INTRODUCTION

In Philadelphia chromosome (Ph)-positive leukemia, BCR-ABL tyrosine kinase inhibitors (TKI) have led to a new paradigm of targeted cancer therapy. However, some patients with CML or Ph-positive acute lymphoblastic leukemia (ALL) experience treatment failure after an initial response. The resistance to TKI is frequently associated with the development of point mutations within the kinase domain of BCR-ABL. TKI bind to the ATP-binding site of BCR-ABL kinase, which traps the kinase in an inactive conformation. At the protein level, point mutations result in inhibition of the interface between BCR-ABL kinase and TKI. In particular, the T315I mutant is uniformly resistant to TKI other than ponatinib. Ponatinib has efficacy in CML patients after failure with other TKI or with T315I.
mutation; however, resistance or intolerance can develop in some cases.7 New treatment options are required for patients with ponatinib failure.

We previously reported medium-chain fatty-acid derivative AIC-47 as a novel anti-cancer agent for the treatment of CML.8,9 AIC-47 induces autophagic cell death through transcriptional suppression of BCR-ABL and perturbation of cancer-specific energy metabolism, including the Warburg effect.8 TKI inhibit phosphorylation of BCR-ABL protein only, whereas AIC-47 suppresses expression of BCR-ABL itself through transcriptional suppression of the BCR-ABL gene.8,9 This suggests that AIC-47 could affect BCR-ABL-mutant cells.

Cancer cells efficiently use a limited energy source by modulating cellular signaling and reprogramming metabolic pathways.10 These alterations including the Warburg effect confer many advantages to cancer cells, including the promotion of biosynthesis, ATP generation, detoxification and support of rapid proliferation.11 The Warburg effect is a well-known metabolic switch that is partly achieved through regulated expression of pyruvate kinase muscle isoforms PKM1 and PKM2.12 These isoforms are expressed by alternative splicing of the PKM mRNA precursor.12 PKM is alternatively spliced to produce either the PKM1 or the PKM2 isoform, which contains exon 9 or exon 10, respectively.13,14 Previous studies showed that heterogeneous nuclear ribonucleoproteins (hnRNP) [ie, polypyrimidine tract-binding protein 1 (PTBP1, also known as hnRNPJ), hnRNPA1, and hnRNPA2] are alternative splicing repressors of PKM114,15 and that serine/arginine-rich protein SRSF3 activates PKM2 expression.16,17 We found that knockdown of BCR-ABL leads to perturbation of the Warburg effect through the hnRNP/PKM cascade.8

We have already shown that AIC-47 showed cytotoxicity in wild type (WT)-BCR-ABL-harbouring cells and leukemia stem cells;8,9 however, the effects on BCR-ABL-mutated cells have not been clarified. Our previous data suggested that the effects of AIC-47 were independent of the configuration of BCR-ABL kinase.9 In the present study, we examined the efficacy of AIC-47 in mutated-BCR-ABL-harbouring cells in vitro and in vivo.

2 | MATERIALS AND METHODS

2.1 | Patient blood samples

Blood samples from newly diagnosed CML patients were collected following protocol approval by the institutional review board of Kobe University and with informed consent.

2.2 | Cell culture and treatment

WT-, M351T-, Y253F- or T315I-BCR-ABL-transformed clones of mouse pro-B Baf3 cells (Baf3p210 cells) were gifted from Brian J. Druker, Oregon Health and Science University Cancer Institute.18 WT-BCR-ABL positive human ALL cell line TCCY was established as reported previously.19 To establish imatinib-resistant clone having T315I-mutated BCR-ABL, the WT-BCR-ABL-harbouring TCCY cells were treated with imatinib by gradually increasing the concentration (3–20 μM). The dead cells were washed out every 3 to 4 days, and the resistant subclones were isolated by limiting dilution. Cells were tested for Mycoplasma contamination by using a MycoAlert Mycoplasma Detection Kit (LT07-118; Lonza, Rockland, ME, USA). Cells were cultured under an atmosphere of 95% air and 5% CO2 at 37°C in RPMI-1640 medium (189-02025; Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated FBS (Sigma-Aldrich, St Louis, MO, USA). Chemical structure and synthesis of AIC-47 were reported previously.8 AIC-47 and imatinib (10936; Tokyo Chemical Industry, Tokyo, Japan) were dissolved in DMSO and added to the cell culture medium at a final concentration of DMSO (<0.3%), which showed no significant effect on the growth and differentiation of the cells (data not shown). Viable cell numbers were measured by carrying out the Trypan-blue dye-exclusion test.

2.3 | Real-time RT-PCR

Total RNA was isolated from cells by using a NucleoSpin miRNA kit (TaKaRa, Otsu, Japan) according to the manufacturer’s protocol. Expression levels of mRNAs were determined as described previously.8 Sequences of the primers used in this study were as follows: BCR-ABL-sense, 5′-TTTCAAGAGCTTTCTCCTGCAT-3′; BCR-ABL-antisense, 5′-TGTGTACGGGTGTAGTATGTCCTGG-3′; PKM1-sense, 5′-CCG CCAATGACTCCACCAT-3′; PKM2-antisense, 5′-GATGACGACCTGTCCAGACT-3′; PKM2-sense, 5′-ATTACCTGAGAACCTCGCCGG CCT-3′; PKM2-antisense, 5′-ATTCCGCGTCCACAGCAATGATGGG-3′; PKM1-sense, 5′-CATGATTTCACCACCCATCA-3′; PKM1-antisense, 5′-AGACAGGACACATTCCATTTCC-3′; GAPDH-sense, 5′-CCTCCTGGAATTTCCATTGGCA-3′; GAPDH-antisense, 5′-TCTAGAGCGCAGGTCTAGGTCCACCAC-3′; mmu-ACTB-sense, 5′-GGAATCATGTCG TGACATC-3′; mmu-ACTB-antisense, AAGGAAGGCTGGAAAAGAAGC-3′; GAPDH and ACTB were used as an internal control. Relative expression level of mRNA was calculated by the ΔΔCt method.

2.4 | Western blotting

Protein extraction and western blotting experiments were carried out as described previously.9 Antibodies against the following proteins were purchased from Cell Signaling Technology (Danvers, MA, USA): PTBP1 (#8776), hnRNPA1 (#4296), PKM1 (#7067), PKM2 (#4053), and PKM1/2 (#3190). Anti-SRSF3 antibody (PN080PW) was obtained from MBL (Nagoya, Japan). The loading control was prepared by reincubating the same membrane with anti-β-actin antibody (A5316; Sigma-Aldrich).

2.5 | Transfections

Cells were transfected with siRNA for PTBP1 (siR-PTBP1; 5′-AUCUCUGGUCUGCUAAAGUCUCACUUCC-3′; Invitrogen), hnRNPA1 (siR-hnRNPA1; sc-270345; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or SRSF3 (siR-SRSF3; 5′-UUACACGCGCCGCAAAUGUGUUCU-3′; Invitrogen) by using Lipofectamine RNAiMAX (Invitrogen).
according to the manufacturer’s protocol. Sequence of the non-specific control RNA (Hokkaido System Sciences, Sapporo, Japan) was 5’-GGCCUUUCACUACUCCUCA-3’.

2.6 | Animal experiments

Our institute’s committee for ethics in animal experimentation approved all animal experimental protocols. Animal experiments were conducted in accordance with the guidelines for Animal Experiments of Gifu International Institute of Biotechnology.

BALB/cScSlc-nu/nu nude mice were obtained from Japan SLC (Hamamatsu, Japan). WT- or T315I-BCR-ABL-harboring Baf3p210 cells (1 x 10^6 cells in 100 μL PBS) were i.v. injected into each of 12 mice (day 0). After 3 days (day 3), blood samples were taken, and expression levels of BCR-ABL mRNA were determined by carrying out real-time RT-PCR. AIC-47 (75 mg/kg) was given i.v. every fourth day. Collection of spleen and liver samples was done on day 18.

2.7 | Statistical analysis

Each examination was carried out in triplicate. Data are presented as means ± SD. Unless otherwise stated, differences were statistically evaluated by use of one-way ANOVA followed by t test. Statistical evaluation was carried out by using GraphPad 6.0 software. For all statistical data, level of significance was set at P < .05.

3 | RESULTS

3.1 | Medium-chain fatty-acid derivative AIC-47 inhibited growth even in BCR-ABL mutant cells

As previously reported,3-5 sensitivity to imatinib was reduced in mutant BCR-ABL (M351T, Y253F or T315I)-harboring cells (Figure 1A). In contrast, AIC-47 showed significant growth inhibition even in T315I-mutated cells at the same concentration as found effective for the cells with WT-BCR-ABL (Figure 1B). We also examined the effect of AIC-47 on primary cells obtained from CML patients. Imatinib showed cytotoxicity only in WT-BCR-ABL-harboring cells (Figure 1C). In contrast, AIC-47 led to a significant reduction in the viability of both WT- and T315I-BCR-ABL cells (Figure 1C). These results indicated that AIC-47 was effective regardless of the BCR-ABL mutation. In normal human bone marrow cells, imatinib showed cytotoxicity at 24 hours (P = .002), whereas AIC-47 had no effect (P = .968; Figure 1D), suggesting that AIC-47 may be safe for use.

3.2 | AIC-47 suppressed transcription of BCR-ABL regardless of the point mutation

The greatest difference between AIC-47 and TKI was the effect of each on the expression of BCR-ABL. TKI inhibit the phosphorylation of BCR-ABL protein only, whereas AIC-47 suppresses transcription of the BCR-ABL gene.9 Previous studies showed that c-Myc and its partner MAX bind to the promoter region of the BCR-ABL gene and play a role as a transcriptional activator of BCR-ABL.8,20 AIC-47 decreased c-Myc expression, leading to transcriptional suppression of WT-BCR-ABL.8 We then examined the effects of AIC-47 on the expression of mutated-BCR-ABL mRNA. As a result, expression levels of both BCR-ABL mRNA (Figure 2A) and c-Myc (Figure 2B) were also decreased in BCR-ABL-mutated cells after treatment with AIC-47. AIC-47 also decreased the expression level of ABL1 (also known as cABL) mRNA in TCCY cells (Figure S1). We previously confirmed that a binding site for c-Myc is present in the promoter region of the ABL1 gene. Thus, we speculated that AIC-47 suppressed the transcription of both BCR-ABL and ABL1 through the downregulation of c-Myc. In primary cells obtained from CML patients, AIC-47 decreased the expression level of WT- and T315I-BCR-ABL mRNA, whereas imatinib did not affect BCR-ABL expression (Figure 2A). These results suggested that AIC-47 suppressed transcription of BCR-ABL regardless of the mutation, which made a large difference compared to that of TKI.

3.3 | AIC-47 perturbed the Warburg effect through dysregulation of the PKM1/PKM2 ratio

Cancer cells efficiently use limited energy sources and anti-cancer drugs targeting cellular metabolism are expected to be useful for overcoming drug resistance.21,22 We reported that AIC-47 inhibits growth of CML cells through perturbation of the Warburg effect.5 The Warburg effect is promoted by the c-Myc/BCR-ABL/hnRNP/PKM2 signaling cascade.8,14,15 In BCR-ABL-mutated cells, PKM2 protein was dominant as it was in WT-BCR-ABL-harboring cells (Figure 3A). In primary CML cells, the expression of PKM2 was higher than that of PKM1 (Figure 3A). Expression level of PKM2 mRNA was also approximately fivefold higher than that of PKM1 (Figure 3B). There was no significant difference in the expression levels of PKM1 (P = .6715), PKM2 (P = .8470), and PKM (P = .4786) between WT-BCR-ABL cells and T315I-BCR-ABL cells (Figure 3B). Reprogramming of the metabolic pathway has been reported to be associated with resistance to anti-cancer drugs;23 however, these results suggested that the Warburg effect was achieved regardless of the point mutations in BCR-ABL and the signaling cascade was useful as a therapeutic target for overcoming TKI resistance. Consistent with previous results, AIC-47 increased the expression of PKM1, whereas it decreased that of PKM2, indicating that the PKM1/PKM2 ratio was increased in both WT- and T315I-BCR-ABL-harboring cells (Figure 3C,D). PKM splicers PTBP1 and hnRNP A1 were decreased after treatment with AIC-47 (Figure 3C). Expression of SRSF3, another PKM splicer, was also markedly decreased by AIC-47 treatment (Figure 3C). Increase in the PKM1/PKM2 ratio was also observed by silencing of PTBP1, hnRNP A1 or SRSF3 (Figure 4), suggesting that AIC-47 dysregulated the Warburg effect-related signaling in both WT- and T315I-BCR-ABL-harboring cells.

3.4 | AIC-47 showed anti-leukemic effects in a leukemic mouse model

To investigate the anti-leukemic effects of AIC-47 in vivo, we established an engraftment mouse model. WT-BCR-ABL-harboring
Baf3p210 cells were injected i.v. into nude mice. After 10 days, expression level of BCR-ABL mRNA was significantly increased in the spleen and blood of the engrafted mice (Figure S2A), suggesting that the model partly showed the phenotype of Ph-positive leukemia.

We then injected WT- or T315I-BCR-ABL-harboring Baf3p210 cells into mice and then gave AIC-47 i.v. every fourth day. Treatment with AIC-47 significantly reduced the expression level of BCR-ABL mRNA in the spleen compared with the control (Figure 5A). In the blood samples, AIC-47 also abrogated the increase in BCR-ABL mRNA (Figure 5B). Splenomegaly is a characteristic feature of the CML-like myeloproliferative disorder and is a useful indicator of leukemic burden.24,25 Spleens of control mice were extremely enlarged, with median spleen volumes of 5.06 mm³ (WT) and 4.00 mm³ (T315I), respectively. In contrast, those of AIC-47-treated mice were almost the same size as the spleens of normal mice, with median spleen volumes of 1.32 mm³ (both WT and T315I) (Figure 5C,D). Hepatomegaly was also significantly improved in AIC-47-treated mice (Figure 5E). Median liver volumes of control mice were 16.7 mm³ (WT) and 18.3 mm³ (T315I), respectively. AIC-47 treatment resulted in median liver volumes of 11.2 mm³ (WT) and 11.6 mm³ (T135I). The spleens of control mice were massively infiltrated with leukemia cells, whereas AIC-47 partly lessened this splenic infiltration (Figure 5F). Together, these results suggested that AIC-47 had anti-leukemic effects in vivo regardless of BCR-ABL mutations.

**DISCUSSION**

In the current study, we demonstrated the effects of AIC-47 on mutated-BCR-ABL-harboring cells in vitro and in vivo. AIC-47 showed anti-leukemic effects on either WT- or mutated-BCR-ABL-harboring cells (Figure 1B,C). Notably, imatinib showed cytotoxicity in normal human bone marrow cells, whereas AIC-47 had no such effect (Figure 1D). These data suggest that AIC-47 may have a higher degree of safety than TKI, but further safety testing will be needed. Our previous studies indicated that AIC-47 had a central effect on the transcriptional suppression of BCR-ABL partly through down-regulation of c-Myc, the effect of which was different from that of TKI.9 In mutated-Myc harboring cells, the expression levels of BCR-ABL mRNA were also suppressed by AIC-47 as in the case of normal human bone marrow cells treated with DMSO (Control), AIC-47 or imatinib (IM). Cells were counted after staining with Trypan-blue. Data are expressed as means ± SD of three different experiments. Differences were statistically evaluated by two-way ANOVA followed by Tukey’s multiple comparisons test.
FIGURE 3  AIC-47 disrupted the Warburg effect through dysregulation of the PKM1/PKM2 ratio. A, Expression profile of PKM isoforms in WT- and mutated-BCR-ABL-harboring cells at the steady state. PKM1 and PKM2 were detected by western blotting analysis under the same experimental conditions at the same time. B, Expression levels of PKM1, PKM2, and PKM mRNAs in TCCY cells at steady state. Expression level of mRNA was calculated by the ΔΔCt method. Data are expressed as means ± SD of three different experiments. C, Expression of the Warburg effect-related proteins after treatment with DMSO (Control) or AIC-47 for 48 h. Expression levels of PTBP1, hnRNP A1, SRSF3, PKM1, PKM2 and PKM1/2 were examined by western blotting analysis. D, Ratio of PKM1/PKM2 was quantified by densitometry scanning. hnRNP, heterogeneous nuclear ribonucleoprotein; PKM, pyruvate kinase muscle; PTBP1, polypyrimidine tract-binding protein 1.
WT-BCR-ABL-harboring cells (Figure 2A). Based on these results, we speculate that AIC-47 might prevent the generation of other mutations in BCR-ABL. Moreover, AIC-47 decreased the expression level of ABL1 mRNA. ABL1 kinase controls cell growth, survival and invasion.\textsuperscript{26} In Ph-positive leukemia cell lines, the expression levels of ABL1 were more than 50-fold lower compared with that of the BCR-ABL fusion gene (data not shown); however, promotion of cancer growth by ABL1 has been reported in solid tumors.\textsuperscript{26,27} AIC-47 might be effective in ABL1-activated solid tumors.

In the present study, we carried out the first examination of the effects of AIC-47 in a leukemic mouse model. AIC-47 suppressed the increase in BCR-ABL mRNA and hepatosplenomegaly regardless of the BCR-ABL mutation (Figure 5). Notably, the improvement of splenomegaly by AIC-47 was remarkable. Median spleen volumes in AIC-47-treated groups were 3.8-fold (WT) and threefold (T315I) less than those in the control group (Figure 5D). Imatinib (100 or 150 mg/kg) treatment induced 1.5- or 2.8-fold reduction in spleen weight.\textsuperscript{24,28} A third-generation TKI ponatinib (20 mg/kg) has been reported to halve the splenomegaly.\textsuperscript{25} These findings suggested that the improvement of splenomegaly by AIC-47 might be equal to or greater than that of TKI. Survival of AIC-47-treated mice tended to be longer than that of the control mice; however, there was no significant difference between them (Figure S2B). To determine the effect of AIC-47 on survival, further long-term experiments will be needed. In this leukemic mouse model, AIC-47 did not show any side-effects, including hepatotoxicity. Furthermore, no abnormal body weight loss was observed in the AIC-47-treated mice (Figure S2C).

We also showed that the expression of PKM2 was dominant even in BCR-ABL-mutated cells (Figure 3A,B). Consistently, AIC-47 induced switching of the PKM isoform from PKM2 to PKM1 in BCR-ABL-mutated cells (Figure 3C,D). Previous reports showed that reprogramming of metabolic pathways is associated with resistance and that PKM1 is commonly upregulated in various chemoresistant cells, including CML cells.\textsuperscript{23,29} However, PKM2 was dominantly expressed in BCR-ABL mutated cells as in WT-BCR-ABL-harboring cells, suggesting that TKI resistance of cells with a point mutation of BCR-ABL is
independent of metabolism reprogramming. These novel findings encourage the discovery of new drugs targeting cancer-specific energy metabolism for overcoming TKI resistance. We previously reported that AIC-47 and imatinib disrupted the Warburg effect through downregulation of the BCR-ABL/PTBP1/PKM2 signaling cascade.\textsuperscript{8,9} Glucose metabolism is a central source of energy for cancer, and
FIGURE 5 AIC-47 showed anti-leukemic effects in a leukemic mouse model. A, Expression level of BCR-ABL mRNA in spleen at day 18. Expression level of mRNA was calculated by the ΔΔCt method. Data are expressed as means ± SD of three different experiments. Expression level of AIC-47 is indicated as "1". **P < .01 vs Control (Student’s t-test). B, Expression level of BCR-ABL mRNA in blood at day 3 and day 18. Expression level of mRNA was calculated by the ΔΔCt method. Data are expressed as means of three different experiments. C, D, Spleen volume of Normal, Control or AIC-47-treated mice. Representative images are shown. Scale bars, 5 mm. E, Liver volume of Control or AIC-47-treated mice. F, H&E-stained spleens of Normal, WT-BCR-ABL-harboring Control or AIC-47-treated mice. Representative photomicrographs are shown. Normal (low magnification ×4); Normal splenic tissue showing intact white and red pulp. Normal (high magnification ×20-40); White pulp showing normal structure including the periarteriolar lymphoid sheath, follicles, and marginal zone. Abundant normal lymphocytes and erythrocytes are seen in the white and red pulp, respectively. Control (low magnification ×4); Proliferative leukemia cells destroying normal structure of spleen. Normal structures of white and red pulp are hardly seen in the control spleen. Control (high magnification ×20-40); Leukemia cells proliferating in the spleen. Round to polygonal-shaped leukemia cells show prominent atypia including anisokaryosis, prominent nucleoli, and varied chromatin distribution. Numerous mitotic figures are seen in the high magnification. AIC-47 treated mice (low magnification ×4); AIC-47 treatment eliminated leukemia cells from the spleen. Spleens of mice treated with AIC-47 have intact structures of white and red pulp. AIC-47 treated mice (high magnification ×20-40); White and red pulp retain their normal structures containing abundant normal lymphocytes and erythrocytes. Leukemia cells are hardly seen. Scale bars, 500 μm (x4), 100 μm (x20), 50 μm (x40).

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AUTHOR CONTRIBUTIONS

H.S. designed the study and carried out the majority of experiments, data collection and analysis, manuscript writing, and final approval of the manuscript. N.S., Y.K. and K.H. carried out experiments, collected data, and approved the manuscript. Y.M. and T.N. provided cell lines and primary cells, and approved the manuscript. Y.A. contributed to the designing of the experiments, manuscript writing, and assisted in the final approval of the manuscript.

CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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