

Effects of Combination of Notch Inhibitor plus Hedgehog Inhibitor or Wnt Inhibitor on Growth of Leukemia Cells

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Abstract. *Background:* The Notch inhibitors, γ -secretase inhibitors (GSIs), are promising candidates for molecular targeted therapy against leukemia. However, they show only limited effectiveness. We thought that the efficacy of GSIs might be improved by their combination with Hedgehog inhibitors and Wnt inhibitors because these signaling pathways are also important for the growth of leukemia cells. *Materials and Methods:* The effects of the combination of GSI-XXI plus the Hedgehog inhibitor, cyclopamine (Cy), or the Wnt inhibitor, quercetin (Qu), on the *in vitro* cell growth, colony formation and Notch1 protein expression of three T-cell acute lymphoblastic leukemia (T-ALL) cell lines with NOTCH1 mutations and three acute myeloid leukemia cell lines were examined. *Results:* The addition of Cy or Qu to GSI suppressed the growth of DND-41 T-ALL cells additively or synergistically, respectively. Interestingly, Cy treatment and Qu treatment reduced Notch1 protein and its active fragment in DND-41 cells, which suggests a relationship between Notch signaling and Hedgehog or Wnt signaling. The addition of Cy or Qu to GSI promoted the decrease of Notch1 activation and expression. *Conclusion:* The anti-leukemic effects of a GSI could be promoted by its combination with Cy or Qu, in appropriate cases selected by *in vitro* drug sensitivity test.

Notch activation is involved in the growth of leukemia cells (1), especially T-cell acute lymphoblastic leukemia (T-ALL) cells (2). Activating NOTCH1 gene mutations are present in half of T-ALL cases. This suggests that γ -secretase inhibitors (GSIs), which block Notch activation, may be candidates for molecular targeted therapy against T-ALL. However, a clinical trial of a GSI for refractory T-ALL showed only limited effectiveness (3). It is possible that the efficacy of

GSI may be improved by their combination with other drugs; indeed the combination of a GSI plus a glucocorticoid was reported to improve its effectiveness (4).

Thus far, GSIs have been reported to suppress the *in vitro* growth of various leukemia cell lines, including some acute myeloid leukemia (AML) cells without NOTCH1 mutations (5). We also reported that the steroidal alkaloid cyclopamine (Cy), which blocks Hedgehog signaling, and the flavonoid quercetin (Qu), which blocks Wnt signaling, suppressed the growth of leukemia cells (6). Both signaling pathways are known to be involved in the growth of leukemia cells, especially in the self-renewal regulation of stem cells (7-9). In this study, we examined the effects of a GSI plus relatively low concentrations of Cy or Qu on the growth of human leukemia cell lines in order to find a way to improve the efficacy of GSIs.

Materials and Methods

Cells. Three T-ALL cell lines with NOTCH1 mutations (DND-41, KOPT-K1 and Jurkat) and three AML cell lines (NB4, HL60 and OCI/AML-3) were used. The T-ALL cell lines were gifts from Drs. Harashima and Orita (Fujisaki Cell Center, Okayama, Japan). NB4 (10) was kindly provided by Dr. M. Lanotte (Institut Universitaire d'Hématologie, Paris, France). OCI/AML3 was established at Ontario Cancer Institute (Toronto, Canada). HL-60 was supplied by the Japanese Cancer Research Resources Bank (Tokyo, Japan).

Cell growth assay. We examined the effects of the agents on cell growth using a colorimetric WST-1 assay (5). Briefly, cells were cultured in 96-well culture plates in 10% fetal calf serum-supplemented RPMI-1640 medium in the presence of 10 μ M GSI-XXI (Compound E; Calbiochem, La Jolla, USA), Cy (Toronto Research Chemicals, North York, Canada) and Qu (Sigma Chemical Co., St. Louis, USA) dissolved in dimethylsulfoxide (DMSO), either alone or in combination. After 7 days, WST-1 reagent (Dojindo Laboratories, Kumamoto, Japan) was added and the optical density (OD) was measured using an enzyme-linked immunosorbent plate reader. The experiments were repeated three times independently to verify the reproducibility of the results.

Colony assay. The effects of the agents on the clonogenicity of the cells were then evaluated using colony assays. Cells were cultured

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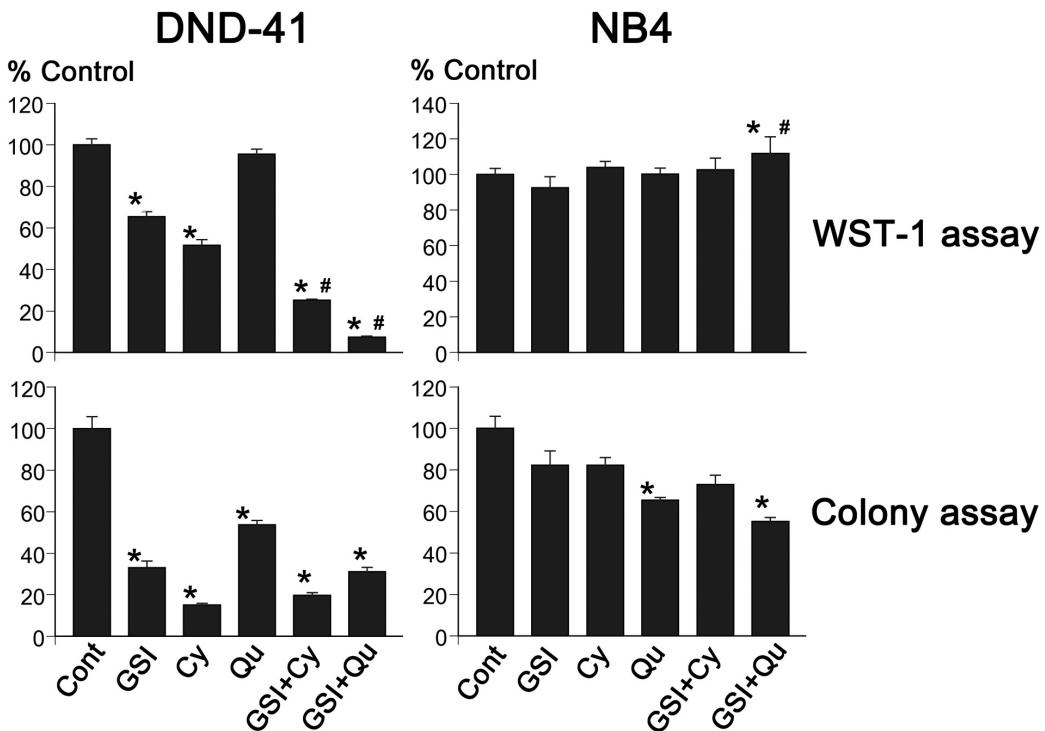


Figure 1. Effects of GSI-XXI, cyclopamine (Cy), and quercetin (Qu), alone or in combination, on the growth of DND-41 and NB4 cells examined using WST-1 assays (upper panel), and on clonogenicity using colony assays (lower panel). Data are shown as a percentage of the mean OD value and mean colony numbers relative to the respective control (treated with DMSO). * $p<0.05$, Significant difference from the control; # $p<0.05$, effect of the combination treatment was significantly different from that of GSI alone.

in methylcellulose in the presence of the three drugs, either alone or in combination. Colony numbers were counted under a microscope after 7 days.

Immunoblot analysis. We examined the molecular effects of the agents on Notch signaling using immunoblot analysis (6). Cells cultured for 24 hours with 10 μ M of each drug, either alone or in combination, were harvested and lysed. The lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with an anti-cleaved NOTCH1 (Val1744) antibody (Ab) (Cell Signaling Technology, Beverly, USA) to selectively detect the active fragment of NOTCH1, an anti-NOTCH1 Ab (C-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti- α -tubulin Ab (Abcam, Cambridge, MA, USA) as a loading control.

Results

Effects of combination of the inhibitors on cell growth and colony formation. The results from representative cells are shown in Figure 1. Data are shown as a percentage of the mean OD values or mean colony numbers relative to the respective control. Growth of DND-41 cells was suppressed by treatment with either GSI or Cy alone. The addition of Cy to GSI additively suppressed cell growth, while the addition

of Qu to GSI synergistically suppressed the growth. The clonogenicity of DND-41 cells was suppressed by all three drugs. The addition of Cy or Qu to GSI did not significantly alter the effect. The growth of NB4 cells was unaffected by the three drugs, although the combination of GSI plus Qu slightly promoted cell growth. The clonogenicity of NB4 cells was suppressed by Qu treatment, suggesting that Qu can suppress leukemic stem/progenitor cells without suppressing the whole cell population of these two cell lines, at least at the concentration used here. The combination of GSI plus Cy or Qu did not promote the suppression of clonogenicity of NB4 cells. In the other four cell lines, the effects of the combinations of GSI with either Cy or Qu were similar to those of GSI alone (data not shown).

Effects of combination of the inhibitors on Notch protein. GSI treatment of DND-41 cells (Figure 2, left panel) decreased active Notch1 fragment without decreasing Notch1 protein itself. Interestingly, Cy treatment decreased Notch1 protein as well as its active fragment. Qu also decreased Notch1 protein and only faintly decreased its active fragment. The combination of GSI plus Cy slightly promoted the decrease of active fragment. The combination of GSI plus Qu promoted the decrease of NOTCH1 protein. NB4 cells, which have no

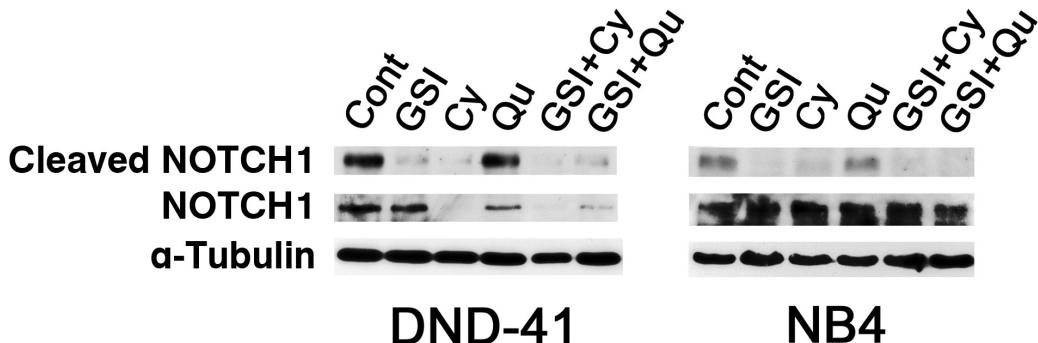


Figure 2. Expression of *NOTCH1* protein and active fragment of *NOTCH1* in cells treated with GSI-XXI, cyclopamine (Cy), and quercetin (Qu), alone or in combination. Lysates from the cells treated for 24 hours were subjected to SDS-PAGE and immunoblotted with anti-cleaved *NOTCH1* (Val1744), anti-*NOTCH1* C-terminus, and anti- α -tubulin antibodies.

NOTCH1 mutations, show slight expression of active NOTCH1 fragment (Figure 2, right panel), and it is possible that the NOTCH ligand, JAGGED1 protein, which is expressed by NB4 cells (11), activates NOTCH1 protein in the cells. GSI treatment reduced active NOTCH1 fragment without reducing NOTCH1 protein. Interestingly, Cy treatment also reduced the active fragment without reduced NOTCH1, in contrast to the effect of Cy in DND-41 cells.

Discussion

Although GSIs looked to be promising new molecularly-targeted drugs for leukemia, they have shown only limited effectiveness to date (3). It is reported that gene mutations in phosphatase and tensin homolog (*PTEN*) (12) and F-box and WD repeat domain containing 7 (*FBW7*) (13) lead to GSI resistance. However, these mutations cannot fully account for GSI-ineffectiveness. To find a method to improve the efficacy of GSIs, we examined the effects of a GSI plus a Hedgehog inhibitor and a Wnt inhibitor on the growth of leukemia cells.

In this study, we showed that the addition of Cy or Qu to GSI promoted the suppressive effect on growth of DND-41 cells. Interestingly, we found that Cy treatment suppressed NOTCH1 expression in DND-41 cells and NOTCH1 activation in NB4 cells. Qu treatment also slightly suppressed Notch1 expression in DND-41 cells, as we showed previously (6). To our knowledge, this is the first report to suggest a relationship between Notch signaling and Hedgehog signaling in leukemia cells. The findings that GSI plus Cy or Qu promoted the decrease of NOTCH1 activation and expression of DND-41 cells can be one of the mechanisms by which GSI plus Cy or Qu promoted growth suppression. In order to elucidate the mechanism by which Cy treatment reduced the active fragment without reduced NOTCH1 protein in NB4 cells, we examined the effect of Cy treatment on JAGGED1 expression, and found that its

expression was unaffected (data not shown). Therefore, the mechanism and the role of Notch activation on the growth of NB4 cells could not be elucidated.

Taken together, these results suggest that the anti-leukemic effects of a GSI could be promoted by its combination with Cy or Qu, in appropriate cases selected using *in vitro* drug sensitivity test. However, the molecular mechanism of how Cy and Qu affect NOTCH1 expression and activation remains to be determined. The mechanism seems to be different among the samples because the findings of immunoblot analysis for DND-41 cells were different from those for NB4 cells. Moreover, the off-target effects of Cy or Qu treatment must be elucidated before their clinical use is feasible. Combinations of these inhibitors, based on a further understanding of the relationships between these signaling pathways, might provide the basis for novel anti-leukemia treatments.

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