Longitudinal inhibition of PI3K/Akt/mTOR signaling by LY294002 and rapamycin induces growth arrest of adult T-cell leukemia cells.

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The abbreviations are: ATL, adult T-cell leukemia; HTLV-1, human T-cell lymphotropic virus type I; PI3-K, phosphatidylinositol 3-kinase; mTOR, mammalian target of rapamycin; p70S6K, p70 S6 kinase; 4E-BP-1, eukaryotic initiation factor 4E-binding protein-1;

Abstract.

This study found that phosphatidylinositol 3-kinase (PI3K)/Akt/ mammalian target of rapamycin (mTOR) signaling was activated in human T-cell lymphotropic virus type I (HTLV-1)-infected leukemia cells. Rapamycin (1-100 nM, 48 h), the inhibitor of mTOR and its analog RAD001 (1-100 nM, 48 h) induced growth inhibition and G0/G1 cell cycle arrest of these cells in association with de-phosphorylation of p70S6K and 4E-BP-1, although IC50 was not achieved. Paradoxically, rapamycin stimulated phosphorylation of Akt at Ser 473. Blockade of Akt signaling by the PI3K inhibitor LY294002 (1-20 μM, 48 h) also resulted in the growth inhibition and G0/G1 cell cycle arrest of HTLV-1-infected cells, with IC50 ranging from 5-20 μM, and it caused de-phosphorylation of p70S6K and 4E-BP-1. Of note, when rapamycin was combined with LY294002, rapamycin-induced phosphorylation of Akt was blocked, and the ability of rapamycin to induce growth arrest of HTLV-1-infected T-cells and suppress the p-p70S6K and p-4E-BP-1 proteins was potentiated. Moreover, both LY294002 and rapamycin down-regulated the levels of c-Myc and Cyclin D1 proteins in these cells, and their combination further decreased levels of these cell cycle-regulating proteins. Taken together, longitudinal inhibition of PI3K/Akt/mTOR signaling represents a promising treatment strategy for individuals with adult T-cell leukemia.

Introduction.

Adult T-cell leukaemia (ATL) is an aggressive malignancy of $CD4^+$ T lymphocytes, in which the human T-cell lymphotropic virus type I (HTLV-I) has been recognized as the etiologic agent [1,2]. Despite the development of intensive combination chemotherapy regimens supported by granulocyte colony-stimulating factor, median survival time of individuals with ATL is less than 13 months [3,4].

Akt is a serine (Ser)/threonine (Thr) protein kinase which plays an important role in controlling cell growth and apoptosis [5]. Upstream of Akt is phosphatidylinositol 3-kinase (PI3K), which activates Akt in part via activation of 3'-phosphoinositide–dependent kinase-1 [5]. The activated Akt phosphorylates target molecules including mammalian target of rapamycin (mTOR), which modulates cell proliferation in part by regulation of initiation of translation [6,7]. mTOR phosphorylates p70 S6 kinase (p70S6K) and eukaryotic initiation factor 4E-binding protein-1 (4E-BP-1) and increases the translation of mRNAs with long, highly structured 5'-untranslated regions, such as cyclin D1 and c-Myc [6,7]. PTEN phosphatase is a major negative regulator of the PI3K/Akt signal pathway [8,9]. In many types of solid tumors including those from prostate, PTEN is inactivated by several mechanisms such as either homozygous deletions, hemizygous deletion and

mutation on the second allele, or methylation of its promoter region. Loss of expression of this phosphatase results in constitutive activation of Akt signaling [8-11. Additional mechanisms which activate PI3K/Akt/mTOR signaling include gain of function mutation of receptor tyrosine kinase (e.g., c-Kit), mutations of an oncogene (e.g., Ras), active mutations in the p110 and p85 subunits of PI3K, and Akt overexpression [12-14]. Thus, PI3K/Akt/mTOR signaling may represent a promising molecular target of cancers. This study found that PI3K/Akt/mTOR signaling was activated in HTLV-1-infected cells. Inhibition of this pathway by the PI3K inhibitor LY294002 and the mTOR inhibitor rapamycin caused growth inhibition of these cells. Of note, when both compounds were combined, more profound growth inhibition was induced.

Materials and Methods.

Cells. HTLV-I-infected T-cell lines, MT-1, MT-2 and MT-4 were kind gifts of I. Miyoshi (Kochi Medical School, Kochi, Japan). HUT102 cells were generously provided by Y. Maeda (Kinki University School of Medicine, Osaka, Japan). Cells were maintained in RPMI1640 medium (GIBCO Life Technologies, Grand Island, NY) with 10 % heat-inactivated fetal bovine serum (FBS, GIBCO). ATL cells were freshly isolated from individuals with acute type of ATL. Clinical characteristics of these individuals are listed on Table 1. $CD4^+$ T lymphocytes were isolated from healthy volunteers by magnetic cell sorting utilizing CD4 MicroBeads as the manufacturer recommended (Miltenyi Biotec GmbH, Germany).

Reagents. Rapamycin and LY294002 were purchased from Wako (Osaka, Japan) and Calbiochem (San Diego, CA), respectively and were dissolved in 100 % DMSO to a stock concentration of 10^{-2} M and stored at -20°C. Rapamycin analogue RAD001 was provided by Novartis (Basel, Switzerland) and was dissolved in 100 % DMSO to a stock concentration of 10^{-2} M and stored at -20°C.

MTT Assays. HTLV-1-infected cells (5×10^5/ml) were incubated with various concentrations of LY294002 or rapamycin either alone or in combination for 2 days in 96-well plates (Flow Laboratories, Irvine, CA). After culture, cell number and viability

were evaluated by measuring the mitochondrial-dependent conversion of the tetrazolium salt, MTT (Sigma), to a colored formazan product, as previously described [15]. All experiments were performed in triplicate and repeated at least three times.

Thymidine Uptake Study. DNA synthesis was measured by tritiated thymidine uptake [^{3}H-TdR] (Perkin Elmer, Boston, MA). HTLV-1-infected cells (5 x 10^5/ml) were cultured with various concentrations of LY294002 or rapamycin either alone or combination for 2 days in 96 well plates. Cells were pulsed with ^{3}H-TdR (0.5 μCi (0.185 MBq)/well) during the last 4 hrs of a 48 hrs culture, harvested onto glass filters with an automatic cell harvester (Cambridge Technology, Cambridge, MA), and ^{3}H-TdR counted using the LKB Beta plate scintillation counter (Wallac, Gaithersburg, MD), as previously described [16]. All experiments were performed in triplicate and repeated at least three times.

Cell Cycle Analysis by Flow Cytometry

Cell cycle analysis was performed on HTLV-1-infected cells incubated for 48 h with LY294002 (5 μM) or rapamycin (10 nM) either alone or in combination. The cells were fixed in chilled methanol overnight before staining with 50 μg/ml propidium iodide in the presence of 1 mg/ml RNase (100 units/ml; Sigma) and 0.1% NP40 (Sigma).

Analysis was performed immediately after staining using a FACScan (Becton Dickinson, Mountain View, CA).

Western Blot Analysis.

Lysates were made by standard methods as previously described [17]. Protein concentrations were quantitated using a Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA). Proteins were resolved on a 4–15% SDS polyacrylamide gel, transferred to an immobilon polyvinylidene difuride membrane (Amersham Corp., Arlington Heights, IL), and probed sequentially with antibodies. Anti-cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-c-Myc (Santa Cruz Biotechnology), anti-p-Akt (Ser473)(Cell Signaling, Beverly, MA), anti-Akt (Santa Cruz Biotechnology), anti-p-p70S6K (Thr389)(Cell Signaling), anti-p70S6K (Cell Signaling), anti-p-4E-BP-1 (Thr70)(Cell Signaling), anti-4E-BP-1 (Cell Signaling), anti-p-mTOR (Ser2448)(Cell Signaling), ant-mTOR (Cell Signaling) and anti-ß-actin (Santa Cruz Biotechnology) antibodies were used. The band intensities were measured using densitometry.

Immunohistochemichal Staining. All specimens were surgically resected, fixed in 10% buffered formalin, and paraffin-embedded. Deparaffinized sections were placed first in plastic Coplin jars filled with preheated citrate buffer (pH 6.0) and

microwave-irradiated for 5 minutes at 650 W. The slides were incubated for 30 minutes in FBS. The tissues were then immunostained with either p-p70S6K (Cell Signaling) or p-4E-BP-1 (Cell Signaling) antibody using a streptavidin-biotin complex peroxidase kit (DAKO LSAB Kit; Dakopatts, Kyoto, Japan). The procedures were performed according to the manufacturer's protocol. The tissue sections were couterstained with hematoxylin and semiquantitatively scored as weakly positive (+; 5> to 20%), moderately positive (++; >20 to 80%), or markedly positive (+++; >80%).

Statistical Analysis. Statistical analysis was performed to assess the difference between two groups under multiple conditions by one-way ANOVA followed by Boneferroni's multiple comparison tests using PRISM statistical analysis software (GraphPad Software, Inc., San Diego, CA).

Results.

Akt signaling is activated in HTLV-1-infected T-cells. We examined whether Akt signaling was activated in HTLV-1-infected MT-1, -2, -4 and HUT102 cells. Western blot analysis showed that all of these cell lines constitutively expressed the phosphorylated form of Akt (Ser473) proteins with HUT102 cells expressing the highest level (Fig 1A). The phosphorylated form of Akt was also detectable in the freshly isolated leukemia cells from individuals with acute type of ATL (Fig 1A, Table 1). On the other hand, p-Akt (Ser473) was not detectable in $CD4^+$ T cells from healthy volunteers (n=3) (figure not shown). We next explored levels of downstream effectors of Akt signaling. All cells expressed phosphorylated forms of p70S6K (Tyr 389) and/or 4E-BP-1 (Tyr 70) proteins (Fig 1A). The phosphorylated forms of p70S6K (Tyr 389) and 4E-BP-1 (Tyr 70) proteins were also detectable in lymph nodes from individuals (n=8) with lymphoma type of ATL, as measured by immunohistochemistry (Fig. 1B,C, Table 2).

Blockade of PI3K/Akt/mTOR signaling causes growth inhibition of HTLV-1-infected T-cells. To explore the impact of activated PI3K/Akt/mTOR signaling on survival of HTLV-1-infected T-cells, we cultured MT-1, -2, -4 and

HUT102 cell with the PI3K inhibitor LY294002 or the mTOR inhibitor rapamycin either alone or in combination. Exposure of these cells to LY294002 (1-20 μM, 48 h) inhibited the growth of MT-1, -2, -4 and HUT102 cells in a dose-dependent manner with IC50 (concentration which induces 50 % growth inhibition) from 10 to 20 μM, as measured by MTT assay (Fig 2A). Rapamycin (0.1-100 nM, 48 h) also inhibited the growth of MT-1, -2, 4, and HUT102 cells, although the IC50 was not achieved (Fig 2B). These cells showed almost identical sensitivities to growth inhibition mediated by RAD001, a rapamycin analog (data not shown). Interestingly, exposure of these cells to both of LY294002 and rapamycin enhanced the growth inhibition compared to either compound alone. For example, LY294002 (5 μM, 48 h) and rapamycin (10 nM, 48 h) alone inhibited the growth of MT-1 cells by 32 ± 3 and 40 ± 4 %, respectively. When these cells were exposed to both at the same concentrations, their growth was inhibited by 60 ± 2 % (p<0.01)(Fig 2C). Similarly, LY294002 (5 μM, 48 h) and rapamycin (10 nM, 48 h) alone inhibited the growth of MT-2 cells by 24 ± 2 and 29 ± 1 %, respectively; and both together inhibited growth by 45 ± 1 % (p<0.01)(Fig 2D). A similar enhanced growth inhibition by the combination of LY294002 and rapamycin also occurred with MT-4 and HUT102 cells (Fig E,F).

We also performed ^{3}[H]-thymidine uptake studies to examine whether LY294002 enhanced the ability of rapamycin to inhibit the proliferation of HTLV-1-infected cells (Fig 2G,H). LY294002 (10 μM) or rapamycin (20 nM) alone inhibited the proliferation of HUT102 cells by approximately 40 % and 50 %, respectively (Fig 2H). Combination of both at the same concentration inhibited their growth by 67 % (p<0.05)(Fig 2H).

We next examined the effect of LY294002 and the rapamycin analog RAD001 on freshly isolated leukemia cells from individuals with acute type of ATL. LY294002 and RAD001 inhibited the proliferation of freshly isolated leukemia cells (case #1) in a dose-dependent manner with IC50 of approximately 3 μM and 10 nM, respectively, as measured by ^{3}[H]-thymidine uptake (Fig 2I). Combination of both enhanced the inhibition of growth. For example, LY294002 (1 μM) or RAD001 (1 nM) inhibited the proliferation of ATL cells by 30 % (case #1)(Fig 2I). Combination of both compounds at the same concentrations inhibited proliferation of these cells by 50 % (p<0.01)(Fig 2I). ATL cells from case #2 were more sensitive to these agents. LY294002 (1 μM, 48 h) and RAD001 (1 nM, 48 h) alone inhibited proliferation by 64 ± 3 % and 55 ± 2 %, respectively (Fig 2J). Combined at the same concentrations, proliferation of these cells was inhibited by 77 ± 4 % (p<0.05) (Fig 2J).

Blockade of PI3K/Akt/mTOR signaling causes G0/G1 cell cycle arrest of HTLV-1-infected T-cells. To understand better the mechanism by which LY294002 and rapamycin inhibited growth of HTLV-1-infected cells, cell cycle changes were examined (Fig 3). A prominent accumulation of the percent of MT-2 and -4 cells in the G0/G1 phase of the cell cycle occurred with a concomitant decrease in the proportion of those in S phase after culture with either LY294002 (5 μM, 48 h) or rapamycin (10 nM, 48 h). Combination of both at the same concentrations augmented the populations in the G0/G1 phase of the cell cycle in conjunction with a decrease in those in S phase ($p<0.01$)(Figs 3B,C). Neither LY294002 nor rapamycin alone significantly affect the MT-1 and HUT102 cells; however, their combination at the same concentrations caused a significant accumulation of these cells in G0/G1 with a concomitant decrease in those cells in S phase ($p<0.01$)(Figs 3A,D).

Effects of LY294002 and rapamycin on the phosphorylated forms of Akt, p70S6K and 4E-BP-1 in HTLV-1-infected T-cells. To explore whether the LY294002 and rapamycin-induced growth inhibition of HTLV-1 infected cells was correlated with impaired mTOR signaling, we measured levels of the phosphorylated forms of mTOR targets p70S6K and 4E-BP-1 after exposure of these cells to LY294002 (5 μM, 1 h) and/or rapamycin (10 nM, 1 h). Effects varied between each cell type (Fig 4A-C);

nevertheless, the combination of LY294002 and rapamycin enhanced the downregulation of p-p70S6K and/or p-4E-BP-1 in all cell types compared to either compound alone. For example, exposure of MT-1 cells to rapamycin (10 nM, 1 h) decreased the level of p-p70S6K by 20 %. It did not affect the level of p-4E-BP-1. Exposure of these cells to LY294002 (5 μM, 1 h) did not modulate the levels of either p-p70S6K or p-4E-BP-1; however, combination of both at the same concentrations dramatically downregulated by 80 and 90 % the levels of p-p70S6K and p-4E-BP-1, respectively (Fig 4A). Similarly, exposure of HUT102 cells to either LY294002 (5 μM, 1 h) and rapamycin (10 nM, 1 h) decreased the levels of p-4E-BP-1 by 60 and 50 %, respectively (Fig 4C). Combination of both almost completely blocked expression of this phosphorylated form of protein (Fig 4C).

LY294002 (5 μM, 1 h) blocked phosphorylation of Akt in all cell types tested (Fig 4A-C). Unexpectedly, rapamycin (10 nM, 1h) increased the levels of p-Akt at Ser 473 by 5- and 1.5-fold in MT-1 and -4 cells, respectively, indicating activated Akt signaling (Fig 4A,B). This rapamycin-stimulated phosphorylation of Akt was completely blocked by LY294002 (5 μM, 1 h) (Fig 4A,B).

Effects of LY294002 and rapamycin on c-Myc and Cyclin D1 proteins. We explored whether blockade of PI3K/Akt/mTOR signaling affects levels of c-Myc and

cyclin D1 in ATL cells whose protein synthesis is regulated by p70S6 and 4E-BP-1. Exposure of MT-1 cells to either LY294002 (5 μM, 2 days) or rapamycin (10 nM, 2 days) decreased the level of c-Myc protein by approximately 60 or 20 %, respectively, as measured by Western blot analysis (Fig 5A). When these cells were exposed to both LY294002 and rapamycin at the same concentrations, the level of this protein decreased by 90 % (Fig 5A). Similarly, exposure of HUT102 cells to either LY294002 (5 μM, 2 days) or rapamycin (10 nM, 2 days) lowered the level of cyclin D1 protein by approximately 70 and 20 %, respectively (fig 5B). Combination of both at the same concentrations downregulated this protein by 90 % (Fig 5B).

Discussion.

This study found that the Akt signal pathway was activated in HTLV-1-infected cells (Fig 1). In the previous studies, other investigators provided the evidence that HTLV-1 Tax stimulated Akt, leading to activation of pro-survival signal nuclear factor κB in HTLV-1-infected cell lines [18,19]. Another group has recently shown that Akt was phosphorylated at Ser473 in MT-2 cells [20]. These results support our observations. In addition, we showed that downstream of Akt, mTOR signaling was activated in HTLV-1 infected cells, although levels of p-Akt did not correlate with those of mTOR targets in these cells (Fig 1). Recent studies showed that other signal pathways such as extracellular signal-regulated kinase (ERK) also regulated p70S6K and 4E-BP-1 via mTOR [21,22]. These observations could explain this discrepancy. Further studies will be required. Blockade of mTOR by rapamycin and its derivative RAD001 inhibited proliferation of HTLV-1 infected cells (Figs 2,3). Also, RAD001 was shown to inhibit proliferation of acute myeloid leukemia cells and sensitize these cells to growth inhibition mediated by cytotoxic agents such as cytarabine [23,24]. A phase 1 clinical study have shown the efficacy of RAD001 in the individuals with solid tumors [25]. These results warrant the clinical study with RAD001 for individuals with ATL.

Exposure of HTLV-1-infected cells to rapamycin stimulated their phosphorylation of Akt (Ser473), which probably activated the pro-survival signals in these cells (Figs 4). This could be one of the reasons why even high concentrations of rapamycin (100 nM, 48 h) failed to inhibit their proliferation by more than 50 % (data not shown). Consistent with our in vitro results, recent clinical studies found that RAD001 increased p-Akt in post-treatment tumor specimens [25]. In addition, other investigators have also shown that inhibition of mTOR by RAD001 increased levels of p-Akt in breast and prostate cancer cells [26]. Moreover, they have found that RAD001-induced p-Akt was mediated by insulin like growth factor I receptor (IGF-1R) signaling, and blockade of IGF-1R potentiated the ability of RAD001 in cancer cells [26]. Another group has also demonstrated that LY294002 enhanced the ability of rapamycin to inhibit the proliferation of T-cells [27]. Recent studies found two mTOR complexes, the rapamycin-sensitive mTOR-Raptor (regulatory-associated protein of mTOR) and the rapamycin-insensitive mTOR-Rictor (rapamycin-insensitive companion of mTOR) complexes [28]. The mTOR-Rictor complexes directly phosphorylate Akt at Ser473 [29]. These two mTOR complexes are involved in the negative feedback of Akt to help prevent uncontrolled cellular proliferation; the activated Akt induces assembly of mTOR-Raptor complex which inhibits assembly of mTOR-Rictor complex and

thereby inhibits Akt [28]. We hypothesize that rapamycin inhibited the mTOR-Raptor complex, which probably promoted the formation of mTOR-Rictor complex, leading to phosphorylation of Akt at Ser473 in HTLV-1-infected cells. When rapamycin was combined with LY294002, rapamycin-stimulated accumulation of p-Akt was blocked, and growth inhibition and G0/G1 cell cycle arrest of these cells were potentiated (Figs 2,3).

Rapamycin effectively suppressed both p-p70S6K and p-4E-BP-1 in HUT102 cells (Fig 4), and these cells were relatively sensitive to rapamycin-mediated growth inhibition compared to the other HTLV-1-infected cells (Fig 1). Among HTLV-1-infected T-cells, HUT102 cells did not have their p-Akt stimulated by rapamycin, which could explain their sensitivity to this compound. Nevertheless, the reason why rapamycin failed to upregulate p-Akt in HUT102 cells remains unknown at the present time.

The combination of LY294002 and rapamycin enhanced their suppression of p-p70S6K and p-4E-BP-1 in HTLV-1-infected cells compared to either compound alone (Fig 4). Consistent with these results, exposure of these cells to the combination of LY294002 and rapamycin resulted in the profound suppression of expression of cell cycle-regulating proteins, cyclin D1 and c-Myc whose protein synthesis is regulated by

p70S6K and 4E-BP-1 (Fig 5). This can explain the enhanced growth inhibition and cell cycle arrest mediated by the combination of both compounds.

Taken together, the Akt/mTOR signaling is activated in ATL. The longitudinal inhibition of PI3K/Akt/mTOR signaling may represent a promising treatment strategy for treatment of individuals with ATL and other types of cancers in which this signal is activated.

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Figure Legends.

Figure 1. Akt signaling is activated in HTLV-1-infected cells. (A), Western blot analysis. Cell lysates from MT-1, -2, -4, HUT102 and freshly-isolated ATL (#1, #2) cells were prepared and subjected to Western blot analysis. The polyvinylidene fluoride membrane was sequentially probed with anti-p-mTOR (Ser 2448), -mTOR, p-Akt (Ser473), -Akt, -p-p70S6K (Tyr389), -p70S6K, p-4E-BP-1 (Tyr70) and -4E-BP-1 antibodies. **(B and C), Representative immunohistochemical staining of lymph node from individuals with lymphoma type of adult T-cell leukemia.** Lymph nodes from case 9 were stained with p-p70S6K (Tyr389) and p-4E-BP-1 (Tyr70) antibodies. Original magnifications were x 400.

Figure 2. The PI3K inhibitor LY294002 and the mTOR inhibitor rapamycin or its analog RAD001 inhibit the proliferation of HTLV-1-infected T-cells. Cells were plated in 96 well plates and cultured with either LY294002 (1-20 μM)(A) or rapamycin (0.1-100 nM)(B). After 2 days, the cells were treated with MTT for 4 h, and absorbance was measured. Results represent the mean ± SD of 3 experiments performed in triplicate. **Combination of LY294002 and rapamycin induces the enhanced growth inhibition of HTLV-1-infected T-cells. MTT assay.** MT-1 (C), -2 (D), -4 (E) and HUT102 (F)

cells were cultured with either LY294002 or rapamycin alone or in combination. After 2 days, their viability was measured by MTT assay. The statistical significance of difference between growth inhibition mediated by either LY294002 or rapamycin alone and that mediated by combination of both was determined by one-way analysis of variance (ANOVA) followed by Boneferroni's multiple comparison tests. Results represent the mean ± SD of 3 experiments performed in triplicate. *, $p < 0.01$; **, $p < 0.05$. rapa, rapamycin.

^{3}[H]-thymidine uptake study. HUT102 (G, H) and freshly-isolated ATL (I, J) cells were cultured with either LY294002, rapamycin or RAD001 alone or the combination of either LY294002 and rapamycin, or LY294002 and RAD001. After 2 days, proliferation was measured by a ^{3}[H]-thymidine uptake study. The statistical significance was determined by one-way analysis of variance (ANOVA) followed by Boneferroni's multiple comparison tests. Results represent the mean ± SD of 3 experiments performed in triplicate. *, $p < 0.01$; **, $p < 0.05$. rapa, rapamycin; RAD, RAD001.

Figure 3. Combination of LY294002 and rapamycin induces the enhanced G0/G1 cell cycle arrest of HTLV-1-infected T-cells. MT-1 (A), -2 (B), -4 (C) and HUT102 (D) cells were cultured with LY294002 (5 μM) or rapamycin (10 nM) either alone or in

combination. After 48 h, cell cycle of these cells was analyzed. The statistical significance of difference between populations in G0/G1 phase of cell cycle induced by either LY294002 or rapamycin alone and those induced by combination of both was determined by one-way analysis of variance (ANOVA) followed by Boneferroni's multiple comparison tests. Results represent the mean ± SD of 3 experiments performed in triplicate. *, $p < 0.01$; rapa, rapamycin; LY, LY294002.

Figure 4. The blockade of PI3K/Akt/mTOR signaling suppresses p-p70S6K and p-4E-BP-1 in HTLV-1-infected T-cells. Western blot analysis. MT-1 (A), -4 (B) and HUT102 (C) cells were cultured with either LY294002 (5 μM) or rapamycin (10 nM) alone or in combination. After 1 h, cells were harvested, cell lysates prepared and subjected to Western blot analyses. The polyvinylidene fluoride membrane was sequentially probed with anti-p-Akt (Ser473), -p-p70S6K (Thr389), -p-4E-BP-1 (Thr70), -Akt, -p70S6K, -4E-BP-1 and β-actin antibodies. The band intensity was measured by densitometry. Two identical experiments yielded similar results.

Figure 5. The blockade of PI3K/Akt/mTOR signaling downregulates levels of c-Myc and cyclin D1 in HTLV-1-infected T-cells. MT-1 (A) and HUT102 (B) cells were cultured with LY294002 (5 μM) or rapamycin (10 nM) alone or combination. After 48 h, cells were harvested, cell lysates prepared and subjected to Western blot

analyses. The polyvinylidene fluoride membrane was sequentially probed with either anti-c-Myc (A) or cyclin D1 (B) and β-actin. The band intensity was measured by densitometry. Two identical experiments yielded similar results.

Table 1. Patient Clinical Characteristics

Patient	Age	Sex	WBC ($x10^9$/L)	% of ATL cells	Type	Disease Status
#1	67	M	141,300	52 %	Acute	Newly diagnosed
#2	63	M	32,100	71 %	Acute	Newly diagnosed

Table 2. Immunohistochemical Staining of Lympho Nodes from ATL

Patient	Age	Sex	Type	p-p70S6K	p-4E-BP-1
#3	73	M	lymphoma type	++	+
#4	69	F	lymphoma type	++	+
#5	67	M	lymphoma type	++	++
#6	48	F	lymphoma type	++	+
#7	60	M	lymphoma type	++	+
#8	50	M	lymphoma type	+++	+++
#9	66	M	lymphoma type	+++	+
#10	62	M	lymphoma type	++	+

The relative intensity of staining of lympho nodes with anti-p-p70S6K and -p-4E-BP-1 antibodies was semiquantitatively scored as weakly positive (+; 5> to 20%), moderately positive (++; >20 to 80%), or markedly positive (+++; >80%).

Figure1

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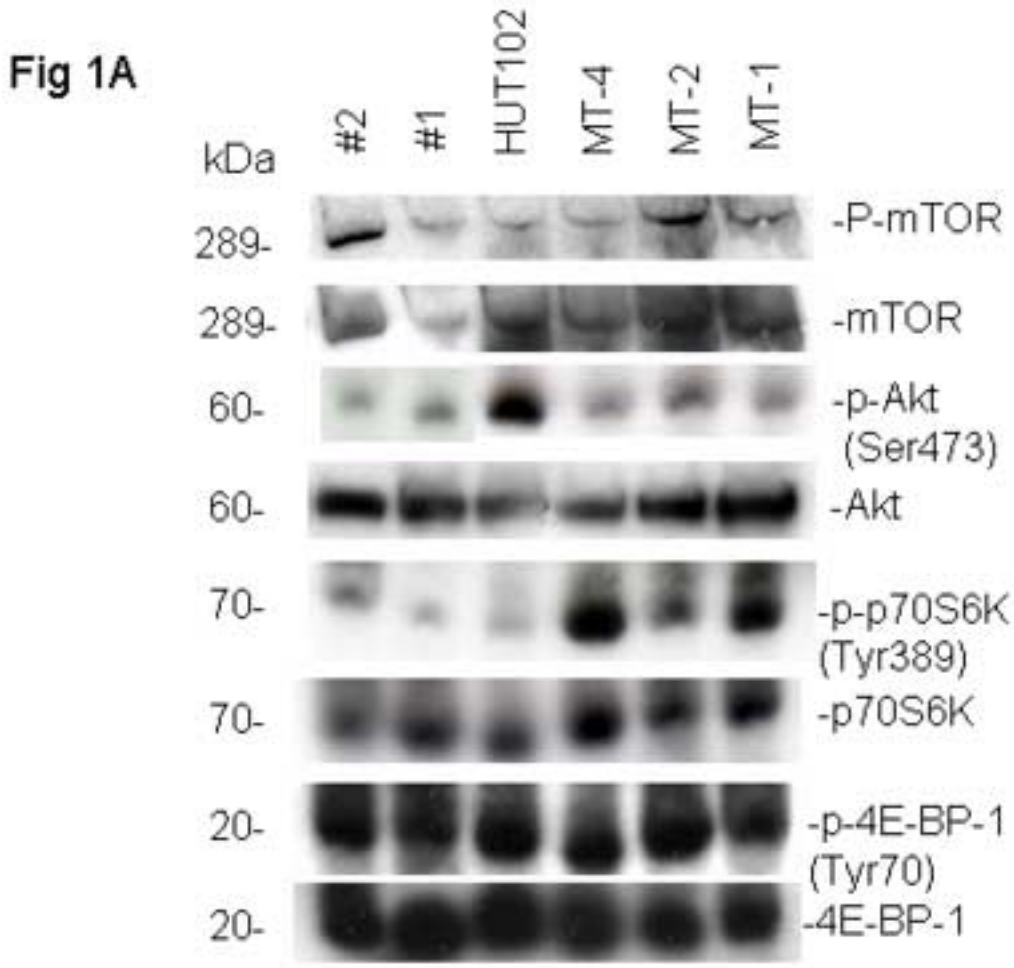

Figure1

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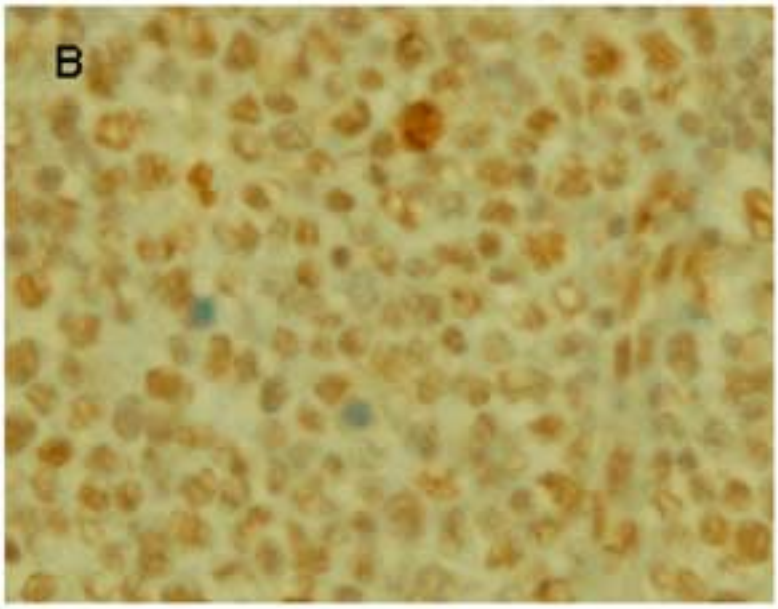

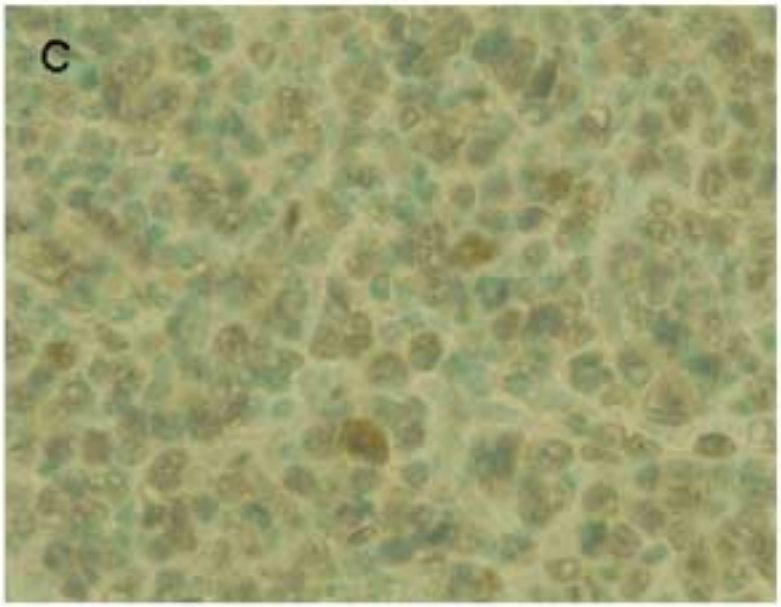

Figure2

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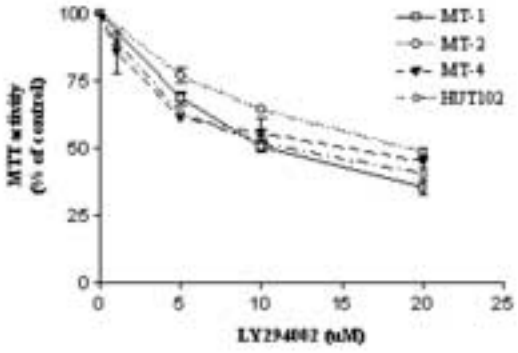
Fig 2A



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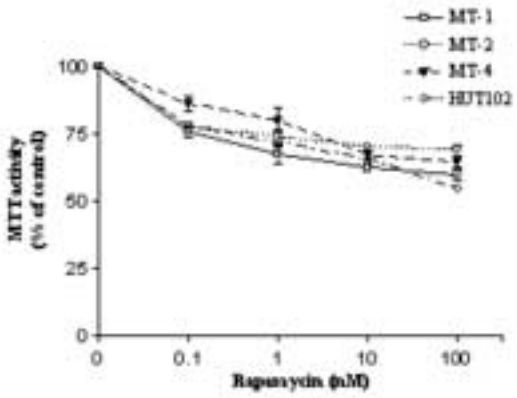

Figure2

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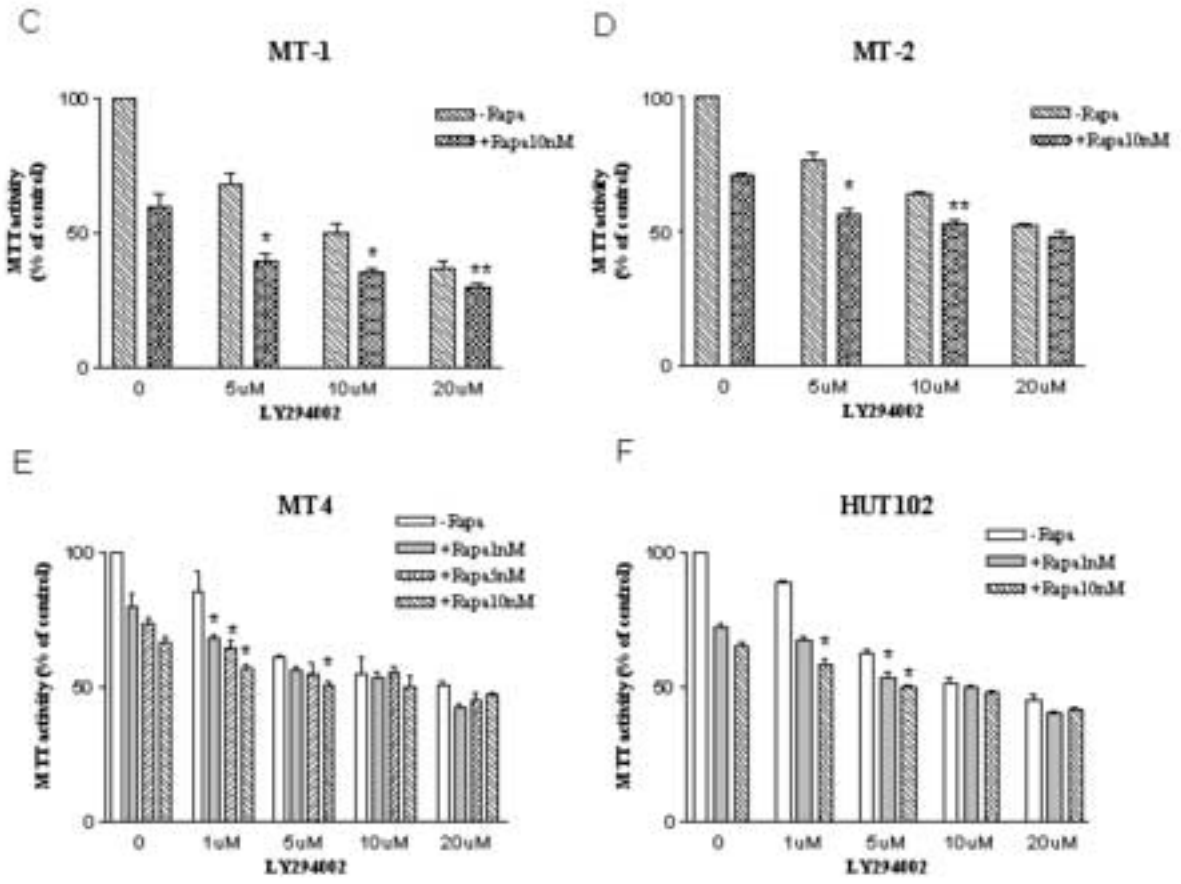

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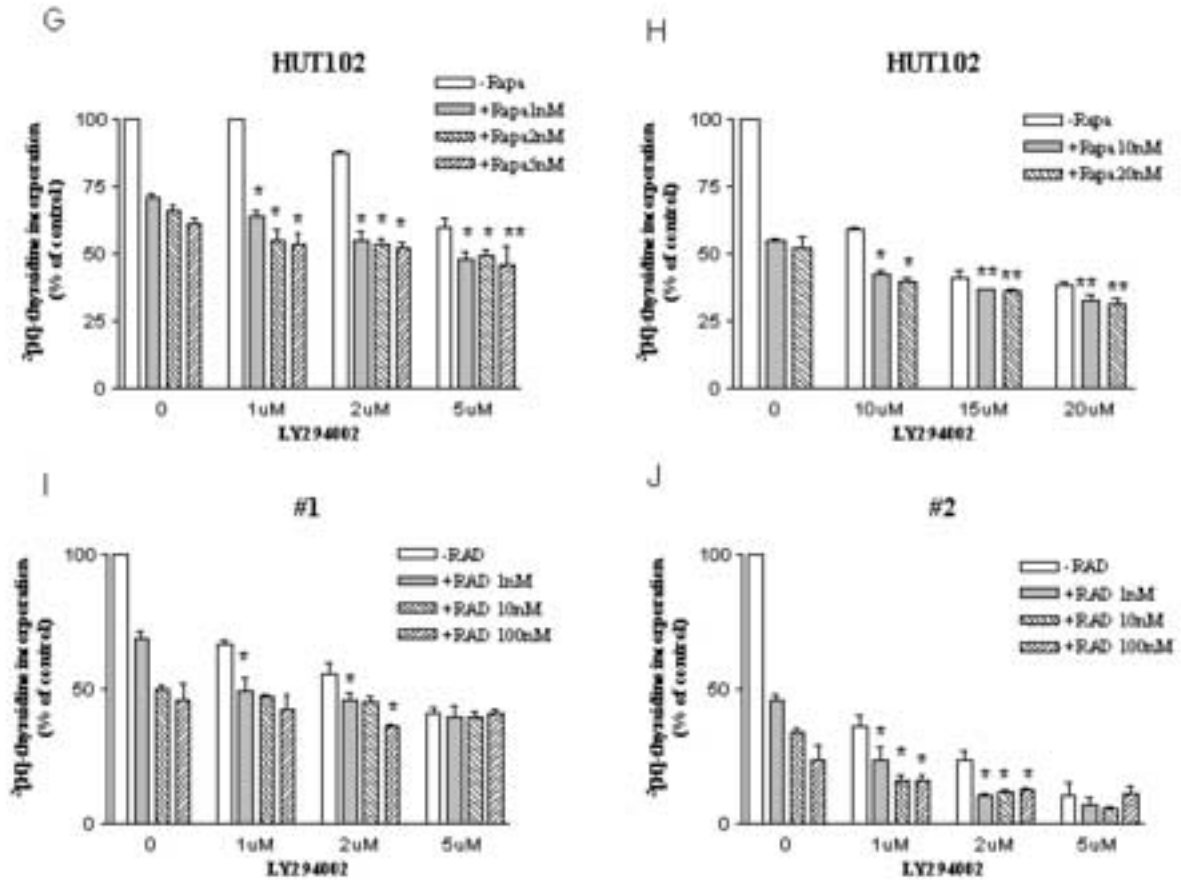

Figure3

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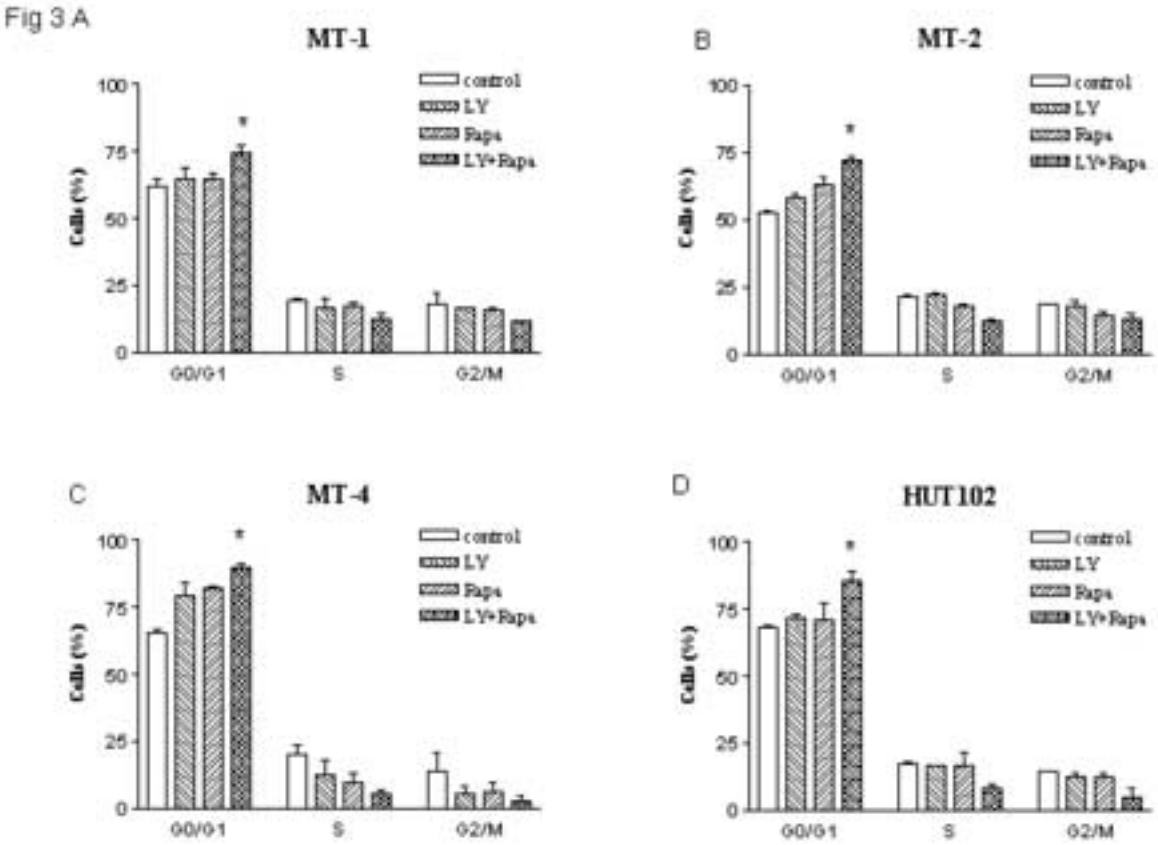

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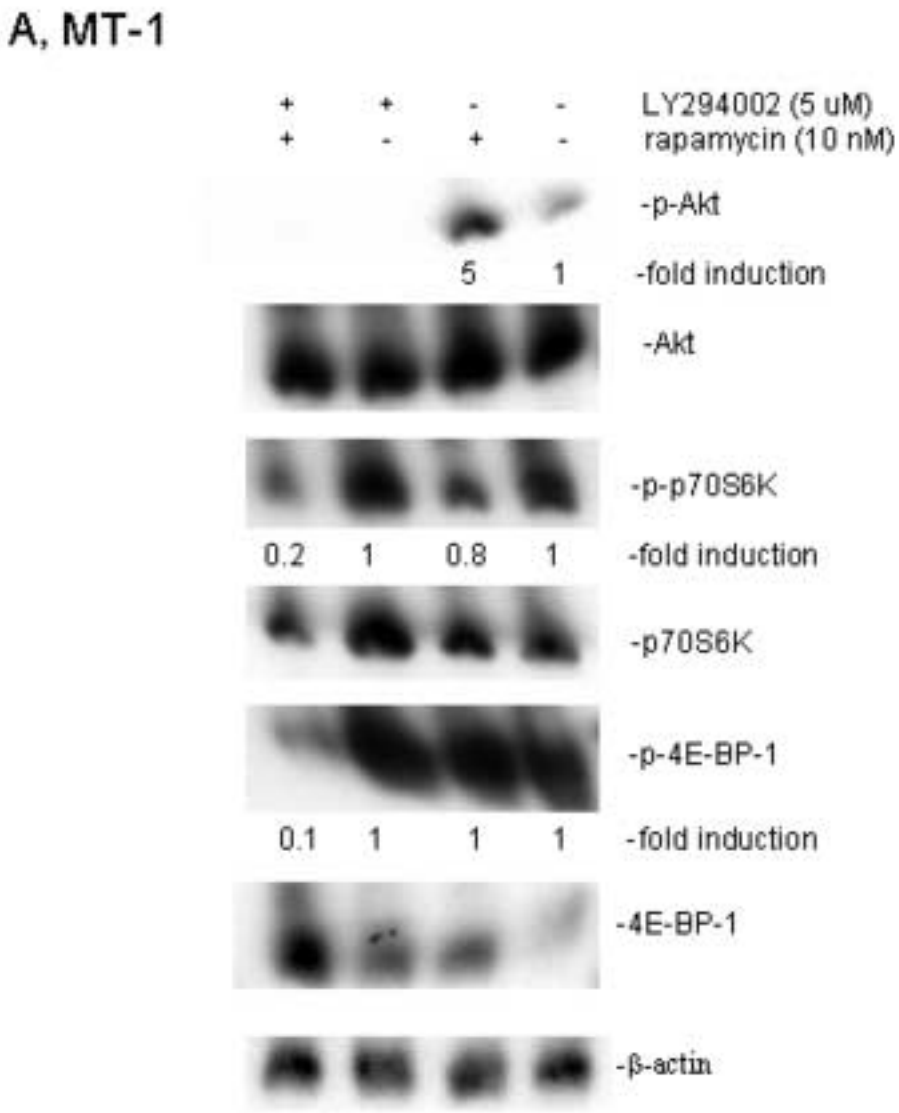

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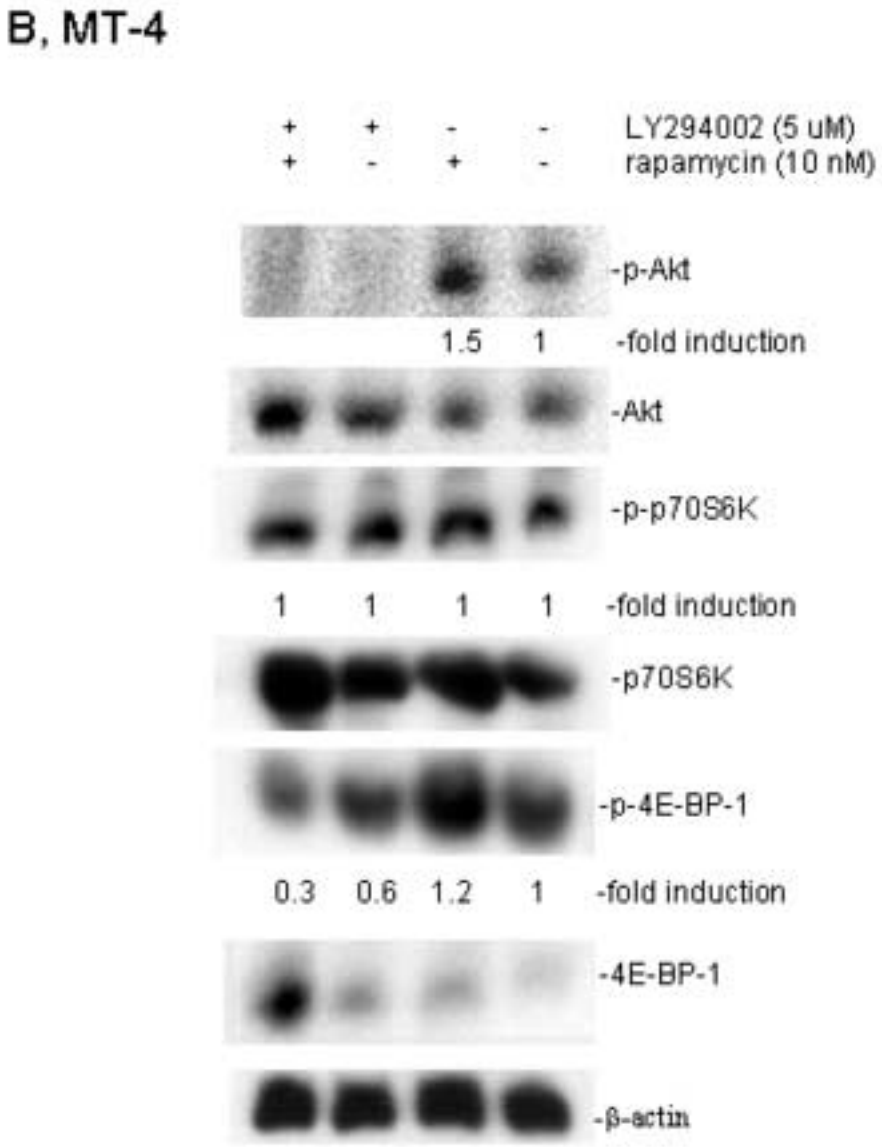

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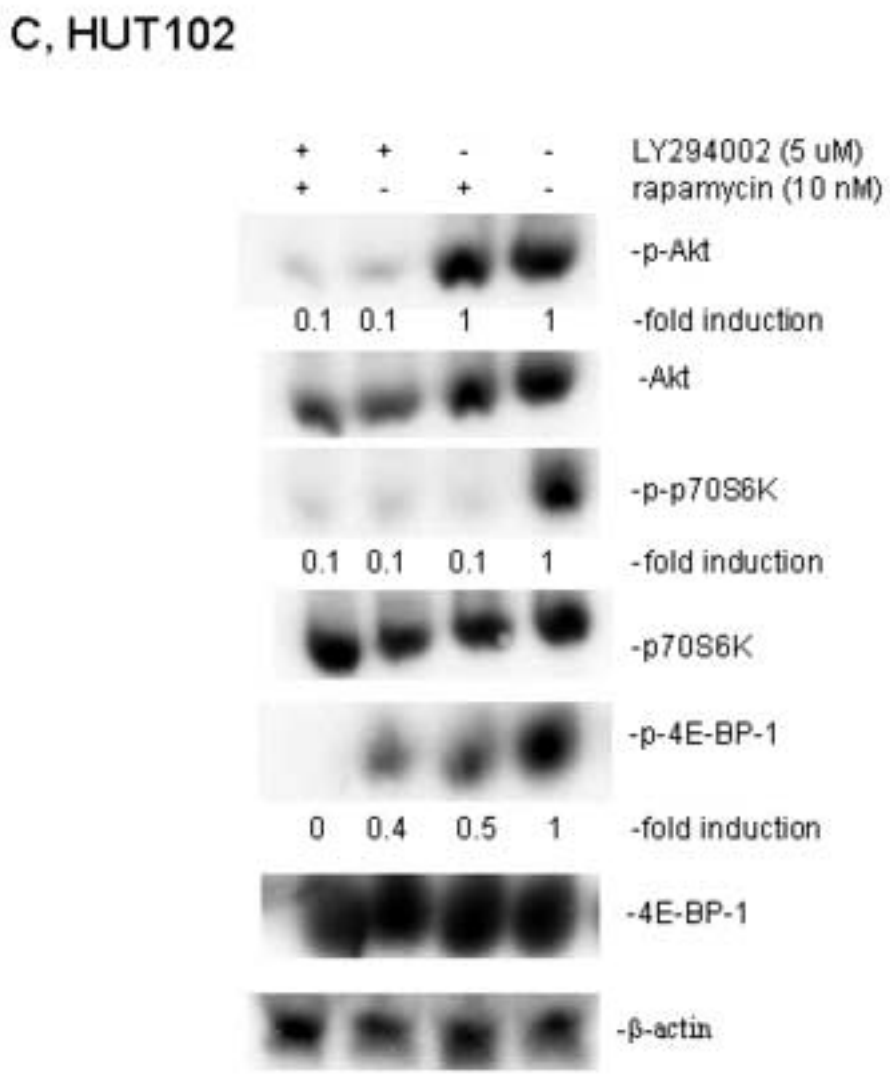

Figure5

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