The mTOR inhibitor, everolimus (RAD001), overcomes resistance to imatinib in quiescent Ph-positive acute lymphoblastic leukemia cells

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In Ph-positive (Ph+) leukemia, the quiescent cell state is one of the reasons for resistance to the BCR-ABL-kinase inhibitor, imatinib. In order to examine the mechanisms of resistance due to quiescence and the effect of the mammalian target of rapamycin inhibitor, everolimus, for such a resistant population, we used Ph+ acute lymphoblastic leukemia patient cells serially xenotransplanted into NOD/SCID/IL2rnull (NOG) mice. Spleen cells from leukemic mice showed a higher percentage of slow-cycling G0 cells in the CD34+ CD38− population compared with the CD34+ CD38+ and CD34− populations. After ex vivo imatinib treatment, more residual cells were observed in the CD34+ CD38− population than in the other populations. Although slow-cycling G0 cells were insensitive to imatinib in spite of BCR-ABL and CrkL dephosphorylation, combination treatment with everolimus induced substantial cell death, despite BCR-ABL and CrkL dephosphorylation, combination treatment with everolimus induced substantial cell death, including that of the CD34+ CD38− population, with p70-S6K dephosphorylation and decrease of MCL-1 expression. The leukemic non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice system with the in vivo combination treatment with imatinib and everolimus showed a decrease of tumor burden including CD34+ cells. These results imply that treatment with everolimus can overcome resistance to imatinib in Ph+ leukemia due to quiescence.

Materials and methods

Leukemic cells

Xenografts were established in NOD/SCID/IL2rnull (NOG) mice as previously described.15 Briefly, Ph+ ALL patient cells were serially xenotransplanted into immunodeficient NOG mice, and engrafted spleen cells were obtained 8–10 weeks after injection. Erythrocytes were removed by erythrocyte lysis buffer (EL-buffer; Qiagen, Hilden, Germany), and the remaining leukemic cells were preserved in liquid nitrogen until use. Leukemic repopulated cells were thawed and washed, resuspended in RPMI containing 10% fetal bovine serum, 5 mM MgCl2 and 0.2 mg/ml DNase I (Roche Diagnostics, Mannheim, Germany) and incubated at 37°C for 10 min. Cells were washed and resuspended at 1 million cells per ml in RPMI containing 20% fetal bovine serum with cytokines (human stem cell factor, 50 ng/ml, human interleukin-3 20 ng/ml and human granulocyte/macrophage-colony-stimulating factor, 20 ng/ml), and incubated with imatinib for 48 h at 37°C in a CO2 incubator. In an in vitro long-term culture, spleen (SP) cells derived from leukemic NOG mice were co-cultured with S17 stromal cells and treated with imatinib and everolimus.16 S-17 cells and leukemic cells were passaged twice weekly.

Reagents

Everolimus and imatinib were supplied by Novartis Institutes for Biomedical Research (Basel, Switzerland). Imatinib was dissolved in dH2O and used for in vitro and in vivo experiments.
Everolimus was stored as $10^{-2}$ M stock solution in dimethylsulfoxide for an in vitro experiment. For in vivo experiments, everolimus was formulated at 2% (wt/wt) in a microemulsion vehicle. Aliquots of everolimus and control vehicle were stored at $-20^\circ$C.

**Immunoblotting**

Antibodies against the phospho(p)-Abl (Tyr245), p-CrkL (Tyr245), p-mTOR (Ser2448), p-p70 S6 kinase (Thr389), p-4EBP1 (Thr70), MCL-1, p-akt (Ser473), AKT and p-FOXO1 (Thr24/FOXO3a/Thr32) were from Cell Signaling (Boston, MA, USA). Immunoblotting was performed with the standard protocols as previously described.

**Flow cytometric analysis and cell sorting**

After the treatment period, cells were washed at 4°C and then stained with anti-CD34-allophycocyanin (APC), anti-CD38-PE-Cy7 (Becton Dickinson, San Jose, CA, USA), and anti-CD45-APC-Cy7 antibodies and labeled with PI. PI staining with anti-CD38-APC, and flow-through cells containing CD34$^+$ cells. MACS-separated cells or drug-treated cells on S17 cells were then sorted and washed at 4°C. MACS-separated CD34$^+$ cells were stained with anti-CD34-APC, anti-CD38-PE-Cy7 and anti-CD45-APC-Cy7 antibodies and labeled with PI. PI staining with anti-CD38-APC, and flow-through cells containing CD34$^+$ cells were stained with anti-CD34-APC, anti-CD38-PE-Cy7 and anti-CD45-APC-Alexa Fluor 750, and were subsequently labeled with annexin-V–fluorescein isothiocyanate and propidium iodide (PI) according to the manufacturer’s protocol (Annexin-V–FLUOS Staining Kit; Roche Diagnostics). The cells were acquired by FACS Aria (Becton Dickinson) and analyzed by FlowJo software. DNA contents analysis was assessed using the standard procedure as previously described.

**Hematoxylin and eosin**

Of each CD34$^+$/CD38$^+$ subpopulation, a z-statistic was calculated for each experiment.

**Statistical analysis**

Differences among more than two groups were analyzed with the Bonferroni test followed by one-way analysis of variance. Statistical analyses were performed with STATA 9.2 software (StataCorp, College Station, TX, USA).

**Results**

Ex vivo treatment with imatinib for more residual quiescent CD34$^+$ CD38$^+$ population in Ph$^+$ ALL cells

We analyzed the cell cycle status of untreated spleen cells derived from the humanized Ph$^+$ ALL leukemia murine model reported previously. In the CD34$^+$ CD38$^+$ population, a higher percentage of Hoechst$^+$/Pyronin Y$^+$ slow-cycling quiescent cells was observed than in the CD34$^+$ CD38$^+$ population ($P < 0.05$) and CD34$^+$ population ($P < 0.01$). A lower percentage of the S + G2/M population was also observed among CD34$^+$ CD38$^−$ cells ($P < 0.05$; Figure 1a, Supplementary Figure S1a).

We next treated these cells with imatinib for 48 h and analyzed the distribution of CD34/CD38 in residual viable cells. After treatment with imatinib at 0.3, 1 and 3 μM, more residual CD34$^+$ CD38$^+$ cells were observed than non-treated cells (12.8 vs 27.2%; Figure 1b, $P < 0.05$; Supplementary Figure S1b). Significantly more slow-cycling quiescent ($G_0$) cells were observed within CD34$^+$ CD38$^−$ population after treated with 3 μM of imatinib ($P = 0.03$), and less $G_0$ cells in CD34$^+$ population ($P = 0.02$, Figure 1c).

Treatment of CD34/CD38 sorted cells for 6 h with 3 μM imatinib caused equivalent inhibition of the phosphorylation of BCR-ABL and direct-substrate CrkL in each CD34/CD38 sub-population (Figure 1d). Inhibition of phosphorylation with imatinib (1 and 3.3 μM) was also observed with intracellular staining of phospho-CrkL (Supplementary Figure S1c). Expressions of BCR-ABL and ABL were equivalent in each CD34/CD38 sub-population (Figure 1d). These results suggested that the slow-cycling population derived from Ph$^+$ leukemia NOG mice was insensitive to imatinib in spite of BCR-ABL dephosphorylation.

Ex vivo effects of everolimus on leukemic spleen cells, alone and in combination with imatinib

Furthermore, we have introduced S-17 murine stromal cell lines to support the leukemic spleen cells in order to assess longer-term effect of treatment drugs, as the CD34$^+$ population of leukemic cells from the NOG mice eventually differentiated into CD34$^−$ cells and could not be maintained only with cytokines.
for a longer period. If cultured with S17 cells, leukemic spleen cells were viable for more than 30 days (Figure 2a).

To examine the potential of everolimus to overcome resistance due to quiescence in Ph

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leukemia cells, everolimus treatment was investigated ex vivo alone and in combination with imatinib on S17 stromal cells. Everolimus treatment at 100 nm for 5 days increased the sub-G1 population (14 vs 7.9%; control), and combination of everolimus (100 nm) and imatinib (1 µM) further increased the sub-G1 population (68%, Figure 2b, upper panel). Cell cycle status was also investigated after

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**Figure 1** Ex vivo analysis of humanized mouse positive (Ph

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) acute lymphoblastic leukemia cells. (a) Leukemic spleen cells were CD34 positively selected with MACS column. CD34

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 cells were stained with Hoechst, Pyronin Y and CD38-allophycocyanin (APC). Cells including CD34

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 population that had flowed through the column were stained with Hoechst, Pyronin Y and CD34-APC. (b) Leukemic spleen cells were ex vivo cultured with cytokines and treated with or without imatinib (IM) for 48h. Human CD45

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 propidium iodide (PI)/Annexin-V viable population was analyzed for CD34 and CD38 distribution. Panels show a representative experiment. (c) After treated with IM for 48 h, CD34

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 cells were positively selected with MACS column, and stained with Hoechst, Pyronin Y and CD34-APC. Cells including CD34

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 population that had flowed through the column were stained with Hoechst, Pyronin Y and CD34-APC. Graphs show the number of forward scatter/side scatter gated G0 cells in each CD34/CD38 sub-population, each relative to the untreated control. Bars indicate mean ± s.d. values of three independent experiments (*P = 0.03 between control and IM 3 µM for CD34

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CD38

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, and **P = 0.02 between control and IM 3 µM for CD34

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, by one-way analysis of variance followed by Bonferroni). (d) CD34/CD38 sorted populations were treated with or without IM 3 µM for 6 h. Expression of BCR-ABL and phosphorylation of BCR-ABL and CrkL in each population was examined by western blotting analysis.
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Treatment with S-17 for 5 days. Although the untreated control and imatinib-treated cells showed 35% of Hoechst⁺/Pyronin Y⁻ cells in total acquired cells, combination of imatinib and everolimus decreased these quiescent cells to 13% of total acquired cells (Figure 2b, lower panels). Significant difference was found between imatinib alone and combination of imatinib plus everolimus (Figure 2c). Treatment with everolimus and imatinib for 5 days induced substantial cell death in CD38⁻/CD34⁻ population relative to dimethylsulfoxide control (Figure 2d). These results indicated that ex vivo combination treatment with imatinib and everolimus was also effective for the quiescent CD34⁻/CD38⁻ cells.

Evaluation of molecular biomarkers during cell death induced by treatment with imatinib and everolimus

We next investigated the effects of imatinib and everolimus on BCR-ABL and mTOR signaling. Separated CD34⁺ cells were treated with and without imatinib (1 μM) or everolimus (100 nM) for 4 h. After imatinib treatment, phosphorylation of BCR-ABL was clearly inhibited in each population, but it was not affected after everolimus treatment (Figure 3a). After everolimus treatment, the phosphorylation of S6K, which is a direct substrate of mTOR, was clearly inhibited; however, the phosphorylation of mTOR and 4EBP1 was not changed (Figure 3b). These results imply that everolimus inhibited mTOR signaling of CD34⁺ cells and induced cell death independently of the BCR-ABL signaling pathway. Both imatinib alone and in combination treatment inhibited phosphorylation of BCR-ABL. Conversely, everolimus alone and in combination both inhibited phosphorylation of S6K in both CD34⁻/CD38⁻ and CD34⁺/CD38⁻ sub-populations (Figure 3c). Everolimus alone or in combination with imatinib decreased the expression of the antiapoptotic BCL-2 family protein, MCL-1, after 4 h, and the combination of everolimus and imatinib also decreased the expression of MCL-1, not BCL-2, after 12 h (Figure 3c). These results implied that combination treatment with imatinib and everolimus induced cell death in quiescent Ph⁺ leukemia cells.

In vivo investigation of effects of everolimus, alone and in combination with imatinib

To elucidate the in vivo efficacy of everolimus treatment, its effects were investigated alone (5 mg/kg) and in combination with imatinib (100 mg/kg + everolimus 5 mg/kg) using NOD/SCID mice intravenously injected with leukemic spleen cells from...
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Figure 3 Western blot analysis of ex vivo-treated leukemic cells. (a) CD34⁺ cells were separated with MACS column and cells were treated with and without imatinib (IM, 3 μM) or everolimus (Eve, 300 nm) for 4 h. Each sample was lysed and western blotting analysis was performed with each antibody. (b) MACS-separated cells were treated with or without everolimus (100 nm) for 4 h. Each sample was lysed and western blotting was performed with p-mTOR, p-S6 K, p-4EBP1 antibodies. Immunoblotting by antitubulin was performed for the control. (c) CD34⁺ 38⁺ sorted cells were treated with treatment drugs for 4 or 12 h. Each sample was lysed and western blotting analysis was performed with each antibody.

Figure 4 In vivo effects of everolimus on leukemic spleen cells in combination with imatinib. (a) Non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice were irradiated (IR), and leukemic spleen (SP) cells from NOG (2 x 10⁷) were injected. Experiments were performed twice with three in each treatment group. Control vehicle (n=6), imatinib 100 mg/kg (n=6), everolimus 5 mg/kg (n=6) or a combination of both (n=6) were administered for 10 days, and mice were dissected 24 h after the last administration on day 28 following the tumor injection. (b, c) Percentages of CD19⁺ cells in peripheral blood (b) and bone marrow (c) are shown, respectively. (d) Spleen weight was relatively compared with the average of control mice in each experiment. Bars indicate average of spleen weight in each study group. (e) CD19⁺ leukemic spleen cell numbers were relatively compared with the average of control. *P<0.001, **P<0.005 and ***P<0.05 by one-way analysis of variance followed by Bonferroni. (f) Hematoxylin and eosin (HE) staining (left panels) and immunohistochemical analysis with CD34 (right panels) of the spleen from control mice and imatinib and/or everolimus-treated mice were performed. A charge-coupled device camera provided images at approximately ×100 of the original magnification.

humanized NOG mouse (Figure 4a). Percentage of CD19⁺ leukemic cells in peripheral blood was lowest in the imatinib-plus-everolimus-treated group, compared with the vehicle or imatinib alone (Figure 4b). Overall tumor burden, as assessed by spleen weight (Figure 4d) and the total number of splenic human CD19⁺ leukemic cells (Figure 4e), was observed to be lowest in the imatinib-plus-everolimus-treated group. Immunohistochemistry showed that the combination of imatinib plus everolimus...
decreased the infiltrated CD34⁺ human leukemic cells in spleen, liver and bone marrow (Figure 4f, Supplementary Figure S2). Everolimus alone also decreased the percentage of G0 cells in the CD34⁺ leukemic cells of the treated bone marrow (Supplementary Figure S3). These results indicated the in vivo efficacy of everolimus treatment in a Ph⁺ leukemia murine model.

Discussion

In this study, we showed the effects of everolimus in combination with imatinib against Ph⁺ ALL quiescent cells. Ex vivo imatinib treatment of Ph⁺ leukemia cells from a humanized mouse model showed more residual cells in the CD34⁺ CD38⁻ population, which contains significantly more quiescent cells. Our data showed an ex vivo effect against these residual cells, and the combination of imatinib and everolimus showed an in vivo effect. These data have shown the potential of everolimus to overcome imatinib resistance in quiescent cells. LSCs are reported to be responsible for the resistance to chemotherapy and molecular targeting agents. In chronic myeloid leukemia, non-proliferating quiescent CD34⁺ cells have been found to be more resistant than proliferating leukemic cells after treatment with several chemotherapeutic agents. Other studies have shown that inhibitors of mTOR with conventional therapies induced apoptosis and reduced LSCs. The definition of LSCs or cancer stem cells is sometimes controversial in certain diseases. In human AML, LSCs have been phenotypically identified within a CD34⁺CD38⁻ fraction. In contrast, it is controversial whether all LSCs exist within the CD34⁺ fraction and how CD34, CD38, CD19 and CD13 relate to all LSCs. In our current study, the potential of everolimus to overcome imatinib-resistant quiescent cells was demonstrated by using a humanized leukemic mouse model that maintains the differentiation hierarchy of Ph⁺ leukemia. However, it cannot be determined at this point if the real LSCs of Ph⁺ ALL can be diminished until the LSCs in this disease category are clarified.

MCL-1, an antiapoptotic member of the BCL-2 protein family, reportedly regulates the self-renewal of human hematopoietic stem cells as well as LSCs. Mills et al. also reported that MCL-1 was translationally regulated by mTORC1. Together with these reports, our results showing decreased expression of MCL-1 by combination treatment of imatinib and everolimus suggested that the combination treatment induced cell death of quiescent Ph⁺ leukemia cells by interfering with the mitochondrial-mediated cell death pathway. Rapamycin and its analogs are also known to induce autophagic cell death, and Bellodi et al. reported that target autophagy potentiates tyrosine kinase-induced cell death in Ph⁺ leukemia cells. We are also investigating the relation of autophagy in cell death in our experimental systems.

In this study, everolimus treatment of Ph⁺ leukemia cells from a humanized mouse model decreased the phosphorylation of S6K, but it increased the phosphorylation of AKT (Ser473) and FOXO1/3a (Supplementary Figure S4a). Rapamycin and its analogs, such as everolimus and temsirolimus, are allosteric mTOR inhibitors that function at a distance from the adenosine triphosphate-catalytic binding site. Of the two cellular protein complexes of mTOR, mTORC1 and mTORC2, mTORC1 is sensitive to these allosteric mTOR inhibitors and mTORC2 is resistant. mTORC2 directly activates AKT, and this AKT activation in a feedback loop has been reported to correlate with rapamycin failure. This feedback loop might also be related to our data on upregulated AKT.

Recently, a new generation of mTOR inhibitors has been developed. Dual PI3K/mTOR inhibitors, such as BEZ235, EX147 and PI-103, inhibit PI3K and both small molecules of mTORC1/2. Adenosine triphosphate-competitive mTOR inhibitors that selectively inhibit TORC1/2 molecules also have been reported to be effective against Ph⁺ transformed leukemia cells and to be less immunosuppressive than PI3K/mTOR inhibitors. The effectiveness of a new generation of mTOR inhibitors should also be investigated in our future studies, in particular, the efficacy of these inhibitors against quiescent or leukemic stem cells using a humanized leukemic mouse model. However, it was suggested that dual PI3K/mTOR inhibitors may cause a greater degree of immune suppression by affecting normal cell functions. Although we have examined the colony formation of CD34⁺ human umbilical cord blood and it was suggested that everolimus did not severely interfere with hematopoietic colony formation (data not shown), the effects of everolimus and the new-generation mTOR inhibitors on normal cells and immune functions must be investigated in future studies.

Acquired mutation in the BCR-ABL gene also causes primary and secondary treatment failure in Ph⁺ leukemia. Our data suggest that imatinib-resistant cell lines with T315I mutation (Supplementary Figures S4b and c) can be inhibited with everolimus with downregulation of the mTOR pathway (Supplementary Figure S4d). The in vivo effect of everolimus on T315I-mutated Ph⁺ leukemic cells is also indicated (Supplementary Figure S4e). Further study is needed to determine the effect of everolimus on T315I-mutated leukemia, especially in combination with a T315I inhibitor such as AP24534 (ponatinib).

In conclusion, we have investigated the imatinib and everolimus combination effect against human Ph⁺ quiescent leukemia cells utilizing a mouse model. Everolimus can improve the treatment of resistant Ph⁺ leukemia. These mice also provide the opportunity to evaluate the effects of new therapeutic modalities on leukemic cells in different stages of cell cycle.

Conflict of interest

Hitoshi Kiyoi received research grants from Novartis Pharma, Kyowa-Hakko Kirin Co. Ltd and Chugai Pharmaceutical Co. Ltd. Tomoki Naoe received research grants from Janssen Pharma, Novartis Pharma, Kyowa-Hakko Kirin Co. Ltd and Chugai Pharmaceutical Co. Ltd. The other authors have no conflict of interest.

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