ANTICANCER RESEARCH *41*: 1841-1847 (2021)
doi:10.21873/anticanres.14950

Effects of HOXA9 Inhibitor DB818 on the Growth of Acute Myeloid Leukaemia Cells

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Abstract. *Background/Aim: Homeobox A9 (HOXA9), a transcription factor regulating haematopoiesis and leukaemia cell proliferation, is suggested as a driver of acute myeloid leukaemia (AML). The aim of this study was to examine the effects of a synthetic HOXA9 inhibitor DB818 on AML cells in vitro. Materials and Methods: AML cell lines OCI/AML3, MV4-11, and THP-1 with gene mutations up-regulating HOXA9 expression were treated with DB818 and analysed for cell proliferation and gene expression. The effects of HOXA9 knockdown were also evaluated. Results: In the three AML cell lines, DB818 suppressed growth, induced apoptosis, and down-regulated the expression of HOXA9 transcriptional target genes: MYB proto-oncogene, transcription factor (MYB), MYC proto-oncogene, bHLH transcription factor (MYC), and BCL2 apoptosis regulator (BCL2), while up-regulating that of Fos proto-oncogene, AP-1 transcription factor subunit (FOS). HOXA9 knockdown showed similar effects, except for MYC expression, which differed between DB818-treated and HOXA9-deficient OCI/AML3 cells, suggesting an off-target effect of DB818. Conclusion: DB818 has potential as a novel molecular targeted drug for treating AML associated with HOXA9 overexpression.*

Homeobox A9 (HOXA9) is a transcription factor that regulates cell stemness and differentiation during normal haematopoiesis. The expression of HOXA9 is strong in haematopoietic stem cells but is silenced in mature differentiated cells (1, 2). Mechanistically, HOXA9, along with cofactor proteins such as Meis homeobox 1 (MEIS1), binds to the regulatory elements of the target genes, including oncogenes BCL2 apoptosis regulator (*BCL2*), MYB proto-oncogene, transcription factor (*MYB*), and LIM domain only 2 (*LMO2*) and controls their expression (3).

It has been reported that HOXA9 is overexpressed in more than 50% of acute myeloid leukaemia (AML) cases and is strongly associated with poor prognosis (4). The up-regulation of HOXA9 expression is a consequence of genetic alterations in the upstream factors, including fusion of mixed lineage leukaemia 1 (*MLL1*, also called *KMT2A*) gene with various partners and mutations in the nucleophosmin-encoding gene nucleophosmin 1 (*NPM1*) (5). As HOXA9 overexpression results in the blockage of haematopoietic stem cell differentiation, this transcription factor can represent a therapeutic target for AML treatment.

Recently, it has been reported that a small-molecule compound, diamidine phenyl-thiophene-benzimidazole (DB818), inhibits HOXA9–DNA interaction by binding to the minor groove on the HOXA9 cognate nucleotide sequence, thus reducing HOXA9-mediated transcription (6). The aim of this study was to elucidate the regulatory role of HOXA9 and examine the molecular mechanism of DB818 activity in AML cells *in vitro*. To confirm the specificity of the effects, we also performed *HOXA9* knockdown experiments using small interfering RNA (siRNA).

Materials and Methods

Cell lines and HOXA9 inhibitor. Most experiments were conducted with three human AML cell lines. The OCI/AML3 cell line, which harbours *NPM1* mutations, was established at the Ontario Cancer Institute (7). MV4-11 cells carrying *MLL*–ALL1-fused gene from chromosome 4 (*AF4*) (*KMT2A–AFF1*) and THP-1 cells carrying *MLL–AF9* (*KMT2A–MLLT3*) fusion genes were purchased from the American Type Culture Collection (Manassas, VA, USA) and the European Collection of Authenticated Cell Cultures (Salisbury, UK), respectively. We also investigated the growth of six other AML cell lines and three acute lymphoblastic leukaemia (ALL) cell lines: HL60 (AML-M2), NB4 (AML-M3), OCI/AML2, OCI/AML5 (AML-M4), U937 (AML-M5), AA (AML-M6), Jurkat, KOPT-K1 (T-ALL), and NALM-6 (B-ALL); their characteristics are presented in the cell line guidebook (8) and our previous report (9). NB4 was provided by Dr. M. Lanotte (France). AA was provided by Dr. A. Arai (Japan). Jurkat and KOPT-K1 were gifted from Drs. Harashima

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Key Words: HOXA9, leukaemia, inhibitor, gene knockdown.

and Orita (Fujisaki Cell Centre, Japan). All cell lines, except HL60, were confirmed to express HOXA9 by immunoblotting (data not shown). Normal lymphocytes from three healthy volunteers who provided informed consent were also used.

The HOXA9 inhibitor DB818 was purchased from Glixx Laboratories Inc. (Hopkinton, MA, USA) and dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 5 mM.

Cell growth assay. Short-term cell growth was evaluated using a colorimetric WST-8 assay kit (Dojindo Laboratories, Kumamoto, Japan). Cells were cultured in 96-well plates in RPMI-1640 medium supplemented with 10% foetal bovine serum, with 10 or 20 μM DB818; after 96 h, WST-8 was added and the optical density was measured in an enzyme-linked immunosorbent assay reader. Relative cell proliferation was calculated as the percentage of the mean optical density value normalized to that of control cells cultured with vehicle (DMSO). In some experiments, cells were harvested after 96 h and counted under a microscope. Cell morphology was analysed in cytospin preparations stained with Wright stain and observed under a microscope.

Flow cytometric analysis. The effects of DB818 on cell differentiation were examined by staining cells with phycoerythrin-conjugated antibodies against CD11b and control IgG1 (BD Biosciences, Franklin Lakes, NJ, USA), whereas those on apoptosis were evaluated after staining with annexin V-fluorescein isothiocyanate and propidium iodide. The stained cells were analysed by flow cytometry in a FACSCalibur system (BD Biosciences).

Electrophoretic mobility shift assay (EMSA). Nuclear extracts from cells cultured with 10 or 20 μM DB818 or vehicle for 2 days were incubated with a biotin-labelled HOXA9 consensus oligonucleotide (CTGCGATGATTTACGACCGC) in the binding buffer from the Lightshift Chemiluminescent EMSA Kit (Pierce Biotechnology, Rockford, IL, USA). For specific competition, a 200-fold excess of the unlabelled probe was added. The samples were separated on 6% polyacrylamide gels, transferred to membranes, and the labelled bands were detected by chemiluminescence on X-ray films.

Immunoblotting analysis. Cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analysed by immunoblotting with antibodies against HOXA9 (Merck, Darmstadt, Germany), BCL2, MYB, and MYC (Cell Signaling Technology, Danvers, MA, USA); anti-GAPDH (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) was used for loading control. Immunoreactive bands were qualitatively detected using Pierce Enhanced Chemiluminescent Western Blotting Substrate (Pierce Biotechnology). Each assay was repeated at least three times to ensure reproducibility.

HOXA9 knockdown by siRNA. HOXA9 knockdown was performed with three different pre-designed *HOXA9*-specific siRNAs (Stealth siRNA™; Life Technologies, Carlsbad, CA, USA): HSS 142495 (5'-GCUUCCAGUCCAAGGCGACGGUGUU-3'), HSS 142496 (5'-CCCAGCGAAGGCGCCUUCUCUGAAA-3'), and HSS 142497 (5'-GGUACGAGGUGGCUCGACUGCUCAA-3'); a Stealth RNAi negative control duplex was used as a control. Cells were transfected with each siRNA using the Neon™ pipette tip chamber-based electroporation system (Life Technologies) and immediately transferred to the culture medium.

Quantitative reverse transcription polymerase chain reaction. Total RNA was extracted from cells transfected with *HOXA9* siRNA or control siRNA, and used to synthesize complementary DNA. Quantitative reverse transcription polymerase chain reaction was performed with FastStart Essential DNA Green Master mix (Roche Diagnostics, Mannheim, Germany) and *HOAXA9*-specific primers (QuantiTect Primer Assay QT01002372; QIAGEN, Hilden, Germany). The relative expression of *HOXA9* mRNA was determined after normalization to that of β-actin-encoding (*ACTB*) mRNA (Roche Diagnostics).

Microarray analysis. Comprehensive gene-expression analysis was performed using microarray. Cells were treated with 20 μM DB818, DMSO (vehicle), 80 nM *HOXA9* siRNA, or control siRNA for 24 h. Total RNA was then extracted with the High Pure RNA isolation kit (Roche Diagnostics) and used to prepare cyanine-3-labelled cRNA, which was hybridized to a SurePrint G3 Human GE microarray 8×60K v3 (Agilent Technologies, Santa Clara, CA, USA). The expression profile was analysed with the Agilent Feature Extraction 11.5.1.1 software.

Statistical analysis. Statistical significance of differences in cell growth was evaluated by Student's *t*-test and *p*-values less than 0.05 were considered to indicate significant difference.

Results

Effects of DB818 on cell growth. Treatment with DB818 suppressed the growth of five AML and two ALL cell lines in a dose-dependent manner (Figure 1, upper panels). MV4-11, THP-1, and OCI/AML3 cells were more susceptible to DB818 than the other cell lines. DB818 slightly inhibited the growth of normal lymphocytes from two out of three volunteers. Time-course curves confirmed the growth suppression of OCI/AML3, MV4-11, and THP-1 cells by DB818 (Figure 1, lower panels).

Analysis of cytospin preparations indicated that DB818 induced apoptosis in some MV4-11 and OCI/AML3 cells as evidenced by the appearance of nuclear condensation and apoptotic bodies, and caused cytoplasmic swelling and volume increase suggestive of macrophage-like differentiation in THP-1 cells (Figure 2).

Flow cytometric analysis showed that DB818 treatment increased the proportion of annexin-V-positive MV4-11, OCI/AML3, and THP-1 cells (Figure 3) and up-regulated the expression of CD11b, a monocyte differentiation marker (Figure 4) in THP-1 cells, thus supporting the results of morphological analysis shown in Figure 2.

Inhibition of HOXA9–DNA binding by DB818. To examine whether DB818 prevented HOXA9–DNA interaction, we performed EMSA, which revealed that DB818 treatment reduced the formation of HOXA9–DNA complex (Figure 5). The same decrease was observed after the addition of the unlabelled probe, indicating the specificity of the inhibitory effect of DB818 on HOXA9 binding to DNA.

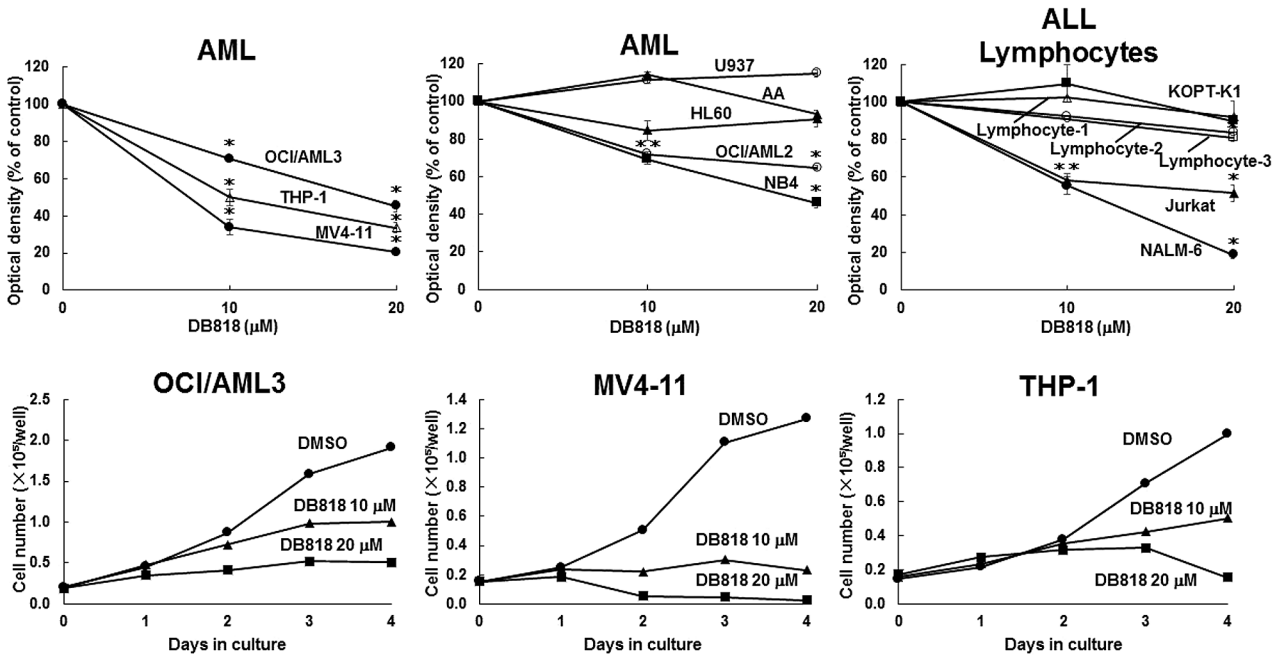

Figure 1. *Effects of homeobox A9 (HOXA9) inhibitor DB818 on the growth of leukaemia cells. DB818 (0, 10, or 20 μM) was added to acute myeloid leukaemia (AML) and acute lymphoblastic leukaemia (ALL) cells and normal lymphocytes for 4 days to assess dose response (upper panels) and to three representative AML cell lines for 1 to 4 days to analyse the time course of effects (lower panels). Cell growth was evaluated in a colorimetric assay and the results are expressed as the percentage of the mean optical density in DB818-treated cells relative to that in dimethyl sulfoxide (DMSO)-treated control cells. *Significantly different at p<0.05 compared to the control.*

DB818 effect on the expression of signaling proteins. DB818 treatment down-regulated the protein expression of MYB and MYC in OCI/AML3, MV4-11 and THP-1 cells and also slightly reduced that of BCL2 in OCI/AML3 cells (Figure 6, left panel). DB818 also weakly down-regulated HOXA9 expression, although the main activity of DB818 is inhibition of HOXA9 binding to DNA rather than its expression.

Effects of HOXA9 knockdown on cell growth and protein expression. To determine whether the DB818-induced changes were due to its inhibition of HOXA9 function or to off-target effects, we performed *HOXA9* knockdown experiments. Among the three *HOXA9* siRNAs, HSS 142495 most effectively inhibited *HOXA9* mRNA expression in OCI/AML3 cells (to 30%) and HSS 142496 in MV4-11 and THP-1 cells (to 35% and 15%, respectively). *HOXA9* knockdown by these siRNAs significantly reduced the growth of OCI/AML3, MV4-11, and THP-1 cells 4 days after transfection: to 58%, 32%, and 60% of control, respectively ($p<0.05$; Figure 7).

The knockdown of *HOXA9* also affected protein expression in AML cells as evidenced by the reduction of MYB and BCL2 levels in OCI/AML3 and MV4-11 cells, respectively; however, the expression of MYC was not affected (Figure 6, right panel).

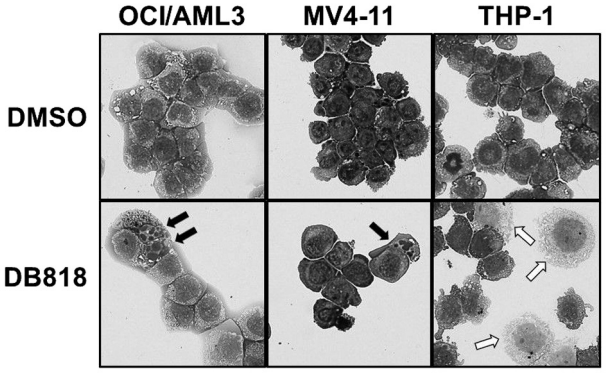

Figure 2. *Morphology of acute myeloid leukaemia cells treated with DB818. Cells were cultured with 20 μM DB818 for 48 h and their cytospin preparations were stained with Wright stain and observed under a microscope. Black arrows: Apoptotic bodies; white arrows: macrophage-like differentiation.*

Comprehensive mRNA expression analysis. The effects of DB818 treatment and *HOXA9* knockdown on gene-expression profiles of AML cells were examined by microarray assay. The results indicated that DB818 treatment and *HOXA9* knockdown down-regulated the mRNA

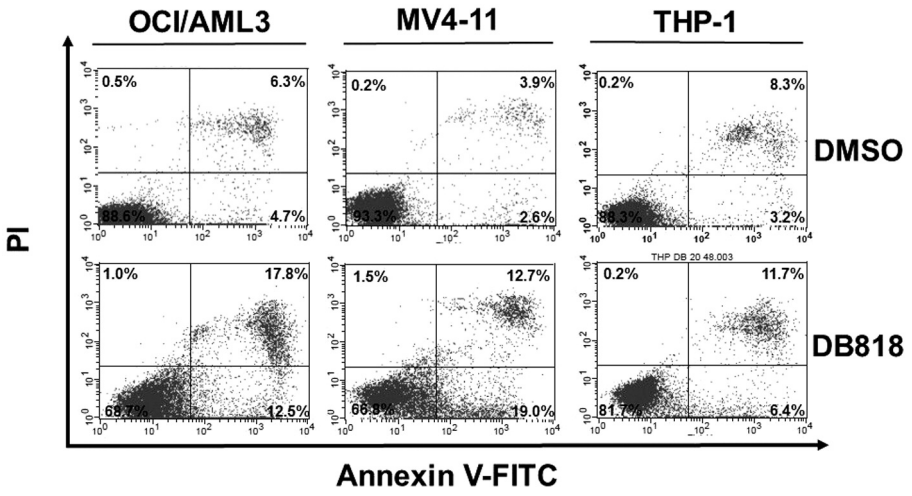

Figure 3. *Apoptosis assay of acute myeloid leukaemia cells treated with DB818. Cells were cultured with 20 μM DB818 for 48 h, stained with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI), and analysed by flow cytometry.*

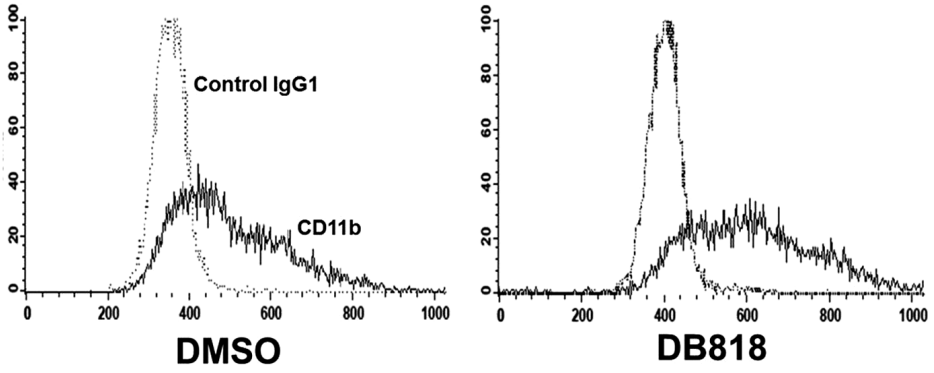

Figure 4. *Induction of macrophage-like differentiation in THP-1 cells by DB818. Cells were cultured with 20 μM DB818 for 48 h, stained with phycoerythrin-conjugated anti-CD11b antibodies and control IgG1, and analysed by flow cytometry.*

expression of *BCL2* and *MYB*, while up-regulating that of Fos proto-oncogene, AP-1 transcription factor subunit (*FOS*) in OCI/AML3, MV4-11, and THP-1 cells (Table I). For the other HOXA9-targeted genes, the effects of DB818 and *HOXA9* knockdown differed among the three cell lines. Thus, DB818 treatment reduced mRNA levels of *MYC* in the three cell lines and increased that of CD11b-encoding integrin subunit alpha M (*ITGAM*) in THP-1 cells, whereas *HOXA9* knockdown did not change *MYC* expression in OCI/AML3 cells or *ITGAM* expression in THP-1 cells.

Discussion

This study showed that the HOXA9 inhibitor DB818 suppressed the growth of several leukaemia cell lines, particularly three AML cell lines with gene mutations up-regulating HOXA9 expression, which was consistent with the effect of *HOXA9* knockdown. Furthermore, DB818 treatment induced the differentiation of THP-1 cells and apoptosis in the three AML cell lines. These findings suggest that HOXA9 drives the proliferation of AML cells, probably through suppression of differentiation and apoptosis.

To investigate the mechanisms underlying these effects, we examined the mRNA and protein expression of HOXA9-regulated genes, and found that, consistent with the DB818-induced inhibition of HOXA9–DNA binding, DB818 reduced the expression of HOXA9-targeted genes. To our knowledge, this is the first report of DB818 effects on AML cell proliferation and gene expression.

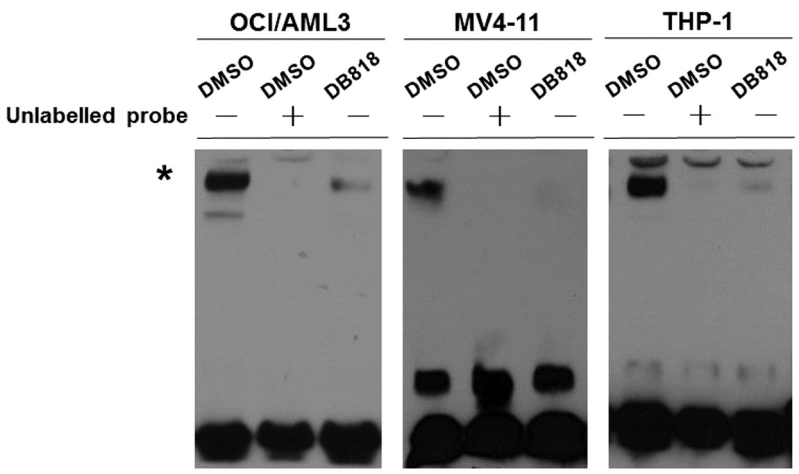

Figure 5. *Electrophoretic mobility shift assay to detect the binding of homeobox A9 (HOXA9) to a HOXA9 consensus oligonucleotide. Nuclear extracts of OCI/AML3 and THP-1 cells treated with 20 μM DB818 for 48 h and MV4-11 cells treated with 10 μM DB818 for 24 h were incubated with the biotin-labelled HOXA9 consensus oligonucleotide; cells treated with dimethyl sulfoxide (DMSO) were used as control. Middle lanes show competition for specific binding using a 200-fold excess of the unlabeled probe. Specific nuclear complexes are indicated by an asterisk.*

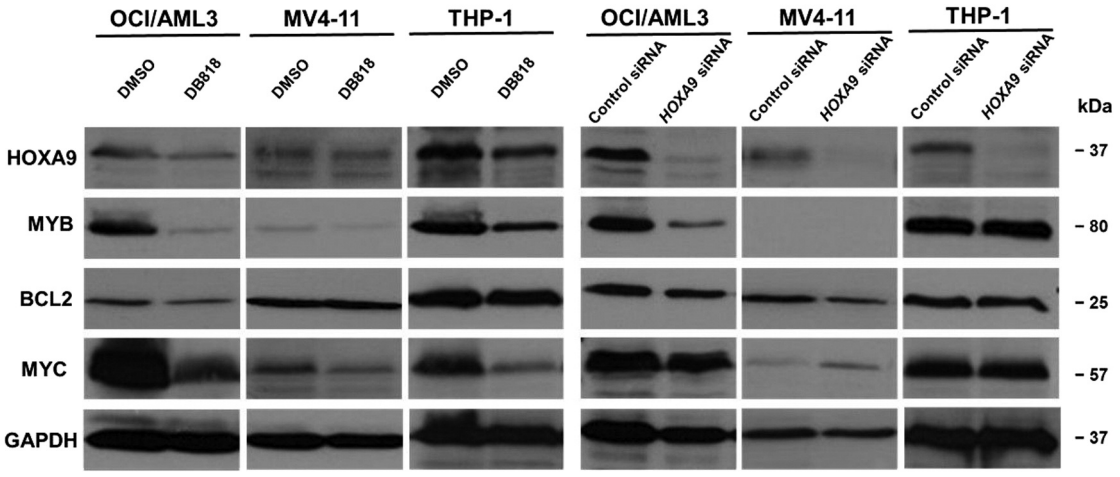

Figure 6. *Protein expression of Homeobox A9 (HOXA9) and its target genes in acute myeloid leukaemia cells after DB818 treatment or HOXA9 knockdown. OCI/AML3, MV4-11, and THP-1 cells were treated with 20 μM DB818 for 48 h, 10 μM DB818 for 24 h, and 20 μM DB818 for 24 h, respectively (left panels), or transfected with HOXA9 siRNA (right panels) and analysed for the expression of HOXA9, MYB proto-oncogene, transcription factor (MYB), BCL2 apoptosis regulator (BCL2), MYC proto-oncogene, bHLH transcription factor (MYC), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control, by immunoblotting.*

However, there were certain differences in the influence on the expression of HOXA9-regulated oncogenes between DB818-treated and HOXA9-deficient AML cells. Thus, *MYC* mRNA and protein levels were reduced by DB818, but not by *HOXA9* siRNA. The limitation of our study was that we did not analyse the time-course of expression changes; therefore, the observed discrepancy between the effects of DB818 and *HOXA9* knockdown may be attributed to the difference in the duration of particular experiments. Otherwise, the DB818-induced down-regulation of MYC might be due to the off-target effects of DB818, although the induction of *MYC* expression through HOXA9 transcriptional activity has been documented in previous studies (10, 11). Another example of discordant results was the expression of spleen associated tyrosine kinase (*SYK*), which was reported to be an important HOXA9 downstream target in a mouse model of

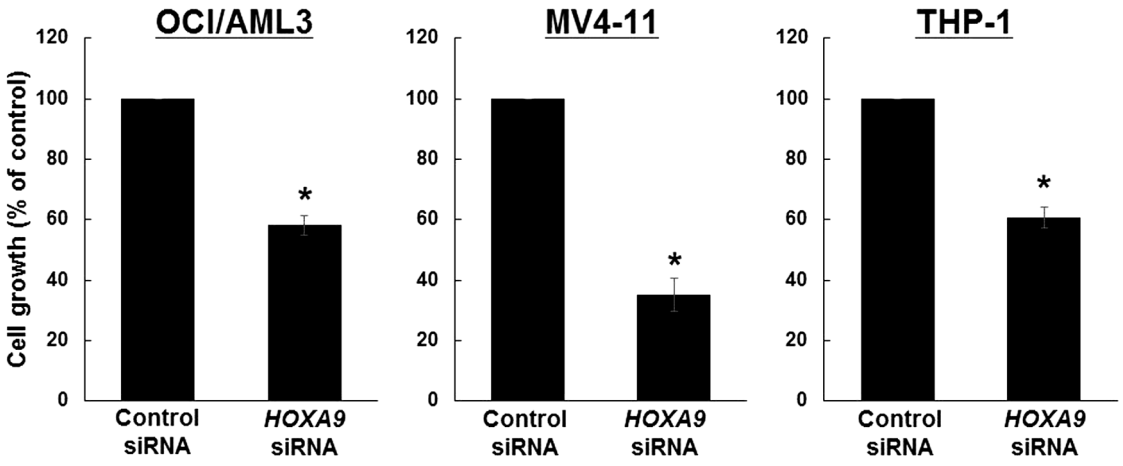

Figure 7. *Effects of Homeobox A9 (HOXA9) knockdown on the growth of acute myeloid leukaemia cells. OCI/AML3, MV4-11, and THP-1 cells were transfected with 80 nM HOXA9 siRNA or control siRNA and analysed for proliferation after 96 h using a colorimetric assay. The results are expressed as the percentage of the mean optical density in HOXA9 siRNA-transfected cells relative to that in control siRNA-transfected cells. Mean values±standard deviation are shown. *Significantly different at p<0.05 compared to the control.*

Table I. *Microarray analysis of the effects of DB818 treatment and homeobox A9 (HOXA9) knockdown on gene expression in acute myeloid leukaemia cells. Log2 ratios of mRNA expression in DB818-treated and HOXA9 siRNA-transfected cells were normalized to those in dimethyl sulfoxide-treated and control siRNA-transfected cells, respectively.*

		DB818			*HOXA9* siRNA		
Gene symbol	Encoded protein	OCI/AML3	MV4-11	THP-1	OCI/AML3	MV4-11	THP-1
HOXA9	Homeobox A9	0.260	–0.009	–0.188	–1.319	–0.569	–1.751
BCL2	BCL2 apoptosis regulator	–0.172	–0.361	–0.491	–1.488	–0.870	–0.405
MYB	MYB proto-oncogene, transcription factor	–0.682	–0.281	–0.696	–1.012	–0.019	–0.076
FOS	Fos proto-oncogene, AP-1 transcription factor subunit	0.282	1.293	2.190	0.717	0.286	0.031
LMO2	LIM domain only 2	–0.032	–0.166	–0.143	0.148	0.054	–0.070
FLT3	Fms-related receptor tyrosine kinase 3	–0.791	0.077	0.382	–0.159	–0.068	–0.177
IGF1	Insulin like growth factor 1	–0.046	–0.211	–1.016	0.372	0.684	–1.292
SYK	Spleen associated tyrosine kinase	0.169	–0.129	0.212	–0.065	–0.072	–0.138
MYC	MYC proto-oncogene, bHLH transcription factor	–0.366	–0.324	–0.828	0.172	–0.266	–0.086
ITGAM	Integrin subunit alpha M	–0.078	1.650	1.037	0.668	0.567	–0.127

Hoxa9/Meis1-driven leukaemia (12). However, our findings indicated that *HOXA9* knockdown tended to reduce *SYK* expression only slightly in the three cell lines, whereas DB818 treatment did not affect it in two of them. Moreover, microarray analysis showed that the effects of *HOXA9* deficiency on the transcription of some genes were the opposite among the cell lines. Cumulatively, these results suggest that the genes targeted by HOXA9 and the related downstream pathways can differ among leukaemia cell lines, which is consistent with the view expressed in a previous study (11).

Nevertheless, our analysis indicates that DB818 can suppress the proliferation of different AML cells without causing toxic effects on normal lymphocytes. Thus, DB818 might be a candidate molecular-targeted drug against AML cells, particularly those carrying gene mutations up-regulating *HOXA9* expression. However, considering that the effects of DB818 on gene expression vary depending on the leukaemia cell line, the therapeutic activity of DB818 may also vary from patient to patient. Therefore, further thorough investigation of DB818 on- and off-target effects should be performed in pre-clinical studies. Moreover, the development of drug sensitivity tests is required to predict the treatment outcomes and appropriately stratify AML cases.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

YS and ST designed the study. YS and MI performed the analysis. YS, MI, and ST wrote the article.

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 (No. 17K09005).

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Received December 28, 2020
Revised March 1, 2021
Accepted March 4, 2021