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Hypoxia-inducible factor 1α inhibitor induces cell death via suppression of BCR-ABL1 and Met expression in BCR-ABL1 tyrosine kinase inhibitor sensitive and resistant chronic myeloid leukemia cells

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Chronic myeloid leukemia (CML) has a markedly improved prognosis with the use of breakpoint cluster region-abelson 1 (BCR-ABL1) tyrosine kinase inhibitors (BCR-ABL1 TKIs). However, approximately 40% of patients are resistant or intolerant to BCR-ABL1 TKIs. Hypoxia-inducible factor 1α (HIF-1α) is a hypoxia response factor that has been reported to be highly expressed in CML patients, making it a therapeutic target for BCR-ABL1 TKI-sensitive CML and BCR-ABL1 TKI-resistant CML. In this study, we examined whether HIF-1α inhibitors induce cell death in CML cells and BCR-ABL1 TKI-resistant CML cells. We found that echinomycin and PX-478 induced cell death in BCR-ABL1 TKIs sensitive and resistant CML cells at similar concentrations while the cell sensitivity was not affected with imatinib or dasatinib in BCR-ABL1 TKIs resistant CML cells. In addition, echinomycin and PX-478 inhibited the c-Jun N-terminal kinase (JNK), Akt, and extracellular-regulated protein kinase 1/2 (ERK1/2) activation via suppression of BCR-ABL1 and Met expression in BCR-ABL1 sensitive and resistant CML cells. Moreover, treatment with HIF-1α siRNA induced cell death by inhibiting BCR-ABL1 and Met expression and activation of JNK, Akt, and ERK1/2 in BCR-ABL1 TKIs sensitive and resistant CML cells. These results indicated that HIF-1α regulates BCR-ABL and Met expression and is involved in cell survival in CML cells, suggesting that HIF-1α inhibitors induce cell death in BCR-ABL1 TKIs sensitive and resistant CML cells and therefore HIF-1α inhibitors are potential candidates for CML treatment. [BMB Reports 2023; 56(2): 78-83]

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INTRODUCTION

Chronic myeloid leukemia (CML) is a myeloproliferative disease of monoclonal undifferentiated hematopoietic stem cells and accounts for 15% of all the leukemias in adults (1). A major pathogenesis of CML is the Philadelphia chromosome, which is formed by the reciprocal translocation of chromosomes 9 and 22. The breakpoint cluster region-abelson 1 (BCR-ABL1) protein encoded by the Philadelphia chromosome constitutively activates survival signaling factors associated with cell survival and proliferation, such as phosphoinositide 3-kinase (PI3K)/Akt pathway, Janus kinase (JAK)/signal transducer and activator of transcription (STAT), and mitogen-activated protein kinase kinase (MEK)/extracellular regulated protein kinase (ERK) (2).

The BCR-ABL1 tyrosine kinase inhibitors (BCR-ABL1 TKIs), such as imatinib, dasatinib, nilotinib, and bosutinib, which are currently the first-line CML therapeutic agents, have markedly improved the survival rates of CML patients (3). However, approximately 40% of CML patients exhibit intolerance or resistance to BCR-ABL1 TKIs, consequently affecting prognosis (4). Point mutations in the BCR-ABL1 kinase domain have been reported to develop BCR-ABL1 TKIs resistance. For example, the T315I mutation develops resistance to imatinib, dasatinib, nilotinib, and bosutinib (5). While currently, the prognosis is improving with the advent of ponatinib for T315I mutation; resistance to ponatinib has also been reported (5). In addition, the reasons for resistance to the second- and third-generation BCR-ABL1 TKIs without point mutations in the BCR-ABL1 kinase domain, which is the cardinal reason for resistance, are largely unknown, and therefore no effective treatments are available.

In the previous study, we established imatinib-resistant strains, which are also resistant to most BCR-ABL1 TKIs, and investigated the cause of resistance in these CML cells. We found that the activation of JNK and ERK1/2 through Met activation is responsible for developing resistance; however, the contribution of other factors has not been clarified (6). In addition, hypoxia-inducible factor 1α (HIF-1α) expression is augmented

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in patients with CML compared to healthy donors and is suggested to be an important factor in maintaining CML stem cells (7, 8). HIF-1α activates the gene transcription associated with crucial processes of cancer biology, including cell survival, invasion, angiogenesis, and glucose metabolism, thereby promoting cancer progression (9). Moreover, since HIF-1α has been shown to regulate Met transcription (10), it is believed that HIF-1α may be associated with the development of BCR-ABL1 TKIs resistance. Therefore, HIF-1α is a potential new therapeutic target for CML, and HIF-1α inhibitors may be unsusceptible to resistance by acting via the Met pathway.

In this study, we investigated whether HIF-1α is associated with imatinib resistance and whether these results can be extrapolated to develop new treatments for CML.

RESULTS

Elevated expression of HIF-1α in imatinib and dasatinib resistant CML cells

We investigated whether HIF-1α expression differed between imatinib, nilotinib, and dasatinib non-responder and responder patients using the GSE77573 and GSE33224 datasets. We found higher expression levels of HIF-1α in imatinib, imatinib and nilotinib, and dasatinib non-responders than in imatinib or dasatinib responders (Fig. 1A, B). In addition, HIF-1α expression was increased in K562 cells compared with that in non-BCR-ABL1-expressing leukemia cell lines, such as HL60, U937, Jurkat, and CCRF-SB (Fig. 1C). We also found elevated expression levels of HIF-1α in K562/IR and K562/DR cells compared to K562 cells (Fig. 1D). Moreover, HIF-1α inhibitors, echinomycin and PX-478, markedly induced cell death in K562, K562/IR, and K562/DR cells; however, they did not overcome imatinib and dasatinib resistance (Fig. 1E, F). siRNA-mediated BCR-ABL1 silencing induced cell death in K562 cells but not in K562/IR cells (Fig. 1G, H). These results indicated that HIF-1α inhibitors promote cell death in BCR-ABL1 TKIs sensitive and resistant cells.

Echinomycin and PX-478 suppressed the BCR-ABL1 and Met expression in imatinib sensitive and resistant cells

Our previous study demonstrated that overexpression and activation of Met are related to imatinib resistance in K562/IR and KU812/IR cells (6). In addition, BCR-ABL1 plays a cardinal role in the survival and proliferation of CML cells (2). Moreover, as observed in this study, HIF-1α inhibitors did not enhance the sensitivity of K562/IR and K562/DR cells to imatinib and dasatinib. Therefore, we investigated whether echinomycin and PX-478 inhibited the BCR-ABL1 activation/expression in K562 and K562/IR cells and the Met activation/expression in K562/IR cells. Our results revealed that echinomycin and PX-478 suppressed the BCR-ABL1 mRNA expression in K562 and K562/IR cells and Met mRNA expression in K562/IR cells (Fig. 2A, B). In addition, echinomycin and PX-478 inhibited BCR-ABL1 phosphorylation and protein expression in K562 and K562/IR cells, and Met phosphorylation and protein expression in K562/IR cells (Fig. 2C, D). These results indicated that HIF-1α inhibitors induced cell death by suppressing BCR-ABL1 and Met expression in BCR-ABL1 TKIs sensitive and resistant cells.

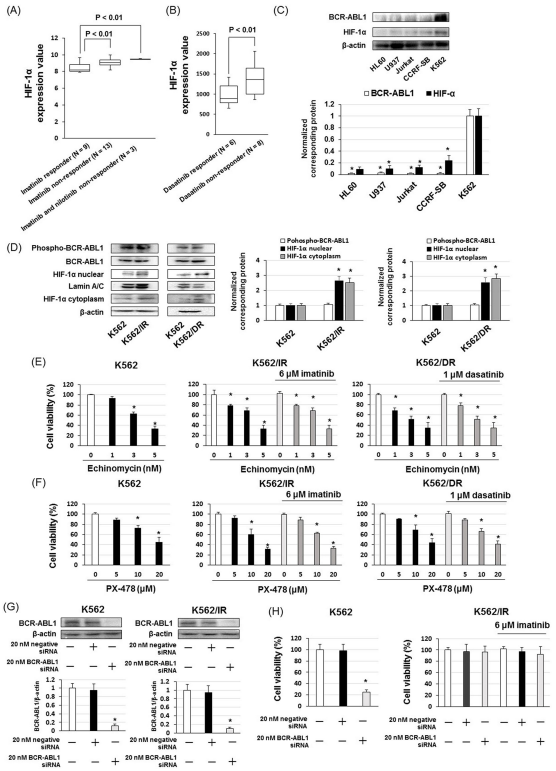

Fig. 1. Effect of echinomycin and PX-478 on K562, K562/IR, and K562/DR cell survival. (A) The expression of HIF-1α in imatinib responder, imatinib non-responder, and imatinib and nilotinib non-responder with CML was analyzed using the GSE77573 dataset. (B) The expression of HIF-1α in dasatinib responder and dasatinib non-responder with CML was analyzed using the GSE33224 dataset. (C) Cell lysates were evaluated by immunoblotting using the indicated antibodies. The content of BCR-ABL1 and HIF-1α, is normalized to the content of β-actin. The results are exemplary of three independent experiments. *P < 0.01 vs. K562 cells. (D) Cell lysates were evaluated by immunoblotting using the previously listed antibodies. The content of nuclear HIF-1α and cytoplasmic HIF-1α normalized to the content of Lamin A/C or β-actin. The results are exemplary of three independent experiments. *P < 0.01 vs. K562 cells. (E, F) The cell survival rate of echinomyacin (E) and PX-478 (F) or combined treatment with echinomycin or PX-478 and imatinib or dasatinib, as determined by the trypan blue staining assay. Cells were administered with the indicated concentrations of echinomycin, PX-478, imatinib, or dasatinib for three days. The results are exemplary of five independent experiments. *P < 0.01 vs. untreated K562, K562/IR, or K562/DR cells. (G) Protein expression in cell lysates was analyzed using immunoblotting. β-Actin was used as the loading control to normalize BCR-ABL1 expression. The results are exemplary of three independent experiments. *P < 0.01 vs. untreated K562 or K562/IR cells. (H) The survival rate of BCR-ABL1 siRNA cells, as determined by trypan blue staining. The results are exemplary of five independent experiments. *P < 0.01 vs. untreated K562 or K562/IR cells.

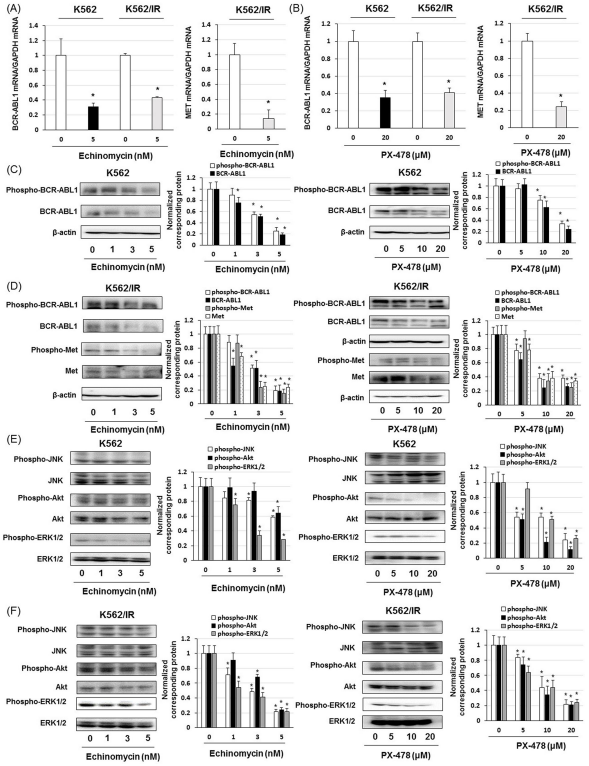

Fig. 2. Echinomycin and PX-478 inhibited BCR-ABL1 and Met expression in K562 and K562/IR cells. (A, B) K562 and K562/IR cells were treated with echinomycin (A) and PX-478 (B) at indicated concentrations for 3 days. RNA was extracted, and BCR-ABL1 and Met levels were examined using real-time PCR. The results were standardized using GAPDH values and then expressed as a test:-control ratio. These results are the average of four independent experiments. *P < 0.01 vs. untreated K562 or K562/IR cells. (C-F) K562 (C, E) and K562/IR cells (D, F) were treated with echinomycin and PX-478 at the indicated concentration for three days. (C, D) Cell lysates were evaluated by immunoblotting using the antibodies. The content of phospho-BCR-ABL1, BCR-ABL1, and Met, normalized to the content of β-actin. The results are exemplary of three independent experiments. *P < 0.01 vs. untreated K562 or K562/IR cells. (E, F) Cell lysates were evaluated by immunoblotting using the antibodies. The content of phospho-ERK1/2, phospho-JNK, and phospho-Akt, normalized to the content of ERK1/2, JNK, and Akt. The results are exemplary of three independent experiments. *P < 0.01 vs. untreated K562 or K562/IR cells.

Echinomycin and PX-478 inhibited the JNK, Akt, and ERK1/2 activation in K562 and K562/IR cells

BCR-ABL1 and Met promote JNK, Akt, and ERK1/2 phosphorylation of in tumor cells (2, 11). Echinomycin or PX-478 inhibited the expression of phosphorylated JNK, Akt, and ERK1/2 in K562 and K562/IR cells (Fig. 2E, F). These results indicated that HIF-1α inhibitors inhibited the JNK, Akt, and ERK1/2 activation via suppression of BCR-ABL1 and Met expression in BCR-ABL1 TKIs sensitive and resistant cells.

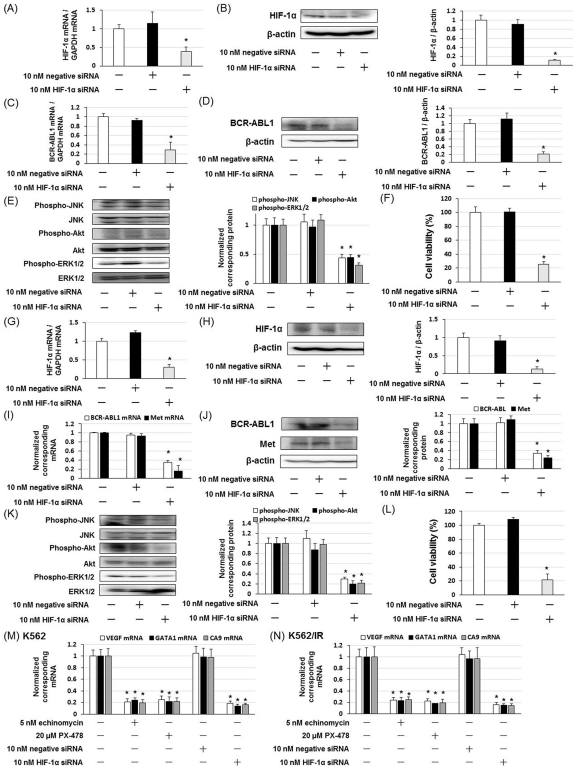

Fig. 3. HIF-1α siRNA inhibited ERK1/2, JNK, and Akt activation by suppressing BCR-ABL1 and Met expression in K562 and K562/IR cells. (A-L) K562 and K562/IR cells were treated with 10 nM HIF-1α or negative siRNA for three days. (A, G) RNA was extracted, and HIF-1α levels were examined using real-time PCR. The results were standardized using GAPDH values and then expressed as a test:control ratio. These results are the average of four independent experiments. *P < 0.01 vs. untreated K562 or K562/IR cells. (B, H) Cell lysates were evaluated by immunoblotting using the antibodies. The content of HIF-1α, is normalized to the content of β-actin. The results are exemplary of three independent experiments. *P < 0.01 vs. untreated K562 or K562/IR cells. (C, I) RNA was extracted, and BCR-ABL1 or Met levels were examined using real-time PCR. The results were standardized using GAPDH values and then expressed as a test-:control ratio. These results are the average of four independent experiments. *P < 0.01 vs. untreated K562 or K562/IR cells. (D, J) Cell lysates were evaluated by immunoblotting using the antibodies. The content of BCR-ABL1 or Met, is normalized to the content of β-actin. The results are exemplary of three independent experiments. *P < 0.01 vs. untreated K562 or K562/IR cells. (E, K) Cell lysates were evaluated by immunoblotting using the indicated antibodies. The content of phospho-ERK1/2, phospho-JNK, and phospho-Akt, normalized to the content of ERK1/2, JNK, and Akt. The results are exemplary of three independent experiments. *P < 0.01 vs. untreated K562 or K562/IR cells. (F, L) The cell survival rate of HIF-1α siRNA, was determined by the trypan blue staining assay. The results are exemplary of five independent experiments. *P < 0.01 vs. untreated K562 or K562/IR cells. (M, N) RNA was extracted, and VEGF, GATA1, or CA9 levels were assessed using real-time PCR. GAPDH was used as the endogenous control to normalize the gene expression data. The results are the average of four independent experiments. *P < 0.01 vs. untreated K562 or K562/IR cells.

HIF-1α silencing promotes cell death via inhibition of BCR-ABL1 and Met expression in K562 and K562/IR cells

Since HIF-α inhibitors inhibited the activation/expression of BCR-ABL1 and Met, we examined whether HIF-1α knockdown with small interfering RNA (siRNA) could similarly induce cell death by suppressing BCR-ABL1 and Met expression. We found that treatment with HIF-1α siRNA suppressed the BCR-ABL1 expression by inhibiting HIF-1α expression in K562 cells (Fig. 3A-D). Moreover, HIF-1α knockdown by siRNA inhibited the JNK, Akt, and ERK1/2 activation consequently inducing cell death in K562 cells (Fig. 3E-F). Furthermore, administration of HIF-1α siRNA inhibited the activation of JNK, Akt, and ERK1/2 by suppressing BCR-ABL1 and Met expression, thereby inducing cell death in K562/IR cells (Fig. 3G-L). Echinomycin, PX-478, and HIF-1α siRNAs suppressed the expression of HIF-1α target genes, such as vascular endothelial growth factor (VEGF), GATA1, and carbonic anhydrase IX (CA9) (Fig. 3M, N).

To validate these findings, we investigated whether echinomycin induced cell death via suppression of BCR-ABL1 and Met expression in a different CML cell line, KU812 and KCL-22, and imatinib-resistant cell line, KU812/IR and KCL-22/IR. Echinomycin markedly promoted cell death in KU812, KU812/IR, KCL-22, and KCL-22/IR cells without affecting the sensitivity of KU812/IR and KCL-22/IR cells to imatinib (Fig. 4A-D). In addition, echinomycin suppressed the BCR-ABL1 expression in KU812, KU812/IR, KCL-22, and KCL-22/IR cells and suppressed Met expression in KU812/IR and KCL-22/IR cells (Fig. 4E-H). These results indicated that HIF-1α regulates the BCR-ABL1 and Met expression and promotes cell survival in BCR-ABL1 TKIs sensitive and resistant cells.

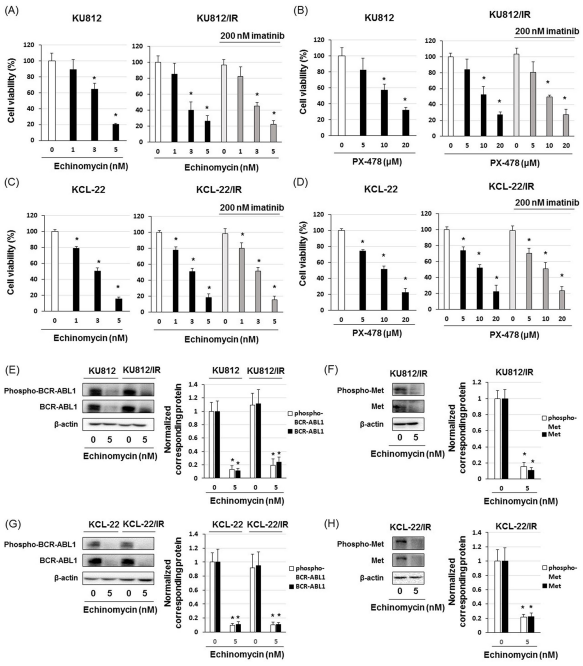

Fig. 4. Effect of echinomycin and PX-478 on cell survival and expression of BCR-ABL1 and Met in KU812, KU812/IR, KCL-22, and KCL-22/IR cells. (A-D) The cell survival rate after treatment with echinomyacin (A, C) and PX-478 (B, D) or combined treatment with echinomycin or PX-478 and imatinib, as determined by the trypan blue staining assay. Cells were administered with echinomycin, PX-478, or imatinib at concentrations mentioned below for three days. The results are exemplary of five independent experiments. *P < 0.01 vs. untreated KU812 or KU812/IR cells. (E-H) KU812, KU812/IR, KCL-22, and KCL-22/IR cells were treated with 5 nM echinomycin for three days. (E, G) Cell lysates were evaluated by immunoblotting using the antibodies. The content of BCR-ABL1, is normalized to the content of β-actin. The results are exemplary of three independent experiments. *P < 0.01 vs. untreated KU812, KU812/IR, KCL-22, or KCL-22/IR cells. (F, H) Cell lysates were evaluated by immunoblotting using the antibodies. The content of Met, is normalized to the content of β-actin. The results are exemplary of three independent experiments. *P < 0.01 vs. untreated KU812/IR or KCL-22/IR cells.

DISCUSSION

We demonstrated that elevated expression of HIF-1α in imatinib, nilotinib, and dasatinib non-responder was higher than that in imatinib and dasatinib responders. It has been suggested that the expression of HIF-1α increases as the disease progresses in CML patients (12). It has also been reported that HIF-1α is related to imatinib resistance and elevated BCR-ABL1 expression is correlated to HIF-1α activation/expression in imatinib-resistant cells (13). These findings indicated that elevated expression of HIF-1α may be associated with BCR-ABL1 TKIs resistance. However, we found that echinomycin and PX-478 induced cell death at the similar concentration in K562, K562/IR, K562/DR, KU812, KU812/IR, KCL-22, and KCL-22/IR cells and that echinomycin and PX-478 did not enhance the sensitivity of K562/IR, K562/DR, KU812/IR, and KCL-22/IR cells to imatinib and dasatinib. In addition, echinomycin and PX-478 abrogated the expression of BCR-ABL1 and Met in K562, K562/IR, KU812, KU812/IR, KCL-22, and KCL-22/IR cells. Moreover, echinomycin, PX-478, and HIF-1α siRNAs suppressed the expression of HIF-1α-induced genes, such as VEGF, GATA1, and CA9 in K562 and K562/IR cells. Furthermore, HIF-1α expression in K562 cells was increased compared with that in non-BCR-ABL1-expressing cell lines, such as HL60, U937, Jurkat, and CCRF-SB. It has been suggested that HIF-1α regulates BCR-ABL1 expression in imatinib-resistant cells (13). It has also been reported that the suppression of HIF-1α, such as by up-regulation of HIF-1α antisense RNA by SC144, a gp130 inhibitor, and arylsulfonamide 64B, which is a HIF-1α inhibitor, inhibited Met expression in human ovarian cancer and uveal melanoma cells (14, 15). In addition, HIF-1α promotes the expression of Met in solid tumors, and CML progenitor cells appear to be more dependent on HIF-1α signaling for survival than normal progenitor cells; this leads to a drastic reduction in the repopulation of HIF-1α knockout CML cells transplanted into mice compared to that of HIF-1α wild type CML cells (16). These findings indicated that HIF-1α inhibitors, such as

echinomycin and PX-478, induced cell death via suppression of the BCR-ABL1 and Met expression in BCR-ABL1 TKIs sensitive and resistant cells.

BCR-ABL1 and Met promote cell proliferation and survival by activating the survival signaling pathway, including MEK/ERK, JNK, and PI3K/Akt pathways (6, 17). We found that echinomycin and PX-478 inhibited the JNK, Akt, and ERK1/2 phosphorylation in K562 and K562/IR cells by suppressing BCR-ABL1 and Met activation/expression. In addition, treatment with HIF-1α siRNA inhibited the activation of JNK, Akt, and ERK1/2 via suppression of BCR-ABL1 and Met expression in K562 and K562/IR cells. Our previous findings indicated that Met activation is associated with low sensitivity of K562/IR and KU812/IR cells to BCR-ABL1 TKIs via activation of JNK and MEK/ERK pathways (6). In addition, depletion of BCR-ABL1 by pterostilbene, induced apoptosis in expressing the BCR-ABL1 wild-type and BCR-ABL1 T315I-mutated cells (18). It has also been reported that inhibition of Heat shock protein family A member 8 (HSPA8) by HSPA8 siRNA inhibited cell proliferation and tumor growth via abrogation of BCR-ABL1 expression and activation of STAT5 and Akt activation in BCR-ABL1 wild type, BCR-ABL1 T315I-mutated, and BCR-ABL independent imatinib-resistant CML cells (19). These findings suggested that the inhibition of JNK, Akt, and ERK1/2 by downregulation of BCR-ABL1 and Met expression using HIF-1α inhibitors induced cell death in BCR-ABL1 wild type and BCR-ABL TKIs resistant CML cells.

In conclusion, HIF-1α was inhibited by inhibitors and siRNA induced cell death by suppressing BCR-ABL1 and Met expression in BCR-ABL1 TKIs sensitive and resistant CML cells. These findings conclude that HIF-1α inhibitors are potential therapeutic agents for CML.

MATERIALS AND METHODS

Materials

Echinomycin and PX-478 were obtained from FUJIFILM Wako (Tokyo, Japan) and Selleckchem (Houston, TX, USA), and were dissolved in dimethyl sulfoxide to make stock solutions.

Cell culture

The K562, KU812, and KCL-22 cells were procured from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). These cell lines were used to establish imatinib and dasatinib resistant lines (K562/IR, K562/DR, KU812/IR, and KCL-22/IR), as described previously (6, 20). The HL-60, U937, Jurkat, and CCRF-SB cell lines were obtained from Riken Cell Bank (Ibaraki, Japan).

Cell survival assay

Cell survival after treatment with echinomycin and PX-478 was measured using the trypan blue dye staining assay, as reported earlier (21, 22).

Immunoblotting

Nuclear and Cytoplasmic fractions extracted from each cell using the ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem, San Diego, CA, USA) were appreciated by immunoblotting, as reported previously (6). The following primary antibodies were used: anti-HIF-1α antibody (#3716), anti-phospho-ABL1 antibody (#2865), anti-ABL1 antibody (#2862), anti-phospho-MET antibody (#3126), anti-MET antibody (#4560), anti-phospho-JNK antibody (#3073), anti-JNK antibody (#9252), anti-phospho-Akt antibody (#9271), anti-Akt antibody (#9272), anti-phospho-ERK1/2 antibody (#3073), anti-ERK1/2 antibody (#4370) (Cell Signaling Technology, Beverly, MA, USA), anti-β-actin antibody (A2228, clone AC-74) (Sigma-Aldrich, St Louis, MO, USA), and anti-Lamin A/C antibody (sc-7293) (Santa Cruz Biotechnologies, CA, USA).

RNA interference

The siRNAs targeting HIF-1α (HSS104774), ABL1 (HSS100045), and Stealth RNAi™ negative control duplex with low GC construct (negative control) were procured from Invitrogen. Transfection of siRNA was performed according to the manufacturer's protocol, as described previously (6).

Quantitative real-time (PCR)

Total RNA was extracted using RNAiso (Takara Biomedical), and cDNA was synthesized from the extracted RNA using the PrimeScript First-Strand Synthesis System (Takara Biomedical). The cDNA was analyzed by real-time PCR using Thermal Cycler Dice Real-Time System (Takara Biomedical) and SYBR Premix Ex Taq (Takara Biomedical). The PCR settings to amplify GAPDH, BCR-ABL1, MET, and HIF-1α gene were 94°C for 2 min followed by 40 cycles of 94°C for 0.5 min, 50°C for 0.5 min, and 72°C for 0.5 min. The following primer sequences were used: BCR-ABL1: 5′- GTG TGA AAC TCC AGA CTG TC -3′ (5′-primer) and 5′- CAA AAT CAT ACA GTG CAA CGA -3′ (3′-primer), MET: 5′- GGT CAA TTC AGC GAA GTC CT -3′ (5′-primer) and 5′- CCA GTG TGT AGC CAT TTT GG -3′ (3′-primer), HIF1A: 5′- TGA TTG CAT CTC CAT CTC CTA CC -3′ (5′-primer) and 5′- GAC TCA AAG CGA CAG ATA ACA CG -3′ (3′-primer), VEGF: 5′- AGG GCA GAA TCA TCA CGA AGT -3′ (5′-primer) and 5′- AGG GTC TCG ATT GGA TGG CA -3′ (3′-primer), GATA1: 5′- TTG TCA GTA AAC GGG CAG GTA -3′ (5′-primer) and 5′- CTT GCG GTT TCG AGT CTG AAT -3′ (3′-primer), CA9: 5′- GGA TCT ACC TAC TGT TGA GGC T -3′ (5′-primer) and 5′- CAT AGC GCC AAT GAC TCT GGT -3′ (3′-primer), and GAPDH: 5′-GAC ATC AAG GTG AA-3′ (5′-primer) and 5′-TGT CAT ACC AGG AAA TGA GC-3′ (3′-primer). For standardization, GAPDH was used as an internal control for each sample; Cycle threshold (Ct) values were archived, and the standardized expression of each gene in cells was evaluated using the $2^{-\Delta\Delta Ct}$ method.

Gene expression omnibus (GEO) dataset

The gene expression profiling with microarray dataset, acces-

sion number GSE77573 and GSE33224, was procured from the National Center of Biotechnology Information (NCBI) GEO database (http://www.ncbi.nlm.nih.gov/geo/). HIF-1α gene expression in imatinib, nilotinib, or dasatinib responders or non-responders with CML patients was analyzed.

Statistical analysis

All data are presented as the mean ± standard deviation of several independent experiments, and the data were analyzed using analysis of variance with the Dunnet test. Significant differences were determined at $P < 0.05$.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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