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Dual inhibition of the mTORC1 and mTORC2 signaling pathways is a promising therapeutic target for adult T-cell leukemia

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Adult T-cell leukemia (ATL) has a poor prognosis as a result of severe immunosuppression and rapid tumor progression with resistance to conventional chemotherapy. Recent integrated-genome analysis has revealed mutations in many genes involved in the T-cell signaling pathway, suggesting that the aberration of this pathway is an important factor in ATL pathogenesis and ATL-cell proliferation. We screened a siRNA library to examine signaling-pathway functionality and found that the PI3K/Akt/mTOR pathway is critical to ATL-cell proliferation. We therefore investigated the effect of mammalian target of rapamycin (mTOR) inhibitors, including the dual inhibitors PP242 and AZD8055 and the mTORC1 inhibitors rapamycin and everolimus, on human T-cell leukemia virus type 1 (HTLV-1)-infected-cell and ATL-cell lines. Both dual inhibitors inhibited the proliferation of all tested cell lines by inducing G1-phase cell-cycle arrest and subsequent cell apoptosis, whereas the effects of the 2 mTORC1 inhibitors were limited, as they did not induce cell apoptosis. In the ATL-cell lines and in the primary ATL samples, both dual inhibitors inhibited phosphorylation of AKT at serine-473, a target of mTORC2, as well as that of S6K, whereas the mTORC1 inhibitors only inhibited mTORC1. Furthermore, AZD8055 more significantly inhibited the in vivo growth of the ATL-cell xenografts than did everolimus. These results indicate that the PI3K/mTOR pathway is critical to ATL-cell proliferation and might thus be a new therapeutic target in ATL.

KEYWORDS
adult T-cell leukemia, Akt, HTLV-1, mTORC, mTOR

1 | INTRODUCTION

Adult T-cell leukemia (ATL) is an aggressive peripheral T-cell neoplasm caused by human T-cell leukemia virus type 1 (HTLV-1). ATL has a very poor prognosis because of resistance to chemotherapy and opportunistic infections.[1-3] Although allogeneic hematopoietic stem-cell transplantation has the potential to provide long-term remission for ATL, only a small percentage of patients have the opportunity to benefit from transplantation because ATL often develops in the elderly.[4] A more effective and less toxic therapeutic option for ATL is therefore highly desired.

Tax, one of the HTLV-1-encoded proteins, is a regulatory protein for viral replication and activates the PI3K/Akt pathway by binding to the p85α inhibitory subunit of PI3K and releasing the active p110α catalytic subunit, resulting in cell proliferation and survival.[5] Inhibition of Akt induces apoptosis in HTLV-1-infected cells.[6] Tax is important for the infection cycle and clonal expansion, but Tax-expressing cells are rapidly eliminated because of its strong immunogenicity.[7-9] Thus, Tax is suppressed or not expressed in ATL.[10-12] In contrast to Tax, HTLV-1- basic-leucine-zipper factor (*HBZ*), an antisense mRNA transcribed from the 3′LTR, has been shown to be consistently expressed in ATL cells.[12-14] The HBZ protein binds to a variety of host factors, such as c-Jun, JunB, JunD, CREB, and the p65 subunit of nuclear factor-kappa B (NK-κB), and has been shown to affect several cellular-signaling pathways in ATL cells.[15] HBZ interacts with GADD34 and positively regulates the mammalian target of rapamycin (mTOR) signaling pathway.[16] Both Tax and HBZ play pivotal roles for HTLV-1 and these findings indicate that the mTOR pathway might be a therapeutic target for ATL.[17,18]

mTOR, a serine/threonine kinase, plays a pivotal role in cancer-cell proliferation and is a promising target for cancer therapy. mTOR comprises 2 distinct protein complexes, mTORC1 and mTORC2, which have different substrates and functions. mTORC1 phosphorylates p70 ribosomal protein S6 kinase (p70S6K) and translation-initiation regulator 4E binding protein 1 (4E-BP1) and regulates protein translation and cell proliferation.[19] mTORC2 phosphorylates Akt and SGK1 and regulates cell survival, apoptosis, and cytoskeletal organization. Studies have shown that the mTORC1 inhibitors rapamycin and its rapalogs (everolimus and temsirolimus) are effective against several cancers, such as renal-cell carcinoma, multiple myeloma, acute myeloid leukemia, and B-cell lymphoma. However, the efficacy of the mTORC1 inhibitors is limited, because they suppress the mTORC1-dependent negative feedback loop and paradoxically activate Akt signaling,[20] resulting in resistance. By contrast, mTORC2 directly phosphorylates Akt at a regulatory site critical to maximal Akt-kinase activity.[21] Thus, targeting both mTORC1 and mTORC2 would seem to be necessary to completely block the PI3K/Akt/mTOR signaling pathway.

Second-generation mTOR inhibitors have recently been developed and these dual mTOR inhibitors target the ATP-binding site of mTOR, resulting in the inhibition of both mTORC1 and mTORC2 activity.[22] These inhibitors have been shown to inhibit mTORC2-mediated Akt Ser-473 phosphorylation and Akt signaling, and used as anticancer agents for hematological malignancies including ATL and other cancers.[22-32]

We herein show that the PI3K/Akt/mTOR signaling pathway is crucial for the proliferation of ATL cells and that the mTOR inhibitors are cytotoxic to ATL-cell lines and primary ATL samples. Dual mTOR inhibitors such as PP242 and AZD8055, in particular, block the phosphorylation of Akt at Ser-473, strongly induce apoptosis, and inhibit the growth of ATL-cell xenografts in mice. We conclude that dual mTOR inhibitors are a promising therapeutic agent for ATL.

2 | MATERIALS AND METHODS

2.1 | Cell culture

ED-40515(+) is an interleukin-2 (IL-2)-dependent T-cell line of leukemic-cell origin established from an ATL patient.[33] ED-40515(−) is an IL-2 independent subclone of ED40515(+).[34] SY is an IL-2-dependent HTLV-I-infected T-cell line derived from a non-leukemic cell clone of ED-40515(+) from the same ATL patient. ATL-43T is an IL-2-dependent T-cell line established from double-negative ($CD4^-$, $CD8^-$) leukemic cells from another ATL patient.[35] SYK-11L(+) is a leukemic-cell line from ATL tumor cells in an in vivo cell-proliferation model using severe combined-immunodeficiency mice.[36] MT-1 was established from human cord-blood T-cell coculture with PBMC from another ATL patient. HUT102 is an IL-2-independent T-cell line from a patient with mycosis fungoides, from which HTLV-1 was isolated.[37] MT-2 is a stably transformed HTLV-I-infected cell line, as previously reported.[33] ATL and HTLV-1 infected cell lines used in this study are listed in Table S1. Jurkat and H9 are IL-2-independent non-HTLV-1-infected human T-cell lines. Cells were maintained in RPMI-1640 medium (Nacalai Tesque, Kyoto, Japan) containing 10% FBS (Sigma-Aldrich, St Louis, MO, USA), 100 units/mL penicillin, 100 μg/mL streptomycin and 0.292 mg/mL L-Glutamine (Invitrogen, Carlsbad, CA, USA). IL-2-dependent cell lines were maintained in the same medium with 0.5 nmol/L recombinant human IL-2 (kindly provided by Shionogi Pharma, Osaka, Japan).

2.2 | Reagents

Rapamycin and LY294002 were purchased from Calbiochem (San Diego, CA, USA); everolimus and AZD8055 from Selleckchem (Newmarket, UK) and MedChem Express (Monmouth Junction, NJ, USA). PP242 was synthesized by SAI Advantium Pharma Ltd (Hyderabad, Telangana, India). For in vitro assays, these chemicals were dissolved in 100% DMSO to a stock concentration of 10 mmol/L and stored at −20°C. FITC-annexin V was purchased from BioLegend (San Diego, CA, USA). Propidium iodide (Nacalai Tesque) was dissolved in PBS to a stock concentration of 2 mg/mL. Rabbit anti-phospho-mTOR (Ser2448) (D9C2), rabbit anti-phospho-mTOR (Ser2481), rabbit anti-mTOR (7C10), rabbit anti-phospho-Akt (Ser473) (D9E), mouse anti-Akt, mouse anti-phospho-p70 S6 kinase (Thr389) (1A5), rabbit anti-p70 S6 kinase, and mouse anti-phospho-p44/42 MAPK (Thr202/Tyr204) (E10) were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-β-actin (AC-15, A5441) was purchased from Sigma-Aldrich.

2.3 | siRNA transfection

The siRNA library was kindly provided by Dr Sato (National Institute of Infectious Diseases, Japan).[38] Transfection of 20 pmol/μL siRNA to 5×10^6 cells of ED-40515(−) was carried out using an Amaxa human T-cell nucleofector kit (Lonza, Basel, Switzerland) as per manufacturer protocols. MISSION® siRNA Universal Negative Control (Sigma-Aldrich) was used as a negative control.

2.4 | Human-cell-killing assay

To assess the proliferation of the human-cell lines transfected with each siRNA, 1×10^4 cells were seeded in 96-well flat-bottomed culture plates (final volume 100 μL/well) and cultured for 48 hour at 37°C. Cell Titer 96 Aqueous One solution (20 μL) from a cell-proliferation assay kit (Promega, Madison, WI, USA) was added to each well, after which cell survival was determined by measuring absorbance intensity at 490 nm using ALVO™ X-3 (PerkinElmer, Waltham, MA, USA). Absorbance values were normalized to controls.

2.5 | Cell-cycle analysis

Cell-cycle analysis was carried out on ED-40515(−) cells treated with rapamycin (100 nmol/L) or PP242 (10 μmol/L) for 24 hour. Pretreated cells were fixed with chilled 70% ethanol for 60 minute at 4°C, then stained with 5 μg/mL PI (Nacalai Tesque) in the presence of 2% BSA (Nacalai Tesque) and 100 μg/mL RNase (Sigma-Aldrich) for 60 minute at room temperature. Immediately after staining, cells were evaluated using a FACSCalibur analyzer (BD Biosciences, Bedford, MA, USA). The resulting data were analyzed using ModFit software (Verity Software House, Topsham, ME, USA). DMSO treatment was used as a negative control. A 0.5 μg/mL Adriamycin (ADR) treatment was used as a positive control.

2.6 | Apoptosis assay

An apoptosis assay was carried out on the ED-40515(−), MT-2 and ATL43T cells after 48-hour treatment with rapamycin (100 nmol/L), everolimus (100 nmol/L), LY294002 (10 μmol/L), or PP242 (10 μmol/L). Pretreated cells were stained with FITC-annexin V (BioLegend) and PI for 10 minute at 4°C. Analysis was carried out immediately after staining using a FACSCalibur analyzer. DMSO treatment was used as a negative control. A 1 μg/mL ADR treatment was used as a positive control.

2.7 | Isolation of PBMC and primary ATL-cell samples

This project was approved by the institutional review board of Kyoto University Hospital, and all participants provided written informed consent in accordance with the Declaration of Helsinki. PBMC were isolated from healthy donors and 2 ATL patients by Ficoll gradient centrifugation. Cells thus isolated were analyzed by western blot. Characteristics of the 2 patients are shown in Table S2.

2.8 | Immunoblotting

A total of 5×10^6 cells were lysed using ice-cold lysis buffer (MPER; Thermo Scientific, Rockford, IL, USA) containing 1 mmol/L PMSF, phosphatase inhibitor cocktail (Roche, Applied Sciences, Basel, Switzerland), and protease inhibitor cocktail (Nacalai Tesque), followed by centrifugation at 10 000 *g* for 15 minute at 4°C. Cell lysates were mixed with an equal volume of 2-fold concentrated sample buffer (Bio-Rad Laboratories, Hercules, CA, USA) containing β-mercaptoethanol (Nacalai Tesque) and treated for 5 minute at 100°C. Western blot analysis was carried out as described previously.[39]

2.9 | Preparation of mouse ATL model

Rapid tumor formation by the ATL-cell line in NOD/SCID mice has been previously established.[35,40,41] In brief, 5-week-old NOD/SCID mice were purchased from CLEA Japan (Tokyo, Japan). Mice were anesthetized with isoflurane, and 3×10^7 of ED-40515(−) cells were s.c. inoculated into the posterior cervical lesion. Beginning

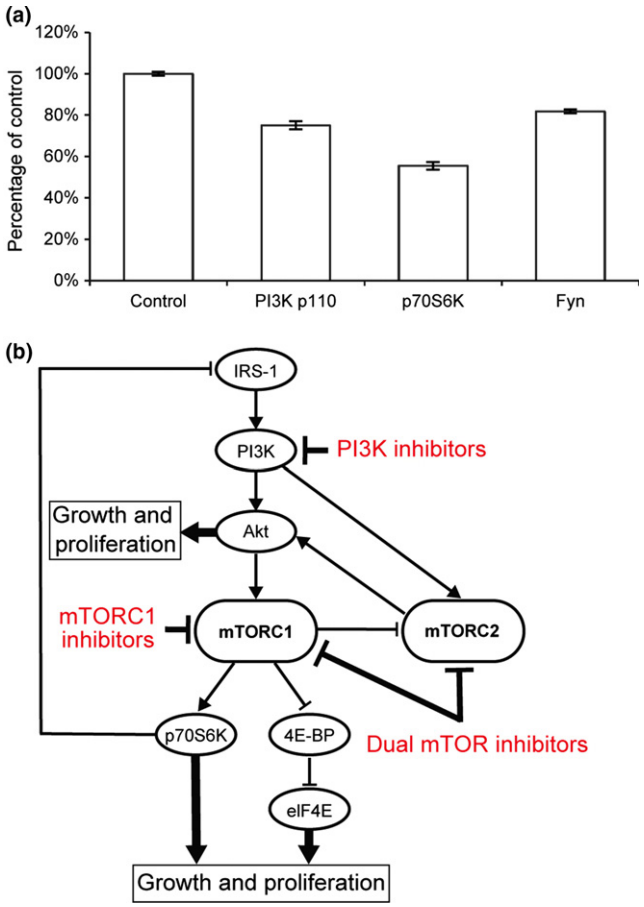

FIGURE 1 Introduction of siRNA of Fyn, PI3K, and S6K inhibits growth in adult T-cell leukemia (ATL) cells. A, siRNA of control, PI3K p110, p70S6K, and Fyn were introduced into ED40515(−) cells by human T-cell nucleofector. Cells were cultured for 48 h in 96-well plate followed by analysis of cell numbers by MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt)). Data shown are for 3 independent experiments. B, Signaling cascade of PI3K/Akt/mTOR, including negative feedback from P70S6K to insulin receptor substrate-1 (IRS-1). mTORC1 inhibitors suppress the negative feedback loop, resulting in paradoxical Akt activation and mTORC2-mediated compensatory activation

2 weeks after inoculation, the long and short axes were measured weekly. Tumor volume was approximated as (long axis) × (short axis)^2. All experiments were carried out under the approved protocols of the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University.

2.10 | Administration of everolimus and AZD8055

Everolimus or AZD8055 was dissolved in 30% (w/v) Captisol (Cydex, Lenexa, KS, USA) and given orally to mice at a dose of 5 mg/kg (everolimus) or 20 mg/kg (AZD8055) per day on weekdays from day 2 to day 20. The control mice received the vehicle only.

2.11 | Statistical analysis

Analyses were done using GraphPadPrism software (GraphPad Software, Inc, San Diego, CA, USA).

3 | RESULTS

3.1 | siRNA library screening identified the importance of the PI3K/mTOR signaling pathway for ATL-cell proliferation

We carried out siRNA screening to identify the genes required for the proliferation and survival of ATL cells using a library of siRNAs targeting 247 human genes (mainly related to signal transduction). Each siRNA was introduced into the ED-40515(−) cells using an Amaxa human T-cell nucleofector kit. Transfection efficiency was 30%-40%, as confirmed by control GFP positivity (data not shown). After the first screening of 247 siRNAs, we found that 35 siRNAs efficiently inhibited cell proliferation compared to the control siRNA (Fig. S1; Table S3). Interestingly, these siRNAs contained several molecules involved in the PI3K/Akt/mTOR signaling pathway, such as PI3K p110, p70S6K, and Fyn (Figure 1A), suggesting that this pathway is important for ATL-cell proliferation.

3.2 | PI3K/Akt/mTOR pathway inhibitors suppress proliferation of ATL and HTLV-1-infected cells

To confirm the importance of the PI3K/Akt/mTOR signaling pathway (Figure 1B) in ATL-cell proliferation, we examined the effect of the mTORC1 inhibitor (rapamycin), dual mTOR inhibitor (PP242) and a PI3K inhibitor (LY294002) on ATL-cell lines (ED-40515(−), ED-40515(+), Hut-102, SYK-11L(+), ATL-43T, and MT-1) and on non-leukemic HTLV-1-infected cell lines (SY and MT-2).

In the rapamycin-treated group, cell lines were rigidly divided into 2 groups based on its efficacy. Rapamycin suppressed the proliferation of the ED-40515(−), ED-40515(+), Hut-102, SY, and MT-2 cells, and to a lesser extent the proliferation of the SYK-11L(+), ATL-43T, and MT-1 cells (Figure 2A). The dose-response was rather flat, plateauing at a low concentration. By contrast, LY294002 and PP242 effectively and uniformly suppressed the proliferation of all cell lines according to dose. We observed similar results in Jurkat T

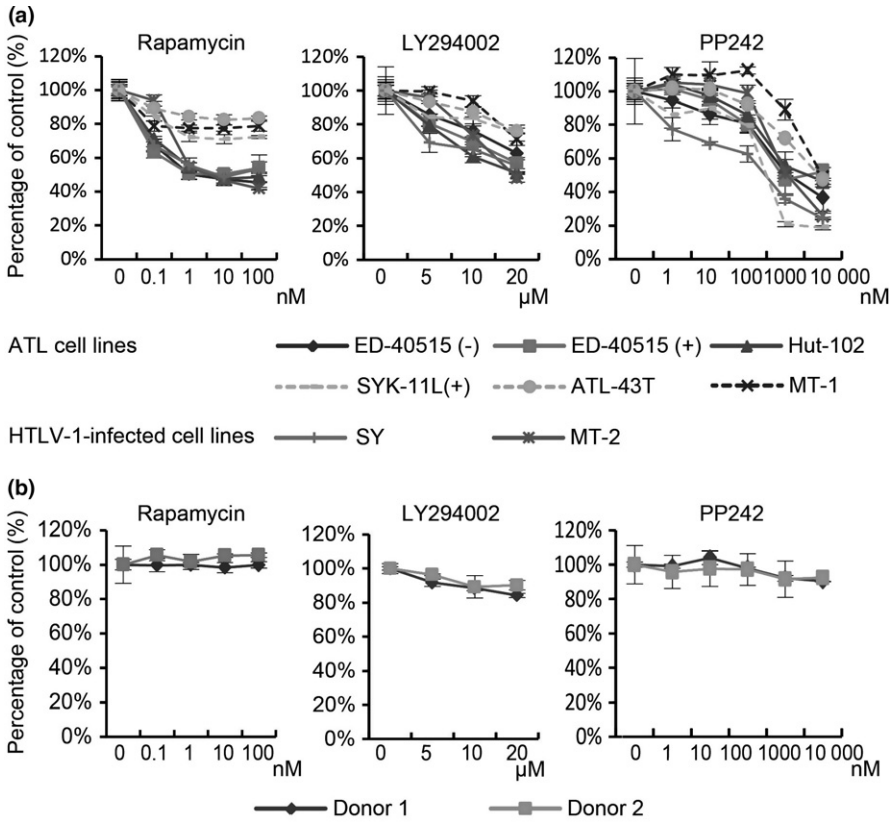

FIGURE 2 The PI3K/Akt/mTOR signaling pathway is involved in adult T-cell leukemia (ATL)-cell proliferation. A, Eight ATL and human T-cell leukemia virus type 1 (HTLV-1)-infected cell lines were treated with increasing concentrations of rapamycin, LY294002, or PP242 for 48 h, after which the number of cells was assessed by MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt)). Data are presented as percentage of control (no inhibitor treatment). In higher concentrations (>1 nmol/L) of rapamycin, significant differences between the 2 groups appeared ($P < .0001$). *P*-values were calculated by a 1-way ANOVA test and Tukey-Kramer correction. B, Cell numbers of 2 similarly treated PBMC derived from normal healthy donors, assessed by MTS assay. Data are presented as percentage of control (no inhibitor treatment)

and H9 cells, both of which are non-HTLV-1-infected cell lines (Fig. S2). Interestingly, in the short term, rapamycin, LY294002, and PP242 were less toxic to PBMC derived from normal, healthy donors (Figure 2B). These results suggest that the PI3K/Akt/mTOR signaling pathway is crucial for ATL-cell proliferation; thus the mTOR inhibitors could be used as therapeutic agents for ATL with less adverse effects on normal cells.

3.3 | PP242 induced G0/G1 cell-cycle arrest and apoptosis in ATL and HTLV-1-infected cells

We examined the effect of the studied inhibitors on cell cycle and apoptosis in ATL cells. It is widely believed that rapamycin inhibits the translation of key mRNAs required for progression from the G1 to the S phase, inducing cell-cycle arrest at G0/G1.[42] Although both rapamycin and PP242 induced G0/G1 arrest in ED-40515(−) cells effectively (Figure 3A,B), only PP242 induced apoptosis compared to the control and the mTORC1 inhibitors in ED-40515(−), MT-2, and ATL-43T cells (Figures 3C, S3). These results suggest that the mTORC1 inhibitors are not potent enough to induce apoptosis in ATL cells and are thus not suitable as anti-ATL agents.

3.4 | PP242 and AZD8055 block the feedback activation of PI3K/Akt/mTOR signaling

To characterize the biochemical activity of the mTOR and PI3K inhibitors, we checked phosphorylation of the PI3K/Akt signaling molecules in rapamycin-sensitive ED-40515(−), Hut-102, and MT-2 cells as well as in rapamycin-resistant ATL-43T cells. All mTOR inhibitors inhibited the phosphorylation of p70S6K in all cell lines except for ATL-43T, which did not show phosphorylation. The PI3K inhibitor (LY294002) and the dual mTORC inhibitors (PP242 and AZD8055) inhibited the phosphorylation of Akt at Ser-473 in all cell lines, whereas the mTORC1 inhibitors (rapamycin and everolimus) did not (Figure 4). These results indicate that the phosphorylation status of p70S6K is not an indicator of the inhibitory effect on ATL-cell growth. Inhibition of Akt phosphorylation at Ser-473 may be crucial for ATL-cell survival and/or proliferation.

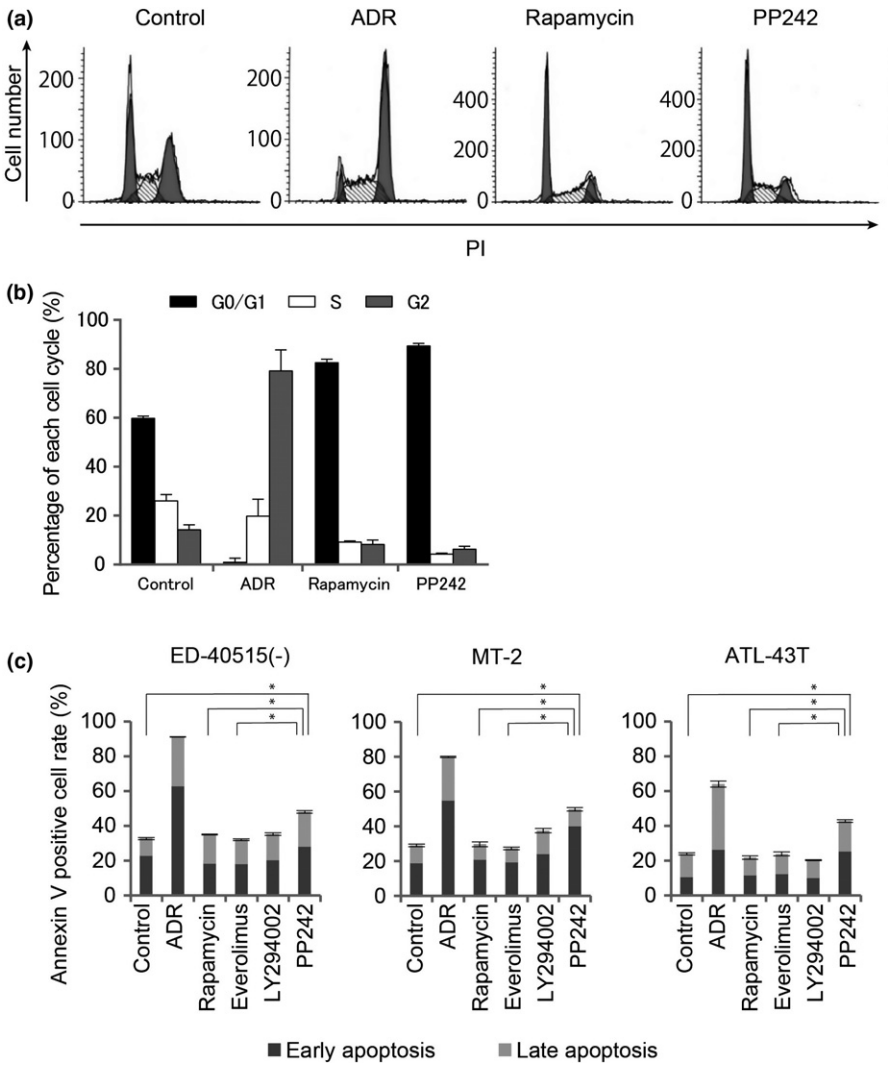

FIGURE 3 PP242 induced G0/G1 cell-cycle arrest and apoptosis in adult T-cell leukemia (ATL) and human T-cell leukemia virus type 1 (HTLV-1)-infected cells. Cells were treated with Adriamycin (ADR), rapamycin (100 nmol/L), everolimus (100 nmol/L), LY294002 (10 μmol/L), or PP242 (10 μmol/L) for 48 h. A, B, ED-40515(−) was treated and followed by propidium iodide (PI) staining. Percentage of each cell-cycle phase is shown. In rapamycin- and PP242-treated groups, G0/G1-phase cells significantly increased ($P < .0001$) and, furthermore, S-phase and G2-phase cells decreased compared to control (S-phase, $P < .0001$; G2-phase, $P < .001$). *P*-values were calculated by a 1-way ANOVA test and Tukey-Kramer correction. C, Cells were treated and stained with PI and annexin V analyzed by flow cytometry. Combination of FITC-Annexin V and PI allowed us to distinguish between early apoptotic cells (FITC-Annexin V positive and PI negative), late apoptotic and/or necrotic cells (FITC-Annexin V and PI positive), and viable cells (unstained). PP242 induced a higher percentage of apoptosis in each cell line, compared to the control, rapamycin, and everolimus (*$P < .0001$). *P*-values were calculated by a 1-way ANOVA test and Tukey-Kramer correction

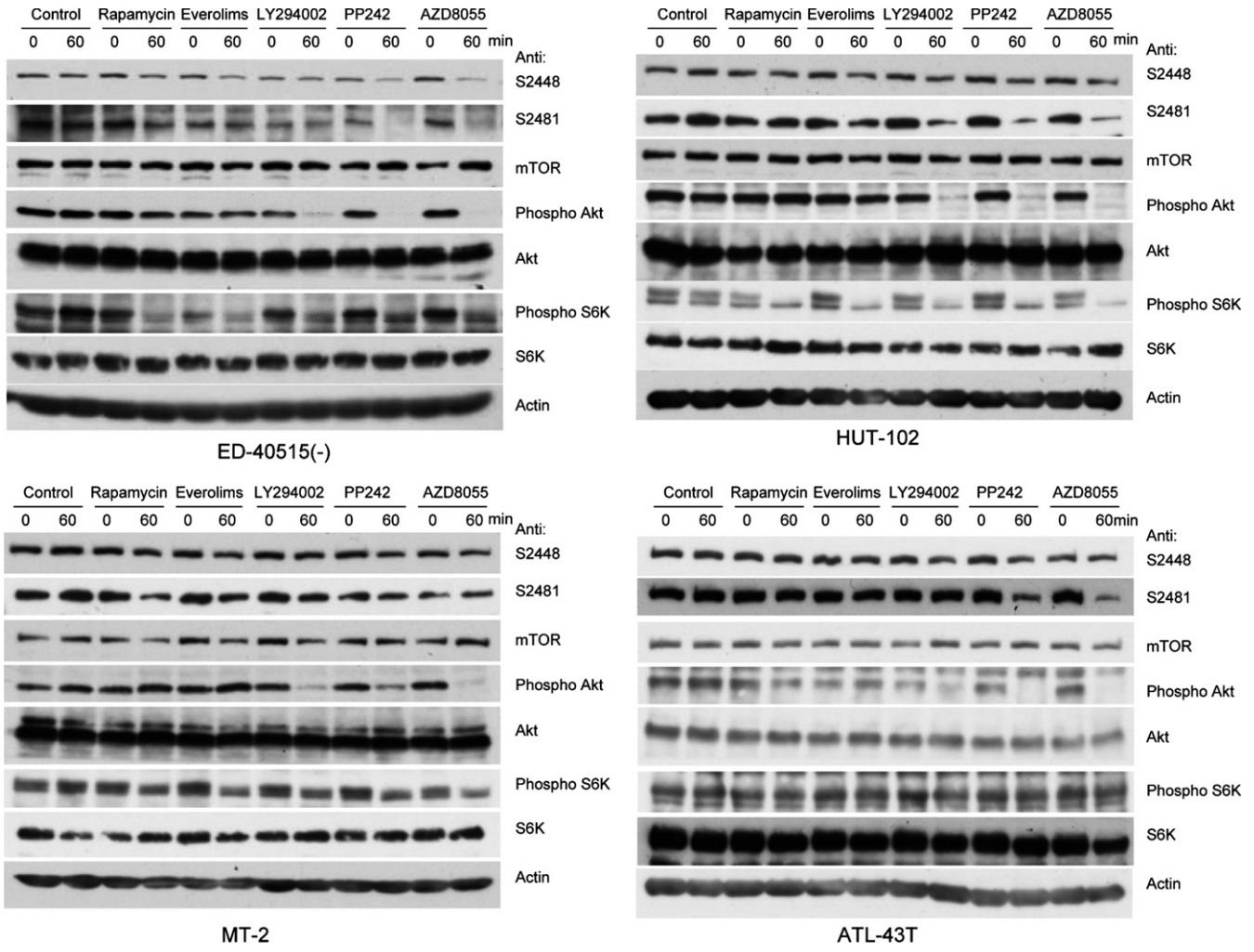

FIGURE 4 PP242 and AZD8055 inhibited mTORC1 and mTORC2 activity in adult T-cell leukemia (ATL) and human T-cell leukemia virus type 1 (HTLV-1)-infected cell lines. Four cell lines were treated for 0 and 60 min with control (DMSO), rapamycin, everolimus, LY294002, PP242 and AZD8055. After treatment, protein lysates were immunoblotted for expression of phosphorylated mTOR S2448 (mTORC1), S2481 (mTORC2), total mTOR, phosphorylated Akt S473, total Akt, phosphorylated p70S6k, total p70S6k, and actin

3.5 | PP242 and AZD8055 inhibit mTORC2-mediated phosphorylation of Akt at Ser-473 in primary ATL cells

We examined the phosphorylation of Akt at Ser-473 in primary ATL cells treated with mTOR inhibitors. Two primary ATL samples were isolated from the peripheral blood of a primary refractory acute-type-ATL patient and from the skin tumor of an acute-type-ATL patient who had relapsed after hematopoietic stem-cell transplantation (Table S2). As shown in Figure 5, the PI3K inhibitor (LY294002) and the dual mTORC inhibitors (PP242 and AZD8055) clearly inhibited Akt phosphorylation at Ser-473 in both primary ATL-cell types, whereas the mTORC1 inhibitor (rapamycin) did not, suggesting that the dual inhibitors are also effective anti-ATL agents to primary ATL cells.

3.6 | AZD8055 inhibited the growth of ATL-cell xenografts in NOD/SCID mice

Finally, we examined in vivo the effect of everolimus and AZD8055 on ATL-cell growth using an ATL-xenograft model. giving oral AZD8055 significantly inhibited the growth of ATL-tumor grafts (Figure 6A) and improved survival (Figure 6B). These results support the critical importance of the PI3K/Akt/mTOR signaling pathway for the proliferation of ATL cells and the therapeutic advantage of the dual mTORC inhibitors.

4 | DISCUSSION

In the present study, we showed that the PI3K/Akt/mTOR signaling pathway is crucial for ATL cells to proliferate and that this pathway could be a novel therapeutic target for ATL. We identified the pathway using siRNA screening, and confirmed it by experimenting with specific inhibitors.

Many previous reports have shown that mTORC1 and mTORC2 represent a promising therapeutic target in a number of cancers and hematological malignancies.[43] Rapamycin and its derivatives (rapalogs) have been clinically approved for renal transplantation, advanced renal cell carcinoma, and other purposes.[44] However, the

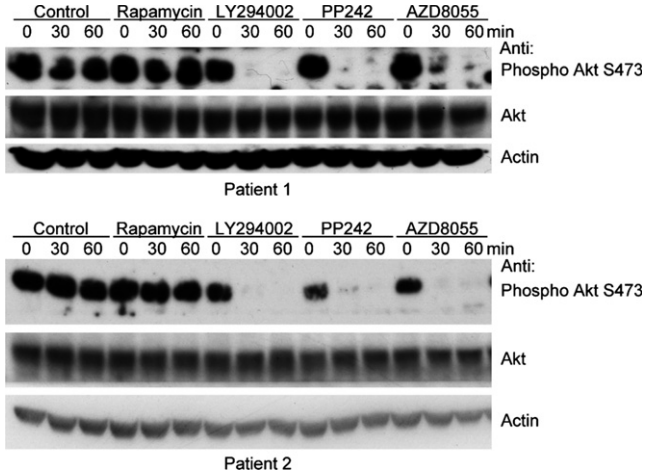

FIGURE 5 PP242 and AZD8055 inhibited mTORC1 and mTORC2 activity in primary adult T-cell leukemia (ATL) cells. Two primary ATL samples derived from ATL patients were treated for 0, 30, and 60 min with control (DMSO), rapamycin, LY294002, PP242, and AZD8055. After treatment, protein lysates were immunoblotted for expression of phosphorylated Akt S473, total Akt, and actin

clinical efficacy of these drugs is somehow limited when used as a single agent for anti-cancer therapy. Several factors may explain this lack of efficacy. For example, the rapalogs cannot completely block the phosphorylation of mTORC1 substrates, especially when the phosphorylation of 4E-BP is insensitive to them. Both rapamycin and rapalogs also suppresses the negative-feedback loop of molecules downstream from mTORC1, including S6K and Grb10.[45,46] This results in the activation of Akt and paradoxically promotes cell growth.[47] Moreover, as mTORC1 also negatively regulates mTORC2, rapamycin and the rapalogs lower inhibition against mTORC2, resulting in compensatory Akt activation by mTORC2. We herein demonstrate that the mTORC1 inhibitors induce cell-cycle arrest but not apoptosis in ATL cells. Everolimus inhibited the growth of ATL-cell xenografts at first, but after the dosing was completed, the tumor cells might be recruited into the cell cycle again, resulting in progressive growth similar to the control group. We suggest that this cytostatic but not cytotoxic effect might be attributable to activation of Akt.

In contrast, PP242 and AZD8055 are ATP-competitive inhibitors of mTOR kinase that suppress both mTORC1 and mTORC2 activity. Akt Ser-473 is essential for full activation of Akt and is a direct substrate of mTORC2. The mTORC1 and mTORC2 dual inhibitors inhibit the phosphorylation of Akt Ser-473 by blocking mTORC2 activity.[21,48] We demonstrate that PP242 and AZD8055 inhibit ATL-cell proliferation more effectively than does rapamycin and everolimus; they also clearly inhibit phosphorylation of Akt Ser-473 and induce apoptosis, resulting in poor tumor growth and longer survival in a xenograft mouse model.

The PI3K/Akt/mTOR pathway in T cells is primarily activated by IL-2, and HTLV-1 Tax oncoprotein makes this process IL-2-independent, resulting in constitutive activation of Akt in HTLV-1-infected cells, leading to their immortalization.[5,49,50] Thus, inhibition of Akt induces apoptosis in HTLV-1-infected cells.[6,51] Akt and S6K were not phosphorylated in the ATL-43T cells, whereas Akt was highly activated in the ED-40515(−) cells, although neither of these cell lines expresses Tax. This discrepancy may be explained by the fact that many genomic and epigenetic alterations occur during ATL development by replacing Tax expression,[12,52] with the PI3K/Akt/mTOR pathway activated differently in each cell line. For example, as a result of the epigenetic silencing of N-myc downstream-regulated gene 2 (NDRG2), we find high phosphorylation of the PTEN protein rather than genetic alterations in the PTEN gene, resulting in activation of both the PI3K/Akt and NF-kB pathways.[53,54] We also see that human telomerase reverse transcriptase is activated through the JAK/STAT and the JAK/PI3K/Akt/HSP90/mTORC1 pathways in IL-2-responsive ATL cells.[55] As mentioned above, HBZ plays a crucial role in the activation of the mTOR pathway. Whatever mechanisms activate the PI3K/Akt/mTOR pathway, our data clearly show that, despite Tax expression, the mTORC1 and mTORC2 dual inhibitors

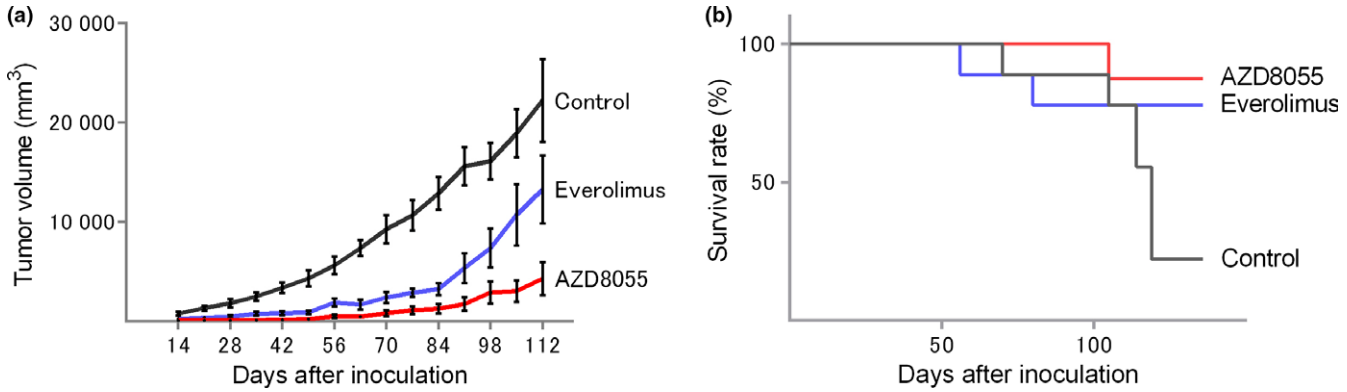

FIGURE 6 AZD8055 effectively inhibits the growth of adult T-cell leukemia (ATL)-cell xenografts in NOD/SCID mice. A, Tumor sizes in xenografted NOD/SCID mice treated with vehicle or everolimus or AZD8055 (control group, n = 9; everolimus group, n = 9; AZD8055 group, n = 8). Mean sizes of individual tumors were plotted; error bars represent SEM. Significant differences between control and everolimus, and between control and AZD8055 appeared after day 14 ($P < .05$). *P*-values were calculated by a 1-way ANOVA test and Tukey-Kramer correction. B, Kaplan-Meier survival curves for everolimus- or AZD8055-treated and control mice. Log-rank testing showed a significant difference between the control and AZD8055 ($P < .017$ after Bonferroni correction)

are effective on ATL cells and the phosphorylation of Akt Ser-473 is crucial for ATL-cell proliferation.

The PI3K and Akt inhibitors also effectively inhibit phosphorylation of Akt Ser-473, so combined therapy with these inhibitors might be more effective than the mTOR inhibitors alone, but might also result in an increase of toxic side-effects because of the broadening of downstream impacts. We show herein that PP242 is less toxic to normal PBMC in the short term. PP242 and AZD8055 target both mTORC1 and mTORC2 without affecting PI3K activity and, theoretically, by inhibiting only their limited targets, supposedly lead to a reduction in toxicity. Moreover, AZD8055 has been tested on lymphoma patients and its safety was confirmed in a phase I study.[56] Recently, the use of the dual PI3K/mTOR inhibitor to treat T-cell acute lymphoblastic leukemia gave promising results,[57] and a phase I study found that combined therapy with Akt inhibitors was safe and feasible for recurrent pediatric solid tumors.[58] However, as ATL often develops in the elderly, and it is not certain how well these therapies are tolerated by seniors, further studies are needed to establish the value of the mTOR inhibitors as single agent or combined therapies for ATL.

In conclusion, the PI3K/Akt/mTOR pathway is crucial for ATL-cell survival and proliferation. Dual mTORC1 and mTORC2 inhibitors show a potent cytotoxic effect on ATL cells and are promising agents for the treatment of ATL.

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CONFLICT OF INTEREST

Authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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