

Effects of MERTK Inhibitors UNC569 and UNC1062 on the Growth of Acute Myeloid Leukaemia Cells

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Abstract. *Background:* MERTK proto-oncogene tyrosine kinase (MERTK) is a receptor tyrosine kinase that affects cancer cell proliferation. This study evaluated the effects of the synthetic MERTK inhibitors UNC569 and UNC1062 on *in vitro* growth of acute myeloid leukaemia (AML) cells. *Materials and Methods:* Four AML cell lines expressing MERTK were treated with UNC569 and UNC1062 and analyzed for cell proliferation, immunoblotting, and gene expression. The effects of MERTK knockdown were also evaluated. *Results:* Treatment with the inhibitors suppressed cell growth and induced apoptosis in all cell lines. OCI/AML5 and TMD7 cells, in which MERTK was constitutively phosphorylated by autocrine mechanisms, were highly susceptible to these inhibitors. The treatment reduced the phosphorylation of MERTK and its downstream signalling molecules, v-akt murine thymoma viral oncogene homolog 1 (AKT) and extracellular signal-regulated kinase (ERK). Similar effects were observed after MERTK knockdown. The inhibitors and the knockdown caused similar changes in mRNA expression. *Conclusion:* These MERTK inhibitors are potential molecular-targeted drugs for treating AML expressing constitutively phosphorylated MERTK.

MERTK proto-oncogene tyrosine kinase (MERTK) is a member of the TYRO3 protein tyrosine kinase (TYRO3)/AXL receptor tyrosine kinase (AXL)/MERTK family of receptor tyrosine kinases (collectively referred to as TAM). These kinases play important roles in cell growth in various organs and types of cancer. Binding of MERTK to its ligands, growth arrest-specific 6 (GAS6) and protein S, induces the phosphorylation of MERTK and its downstream signalling molecules, including v-akt murine thymoma viral oncogene homolog 1

(AKT) and extracellular signal-regulated kinase (ERK) (1). Overexpression of MERTK has been reported in acute myeloid leukaemia (AML) and acute lymphoblastic leukaemia (ALL) cells (2-4). However, the role of MERTK in leukaemia cell growth remains unclear.

Two small-molecule MERTK-selective inhibitors, UNC569 and UNC1062, have been developed (5-7) that inhibit the phosphorylation of MERTK by occupying its adenine pocket. The effects of UNC1062 are more specific to MERTK than those of UNC569. UNC569 was reported to suppress ALL cell growth *in vitro* (5). However, the effects of these inhibitors on AML cells and their molecular mechanisms have not been elucidated.

To elucidate the role of MERTK in the growth of AML cells and the molecular mechanism of action of these MERTK inhibitors, we evaluated the effects of these MERTK inhibitors on AML cell growth *in vitro*. To confirm the specificity of the effects, MERTK knockdown experiments using small interfering RNA (siRNA) were also performed. We aimed to determine whether MERTK inhibitors are candidates for use as novel molecularly-targeted drugs for the treatment of AML.

Materials and Methods

Cell lines and MERTK inhibitors. Four human AML cell lines were used in this study. The TMD7 cell line was established in our laboratory (8). The OCI/AML5 cell line was established at Ontario Cancer Institute (9). The THP-1 and HEL cell lines were obtained from the Health Science Research Resource Bank (Osaka, Japan). Normal lymphocytes from two healthy volunteers who provided informed consent were also used. UNC569 and UNC1062 were purchased from Calbiochem (San Diego, CA, USA) and Glxxx Laboratories (Southborough, MA, USA), respectively. These inhibitors were dissolved in dimethyl sulfoxide (DMSO) at concentrations of 20 mM and 3 mM, respectively.

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 or without inhibitors, in a humidified atmosphere with 5% CO₂. After 72 h, WST-8 and 1-methoxy-5-methylphenazinium methyl sulphate were added and the optical density (OD) was measured using an enzyme-linked immunosorbent assay plate reader to determine the relative cell

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Key Words: MERTK, leukaemia, apoptosis.

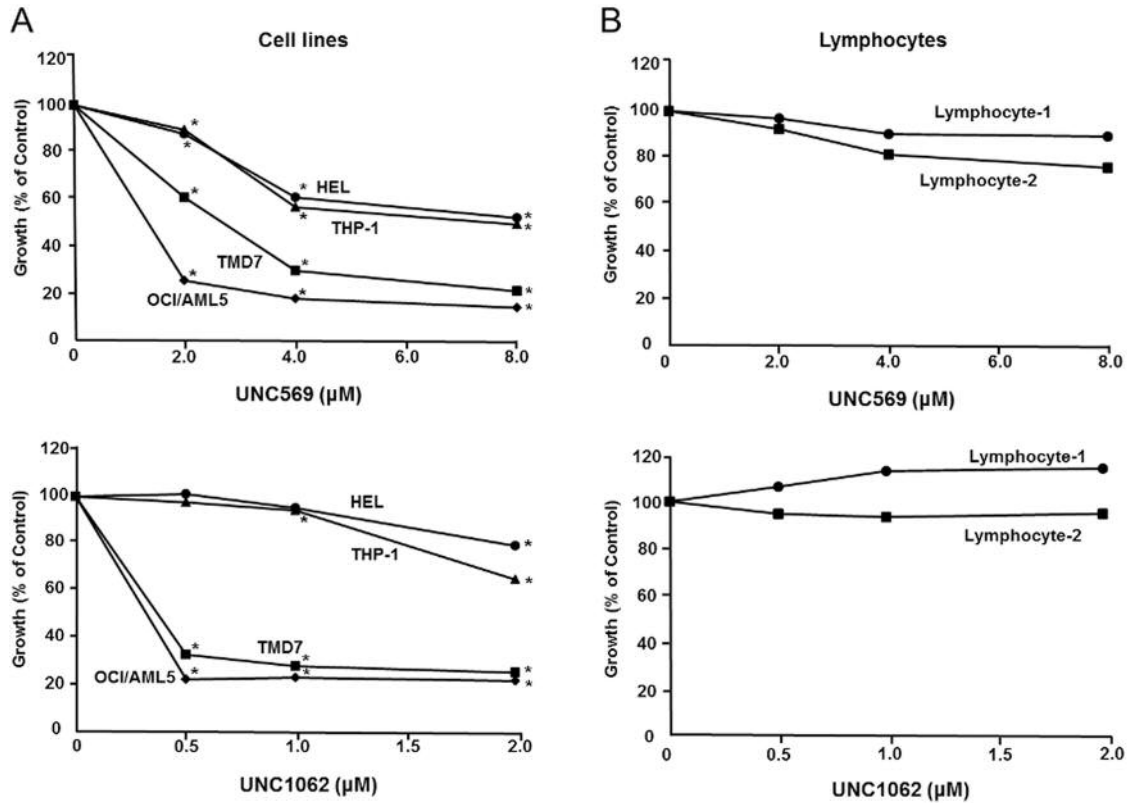


Figure 1. Effects of *MER* proto-oncogene tyrosine kinase inhibitors UNC569 and UNC1062 on growth of leukaemia cell lines (A) and lymphocytes from two healthy donors (B). Cells were cultured with the indicated concentrations of the inhibitors for three days and cell growth was evaluated using a colorimetric assay. The results are expressed as a percentage of the mean optical density in inhibitor-treated cells normalized to that in control (dimethyl sulfoxide-treated) cells. *Significantly different at $p < 0.05$ compared to the control.

number. Relative cell proliferation was calculated as the percentage of the mean OD value normalized to that of the control cells cultured with DMSO. Student's *t*-test was used to determine statistical significance of differences in growth. To examine cell morphology, the harvested cells were stained with May-Grünwald-Giemsa stain and observed on cytospin preparations under a microscope.

Apoptosis assay. Cells treated with the inhibitors for 48 h were stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI), then analysed using a Navios cytometer (Beckman Coulter, Brea, CA, USA) to evaluate the induction of apoptosis.

MERTK knockdown by siRNA. MERTK knockdown by siRNA was performed to confirm the specificity of the effects of the inhibitors. Three different pre-designed *MERTK* siRNAs (Stealth siRNA™, HSS 116030, 116031, and 173654) were purchased from Life Technologies (Carlsbad, CA, USA). 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.

Immunoblot analysis. The lysates from the cells cultured with the inhibitors or siRNA were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analysed by immunoblotting with antibodies against MERTK, AKT, phospho-AKT, ERK,

phospho-ERK, caspase-3, cleaved caspase-3 (Cell Signaling Technology, Danvers, MA, USA), GAS6 (R & D systems, Minneapolis, MN, USA), phospho-MERTK, protein S, and α -tubulin (Abcam, Cambridge, MA, USA), which was used as a loading control. Immunoreactive bands were detected using a Pierce Enhanced Chemiluminescent Western Blotting Substrate (Pierce Biotechnology, Rockford, IL, USA). Each assay was repeated more than twice to ascertain qualitative reproducibility.

Microarray analysis. Microarray analysis was performed to assess the effects of the inhibitors and siRNA on comprehensive gene expression. OCI/AML5 and TMD7 cells were treated with UNC569, UNC1062, DMSO (solvent control), *MERTK* siRNA, or control siRNA for 24 h. Total RNA was extracted using a High Pure RNA isolation kit (Roche Diagnostics, Mannheim, Germany) 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 using Agilent Feature Extraction 11.5.1.1 software.

Results

Effects of MERTK inhibitors on cell growth. Treatment with UNC569 and UNC1062 suppressed growth in all AML cell

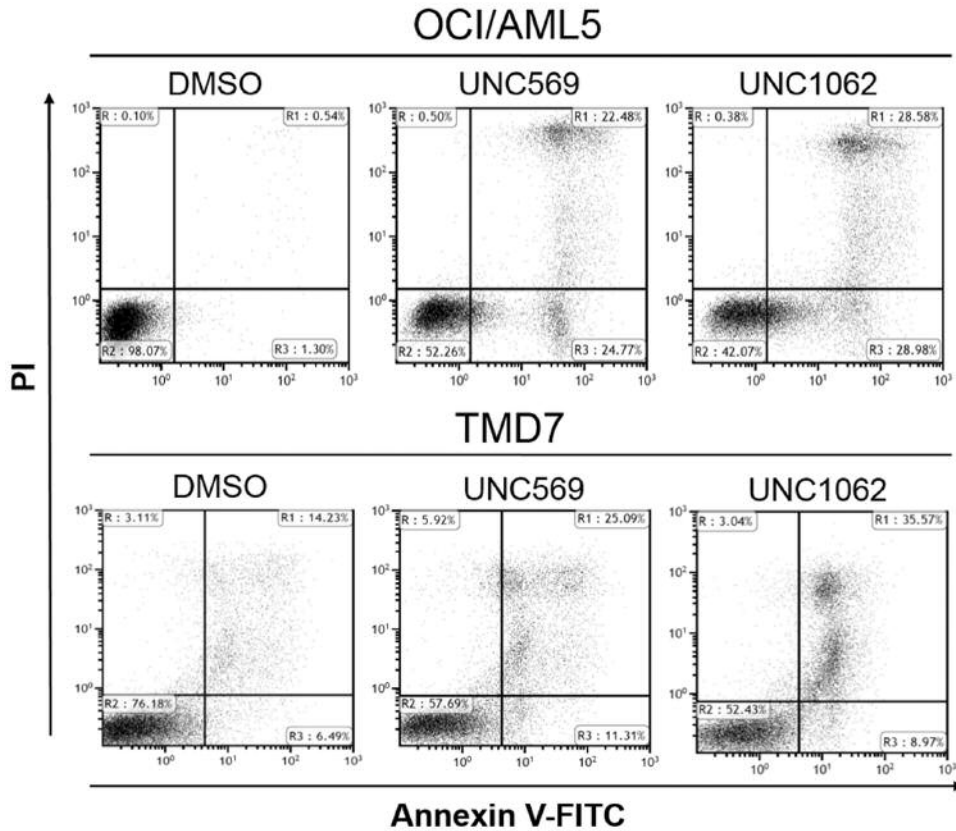


Figure 2. Apoptosis assay of acute myeloid leukaemia cells treated with *MER* proto-oncogene tyrosine kinase inhibitors *UNC569* and *UNC1062*. Cells were cultured with 4 μ M *UNC569* and 2 μ M *UNC1062* for 48 h, stained with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI), and analyzed for apoptosis using flow cytometry. DMSO: Dimethyl sulfoxide.

Table 1. Microarray analysis of the effects of *MER* proto-oncogene tyrosine kinase (*MERTK*) inhibitors *UNC569* and *UNC1062*, and *MERTK* siRNA on gene expression in leukaemia cell lines in vitro. Scale signals indicate the expression of each gene in dimethyl sulfoxide (DMSO)-treated control cells. Log₂ ratios of mRNA expression in inhibitor-treated and *MERTK* siRNA-transfected cells were normalized to those in DMSO-treated and control siRNA-transfected cells, respectively.

Gene symbol	OCI/AML5				TMD7			
	Scale signal	Log ₂ ratio			Scale signal	Log ₂ ratio		
		UNC569	UNC1062	siRNA		UNC569	UNC1062	siRNA
<i>MERTK</i>	151	0.90	1.21	-3.58	1,171	0.27	0.37	-2.63
<i>TYRO3</i>	11,436	-0.27	-0.43	-0.13	507	-0.11	-0.47	-0.18
<i>AXL</i>	750	0.33	0.25	-0.14	860	-0.32	-0.31	-0.02
<i>GAS6</i>	1,402	-0.27	-0.48	0.16	16	NS	NS	0.67
<i>PROS1</i>	2,091	0.65	0.87	0.04	7,230	0.37	0.16	0.08
<i>ADM</i>	1,590	-4.10	-3.87	1.91	26,235	-1.28	-1.11	0.10
<i>BCL2</i>	17,191	-0.06	-0.23	-0.20	1,844	0.33	-0.19	-0.46
<i>CCND1</i>	1,463	-0.74	-1.11	-0.06	123	-0.42	-0.45	-0.45
<i>IL1B</i>	80	-1.52	-2.30	-1.79	858	0.70	0.70	-1.46
<i>MYC</i>	70,188	-0.51	-0.71	0.04	74,542	-0.24	0.12	0.14
<i>RAG1</i>	56	1.47	2.21	1.34	237	0.59	1.27	1.36

NS: Not significantly expressed; *TYRO3*: TYRO3 protein tyrosine kinase; *AXL*: AXL receptor tyrosine kinase; *GAS6*: growth arrest-specific 6; *PROS1*: protein S; *ADM*: adrenomedullin; *BCL2*: B-cell leukaemia/lymphoma 2; *CCND1*: cyclin D1; *IL1B*: interleukin 1 beta; *MYC*: myelocytomatosis oncogene; *RAG1*: recombination activating 1.

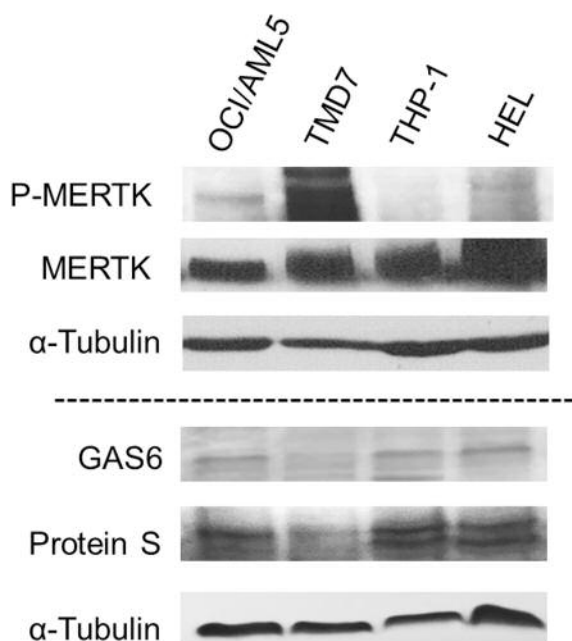


Figure 3. Expression of *MER* proto-oncogene tyrosine kinase (*MERTK*), phospho (*P*)-*MERTK*, growth arrest specific 6 (*GAS6*), protein *S*, and α -tubulin in acute myeloid leukaemia cells analyzed by immunoblotting.

lines in a dose-dependent manner (Figure 1A). OCI/AML5 and TMD7 cells were more susceptible to the inhibitors than THP-1 and HEL cells. The treatment did not significantly affect the viability of normal lymphocytes in the range of concentrations tested (Figure 1B).

Cytospin preparations of cells treated with UNC569 and UNC1062 indicated there were apoptotic cells with nuclear condensation and apoptotic bodies in all AML cell lines (data not shown). Consistent with these results, the flow cytometric apoptosis assay revealed that the treatment increased the annexin-V positive fraction of OCI/AML5 and TMD7 cells (Figure 2). These results indicated that treatment with the inhibitors induced apoptosis.

***MERTK*, *GAS6*, and protein *S* expression in AML cell lines.** *MERTK* protein was expressed in all four cell lines (Figure 3). *MERTK* protein was constitutively phosphorylated in OCI/AML5 and TMD7 cells and weakly phosphorylated in HEL cells. *GAS6* and protein *S* were strongly expressed in OCI/AML5, THP-1, and HEL cells, but weakly expressed in TMD7 cells. In the subsequent experiments to examine the effects of the inhibitors on signalling proteins, we used OCI/AML5 and TMD7 cells, in which *MERTK* was constitutively activated.

Effects of *MERTK* inhibitors on signalling proteins. Phosphorylation of *MERTK* in OCI/AML5 and TMD7 cells

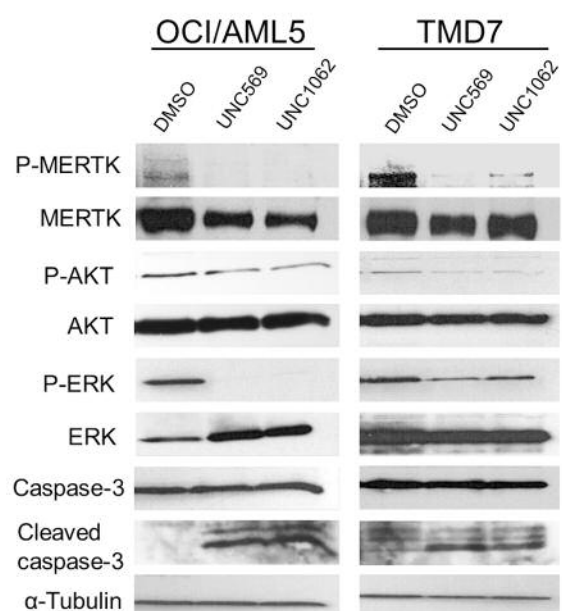


Figure 4. Expression and phosphorylation (*P*) of *MER* proto-oncogene tyrosine kinase (*MERTK*) and down-stream signalling proteins in OCI/AML5 and TMD7 cells treated with *MERTK* inhibitors UNC569 and UNC1062. Cells were cultured with 4 μ M UNC569 and 2 μ M UNC1062 for 24 h and analysed by immunoblotting. Dimethyl sulfoxide (DMSO) was used as a vehicle control. (*P*-)AKT: (Phospho)-v-akt murine thymoma viral oncogene homolog 1; (*P*-)ERK: (phospho)-extracellular signal-regulated kinase.

was suppressed by treatment with the inhibitors without significantly affecting the amount of *MERTK* protein (Figure 4). The treatment also suppressed phosphorylation of AKT and ERK, which are down-stream proteins of *MERTK* (1), and induced cleavage of caspase-3.

Effects of *MERTK* siRNA on cell growth and signalling proteins. To ascertain whether the above effects of the inhibitors were due to inhibition of *MERTK* function or off-target effects, we performed *MERTK* knockdown experiments. Because siRNA HSS 173654 (5'-GCCGCAUUGCUAAG AUGCCUGUAAA-3') suppressed *MERTK* expression most potently among the three *MERTK*-specific siRNAs (data not shown), its effects on cell growth are shown in Figure 5. *MERTK* knockdown suppressed the growth of OCI/AML5 and TMD7 cells. In addition, *MERTK* knockdown suppressed the expression of *MERTK* and slightly suppressed the phosphorylation of AKT and ERK, without a decrease in AKT and ERK expression (Figure 6). *MERTK* knockdown also induced cleavage of caspase-3.

Microarray analysis. The effects of the inhibitors and *MERTK* siRNA on comprehensive gene expression were examined

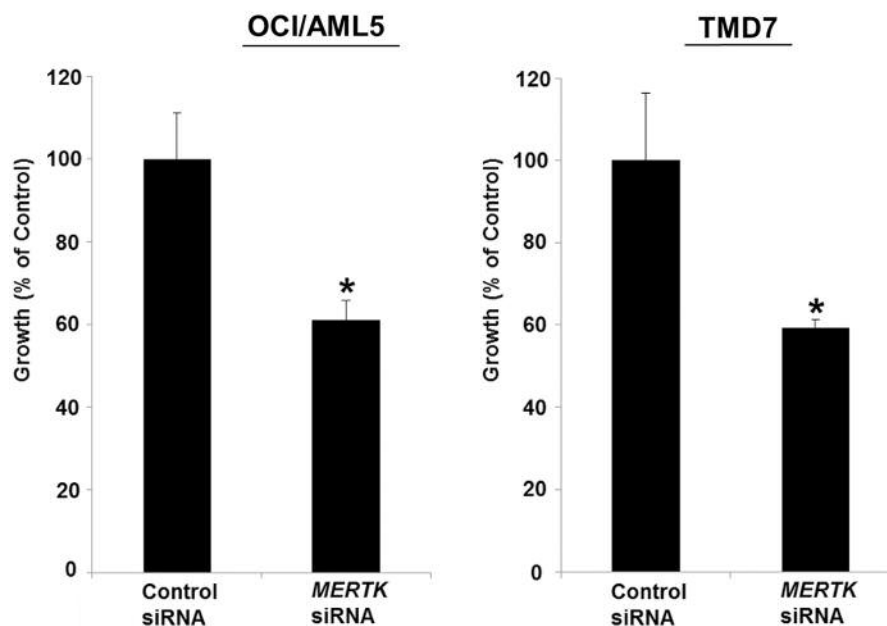


Figure 5. Effects of MER proto-oncogene tyrosine kinase (*MERTK*) knockdown on cell growth. The growth of OCI/AML5 and TMD7 cells transfected with 40 nM of siRNA against *MERTK* was evaluated after 3 and 5 days, respectively, using a colorimetric assay. The results are expressed as a percentage of the mean optical density for *MERTK*-knockdown cells normalized to that in vector control cells. *Significantly different at $p < 0.05$ compared to control.

using microarray analysis. The data from the representative genes are shown in Table I.

MERTK siRNA potently suppressed *MERTK* mRNA expression without affecting the expression of *TYRO3* and *AXL*. Treatment with the inhibitors down-regulated some genes such as cyclin D1 (*CCND1*), but up-regulated others such as recombination activating 1 (*RAG1*). Adrenomedullin (*ADM*) was down-regulated by both inhibitors, but up-regulated by *MERTK* siRNA in OCI/AML5 cells. Expression of *GAS6* and *PROS1*, which encodes protein S, was not significantly affected by the inhibitors or siRNA.

Discussion

This study indicated that the *MERTK* inhibitors UNC569 and UNC1062 suppressed the growth of AML cells and induced their apoptosis. The suppressive effects of the inhibitors were more potent in OCI/AML5 and TMD7 cells in which *MERTK* was constitutively phosphorylated. A possible mechanism for the constitutive phosphorylation of *MERTK* in the cells was autocrine activation by the *MERTK* ligands *GAS6* and protein S produced by the leukaemia cells themselves. We confirmed that the use of a non-serum culture medium did not affect the intensity of their phosphorylation (data not shown). Therefore, *GAS6* and protein S contained in the serum of the culture medium did not act as ligands. The reason that *MERTK* in THP-1 cells was not phosphorylated, even though the cells expressed *GAS6* and protein S, is not clear.

As indicated above, treatment with *MERTK* inhibitors suppressed phosphorylation of its down-stream proteins and induced cleavage of caspase-3. *MERTK* knockdown resulted in the same effects. These results suggest that the effects of the inhibitors were due to on-target effects.

Conversely, the changes in the expression of some genes caused by the inhibitors and those caused by *MERTK* knockdown were discrepant, although the discrepancies were not necessarily due to off-target effects because gene expression was examined only 24 h after inhibitor treatment and siRNA transfection. For example, *ADM* expression was down-regulated by UNC569 treatment but up-regulated by siRNA transfection. *ADM* was previously reported to be involved in the proliferation of AML cells (10). Therefore, this effect of UNC569 may be an advantage of an anti-leukaemia drug. However, because *ADM* is also involved in vasodilation (11), administration of UNC569 might result in changes in blood pressure.

Recently, various novel TAM-targeting drugs have been developed and clinical trials using TAM-targeting drugs against leukaemia and various cancer types have been performed (12, 13). Considering the above effects of UNC569 and UNC1062 and the fact that they did not significantly affect the viability of normal lymphocytes, these inhibitors may be useful as novel molecularly-targeted drugs against AML cells, particularly AML cells in which *MERTK* is constitutively activated. Before their clinical use, further investigation for the possibility of adverse effects due to their

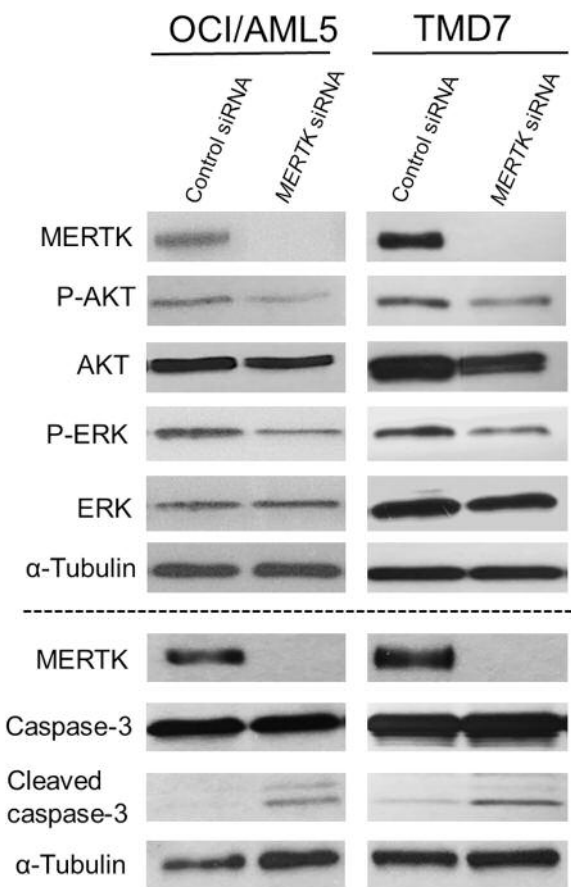


Figure 6. Effects of MER proto-oncogene tyrosine kinase (MERTK) knockdown on the expression and phosphorylation (P) of MERTK and down-stream proteins. OCI/AML5 and TMD7 cells were transfected with 80 nM siRNA or 40 nM siRNA, respectively. After 2 days, cells were harvested for immunoblotting. (P-)AKT: (Phospho)-v-akt murine thymoma viral oncogene homolog 1; (P-)ERK: (phospho)-extracellular signal-regulated kinase.

off-target and on-target effects on normal cells and organs is required. Moreover, development of drug-sensitivity tests to predict validity and select appropriate AML cases are required.

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