

Cyclopamine and Quercetin Suppress the Growth of Leukemia and Lymphoma Cells

TATSUO KAWAHARA, NORIKO KAWAGUCHI-IHARA, YUKI OKUHASHI,
MAI ITOH, NOBUO NARA and SHUJI TOHDA

*Department of Laboratory Medicine, Tokyo Medical and Dental University,
1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan*

Abstract. *Background: Hedgehog (Hh) and Wnt signaling pathways are involved in the stimulation of growth of leukemia and lymphoma cells. In the present study, whether or not the Hh inhibitor, cyclopamine, and the Wnt inhibitor, quercetin, suppress cell growth was investigated. Materials and Methods: The effects of cyclopamine and quercetin on the in vitro growth and protein expression of ten acute leukemia and B-cell lymphoma cell lines were examined. Results: Cyclopamine and quercetin suppressed cell growth and induced apoptosis in seven and eight cell lines respectively. Cyclopamine decreased the level of Gli1 protein, a target gene product of Hh signaling. Quercetin decreased the level of Notch1 protein and its active fragment in the DND-41 T-lymphoblastic leukemia cell line with constitutive Notch activation. Conclusion: Cyclopamine and quercetin suppress the growth of a number of leukemia and lymphoma cells. This finding suggests the potential use of these compounds in molecularly-targeted therapy for leukemia and lymphoma.*

Notch, Hedgehog (Hh), and Wnt signaling are involved in the self-renewal of hematopoietic stem cells and the growth of hematological malignancies (1-4). We have previously reported that Notch and Wnt signaling were involved in the self-renewal capacity of acute myeloblastic leukemia (AML) cells (4, 5). Sonic Hh has also been found to be expressed in cells derived from hematological malignancies (6). These pathways are considered as potential candidate targets for molecular therapy. We have previously demonstrated that

Correspondence to: Shuji Tohda, MD, Department of Laboratory Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. Tel: +81 358035334, Fax: +81 358035629, e-mail: tohda.mlab@tmd.ac.jp

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Notch inhibitors suppressed the growth of leukemia cells (7). It is therefore possible that Hh and Wnt inhibitors might also suppress their growth.

The Hh signaling cascade is as follows: in the absence of Hh ligands, the activity of Smoothed (Smo) is suppressed by Patched (Ptch), which is a receptor of Hh ligands; ligand binding to Ptch releases Smo, which then activates the Gli transcription factor. Gli induces the transcription of target genes such as Ptch1, cyclin D and Gli1 itself. The steroidal alkaloid cyclopamine blocks Hh signaling by direct binding to Smo (8).

The Wnt signaling pathway is as follows: in the absence of Wnt ligands, glycogen synthase kinase 3 β (GSK3 β) phosphorylates β -catenin, which is then degraded by proteasomes; Wnt ligand binding to receptors inhibits the activity of GSK3 β , resulting in the accumulation of β -catenin; the accumulated β -catenin translocates to the nucleus and induces the expression of growth-related genes (5). The flavonoid quercetin blocks Wnt signaling by inhibiting the transcriptional activity of β -catenin (9).

In this study, the effects of cyclopamine and quercetin on the growth of leukemia and lymphoma cell lines in culture were investigated. Furthermore, the mechanisms responsible for their effects were examined, with the aim of establishing a potential therapeutic use for these drugs for the treatment of leukemia and lymphoma.

Materials and Methods

Cells. Five myeloid leukemia cell lines, HL60, TMD7 (AML), NB4 (acute promyelocytic leukemia), HEL (erythroleukemia) and K562 (chronic myeloid leukemia), three T-lymphoblastic leukemia (T-ALL) cell lines, Jurkat, KOPT-K1 and DND-41, and two B-cell lymphoma cell lines, Daudi (Burkitt lymphoma) and TMD8 (diffuse large B-cell lymphoma) were used. TMD7 (10) and TMD8 (11) were established in our laboratory. NB4 was kindly provided by Dr. Lanotte (12). The T-ALL cell lines were donated by Drs. Harashima and Orita (Fujisaki Cell Center, Okayama, Japan). The other cells were supplied by the Japanese Collection of Research Bioresources (Ibaraki, Japan).

Inhibitors. Cyclopamine, tomatidine hydrochloride and quercetin were purchased from Toronto Research Chemicals (North York, Canada), Sigma Chemical Co. (St. Louis, USA) and Calbiochem (La Jolla, USA), respectively. Tomatidine is a steroidal alkaloid that does not inhibit Hh signaling and was used as a negative control for cyclopamine. The drugs were dissolved in dimethyl sulphoxide (DMSO), or in ethanol in the case of cells whose growth was affected by DMSO.

Cell growth assay. The effects of the inhibitors on short-term growth were examined using a colorimetric assay (WST-1 assay). Briefly, cells (1×10^4 cells/well) were cultured in 0.1 ml of 10% fetal calf serum (FCS)-supplemented RPMI-1640 medium (GIBCO, Grand Island, USA) in the absence or presence of increasing concentrations of inhibitors. After three days, WST-1 and 1-methoxy PMS (Dojindo Laboratories, Kumamoto, Japan) were added according to the manufacturer's protocol. The optical density (OD) was measured using an enzyme-linked immunosorbent assay plate reader and growth was expressed as a percentage of the mean OD value of the control cells. The experiments were repeated at least three times, independently, to verify the reproducibility of the results.

Colony assay. The effects of the inhibitors on colony formation were examined in methylcellulose cultures. In 96-well culture plates, 1×10^4 cells/well were plated in 0.1 ml of RPMI-1640 medium containing 0.8% methylcellulose and 10% FCS, with or without the inhibitors. After 7 days in culture, colonies containing more than 20 cells were counted under an inverted microscope.

Morphology. Cytospin preparations of cells cultured with the inhibitors were stained with Wright stain and observed under a light microscope to evaluate differentiation and apoptosis.

Immunoblotting. The effects of the inhibitors on protein expression in the cells were examined by immunoblotting. Cells were harvested and lysed before or after culture with the inhibitors for 24 or 48 hours. The lysates from 1×10^6 cells/lane were then subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with an anti-Gli1 antibody (Ab), anti-cleaved Notch1 (Val1744) Ab (Cell Signaling Technology, Beverly, USA) to selectively detect the active fragment of Notch1 (7), an anti-Notch1 C-terminus Ab (Santa Cruz Biotechnology, Santa Cruz, USA), or an anti- α -tubulin Ab (Abcam, Cambridge, USA) as a loading control. Immunoreactive bands were detected using an enhanced chemiluminescent detection kit (GE Healthcare Biosciences, Buckinghamshire, UK). The experiments were repeated at least twice to verify their reproducibility.

Results

Growth suppression by cyclopamine and quercetin. Dose-response curves for the effects of the two inhibitors on the growth of myeloid and lymphoid leukemia and lymphoma cells are shown in Figure 1. Cyclopamine and quercetin dose-dependently suppressed the growth of seven and eight out of the ten cell lines, respectively. Tomatidine had no significant effect on the growth of these cells (data not shown). Treatment of the cells with cyclopamine and quercetin together demonstrated that their suppressive effects were additive, not synergistic (data not shown).

Table I. Effects of cyclopamine and quercetin on colony formation.

Cells	Cyclopamine	Quercetin
NB4	101 (%)	10*(%)
HL60	47*	3*
K562	10*	48*
Jurkat	70	93
Daudi	12*	0*

Cells were cultured in methylcellulose with 15 μ M cyclopamine or 50 μ M quercetin. Colony numbers were counted after 7 days in culture. Values are presented as the percentage relative to the number of colonies of control cells cultured without the inhibitors. * $p < 0.05$, Significantly different from the control.

Suppression of colony formation by cyclopamine and quercetin. Each inhibitor suppressed colony formation in a number of cell lines as shown in Table I. The susceptibility of colony formation to each inhibitor was similar to the susceptibility of cell growth shown in Figure 1.

Apoptosis induced by cyclopamine and quercetin. As shown in Figure 2, apoptotic cells with nuclear condensation and apoptotic bodies were observed in cultures whose growth was suppressed by the inhibitors. No morphological differentiation was seen in any cell line.

Effects of cyclopamine and quercetin on protein expression. Figure 3 shows the results of immunoblotting with anti-Gli1 Ab. Cyclopamine treatment decreased the amount of Gli1 protein in KOPT-K1 cells in a dose-dependent manner. It also decreased Gli1 protein in HL60 cells. However, it increased Gli1 protein in TMD8 cells in a dose-dependent manner.

The effect of quercetin on the expression and activity of Notch1 protein was examined (Figure 4). Quercetin suppressed the expression of Notch1 protein and decreased the active fragment of Notch1 in DND-41 cells, but had no effect on Notch1 in Jurkat cells.

Discussion

In this study, cyclopamine suppressed the growth of some leukemia and lymphoma cells, including AML, T-ALL and B-lymphoma cells, through the induction of apoptosis, though some cell lines were resistant to cyclopamine. Cyclopamine has previously been reported to suppress the growth of endometrial carcinoma cells (13), K562 cells and RS4; 11 acute B-lymphoblastic leukemia cells (14). In the present study, cyclopamine decreased the expression of Gli1 protein, which is the target gene product of Hh signaling, suggesting that cyclopamine inhibited Hh signaling. Interestingly, cyclopamine increased the expression of Gli1

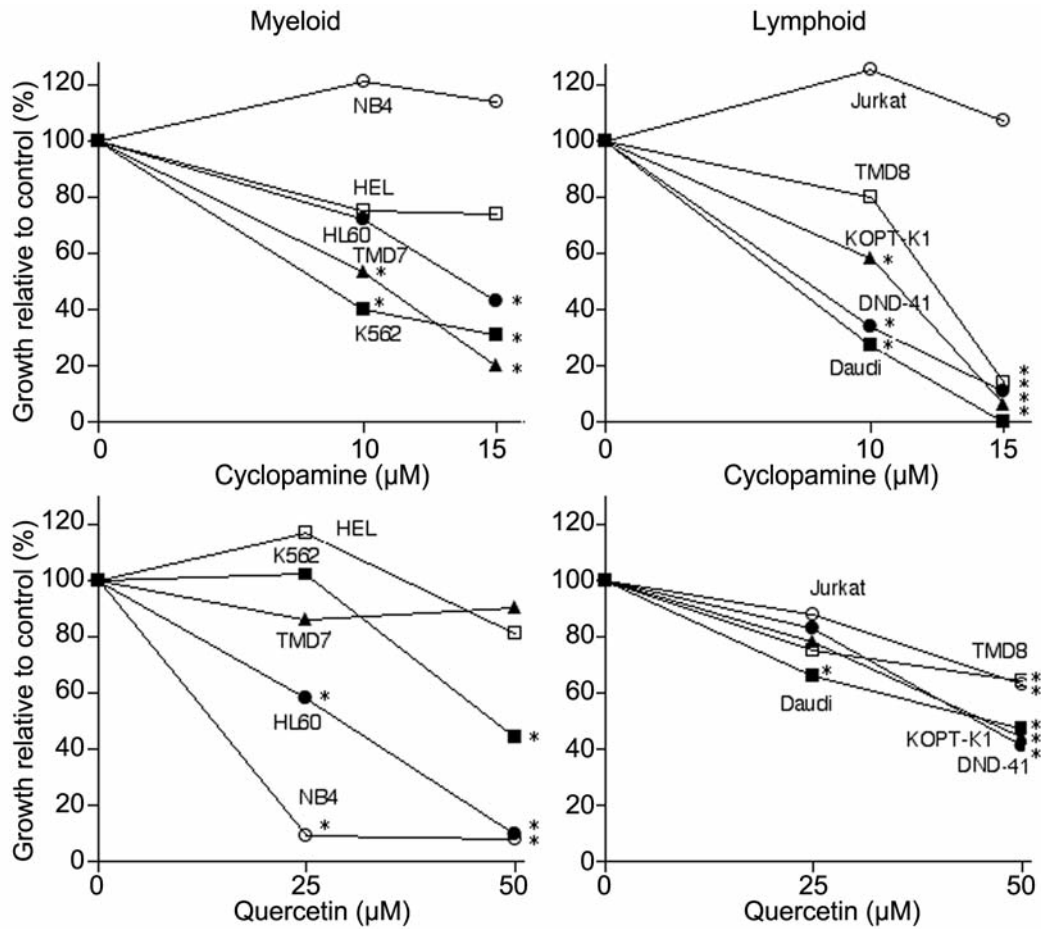


Figure 1. Dose-response curves of the effects of cyclopamine and quercetin on growth of leukemia and lymphoma cells. Growth was examined using a WST-1 assay after 3 days' culture. Growth is shown as a percentage relative to the mean value of control cells cultured without the inhibitors. * $p < 0.05$, Significantly different from the control.

protein in TMD8 cells, but also suppressed their growth. The reason for this phenomenon remains to be clarified. The suppression of cell growth by cyclopamine implies that Hh signaling is constitutively activated in those cells. Because sonic Hh has been reported to be expressed in leukemia cells (5), it is likely that Hh signaling is constitutively activated by autocrine mechanisms.

Quercetin has been shown to suppress the growth of colon cancer cells (9) and the results of the current study showed that it was also able to suppress the growth of some leukemia and lymphoma cells through the induction of apoptosis. Wnt signaling is known to be constitutively activated in leukemia cells (15, 16). It is possible that quercetin acts *via* effects on the Wnt pathway.

Because Wnt and Notch signaling have an interactive relationship in the regulation of stem cells (2), Notch protein expression was evaluated in the present study. Quercetin

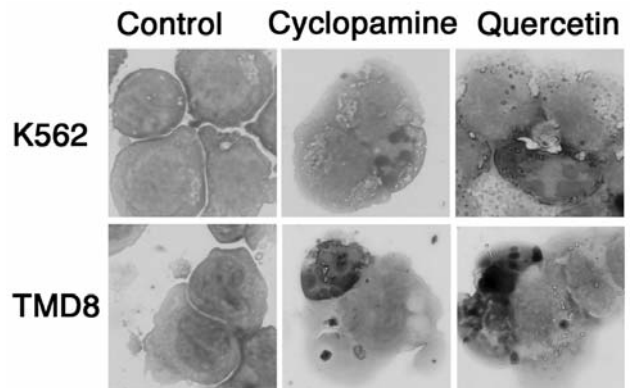


Figure 2. Effects of cyclopamine and quercetin on the morphology of representative cell lines. Cytospin preparations of the cells were made after culture with 10 μM cyclopamine or 25 μM quercetin for 3 days. Preparations were then stained with Wright stain and observed under a light microscope (original magnification $\times 400$).

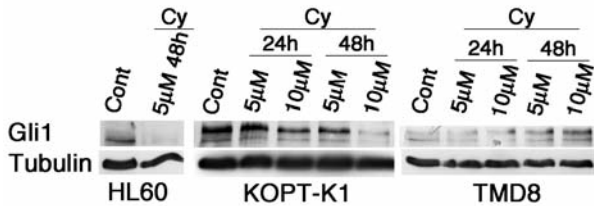


Figure 3. Expression of Gli1 protein in cells treated with cycloamine (Cy). Cell lysates were subjected to SDS-PAGE and immunoblotted with anti-Gli1 and anti- α -tubulin (loading control) antibodies.

suppressed the expression and activity of Notch1 in DND-41 cells, in which Notch signaling is constitutively activated due to *NOTCH1* gene mutations. This finding demonstrated the relationship between Wnt and Notch and this may be one of the mechanisms whereby quercetin suppresses cell growth. However, quercetin had no effect on Notch1 protein in Jurkat cells, in which Notch is also activated. The reason for this functional discrepancy remains to be determined.

In conclusion, cycloamine and quercetin suppress the growth of a number of leukemia and lymphoma cells. Further studies are needed to clarify the mechanisms responsible for the effects of the drugs. The results in this study could lead to the development of novel molecularly-targeted therapy for chemotherapy-resistant leukemia and lymphoma.

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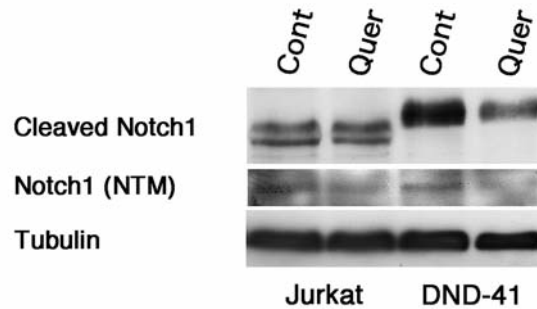


Figure 4. Expression of Notch proteins in cells treated with quercetin. Cells cultured with 25 μ M quercetin (Quer) for 24 hours were harvested. The lysates were subjected to SDS-PAGE and immunoblotted with anti-cleaved Notch1 (Val1744), anti-Notch1 C-terminus and anti- α -tubulin antibodies. NTM, Notch1 transmembrane subunit.

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