemia/lymphoma (ATL) is a malignancy of mature T lymphocytes induced by human T-cell leukemia virus-1 and has a poor outcome. New molecular targets for the prevention and treatment of ATL are needed urgently. We previously reported high expression of Sirtuin 1, a nicotinamide adenine dinucleotide (NAD^+)-dependent histone/protein deacetylase, in primary acute-type ATL cells. NAD^+ biosynthesis via nicotinamide phosphoribosyltransferase (NAMPT) modulates Sirtuin 1 activity. Here, we examined the expression and effects of inhibiting NAMPT, a rate-limiting enzyme in NAD^+ biosynthesis, in ATL cells. We found that peripheral blood mononuclear cells from patients with acute-type ATL expressed significantly higher levels of NAMPT protein than cells from healthy subjects. FK866, a NAMPT inhibitor, induced apoptosis of freshly isolated ATL cells *ex vivo* and HTLV-1-infected T-cell lines *in vitro*, which was accompanied by activation of caspases, DNA fragmentation, and disruption of mitochondrial transmembrane potential. However, a pan-caspase inhibitor failed to prevent this FK866-induced cell death, while FK866 increased the caspase-independent cell death mediator endonuclease G. Intriguingly, FK866 also activated autophagy, as demonstrated by increases in protein levels of autophagosome marker LC3-II. Thus, FK866 simultaneously activated apoptosis and autophagy. Finally, FK866 treatment markedly decreased the growth of human ATL tumor xenografts in immunodeficient mice. We showed that NAMPT is highly expressed in primary ATL cells *ex vivo*, and that FK866 induces autophagy and caspase-dependent and -independent cell death pathways *in vitro* and has an anti-tumor activity *in vivo*. These results suggest a novel therapeutic strategy for patients with this fatal disease.

1. Introduction

Adult T-cell leukemia/lymphoma (ATL) is a malignancy of mature T lymphocytes caused by infection with human T-cell leukemia virus-1 (HTLV-1) and has a poor outcome(Ishitsuka and Tamura, 2014; Uchiyama et al., 1977; Yoshida, 2001). Epigenetic abnormalities and the host genetic/immunological status should be considered in attempts to understand the mechanism of ATL development, although the underlying mechanisms of oncogenesis have not been elucidated fully (Kogure and Kataoka, 2017; Kozako et al., 2006; Matsuoka and Jeang, 2007). Despite recent advances in chemotherapy, allogeneic hematopoietic stem cell transplantation, and monoclonal antibody therapies, the prognosis for aggressive subtypes is among the poorest of hematological malignancies(Katsuya and Ishitsuka, 2017; Tsukasaki and Tobinai, 2012; Yoshimitsu et al., 2014). Thus, new therapeutic strategies are required for ATL, such as novel molecular targeted agents (Hermine et al., 2018).

Sirtuin 1 (SIRT1), a nicotinamide adenine dinucleotide (NAD^+)-dependent histone/protein deacetylase, plays critical roles in various physiological processes, including aging and apoptosis(Bordone and Guarente, 2005; Imai, 2009; Kozako et al., 2014). We previously reported that peripheral blood mononuclear cells (PBMCs) from acute-type ATL patients express significantly higher levels of SIRT1 protein than cells from healthy individuals, and novel small-molecule SIRT1 inhibitors are highly effective suppressors of ATL cell viability(Kozako et al., 2012, 2015). However, nicotinamide phosphoribosyltransferase

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(NAMPT)-mediated NAD^+ biosynthesis tunes SIRT1 activity(Imai, 2016). NAMPT, also known as pre-B-cell colony-enhancing factor 1 and visfatin, is a rate-limiting enzyme in NAD^+ biosynthesis that regulates intracellular adenosine triphosphate (ATP) levels in mammalian cells (Garten et al., 2009). NAMPT expression is upregulated in several human cancers in which it regulates a variety of signaling pathway components including AMP-activated protein kinase (AMPK), PI3K/Akt, and ERK1/2(Bi and Che, 2010; Lucena-Cacace et al., 2017a, 2017b; Salminen et al., 2016). Thus, NAMPT may be a new treatment target for cancer(Zhao et al., 2017). Cytotoxicity assays have shown that most types of cancer, including solid tumors and leukemia, are sensitive to low concentrations of the NAMPT inhibitor FK866(Bi and Che, 2010; Nahimana et al., 2009; Zhang et al., 2012), that acts via NAD^+ depletion and inhibition of glycolysis(Cea et al., 2012; Tan et al., 2013; Wilsbacher et al., 2017). It is also anticipated that NAMPT inhibitors may be a novel non-antimitotic payload for antibody-drug conjugates(Karpov et al., 2018). However, the precise mechanisms underlying NAMPT regulation in ATL cells and the associated anti-tumor activity are unclear.

In this study, we examined the expression of NAMPT and the effects of FK866 in HTLV-1-infected T-cell lines, primary ATL cells, and an ATL model using an improved NOD/SCID/gamma (NSG) mouse. We found that NAMPT is highly expressed in primary ATL cells *ex vivo*, and that FK866 induces autophagy and caspase-dependent and -independent cell death pathways *in vitro*. FK866 also has an anti-tumor activity against ATL cells *in vivo*. This is the first demonstration of the mechanism-of-action of FK866 in ATL and suggests that NAMPT may be a useful therapeutic target for this disease.

2. Materials and methods

2.1. Clinical samples

Twenty subjects were enrolled in the study, **16** acute-type ATL patients (10 patients for Western blot analysis, **six** for cell viability and apoptosis assays) and six healthy individuals who were negative for HTLV-1. The ATL patients were tested for the presence of HTLV-1 by standard serology, and the diagnosis of ATL was made by hematological analysis(Shimoyama, 1991). PBMCs were obtained the ATL patients and health individuals in accordance with the approved guidelines of the Committees for Ethical Review of Research involving Human Subjects at Kagoshima University Hospital (approval number: 28–138) and Fukuoka University (approval number: 18-04-04), respectively. All subjects provided written informed consent for participation in the study, review of their medical records, and peripheral blood sampling in accordance with the guidelines set by the Declaration of Helsinki. PBMCs were obtained from fresh peripheral blood samples by centrifugal separation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). (Kozako et al., 2009).

2.2. Cell lines

S1T (an HTLV-1-infected T-cell line derived from an ATL patient) was kindly provided by Dr. Arima (Kagoshima University) (Arima et al., 2004), and MT-2 (an HTLV-1-infected T-cell line derived from normal human leukocytes transformed by leukemic T-cells from a patient with ATL) was purchased from JCRB (Osaka, Japan; JCRB1210)(Miyoshi et al., 1981). Cells were cultured in RPMI-1640 medium (Wako, Osaka, Japan) supplemented with 10% heat-inactivated fetal calf serum (Biocera, Kansas City, MO, USA), 0.1 mg/ml streptomycin, 100 U/ml penicillin, and 2 mM L-glutamine (all from Nacalai Tesque, Kyoto, Japan), as described previously(Kozako et al., 2018b).

2.3. Mice

Immunodeficient NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ (NSG) female mice at 4 weeks of age were obtained from Charles River Japan (Tokyo, Japan). The mice were housed in autoclaved polycarbonate cages with paper chip bedding (Japan SLC, Inc., Hamamatsu, Japan) covered with filter caps (CLEA Japan, Inc. Tokyo, Japan) within isolation cabinets (BBH Unit, Seobit Inc., Tokyo, Japan) and fed a sterile, irradiated diet (CLEA Japan) with free access to acidified, autoclaved water. The animal room was maintained under barrier-sustained conditions with controlled temperature (23 ± 2 °C) and lighting (12-h light/dark cycle)(Ohsugi et al., 2005). Mice were acclimated for 1 week before experiments. This study was carried out in strict accordance with the Guidelines for Proper Conduct of Animal Experiments, Science Council of Japan (http://www.scj.go.jp/en/animal/index. html). All animal procedures and their care were approved by the Animal Care and Use Committee of Rakuno Gakuen University in accordance with the Guide for the Care and Use of Laboratory Animals (approval number: VH16A21).

2.4. Inoculation of cells in SCID mice

S1T cells were passaged twice weekly, harvested, washed three times with phosphate-buffered saline, and resuspended in phosphate-buffered saline. Then, 5×10^7 cells were injected subcutaneously into the postauricular region of 5-week-old NSG mice(Ohsugi et al., 2007). There was no extreme weight loss or lack of energy observed throughout the study.

2.5. Reagents

FK866 (APO866, N-[4-(1-benzoyl-4-piperidinyl)butyl]-3-(3-pyridinyl)-2E-propenamide) was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). A monoclonal anti-Fas antibody, CH11, and caspase inhibitors, Z-VAD-FMK (pan-caspase), Z-WEHD-FMK (caspase-1), Z-DEVD-FMK (caspase-3), Z-IETD-FMK (caspase-8), and Z-LEHD-FMK (caspase-9) were purchased from Medical and Biological Laboratories (MBL; Nagoya, Japan). Western blot analysis was performed with primary antibodies against NAMPT (H-300; Santa Cruz Biotechnology, Santa Cruz, CA, USA), AMPKα (23A3), phospho-AMPKα (p-AMPKα; 40H9), AMPKβ1/2 (57C12), p-AMPKβ1 (S108), apoptosis-inducing factor (AIF; D39D2), endonuclease G (4969), histone H3 (3H1), mammalian target of rapamycin (mTOR; 7C10), p-mTOR (Ser2448, D9C2), poly (ADP-ribose) polymerase (PARP; 9542), caspase-1 (D7F10), β-actin (4967, all from Cell Signaling Technology, Beverly, MA, USA), and LC-3 (PM-036, MBL). Horseradish peroxidase-conjugated secondary antibodies were purchased from Vector Laboratories (PI-1000, Burlingame, CA, USA).

2.6. Protein extraction and Western blot analysis

Whole cell lysates were obtained using RIPA Lysis Buffer (Santa Cruz Biotechnology). Nuclear and cytoplasmic extracts were obtained using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL, USA), according to the manufacturer's protocol(Kozako et al., 2012, 2016). Cell extracts were subjected to sodium dodecyl sulfate-poly-acrylamide gel electrophoresis, electroblotted onto Immobilon-P membranes (Millipore, Billerica, MA, USA), and analyzed with appropriate primary and secondary antibodies, as indicated in the figures. Blots were developed using Chemi-Lumi One Super (Nacalai Tesque), according to the manufacturer's protocol. Images were visualized using a ChemiDoc XRS instrument (Bio-Rad, Hercules, CA, USA).

2.7. Cell viability and NAD^+ measurements

The effects of FK866 on cell viability were examined using Cell Count Reagent SF (Nacalai Tesque), according to the manufacturer's protocol(Kozako et al., 2018b). Briefly, aliquots containing

2×10^5 cells/ml were incubated in 96-well plates in the absence or presence of FK866. Cell Count Reagent SF (10 µl) was added for the last 2 h of incubation, and optical absorbance at 450 nm (A_{450}) was measured using an Infinite 200 PRO instrument (Tecan, Männedorf, Switzerland). Cell viability was also measured by trypan blue dye exclusion with a TC10 automated cell counter (Bio-Rad). The NAD^+ concentration was measured using an NAD/NADH Assay Kit-WST (DOJINDO, Kumamoto, Japan), according to the manufacturer's instructions.

2.8. Apoptosis analysis

Apoptotic cells were analyzed by staining with annexin V-FITC (MBL) and 7-amino-actinomycin D (7-AAD; Beckman Coulter), followed by flow cytometry using a Cell Analyzer EC800 (Sony, Tokyo, Japan), as described previously(Kozako et al., 2012). DNA fragmentation was detected by TUNEL staining (MEBSTAIN Apoptosis TUNEL Kit Direct; MBL). The percentage of apoptotic cells was calculated as follows: % specific apoptotic cells = (annexin V-positive cells − spontaneous annexin V-positive cells)/(100 − spontaneous annexin V-positive cells) × 100.

2.9. Detection of caspase activity

Caspase activity was evaluated using fluorochrome-labeled inhibitors of caspases(Pozarowski et al., 2003). Caspase-3, -8, and -9 activities were assessed using an APOPCYTO Intracellular Caspase-3, -8 Activity Detection Kit (MBL) and CaspGLOW™ Fluorescein Active Caspase-9 Staining Kit (BioVision, Milpitas, CA, USA), according to the manufacturers' instructions(Kozako et al., 2015).

2.10. Mitochondrial transmembrane potential assay

Mitochondrial transmembrane potential (Δψm) was assessed using a JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman Chemical Co.). Briefly, cultured cells were incubated with JC-1 Staining Solution for 30 min. Functional mitochondria then contain red JC-1 J-aggregates, whereas apoptotic cells and cells with a disrupted Δψm contain green JC-1 monomers. Cells were analyzed according to the manufacturer's recommended method(Kozako et al., 2015).

2.11. Analysis of autophagy by flow cytometry

Autophagy was evaluated using a Cyto-ID Autophagy Detection Kit (Enzo Life Sciences, Farmingdale, NY, USA), according to the manufacturer's instructions(Chan et al., 2012; Stankov et al., 2014). Because autophagy is a constitutive cellular degradation process, pretreatment of 72-h cultures with a lysosomal inhibitor, bafilomycin A1, was required for 30 min at 37 °C to prevent lysosomal degradation of autophagic vesicles. Cells were then treated with CYTO-ID Green Detection Reagent to stain the autophagic vesicles. After washing, the cells were analyzed by flow cytometry using the Cell Analyzer EC800, as described previously(Kozako et al., 2018a).

2.12. Statistical analysis

Data are expressed as the means ± standard deviation (S.D.). For data analysis, the Mann-Whitney U-test and two-tailed Student's *t*-test were performed using Excel 2007 (Microsoft Japan, Tokyo, Japan) and Statcel2 software (OMS Publishing Inc., Tokyo, Japan). In all tests, $P < 0.05$ was considered as statistically significant.

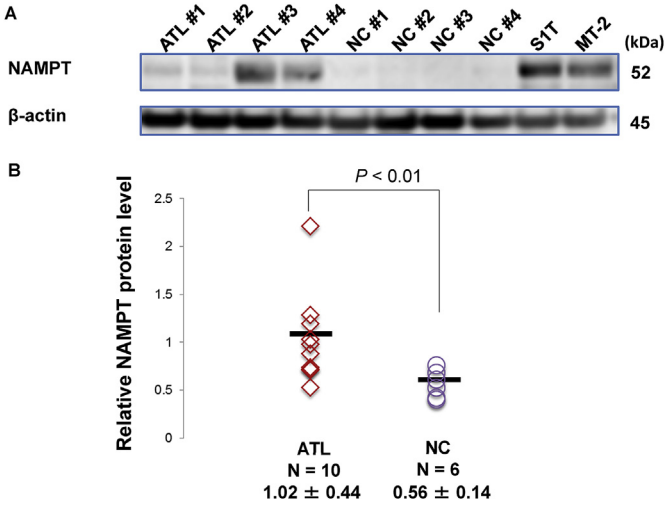

Fig. 1. Expression of NAMPT in PBMCs from ATL patients. (A) Representative western blots of NAMPT protein in PBMCs from four ATL patients (ATL#1–#4), four normal controls (NCs), and HTLV-1-infected T-cell lines. β-Actin was probed as a loading control. (B) Quantification of NAMPT protein levels in PBMCs from 10 ATL patients and six NCs. Expression levels were normalized against β-actin and MT-2 cells as an inter-experiment control. Horizontal bars indicate mean expression levels. Lower numbers represent means ± S.D. of each subject group. Differences in NAMPT expression between ATL patients and NCs were evaluated by the nonparametric Mann-Whitney U-test.

3. Results

3.1. Expression of NAMPT in PBMCs from ATL patients and healthy subjects

We first evaluated the expression of NAMPT in PBMCs isolated from 10 acute-type ATL patients and six HTLV-1-uninfected normal controls (NCs) by western blotting. Representative results are shown in Fig. 1A. NAMPT levels were significantly higher in acute-type ATL patients than NCs (Fig. 1B). In the same subject who progressed from chronic-to acute-type ATL, the NAMPT expression level was undetectable in cells in the chronic phase, but increased markedly in the acute phase (Fig. S1).

3.2. Effects of FK866 on primary ATL cell viability and apoptosis

We next investigated the effect of FK866 on the viability of primary ATL cells *ex vivo*. PBMCs from acute-type ATL patients and NCs were incubated for 72 h with the vehicle [dimethyl sulfoxide (DMSO)] or FK866, and then their viability was assessed. Fresh PBMCs from acute-type ATL patients were more sensitive to FK866 than control PBMCs (Fig. 2A). FK866 showed a potent inhibitory activity with a mean ± S.D. half maximal effective concentration (EC_{50}) value of 10.3 ± 14.0 nM.

To investigate whether FK866 decreases cell viability by enhancing apoptosis, PBMCs were treated with FK866, stained with annexin V, 7-AAD, and DEVD (caspase-3 substrate)-7-amino-7-methylcoumarin, and then analyzed by flow cytometry. Representative data of PBMCs from six acute-type ATL patients in Fig. 2A, showing dose-dependent increases in annexin V-positive cells and caspase-3 activity without 7-AAD-positive cells, are shown in Fig. 2B. FK866 did not increase annexin V-positive cells or caspase-3 in NCs. Thus, FK866 induces primary ATL cell death via caspase-dependent apoptosis.

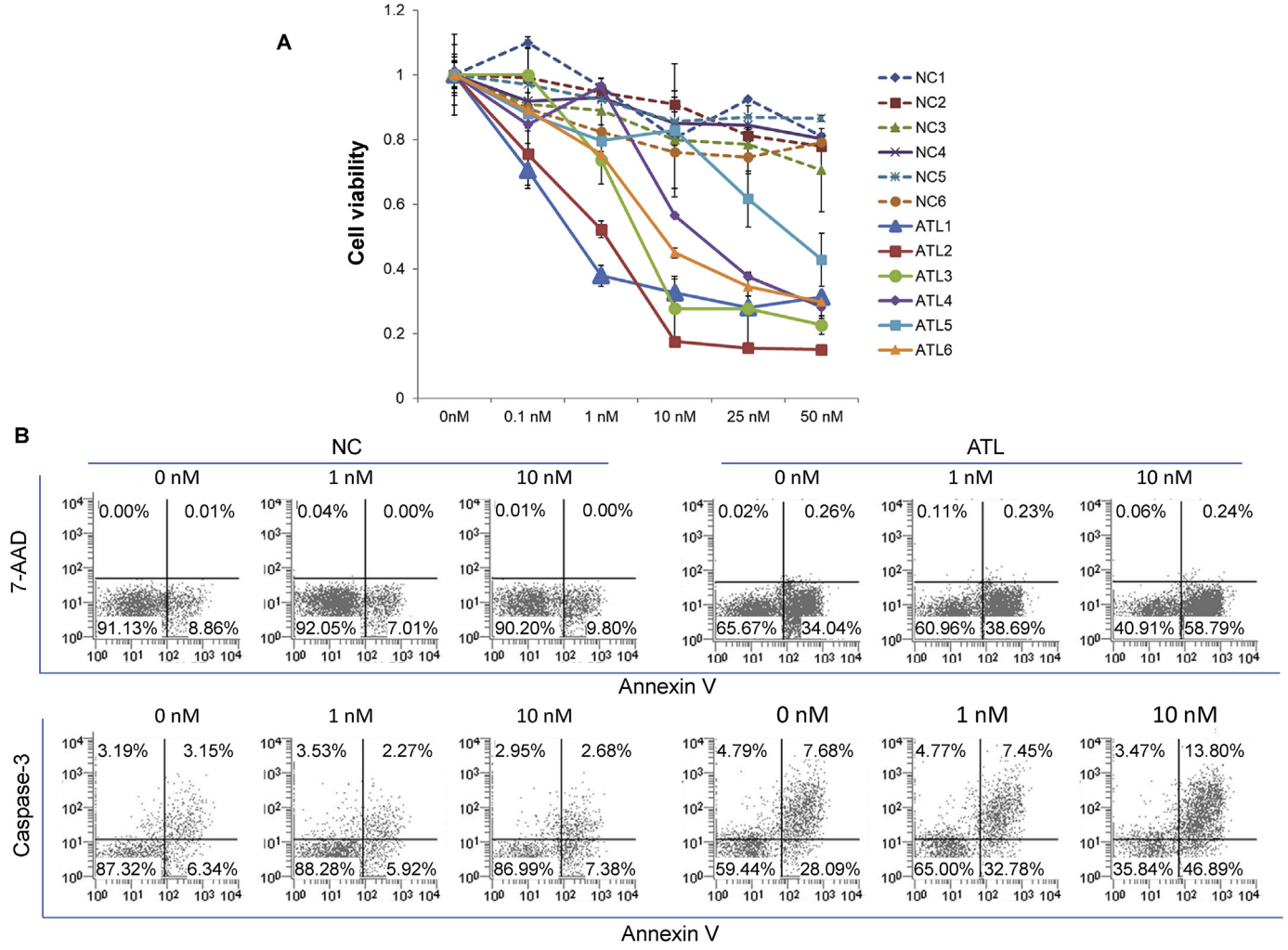

Fig. 2. Effects of FK866 on primary ATL cell viability and apoptosis. (A) Cell Count Reagent SF assay of cell viability after 72 h of incubating PBMCs from four acute ATL patients and six normal controls with various concentrations of FK866. Cells cultured in the absence of FK866 were assigned a viability of 1. (B) Flow cytometric analysis of apoptotic cells after 72 h of incubating of PBMCs from NCs and acute ATL patients detected by annexin, V 7-AAD, and DEVD (caspase-3 substrate)-7-amino-7-methylcoumarin staining. Data represent the means ± S.D. of three independent experiments (A) or are representative of six experiments (B).

3.3. Effect of FK866 on cell viability and apoptosis of HTLV-1-infected T-cell lines

HTLV-1-infected S1T and MT-2 cell lines expressed high levels of NAMPT protein (Fig. 1A). Similar to the results from fresh PBMCs, we found that incubation for 72 h with FK866 significantly reduced the viability of S1T and MT-2 cells (EC_{50} values: 0.63 and 3.7 nM, respectively; Fig. 3A). S1T is derived from an ATL patient with no Tax expression, whereas MT-2 is a HTLV-1 Tax-producing HTLV-1-infected T-cell line. HTLV-1–derived proteins such as Tax, which plays a crucial role in viral replication and cell proliferation(Ishitsuka and Tamura, 2014; Uchiyama et al., 1977; Yoshida, 2001), may be involved in the differences of sensitivity to FK866. S1T and MT-2 cells in the early phase of apoptosis (annexin V + /7-AAD −) were detectable within 48 h of FK866 treatment (Fig. 3B). FK866-induced apoptosis was also detected by three other assays of cell death: DNA fragmentation (TUNEL assay; Fig. 2C), activation of the apoptosis effector enzymes, caspase-3, -8, and -9 (Fig. 3D), and PARP cleavage (Fig. 3E). Furthermore, FK866 induced NAD^+ depletion of S1T and MT-2 cells (data not shown).

3.4. Induction of caspase-independent cell death with FK866

Next, we examined the effect of pan-caspase inhibitor Z-VAD-FMK, on FK866-induced cell death. We found no significant effects of Z-VAD-FMK on the FK866-induced decrease in cell viability or increase in annexin V-positive cells, DNA fragmentation, or caspase activation (Fig. 4). Moreover, a caspase-3 inhibitor (Z-DEVD-FMK), caspase-8 inhibitor (Z-IETD-FMK), and caspase-9 inhibitor (Z-LEHD-FMK) did not inhibit FK-866-induced cell death (data not shown). However, as a positive control, Z-VAD-FMK suppressed anti-Fas antibody-induced cell death (Fig. S2).

Caspase-independent cell death (CICD) is mediated in part by loss of mitochondrial functions induced by mitochondrial outer membrane permeabilization (MOMP)(Tait and Green, 2008). Therefore, we measured the effects of FK866 on Δψm in leukemic cells labeled with JC-1, a Δψm-sensitive dye that forms red-fluorescing aggregates in the mitochondria of healthy cells. We found that FK866 induced a shift from red to green fluorescence of JC-1 monomers, indicating disruption of the Δψm (Fig. 5A).

AIF and endonuclease G, which are CICD mediators, are released from mitochondria following MOMP in a caspase-independent manner, although caspase activity may promote their release(Tait and Green, 2008). FK866 treatment of S1T and MT-2 cells increased nuclear endonuclease G levels (Fig. 5B). However, AIF levels in cytosolic and nuclear extracts were unaffected by FK866 treatment.

Cell death is divided into the three types, type I (apoptosis), type II (autophagic cell death), and type III (necrosis) (Garrido and Kroemer, 2004; Shimizu et al., 2014). Necroptosis is characterized by cell

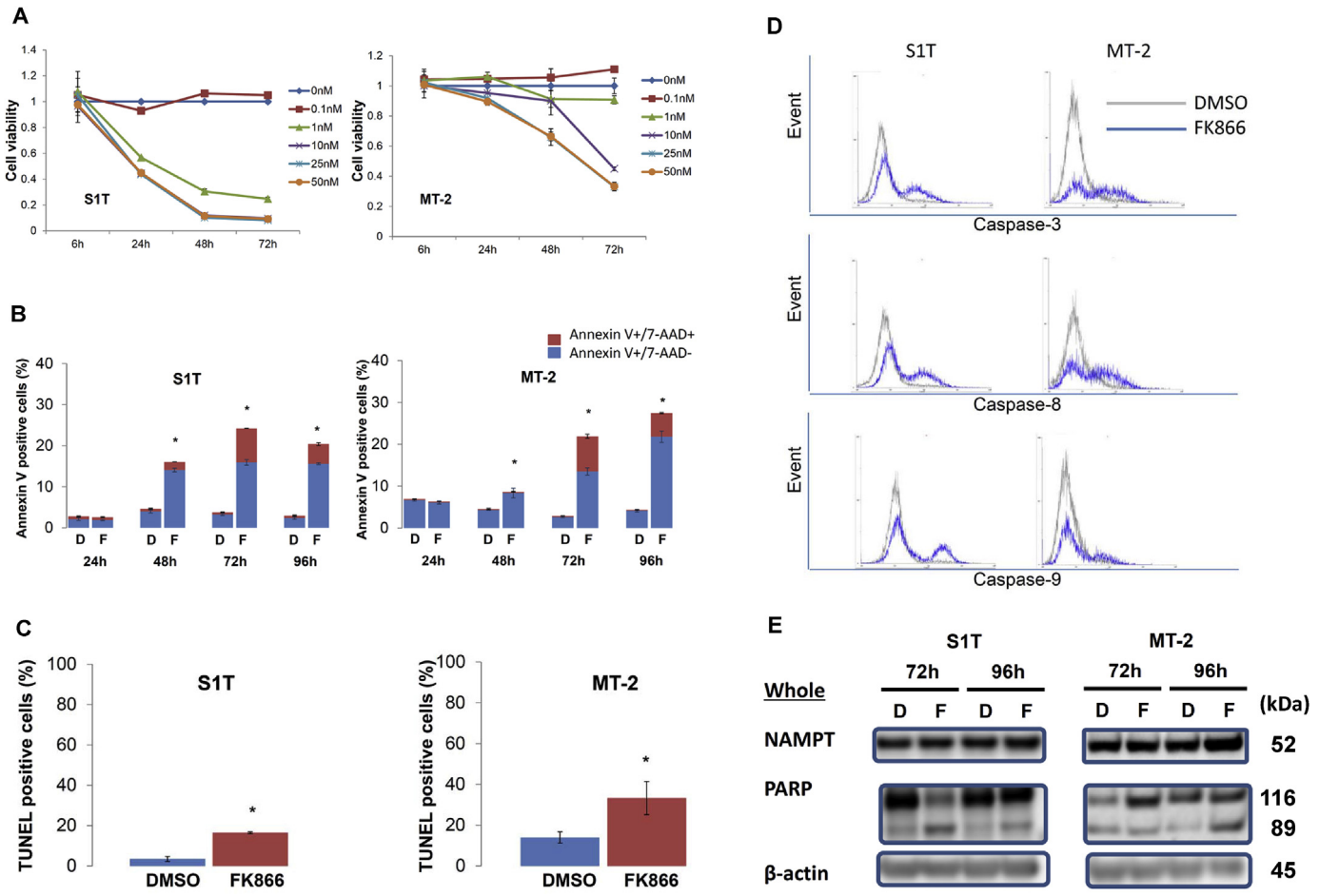

Fig. 3. Effect of FK866 on cell viability and apoptosis of HTLV-1-infected T-cell lines. (A) Cell Count Reagent SF assay of cell viability after 72 h of incubating cell lines with various concentrations of FK866. Cells cultured in the absence of FK866 were assigned a viability of 1. (B–D) Flow cytometric analysis of apoptotic cells after incubation with DMSO (*D*) or FK866 (*F*; S1T, 1 nM; MT-2, 50 nM) for 72 h. (B) Annexin V ± 7-AAD staining. (C) TUNEL staining. (D) Activated caspase-3, -8, and -9 staining. Data represent the means ± S.D. of n = 3 replicates in three independent experiments (A–C) or are representative of three experiments (D and E). $^{*}P < 0.05$ versus DMSO. Significant differences between positive and negative groups were determined by the nonparametric Mann-Whitney U-test. (E) Western blot analysis of the indicated proteins in whole cell lysates prepared at 72 and 96 h after incubation with DMSO (*D*) or FK866 (*F*; S1T, 1 nM; MT-2, 50 nM). β-Actin was probed as a loading control.

swelling similar to necrosis and nuclear condensation as observed during apoptosis, and induced by cleavage of gasdermin D following caspase-1 activation(Shi et al., 2015). FK866 did not activate caspase 1, while caspase 1 were increased by FK866 treatment (Fig. 5B). Next, we examined the effect of a caspase-1 inhibitor, Z-WEHD-FMK, on FK866-induced cell death. We found no significant effects of Z-WEHD -FMK on the FK866-induced decrease in cell viability (Fig. S3A). FK866 treatment of S1T and MT-2 cells induced cell death without caspase-1 activation under Z-WEHD-FMK treatment (Fig. S3B). These data indicate that FK866-induced cell death is independent of pyroptosis.

3.5. Effect of FK866 on autophagy in HTLV-1-infected T-cell lines

Despite the many links between apoptosis and autophagy, the strong causality of controlling one or the other has not been fully demonstrated(Gump and Thorburn, 2011). The incidence of CICD concomitant with increased autophagic activity may be indicative of autophagic type II cell death(Tait and Green, 2008). NAMPT expression is upregulated in colon cancer and glioma in which it regulates a variety of signaling pathways such as AMPK(Bi and Che, 2010; Lucena-Cacace et al., 2017a, 2017b; Salminen et al., 2016). AMPK and mTOR regulate autophagy(Kim et al., 2011). FK866 treatment of S1T and MT-2 cells decreased the expression levels of AMPKα, p-AMPKα, AMPKβ1/2, p-AMPKβ1, and p-mTOR in the cytosol or nucleus (Fig. 5B). We therefore assessed the effects of FK866 on autophagy (Fig. 6). A shift of the soluble form of a microtubule-associated protein, LC3-I, to the autophagic vesicle-associated form LC3-II is a specific marker for autophagosome formation. In immunoblotting to detect autophagic flux, inhibition of lysosomal degradation by bafilomycin A1 induces accumulation of LC3-II, suggesting that the amount of LC3-II has been degraded by autophagy(Yoshii and Mizushima, 2017). FK866 increased the LC3-II-enriched protein fraction induced by bafilomycin A1 treatment (Fig. 6A), indicating autophagosome accumulation. Autophagy was also detected using a Cyto-ID Autophagy Detection Kit. The Cyto-ID Green autophagy dye was validated by observing colocalization of the dye with autophagic vesicles in HeLa cells using fluorescence microscopy(Chan et al., 2012). Autophagy levels in S1T and MT-2 cells were increased in the presence of FK866 (Fig. 6B). Thus, FK866 simultaneously caused apoptosis and autophagy.

3.6. Effect of FK866 on ATL tumor growth in vivo

To determine whether the inhibitory effect of FK866 on ATL cell growth *in vitro* is also observed *in vivo*, we subcutaneously injected immunodeficient NSG mice with S1T cells. The mice were then treated with intraperitoneal injections of the vehicle or FK866 (20 mg/kg) once daily. FK866 effectively suppressed the tumor volume over the entire 15-day experiment (Fig. 7A and B). In control mice, tumors were

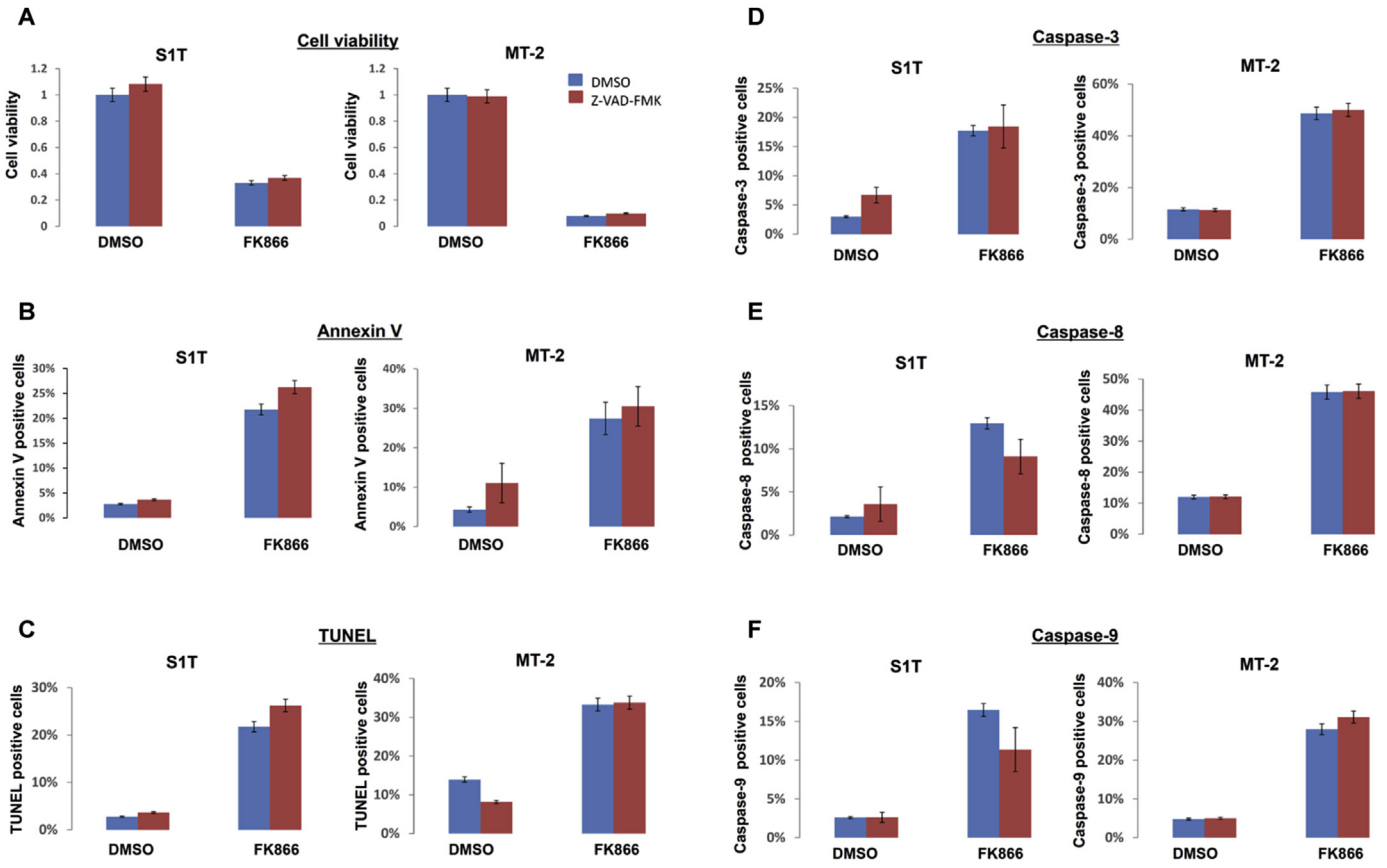

Fig. 4. Effect of pan-caspase inhibitor Z-VAD-FMK on FK866-induced cell death. **(A)** Cell Count Reagent SF assay of cell viability after incubation for 72 h with DMSO or FK866 (S1T, 1 nM; MT-2, 50 nM) and Z-VAD-FMK (40 μM). Cells cultured in the absence of FK866 and Z-VAD-FMK were assigned a viability of 1. (B–F) Analysis of apoptosis by flow cytometric detection of annexin V staining (B), TUNEL staining (C), and activated caspase-3 (D), −8 (E), and −9 (F) staining. Data represent the means ± S.D. of n = 3 replicates in three independent experiments.

palpable at 10 days after cell injection and had encompassed the surrounding cervical and thoracic vertebral regions by 15 days, whereas the tumors were markedly smaller in mice treated with FK866 (Fig. 7C, $P < 0.01$). To ensure that the tumors originated from the injected cells, they were excised on day 28 and analyzed by polymerase chain reaction. This analysis confirmed the presence of HTLV-1 *tax* and human β-globin in the tumors (data not shown).

4. Discussion

NAD^+ plays a crucial role in tumor cell metabolism, survival, and growth(Garten et al., 2009). In tumor cells, NAD^+ is rapidly turned over by degrading enzymes such as SIRT1, and replenishment of NAD^+ pools through salvage pathways is essential for cell survival. NAMPT expression is increased in a number of cancers, suggesting that it is a therapeutic target(Garten et al., 2009; Sampath et al., 2015). NAMPT-mediated NAD^+ biosynthesis plays an important role in regulating the NAD^+-dependent deacetylase activity of SIRT1(Imai, 2016) that is highly expressed in primary acute-type ATL cells(Kozako et al., 2012). In this study, we found that NAMPT is also expressed at high levels in primary ATL cells compared with normal PBMCs. Interestingly, in one patient with chronic-type disease who progressed to acute-type ATL, NAMPT was undetectable in the chronic phase, but highly expressed in the acute phase. Thus, NAMPT is a promising target for innovative molecular treatment strategies of ATL.

We found that FK866 induced apoptosis of HTLV-1-infected T-cell lines. Previous studies have shown that FK866 induces apoptosis by depleting NAD^+ in some tumor cell types such as HepG2 cells (hepatocellular carcinoma) and malignant hematologic cell lines(Hasmann and Schemainda, 2003; Muruganandham et al., 2005; Nahimana et al., 2009). It induces cell death via caspase-independent pathways in neuroblastoma and human hematologic malignancies including acute myeloid leukemia, acute lymphoblastic leukemia, chronic lymphocytic leukemia, and various T- and B-cell lymphomas(Billington et al., 2008; Ginet et al., 2014; Nahimana et al., 2009). In the present study, we found that a pan-caspase inhibitor and caspase-3, -8, and -9 inhibitors did not prevent FK866-mediated apoptosis of ATL cells, suggesting that this NAMPT inhibitor acts via a caspase-independent pathway. CICD is accompanied by a gradual loss of the Δψm and reduction in ATP generation, which precedes the release of AIF from mitochondria. Thus, MOMP may contribute to CICD primarily through a loss of mitochondrial functions(Tait and Green, 2008). AIF and endonuclease G are released from mitochondria following MOMP in a caspase-independent manner, although caspase activity may promote their release. Here, we found that FK866 treatment disrupted the Δψm and increased endonuclease G levels in the nucleus. These results suggest that FK866 induces caspase-dependent or -independent cell death via endonuclease G.

A recent study suggests that the manipulation of autophagy, a process for the degradation of cellular materials, might have broad physiological relevance in health and disease(Ohsumi, 2014). In this study, FK866 induced CICD and increased LC3-II expression, both of which are indicative of activated autophagy. Autophagy sequesters cytoplasmic materials and has a cytoprotective function. Tumor cells with activated autophagy exhibit prolonged survival, and many anti-tumor agents induce autophagic cell death(Cheng et al., 2013; Wang and Levine, 2010), although the mechanisms by which this occurs are unclear.

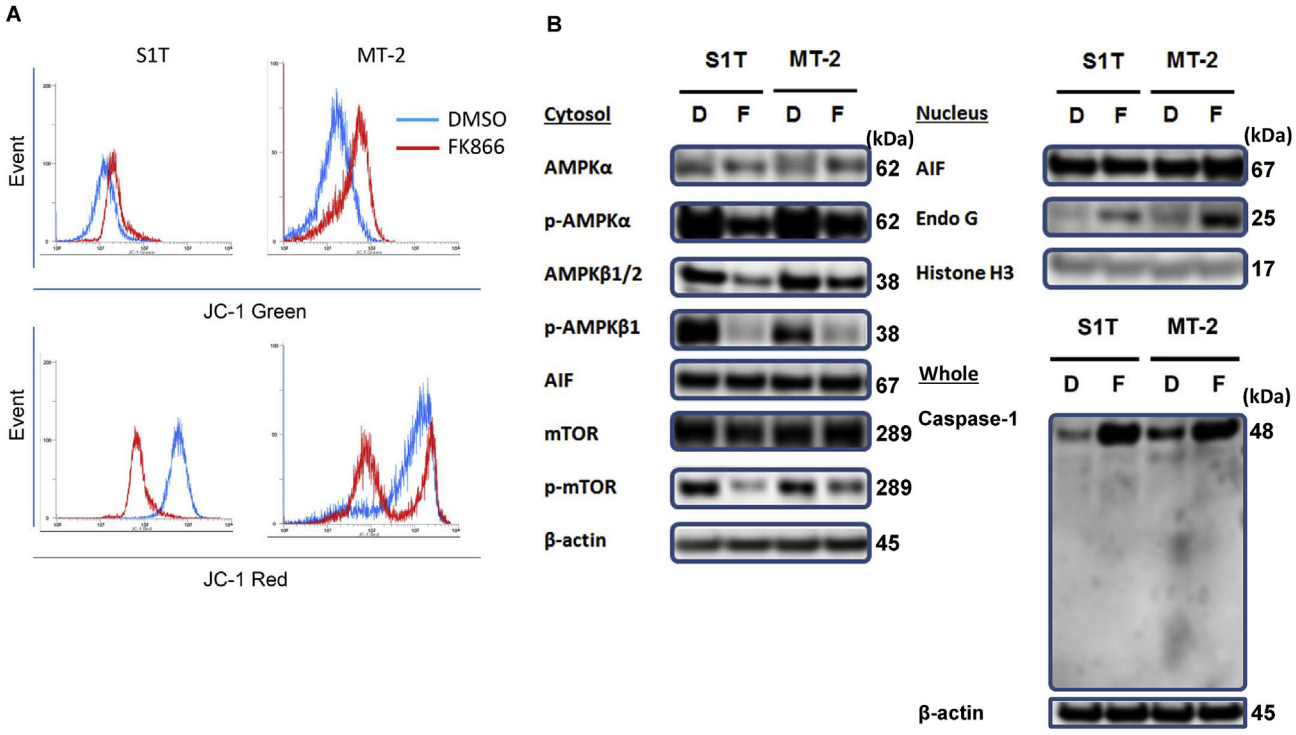

Fig. 5. Effect of FK866 on the mitochondrial transmembrane potential and caspase-independent cell death of HTLV-1-infected T-cell cell lines. (A) Flow cytometric analysis of JC-1 fluorescence after incubation of cells with DMSO or FK866 (S1T, 1 nM; MT-2, 50 nM) for 24 h. JC-1 fluorescence shifts from red to green as the mitochondrial membrane potential is disrupted. (B) Western blot analysis of the indicated proteins in cytosolic, nuclear and whole cell extracts of cells incubated for 96 h with DMSO (*D*) or FK866 (*F*; S1T, 1 nM; MT-2, 50 nM). Data are representative of three experiments.

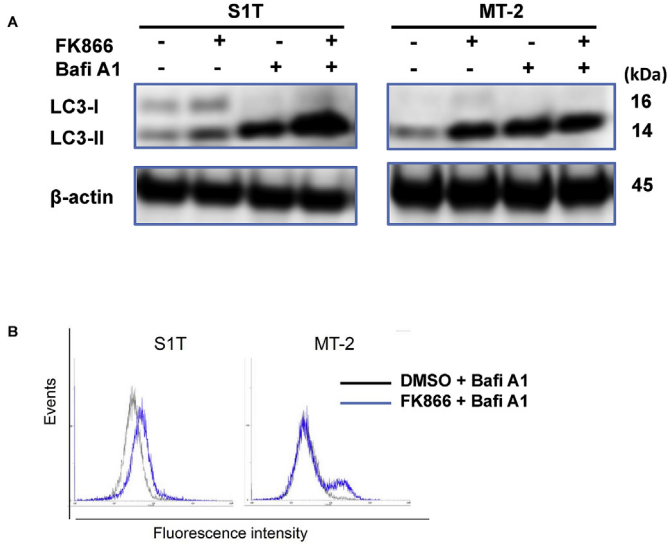

Fig. 6. Effect of FK866 on autophagy of HTLV-1-infected T-cell lines. (A) Western blot analysis of the indicated proteins in whole cell lysates of cell lines treated with FK866 (S1T, 1 nM; MT-2, 50 nM) and bafilomycin A1 (Bafi A1: 10 μM) for 72 h. (B) Autophagy after 72 h of incubation was measured by flow cytometry using a Cyto-ID Autophagy Detection Kit. Data are representative of three experiments.

FK866-induced cell death in Jurkat (a HTLV-1-negative T-lineage acute lymphoblastic leukemia cell line) is accompanied by AMPK phosphorylation and is independent of caspase activation(Nahimana et al., 2009; Zucal et al., 2015). Here, we observed that AMPKα, p-AMPKα, AMPKβ1/2, p-AMPKβ1/2, and p-mTOR protein levels were decreased together with caspase activation in HTLV-1-related T-cell lines by FK866 (Fig.s 3B and 5B). AMPK promotes autophagy by direct Ulk1 phosphorylation, while high activity of mTOR, a serine/threonine protein kinase, prevents Ulk1 activation by phosphorylation and disrupts the interaction between Ulk1 and AMPK(Kim et al., 2011; Wang et al., 2015). mTOR has two subtypes, mTORC1 (TOR complex 1) and mTORC2. mTORC1, which is associated with autophagy, consisting of regulatory-associated protein of mTOR (raptor), mLst8, proline-rich Akt substate 40 kDa, and DEP domain containing mTOR-interacting protein. TSC2 inhibits Rheb that activates mTOR. AMPK inactivates mTOR activity via phosphorylation of raptor (Ser722 and Ser792) and TSC2 (Ser1387), but both p-AMPKα and p-mTOR protein levels were decreased (Fig. 5B). mTORC1 is also inhibited by phosphorylation through PI3K/AKT and Ras/Erk signaling pathways (Kim and Guan, 2015). Therefore, degradation of p-mTOR via PI3K/AKT and Ras/Erk pathways may lead to autophagy. Furthermore, degradation of a negative regulator of NF-κB (p47) by autophagy contributes to overexpression of cell adhesion molecule 1, which is transcriptionally upregulated in HTLV-1-infected cells, through activation of NF-κB(Sarkar et al., 2019). Thus, HTLV-1-related proteins may contribute to autophagy regulation through unknown mechanisms.

We found that FK866 significantly decreased the viability of primary ATL cells *ex vivo* and decreased the volume of human ATL tumor xenografts in NSG mice. Hematological and retinal toxicities have hampered clinical development of FK866(Holen et al., 2008; von Heideman et al., 2010). NAD^+ is synthesized via the nicotinic acid phosphoribosyltransferase domain containing 1-dependent salvage pathway(Imai, 2016). Effective approaches to reduce these toxicities include coadministration of FK866 with nicotinic acid(Olesen et al., 2010). Furthermore, dual NAMPT and histone deacetylase inhibitors have shown potent anti-tumor efficacy *in vivo*(Chen et al., 2018). We previously showed that SIRT1 is overexpressed in PBMCs of acute-ATL patients(Kozako et al., 2012). Thus, dual NAMPT and histone deacetylase inhibitors may have a novel therapeutic potential for the treatment of ATL.

In conclusion, we showed that NAMPT is highly expressed in

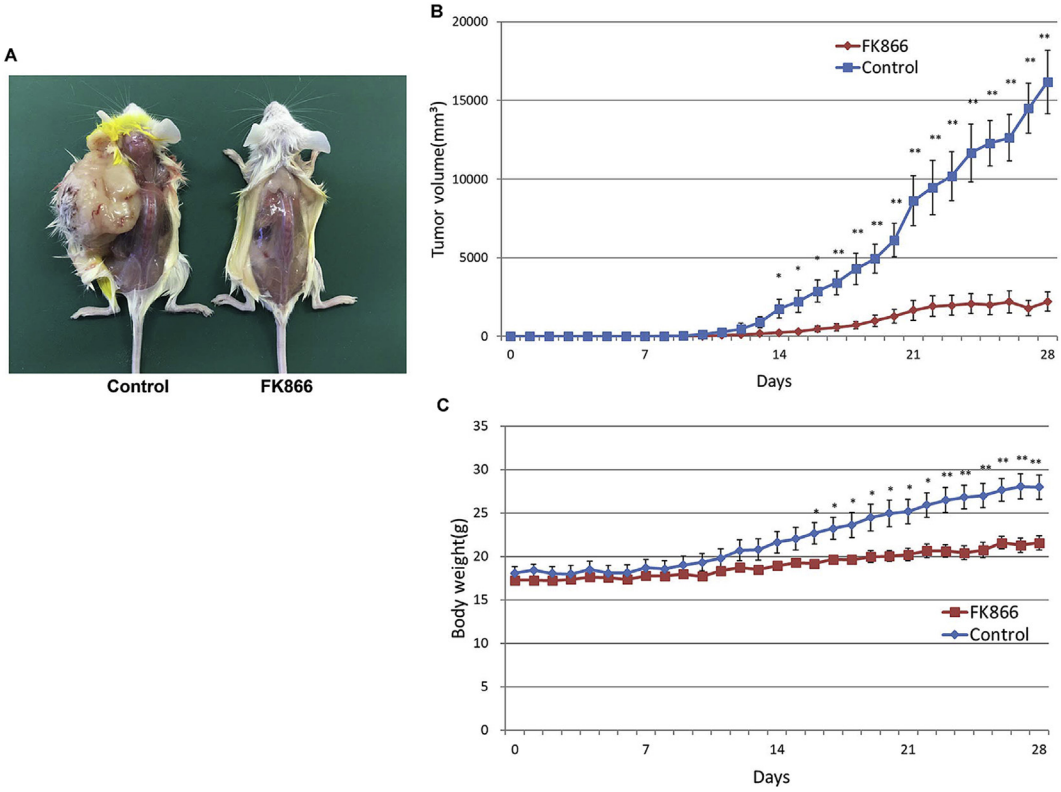

Fig. 7. Effect of FK866 on ATL tumor growth *in vivo*. (A–C) Five-week-old NSG mice were injected with S1T cells and then treated with the vehicle or FK866 (20 mg/kg body weight) once daily for 28 days. Tumor volumes and mouse body weights were measured at the indicated times. (A) Images of representative mice on day 28 post-tumor cell injection. (B–C) Tumor volume (B) and body weight (C) of mice treated for up to 28 days. Data represent the means ± S.D. of five mice/group. Statistical analysis was performed by the Student's *t*-test. $*P < 0.05$, $**P < 0.01$ versus control.

primary acute-type ATL cells. Moreover, NAMPT inhibition by FK866 induced growth inhibition of freshly isolated ATL cells and HTLV-1-infected T-cell lines, induced apoptosis, CICD, and autophagy in leukemic cell lines, and decreased the growth of ATL tumor xenografts in NSG mice. This is the first evidence indicating that FK866 inhibits cell growth, induces caspase-dependent and -independent cell death, and activates autophagy in these leukemic cells. Collectively, our results suggest that NAMPT is a therapeutic target for ATL.

Authors' contributions

T.K. designed and performed the experiments, analyzed the data, and wrote the manuscript. T.O., Y.U., N.K., K.S., and A.A. performed experiments and analyzed data. M.Y. and K.I. supervised the project and provided clinical samples. S.H. supervised the project and contributed to manuscript development.

Declaration of competing interest

The authors declare there are no potential conflicts of interest relevant to this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2019.172738.

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