

Effects of γ -Secretase Inhibitors on the Growth of Leukemia Cells

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Abstract. *Background:* Notch activation is involved in the growth of leukemia cells. γ -Secretase inhibitors (GSIs), which block Notch activation, may be candidates for molecular target therapy against leukemia. *Materials and Methods:* The effects of three kinds of GSIs: GSI-IX, GSI-XII and GSI-XXI, on the *in vitro* growth of various leukemia cell lines were examined. *Results:* The effects of GSI were diverse depending upon the combination of cells and GSI. GSI treatment suppressed the growth of most of the cell lines examined. Conversely, the growth of some cell lines were promoted by GSI-XXI. GSI-XXI treatment reduced the amount of cleaved Notch1 protein and HES1 mRNA in the cells, which means that it suppressed Notch activity. The treatment up-regulated mRNA of nuclear factor kappa-B1 (NFKB1) and *v-rel* reticuloendotheliosis viral oncogene homolog A (RELA), which can be a cause of growth promotion. *Conclusion:* The diverse effects of GSIs must be elucidated before clinical use because they can stimulate the growth of leukemia cells.

The fate of hematopoietic stem cells is regulated by Notch signaling. In bone marrow, Notch protein on hematopoietic stem cells is activated by the Notch ligand (such as Jagged1) on stromal cells. Namely, the binding of Notch ligands to Notch protein causes cleavage of the Notch transmembrane subunit using γ -secretase, and releases an intracellular fragment of Notch (ICN). ICN is translocated to the nucleus where it induces expression of hairy and enhancer of split 1 (HES1) and other genes (1).

Notch activation is involved in the abnormal growth of various hematological malignancies (2). It was reported that activating *NOTCH1* gene mutations are present in more than half of all acute T-lymphoblastic leukemia (T-ALL) cases (3).

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This fact indicates that Notch inhibitors may be attractive candidates for a novel molecular target therapy in hematological malignancies (4). Thus far, we and other investigators reported that γ -secretase inhibitors (GSIs), which block the release of ICN, suppress growth through induction of apoptosis (3, 5, 6).

In this study, we examined the effects of three kinds of GSI, with different molecular structures, on the *in vitro* growth of various leukemia cell lines.

Materials and Methods

Cells and GSIs. Nine leukemia cell lines which have been growing in culture in our laboratory were used. NB4 (derived from acute promyelocytic leukemia) was kindly provided by Dr. Lanotte (7). OCI-AML3 (acute myelomonocytic leukemia) was established at the Ontario Cancer Institute (Canada). THP-1 (acute monocytic leukemia), K562 (chronic myeloid leukemia) and Daudi (Burkitt leukemia/lymphoma) were supplied by the Japanese Cancer Research Resources Bank. TMD2 (chronic lymphoid leukemia in acute phase) was established in our laboratory (8). T-ALL cell lines, KOPT-K1, DND-41, and Jurkat were donated by Drs. Harashima and Orita (Fujisaki Cell Center, Japan). Three kinds of GSI, namely GSI-IX (DAPT), GSI-XII (Z-IL-CHO) and GSI-XXI (Compound E) were purchased from Calbiochem (USA). They were dissolved in dimethyl sulfoxide (DMSO).

Cell growth assay. The effects of GSI on cell growth were examined using a colorimetric WST-1 assay. Briefly, 1×10^3 cells were cultured in 0.1 ml of 10% fetal calf serum-supplemented RPMI-1640 medium (GIBCO BRL, USA) in the presence of increasing concentrations of GSI in 96-well culture plates. After 6-7 days, WST-1 and 1-methoxy-5-methylphenazinium methylsulfate (Dojindo Laboratories, Japan) were added at the manufacturers recommended concentrations. The optical density (OD) was measured using an ELISA plate reader. The growth is shown as a percentage of the mean OD value of control cells cultured without GSI.

Morphology. Cytospin preparations of cells cultured with GSI were stained with Wright stain and observed under light microscopy to evaluate morphological differentiation and apoptosis.

Immunoblotting. The effects of GSI-XXI on activation of Notch1 protein were examined by immunoblotting. Before and after culture

with 10 μ M GSI-XXI for 24 hours, cells were harvested and lysed. The lysates from 1×10^6 cells/lane were subjected to SDS-PAGE and immunoblotted with an anti-cleaved Notch1 (Val1744) antibody (Ab) (Cell Signaling Technology, USA) to selectively detect ICN, an anti-jagged1 Ab (Santa Cruz Biotechnology, USA), and anti- α -tubulin Ab (Abcam, USA) as a loading control.

Quantitative reverse transcription polymerase chain reaction (RT-PCR). The effects of GSI-XXI on gene expression were examined using quantitative RT-PCR. First-strand cDNAs were synthesized from RNA extracted from cells treated with 10 μ M GSI-XXI for 6 and 24 hours. Quantitative PCR was performed using QuantiTect primers (QIAGEN, Germany), LightCycler primer sets, a FastStart DNA Master SYBR Green I kit, and a LightCycler (Roche Diagnostics, Germany). The expression level of each mRNA was normalized by the corresponding β -actin (*ACTB*) mRNA level, which was measured concurrently.

Results

Effects of GSI on cell growth. Dose-response curves showing the effects of GSI on the growth of myeloid and lymphoid leukemia cells are shown in Figure 1. The growth of K562, KOPT-K1, and DND-41 cells was suppressed by the three kinds of GSI in a dose-dependent manner. The growth of OCI-AML3 and Daudi cells was suppressed by GSI-IX and GSI-XII, but not by GSI-XXI. The growth of NB4 was suppressed only by GSI-XII.

Interestingly, the growth of Jurkat cells was promoted by GSI-XXI whereas GSI-XII suppressed growth and GSI-IX had no significant effect. Similarly, the growth of THP-1 and TMD2 cells was promoted by GSI-IX and GSI-XXI, whereas GSI-XII suppressed the growth of THP-1 and had no significant effect on the growth of TMD2. We repeated the experiments at least three times to verify reproducibility. Thus, we found that GSI-IX and GSI-XXI had promoting effects on the growth of some cells. Therefore, we further examined the growth promotive effects of GSI, focusing on GSI-XXI and Jurkat cells.

Effects of GSI on morphology. Figure 2 shows the cytospin preparations of the representative cells treated with GSI-XXI. Apoptotic cells with nuclear condensation and apoptotic bodies were observed in K562 and KOPT-K1 cells treated with GSI-XXI. In THP-1 and Jurkat cells, increased mitosis was recognized in GSI-XXI-treated cells compared to control cells. Morphological differentiation was not recognized in any of the cell lines examined.

Effects of GSI on the cleavage of ICN. Figure 3 shows the results of immunoblotting in Jurkat cells treated with GSI-XXI. GSI-XXI treatment reduced the amount of ICN expressed, which indicates that the treatment suppressed Notch1 activation. The treatment did not affect the expression of Notch ligand Jagged1. These results suggest

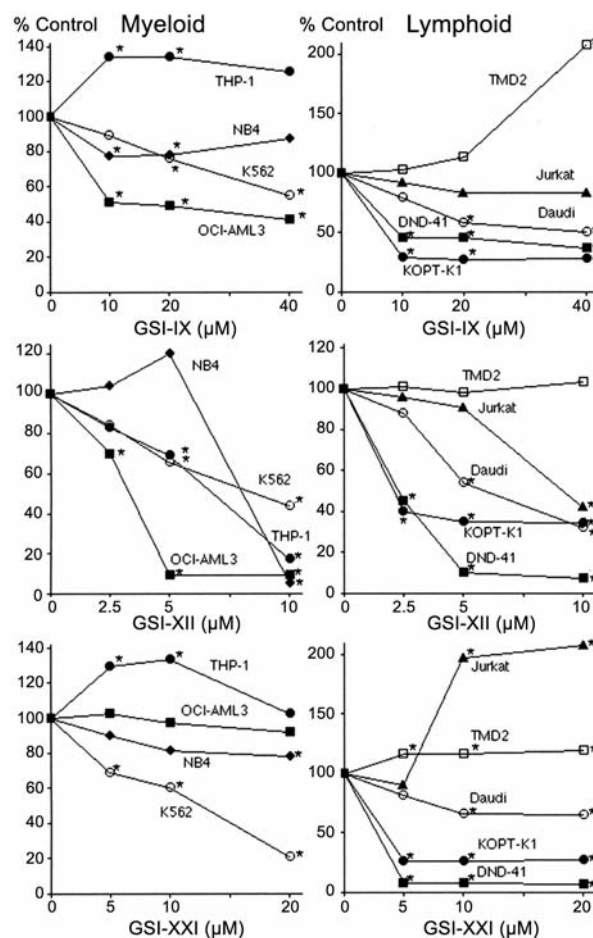


Figure 1. Dose-response curves of the effects of GSI-IX, GSI-XII and GSI-XXI on growth of myeloid and lymphoid leukemia cell lines. Cells were cultured with increasing concentrations of GSI. After 6-7 days, growth was examined using a colorimetric assay. Growth is shown as a percentage of the mean OD value of control cells cultured without GSI. * $p < 0.05$, significantly different from the control.

that the suppression of Notch activity is not due to the decrease of ligand protein but due to the inhibition of γ -secretase.

Effects of GSI on gene expression. To elucidate the mechanisms of how GSI-XXI exhibited different effects on the growth of Jurkat and KOPT-K1 cells, we examined gene expression in these cells treated with GSI-XXI (Figure 4). The expression levels are shown as a percentage of the level in control cells. Treatment with GSI-XXI reduced the expression of *HES1*, a well-known target gene of the Notch signal, in both cell types. Regarding the cell cycle-related genes (upper panel), the expression of *p18^{INK4C}* was up-regulated and that of cyclin D1 (*CCND1*) was down-regulated in KOPT-K1 cells. The changes in the expression

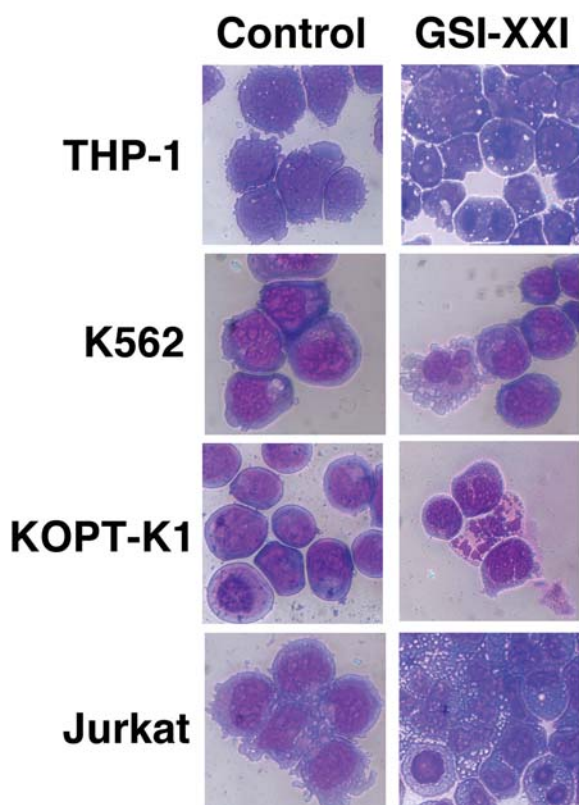


Figure 2. Effect of GSI-XXI on cell morphology of the representative cell lines. After culture with or without 10 μ M GSI-XXI for 6-7 days, cytopsin preparations of the cells were made, stained with Wright stain and observed under a light microscope (original magnification $\times 400$).

of these two genes in Jurkat cells were within 50% and 200%. Such changes are generally considered not significant in PCR experiments. Regarding the nuclear factor kappa B (NF- κ B)-related genes (lower panel), the expressions of *NFKB1* and v-rel reticuloendotheliosis viral oncogene homolog A (*RELA*) were up-regulated in Jurkat cells. The expression of *NFKB2* was down-regulated in Jurkat cells, while it was up-regulated in KOPT-K1 cells.

Discussion

In this study, we showed that the effects of GSIs are diverse depending upon the combination of cells and GSI used. Notably, GSI-XXI, a selective and potent GSI, stimulated the growth of three cell lines examined. Regarding T-ALL cells, GSI-XXI treatment suppressed the growth of KOPT-K1 and DND-41 cells, while it promoted the growth of Jurkat cells. Notch signaling is constitutively activated in these three cell lines, due to mutations in heterodimerization domain and proline, glutamic acid, serine, and threonine (PEST) domain

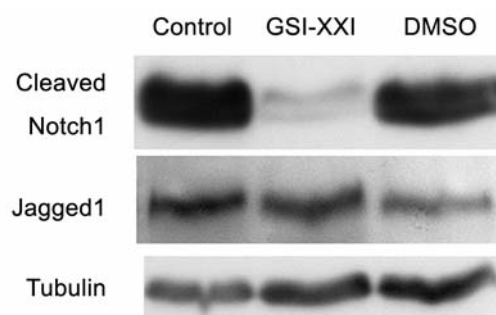


Figure 3. Effects of GSI-XXI on Notch1 and Jagged1 protein in Jurkat cells. Cells were cultured with GSI-XXI and DMSO as a vehicle control for 24 hours. The lysates were subjected to SDS-PAGE and immunoblotted with anti-cleaved Notch1 Ab, anti-Jagged1 Ab and anti- α -Tubulin Ab.

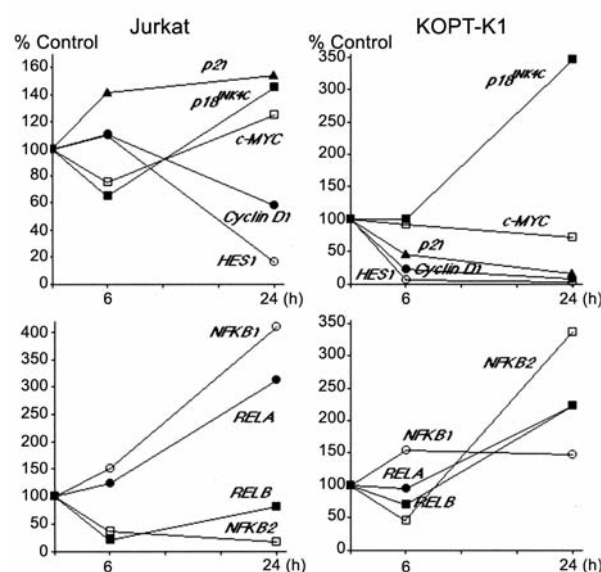


Figure 4. Expression of mRNA in Jurkat and KOPT-K1 cells treated with GSI-XXI. RNA was extracted from cells cultured with GSI-XXI for 6 and 24 hours. Quantitative RT-PCR for cell cycle-related genes (upper panel) and NF- κ B-related genes (lower panel) was performed. The expression level of each gene was normalized to the β -actin (*ACTB*) mRNA expression level. The expression levels at 6 hours and 24 hours are shown as a percentage of the levels in control cells.

in KOPT-K1 and DND-41 cells (3) and extracellular juxtamembrane expansion mutation in Jurkat cells (9). Therefore, we investigated the mechanisms behind this phenomenon focusing on GSI-XXI and Jurkat cells.

In general, GSI treatment inhibits Notch activation and suppresses growth through induction of apoptosis (5). As shown here, GSI-XXI treatment reduced ICN fragments and *HES1* mRNA in Jurkat cells, which means that GSI-XXI suppresses Notch activity, yet growth was promoted. It is

reported that gene mutations in phosphatase and tensin homolog (*PTEN*) (10) and F-box and WD repeat domain containing 7 (*FBW7*) (11) in Jurkat cells lead to GSI-resistance. However, these facts cannot explain the growth promotion observed. Therefore, to elucidate the mechanisms, we examined the expression of various genes in cells treated with GSI-XXI. The changes in the expression of some genes were different between KOPT-K1 cells and Jurkat cells. The relation between Notch inhibition and cell cycle was reported by others(12). We found that GSI-XXI treatment up-regulated the expression of *p18^{INK4C}*, one of the genes of inhibitors of the CDK4 family, in KOPT-K1 cells but not in Jurkat cells.

We also found that expressions of *NFKB1* and *RELA*, components of the canonical pathways in NF- κ B signaling, were up-regulated in GSI-XXI-treated Jurkat cells. The expression of *NFKB2*, a component of non-canonical pathways in NF- κ B signaling, was up-regulated in GSI-XXI-treated KOPT-K1 cells, while it was down-regulated in Jurkat cells. NF- κ B signaling plays important roles in cell growth (13). We reported the diverse relationship between NF- κ B and Notch (14). The activation of canonical NF- κ B pathways could be one of the causes of growth promotion of GSI-XXI-treated Jurkat cells. Further studies are needed to prove this hypothesis.

We showed the diversity of the effects of GSIs. We have to consider that these GSIs may act on pathways other than Notch signaling because many proteins, such as cadherin and CD44, are γ -secretase substrates (15). Growth promotion by GSI may involve growth-related proteins other than Notch and may effect the system other than by γ -secretase. GSIs may become excellent drugs to be used for molecularly-targeted therapy. However, we should be cautious about their clinical use because, as shown here, GSI can also stimulate the growth of leukemia cells. Recently, it was also reported that GSI treatment abrogates chemotherapy-induced apoptosis in T-ALL cells (16). Before clinical use of GSIs, their effects and the mechanisms behind them need to be fully clarified.

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