Comparative Effects of PP242 and Rapamycin on mTOR Signalling and NOTCH Signalling in Leukemia Cells

AYA ONO, RYO OIKE, YUKI OKUHASHI, YUSUKE TAKAHASHI, MAI ITOH, NOBUO NARA and SHUJI TOHDA

Department of Laboratory Medicine, Tokyo Medical and Dental University, Yushima, Tokyo, Japan

Abstract. *Aim: PP242 is a compound which inhibits both mammalian target of rapamycin complex-1 (mTORC1) and mTORC2. We examined the effects of PP242 and rapamycin on mTOR signalling and evaluated potential crosstalk with the NOTCH signalling in eight leukemia cell lines. Materials and Methods: We examined the effects of treatment with these inhibitors on cell growth and protein expression. Results: PP242 suppressed growth more potently than did rapamycin. In two cell lines poorly sensitive to PP242, PP242 failed to inhibit v-akt murine thymoma viral oncogene homolog (AKT) phosphorylation. Suppression of mTOR phosphorylation was weaker in myeloid cell lines. Rapamycin induced eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) hyper-phosphorylation in three cell lines. Phosphorylation of both isoforms (p70 and p85) of S6 kinase (S6K) was suppressed in three cell lines; only p70 was suppressed in the others. NOTCH1 expression and activation were up-regulated by PP242 in one cell line but down-regulated in another. Conclusion: PP242 is a candidate for molecular-targeted leukemia therapy, although its effects must be evaluated on a case-by-case basis. Crosstalk was found between the mTOR and NOTCH signalling pathways.*

Abnormal activation of the mammalian target of rapamycin (mTOR) pathway is involved in the growth of leukemia cells (1). mTOR resides in two multiprotein complexes, mTORC1 and mTORC2. The former contains a regulatory-associated protein of mTOR (RAPTOR), and its activation leads to the phosphorylation of S6 kinase (S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1). The latter contains a rapamycin-insensitive companion of mTOR (RICTOR), and its activation leads to the phosphorylation of v-akt murine thymoma viral oncogene homolog (AKT) protein. In both cases, mTOR activation engenders protein synthesis and cell growth (Figure 1).

Therefore, mTOR inhibitors have potential utility as drugs for molecular-targeted therapy against various malignancies, including leukemia. An allosteric inhibitor, rapamycin, generally inhibits the activity of mTORC1 by binding to the FK506-binding protein (FKBP)-rapamycin-binding domain of mTOR protein, resulting in the dissociation of mTOR from RAPTOR. A small-molecule compound, PP242, is a second-generation mTOR inhibitor which inhibits mTOR catalytic activity in the context of both mTORC1 and mTORC2 (2). In this study, we examined the effects of these inhibitors on cell growth and mTOR signalling proteins in various leukemia cell lines.

NOTCH signalling is also involved in the growth of leukemia cells (3, 4). There are hints of crosstalk between mTOR and NOTCH pathways, as has been reported that NOTCH activation induces the expression of hairy and enhancer of split-1 (HES1) protein, which then down-regulates the transcription of phosphatase and tensin homolog (*PTEN*) gene (5, 6). Because PTEN works as a suppressor of mTOR signalling, the down-regulation of *PTEN* results in mTOR activation. However, whether mTOR signalling affects NOTCH signalling is unclear. In this study, we therefore also examined whether suppression of mTOR signalling by rapamycin and PP242 affects NOTCH signalling in leukemia cells.

Materials and Methods

Cells and mTOR inhibitors. Eight human leukemia and lymphoma cell lines that have been cultured in our laboratory were used. T-Lymphoblastic leukemia cell lines, Jurkat, KOPT-K1, and DND-41 were donated by Drs. Harashima and Orita (Fujisaki Cell Center, Japan). Acute promyelocytic leukemia cell line NB4 (7) was kindly provided by Dr. Lanotte (Hôpital Saint-Louis, Paris, France). THP-1 (acute monocytic leukemia) and HEL (erythroleukemia) were supplied by the Japanese Collection of Research Bioresources (Ibaraki, Japan). TMD7 (acute myeloid leukemia with trilineage myelodysplasia) and TMD8 (diffuse large B-cell lymphoma) were established in our laboratory (8, 9). Rapamycin and PP242 were purchased from Calbiochem (La Jolla, CA, USA) and Sigma Chemical Co. (St. Louis, MO, USA), respectively. Both were dissolved in dimethyl sulphoxide (DMSO).

Correspondence to: Shuji Tohda, MD, Ph.D., Department of Laboratory Medicine, Tokyo Medical and Dental University, Yushima 1-5-45, Bunkyo-Ku, Tokyo 113-8519, Japan. Tel: +81 358035334, Fax: +81 358035629, e-mail: tohda.mlab@tmd.ac.jp

Key Words: mTOR, rapamycin, NOTCH, leukemia.

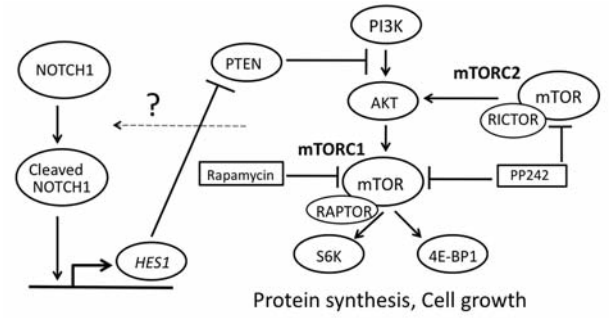

Figure 1. *Overview of mammalian target of rapamycin (mTOR) pathway, NOTCH pathway, and their crosstalk. The dotted line represents one of the goals of this study, namely, to determine whether mTOR signalling affects the NOTCH pathway.*

Cell growth assay. The effects of inhibitors on short-term growth were examined using a colorimetric WST-1 assay. Cells (2×10^4 cells/well) were cultured in 0.1 ml of RPMI-1640 medium supplemented with 10% fetal calf serum in the presence of increasing concentrations of each inhibitor. After three days, WST-1 and 1-methoxy-5-methylphenazinium methyl sulphate (Dojindo Laboratories, Kumamoto, Japan) were added to the cells. Optical density (OD) was measured using an enzyme-linked immunosorbent assay (ELISA) plate reader to determine the cell number. The cell growth is shown as a percentage of the mean OD value of the control. To examine the effects of inhibitors on differentiation and apoptosis, cytospin preparations were prepared from the harvested cells, stained with Wright stain, and observed under a microscope.

Immunoblotting. The effects of inhibitors on the expression and phosphorylation of proteins in the mTOR and NOTCH pathways were examined by immunoblotting. After culture with 0.5 μM of each inhibitor for 24 h, cells were harvested and lysed. The lysates were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with antibodies against AKT, p-AKT (Ser473), mTOR, p-mTOR (Ser2481), 4E-BP1, p-4E-BP1 (Ser65), S6K, p-S6K (Thr389), PTEN, NOTCH1, cleaved NOTCH1 (Val1744), NOTCH2 (Cell Signaling Technology, Danvers, MA, USA), and α-tubulin (Abcam, Cambridge, MA, USA) as a loading control. Each assay was repeated at least three times independently to verify their reproducibility.

Reverse transcription-polymerase chain reaction (RT-PCR). The effects of inhibitors on the expression of mTOR and NOTCH-related genes were examined by quantitative RT-PCR using a FastStart DNA Master SYBR Green I kit, LightCycler primer sets (Roche Diagnostics, Mannheim, Germany) and QuantiTect primers (QIAGEN, Hilden, Germany). RNA was extracted after culture with 0.5 μM of each inhibitor for 24 h. The expression level of each mRNA was normalised to that of β-actin mRNA which was measured concurrently.

Results

Effects of mTOR inhibitors on cell growth. Dose response curves showing the effects of PP242 and rapamycin on cell growth are shown in Figure 2. Treatment with the inhibitors suppressed growth in six cell lines, except for DND-41 and HEL cells whose growth was not significantly suppressed by rapamycin treatment. The suppressive effect of PP242 was more potent than that of rapamycin in all cell lines except NB4 cells. Cytospin preparations from the cells in which growth was suppressed revealed apoptotic cells with nuclear condensation and apoptotic bodies (photograph not shown). Morphological differentiation was not observed in any of the cell lines examined.

Effects of inhibitors on mTOR signalling proteins. Figure 3 shows the expression and phosphorylation of mTOR signalling proteins in the representative four cell lines treated with PP242, rapamycin, or DMSO as a vehicle control. PTEN was deficient in Jurkat cells, as previously reported (10), and 4E-BP1 was not detected. In all cell lines, AKT, mTOR, 4E-BP1, and S6K were constitutively phosphorylated. Treatments affected the expression and phosphorylation of these markers in a cell line- and inhibitor-dependent manner. The phosphorylation of AKT was suppressed by PP242 in DND-41, THP-1, and KOPT-K1* cells (asterisks represent the cell lines for which figures are not shown), but not by rapamycin. In the other cell lines, neither inhibitor significantly affected AKT phosphorylation.

mTOR phosphorylation was suppressed by both inhibitors in all cell lines except HEL, NB4*, and TMD7*. PP242 was more potent than rapamycin in DND-41, THP-1, and KOPT-K1* cells, whereas rapamycin was more potent in Jurkat and TMD8* cells.

The phosphorylation of 4E-BP1 was suppressed by PP242 in all cell lines except in Jurkat cells. In DND-41, THP-1, HEL, KOPT-K1* cells, 4E-BP1 protein was also reduced upon treatment with the inhibitor. Interestingly, rapamycin actually increased 4E-BP1 phosphorylation in THP-1, HEL, and NB4* cells.

S6K has two isoforms, namely, p70 and p85, based on their molecular weight (11, 12). In THP-1, HEL, and TMD8* cells, phosphorylation of both p70 and p85 were suppressed by the inhibitors. In the other five cell lines, only p70 was suppressed. In Jurkat, HEL, NB4*, and TMD8* cells, S6K was more potently suppressed by rapamycin than PP242.

Effect of PP242 and rapamycin on NOTCH signalling proteins. Figure 4 shows the expression of NOTCH1 (transmembrane subunit), the active form of NOTCH1 (cleaved NOTCH1 fragment), and NOTCH2 (transmembrane subunit) proteins in four representative cell lines. In Jurkat cells, PP242 treatment up-regulated NOTCH1 and cleaved NOTCH1. Conversely, PP242 slightly suppressed NOTCH1, cleaved NOTCH1, and NOTCH2 in DND-41 cells. In TMD7 cells, rapamycin treatment slightly down-regulated NOTCH1 and NOTCH2. Cleaved NOTCH1 was below the detectable level in TMD7 cells. In the other cell lines, neither inhibitor significantly affected the expression of NOTCH proteins.

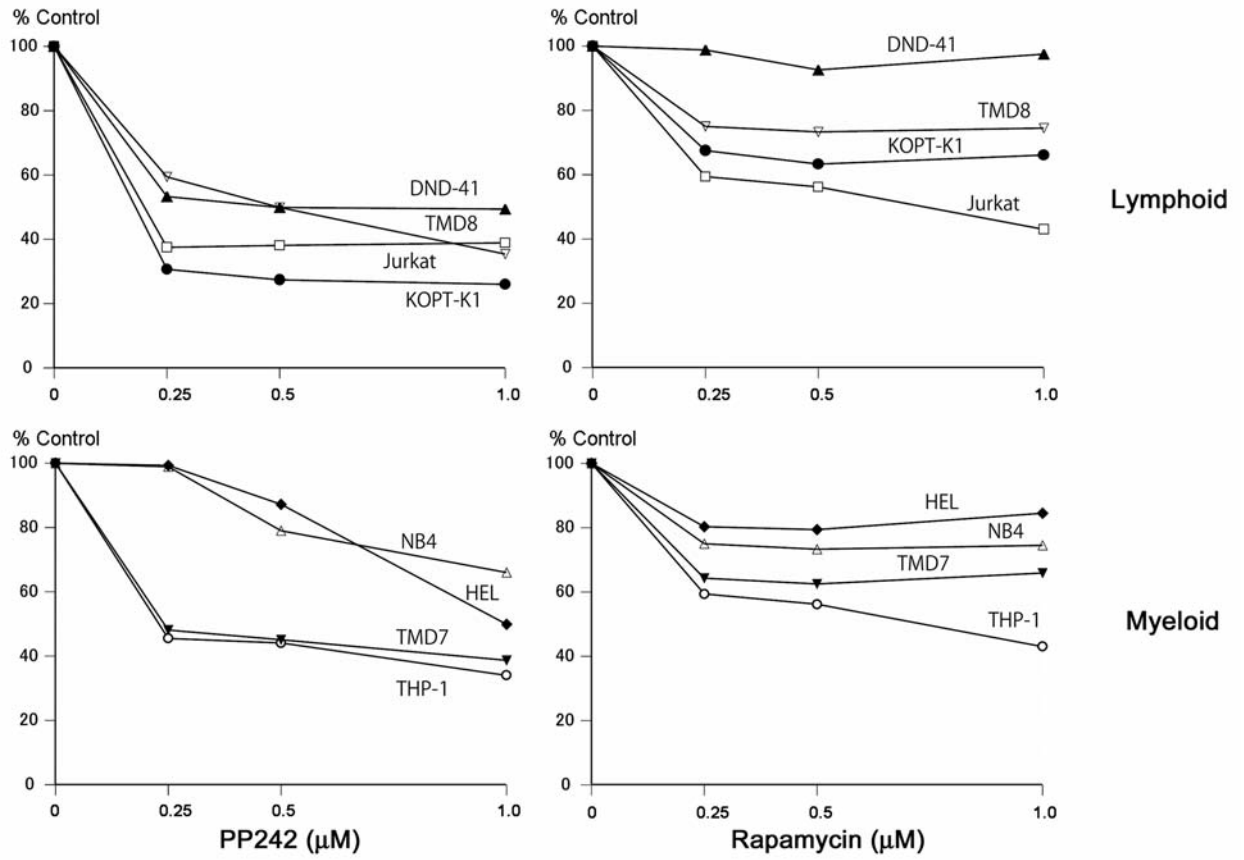

Figure 2. *Dose response curves of the effects of PP242 and rapamycin on growth of lymphoid and myeloid leukemia cell lines. Cells were cultured with increasing concentrations of inhibitors. After three days, cell growth was examined using a colorimetric assay. Growth is shown as a percentage of the mean OD value of control cells.*

Effects of inhibitors on gene expression. To examine the possible causes of the changes in the proteins described above, the expression of mTOR-related genes (*AKT*, *MTOR*, *4EBP1*, and *S6K*) and NOTCH-related genes (*NOTCH1*, *NOTCH2*, and *HES1*) was examined. PP242 treatment up-regulated the expression of *NOTCH1* and *HES1* by 493% and 255% compared to those of control, respectively in Jurkat cells. In the other cell lines, treatment with inhibitors did not significantly affect gene expression (data not shown).

Discussion

In this study, we showed that mTOR inhibitors suppressed the growth of several leukemia cell lines and altered mTOR signalling, which is constitutively activated in these cells. The suppressive effects of PP242 were more potent than those of rapamycin. The increased efficacy of PP242 may be observed because this compound suppresses both mTORC1 and mTORC2, whereas rapamycin suppresses only mTORC1 (2); this finding was supported by our observation that AKT phosphorylation was suppressed by PP242. In HEL and NB4 cells, AKT phosphorylation was not suppressed by 0.5 μM of PP242. This may explain why in some instances PP242 did not out-perform rapamycin in the suppression of cell growth.

The degree of suppression of mTOR, 4E-BP1, and S6K phosphorylation by the inhibitors varied among the cell lines. mTOR phosphorylation was not suppressed by the inhibitors in three out of four myeloid cell lines, whereas phosphorylation was inhibited in all four lymphoid cell lines. Although 4E-BP1 and S6K are direct mTORC1 substrates, PP242 tended to suppress 4E-BP1 phosphorylation more potently, whereas rapamycin preferentially blocked S6K phosphorylation. It was reported that rapamycin induced 4E-BP1 hyperphosphorylation in the human embryonic kidney 293 cell line, HEK293 (13). We found that rapamycin also induced 4E-BP1 hyperphosphorylation in three leukemia cell lines. This may explain the limited potency of rapamycin with respect to leukemia cell growth.

Regarding suppression of S6K phosphorylation by the inhibitors, we found that cell lines could be divided into two

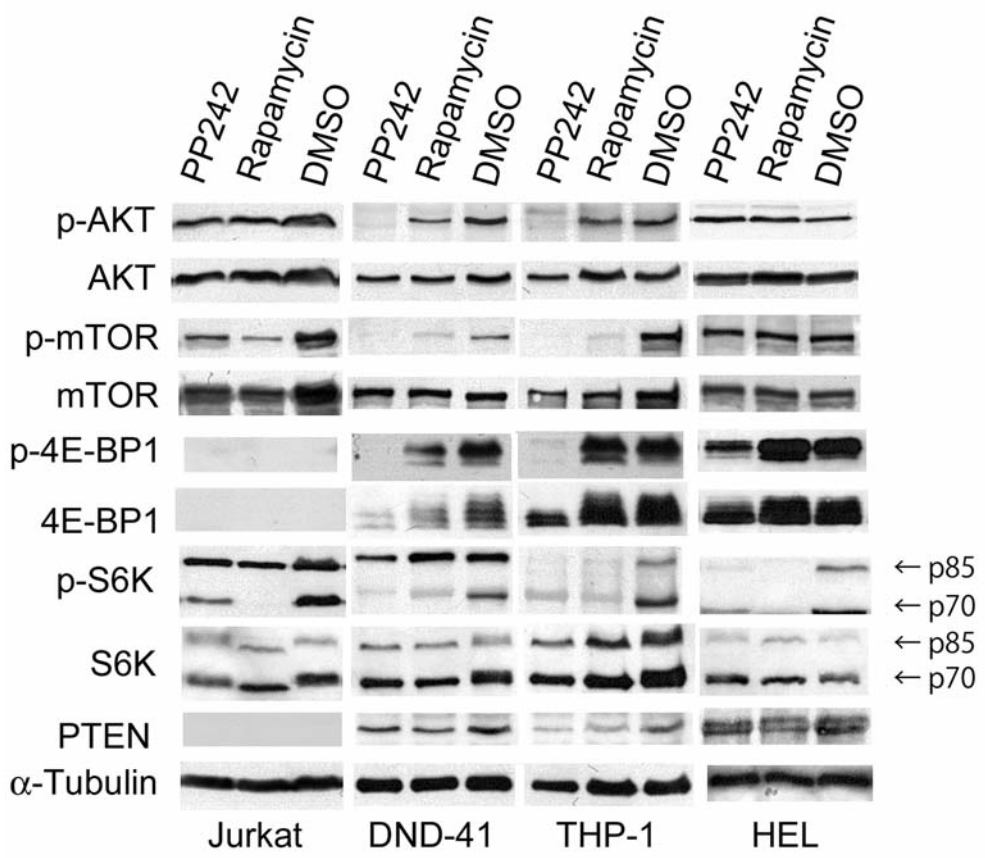

Figure 3. *Effects of PP242 and rapamycin on the expression and phosphorylation of proteins in mammalian target of rapamycin (mTOR) pathway. Cells were cultured with 0.5 μM inhibitors and dimethyl sulphoxide (DMSO) as a vehicle control for 24 h. The lysates were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoblotted with the indicated antibodies.*

groups. Both p70 S6K and p85 S6K were suppressed in two myeloid cell lines and one B-cell line, and only p70 S6K was suppressed in the other cell lines. The two isoforms are transcribed by a single gene due to alternative splicing. p70 is localised in the cytoplasm and p85, which has an additional 23 residues encoding a nuclear localising signal, is localised in the nucleus (12). The biological significance of our findings remains to be determined.

We also found that the suppression of mTOR signalling affects the expression and/or activation of the NOTCH protein. In Jurkat cells, PP242 up-regulated *NOTCH1* mRNA, NOTCH1 protein, and activated NOTCH1 fragment, leading to the subsequent induction of *HES1* mRNA. In contrast, in DND-41 cells, PP242 down-regulated NOTCH1 and NOTCH2 proteins, and reduced the amount of activated NOTCH1 without significant changes in *NOTCH1* and *NOTCH2* mRNA expression. The latter phenomenon may be due to the suppressive effect of PP242 on protein synthesis. The mechanism that underlies the former has not been determined thus far. Considering these findings and the report that Notch signalling activates mTOR signalling through the suppression of PTEN (5,6), we infer that the NOTCH and mTOR signalling modules participate in bi-directional crosstalk.

Recently, the effects of PP242 on the growth of BCR-ABL–positive leukemia cells (2), T-acute lymphoblastic leukemia (ALL) cell lines (14), and primary acute myeloid leukemia (AML) cells (15) were reported. Here, we compared the effects of PP242 and rapamycin on the growth and levels of signalling proteins of several leukemia cell lines. To our knowledge, this is the first report that shows the effect of mTOR signalling on NOTCH signalling. On the basis of these findings, we suggest that PP242 may find future applications as a molecular-targeted therapy in leukemia. Before its clinical use, however, its effects must be reviewed on a case-by-case basis.

Acknowledgements

This study was supported in part by a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science.

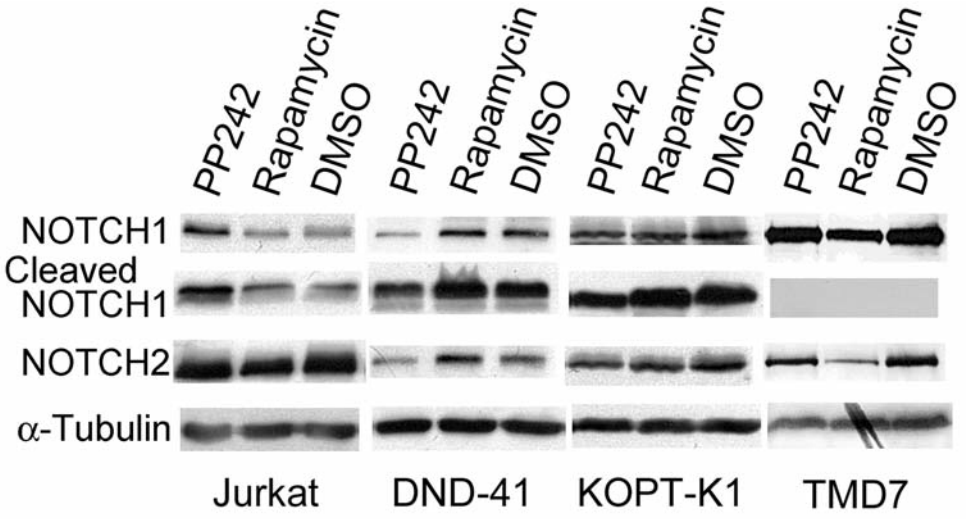

Figure 4. *Effects of PP242 and rapamycin on the expression and activation of NOTCH proteins. Cells were cultured with 0.5 μM inhibitors for 24 h. The lysates were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoblotted with the indicated antibodies.*

References

1 Chapuis N, Tamburini J, Green AS, Willems L, Bardet V, Park S, Lacombe C, Mayeux P and Bouscary D: Perspectives on inhibiting mTOR as a future treatment strategy for hematological malignancies. Leukemia *24*: 1686-1699, 2010.

2 Janes MR, Limon JJ, So L, Chen J, Lim RJ, Chavez MA, Vu C, Lilly MB, Mallya S, Ong ST, Konopleva M, Martin MB, Ren P, Liu Y, Rommel C and Fruman DA: Effective and selective targeting of leukemia cells using a TORC1/2 kinase inhibitor. Nat Med *16*: 205-213, 2010.

3 Okuhashi Y, Nara N and Tohda S: Effects of gamma-secretase inhibitors on the growth of leukemia cells. Anticancer Res *30*: 495-498, 2010.

4 Pancewicz J and Nicot C: Current views on the role of NOTCH signaling and the pathogenesis of human leukemia. BMC Cancer *11*: 502, 2011.

5 Palomero T, Sulis ML, Cortina M, Real PJ, Barnes K, Ciofani M, Caparros E,Buteau J, Brown K, Perkins SL, Bhagat G, Agarwal AM, Basso G, Castillo M, Nagase S, Cordon-Cardo C, Parsons R, Zúñiga-Pflücker JC, Dominguez M and Ferrando AA: Mutational loss of PTEN induces resistance to NOTCH1 inhibition in T-cell leukemia. Nat Med *13*: 1203-1210, 2007.

6 Wong GW, Knowles GC, Mak TW, Ferrando AA and Zúñiga-Pflücker JC: HES1 opposes a PTEN-dependent check on survival, differentiation, and proliferation of TCRβ-selected mouse thymocytes. Blood *120*: 1439-1448, 2012.

7 Lanotte M, Martin-Thouvenin V, Najman S, Balerini P, Valensi F and Berger R: NB4, a maturation-inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3). Blood *77*: 1080-1086, 1991.

8 Tohda S, Sakano S, Ohsawa M, Murakami N and Nara N: A novel cell line derived from de novo acute myeloblastic leukaemia with trilineage myelodysplasia which proliferates in response to a NOTCH ligand, Delta-1 protein. Br J Haematol *117*: 373-378, 2002.

9 Tohda S, Sato T, Kogoshi H, Fu L, Sakano S and Nara N: Establishment of a novel B-cell lymphoma cell line with suppressed growth by gamma-secretase inhibitors. Leuk Res *30*: 1385-1390, 2006.

10 Shan X, Czar MJ, Bunnell SC, Liu P, Liu Y, Schwartzberg PL and Wange RL: Deficiency of PTEN in Jurkat T-cells causes constitutive localization of Itk to the plasma membrane and hyperresponsiveness to CD3 stimulation. Mol Cell Biol *20*: 6945-6957, 2000.

11 Ming XF, Burgering BM, Wennström S, Claesson-Welsh L, Heldin CH, Bos JL, Kozma SC and Thomas G: Activation of p70/p85 S6 kinase by a pathway independent of p21ras. Nature *371*: 426-429, 1994.

12 Laser M, Kasi VS, Hamawaki M, Cooper G 4th, Kerr CM and Kuppuswamy D: Differential activation of p70 and p85 S6 kinase isoforms during cardiac hypertrophy in the adult mammal. J Biol Chem *273*: 24610-24619, 1998.

13 Choo AY, Yoon SO, Kim SG, Roux PP and Blenis J: Rapamycin differentially inhibits S6Ks and 4E-BP1 to mediate cell-type-specific repression of mRNA translation. Proc Natl Acad Sci USA *105*: 17414-17419, 2008.

14 Evangelisti C, Ricci F, Tazzari P, Tabellini G, Battistelli M, Falcieri E, Chiarini F, Bortul R, Melchionda F, Pagliaro P, Pession A, McCubrey JA and Martelli AM: Targeted inhibition of mTORC1 and mTORC2 by active-site mTOR inhibitors has cytotoxic effects in T-cell acute lymphoblastic leukemia. Leukemia *25*: 781-791, 2011.

15 Zeng Z, Shi YX, Tsao T, Qiu Y, Kornblau SM, Baggerly KA, Liu W, Jessen K, Liu Y, Kantarjian H, Rommel C, Fruman DA, Andreeff M and Konopleva M: Targeting of mTORC1/2 by the mTOR kinase inhibitor PP242 induces apoptosis in AML cells under conditions mimicking the bone marrow microenvironment. Blood *120*: 2679-2689, 2012.

Received December 25, 2012
Revised February 5, 2013
Accepted February 5, 2013