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Enhanced efficacy of JAK1 inhibitor with mTORC1/C2 targeting in smoldering/chronic adult T cell leukemia

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ABSTRACT

Adult T-cell leukemia (ATL) is an aggressive T-cell lymphoproliferative malignancy of regulatory T lymphocytes (Tregs), caused by human T-cell lymphotropic virus 1 (HTLV-1). Interleukin 2 receptor alpha (IL-2Rα) is expressed in the leukemic cells of smoldering/chronic ATL patients, leading to constitutive activation of the JAK/STAT pathway and spontaneous proliferation. The PI3K/AKT/mTOR pathway also plays a critical role in ATL cell survival and proliferation. We previously performed a high-throughput screen that demonstrated additive/synergistic activity of Ruxolitinib, a JAK1/2 inhibitor, with AZD8055, an mTORC1/C2 inhibitor. However, effects of unintended JAK2 inhibition with Ruxolitinib limits it therapeutic potential for ATL patients, which lead us to evaluate a JAK1-specific inhibitor. Here, we demonstrated that Upadacitinib, a JAK-1 inhibitor, inhibited the proliferation of cytokine-dependent ATL cell lines and the expression of p-STAT5. Combinations of Upadacitinib with either AZD8055 or Sapanisertib, mTORC1/C2 inhibitors, showed anti-proliferative effects against cytokine-dependent ATL cell lines and synergistic effect with reducing tumor growth in NSG mice bearing IL-2 transgenic tumors. Importantly, the combination of these two agents inhibited *ex vivo* spontaneous proliferation of ATL cells from patients with smoldering/chronic ATL. Combined targeting of JAK/STAT and PI3K/AKT/mTOR pathways represents a promising therapeutic intervention for patients with smoldering/chronic ATL.

Introduction

Adult T-cell leukemia (ATL) is a malignancy of regulatory T cells (Tregs), associated with human T-lymphotropic virus type 1 (HTLV-1) infection that is curable only with an allogeneic stem cell transplantation [1]. ATL is classified into four clinical subtypes: acute, lymphoma, smoldering, and chronic subtypes [2]. Although various molecular pathophysiological characteristics of ATL have been explored, the efficacy of current treatments is still limited, particularly in relapsed and refractory disease [3]. The HTLV-1-encoded transactivating protein Tax, interacts with numerous cellular factors that promote survival and immortalization of HTLV-1-infected T-cells [4]. Our group demonstrated that Tax transactivates two autocrine pathways of IL-2/IL-2Rα, IL-15/IL-15Rα and one paracrine pathway of IL-9 [5–7]. This activation led to the phosphorylation of JAK1, JAK3, and STAT5 with the subsequent entry of phosphorylated STAT5 into the nucleus. Abnormal activation of the JAK/STAT pathway is also prevalent in other types of T-cell malignancies [8]. Independently, JAK activation or STAT mutations are not adequate to initiate leukemic cell proliferation, but rather required the complete cascade of JAK activation and STAT phosphorylation. Therapeutic strategies targeting this pathway using small molecules have been conducted in numerous clinical trials [9,10].

Our group has previously demonstrated the additivity/synergy of drug combination between the JAK1/2 inhibitor, Ruxolitinib (Jakafi, ICNB018424), and BCL-XL inhibitor, Navitoclax in ATL models [11]. This combination was shown to inhibit the proliferation and tumor growth in a cytokine dependent ATL model and led to initiation of a Phase I/II clinical trial of Ruxolitinib for the treatment of ATL (NCT01712659). Although Ruxolitinib was previously approved by the FDA for the treatment of patients with myelofibrosis [9], long term ad-

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Abbreviations: ATL, Adult T-cell leukemia; Tregs, Regulatory T lymphocytes; HTLV-1, Human T-lymphotropic virus type 1; JAK, Janus kinase; mTOR, Mammalian target of rapamycin.

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ministration resulted in anemia and thrombocytopenia, likely due to its JAK2-inhibitory effects [12]. Thus, in this study, we focused on examining a specific JAK1 inhibitor to circumvent the off-target effect.

Upadacitinib, also known as ABT-494, is a potent and selective JAK1 inhibitor with a specificity of 60-fold and >100 fold over JAK2 and JAK3 respectively [13]. Upadacitinib was demonstrated to suppress paw swelling and bone destruction in a rat adjuvant-induced arthritis model indicating its efficacy *in vivo*. Moreover, Upadacitinib received FDA approval for the treatment of moderate to severe rheumatoid arthritis after demonstrating significant radiographic and symptomatic improvement in these patients [14]. However, we expected that monotherapy with a JAK inhibitor would be insufficient therapy for smoldering/chronic ATL patients. Thus, we have identified AZD8055, a mammalian target of rapamycin (mTOR) inhibitor from our high-throughput screening analysis as having profound additivity/synergy with Ruxolitinib. [11]. The therapeutic efficacy of Upadacitinib combined with mTOR inhibitors remain to be explored, particularly in smoldering and chronic ATL patients.

AZD8055 is a first-in-class agent of a second generation of mTOR inhibitor that was designed to target both mTORC1 and mTORC2 and act as an ATP-competitive inhibitor. mTOR is a conserved serine/threonine kinase that regulates many major cellular processes such as survival, proliferation and metabolism [15]. Constitutive activation of the mTOR pathway has been found in multiple types of cancer and two of the first generation mTOR inhibitors have been approved by FDA, temsirolimus for the treatment of advanced-stage renal cell carcinoma [16] and Everolimus for tuberous sclerosis [17]. These two agents are rapamycin analogs and only inhibit mTORC1, whereas mTORC2 is known to directly activate AKT on Ser473 that regulates cell survival, apoptosis [18]. Thus, treatment with these rapamycin analogs have limited clinical efficacy and are associated with drug resistance [19,20].

In contrast to the rapamycin analogs, AZD8055 inhibits the phosphorylation of both mTORC1 and mTORC2 substrates; p70S6K and 4E-BP1 and p-AKT, respectively [21]. The safety profile, including pharmacokinetics and pharmacodynamics of AZD8055, has been evaluated in advanced solid tumors and lymphomas and demonstrated acceptable toxicity [22]. Another dual ATP-competitive mTORC1/C2 inhibitor, Sapanisertib, also exhibited therapeutic effects in phase I trials in multiple myeloma, non-Hodgkin's lymphoma, and Waldenström's macroglobulinemia patients [23].

In the current study, we demonstrated that Upadacitinib suppressed cell survival and proliferation of cytokine-dependent ATL cell lines and dual inhibition of mTORC1/C2 with AZD8055 or Sapanisertib was more effective than Everolimus's mTORC1 inhibition alone. The combination of Upadacitinib with either AZD8055 or Sapanisertib showed synergistic effects on cytokine-dependent ATL cell lines, ATL cells from smoldering/chronic ATL patients, and in two xenograft models with NSG mice bearing tumors. These results indicate that the combination of JAK1i and mTORC1/2i has a significant promise as multi-agent therapy for patients with smoldering/chronic ATL.

Materials and methods

Reagents and cell lines

Upadacitinib (ABT494), AZD8055 and Sapanisertib were purchased from Medchem Express (Monmouth Junction, NJ). Human IL-2-dependent ATL cell lines; ED40515(+), ED41214(+), ATL55T(+) were kindly provided by Dr. Michiyuki Maeda (Kyoto University), KOB, LM-Y1, KK1 were provided by Dr. Yasuaki Yamada (Nagasaki University). Cells were maintained in RPMI 1640 medium plus 10% FBS with 100U/mL recombinant human IL-2. Human IL-2- independent ATL cell lines; ED40515(-), ED41214(-) and ATL43Tβ(-) were obtained from (Dr. Michiyuki Maeda, Kyoto University), ST1 (Dr. Tomoko Hata, Nagasaki University), Su9T01 (Dr. Naomichi Arima, Kagoshima University), ATN1 (Dr. Tomoki Naoe, Nagoya University) and MT1 (Dr. Miyoshi, Okayama University) and ALK(−) Anaplastic large cell lymphoma (ALCL) cell lines; FEPD, Mac-1, Mac2A, Mac2B, TLBR1, TLBR2 were maintained in RPMI complete medium without cytokine. Human foreskin fibroblast cells were purchased from ATCC (Gaithersburg, MD).

Cell proliferation assay

Twenty thousand ATL cells were seeded in 96-well plates and cultured for 72 h either in RPMI 1640 medium plus 10% FBS with 100U/mL recombinant human IL-2 alone or in serial dilutions of drug combinations. On day 3, the cells were pulsed with 1 μCi (0.037 MBq) of ^{3}H-thymidine during the last 6 h of culture. The cells were then harvested with a Tomtec cell harvester (Hamden, CT) and counted with a MicroBeta2 microplate counter (PerkinElmer, Shelton, CT). The assay was performed in three independent experiments.

Intracellular staining of p-STAT5

Human IL-2-dependent ATL cell lines were cultured with 1 μM of Upadacitinib for 1, 4 and 24 h. Control groups were incubated with DMSO. The cells were harvested at different time points and fixed with BD Phosflow^{TM} Lyse/Fix Buffer at 37°C for 10 min. After washing, the cells were permeabilized with cold BD Phosflow^{TM} Perm Buffer III on ice for 30 min followed by staining with Anti-Stat5 (pY694), Clone 47/Stat5 (pY694) for 1 h in staining buffer at room temperature. Cells were collected on FACS Calibur analysis (BD Biosciences, San Jose, CA, USA). and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

Annexin V staining

Cell apoptosis assay was performed according to the manufacturer's instruction using an Annexin V apoptosis detection kit (BD Biosciences, San Jose, CA) and detected by flow cytometry. Cells were cultured with 1 μM of Upadacitinib or DMSO for 48 h and then labeled with Annexin V followed by FACS Calibur analysis. For patient's peripheral blood mononuclear cells (PBMCs), after 6-day *ex vivo* culture without cytokine, the enriched tumor cells were further cultured with either DMSO, Upadacitnib, AZD8055 or in combinations for an additional of 48 h and cells were subjected for Annexin V staining.

Cell cycle analysis

ATL cell lines were cultured with 1 μM of AZD8055 or Everolimus or DMSO for 24 h. The cells were then labeled with 10 μM BrdU for 45 min. The BrdU-pulsed cells were stained according to the BrdU Flow Kit staining protocol (BD Biosciences, San Jose, CA, USA) and analyzed using a FACS Calibur flow cytometry.

Mouse model of ED40515(+)/IL-2 and therapeutic study

The ED40515(+)/IL-2 cell line was generated as previously described [11]. The xenograft tumor model of human IL-2–dependent ATL was established by subcutaneous injection of 1×10^7 ED40515 (+)/IL-2 cells into the right flank of female NOD.Cg-Prkdc^{scid}IL2rg^{tm1Wjl}/SzJ (NSG) mice (The Jackson Laboratory, Bar Harbor, ME). Treatment was started ten days after tumor inoculation when the average tumor volume reached approximately 100 mm^3. Upadacitinib was dissolved in 30% PEG300 (Sigma-Aldrich, St. Louis, MO) at a dose of 6 mg/kg per day by osmotic pump insertion or via the oral route daily for two weeks. AZD8055 (15 mg/kg/day) or Sapanisertib (1 mg/kg/day) were dissolved in 30% PEG300 and given orally for five times/week. Combination treatment was administered at the same dose and dosing schedules. Control mice receiving 30% PEG300 dissolved in water were used as a vehicle group. Tumor growth was monitored by measuring tumor size in two orthogonal dimensions with tumor volume calculated using the formula $\frac{1}{2}$ (long dimension) x (short dimension)^2. The level of human sIL-2Rα in serum of treated mice was measured using enzyme-linked immunosorbent assays (ELISA) (R&D Systems, Minneapolis, MN). All animal experiments were approved by the National Cancer Institute Animal Care and Use Committee (NCI ACUC) and were performed in accordance with NCI ACUC guidelines.

Ex vivo *cultures of PBMCs from ATL patients*

Peripheral blood samples were obtained from patient volunteers with chronic and smoldering ATLs under the care of the Clinical Trials Team, Lymphoid Malignancies Branch, NCI. This study protocol was approved by the Institutional Review Board of the NCI. Informed consent was obtained in writing from patients. The proliferation assay of *ex vivo* 6-day culture was performed as described previously [11]. Prior to *ex vivo* culture, we screened for the smoldering/chronic ATL patients by measuring $CD4^+CD25^+$ in patient's blood using flow cytometry. PBMCs were isolated from patient blood by ficoll density gradient centrifugation and then cultured *ex vivo* in RPMI 1640 medium containing 10% FBS without cytokines either with DMSO or increasing doses of Upadacitinib, AZD8055 or in combination for 6 days. No stimuli were added to the culture to let the IL-2Rα^+ leukemic cells activate, expand and enrich. The leukemic cells were pulsed during the last 6 h of incubation with 1 μCi of ^{3}H-thymidine, and then harvested and counted with a Micro Beta2 microplate counter.

Western blot analysis

Whole-cell lysates were collected using lysis buffer supplemented with the protease inhibitor cocktail according to the manufacturer's protocol, MCL-1 from Sigma-Aldrich (St. Louis, MO). Cell lysates were electrophoresed on 4-12% Bis-Tris Novex gel and blotted onto polyvinylidene difluoride membranes from Invitrogen (Carlsbad, CA). Proteins were detected by immunoblotting after blocking. Antibodies were from Cell Signaling Technology Inc. (Danvers, MA); p-STAT5 (#9359), STAT5 (#94205), p-STAT3 (#9145), p-AKT-Ser473 (#4060), AKT (#9272), p-4E-BP1 (#2855), GAPDH (#5174). Monoclonal anti-β-actin antibody (AC-74) was purchased from Sigma-Aldrich. Signal intensity was quantified with ImageJ software.

Statistical analysis

For comparison between control, single agent and combination groups, one-way ANOVA was used to determine statistical significances. The two-way ANOVA was performed in the cell cycle analysis assay (GraphPad Prism software, version 7). For patient data, Mann-Whitney test was performed to determine statistical differences between groups. $p\text{-value}<0.05$ was considered statistically significant.

Results

JAK1 inhibition with Upadacitinib inhibited proliferation and phosphorylation of STAT5 in cytokine-dependent but not cytokine-independent ATL cell lines

We previously demonstrated that the JAK1/2 inhibitor, Ruxolitinib diminished cell growth and proliferation of cytokine-dependent ATL but has limited potential as a therapeutic strategy [11]. To address the dose-limiting effects of JAK2 inhibition with Ruxolitinib, we evaluated a JAK1 inhibitor (JAK1i) with its better pharmacodynamics and specificity as an alternative to Ruxolitinib. Upadacitinib was highly effective as an inhibitor that inhibited the survival and proliferation of IL-2-dependent ATL cell lines; ED40515(+), ED41214 (+), ATL55T (+), KK1, KOB and LMY1. Limited or no inhibition was observed in the seven cytokine-independent ATL cell lines; ED40515 (-), ED41214 (-), ATL43Tβ (-), ATN1, MT1, ST1 and Su9T01, which further demonstrating its specificity for JAK1 (**Fig. 1A**). Furthermore, p-STAT5 expression was reduced at 24 h in the IL-2-dependent cell lines after JAK1 blockade (**Fig. 1B**). These results are consistent with our previous study [11] demonstrated that six IL-2-independent ATL cell lines did not express p-STAT5, therefore, JAK1i had no effect on the cytokine-independent ATL cell proliferation.

Ruxolitinib has a short half-life of approximately 3 h, which requires at least twice daily (b.i.d.) administration [24] whereas with Upadacitinib's half-life of 6-16 h would probably allow for less frequent dosing [10]. Thus, we sought to determine the pharmacodynamics of Upadacitinib in inhibiting p-STAT5 in IL-2-dependent cell lines. Upadacitinib exhibited longer pharmacodynamic effects with a robust inhibition of p-STAT5 at 1 h that was maintained for up to 4 h. The p-STAT5 expression increased at 24 h but had not reached the baseline (**Fig. 1C**). Furthermore, we evaluated the effect of Upadacitinib on cell apoptosis with Annexin V staining. Upadacitnib induced apoptosis in cytokine dependent ATL cell lines, ED40515(+) and LMY1 cell lines after 48 h of treatment, whereas fibroblasts did not respond to the inhibitor (**Fig. 1D**).

Dual inhibition of mTORC1/C2 with AZD8055 was more effective than the single mTORC1 inhibitor Everolimus, in cytokine-dependent ATL cell lines

From our high-throughput screening results, AZD8055, an mTORC1/C2 inhibitor, demonstrated an additive/synergistic effect with Ruxolitinib suggesting that the PI3K/AKT/mTOR pathway was essential for ATL cell survival. We confirmed the screening results by performing a cell proliferation assay with AZD8055. In parallel, we also evaluated another FDA-approved mTORC1 inhibitor, Everolimus. As seen in the dose response curves, AZD8055 had an antiproliferative effect against 13 ATL cell lines regardless of cytokine-dependency. In contrast, Everolimus had little effect on ATL cell line proliferation (**Fig. 2A**). The IC50 of IL-2-dependent and IL-2-independent cell lines treated with AZD8055 or Everolimus were determined and summarized on **Supplementary Table S1**. Similar results were previously described in ATL cell lines with ED40515 (-), Hut102 (-), MT2 (-) and ATL43T(+) by Kawata and colleagues [25] who showed that dual inhibition of mTORC1 and mTORC2 was more effective than single blockade of mTORC1 as mTORC2 has the ability to directly activate AKT that in turn augments cell survival. We also examined phosphorylated AKT at Ser-473, a direct substrate of mTORC2, in the six IL-2 dependent ATL cell lines and found that only AZD8055 but not Everolimus inhibited AKT phosphorylation (**Fig. 2B**) suggesting that dual targeting of mTORC1/C2 is required to suppress ATL cell proliferation. We further examined the effects of AZD8055 and Everolimus on the cell cycle analysis. We demonstrated that AZD8055 markedly induced cell-cycle arrest at the G0/G1 phase in ED40515(+) ($p<0.0001$), ATL55T (+) ($p<0.0001$) and KK1 ($p<0.0395$) cell lines compared to the Everolimus-treated or DMSO groups ($p<0.0001$). Moreover, the cells entering S phase were significantly decreased in the AZD8055-treated group compared to the DMSO control ($p<0.0001$) group or the Everolimus-treated group in ED40515(+) ($p<0.0001$), ATL55T(+) ($p<0.0001$), and KK1 ($p<0.0418$) cell lines (**Fig. 2C**).

The combination of Upadacitinib and AZD8055 showed synergistic effect with cytokine–dependent ATL cell lines

As a result of additive/synergistic effects between Ruxolitinib and AZD8055, we hypothesized that Upadacitinib with AZD8055 would display the same effect. We initially examined these combinations in primary T cells stimulated with anti-CD3 and anti-CD28 in which the activation of JAK/STAT and PI3K/Akt/mTOR pathways are known to be pervasive. The combinations of these two inhibitors, Upadacitinib (15.6 nM to 250 nM) and AZD8055 (31.2 nM to 500 nM) were synergistic in inhibiting T-cell proliferation with increasing doses of this drug combination evaluated in four different PBMCs populations isolated from healthy donors (**Supplementary Fig. 1**). Consistently, Upadacitinib and AZD8055 showed synergistic effect with the six cytokine–dependent

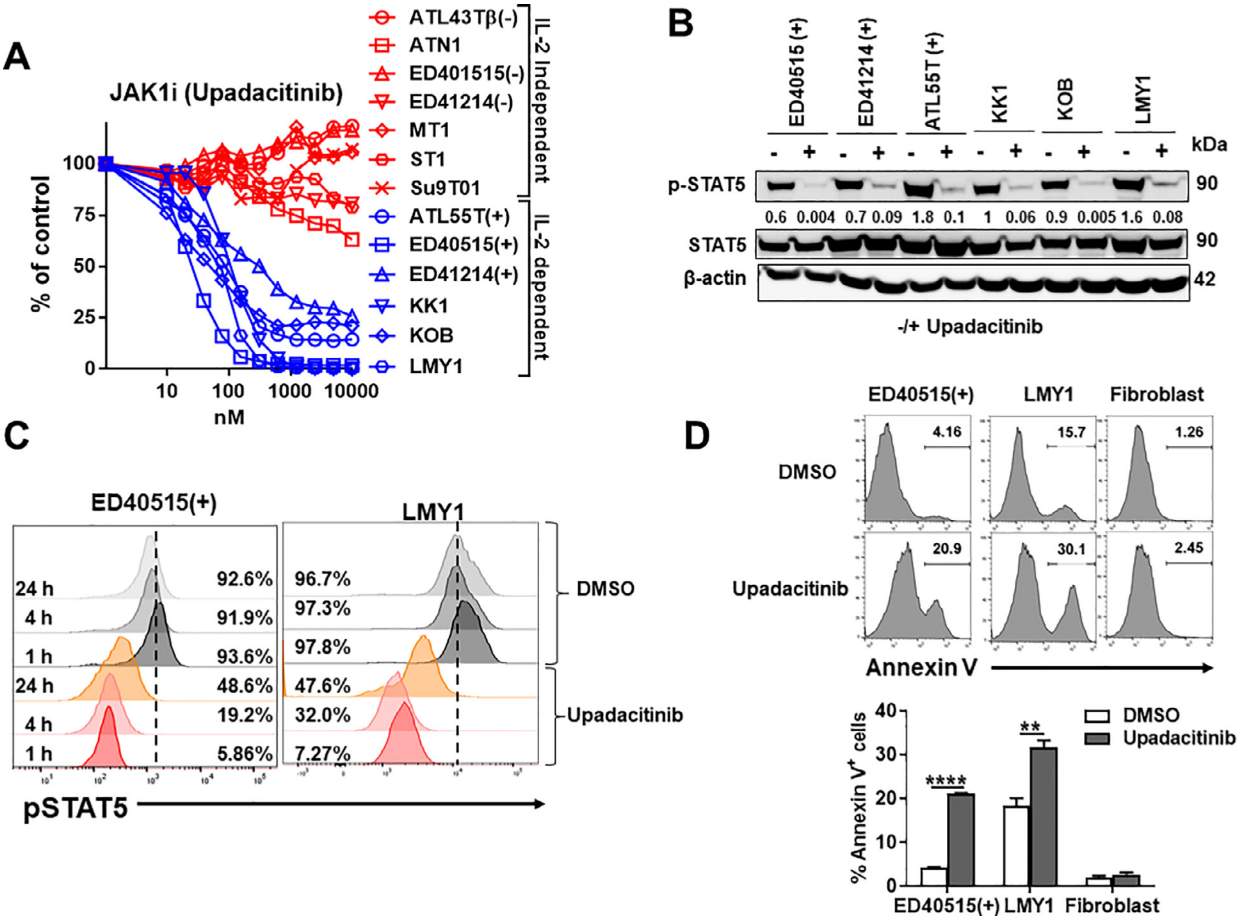

Fig. 1. JAK1 inhibitor, Upadactinib inhibited proliferation and phosphorylation of STAT5 in cytokine dependent but not cytokine independent ATL cell lines.

(A) Dose response curves of Upadacitinib on human IL-2 dependent (blue) and IL-2–independent (red) ATL cell lines. Six IL-2–dependent and seven IL-2–independent ATL cell lines were treated with increasing doses of Upadacitinib for 72 h and the cell proliferation was measured with a thymidine incorporation assay. The figures are representative of three independent experiments. (B) Western blot analysis of p-STAT5 and STAT5 protein at 24 h after treatment with 1 μM of Upadacitinib. The figure images are representative of two biological replicates. (C) The time course of p-STAT5 inhibition by Upadacitinib. ED40515(+) and LMY1 were incubated with 1 μM Upadacitinib for 1 h, 4h or 24 h and analyzed for p-STAT5 expression by flow cytometry. Cells incubated with DMSO were used as a control. The figures are representative of three independent experiments. (D) Induction of cell apoptosis by Upadacitinib. ED40515(+) and LMY1 were treated with Upadacitinib or DMSO for 48 h and the cell apoptosis was measured by Annexin-V staining (n=3). Human fibroblast cells were used as a control. Student *t*-test was performed to determine statistical differences, **p <0.01, ****p<0.0001. The figures are representative of three independent experiments.

ATL cell lines (Fig. 3) while the same treatment had no effect on fibroblast proliferation, demonstrating the specificity of these effects on ATL cell lines. Because cytokine–independent ATL cell lines did not respond to the JAK1 inhibitor, the synergistic effect of these two inhibitors was not observed in these cell lines **(Supplementary Fig. 3)**. To measure synergistic effect between Upadacitinib and AZD8055, we used Chou-Talalay method [26] to quantify whether the interaction is additive or synergistic. The combination index (CI) values were generated when two drugs were combined at a constant ratio. CI values less than 1 indicates synergism. The synergistic effect of this drug combination was observed in six IL-2 dependent ATL cell lines with CI values less than 1 over five different concentrations **(Supplementary Table 2).** CI plots of synergism represents each individual combination data point in **Supplementary Fig. 2**.

Therapeutic effects of the Upadacitinib and AZD8055 combination in suppressing tumor growth in xenograft mice bearing ED40515(+)/IL-2 transgenic tumors and ALK (-) ALCL tumors

To evaluate the efficacy of this combination *in vivo*, we used the ED40515(+) cell line that expresses the human IL-2 transgene with a retroviral system [11]. The ED40515(+)/IL-2 transgenic tumor has the ability to grow in immunodeficient NOD/SCID/$\gamma^{-/-}$ (NSG) mice without an external supply of human IL-2 cytokine. Upadacitinib was administered either by continuous infusion pump **(Fig. 4A)** or orally **(Supplementary Fig. 4A)**. The results of treatment showed that Upadacitinib suppressed the tumor growth via infusion pump better than by the oral route. Single agents of Upadacitinib or AZD8055 delayed the tumor growth in tumor-bearing mice compared to vehicle control mice. The combination therapy with Upadacitinib and AZD8055 dramatically reduced tumor volumes compared with single agents alone, whereas Upadacitinib combined with Everolimus showed little effect **(Supplementary Fig. 4B)**. Furthermore, we determined the level of soluble IL-2Rα, which is a surrogate tumor marker, in the serum of treated mice after two weeks of treatment. Consistent with the decreased tumor volume, the level of sIL-2Rα was meaningfully reduced in the combined-treatment group with Upadacitinib and AZD8055 compared to single agent-treated mice **(Fig. 4B)**.

We further examined another xenograft mouse model that involves an ALK (-) ALCL tumor wherein the JAK/STAT pathway was also reported to be activated in ALK(−) ALCL patients [27]. Similar results were also observed when the TLBR1 cell line was implanted. Treatment with

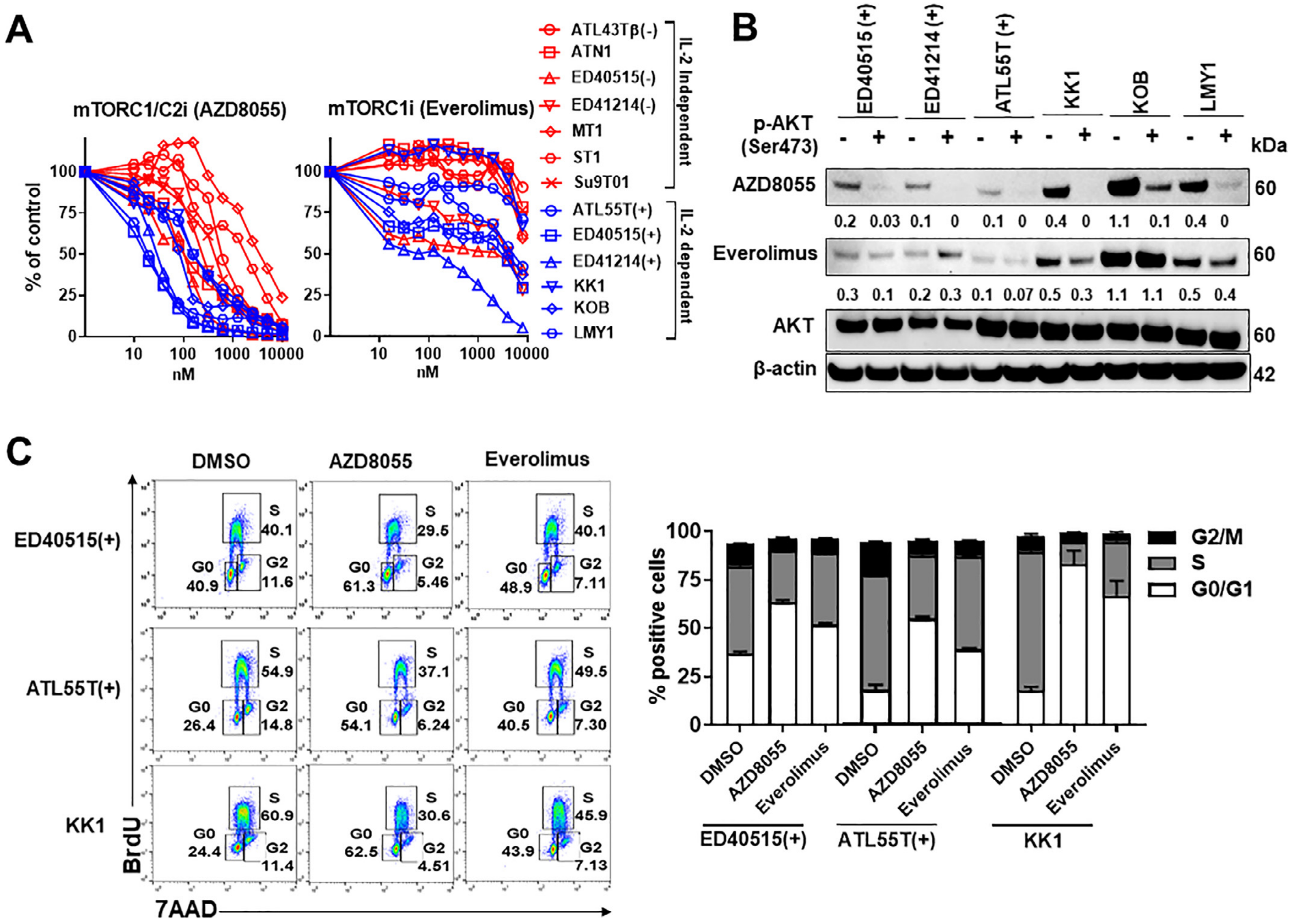

Fig. 2. Dual inhibition of mTORC1/C2 with AZD8055 was more effective than the single mTORC1 inhibitor, Everolimus, with cytokine-dependent ATL cell lines.

(A) Dose response curves of AZD8055 versus Everolimus inhibition on human ATL cell lines. Six IL-2–dependent and seven IL-2–independent ATL cell lines were treated with increasing doses of AZD8055 or Everolimus inhibitors for 72 h and the cell proliferation was measured with a thymidine incorporation assay. The figures are representative of three independent experiments. (B) Western blot analysis of p-AKT and total AKT protein after treatment with 1 μM of AZD8055 or Everolimus for 24 h. The figures are representative of two biological replicates. (C) Effect of AZD8055 versus Everolimus on cell cycle analysis. ED40515(+), ATL55T (+) and KK1 were treated with inhibitors or DMSO for 24 h and labeled with BrdU for 45 min. The percentages of G0/G1, S, or G2/M phases were evaluated with BrdU and 7AAD staining by flow cytometry. Two-way ANOVA was performed to determine statistical differences (n=4). Representative flow plots of three independent experiments are shown.

Upadacitinib or AZD8055 alone moderately diminished tumor growth. Notably, the combination of Upadacitinib and AZD8055 significantly suppressed tumor growth as evidenced by decreased cell proliferation and tumor volume in the combined-treated group **(**Fig. 4**C-D)**. These *in vivo* results correlated with the decreased cell proliferation, p-STAT3 and p-AKT expressions in ALCL cell lines **(Supplementary Fig. 5A-B)**.

Antiproliferative effect of the Upadacitinib and AZD8055 combination on the 6-day ex vivo *spontaneous proliferation of ATL cells isolated from patients with smoldering/chronic ATL*

Our established 6-day *ex vivo* culture of PBMCs containing ATLs from patients with smoldering/chronic ATL demonstrated the activation of JAK/STAT signaling associated with spontaneous leukemic cell proliferation. We previously demonstrated that the leukemic cells of smoldering/chronic ATL patients constitutively expressed IL-2R*α* whereas resting normal T cells do not express this marker [28]. This autonomous proliferation of leukemic cells in smoldering/chronic ATL patients occurs in an IL-2/IL-2R*α* dependent manner but it also requires autologous monocytes to fully induce proliferation via cell-cell contact [7]. In contrast, PBMCs from acute ATL patients do not proliferate or proliferated independent of cytokines. Therefore, autocrine stimulation by IL-2/IL-2R*α* interaction is not observed in acute ATL patients.

Prior to *ex vivo* culture, we screened for smoldering/chronic ATL patients by measuring $CD4^+CD25^+$ in patient's blood by flow cytometry. No stimuli were added throughout 6-day culture to let the IL-$2R\alpha^+$ leukemic cells activate, expand and enrich. Robust inhibition of ATL cells in *ex vivo* culture proliferation was demonstrated in five ATL patients when Upadacitinib and AZD8055 were added to the cultures. Cell proliferation was inhibited more than 95% when the combination of 250 nM Upadacitinib and 500 nM AZD8055 were used **(**Fig. 5**A)**. The CI values indicates synergistic effect of this drug combination are shown on **Supplementary Table 3**. We further examined the molecular pathways present on the leukemic cells when the number of ATL cells were enriched after 6 days. By culturing the ex vivo cultured ATL cells with the drug combination, Upadacitinib potently inhibited p-STAT5 and AZD8055 suppressed the expression of p-AKT and p-4EBP1 which is in line with the results of the cell proliferation assay **(**Fig. 5**B)**. Induction of cell apoptosis was also enhanced in the combination-treated group **(**Fig. 5**C)**.

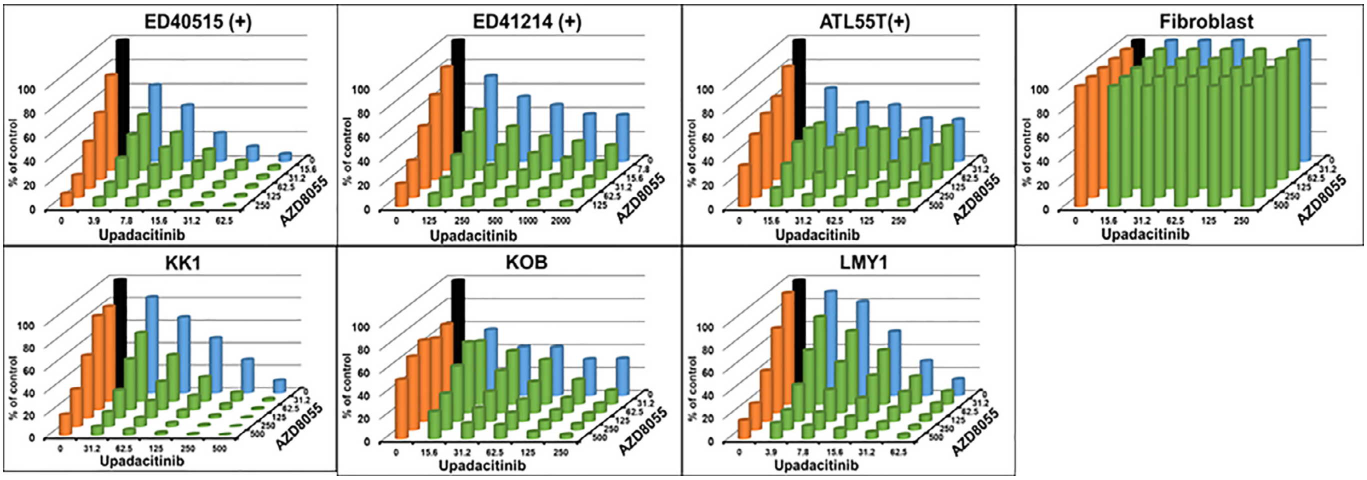

Fig. 3. The combination of Upadacitinib and AZD8055 showed synergy with cytokine–dependent ATL cell lines.
The combined effects of Upadacitinib with AZD8055 inhibitors on six IL-2–dependent ATL cell lines incubated for 72 h in the presence of five increasing concentrations of Upadacitinib (blue), AZD8055 (orange), or their combinations (green). Fibroblast cells were used as a control. A thymidine incorporation assay was performed, and percentages of control were calculated from the counts per minutes (cpm) of samples with drugs divided by the cpm of samples with no inhibitor x 100. Representative figures of three independent experiments are shown.

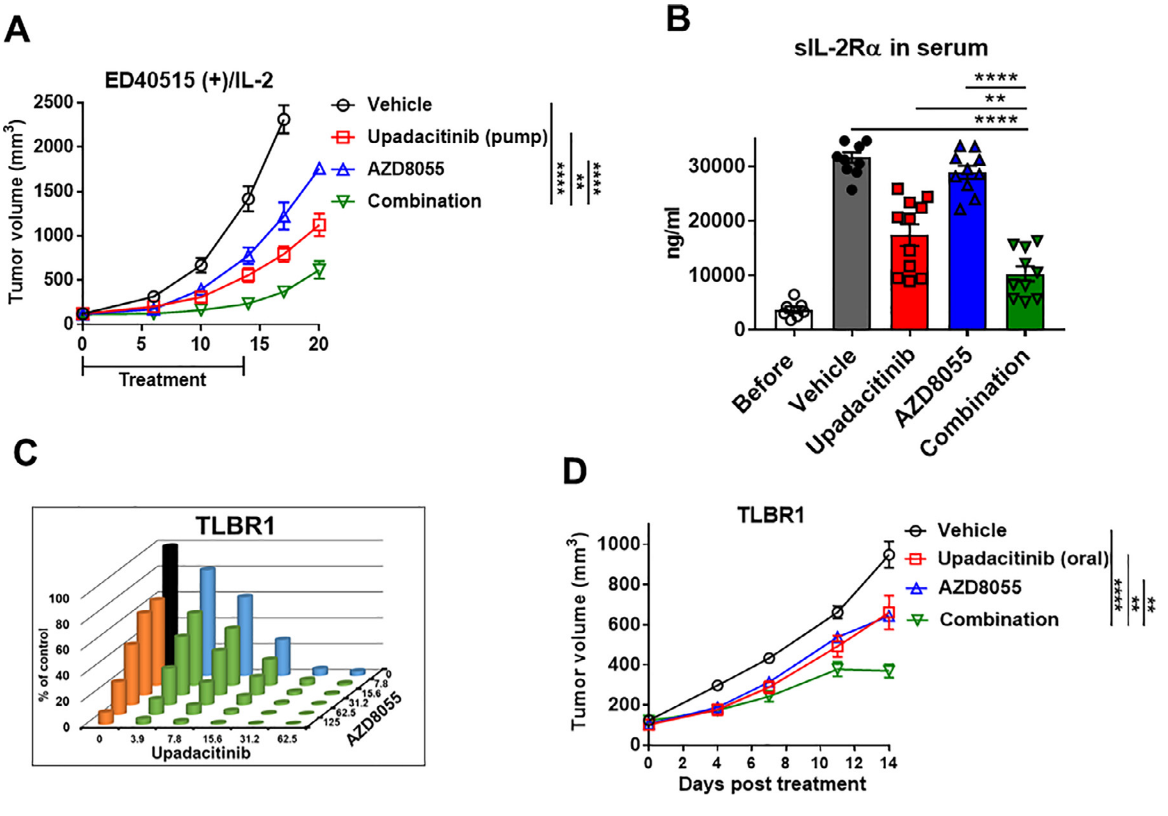

Fig. 4. Therapeutic effects of the Upadacitinib and AZD8055 combination in suppressing tumor growth in xenograft mice bearing ED40515(+)/IL-2 transgenic tumors and ALK (-) ALCL tumor.
Ten million ED40515(+)/IL-2 or 5×10^6 TLBR1 cell lines were subcutaneously implanted in NSG mice. The therapy was started when average tumor volumes reached around 100 mm^3. Upadacitinib was administered by pump at a dose of 6 mg/kg/day and AZD8055 was given orally at a dose of 15 mg/kg for 14 days. The vehicle group received 30% PEG300 dissolved in water. (A) Average tumor volumes during the therapeutic time course were measured twice weekly until the tumor volume reached 2000 mm^3 (n=10-12). (B) Serum levels of human soluble IL-2Rα (sIL-2Rα) were measured on day 14 after treatment with ELISA assay. Two independent experiments were performed, and data were pooled together. (C) The combined effects of Upadacitinb with AZD8055 inhibitors on the TLBR1 cell line incubated for 72 h in the presence of five different drug concentrations and cell proliferation was measured with a thymidine incorporation assay. Representative figures of three independent experiments are shown. (D) Average tumor volumes were measured during the therapeutic time course of drug combination on the TLBR1 model (n=5-6). One-way ANOVA was performed to determine statistical differences $^{**}p < 0.01$, $^{****}p<0.0001$.

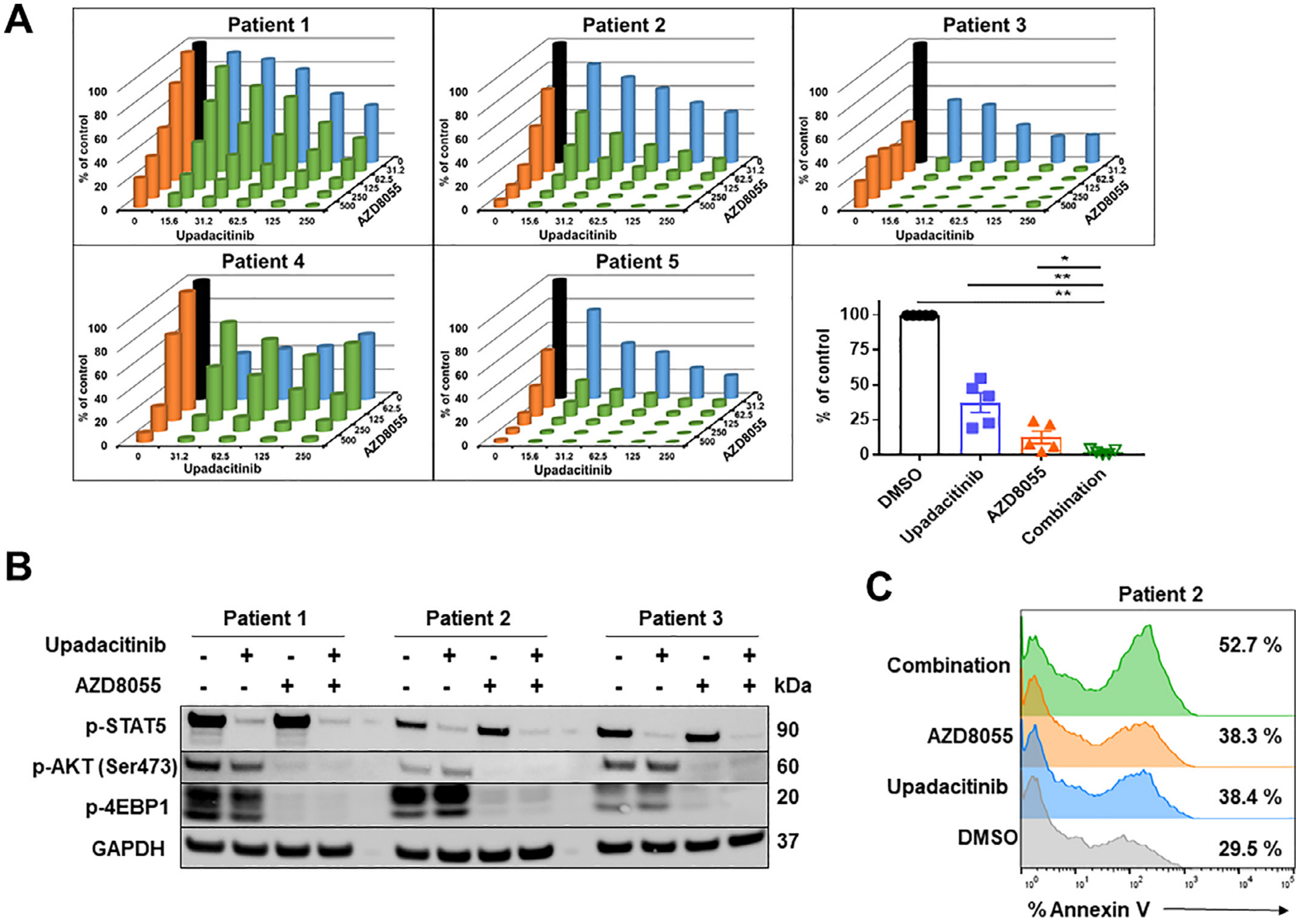

Fig. 5. Antiproliferative effect of the Upadacitinib and AZD8055 combination on the 6-day *ex vivo* spontaneous proliferation of ATL cells in PBMCs isolated from patients with smoldering/chronic ATL.

(A) Anti-proliferative effects of Upadacitinib and AZD8055 combination on ATL cells in PBMCs from five smoldering/chronic ATL patients cultured *ex vivo* for 6 days in the presence of increasing concentrations of Upadacitinib (blue), AZD8055 (orange), or their combinations (green). Average percentages of control are shown from five patients at the concentrations of 250 nM Upadacitinib or 500 nM AZD8055 or in combinations (n=5). Five biological replicates were performed. Error bars represent ± SEM. Mann-Whitney test was performed to determine statistical differences $^*p < 0.05$, $^{**}p < 0.01$. (B) Western blot analysis of p-STAT5, p-AKT and p-4EBP1 after 6-day *ex vivo* culture treated with 1 μM Upadacitinib or AZD8055 or combination for 1 h. Three biological replicates were performed. (C) Annexin V staining, after induction of cell apoptosis by drug combination on ATL cells in patient's PBMC after 6-day *ex vivo* culture.

The combination of Upadacitinib and Sapanisertib showed synergistic effect with cytokine-dependent ATL cell lines, PBMCs containing ATL cells from ATL patients and in a xenograft mouse model

AZD8055 displayed anti-proliferative effects against ATL cell lines, however, the clinical development of this inhibitor has not been advanced meaningfully due to treatment related abnormal liver functions [22]. Therefore, we examined another mTORC1/C2 inhibitor with similar efficacy as AZD8055. Sapanisertib is an oral ATP-dependent mTORC1/C2 inhibitor that showed potent antitumor activity by suppressing in a metastatic prostate cancer model [29]. Moreover, Sapanisertib is currently being evaluated in clinical trials for various types of cancer [23,30]. In examining the dose response curves of Sapanisertib in IL-2 dependent ATL cell lines, Sapanisertib demonstrated anti-proliferative effects similar to those by AZD8055 (**Fig. 6A**). However, the AZD8055 showed higher potency in cytokine-independent ATL cell lines as IC50 of AZD8055 is slightly lower than Sapanisertib (Supplementary Table S1). Fibroblasts were used as a negative control to confirm drug specificity. The level of cell inhibition also correlated with the decreased expression of p-AKT at Ser-473 (**Fig. 6B**). Furthermore, the combination of Upadacitinib with Sapanisertib demonstrated synergistic effect in six cytokine–dependent ATL cell lines **(Supplementary Fig. 6 and Supplementary Table 4)** and in ATL cells from PBMCs isolated from two patients with smoldering/chronic ATL (**Fig. 6C and Supplementary Table 5)**. Importantly, similar therapeutic efficacy *in vivo* of this drug combination was also observed in xenograft mice bearing ED40515(+)/IL-2 transgenic tumors (**Fig. 6D)** comparable to as that observed in the combination with AZD8055.

Discussion

Previously, we demonstrated that the JAK/STAT pathway is critical for smoldering/chronic ATL cell survival and JAK1/2 inhibition with Ruxolitinib blocked STAT5 phosphorylation and reduced proliferation of IL-2–dependent ATL cell lines [11]. However, JAK2 is involved in the regulation of the platelet and red blood cell production as thrombopoietin and erythropoietin receptors utilize JAK2 for signaling, thus, the effects of JAK2 inhibition by Ruxolitinib led to adverse events of anemia and thrombocytopenia [12]. In the present study, we sought to replace Ruxolitinib with a specific JAK1 inhibitor to address this concern while enhancing specificity. Our current work demonstrates that Upadacitinib is highly specific for JAK1 and its efficacy could be enhanced

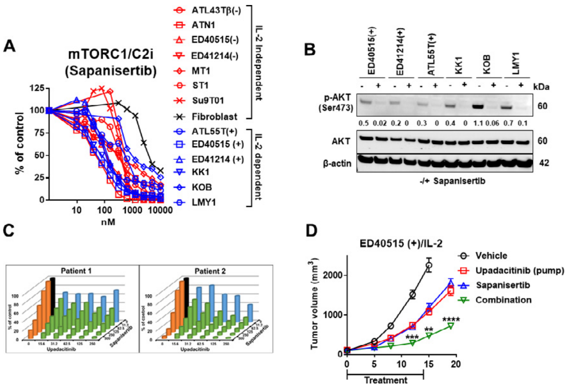

Fig. 6. The combination of Upadacitinib and Sapanisertib showed synergistic effect with cytokine–dependent ATL cell lines, PBMCs containing ATL cells from patients with smoldering/chronic ATL and in a xenograft mouse model.
(A) Dose response curves of mTORC1/C2i, Sapanisertib, on human ATL cell lines. Representative figures of three independent experiments are shown. (B) Western blot analysis of p-AKT after incubation with 1 μM Sapanisertib for 1 h. Two biological replicates were performed. (C) PBMCs from smoldering/chronic ATL patients were cultured *ex vivo* for 6 days in the presence of increasing concentrations of Upadacitinib (blue), Sapanisertib (orange), or their combinations (green) and the cell proliferation was measured with a thymidine incorporation assay. Two biological replicates were performed. (D) Average tumor volumes during the therapeutic time course of Upadacitinib administered by pump at a dose of 6 mg/kg/day and Sapanisertib given orally at 1 mg/kg for 14 days (n=10-12). One-way ANOVA was performed to determine statistical differences **p <0.01, ***p <0.001, ****p<0.0001. Data were pooled from two independent experiments.

by combining with mTOCR1/C2 inhibitors in smoldering/chronic ATL model.

The Tax protein of HTLV-1 transactivates IL-2Rα on smoldering/chronic ATL cells leads to the activation of JAK/STAT pathway and spontaneous cell proliferation. However, with acute ATL or some transformed cells, they do not rely on JAK/STAT for survival and growth. Thus, this combination therapy targeting JAK1 and mTOR pathways could only be applied with smoldering/chronic ATL and not on acute ATL patients [28]. It has been suggested that ATL cells progressing away from cytokine dependence continue to accumulate transforming mutations and epigenetic changes [31] [32]. Deciphering the potential differences between cytokine dependent and independent ATL cells could prove valuable at the level of therapeutics.

The utilization of JAK1 inhibitor may have broader application against other types of T cell malignancies possessing an activated JAK/STAT pathway such as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [33] or ALK (-) ALCL patients [34] as the autocrine IL-2/IL-2Rα loop mediates spontaneous proliferation, is also present in these patients. For ALK (-) ALCL patients, the 5-year overall survival rate was poorer than ALK (+) with 49% and 70% respectively [35]. ALK (-) ALCL patients were generally responsive to standard first-line treatment of chemotherapy regimens but tumor relapse often occurred [36,37]. Our results indicate that the combination of JAK1i and mTORC1/C2i inhibition treatment has significant potential in a disease driven by JAK/STAT activation including ALK (-) ALCL cells (Fig. 4C-D), which rely on IL-6 signaling through JAK1/JAK2/STAT3 pathway [34].

Preclinical evaluation of several FDA-approved mTOR inhibitors, rapamycin analogs, demonstrated limited efficacy suggesting that mTORC1 targeting alone is insufficient [19,20]. Kawata and colleagues [25] demonstrated superior efficacy of the dual inhibitors, PP242 and AZD8055, compared to mTORC1 inhibitors, rapamycin and Everolimus with ATL cell lines which have the benefit of blocking the AKT feedback activation loop. In our study with cytokine-dependent cell lines, we noted similar effects with AZD8055 that also inhibited p-AKT (S-473) and was more efficient in inducing cell cycle arrest and in suppressing the tumor growth than Everolimus. These results suggest that dual inhibition of mTOR is required for effective treatment of ATL patients.

Nevertheless, patients treated in a phase I clinical trial of AZD8055 showed hepatic dysfunction with elevated levels of transaminases that precluded further clinical development [22]. Such liver function abnormalities were not observed with other mTORC1 inhibitors such as Temsorilmus and Everolimus. Therefore, we investigated another dual mTOR inhibitor, Sapanisertib. In the first clinical trial of Sapanisertib in hematologic malignancies, it was well tolerated, and patients exhibited partial responses in relapsed or refractory multiple myeloma, non-Hodgkin lymphoma, or Waldenström macroglobulinemia. This study also suggested that Sapanisertib could be used in combination with other agents as it showed limited efficacy as a single agent [23].

Taken as a whole, our preclinical studies suggest the importance of the JAK/STAT and PI3K/AKT/mTOR signaling pathways in the survival of smoldering or chronic ATL malignant lymphocytes. The combination of Upadacitinib with Sapanisertib demonstrated high potency and synergy and supports a proposed Phase I/II clinical trial with this two-agent combination for smoldering/chronic ATL patients.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2020.100913.

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