

ORIGINAL ARTICLE

Altered intracellular signaling by imatinib increases the anti-cancer effects of tyrosine kinase inhibitors in chronic myelogenous leukemia cells

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Tyrosine kinase inhibitors (TKI), including imatinib (IM), improve the outcome of CML therapy. However, TKI treatment is long-term and can induce resistance to TKI, which often leads to a poor clinical outcome in CML patients. Here, we examined the effect of continuous IM exposure on intracellular energy metabolism in K562 cells, a human Philadelphia chromosome-positive CML cell line, and its subsequent sensitivity to anti-cancer agents. Contrary to our expectations, we found that continuous IM exposure increased sensitivity to TKI. Cancer energy metabolism, characterized by abnormal glycolysis, is linked to cancer cell survival. Interestingly, glycolytic activity was suppressed by continuous exposure to IM, and autophagy increased to maintain cell viability by compensating for glycolytic suppression. Notably, increased sensitivity to TKI was not caused by glycolytic inhibition but by altered intracellular signaling, causing glycolytic suppression and increased autophagy, as evidenced by suppression of p70 S6 kinase 1 (S6K1) and activation of AMP-activated protein kinase (AMPK). Using another human CML cell line (KCL22 cells) and BCR/ABL+ Ba/F3 cells (mimicking Philadelphia chromosome-positive CML cells) confirmed that suppressing S6K1 and activating AMPK increased sensitivity to TKI. Furthermore, suppressing S6K1 and activating AMPK had a synergistic anti-cancer effect by inhibiting autophagy in the presence of TKI. The present study provides new insight into the importance of signaling pathways that affect cellular energy metabolism, and suggests that co-treatment with agents that disrupt energy metabolic signaling (using S6K1 suppressors and AMPK activators) plus blockade of autophagy may be strategies for TKI-based CML therapy.

KEYWORDS

AMPK, autophagy, BCR/ABL, cancer energy metabolism, S6K1

Abbreviations: ALL, acute lymphoblastic leukemia; AMPK, AMP-activated protein kinase; CML, chronic myelogenous leukemia; CQ, chloroquine diphosphate; HIF, hypoxia-inducible factor; IM, imatinib mesylate; LSD1, lysine-specific demethylase-1; mTORC1, mammalian target of rapamycin complex 1; ROS, reactive oxygen species; S6K1, p70 S6 kinase 1; STAT, signal transducers and activator of transcription; TKI, tyrosine kinase inhibitors.

1 | INTRODUCTION

Chronic myelogenous leukemia is a malignant clonal disorder of hematopoietic stem cells characterized by the presence of the

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Philadelphia chromosome, which results from reciprocal translocation of t(9;22).¹ The resulting BCR/ABL fusion protein activates survival pathways, including PI3K/Akt, JAK/STAT, and Ras/Raf.^{2,3} These pathways also trigger abnormal proliferation of CML cells and increase their resistance to apoptosis. IM, a specific BCR/ABL TKI, yields a marked improvement in CML patient survival;⁴ however, suboptimal responses or IM-resistance often makes it difficult to continue therapy long term.⁵⁻⁷

Point mutations within the ABL kinase domain (eg, Y235F/H, E255K/V, T315I, and H396P/R) are associated with IM-resistance.^{8,9} To counter this, second and third generation TKI (eg, dasatinib, nilotinib, bosutinib, and ponatinib) were developed.¹⁰ Alternatively, multiple studies suggest that overexpression of BCR/ABL-dependent or -independent signaling pathways correlates with resistance to or efficacy of TKI, regardless of the point mutations mentioned above.¹¹ For example, BCR/ABL-dependent activation of the PI3K/Akt pathway contributes to the survival of CML cells during the early phase of IM-resistance,¹² and the JAK2/STAT5 pathway induces anti-apoptotic effects that thwart the actions of IM.¹³ Also, deficiency of p53 in BCR/ABL-transformed cells decreases their sensitivity to IM in a BCR/ABL-independent way.¹⁴ Thus, targeting intracellular signaling may be an effective CML therapy.

BCR/ABL tyrosine kinase promotes not only cell proliferation and survival but also glycolysis.¹⁵ IM suppresses glycolysis in human CML cells by inhibiting several glycolytic enzymes and decreasing glucose uptake.^{16,17} Moreover, IM-resistant cells express high levels of HIF-1 α , which activates glucose transporters and many glycolytic enzymes.¹⁸ Thus, the efficacy of IM is closely associated with energy metabolism, particularly glycolysis. In addition, cancer cells obtain most of their energy and ATP from glycolysis, even under aerobic conditions.¹⁹ Targeting hallmarks of energy metabolism has a place in cancer therapy. For instance, suppressing glycolysis in human tumors using 2-deoxy-D-glucose, a hexokinase inhibitor, increases the efficacy of doxorubicin and paclitaxel.²⁰ Therefore, molecules or machineries that interfere with cancer energy metabolism, including aerobic glycolysis, may improve the efficacy of TKI and improve CML therapy.

Here, we first examined changes in the IM-sensitivity of K562 cells, a human CML cell line, continuously exposed to IM. We expected that the K562 cells would become resistant to IM; however, these cells actually became more sensitive to IM and other TKI. In addition, continuous exposure to IM inhibited glycolysis, which induced autophagy as a compensatory mechanism to maintain cell viability. Importantly, continuous exposure to IM increased the sensitivity to TKI by suppressing S6K1 and activating AMPK, both of which play an important role in regulating glycolysis and autophagy. Furthermore, a mTORC1/S6K1 signaling suppressor and an AMPK activator (rapamycin and metformin, respectively) increased sensitivity to TKI, and mediated synergistic anti-cancer effects by inhibiting autophagy in the presence of TKI. Thus, we propose that altering intracellular signaling pathways that affect cancer metabolism (using S6K1 suppressors and AMPK activators) and blocking autophagy will improve TKI treatment for CML.

2 | MATERIALS AND METHODS

2.1 | Reagents

Imatinib (Phoenix Pharmaceuticals, Belmont, CA, USA), 2-deoxy-D-glucose (Wako, Osaka, Japan), CQ (Sigma, St Louis, MO, USA), metformin hydrochloride (LKT Laboratories, St Paul, MN, USA), rapamycin (Adooq Bioscience, Irvine, CA, USA), dasatinib (Cayman Chemical, Ann Arbor, MI, USA), bosutinib (KareBay Biochem, Ningbo, China), nilotinib (Cayman Chemical), ponatinib (Adooq Bioscience), methotrexate (Wako), cytarabine (LKT Laboratories), cisplatin (Sigma), vincristine sulfate (LKT Laboratories), STAT5 inhibitor (Calbiochem, San Diego, CA, USA), LY294002 (LC Laboratories, Woburn, MA, USA), and LY3900120 (Medchem Express, Princeton, NJ, USA).

2.2 | Cell culture

K562 and Ba/F3 cells (RIKEN Cell Bank, Tsukuba, Japan), and KCL22 cells (JCRB Cell Bank, Osaka, Japan), were cultured in RPMI-1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% or 20% (KCL22 cells) FBS (Life Technologies, Grand Island, NY, USA) plus antibiotics (Nacalai Tesque). Prior to introduction of the BCR/ABL gene, Ba/F3 cells were cultured with 10 ng/mL interleukin-3 (Peprotech, Rocky Hill, NJ, USA). K562 cells that survived in medium containing 1 μ mol/L IM (named K562-IM1e ["e" denotes "exposure" to 1 μ mol/L IM]) were generated as described previously,²¹ with minor modifications. Briefly, K562 cells were initially cultured in the presence of 0.1 μ mol/L IM. Then, the concentration of IM in the medium was subsequently increased by 0.1-0.2 μ mol/L every 6-8 weeks. K562-IM1e cells were maintained in medium containing 1 μ mol/L IM.

2.3 | Plasmid construction, transfection, and live cell imaging

A previously constructed GFP-LC3 plasmid was transfected into K562 cells using Lipofectamine™ 2000 (Life Technologies).²² After 48 hours, cells were treated at 37°C with 1 μ mol/L LysoTracker Red (Life Technologies) for 1 hour. Images were obtained under a Carl Zeiss LSM700 laser scanning confocal microscope (Prenzlauer, Berlin, Germany). The BCR/ABL gene, cloned from the cDNA of K562 cells, was inserted into a pAcGFP1-C1 plasmid (Clontech, Palo Alto, CA, USA). The resulting GFP-BCR/ABL DNA was introduced into Ba/F3 cells by electroporation using NEPA21 apparatus (NEPAGENE, Chiba, Japan). After 48 hours, interleukin-3 was depleted from the culture medium and stable lines expressing the BCR/ABL gene were established.

2.4 | Flow cytometric detection of dead cells

Collected cells were stained with the Zombie NIR™ Fixable Viability kit (BioLegend, San Diego, CA, USA) to distinguish dead cells from

live cells. Data acquisition was carried out using an EC800 Flow Cytometry Analyzer (Sony, Tokyo, Japan).

2.5 | Measurement of cellular lactate release

Release of cellular lactate was measured as described previously.²²

2.6 | BrdU incorporation assay

To assess cell proliferation, K562 cells continuously exposed to IM were incubated with 10 $\mu\text{mol/L}$ BrdU (Tokyo Chemical Industry Co., Tokyo, Japan) for 1 hour. BrdU-positive cells were identified using an EC800 Flow Cytometry Analyzer (Sony).

2.7 | Western blot analysis

Cells were lysed on ice in lysis buffer containing PBS (pH 7.4), 1% Triton X-100, and protease and phosphatase inhibitor cocktails (Roche, Mannheim, Germany). Identical amounts of protein from each sample were separated in SDS-PAGE gels and then transferred to PVDF membranes (Merck Millipore, Berlin, Germany). After membrane blocking, the blots were probed with primary antibodies specific for BCR, p-BCR (T177), HIF-1 α , LC3B, p70S6K, p-p70S6K (T389), AMPK α , p-AMPK α (T172), and GAPDH (all purchased from Cell Signaling Technologies, Beverly, MA, USA). Immunolabeled proteins were detected using a HRP-labeled anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.8 | RNA isolation and quantitative real-time PCR

Total RNA was isolated using Sepasol-RNA I reagent (Nacalai Tesque) and reverse transcribed using Rever Tra Ace[®] qPCR RT Master mix (TOYOBO, Osaka, Japan). The resulting cDNA was mixed with THUNDERBIRD[™] SYBR qPCR mix (TOYOBO) and subjected to quantitative real-time PCR using a LightCycler[™] Nano System (Roche) and the following primers: hexokinase 2 forward, 5'-ACAGGTGCTCTCAAGCCCTAAG-3' and reverse, 5'-CGAGGCCGCCATCTCAGAGCGG-3'; lactate dehydrogenase A forward, 5'-GGAGATCCATCATCTCTCC-3' and reverse, 5'-GGCCTGTGCCATCAGTATCT-3'; and 18s ribosomal RNA forward, 5'-CGCCGCTAGAGGTGAAATTC-3' and reverse, 5'-TTGGCAAATGCTTTCGCTC-3'. The reaction was carried out at 95°C for 60 s, followed by 40 cycles at 95°C for 10 s and at 60°C for 60 s. Relative expression of mRNA was calculated after normalization against 18s ribosomal RNA.

2.9 | Transmission electron microscopy

Cells were prepared for transmission electron microscopy and observed as previously described.²³⁻²⁵ Images were obtained under a JEM-1400 electron microscope (JEOL, Tokyo, Japan) at 2500-10 000 \times nominal magnification.

2.10 | Statistical analysis

All data were expressed as the mean \pm SD of at least 3 independent experiments. Statistical analysis was carried out using Student's *t* test or analysis of variance followed by the Bonferroni test where applicable. A *P* value of <0.05 was considered significant.

3 | RESULTS

3.1 | Continuous exposure of K562 cells to IM increases their sensitivity to TKI

To examine the effect of altered intracellular responses in CML cells continuously exposed to IM on subsequent sensitivity to TKI, we first investigated the relationship between continuous exposure to IM and sensitivity using Philadelphia chromosome-positive K562 cells. Based on a previous report that exposure of K562 cells to the concentration of IM (0.1 $\mu\text{mol/L}$) for 96 hours did not cause marked cell death,²⁶ we exposed K562 cells to 0.1 $\mu\text{mol/L}$ IM over a long period. Consistent with the previous report, continuous exposure (4 weeks) to 0.1 $\mu\text{mol/L}$ IM gradually arrested cell proliferation (Figure 1A), but did not cause appreciable cell death (Figure 1B). Although continuous exposure to 0.1 $\mu\text{mol/L}$ IM mildly suppressed auto-phosphorylation of BCR/ABL in K562 cells (Figure 1C), which is an important determinant of CML cell survival, we surmised that the extent of BCR/ABL suppression was insufficient to decrease their viability. Commonsense led us to assume that continuous exposure of K562 cells to IM would reduce subsequent susceptibility to IM. However, K562 cells cultured with 0.1 $\mu\text{mol/L}$ IM became increasingly sensitive to IM (at the dose of 15 $\mu\text{mol/L}$), as reflected by increased cell death (Figure 1D). K562 cells continuously exposed to 0.1 $\mu\text{mol/L}$ IM for 3 weeks are named "K562-IM3w" cells.

Next, to examine whether increased IM-sensitivity of K562-IM3w cells is also true for other TKI, we exposed K562-IM3w cells to dasatinib, nilotinib, bosutinib, or ponatinib, or to the classical anti-cancer agents methotrexate, cytarabine, cisplatin, and vincristine. As observed for IM, other TKI were more effective against K562-IM3w cells than against the parental cells (Figure 1E). By contrast, the classical anti-cancer agents tended to be less effective against K562-IM3w cells (Figure 1E). These findings suggest that continuous exposure to IM sensitizes K562 cells to TKI.

3.2 | Continuous exposure to IM suppresses glycolysis and increases autophagy

Aerobic glycolysis is a hallmark of cancer energy metabolism, and targeting glycolytic pathways sensitizes several cancer cells to chemotherapeutic agents.²⁷ As IM suppresses glycolysis,^{16,17} we hypothesized that altered intracellular energy metabolism, particularly glycolysis, by continuous exposure to IM increases sensitivity to TKI. As in previous reports, we confirmed that lactate production, the final step of glycolysis, was reduced in K562 cells exposed to IM

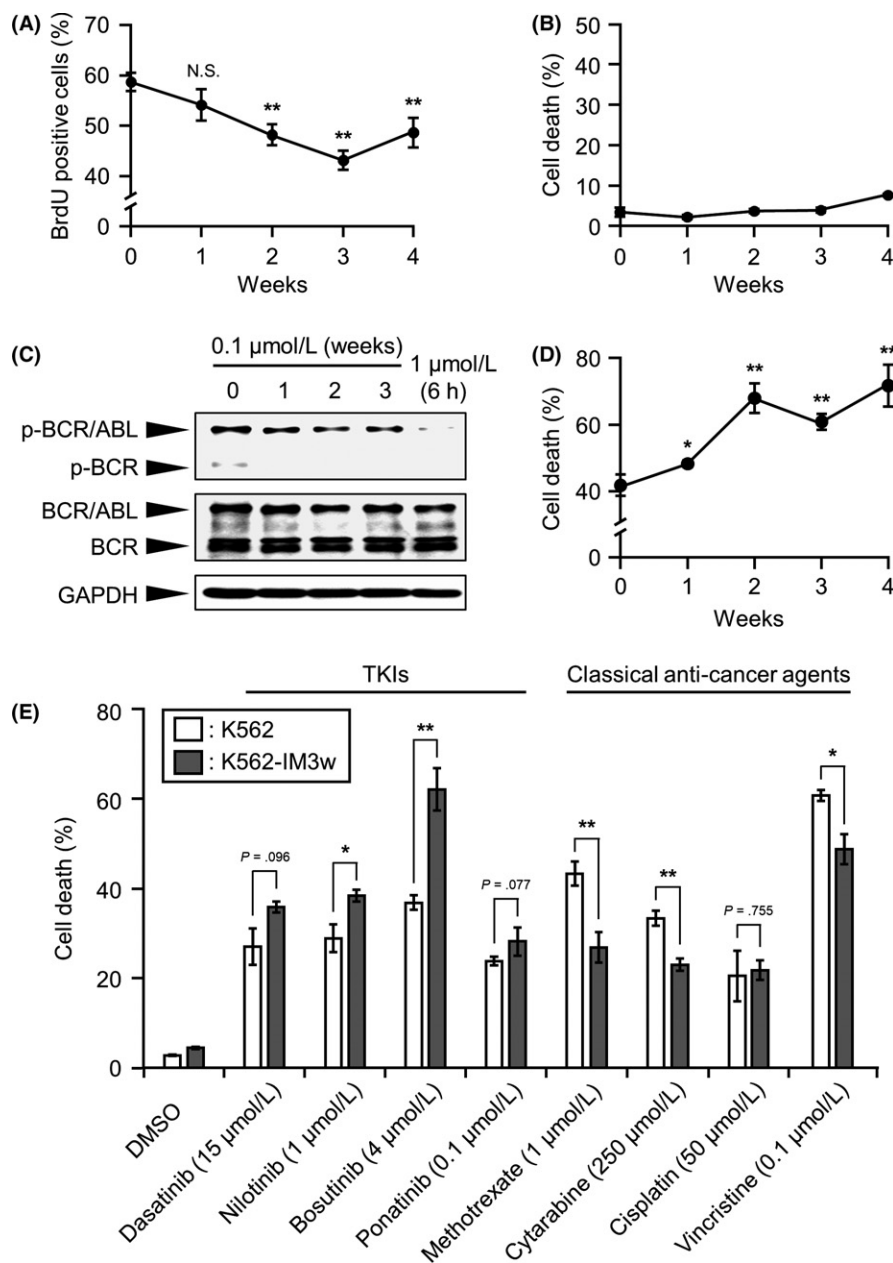


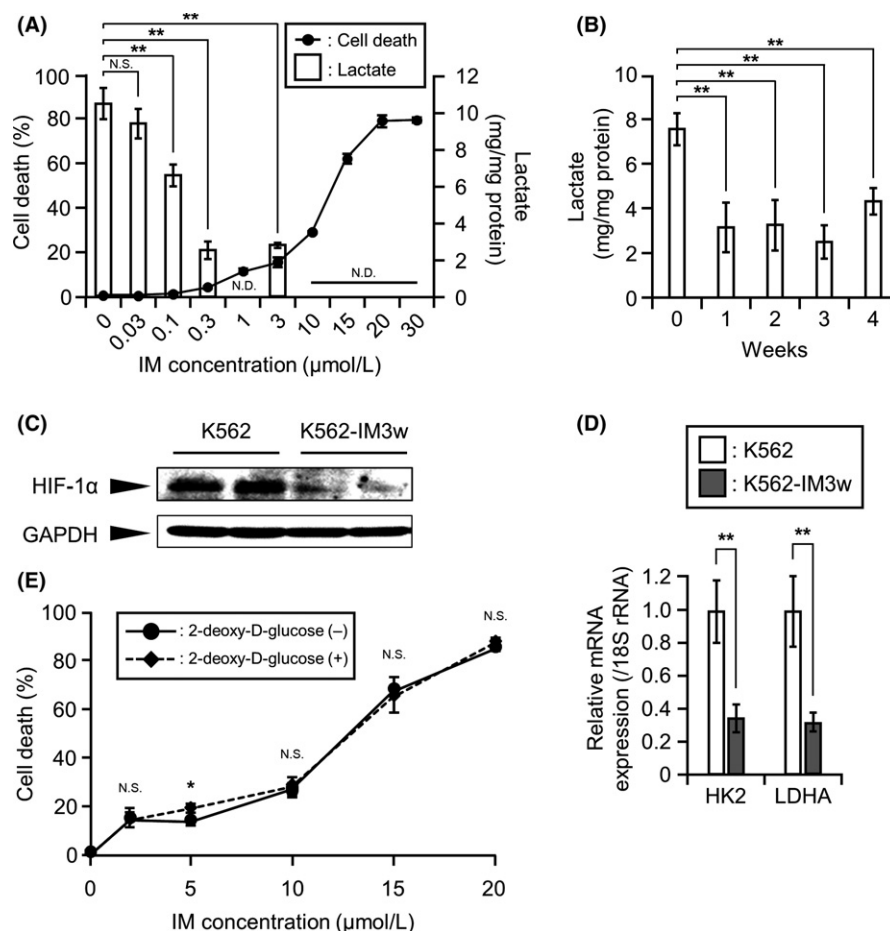
FIGURE 1 K562 cells continuously exposed to imatinib (IM) become sensitive to tyrosine kinase inhibitors (TKI). A, BrdU incorporation into K562 cells continuously cultured with 0.1 $\mu\text{mol/L}$ IM for 0–4 weeks was evaluated. ** $P < .01$ and N.S., not significant, compared with cells not treated with IM. B, Death rate of K562 cells continuously exposed to 0.1 $\mu\text{mol/L}$ IM for 0–4 weeks was evaluated. C, Total cell lysates prepared from K562 cells continuously cultured with 0.1 $\mu\text{mol/L}$ IM for 0–3 weeks and treated with 1 $\mu\text{mol/L}$ IM for 6 hours were subjected to western blotting with anti-phospho-BCR, anti-BCR, and anti-GAPDH antibodies. D, K562 cells were cultured for the indicated periods with 0.1 $\mu\text{mol/L}$ IM and then treated with 15 $\mu\text{mol/L}$ IM for 72 hours. Cell death was evaluated. * $P < .05$ and ** $P < .01$, compared with cells not treated with IM. E, Parental K562 and K562-IM3w cells were treated with the following agents at the indicated concentrations for 72 hours, and cell death was evaluated: dasatinib, nilotinib, bosutinib, and ponatinib (all TKI), and methotrexate, cytarabine, cisplatin, and vincristine (all classical anti-cancer agents). * $P < .05$ and ** $P < .01$

(Figure 2A). Interestingly, a significant decrease in lactate production was observed even at concentrations lower than those at which marked cell death occurred (LD_{50} , $11.72 \pm 0.462 \mu\text{mol/L}$). Also, lactate production remained low when cells were exposed to 0.1 $\mu\text{mol/L}$ IM for 4 weeks (Figure 2B). Furthermore, we observed reduced expression of HIF-1 α protein, which contributes to increased glycolysis and proliferation of cancer cells,^{28,29} and reduced expression of mRNA encoding glycolytic enzymes hexokinase 2 and lactate dehydrogenase A in K562-IM3w cells (Figure 2C,D). Inhibiting glycolysis using 2-deoxy-D-glucose increases the efficacy of several anti-cancer agents;²⁰ however, inhibiting glycolysis in K562 cells using 2-deoxy-D-glucose did not increase their sensitivity to IM (Figure 2E), despite marked inhibition of glycolysis (Figure S1). These results suggested that increased sensitivity of K562 cells to TKI was not directly

related to inhibition of glycolysis; therefore, other factors needed to be considered.

We previously proposed that autophagy is induced to maintain energy homeostasis and survival of ALL cells when glycolysis is suppressed.²² Therefore, we assumed that autophagy would be increased by continuous exposure to IM, which may hint at the cause underlying increased sensitivity to TKI. We found that expression of LC3-II, a characteristic marker of autophagosome formation, was higher in K562-IM3w cells than in parental cells (Figure 3A). Also, the distribution pattern of aggregated GFP-LC3 in K562-IM3w cells supported IM-induced autophagosome formation (Figure 3B). Moreover, autophagic vacuoles in K562-IM3w cells were clearly visible by transmission electron microscopy (Figure 3C). In addition, to rule out the possibility that the appearance of autophagic vacuoles

FIGURE 2 Continuous exposure to imatinib (IM) suppresses glycolysis in K562 cells. A, K562 cells were treated with different concentrations of IM (0–30 $\mu\text{mol/L}$) for 72 hours. Cell death and the amount of lactate released from the cells for the last 24 hours were evaluated. $**P < .01$, N.S., not significant, and N.D., not detected. B, Amount of lactate released from K562 cells continuously exposed to 0.1 $\mu\text{mol/L}$ IM for 0–4 weeks was evaluated. $**P < .01$, compared with cells not treated with IM. C, Total cell lysates prepared from parental K562 and K562-IM3w cells were subjected to western blotting with anti-hypoxia-inducible factor (HIF)-1 α and anti-GAPDH antibodies. D, Relative expression of mRNA encoding hexokinase 2 (HK2) and lactate dehydrogenase A (LDHA) in parental K562 and K562-IM3w cells was evaluated. $**P < .01$. E, K562 cells were preincubated with or without 10 $\mu\text{mol/L}$ 2-deoxy-D-glucose for 48 hours, followed by treatment with IM (0–20 $\mu\text{mol/L}$) for 72 hours. Cell death was then evaluated. $*P < .05$ and N.S., not significant



was because of suppression of fusion between autophagosomes and lysosomes (ie, the final step of autophagy), we examined the effect of CQ, an inhibitor of fusion between autophagosomes and lysosomes, on the survival of IM-treated K562 cells. As expected, CQ reduced the viability of K562-IM3w cells (Figure 3D), indicating that continuous exposure to IM induced autophagy to increase survival. Taken together, the data suggest that continuous exposure to IM dynamically changes energy metabolism in K562 cells without causing cell death; therefore, alterations to intracellular signals that regulate energy metabolism may increase sensitivity to TKI.

3.3 | Continuous treatment with IM alters the activity of 2 key signaling molecules: S6K1 and AMPK

To explore why K562 cells continuously cultured with IM became highly sensitive to TKI, we focused on representative proteins related to changes in energy metabolism (ie, glycolytic suppression and autophagic enhancement). S6K1, a serine/threonine kinase regulated by mTORC1 that induces glycolysis downstream of BCR/ABL,³⁰ may be 1 candidate, because in CML cells, S6K1 is activated in response to abnormal activation of the PI3K/Akt/mTORC1 pathway downstream of BCR/ABL.³ Indeed, immunoblot analysis showed suppressed phosphorylation of S6K1 in K562-IM3w cells (Figure 4A).

We also focused on another signaling molecule, AMPK, which is a sensor of intracellular energy status.³¹ As AMPK is activated by energy metabolic stresses (eg, glucose deprivation) to maintain intracellular ATP levels,³² continuous exposure to IM may activate AMPK by lowering the ATP supply from glycolysis. In addition, AMPK increases autophagy by directly suppressing mTORC1,³³ therefore, we assumed that activation of AMPK increases autophagy by inactivating mTORC1. As expected, the amount of phosphorylated AMPK in K562-IM3w cells increased (Figure 4B). These data indicate that continuous exposure of K562 cells to IM suppresses S6K1 and activates AMPK. To clearly show that continuous exposure of K562 cells to IM increases their sensitivity to TKI by altering energy metabolic signaling, we monitored IM-sensitivity and lactate production by K562-IM3w cells cultured in IM-free medium for 1 week. By removing IM from the culture medium, IM-sensitivity and lactate production by K562-IM3w cells returned to the same level as those in parental K562 cells (Figure 3C,D). These results showed that increased sensitivity of K562 cells to TKI resulting from continuous IM exposure is a result of altered intracellular signaling through mild inhibition of BCR/ABL rather than to genetic modifications.

Prior to investigating the involvement of these 2 molecules in IM-sensitivity, we checked the Oncomine cancer gene expression database (Compendia Biosciences, www.Oncomine.com),³⁴ to check whether CML patients treated with IM showed differential

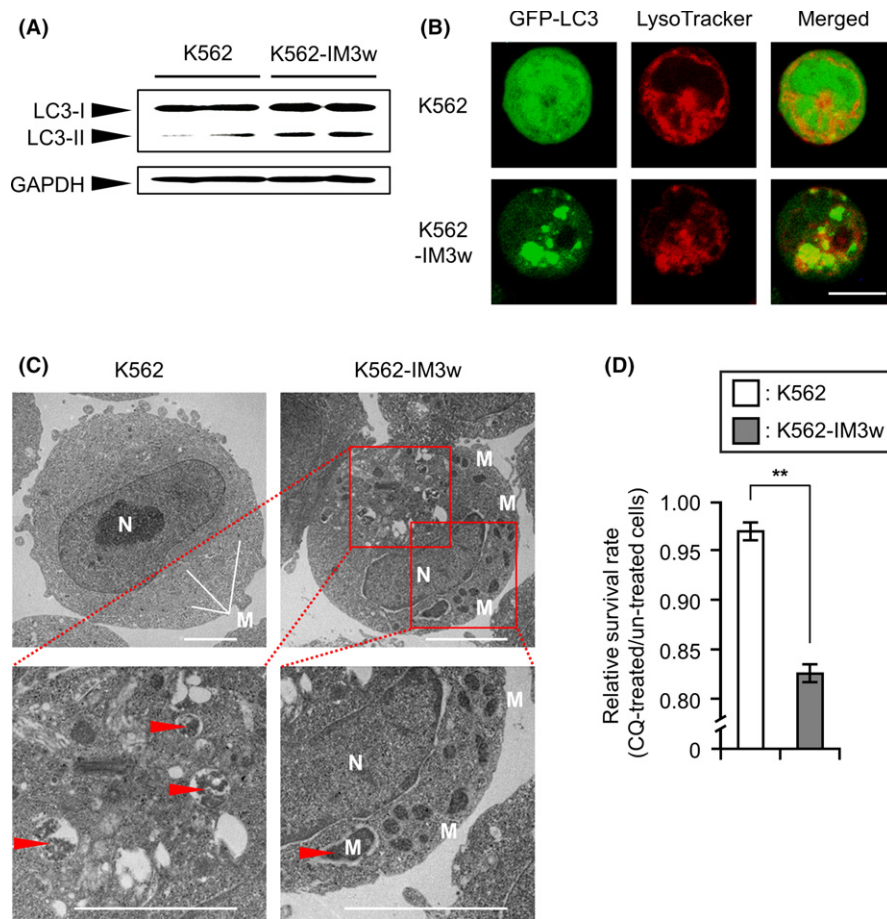


FIGURE 3 Continuous exposure to imatinib (IM) activates autophagy as a survival mechanism in K562 cells. A, Total cell lysates prepared from parental K562 and K562-IM3w cells were subjected to western blotting with anti-LC3 and anti-GAPDH antibodies. B, Subcellular localization of GFP-LC3 and LysoTracker (a lysosomal marker) was observed by confocal microscopy. Scale bars, 8 $\mu\text{mol/L}$. C, Intracellular structures were scanned by transmission electron microscopy. Cells were photographed at different magnifications. Scale bars, 4 $\mu\text{mol/L}$. M, mitochondria; N, nucleus. Arrowheads indicate autophagic bodies. D, Parental K562 and K562-IM3w cells were treated with or without chloroquine (CQ; 10 $\mu\text{mol/L}$) for 72 hours, and cell survival was evaluated. Each bar represents the survival of CQ-treated/untreated cells. ** $P < .01$

expression of S6K1 and AMPK. Database analysis indicated that IM treatment tended to reduce expression of the gene encoding S6K1 and increase that of the gene encoding AMPK (Figure 4E). Consistent with the clinical data, we observed marked changes in expression of S6K1 and AMPK mRNA in K562-IM3w cells (Figure S2; Appendix S1).

3.4 | Suppressing S6K1 and activating AMPK increase the sensitivity of CML cells to TKI

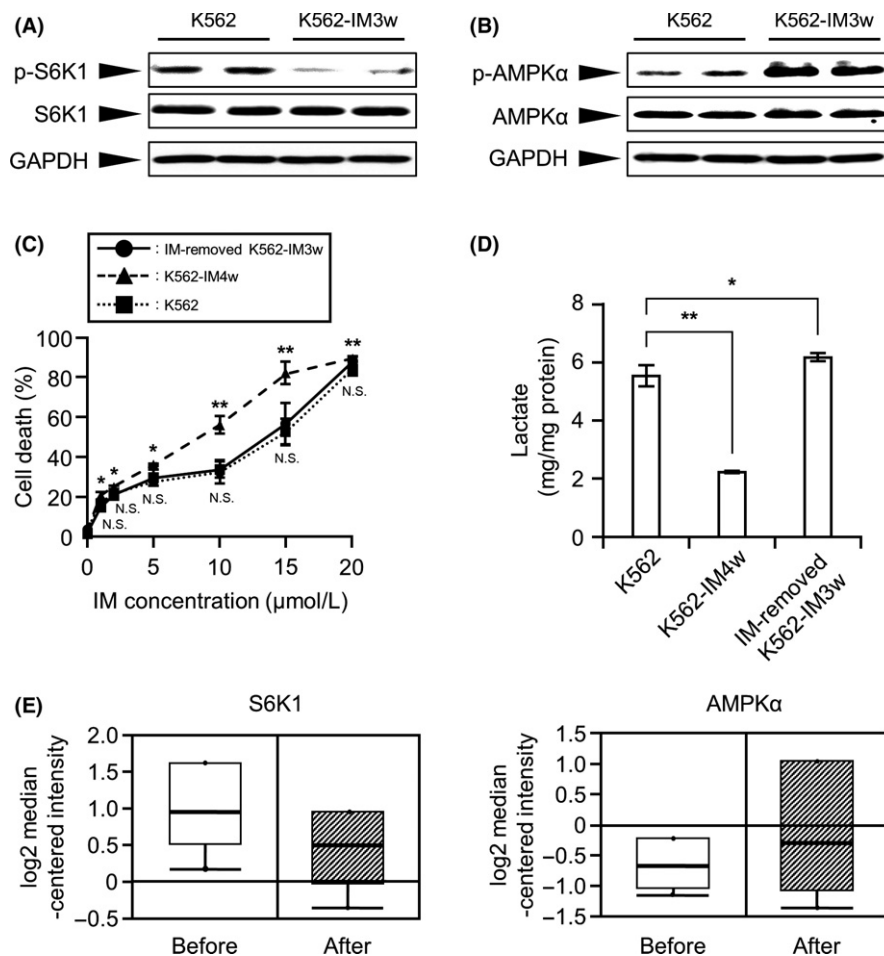
To examine whether IM-induced suppression of S6K1 and activation of AMPK increase the anti-cancer activity of TKI against K562 cells, we prepared K562 cells in which these signaling pathways were disrupted by rapamycin and metformin (a mTORC1 inhibitor and an AMPK activator, respectively) (Figure S3A,C).^{35,36} K562 cells pretreated with rapamycin (10 nmol/L) and metformin (1 mmol/L) were exposed to TKI or classical anti-cancer agents. Consistent with the results observed for K562-IM3w cells, pretreated K562 cells were more sensitive to TKI (Figure 5A), but not to classical anti-cancer agents (Figure S4A). Figure 3(D) shows that autophagy maintains the viability of K562 cells continuously exposed to IM. Therefore, we examined whether suppressing mTORC1/S6K1 signaling and activating AMPK have a synergistic effect on the anti-cancer efficacy of an autophagy inhibitor, CQ, in the presence of TKI; indeed, rapamycin

and metformin increased CQ-mediated cell death in the presence of TKI (Figure 5A white bars vs black bars).

Next, to investigate whether increased sensitivity to TKI caused by suppression of S6K1 and activation of AMPK is common for CML cells, we carried out the same experiments using another human CML cell line, KCL22 cells. KCL22 cells pretreated with rapamycin (1 nmol/L) and metformin (0.5 mmol/L) (concentrations sufficient to suppress S6K1 and activate AMPK, respectively [Figure S3B, D]) become more sensitive to TKI; sensitivity was also increased upon autophagy inhibition (Figure 5B). Sensitivity to classical anti-cancer agents was similar to that of K562 cells (Figure S4B). These data suggest that suppressing S6K1 and activating AMPK increase sensitivity to TKI, and increase the anti-cancer efficacy of autophagic inhibitors in the presence of TKI.

From the viewpoint of clinical application, we next examined whether suppressing S6K1 and activating AMPK increase the efficacy of TKI. For this, we used surviving K562 cells after exposure to IM, which usually show sufficient suppression of BCR/ABL auto-phosphorylation. Figure 1(C) shows that auto-phosphorylation of BCR/ABL was inhibited upon exposure to 1 $\mu\text{mol/L}$ IM; therefore, we generated K562 cells that survived in medium containing 1 $\mu\text{mol/L}$ IM (named K562-IM1e ["e" denotes "exposure" to 1 $\mu\text{mol/L}$ IM] cells) (Figure S5). Pretreatment with rapamycin (10 nmol/L) and metformin (1 mmol/L) restored the sensitivity of K562-IM1e

FIGURE 4 Continuous treatment with imatinib (IM) alters the activity of 2 key signaling molecules: p70 S6 kinase 1 (S6K1) and AMP-activated protein kinase (AMPK). A and B, Total cell lysates prepared from parental K562 and K562-IM3w cells were subjected to western blotting with (A) anti-phospho-S6K1, anti-S6K1, and anti-GAPDH antibodies, and with (B) anti-phospho-AMPK α , anti-AMPK α , and anti-GAPDH antibodies. C, K562 cells cultured continuously in the presence of 0.1 μ M/L IM for 3 weeks were then cultured with (K562-IM4w) or without (IM-removed K562-IM3w) 0.1 μ M/L IM for 1 week. Parental K562, K562-IM4w, and IM-removed K562-IM3w cells were then treated with different concentrations of IM (0–20 μ M/L) for 72 hours, and cell death was evaluated. * P < .05, ** P < .01, and N.S., not significant, compared with parental K562 cells. D, Amount of lactate released from parental K562, K562-IM4w, and IM-removed K562-IM3w cells was evaluated. * P < .05 and ** P < .01. E, S6K1 and AMPK α mRNA expression in samples from 6 CML patients treated with IM for 7 days (from the Bruennert et al.³⁴ study) was examined. Open box represents “before therapy” and the hatched box represents “after therapy”



cells to 1 μ M/L IM and further sensitized them to IM at any concentration examined (Figure 5C). These results suggest that suppressing S6K1 and activating AMPK (using rapamycin and metformin, respectively) might be clinically effective not only at the initial stage of CML therapy but also in patients receiving standard-dose TKI treatment.

3.5 | Suppressing S6K1 and activating AMPK increase the anti-cancer efficacy of IM against BCR/ABL-positive cells

To rule out the possibility that suppressing S6K1 and activating AMPK increase cytotoxicity by affecting BCR/ABL-independent pathways, we investigated whether rapamycin and metformin increase the anti-cancer effects caused by inhibition of signaling pathways downstream of BCR/ABL. Signaling pathways downstream of BCR/ABL, in particular, the JAK2/STAT5, PI3K, and Ras/Raf pathways, maintain proliferation of CML cells and increase their resistance to apoptosis.^{2,3} A previous report suggests that suppressing each of these pathways alone has little effect; however, co-suppression of 2 pathways (in any combination) induces severe apoptosis.² As previously reported, a single inhibitor (ie, STAT5 inhibitor, LY294002 [a PI3K inhibitor], or LY3009120 [a Raf inhibitor]) did not

induce significant cell death in K562 cells pretreated with rapamycin and metformin (data not shown); however, suppression of any 2 signaling pathways led to marked cell death (Figure 6).

Finally, to further investigate whether altered signaling, characterized by suppressed S6K1 and activated AMPK, increases the anti-cancer efficacy of IM by inhibiting BCR/ABL, we established Ba/F3 cells (murine pro-B cells) stably expressing the BCR/ABL protein fused to GFP (Ba/F3-BCR/ABL cells) (Figure 7A). Generally, Ba/F3 cells were dependent on interleukin-3 for their survival and proliferation; however, transduction of the BCR/ABL gene allowed survival in the absence of interleukin-3 (Figure 7B).³⁷ We next confirmed that the sensitivity of Ba/F3-BCR/ABL cells to IM was dose-dependent (Figure 7C). Suppressing S6K1 and activating AMPK using rapamycin (10 nmol/L) and metformin (1 mmol/L), respectively, increased sensitivity to IM (0.7 μ M/L) (Figure 7D). These data suggest that altered intracellular signaling as a result of suppression of S6K1 and activation of AMPK increases the anti-cancer effect of inhibiting BCR/ABL.

4 | DISCUSSION

Here, we show that continuous exposure to IM increases the sensitivity of K562 cells to TKI. We also confirmed that IM-mediated

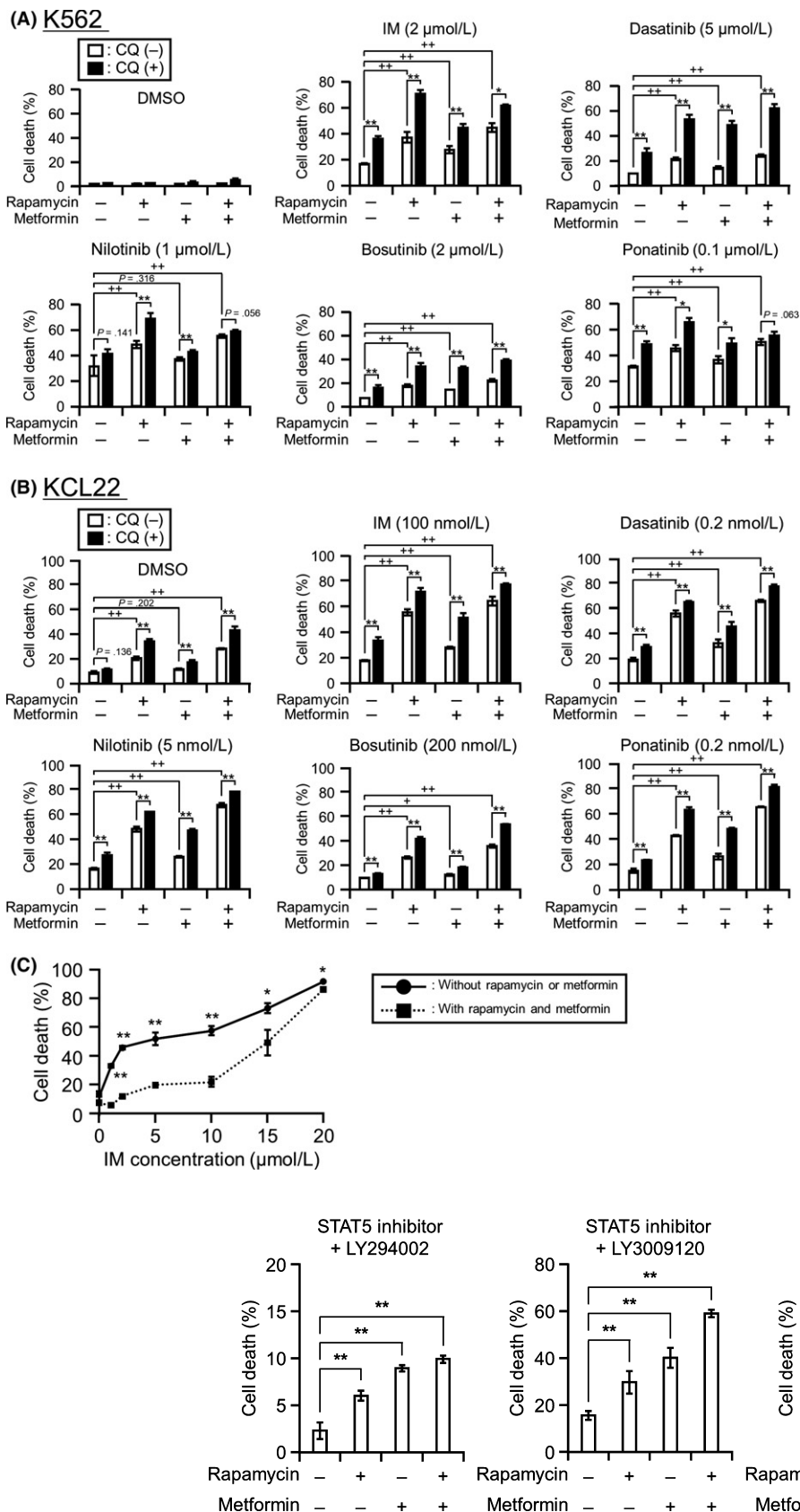


FIGURE 6 Rapamycin and metformin increase cell death by inhibiting signaling pathways downstream of BCR/ABL. K562 cells were preincubated with or without rapamycin (10 nmol/L) and/or metformin (1 mmol/L) for 48 hours, followed by a combination of the following 2 agents for 72 hours: STAT5 inhibitor (200 μ mol/L) plus LY294002 (50 μ mol/L); STAT5 inhibitor plus LY3009120 (0.25 μ mol/L); or LY294002 plus LY3009120. Cell death was then evaluated. $**P < .01$

FIGURE 5 Suppressing p70 S6 kinase 1 (S6K1) and activating AMP-activated protein kinase (AMPK) increase the anti-cancer efficacy of tyrosine kinase inhibitors (TKI). A, K562 or B, KCL22 cells were preincubated with or without rapamycin (10 and 1 nmol/L, respectively) and/or metformin (1 and 0.5 mmol/L, respectively) for 48 hours, followed by exposure to TKI at the indicated concentrations for 72 hours. Cells were cotreated with (black bars) or without (white bars) chloroquine (CQ: 10 and 5 μ mol/L, respectively). Cell death was then evaluated. $*P < .05$ and $**P < .01$, compared with cells not treated with CQ. $+P < .05$ and $++P < .01$, compared with cells not treated with rapamycin and metformin. C, Parental K562 and K562-IM1e cells were preincubated for 48 hours with or without rapamycin (10 nmol/L) and metformin (1 mmol/L), followed by treatment with IM (0-20 μ mol/L) for 72 hours. Cell death was then evaluated. $*P < .05$ and $**P < .01$, compared with cells not treated with rapamycin and metformin

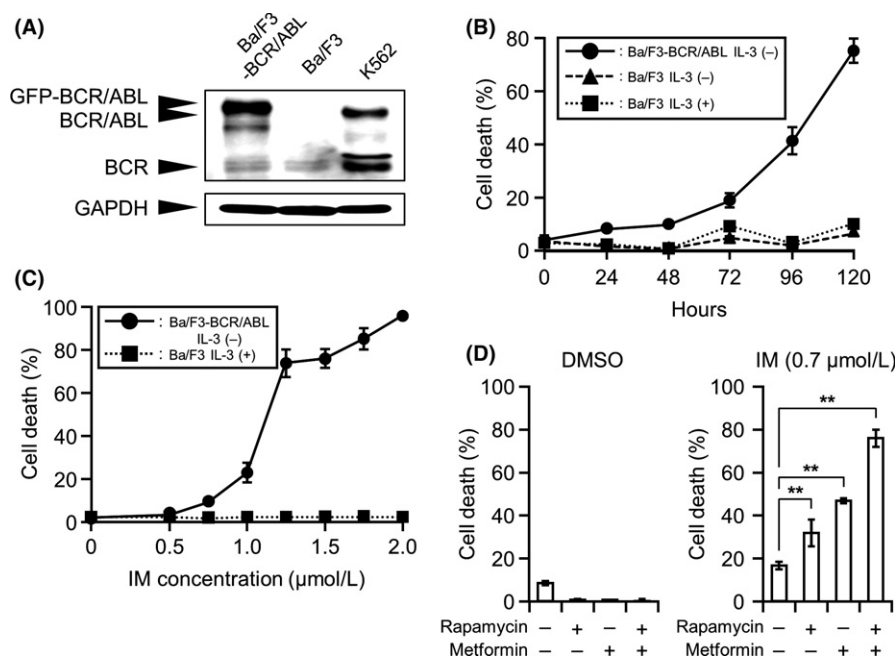


FIGURE 7 Suppressing p70 S6 kinase 1 and activating AMP-activated protein kinase (AMPK) in BCR/ABL-introduced Ba/F3 cells increase the anti-cancer efficacy of imatinib (IM). A, Total cell lysates prepared from Ba/F3-BCR/ABL, Ba/F3, or K562 cells were subjected to western blotting with anti-phospho-BCR, anti-BCR, and anti-GAPDH antibodies. B, Death rate of Ba/F3-BCR/ABL and Ba/F3 cells cultured for the indicated periods in medium with or without 10 ng/mL interleukin-3 (IL-3) was evaluated. C, Viable Ba/F3-BCR/ABL and Ba/F3 cells were treated with different concentrations of IM (0–2 $\mu\text{mol/L}$) for 72 hours. Cell death was then evaluated. D, Ba/F3-BCR/ABL cells were preincubated with or without rapamycin (10 nmol/L) and/or metformin (1 mmol/L) for 48 hours, followed by exposure to IM (0.7 $\mu\text{mol/L}$) for 72 hours. Cell death was then evaluated. ** $P < .01$

inhibition of glycolysis was not in itself responsible for increased IM-sensitivity; rather, altered intracellular signaling (S6K1 suppression and AMPK activation) increased TKI-sensitivity. S6K1, which acts downstream of the PI3K/Akt/mTORC1 pathway, mainly plays a role in promoting protein synthesis and cell proliferation.³⁸ As the PI3K/Akt/mTORC1 pathway is located downstream of BCR/ABL,³ it is unsurprising that S6K1 is directly affected by inhibiting BCR/ABL. Therefore, we assume that altered intracellular signaling is caused by sustained but mild BCR/ABL suppression. Recent evidence suggests that inhibiting PI3K and mTORC1 increases the susceptibility of CML cells to IM.^{39,40} The underlying mechanism is thought to involve increased endoplasmic reticulum (ER) stress and increased nuclear translocation of p-145 c-ABL by activation of the JNK signaling cascade induced by inhibition of the PI3K/Akt/mTORC1 pathway. As we observed that S6K1 was suppressed upon continuous exposure to IM, we considered that the increased efficacy of TKI is induced by a similar mechanism. In general, activation of AMPK acts as a sensor of energy status and a mediator of energy homeostasis;³¹ therefore, it is possible that IM-mediated suppression of glycolysis disturbs the intracellular energy balance and activates AMPK. Several studies suggest that inhibiting the mTORC1 and Wnt/ β -catenin signaling pathways, which act downstream of AMPK activation, has anti-cancer effects.^{41,42} There is little information about the relationship between AMPK and TKI, but we assume that activating AMPK by

continuous exposure to IM might increase sensitivity to TKI by affecting the above signaling pathways.

Here, we showed that continuous exposure to IM suppresses glycolysis and drives autophagy. Furthermore, we found that mitochondrial activity in K562 cells was increased by continuous exposure to IM (Figure S6; Appendix S1). These findings are comparable with those described in our previous report showing that increased autophagy as a result of inhibited glycolysis promotes mitochondrial activity to overcome the energy shortage in ALL cells.²² These findings are supported by other studies arguing that IM increases intracellular glutamate levels, a major intermediate in the mitochondrial Krebs cycle,¹⁷ and that inactivation of BCR/ABL promotes mitochondrial fatty acid oxidation.⁴³ Hence, continuous exposure to IM (probably at concentrations too low to cause cytotoxicity) may dynamically alter intracellular energy metabolism to maintain intracellular ATP levels. Activated mitochondrial oxidative phosphorylation produces a large amount of ATP, but this process generates ROS as a natural byproduct, causing oxidative damage to mitochondrial lipids, cellular DNA, and functional proteins.⁴⁴ We showed that IM-induced autophagy reduces intracellular ROS levels (Figure S7; Appendix S1), suggesting that autophagy might work cytoprotectively by lowering intracellular stress generated by mitochondria as compensation for energy acquisition. Therefore, co-treatment with autophagy inhibitors and TKI is expected to show synergistic anti-cancer effects.

Recent studies suggest that epigenetic patterns are affected by nutrients and their metabolites, which likely change in different environments.⁴⁵ The high reliance of cancer cells on glycolytic pathways may be a consequence of reprogramming energy metabolism.⁴⁶ Although the mechanisms underlying epigenomic control of cancer energy metabolism have not been fully elucidated, some studies provide insight. For example, LSD1, which suppresses gene expression by removing the methyl group from histone H3,⁴⁷ plays a key role in the epigenetic plasticity of cell metabolism. Sakamoto et al⁴⁸ showed that inhibiting LSD1 reduced glycolytic activity and activated mitochondrial respiration in hepatocellular carcinoma cells, and that this metabolic shift was related to expression of HIF-1 α protein. Here, we showed that continuous exposure to IM reduced expression of the HIF-1 α protein, thereby suppressing glycolysis; this is comparable with the findings in LSD1-inhibited cancer cells described above. In addition, IM-treated cells reduced expression of mRNA encoding S6K1 and AMPK; this was also observed in patients subjected to IM treatment. This may be a result of genomic reprogramming, which affects energy metabolism in CML cells. Although knowledge about TKI-mediated epigenetic regulation is scarce, future studies should uncover the mechanism by which altered intracellular signaling pathways/molecules and energy metabolism affect sensitivity to anti-cancer agents.

A previous study shows that various molecular signaling inhibitors have anti-cancer effects.⁴⁹ Here, we showed that rapamycin- and metformin-mediated disturbance of signaling related to intracellular energy metabolism increased sensitivity to TKI. Indeed, mTORC1 inhibitors such as rapamycin and RAD001 show synergistic anti-cancer effects when combined with TKI, and AMPK activators such as metformin and AICAR show anti-leukemic effects against Philadelphia-positive leukemic cells.⁴¹ As activating AMPK leads to cell death when intracellular ATP concentrations are low enough,⁵⁰ the result showing that metformin-induced AMPK activation acts synergistically with TKI, which themselves may alter intracellular energy balance and metabolism, is unsurprising. Moreover, metformin activates AMPK by inhibiting mitochondrial complex I in a direct and/or indirect manner.⁵¹ This agent might exert synergistic anti-cancer effects by inhibiting mitochondrial function in CML cells in which the mitochondrial energy supply is key to maintaining survival upon exposure to TKI. In addition, we also showed that suppressing S6K1 and activating AMPK increased the anti-cancer effect of TKI in the presence of CQ. Thus, disruption of intracellular signaling provides new therapeutic opportunities for CML patients; indeed, co-treatment with molecules that disrupt energy metabolism (rapamycin and metformin) and an autophagy inhibitor (CQ) may be a useful strategy for improved TKI therapy.

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CONFLICT OF INTEREST

Authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION

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