Torin2 Potentiates Anticancer Effects on Adult T-Cell Leukemia/Lymphoma by Inhibiting Mammalian Target of Rapamycin

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Abstract. *Background: Torin2 is a second-generation ATP-competitive inhibitor of the mammalian target of rapamycin (mTOR). Dysregulation of mTOR signaling pathway, consisting of mTOR complexes mTORC1 and mTORC2, is a promising therapeutic target in some human malignancies. We examined antitumor effects of Torin2 in adult T-cell leukemia/lymphoma (ATL)-related cell lines compared to those of rapamycin, a classical mTOR inhibitor. Materials and Methods: Cell growth was monitored by detecting viable cells with Cell Counting Kit-8 or trypan blue. Cell cycle was studied by flow cytometric analysis. The phosphorylation status of proteins in the mTOR signaling pathway was examined by western blot analysis. Results: Torin2 exhibited greater efficacy in cell growth inhibition than rapamycin, associated with a strong reduction of phosphorylated v-akt murine thymoma viral oncogene homolog (AKT) (Ser 473), that is downstream of mTORC2. Conclusion: Since mTORC2 activates AKT, Torin2 might inhibit both mTORC1 and mTORC2, resulting in stronger growth inhibition of ATL cells.*

Adult T-cell leukemia/lymphoma (ATL) is an aggressive hematological malignancy derived from cluster of differentiation 4 (CD4)$^+$ T-cells and is induced by persistent infection with human T-cell lymphotropic virus-1 (HTLV-1) (1-3). After virus infection by breast feeding, sexual transmission, or blood transfusion, it takes several decades for HTLV-1-infected cells to reach the final stage of multi-stage oncogenesis, that is clinically recognized as aggressive ATL (3, 4). ATL cells tend to have intrinsic chemoresistance and the median survival time is limited to only 13 months with vincristine, cyclophosphamide, doxorubicin and prednisone–doxorubicin, ranimustine and prednisone–vindesine, etoposide, carboplatin and prednisone treatment protocol (5), resulting in a poor prognosis. Recent analyses have revealed molecular mechanisms related to development and maintenance of ATL, and new targeting therapies with various molecules such as Tax (one of the major virulence factors of HTLV-1) (6), nuclear factor-kappa B (NF-κB) (7), topoisomerase IIα (8), DNA-dependent protein kinase (8), CD25 (9), and CC chemokine receptor 4 (CCR4) (10) have been developed under clinical or preclinical studies using small molecules or monoclonal antibodies.

Mammalian target of rapamycin (mTOR), a serine/threonine protein kinase that belongs to the phosphoinositide kinase-related family of protein kinases, is a key downstream molecule of the phosphatidylinositol 3-kinase (PI3K)/v-akt murine thymoma viral oncogene homolog (AKT) signaling pathway, that regulates cell growth and survival in a number of human malignancies (11, 12). The AKT/mTOR signaling pathway is activated by interleukin-2 (IL2), regulates survival of T-cells (13, 14) and is frequently dysregulated in leukemia and lymphoma (11, 15). It also contributes to transformation of mouse T-cells to IL2-independent growth triggered by Tax (16). Therefore, here we focused on the AKT/mTOR signaling pathway as a possible target for ATL treatment.

mTOR is the catalytic subunit of two functionally and structurally distinct signaling complexes: mTORC1 and mTORC2. Rapamycin, a classical mTOR inhibitor, inhibits mTORC1 by binding with 12-kDa FK506-binding protein (FKBP12) (12). Because mTORC1, stimulated by growth factors or cytokines, phosphorylates p70 ribosomal S6 kinase (p70 S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), which regulate protein synthesis and cell growth, rapamycin and its derivatives

have drawn attention as antitumor agents and are being evaluated in several clinical trials for human malignancies (12). In pre-clinical studies of ATL, rapamycin inhibited growth of ATL cell lines (17), and everolimus, a rapamycin derivative, induced cell-cycle arrest, apoptosis, and senescence (18). However, the antitumor, mTORC1-inhibiting activity of rapamycin can weaken with prolonged usage (19, 20) because of reactivation of its upstream molecules, such as PI3K and AKT. This re-activation seems to limit the efficacy of rapamycin and its derivatives in cancer treatment (12).

Torin2 ((9-(6-amino-3-pyridinyl)-1-[3-(trifluoromethyl) phenyl]-benzo[*h*]-1,6-naphthyridin-2(*1*)-one)) is a small molecule with molecular weight 432.4 that acts as an mTOR inhibitor in an ATP-competitive manner (21, 22). It has been reported to bind to the ATP pocket of mTOR protein, as observed using PI3Kγ crystal structure. Oral administration of Torin2 to mice reduced phosphorylation of S6K (Thr 389) and AKT (Thr 308) by over 95% in the lung and liver [half-maximal effective concentration (EC_{50})=0.3 nM]. In addition, it had a more than 800-fold selectivity for mTOR over PI3K (EC_{50}=200 nM) (21, 22). Antitumor activity of Torin2 in a single treatment was recently been reported for several cancer cell lines such as of those lung, colorectal, breast, cervical and thyroid cancer (22-24). In the present article, we report our investigations on the effect of Torin2 on ATL-related cell lines compared to rapamycin.

Materials and Methods

Cell culture and reagents. Torin2 was purchased from Calbiochem (La Jolla, CA, USA); rapamycin and etoposide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against mTOR, pmTOR (Ser 2448), pmTOR (Ser 2481), p70 S6K (Thr 389), AKT, pAKT (Ser 473), and p(4E-BP1) (Thr 37/Thr 46) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibody against actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Six different ATL-related T-cell lines (five ATL-derived and one HTLV-1-infected) were used: MT-1, ATN-1, ST-1, KOB, and KK-1 were derived from patients with ATL and MT-2 was established by co-cultivation of human cord blood lymphocytes from a healthy individual with peripheral blood mononuclear cells from a patient with ATL. MT-1, MT-2, and ST-1 cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS). ATN-1 cells were maintained in RPMI-1640 medium containing 10% FBS and 1% nonessential amino acids (Gibco, CA, USA). KOB and KK-1 cells are IL2 dependent and were cultured in RPMI-1640 medium containing 10% FBS supplemented with 10 ng/ml recombinant human IL2 (Takeda Chemical Industries, Osaka, Japan) as described previously (8).

Analysis of cell growth and viability. Cell growth was measured using a Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technology, Kumamoto, Japan) according to the manufacturer's instructions 72 h after treatment of with six different ATL-related T-cell lines with torin2, rapamycin or etoposide. Since MT-1 cell

line is one of the standard ATL cell lines used in many other groups and we found it is sensitive to both of Torin2 and rapamycin, we used this cell line in other experiments. Cell viability was determined by counting the number of MT-1 cells stained with trypan blue as described previously (8).

Analysis of cell apoptosis and cell cycle. MT-1 cells were stained with fluorescein isothiocyanate (FITC)-conjugated annexin V 72 h after treatment with Torin2, rapamycin or etoposide, and FITC fluorescence was measured by flow cytometry. Cell-cycle analysis of MT-1 cells was performed using flow cytometry. Cells treated with Torin2, rapamycin or etoposide for 24 h were fixed with 70% ethanol. After permeabilization with TritonX-100, cells were stained with propidium iodide (PI) and analyzed by flow cytometry.

Western blot analysis. MT-1 cells were treated with rapamycin or Torin2 for 24 h. Each cell lysate was resolved in 10% Nu-polyacrylamide gel electrophoresis. Phosphorylation of proteins in the mTOR signaling pathway was determined by western blotting, as described previously (8). Signal intensity of pAKT (Ser 473) as the result of western blotting was measured by the Image-J software and then divided by the intensity of AKT. The proportion of pAKT in non-treated cells was defined to be 100%. Results are the means of three independent experiments.

Statistical analysis. Statistical analysis was conducted using a two-independent sample Student's *t*-test. *p*-Values lower than 0.05 were considered significant.

Results

Torin2 exhibited more efficacy than rapamycin in cell growth inhibition. In the six ATL-related T-cell lines cultured in the presence of Torin2, rapamycin, or etoposide for 72 h, rapamycin had a strong growth-inhibitory effect compared to Torin2 and etoposide, currently a key drug for ATL treatment, at less than 10 nM for each cell line, as measured by the CCK-8 assay (Figure 1A). The 50% inhibitory concentration (IC_{50}) values for rapamycin ranged from 0.15 to 4.4 nM in four out of the five ATL-related cell lines, except for KK-1 cells (Table I). KK-1 cells showed resistance to rapamycin and etoposide as each IC_{50} value exceeded 100 nM, but Torin2 significantly inhibited their growth, with an IC_{50} value of 31.1 nM (Table I). Although the IC_{50} value for Torin2 was greater than that for rapamycin, 2.4- to 24.7-fold in other cell lines (Table I), it is important to note that the growth-inhibitory effect of Torin2 exceeded that of rapamycin and maintained a dependence on concentration above 20 nM (Figure 1A). The number of viable cells after treatment with each compound showed the same tendency as with the CCK-8 assay; rapamycin only reduced viability of MT-1 cells to around 80% 72 h after treatment at 20 nM but Torin2 and etoposide significantly reduced viability at 200 nM compared with rapamycin (Figure 1B).

Induction of G_1/S phase cell-cycle arrest and apoptosis by Torin2. Treatment of MT-1 cells with etoposide strongly induced apoptosis as assessed by staining with FITC-
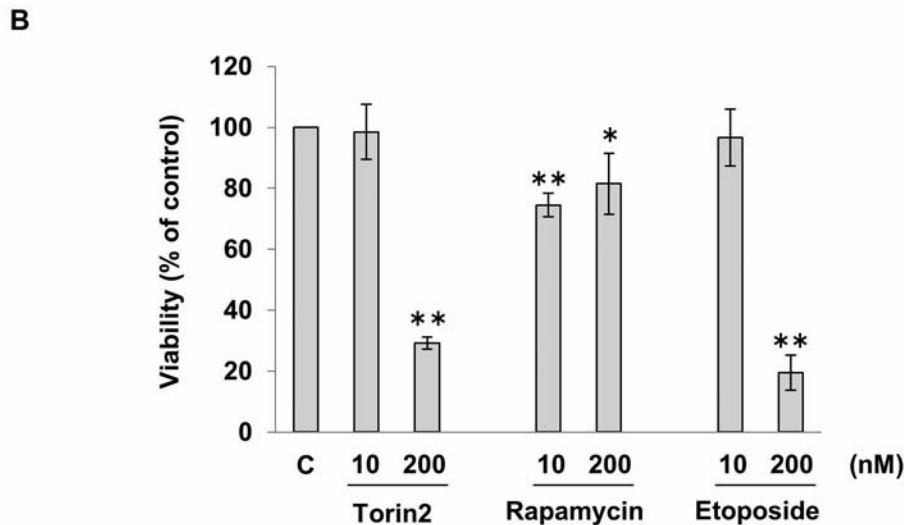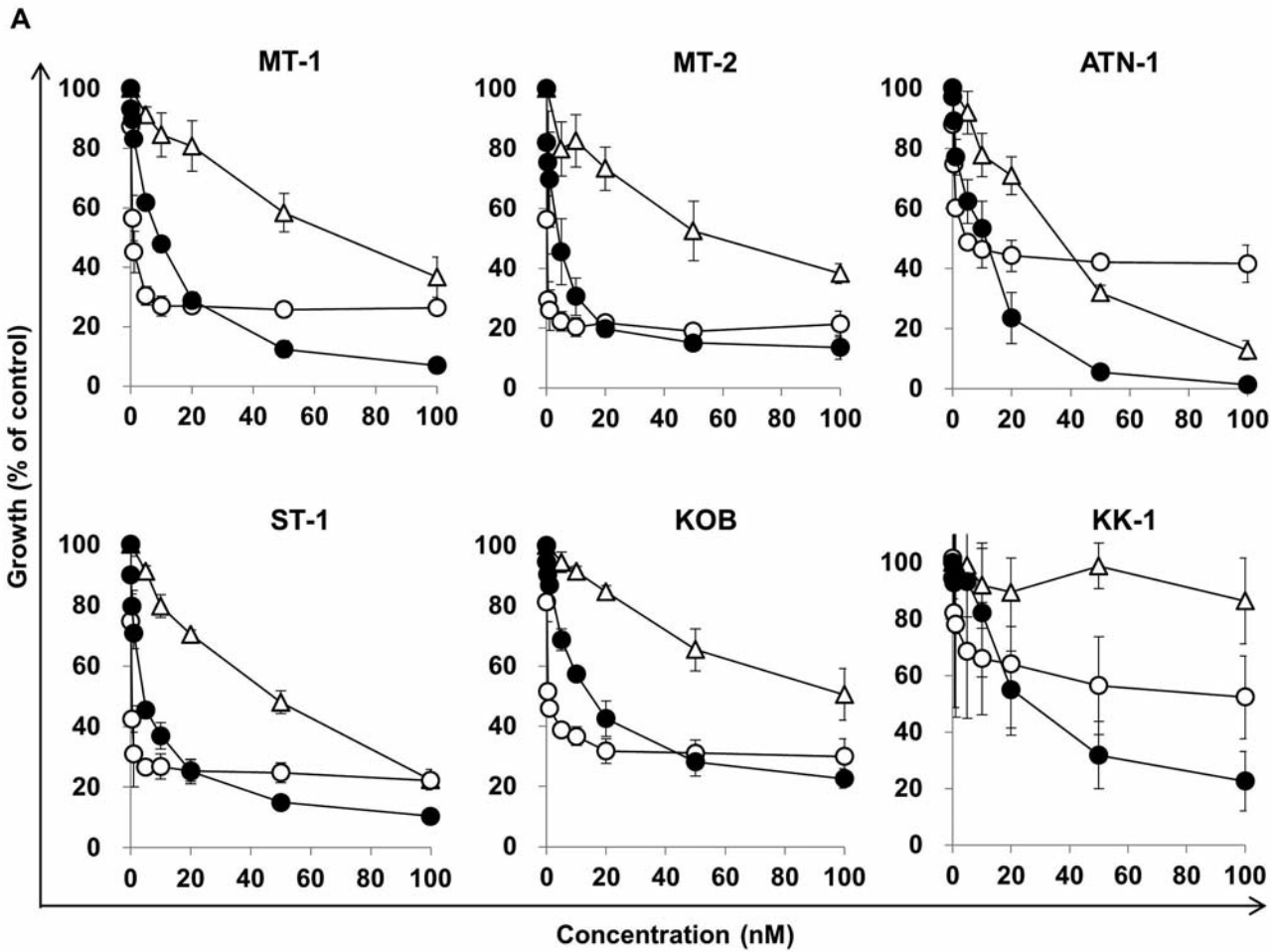
Figure 1. *Growth inhibition of adult T-cell leukemia/lymphoma (ATL)-related cell lines induced by Torin2, rapamycin, and etoposide. A: ATL-related cell lines were treated with Torin2 (●), rapamycin (○), or etoposide (△) for 72 h. Cell growth was determined with CCK-8 kit, as described in the Materials and Methods. The absorbance of non-treated cells was defined as 100%. The results are the means of three independent experiments. B: The viability of MT-1 cells treated with each compound for 72 h was measured by the trypan blue staining, as described in the Materials and Methods. The number of variable cells in controls was defined as 100%. The results are the means of three independent experiments.*

Table I. *The 50% inhibitory concentration (IC$_{50}$) values for Torin2, rapamycin, and etoposide against adult T-cell leukemia/lymphoma-related cell lines as determined by CCK-8 assay 72 h after treatment.*

Cell line	Mean IC$_{50}$ determined by CCK-8 assay (nM)±SD		
	Torin2	Rapamycin	Etoposide
MT-1	8.7±2.0	0.71±0.17	66±13
MT-2	3.7±2.5	0.15±0.09	55±19
ATN-1	10.4±2.1	4.4±1.1	33±3
ST-1	3.2±0.8	0.35±0.05	51±9
KOB	14.6±2.6	0.63±0.11	>100
KK-1	31.1±14.4	>100	>100

conjugated annexin V, whereas Torin2 had a marginal effect with a trend when compared to control (Figure 2A). However, rapamycin did not induce apoptosis (Figure 2A). When the cell cycle of MT-1 cells treated with Torin2, rapamycin, or etoposide was analyzed, the number of cells in sub-G$_1$ phase was increased by treatment with Torin2 for 24 h (Figure 2B and C), indicating that it weakly induced apoptosis of the cells as shown in Figure 2A. The number of cells in the G$_0$/G$_1$ phase was increased after treatment with Torin2 or rapamycin, whereas the number in the S phase was reduced by Torin2, but not rapamycin (Figure 2B and C). Etoposide reduced the number of cells in S phase and increased the number in G$_2$/M phase (Figure 2B and C).

Torin2 inhibits both the mTORC1 and mTORC2 signaling pathways. We next determined inhibition by Torin2 and rapamycin of mTOR and its signaling pathway comprising of mTORC1 and mTORC2, to understand the differences in growth inhibition and cell-cycle arrest. In MT-1 cells treated for 24 h, rapamycin significantly reduced phosphorylation of mTOR on Ser 2448, which is the specific site associated with the activation of mTORC1, and it is interesting that its concentration dependency was maintained at a concentration of 10 nM, as with growth inhibition (Figure 3A). However, the inhibition of phosphorylation of mTOR on Ser 2448 induced by Torin2 was very low compared to rapamycin, and differences in the level of Ser 2448 phosphorylation of mTOR correlated well with the amount of phosphorylated p70 S6K (Thr 389), that is a downstream target of mTORC1 (Figure 3A). On the other hand, phosphorylation of 4E-BP1 (Thr 37/Thr 46) was strongly reduced by treatment with Torin2 compared to rapamycin, although like p70 S6K, it is a downstream target of mTORC1 (Figure 3A). Phosphorylation of 4E-BP1 (Thr 37/Thr 46) is usually resistant to rapamycin and its derivatives and possibly limits their antitumor effect (12). Thus, down-regulation of phosphorylation of 4E-BP1 (Thr 37/Thr 46) by Torin2 might
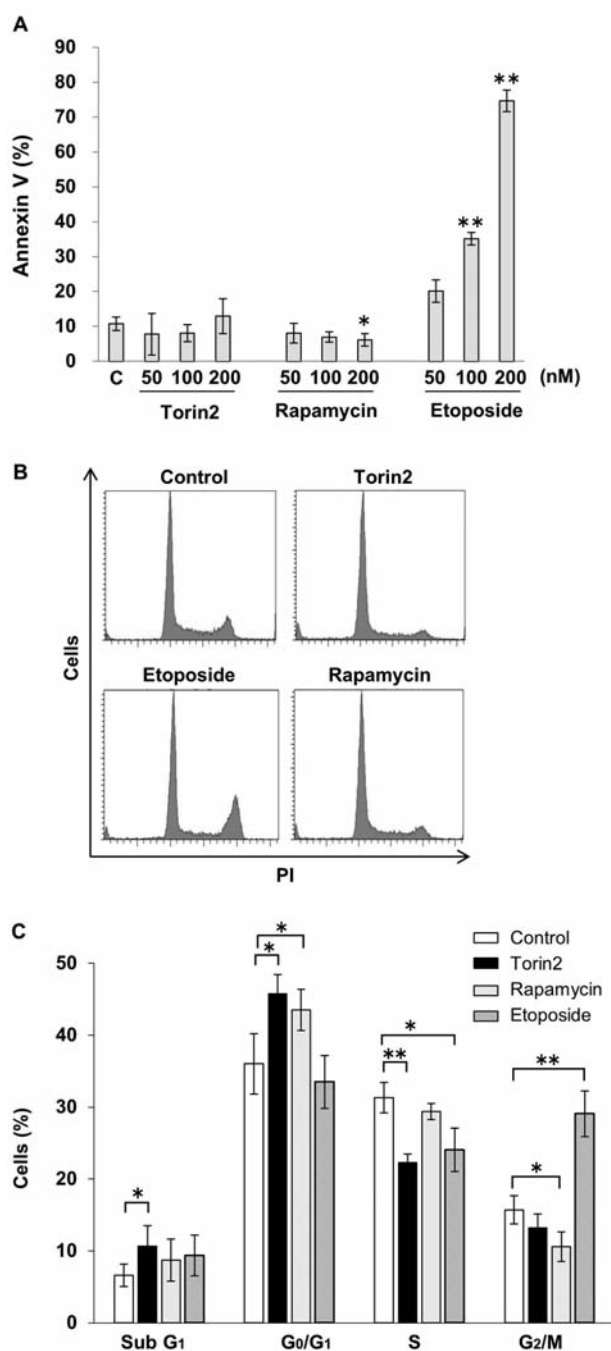
Figure 2. *Induction of apoptosis and cell-cycle arrest by Torin2, rapamycin, and etoposide. A: MT-1 cells were treated with Torin2, rapamycin or etoposide for 72 h. The number of cells stained with fluorescein isothiocyanate (FITC)-annexin V was measured by flow cytometric analysis, as described in the Materials and Methods. The results are the means of three independent experiments with SD. *p<0.05; **p<0.005. B: MT-1 cells were treated with 200 nM of Torin2, rapamycin, or etoposide for 24 h. Cell-cycle distribution was examined by flow cytometry of propidium iodide (PI)-stained cells, as described in the Materials and Methods. C: The percentages in each cell-cycle phase were analyzed by the FlowJo software. The results are the means of three independent experiments with SD. *p<0.05; **p<0.005.*
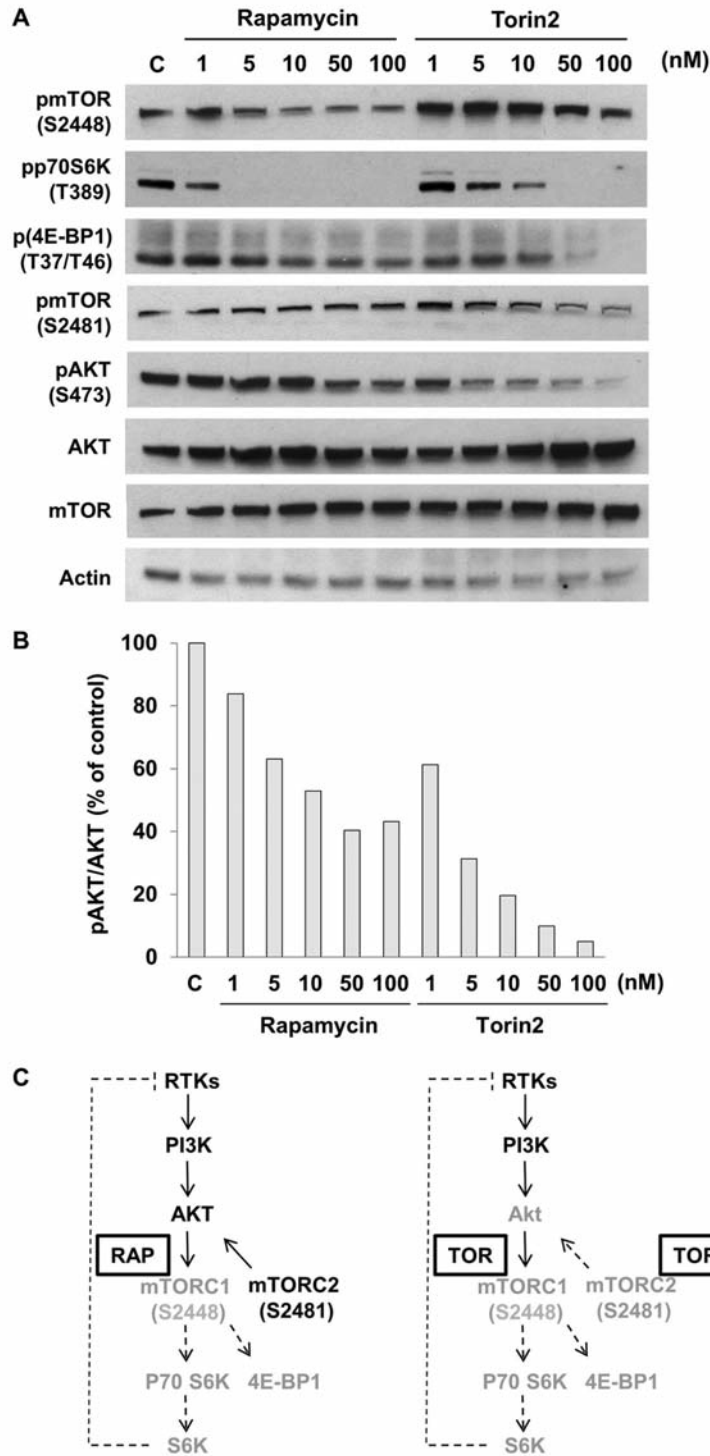
Figure 3. *Down-regulation of phosphorylation of components consisting of the mammalian target of rapamycin (mTOR) signaling pathway induced by Torin2 and rapamycin. A: MT-1 cells were treated with Torin2 or rapamycin for 24 h. Each cell lysate was resolved in 10% Nu-polyacrylamide gel electrophoresis. Phosphorylation of proteins in the mTOR signaling pathway was determined by western blotting. B: Signal intensities of phosphorylated v-akt murine thymoma viral oncogene homolog (Ser 473, pAKT) in the result of western blotting were measured by the Image-J software and then divided by the intensity of AKT. The proportion of pAKT in non-treated cells was defined as 100%. The results are the means of three independent experiments. C: The results from the western blot analysis suggest that Torin2 inhibits both mTORC1 and mTORC2 complexes, whereas rapamycin inhibits only mTORC1 and induces negative feedback re-activation of AKT. PI: Propidium iodide, p70S6K: p70 ribosomal S6 kinase, 4E-BP1: 4E-binding protein 1, RTK: receptor tyrosine kinase, PI3K: phosphatidylinositol 3-kinase, RAP: rapamycin, TOR: Torin2, S6K: ribosomal protein S6 kinase.*

be related to its higher antitumor activity. On the other hand, phosphorylation of mTOR on Ser 2481, a specific site indicating the activation of mTORC2, was dose-dependently down-regulated by treatment with Torin2, but not with rapamycin (Figure 3A). Although the function of mTORC2 in cancer cells has not been explained in detail as much as mTORC1, it activates AKT phosphorylation in concert with phosphoinositide-dependent kinase-1 (11). We therefore determined the phosphorylation status of AKT on Ser 473 using image-J software (Figure 3A and B). Although the down-regulation of AKT phosphorylation on Ser 473 induced by rapamycin lost its concentration dependency above 10 nM, Torin2 reduced AKT phosphorylation to a greater extent than rapamycin in a concentration-dependent manner (Figure 3A and B). Rapamycin and its derivatives specifically down-regulate mTORC1 and cancel the negative feedback loop consisting of S6K, which phosphorylates insulin receptor substrate 1 and 2 and uncouples PI3K from receptor tyrosine kinases, resulting in activation of the PI3K/AKT signaling pathway (11, 12) (Figure 3C). On the other hand, Torin2 has been reported to weakly inhibit PI3K *in vitro* (EC_{50}: 200 nM), although it remains unclear whether this inhibitory effect contributes to prevention of re-activation of AKT (22). Therefore, Torin2 can down-regulate the mTOR signaling pathway without AKT re-activation in MT-1 cells. We believe this is why Torin2 exhibited a stronger dose-dependent growth-inhibitory effect than that of rapamycin up to 100 nM. The previous results that the combination of rapamycin with LY294002 increases antitumor effects associated with reduction of AKT phosphorylation in ATL-related cell lines (25) might be caused by the same mechanisms.

Discussion

In the present study, we first showed that Torin2, an ATP-competitive mTOR inhibitor, inhibits cell growth of ATL-related cell lines in a concentration-dependent manner. On the other hand, although rapamycin, a classical mTORC1 specific inhibitor, had a more potent growth-inhibitory effect compared to Torin2 at low concentration (<5-10 nM), its effect reached a plateau around 5-20 nM in each cell line (Figure 1A and B). The similar loss of dose dependence in growth-inhibitory effect induced by rapamycin and everolimus has been observed in other ATL cell lines (17, 18, 25). Although the mechanism of growth inhibition induced by Torin2 and rapamycin was G_1/S phase cell-cycle arrest, Torin2 induced weak but significant cell apoptosis. Torin2 has been reported to induce G_1/S phase arrest and caspase-dependent apoptosis in an anaplastic thyroid cancer cell line (24) and increase in S-phase cells and apoptosis in HeLa cells (22). Thus, cellular responses to inhibition of the mTOR signaling pathway with Torin2 vary in different types

of cancer cells. However, everolimus induced G_1/S phase arrest but not apoptosis in ATL-related cells 48-72 h after treatment (18) and the combination of rapamycin with LY294002, a PI3K inhibitor, induced G_1/S phase arrest in ATL-related cell lines (25). Thus, in ATL-related cell lines, inhibition of the mTOR signaling pathway with Torin2 or rapamycin induced G_1/S phase arrest, resulting in cell growth inhibition. The effect of Torin2 was stronger than that of rapamycin, in the same way as with growth inhibition induced by these compounds.

The difference of mode of action between rapamycin and Torin2 might originate from different binding targets. There are currently three different types of mTOR inhibitors (12). The classical mTOR inhibitor rapamycin, and its derivatives, bind to FKBP12, resulting in inhibition of the mTORC1 signaling pathway. Since mTOR and PI3K belong to the same kinase family, their catalytic domain has a common structure. Therefore, several dual PI3K/mTOR inhibitors have been developed as second-class inhibitors and are currently in clinical trials as monotherapies against several tumor types. These compounds generally have stronger antitumor effect than rapamycin and its derivatives, associated with inhibition of mTORC1 targets that are resistant to allosteric inhibition, such as phosphorylation of 4E-BP1. The latest class of mTOR inhibitor has been developed as a selective ATP-competitive inhibitor against mTORC1 and mTORC2, and includes Torin2, in order to reduce the side-effects of dual PI3K/mTOR inhibitors. Several compounds belonging to this class have been or are being investigated in clinical trials against several human malignancies, such as CC-223 for glioblastoma, hepatocellular carcinoma and multiple myelomas, and CC-115 for glioblastoma, prostate cancer, Ewing's osteosarcoma and chronic lymphocytic leukemia (12). Since Torin2 is a new compound, there exist no clinical trial reports, but based on our results we think that Torin2 has stronger anti-tumor activity in ATL-related cell lines compared with rapamycin and etoposide.

Based on our these results, we think inhibition of mTOR signaling pathways associated with avoiding AKT reactivation is a promising therapeutic approach for ATL and Torin2 is a potent compound as a monotherapy for inducing this cellular response.

Conflicts of Interest

The Authors declare that there are no conflicts of interest.

Acknowledgements

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