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γ-Secretase Inhibitors Induce Erythroid Differentiation in Erythroid Leukemia Cell Lines

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Abstract. *Background: Notch signaling regulates the fate of hematopoietic stem cells and leukemia cells. However, the role of Notch in erythroid differentiation remains unclear. Materials and Methods: We examined the effects of three γ-secretase inhibitors (GSI-IX, GSI-XII and GSI-XXI) that inhibit Notch signaling on the in vitro growth and differentiation of HEL and AA erythroid leukemia cell lines. Results: GSI treatment induced morphologic erythroid differentiation and promoted hemoglobin production. GSI treatment suppressed short-term growth and colony formation, while treatment with GSI-XXI promoted the growth of AA cells. The degree of differentiation induced by each GSI roughly correlated with the reduction in HES1 mRNA expression. Conclusion: GSIs have potential uses in differentiation induction therapy for erythroid leukemia in the future. Before clinical use, in vitro sensitivity tests should be performed because the effects of GSIs are diverse depending upon the combination of leukemia cells and GSIs.*

Notch signaling regulates the fate of normal hematopoietic stem cells and leukemia cells (1, 2). Binding of Notch protein on stem cells to Notch ligands (such as Jagged1) on stromal cells results in the release of the intracellular fragment of Notch (ICN) *via* the action of γ-secretase. This fragment translocates to the nucleus and induces expression of hairy and enhancer of split 1 (*HES1*) and other genes. The resulting signals regulate cell self-renewal, proliferation and differentiation, depending on the cellular context (2, 3).

The effects of Notch signaling on erythroid differentiation have been investigated by several research groups, although controversial results have been reported (4-7). Jang *et al.* reported that both down-regulation and constitutive activation of Notch-1 prevented pharmacologically induced erythroid differentiation in murine erythroleukemia cells (4). Ishiko *et al.* reported that Notch activation inhibited erythroid differentiation by suppressing GATA-1 activity (5). Henning *et al.* reported that Notch activation induced erythroid differentiation of FDCP-mix cells (6), and Robert-Moreno *et al.* reported that Notch activation induced apoptosis during erythroid differentiation (7).

Because Notch activation is involved in the growth of leukemia cells, Notch inhibitors are potential candidates for novel molecularly targeted therapy. γ-Secretase inhibitors (GSIs), which block the release of ICN, have been reported to suppress the growth of leukemia cells through the induction of apoptosis (8-10). The present study investigated the effects of three kinds of GSIs on the *in vitro* growth of erythroid leukemia cell lines, with the aim of determining the clinical suitability of GSIs for differentiation-induction therapy against erythroid leukemia.

Materials and Methods

Cells and GSIs. Two erythroid leukemia cell lines were used. The AA cell line was established by Dr. A. Arai from blast cells from a 72-year-old male with acute pure erythroid leukemia. AA cells were cultured in 10% fetal calf serum (FCS)-supplemented RPMI-1640 medium without erythropoietin. Flow cytometric analysis showed that AA cells strongly express glycophorin A. AA cells express Notch1 and Jagged1 proteins (data not shown) according to immunoblotting studies (11). HEL cells were supplied by the Japanese Collection of Research Bioresources (Ibaraki, Japan). Three kinds of GSIs, GSI-IX (DAPT), GSI-XII (Z-IL-CHO) and GSI-XXI (Compound E) were purchased from Calbiochem (USA) and dissolved in dimethyl sulphoxide (DMSO).

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

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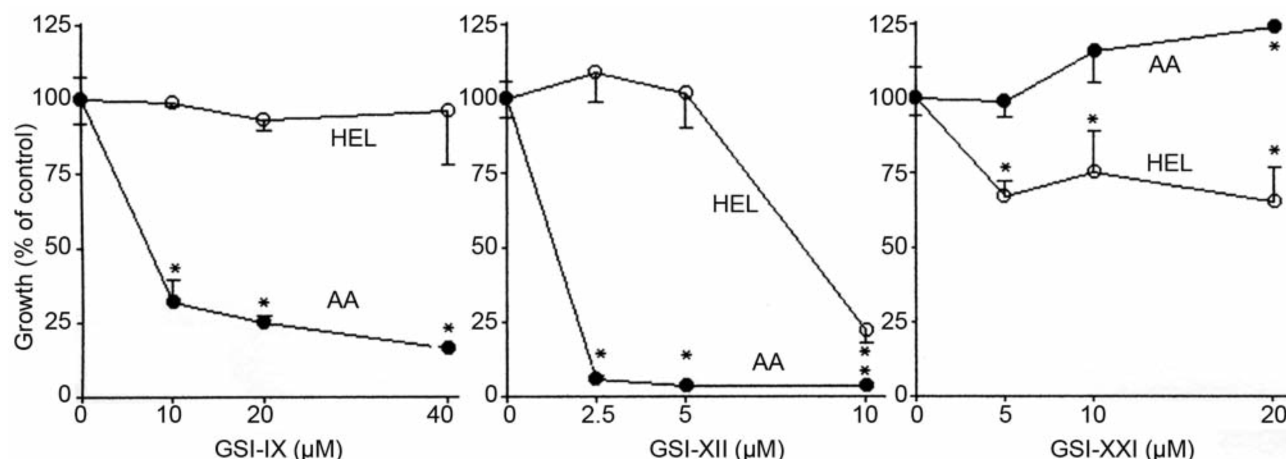

Figure 1. *Dose–response curves for the growth of AA and HEL cells treated with γ-secretase inhibitors (GSIs). Cells were cultured with increasing concentrations of GSIs. Growth was examined after 7 days using a colorimetric assay. Growth is shown as a percentage of the mean optical density value of control cells. *p<0.05, Significantly different from the control.*

Colony assay. The effects of the GSIs on colony formation were examined in methylcellulose cultures. Using 96-well culture plates, 2×10^2 cells/well were plated in RPMI-1640 medium containing 0.8% methylcellulose and 10% FCS, with or without GSIs. Colonies containing more than 20 cells were counted under an inverted microscope after 7 days in culture.

Cell differentiation. Cytospin preparations of cells from 5-day suspension cultures with GSIs were stained with Wright solution and observed under a light microscope to examine cell morphology. Hemoglobin production was evaluated by diaminofluorene (DAF) staining (12). The DAF stock solution contained 1% 2,7-diaminofluorene in 90% acetic acid. The working solution was prepared by mixing 0.1 ml of DAF stock solution with 0.1 ml of hydrogen peroxide and 10 ml of 0.2 M Tris-HCl buffer (pH 7.0). DAF working solution was added to the cell suspension or was layered onto the methylcellulose cultures. Cells or colonies stained blue after 10 minutes were considered to be hemoglobin positive.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR). The effects of GSIs on gene expression were examined using quantitative RT-PCR. First-strand cDNAs were synthesized from RNA extracted from cells treated with GSIs for 24 hours. Quantitative PCR was performed using a FastStart DNA Master SYBR Green I kit, a LightCycler (Roche Diagnostics, Germany) and LightCycler primer sets for *β-ACTIN* and *HES1*. The expression level of mRNA was normalized to the corresponding *β-ACTIN* mRNA level, which was measured concurrently.

Results

Effects of GSIs on cell growth. Dose response curves showing the effects of GSIs on short-term cell growth are shown in Figure 1. The concentrations of the three GSIs were chosen based on a lack of non-specific toxicity in previous studies (10). The growth of HEL cells was suppressed by GSI-XII and GSI-XXI. The growth of AA cells was suppressed by GSI-IX and GSI-XII, but was slightly promoted by 20 μM GSI-XXI.

Effects of GSIs on colony formation. The effects of GSIs on colony formation by AA cells were examined. The mean number of colonies per well from cells treated with DMSO (control), 20 μM GSI-IX, 5 μM GSI-XII and 10 μM GSI-XXI were 83, 71, 5, and 91, respectively. The number of colonies from GSI-XII-treated cells was significantly decreased, while that from GSI-XXI-treated cells was significantly increased compared with DMSO-control cells ($p<0.05$). Colony assays could not be performed for HEL cells because they proliferated diffusely in methylcellulose, and the exact colony numbers could not be counted.

Effects of GSIs on cell differentiation. Cytospin preparations of GSI-treated cells are shown in the upper column in Figure 2. The GSI-treated cells had reddish cytoplasm and round nuclei with condensed chromatin. These findings suggest that GSI treatment induced erythroid differentiation. These changes were especially evident in GSI-IX-treated HEL cells and GSI-XXI- and GSI-XII-treated AA cells.

Representative colonies of GSI-treated AA cells are shown in the lower column in Figure 2. Without staining, the colonies of GSI-treated cells were more reddish in color than the control colonies. After DAF staining, the colonies of GSI-treated cells became blue, compared with the control colonies, indicating an increase in hemoglobin production.

DAF-positive and -negative cells could be distinguished in suspension cultures of AA cells, and the percentages of positively stained cells were therefore counted (Figure 3). In the absence of GSIs, 39% of AA cells were DAF positive, and this percentage was significantly increased following GSI treatment.

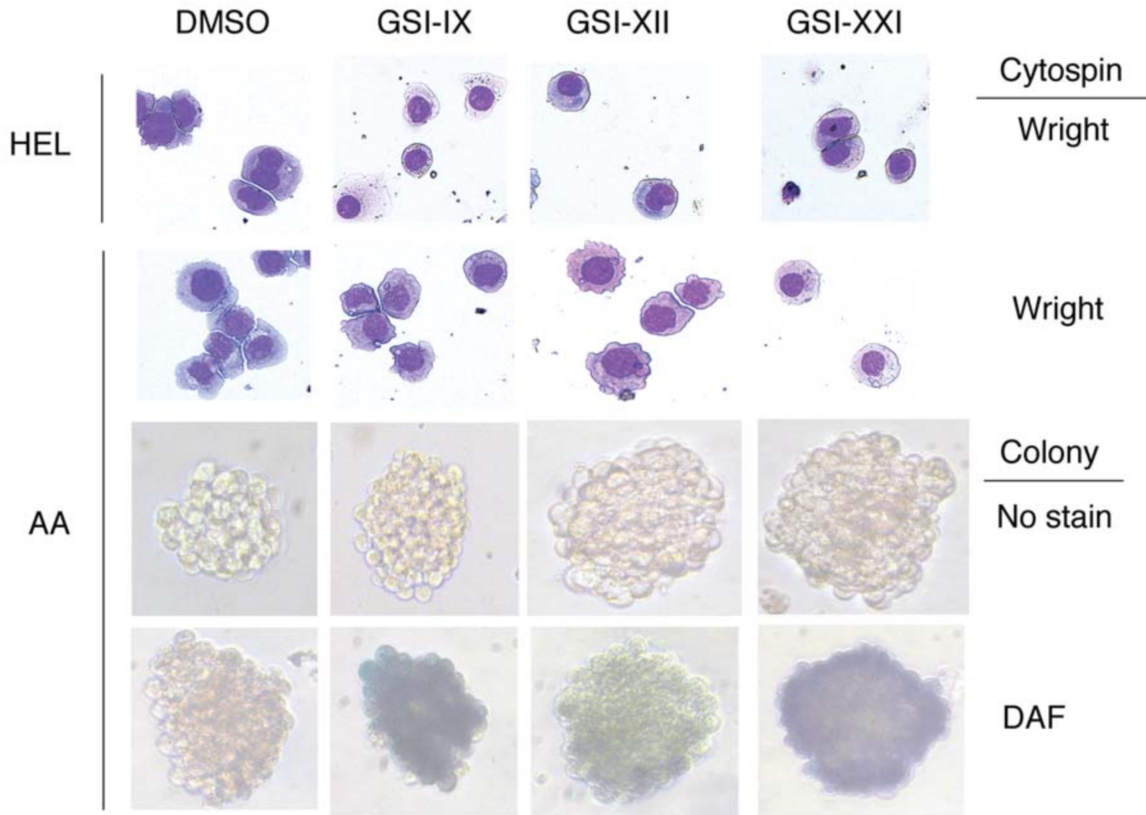

Figure 2. *Effect of γ-secretase inhibitors (GSIs) on morphological differentiation. Cells were cultured in suspension and in methylcellulose with 20 μM GSI-IX, 5 μM GSI-XII or 10 μM GSI-XXI. After 7 days, cytospin preparations of the cells in suspension were stained with Wright solution and observed under a light microscope (original magnification, ×400). Colonies were observed under an inverted microscope before and after diaminofluorene (DAF) staining.*

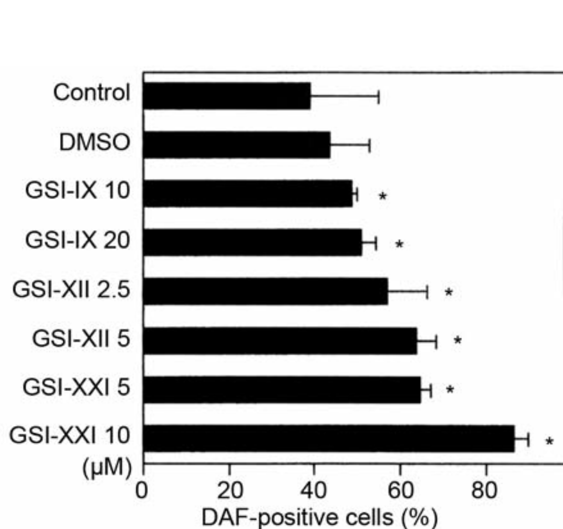

Figure 3. *Effect of γ-secretase inhibitors (GSIs) on hemoglobin production in AA cells. Cells were cultured in suspension with GSIs for 5 days, stained with diaminofluorene (DAF) and observed in counting chambers under a microscope. Blue-stained cells were counted as positive cells. The percentages of positive cells are shown.*

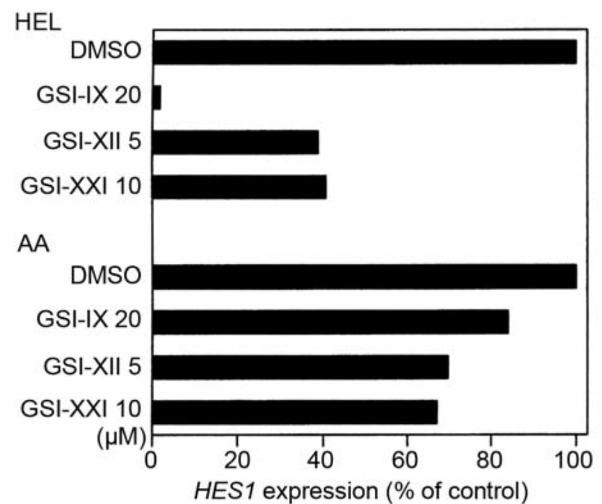

Figure 4. *Effect of γ-secretase inhibitors (GSIs) on HES1 mRNA expression. RNA was extracted from cells cultured with GSIs for 24 hours. Quantitative reverse transcription-polymerase chain reaction was performed. The expression level of HES1 was normalized to the β-ACTIN mRNA expression level. The expression levels are shown as a percentage of the levels in DMSO-treated control cells.*

Effects of GSIs on gene expression. Figure 4 shows the results of quantitative RT-PCR. GSI treatment reduced the expression of *HES1*, a well-known target gene of Notch signaling, in both cell lines, suggesting that treatment with these GSIs suppressed Notch signaling. We also examined the effects of GSIs on GATA binding protein 1 (*GATA1*) mRNA expression, which is known to be involved in erythroid differentiation (5). The expression of *GATA1* was not significantly affected by GSI treatment in either cell line (data not shown).

Discussion

The role of Notch in the activation or inhibition of erythroid differentiation remains unclear. However, the results of the present study demonstrate that GSI treatment induced erythroid differentiation in two erythroid leukemia cell lines, suggesting that inhibition of Notch was associated with this process. The degree of differentiation appeared to be related to the decrease in *HES1* mRNA expression, such that greater *HES1* mRNA suppression was associated with more differentiation, as shown in HEL cells treated with 20 μM GSI-IX and AA cells treated with 10 μM GSI-XXI.

GSI treatment suppressed short-term cell growth and colony formation, while treatment with GSI-XXI promoted the growth of AA cells. The growth suppression did not appear to be related to the suppression of *HES1* mRNA expression, because although the growth of AA cells was potently suppressed by GSI-IX and GSI-XII, the decrease in *HES1* mRNA expression was only slight. This apparent discrepancy could be due to the fact that GSIs act on pathways other than Notch signaling, because many proteins, such as cadherin and CD44, are also γ-secretase substrates (13).

Regarding the possible mechanism of the promotion of AA cell growth by GSI-XXI, GSI-XXI may prompt the differentiation of already differentiating cells, while stimulating the leukemia stem/progenitor cells of AA cells, as we previously reported that GSIs can stimulate the growth of some leukemia cell lines (10).

Although the precise mechanisms responsible for the effects of GSIs remain to be determined, GSIs have potential use in molecularly targeted or differentiation-induction therapy against erythroid leukemia. However, the current study also indicates the possibility that GSIs can stimulate the growth of some leukemia cells. Therefore, *in vitro* drug-sensitivity tests should be performed before GSIs are introduced into clinical use.

Acknowledgements

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