Effects of the HIF1 Inhibitor, Echinomycin, on Growth and NOTCH Signalling in Leukaemia Cells

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Abstract. *Aim: To examine the effects of echinomycin, a compound that inhibits DNA-binding activity of hypoxia-inducible factor-1 (HIF1), on leukaemia cell growth. Materials and Methods: Three acute myeloid leukaemia cell lines and three T-lymphoblastic leukaemia cell lines were cultured with echinomycin. Cell growth, mRNA and protein expression levels were examined by WST-1 assay, reverse-transcription polymerase chain reaction and immunoblotting, respectively. Results: HIF1α protein was expressed in all cell lines under normoxia. Treatment with echinomycin suppressed cell growth and induced apoptosis in association with decreased mRNA expression of HIF1 targets, glucose transporter-1 (GLUT1) and B-cell CLL/lymphoma-2 (BCL2). Echinomycin also suppressed the protein expression of NOTCH1, cleaved NOTCH1, v-myc myelocytomatosis viral oncogene homolog (MYC), v-akt murine thymoma viral oncogene homolog-1 (AKT), phosphorylated AKT, mechanistic target of rapamycin (mTOR), and phosphorylated mTOR and increased that of cleaved caspase-3 in some cell lines. Conclusion: Echinomycin suppresses leukaemia cell growth in association with reduced NOTCH1 expression. This is the first report to show that HIF inhibitor treatment suppresses NOTCH1 signalling. HIF inhibitors could be novel candidates for a molecular-targeted therapy against leukaemia.*

Hypoxia-inducible factor (HIF) is a DNA-binding transcription factor, which transactivates various genes to allow cell adaptation to hypoxic conditions. HIF is a heterodimer comprising of an oxygen-labile α-subunit and a constitutively expressed β-subunit (1). The HIF family has three members, HIF1, HIF2, and HIF3. HIF1-mediated signalling plays a role in maintenance of both normal haematopoietic stem cells and leukaemia stem cells under hypoxic environments such as bone marrow niches (2, 3). It has also been reported that HIF1 signalling is activated in mouse lymphoma stem cells and human acute myeloid leukaemia cells under normoxic conditions (4). To clarify the role of HIF1 on the growth of leukaemia cells under normoxia, we examined the effects of echinomycin (5), a compound that inhibits HIF1 DNA-binding activity, on the *in vitro* growth of various leukaemia cell lines.

Crosstalk between HIF signalling and Notch signalling has been previously reported. Gustafsson *et al*. reported that HIF1α binds to cleaved NOTCH1, the activated form of the NOTCH1 protein, which results in its stabilization and activation of Notch signalling (6). Wang *et al*. reported that HIF1α represses a negative feedback loop of the NOTCH1-hairy and enhancer of split-1 (HES1) system by inhibiting HES1 binding to the *HES1* promoter resulting in the consequent enhancement of NOTCH signalling (4). In our study, to elucidate the mechanisms underlying HIF-mediated effects on cell growth, we examined the effects of echinomycin on both Notch signalling and mammalian target of rapamycin (mTOR) signalling, which also crosstalks with NOTCH as reported by us and others (7, 8). We found that echinomycin-mediated inhibition of HIF impaired leukaemia cell growth in association with reduced Notch signalling.

Materials and Methods

Cells and HIF1 inhibitor. Three T-lymphoblastic leukaemia cell lines, Jurkat, KOPT-K1, and DND-41 (donated by Drs. Harashima and Orita, Fujisaki Cell Center, Japan) and three acute myeloid leukaemia cell lines, THP-1, NB4, and TMD7 (9) were used. Acute promyelocytic leukaemia cell line NB4 (10) was kindly provided by Dr. Lanotte (Hôpital Saint-Louis, Paris, France). Echinomycin was purchased from Calbiochem (La Jolla, CA, USA), and was dissolved in dimethyl sulphoxide (DMSO).

Cell growth assay. The effects of echinomycin on short-term growth were examined using a colorimetric WST-1 assay. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum in the presence of increasing concentrations of echinomycin in a humidified 5% CO_2 atmosphere. After three days, WST-1 and

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Key Words: Leukaemia, HIF, echinomycin, NOTCH, NB4, Jurkat cells.

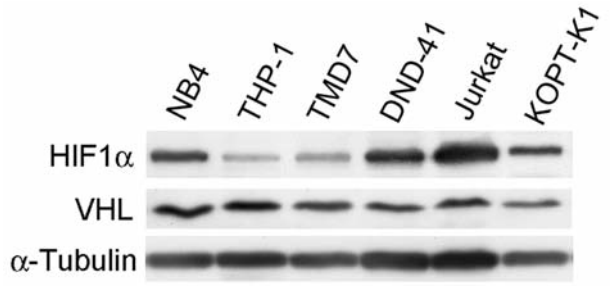

Figure 1. *Expression of hypoxia-inducible factor-1α (HIF1α) and von Hippel-Lindau tumor suppressor (VHL) proteins in leukaemia cell lines under normoxia. Lysates from cells cultured under normoxia were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoblotted with antibodies to HIF1α and VHL. α-Tubulin was used a control for input.*

1-methoxy-5-methylphenazinium methyl sulphate (Dojindo Laboratories, Kumamoto, Japan) were added. Optical density (O.D.) was measured using an enzyme-linked immunosorbent assay plate reader to determine the relative cell number. Cell growth is shown as a percentage of the mean OD value normalized to the control. To examine the effects of echinomycin on morphological differentiation and apoptosis, cytospin preparations were prepared from harvested cells, stained with Wright stain and the DeadEnd colorimetric TUNEL system (Promega, Madison, WI, USA), and observed under a microscope.

Reverse-transcription polymerase chain reaction (RT-PCR). The effects of echinomycin on gene expression were examined by quantitative RT-PCR using a FastStart DNA Master SYBR Green I kit, LightCycler primer sets (Roche Diagnostics, Mannheim, Germany) and QuantiTect primers (Qiagen, Hilden, Germany). RNA was extracted from cells cultured with 5 nM of echinomycin for 12 h. The expression level of each mRNA was normalized to that of β-actin (*ACTB*) mRNA, which was concurrently measured.

Immunoblotting. The effects of echinomycin on the expression and phosphorylation of proteins involved in the HIF, NOTCH and mTOR pathways were examined by immunoblotting. After culture with 5 nM echinomycin for 24 h, cells were harvested and lysed. The lysates were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with antibodies against HIF1α, transcription of phosphatase and tensin homolog (PTEN) (Santa Cruz Biotechnology, Santa Cruz, Ca, USA), von Hippel-Lindau tumor suppressor (VHL), NOTCH1, cleaved NOTCH1 (Val1744), MYC, v-Akt murine thymoma viral oncogene homolog (AKT), p-AKT (Ser473), mTOR, p-mTOR (Ser2481), caspase-3, and cleaved caspase-3 (Cell Signaling Technology, Danvers, MA, USA), and α-tubulin (Abcam, Cambridge, MA, USA) as a loading control. Each assay was repeated at least three times.

Results

Expression of HIF1α protein in leukaemia cells. We found that the HIF1α protein was expressed in all six cell lines under normoxic conditions in a CO_2 incubator (Figure 1). Culture in hypoxic conditions further promoted the expression of HIF1α (data not shown). The VHL protein, which is involved in the degradation of HIF1α under normoxia, was also expressed in all cell lines. In the subsequent experiments, the effects of echinomycin on leukaemia cells were examined under normoxia.

Table I. *Effects of echinomycin on the mRNA expression of hypoxia-inducible factor and NOTCH signalling.*

	GLUT1	*BCL2*	*MYC*	*NOTCH1*
NB4	16±15	37±26	23±14	21±15
Jurkat	46±18	55±10	38±15	62±2

RNA was extracted from NB4 or Jurkat cells cultured with 5 nM echinomycin. The expression of the indicated genes was measured by quantitative RT-PCR. The numbers indicate the mean percentage decrease in gene expression normalized to *ACTB* expression relative to the control±standard deviation. *GLUT1*: glucose transporter 1; *BCL2*: B-cell CLL/lymphoma 2; *MYC*: v-myc myelocytomatosis viral oncogene homolog.

Effects of echinomycin on cell growth. To test the effect of echinomycin on cell growth, dose-response curves were generated. Treatment with echinomycin significantly suppressed growth in all cell lines (Figure 2). Additionally, we examined cytospin preparations of cells with and without treatment of echinomycin. We observed apoptotic cells with nuclear condensation and apoptotic bodies following treatment with echinomycin (Figure 3). These cells were also TUNEL-positive (data not shown). These data indicate that echinomycin induces apoptosis. Morphological differentiation was not observed.

Effects of echinomycin on gene expression. To confirm that echinomycin inhibits HIF1-mediated transcription, we examined the expression of HIF1-target genes. The results from two representative cell lines are shown in Table I. Briefly, expression of the HIF1 target genes, glucose transporter-1 (*GLUT1*) and B-cell CLL/lymphoma-2 (*BCL2*) was significantly suppressed by echinomycin treatment. Echinomycin also suppressed the expression of *NOTCH1* and *MYC*. The suppressive effects were also observed in other cell lines (data not shown).

Effects of echinomycin on signalling proteins. Next, we assessed the effects of echinomycin on HIF-associated signalling pathways (Figure 4). We found that treatment with echinomycin decreased cleaved NOTCH1 along with NOTCH1 protein in NB4 cells and Jurkat cells. Expression of MYC was also suppressed in both cell lines. Echinomycin suppressed expression of proteins associated with the AKT-mTOR signalling cascade including PTEN, AKT, and

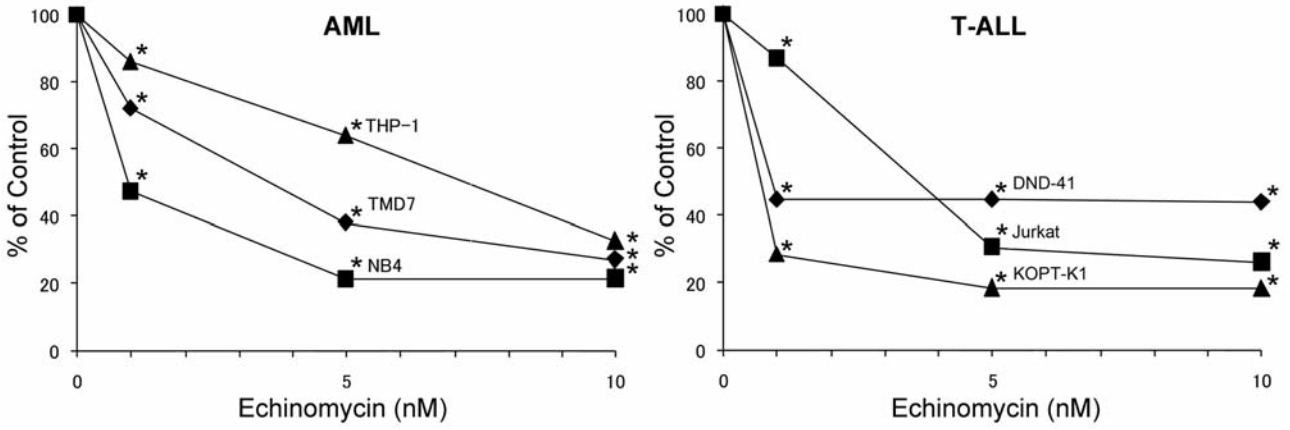

Figure 2. *Effects of echinomycin on growth of acute myeloid leukaemia (AML) and T-lymphoblastic leukaemia (T-ALL) cell lines. Cells were cultured with increasing concentrations of echinomycin. After three days, cell growth was examined using a colorimetric assay. Growth is shown as a percentage of the mean O.D. value normalized to that of control cells. *p<0.05 compared to control.*

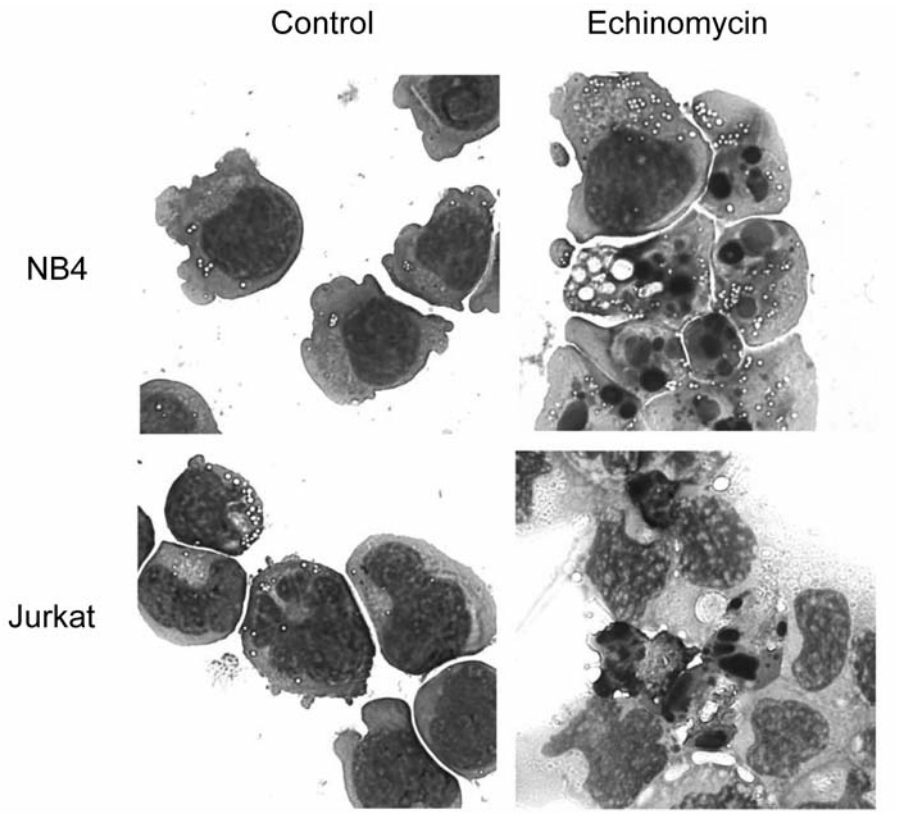

Figure 3. *Echinomycin induced apoptosis in NB4 and Jurkat cells. Cytospin preparations from cells cultured without (control) or with 5 nM echinomycin for 12 h (NB4) or 72 h (Jurkat) were stained with Wright stain (original magnifications, ×600).*

phosphorylated AKT, mTOR and phosphorylated mTOR in NB4 cells. In contrast, echinomycin did not significantly affect the expression levels of AKT or mTOR, nor their phosphorylation, in PTEN-deficient Jurkat cells (11). In both cell lines, caspase-3 was cleaved after echinomycin treatment, suggesting that echinomycin induced the activation of caspase-3-mediated apoptosis. In KOPT-K1 cells, echinomycin reduced the expression of NOTCH1,

cleaved NOTCH1, and MYC, and increased that of cleaved caspase-3. It did not significantly affect AKT and mTOR (data not shown).

Discussion

In this study, we showed that HIF1α was expressed in some leukaemia cell lines under normoxia and that the HIF1 inhibitor, echinomycin, suppressed the growth through induction of apoptosis, in association with suppression of Notch and mTOR signalling. It is known that HIF1α is hydroxylated by prolyl-hydroxylases under normoxic conditions and hydroxy-HIFα is degraded by VHL. HIFα is stable under hypoxia because HIFα is not hydroxylated (12). We hypothesized that the deficit of VHL might be a cause of expression of HIF1α under normoxia. However, VHL was expressed in these cell lines as shown by immunoblot analysis. It has been reported that HIF is up-regulated by mTOR signalling (13). Because mTOR signalling is constitutively activated in the leukaemia cell lines used in this study, as previously reported (7), we speculated that it might be a possible cause of HIF1α expression. On the other hand, functional inhibition of HIF1 by echinomycin suppressed the expression of AKT and mTOR proteins in some cell lines. These findings indicate that a mutual crosstalk exists between HIF and mTOR signalling.

Regarding the crosstalk between HIF and NOTCH, two kinds of mechanisms have been reported (3, 6). We have found another mechanism of crosstalk, namely that functional inhibition of HIF down-regulates both mRNA and protein expression of NOTCH1, which results in down-regulation of NOTCH signalling. MYC protein, a NOTCH1 transcriptional target, was also down-regulated in echinomycin-treated cells (14).

Crosstalk between NOTCH and mTOR has also been previously reported (8). Indeed, NOTCH activation induces the expression of HES1 protein, which down-regulates *PTEN* gene expression. Because PTEN works as a suppressor of mTOR signalling, the down-regulation of *PTEN* results in mTOR activation. In this study, echinomycin-treated NB4 cells exhibited suppression of NOTCH signalling, down-regulation of *PTEN*, and suppression of mTOR signalling, which are inconsistent with the above mechanism. Thus, some different mechanisms might occur in NB4 cells. Indeed, suppression of mTOR was mainly due to the suppression of mTOR protein expression rather than its phosphorylation.

In this study, we propose that echinomycin-treatment induced suppression of *BCL2*, a direct target of HIF1, and indirect suppression of NOTCH1, MYC, and AKT/mTOR causes growth-suppression through apoptosis. On the basis of these findings, we suggest that HIF1 inhibitors, such as echinomycin, could be novel candidates for molecular-targeted therapy against leukaemia. Because some of these phenomena might be HIF-unrelated, off-target effects of echinomycin as well as its precise mechanism of action require further elucidation. Moreover, the effects of echinomycin on normal haematopoietic stem cells need clarification before clinical use.

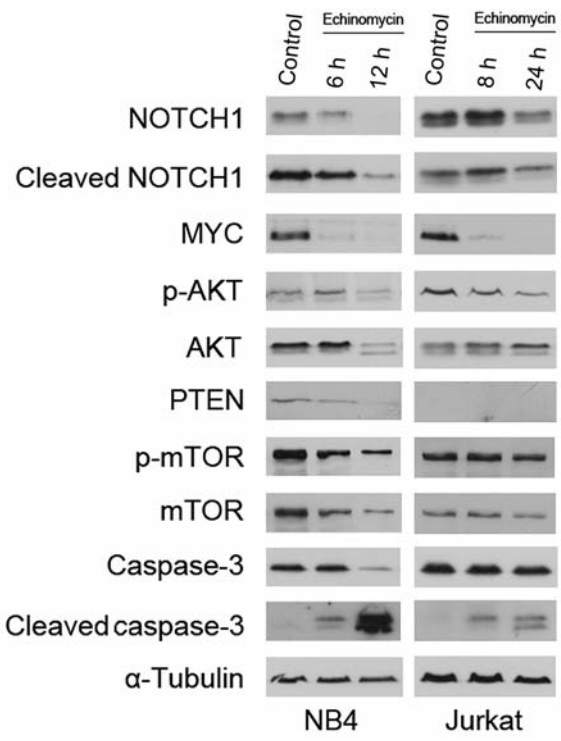

Figure 4. *Effects of echinomycin on expression and activation of various signalling proteins. Cells were cultured without (control) or with 5 nM echinomycin for the indicated hours. Lysates were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoblotted with the indicated antibodies.*

Acknowledgements

This study was supported in part by a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science.

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Received May 14, 2013
Revised June 13, 2013
Accepted June 14, 2013