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

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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 Bioreresources (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).

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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.
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
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.

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References


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