



Full Paper

Aldo-keto reductase inhibitors increase the anticancer effects of tyrosine kinase inhibitors in chronic myelogenous leukemia

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ABSTRACT

Tyrosine kinase inhibitors (TKIs) are widely utilized in clinical practice to treat carcinomas, but secondary tumor resistance during chronic treatment can be problematic. AKR1B1 and AKR1B10 of the aldo-keto reductase (AKR) superfamily are highly expressed in cancer cells and are believed to be involved in drug resistance. The aim of this study was to understand how TKI treatment of chronic myelogenous leukemia (CML) cells changes their glucose metabolism and if inhibition of AKRs can sensitize CML cells to TKIs. K562 cells were treated with the TKIs imatinib, nilotinib, or bosutinib, and the effects on glucose metabolism, cell death, glutathione levels, and AKR levels were assessed. To assess glucose dependence, cells were cultured in normal and low-glucose media. Pretreatment with AKR inhibitors, including epalrestat, were used to determine AKR-dependence. Treatment with TKIs increased intracellular glucose, AKR1B1/10 levels, glutathione oxidation, and nuclear translocation of nuclear factor erythroid 2-related factor 2, but with minimal cell death. These effects were dependent on intracellular glucose accumulation. Pretreatment with epalrestat, or a selective inhibitor of AKR1B10, exacerbated TKI-induced cell death, suggesting that especially AKR1B10 was involved in protection against TKIs. Thus, by disrupting cell protective mechanisms, AKR inhibitors may render CML more susceptible to TKI treatments.

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1. Introduction

Tyrosine kinases specifically phosphorylate tyrosine residues in proteins by transferring the γ -phosphate group from ATP. They activate signal transduction pathways, such as mitogen-activated protein kinase, phosphoinositide 3-kinase/Akt, and Janus kinase/signal transducer and activator of transcription protein, via phosphorylation of tyrosine residues in their substrate proteins.¹ These signals play important roles in the regulation of various cellular processes, such as cell survival, proliferation, and apoptosis, and

their activities are usually precisely regulated. Excessive activation of tyrosine kinases leads to abnormal cell proliferation and longevity, leading to the acquisition of cancer-like properties.² For example, the breakpoint cluster region-abelson (BCR-ABL) chimeric tyrosine kinase, which is encoded by the Philadelphia chromosome, a fusion chromosome derived from the t(9; 22) translocation, is a constitutively activated kinase and a major cause of chronic myelogenous leukemia (CML).³

BCR-ABL is activated through intermolecular phosphorylation, resulting in constitutive cell proliferation and acquisition of anti-apoptotic functions.³ Examples of other tyrosine kinases include the abnormally activated epidermal growth factor receptor (EGFR) identified in various carcinomas, including lung cancer,⁴ as well as human EGFR-related 2, which is often found in breast cancer and is involved in cancer progression.⁵ Since 2000, many drugs have emerged that target these tyrosine kinases (<http://www.brimr.org/PKI/PKIs.htm>). Prior to that time, cancer chemotherapy mainly

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utilized cytotoxic anticancer agents such as antimetabolites, alkylating agents, antibiotics, and microtubule inhibitors.⁶ Although serious side effects due to cytotoxicity have been a major problem with these agents, molecular-targeted drugs have dramatically improved cancer chemotherapy because of their ability to act specifically on cancer cells.^{7,8} Tyrosine kinase inhibitors (TKIs), a class of molecular-targeted drugs, specifically bind to the ATP binding site of tyrosine kinases and inhibit downstream signal transduction to suppress cancer cell growth and induce apoptosis.⁹ To date, TKIs capable of targeting a variety of different carcinomas, such as the BCR-ABL, TKI imatinib, the EGFR TKIs, erlotinib and gefitinib, and the anaplastic lymphoma kinase TKI, crizotinib, have been developed and widely used in clinical practice.¹⁰ Although TKIs have dramatically improved cancer chemotherapy, their use in cancer treatments are generally long-term, and the acquisition of secondary resistance during treatment is a concern.¹¹ Therefore, more effective treatments that use TKIs are needed; in particular, it is important to have a broad understanding of the response of cancer cells to continuous TKI exposure.

In recent years, the unique energy metabolism of cancer cells has garnered attention as a potential target for cancer treatments.¹² Generally, normal cells efficiently produce ATP by metabolizing glucose to pyruvate, followed by oxidative phosphorylation in the mitochondria. In contrast, cancer cells have a characteristic metabolic system that promotes glucose uptake and enhances glycolysis even under adequate oxygen supply.¹³ Continuous exposure of CML cells to imatinib suppresses glycolysis and shifts the cells toward mitochondrial-dependent glycometabolism, and this metabolic reprogramming helps maintain CML cell viability.¹⁴ This change in glycometabolism alters the cell sensitivity to TKIs, including imatinib¹⁴; therefore, the metabolic environment within cancer cells cannot be overlooked when considering cancer treatments, as it also affects cell sensitivity to anticancer agents.

Recently, it was reported that AKR1B1 and AKR1B10 of the aldoketo reductase (AKR) superfamily are highly expressed in liver and lung cancers¹⁵ and are considered to be involved in the drug resistance of cancer cells.¹⁶ AKR superfamily proteins are NAD(H) or NADP(H)-dependent oxidoreductases that metabolize carbohydrates, steroids, endogenous aldehydes, and ketones, with “cell cleaning” properties.¹⁷ Among them, AKR1B1, also called aldose reductase, acts as a rate-determining enzyme in the polyol pathway and uses NADPH as a cofactor to reduce glucose to sorbitol.¹⁸ Augmentation of the polyol pathway is one of the pathologic mechanisms of diabetic complications, which suggests a strong association between glucose metabolic changes and AKR family proteins. Even in cancer cells, through the attenuation of intracellular stress, these proteins may be involved in the malignant transformation of cells. However, many aspects of their functions and fluctuations in activity remain unknown.

In this study, to understand how CML cells exposed to TKIs undergo changes in glucose metabolism and the impact of this exposure on their intracellular milieu, we examined glycolytic suppression during continuous TKI exposure and the activity of AKRs induced by glucose accumulation. Furthermore, we evaluated the *in vitro* therapeutic potential of the suppression of AKRs, which are cytoprotective during TKI treatment of CML.

2. Materials and methods

2.1. Reagents

Imatinib (Phoenix Pharmaceuticals, Belmont, CA, USA), nilotinib (Cayman Chemical, Ann Arbor, MI, USA), bosutinib (KareBay Biochem, Ningbo, China), epalrestat (Tokyo Chemical Industry Co., Ltd., Osaka, Japan), and 7-Hydroxy-2-oxo-2H-chromene-3-carboxylic

acid [3-(4-fluorophenyl)propyl]amide (HCCFA), an AKR1B10 inhibitor ($IC_{50} = 3.5$ nM) that was originally synthesized by our laboratory,¹⁹ were all dissolved in DMSO. The final DMSO concentration in cell cultures did not exceed 0.5% (v/v).

2.2. Cell culture

Human chronic myelogenous leukemia K562 cells (RIKEN Cell Bank, Tsukuba, Japan) were cultured in RPMI-1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Grand Island, NY, USA) and antibiotics (Nacalai Tesque). The glucose concentration in the medium was adjusted to 2 g/L or 0.2 g/L (low-glucose). The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO₂.

2.3. Bicinchoninic acid (BCA) protein assay

Total protein was quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Hanover Park, IL, USA), following the manufacturer's protocol.

2.4. Measurement of lactate production

Cellular lactate release was measured as previously described.²⁰ Briefly, the conditioned medium from cultured cells was deproteinized with perchloric acid and neutralized with potassium hydroxide. The supernatant was mixed with NAD⁺ and glutamate pyruvate transaminase (Roche, Mannheim, Germany). The enzymatic reaction was initiated by adding lactate dehydrogenase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) to each sample and then incubated at 37 °C for 30 min. The absorbance was measured at a wavelength of 340 nm.

2.5. Measurement of intracellular glucose

The glucose levels in K562 cells were measured using a fluorescence-based Amplex® Red Glucose/Glucose Oxidase Assay Kit (Thermo Fisher Scientific), following the manufacturer's protocol.

2.6. Flow cytometric detection of proliferating and dead cells

K562 cells were stained with the Zombie NIR™ Fixable Viability Kit (BioLegend, San Diego, CA, USA) to distinguish dead cells from live cells. To analyze their proliferation rate, the K562 cells were incubated for 15 min with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Dojindo Laboratories, Kumamoto, Japan) dissolved in FBS-free RPMI-1640. After dye loading, the cells were washed by centrifugation and cultured for 72 h. The CFSE dye was retained intracellularly, and following each cell division, the fluorescence intensity was reduced by half in the daughter cells. Fluorescence intensity was detected using an EC800 flow cytometer (SONY, Tokyo, Japan).

2.7. Evaluation of AKR enzymatic activities

AKR enzymatic activities were evaluated using the Aldo-Keto Reductase (AKR) Activity Assay Kit (Abcam, Cambridge, UK), following the manufacturer's protocol. This assay has been validated with AKR1B10, AKR1C1 and AKR1C3. It is based on the ability of AKR to reduce a general substrate and convert NADP⁺ to NADPH, which reacts with the AKR probe.

2.8. RNA isolation and quantitative reverse transcription PCR

Total RNA was isolated using Sepasol-RNA I reagent (Nacalai Tesque) and reverse transcribed using ReverTra Ace® qPCR RT Master Mix (TOYOBO, Osaka, Japan). The resulting cDNA was mixed with THUNDERBIRD™ SYBR qPCR Mix (TOYOBO) and subjected to real-time PCR using a LightCycler™ Nano System (Roche) with the following primers: AKR1B1 forward, 5'-TAC CAT GAG AAG GGC CTG GTG AAA-3' and reverse, 5'-TCC AGA ATG TTG GTG TCA CTG GGA-3'; AKR1B10 forward, 5'-TTC TTT GAG AGA CCC CTT GTG AGG-3' and reverse, 5'-TCC AAG AAC GTT GCT TTT CCA CCG-3'; 18s ribosomal RNA forward, 5'-CGC CGC TAG AGG TGA AAT TC-3' and reverse, 5'-TTG GCA AAT GCT TTC GCT C-3'. Initial denaturation was performed at 95 °C for 60 s, followed by 40 cycles at 95 °C for 10 s and 60 °C for 60 s. Relative mRNA expression was calculated after normalization to 18s ribosomal RNA levels.

2.9. Western blotting

Nuclear and cytosolic proteins from K562 cells were extracted using a LysoPure Nuclear and Cytoplasmic Extractor Kit (Wako Pure Chemical Industries) containing protease inhibitor cocktail (Roche). Identical amounts of protein from each sample were separated by SDS-PAGE and then transferred to polyvinylidene fluoride membranes (Merck Millipore, Berlin, Germany). After membrane blocking, the blots were probed with primary antibodies specific for nuclear factor erythroid 2-related factor 2 (Nrf2), histone deacetylase 2 (HDAC2), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (all purchased from Cell Signaling Technologies, Beverly, MA, USA). Immunolabeled proteins were detected using a horseradish peroxidase (HRP)-labeled anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and ECL Prime detection reagent (GE Healthcare, Buckinghamshire, UK).

2.10. Calculation of GSH/GSSG ratio

Total glutathione and GSSG levels were measured using the GSH/GSSG-Glo™ Assay (Promega, Madison, WI, USA), following the manufacturer's protocol. GSH was calculated as the difference between total glutathione and GSSG, and the GSH/GSSG ratio was determined.

2.11. Statistical analysis

All data are expressed as the means ± standard deviation (SD) of at least three independent experiments. Statistical analysis was performed using Student's *t*-tests or analysis of variance (ANOVA) followed by Bonferroni tests, where applicable.

3. Results

3.1. Glucose accumulates in CML cells during continuous exposure to TKIs

Although cancer cells rely on glycolysis as their primary source of ATP,¹³ exposure of CML cells to TKIs suppresses glycolysis.¹⁴ The suppression of glycolysis occurs at an even lower concentration of TKIs than that used therapeutically.¹⁴ In this study, when the CML-derived cell line K562 was exposed to low-dose imatinib (0.1 and 0.3 μM), a BCR-ABL TKI, for 72 h, lactate release was reduced, thereby confirming the suppression of glycolysis in these cells (Fig. 1A). Furthermore, when glycolysis was continuously suppressed by prolonged exposure of the cells to imatinib for 8 days, glucose accumulated intracellularly (Fig. 1B). Intracellular glucose accumulation also occurred prominently during continuous exposure to second generation BCR-ABL TKIs such as nilotinib and

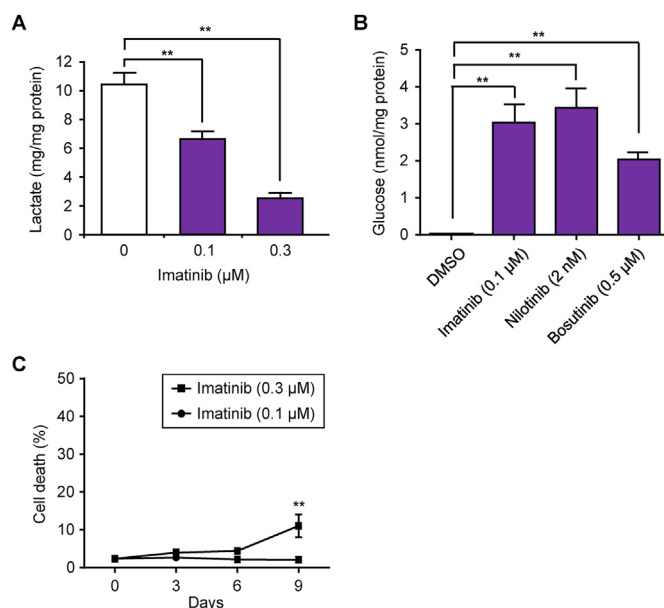


Fig. 1. Continuous exposure to tyrosine kinase inhibitors (TKIs) leads to glucose accumulation in chronic myelogenous leukemia cells (A) K562 cells were treated with (0.1 or 0.3 μM) or without imatinib for 72 h and the amount of lactate released into the culture medium for the last 24 h was evaluated. Data represent means ± SD of three independent cell cultures. One-way ANOVA was followed by Bonferroni's test. ***P* < 0.01 (B) K562 cells were treated with a TKI (imatinib, nilotinib, or bosutinib) at the indicated concentrations for 8 days, and the intracellular glucose level was measured. Data represent means ± SD of three independent cell cultures. One-way ANOVA was followed by Bonferroni's test. ***P* < 0.01 (C) K562 cells were cultured in the presence of imatinib (0.1 or 0.3 μM) for 3, 6, and 9 days, and cell death was evaluated by flow cytometry after propidium iodide uptake. Data represent means ± SD of three independent cell cultures. One-way ANOVA was followed by Bonferroni's test. ***P* < 0.01, compared with the value at 0 days.

bosutinib (Fig. 1B). Thus, continuous suppression of glycolysis by TKIs led to glucose accumulation in CML cells.

Various diabetes studies have indicated that excess glucose stresses cells and causes cytotoxicity.²¹ In this study, continuous exposure of K562 cells to 0.1 μM imatinib for 9 days did not result in significant cell death, although a slight increase was observed with 0.3 μM imatinib (Fig. 1C). These results demonstrate that excessive accumulation of glucose in CML cells during TKI exposure does not induce cell death and suggest that a mechanism exists to attenuate glucose toxicity.

3.2. Expression of AKR1B1 and AKR1B10 increases as CML cells are exposed to TKIs

The AKR1B subfamily uses a wide range of carbonyl compounds as substrates and can reduce highly reactive carbonyl compounds, which can induce "carbonyl stress" by modifying proteins and lipids in a non-enzymatic manner.^{22,23} Based on this, we hypothesized that in CML cells continuously exposed to TKIs, toxic carbonyl compounds generated through the accumulation of glucose would be reduced by AKR1B1/10, thereby attenuating intracellular carbonyl stress. Continuous exposure to 0.1 μM imatinib for 8 days resulted in increased intracellular AKR activity (Fig. 2A). In addition, the levels of AKR1B1 and AKR1B10 mRNA increased as a result of continuous exposure to imatinib, and these increases were concentration-dependent (Fig. 2B and C). These results indicate that exposure of CML cells to TKIs upregulates AKR1B1/10 expression and that these proteins may play an important role in helping the cell avoid stress generated by intracellular glucose accumulation.

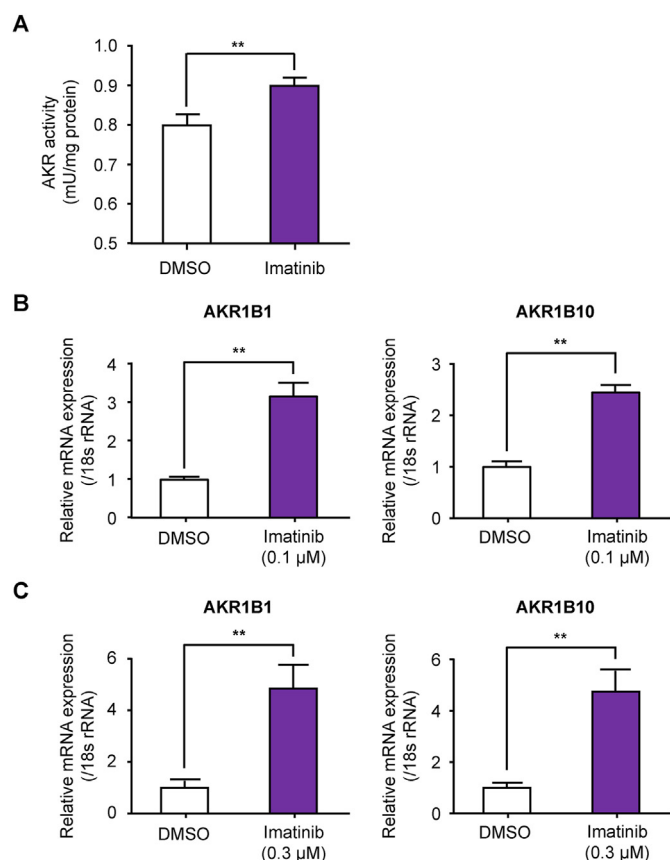


Fig. 2. Exposure to imatinib increases AKR1B expression in chronic myelogenous leukemia cells (A) K562 cells were cultured in the presence (0.1 μM) or absence of imatinib for 8 days, and AKR activity was evaluated. Data represent means ± SD of three independent cell cultures. Differences between groups were analyzed using Student's *t*-test. ***P* < 0.01 (B, C) Quantitative RT-PCR was performed to measure the mRNA levels of AKR1B1 and AKR1B10 in K562 cells treated with (0.1 μM (B) or 0.3 μM (C)) or without imatinib for 5 days. Data represent means ± SD of five independent cell cultures. Differences between groups were analyzed using Student's *t*-test. ***P* < 0.01.

3.3. Glucose accumulation in CML cells enhances AKR1B1 and AKR1B10 expression

Next, we investigated whether the increased expression of AKR1B1/10 accompanying the continuous exposure of CML cells to imatinib was caused by glucose accumulation in the cells. We evaluated whether the increase in AKR1B1/10 expression due to imatinib exposure could be suppressed by lowering the glucose concentration in the culture medium from 2 g/L to 0.2 g/L. We confirmed that the accumulation of glucose in K562 cells exposed to imatinib was suppressed under low glucose culture conditions (Fig. 3A). While AKR activity increased with imatinib exposure when the cells were cultured in medium with a normal glucose concentration (Fig. 2A), it did not change significantly in cells cultured under low glucose conditions (Fig. 3B). We also confirmed that while AKR1B1 and AKR1B10 mRNA levels were upregulated with imatinib exposure in cells cultured under normal glucose conditions, the increase in their mRNA expression was suppressed in cells cultured under low glucose conditions (Fig. 3C). These results suggest that the increased expression of AKR1B1/10 in CML cells upon exposure to imatinib is caused by intracellular glucose accumulation as a result of glycolytic suppression.

Upstream of the AKR1B1 and AKR1B10 gene promoters, there are binding sites for the transcriptional activator Nrf2.^{24,25} Nrf2

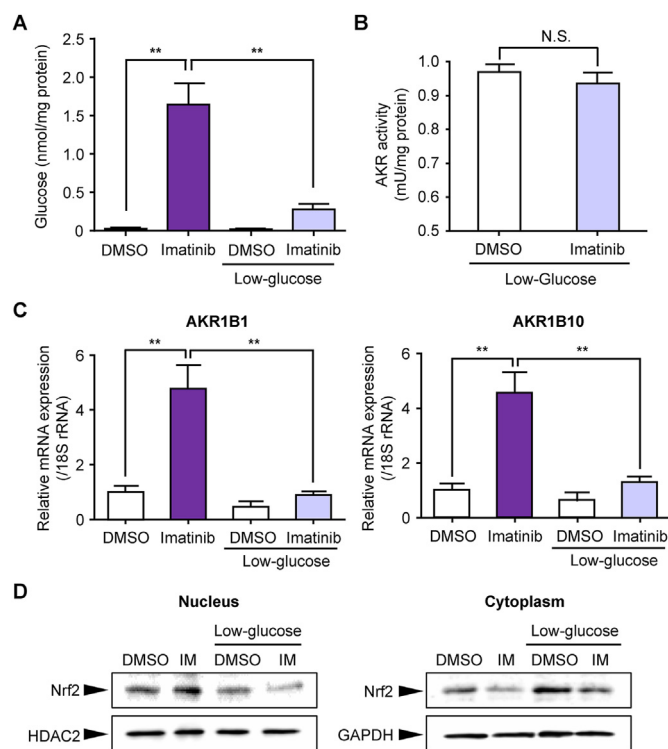


Fig. 3. Higher glucose accumulation by imatinib induces AKR hyperactivation in chronic myelogenous leukemia cells. K562 cells were preincubated in low-glucose or normal medium for 72 h and then treated with (0.3 μM) or without imatinib for 5 days (A) Intracellular glucose levels were measured. Data represent means ± SD of three independent cell cultures. One-way ANOVA was followed by Bonferroni's test. ***P* < 0.01 (B) AKR activity was evaluated. Data represent means ± SD of three independent cell cultures. Differences between groups were analyzed using Student's *t*-test. N.S., not significant (C) Quantitative RT-PCR of AKR1B1 and AKR1B10 mRNA was performed. Data represent means ± SD of five independent cell cultures. One-way ANOVA was followed by Bonferroni's test. ***P* < 0.01 (D) Representative western blotting of nuclear and cytoplasmic protein extracts with anti-Nrf2, anti-HDAC2, and anti-GAPDH antibodies. IM, imatinib-treated lysates.

possesses a stress defense function *in vivo* and enhances the resistance of cells to oxidative stress and drugs by inducing the expression of antioxidant proteins and detoxifying enzymes.²⁶ In addition, Nrf2 functions as a response factor not only to reactive oxygen species, but also to carbonyl stress caused by reactive carbonyl compounds.²⁷ Therefore, we hypothesized that the imatinib-induced AKR1B1/10 upregulation may be the result of the response of Nrf2 to carbonyl stress generated in the cells. We studied the expression level of Nrf2 in cells exposed to imatinib and found an increase in Nrf2 levels in the nucleus (Fig. 3D). This suggests that Nrf2 enhances the transcriptional activity of the AKR1B1/10 promoter. Since an increase in the nuclear level of Nrf2 due to imatinib exposure was not observed under low glucose conditions (Fig. 3D), we also believe that an increase in carbonyl stress due to the intracellular accumulation of glucose stimulated Nrf2 activation.

3.4. Epalrestat, an AKR inhibitor, increases stress in CML cells exposed to imatinib

Generally, carbonyl stress is induced by modified proteins with reactive sugar carbonyl compounds under oxidative conditions.²⁸ Since TKI exposure is well known to increase oxidative stress,²⁹ as we showed in Fig. 4A depicting GSH/GSSG ratio was decreased with exposure to imatinib, it is suggested that TKI initially triggers

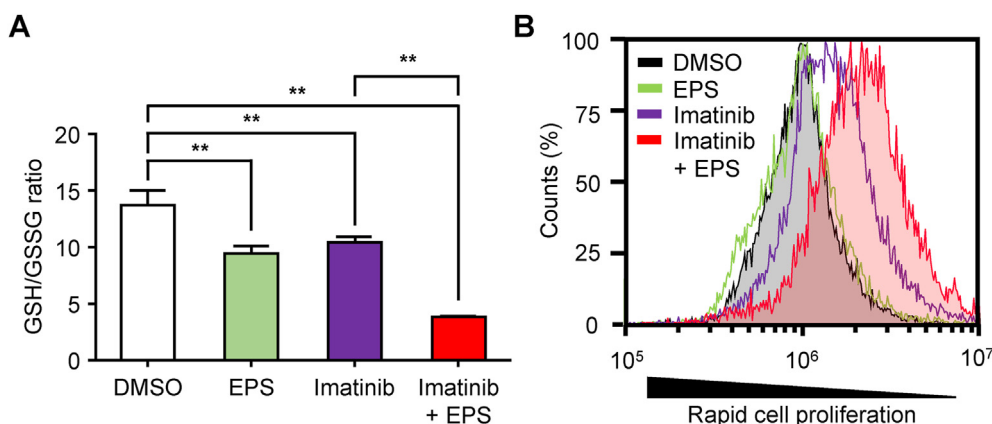


Fig. 4. Epalrestat (EPS) treatment along with imatinib increases intracellular oxidative stress in chronic myelogenous leukemia cells (A) K562 cells pretreated with or without EPS (80 μ M) for 72 h were additionally treated with (0.3 μ M) or without imatinib for 5 days. The intracellular GSH/GSSG ratio was evaluated. Data represent means \pm SD of three independent cell cultures. One-way ANOVA was followed by Bonferroni's test. ** $P < 0.01$ (B) Proliferation of the K562 cells was evaluated by CFSE dye dilution in 3-day cultures using flow cytometry. The CFSE concentration per cell is lower in highly proliferative cells and higher in slower-growing cells. Data are representative of three independent cell culture experiments.

the generation of reactive carbonyl compounds via accumulation of glucose and the induction of carbonyl stress. It has been reported that these reactive compounds are especially reduced by AKR1B10.²² Carbonyl compounds also have potential to induce oxidative stress probably due to their high reactivity.³⁰ Since we believed that increased expression of AKR1B1/10, which accompanies cell exposure to TKI, helps attenuate intracellular carbonyl stress, we next investigated whether inhibiting these AKRs could also increase oxidative stress levels in CML cells. In clinical practice, epalrestat, which inhibits AKR1B1 and AKR1B10,³¹ is used to treat diabetic peripheral neuropathy. K562 cells were preincubated with epalrestat for 72 h and then exposed to imatinib to evaluate intracellular stress. As a result, we found that the GSH/GSSG ratio, which is an indicator of intracellular oxidative stress, was significantly lower in cells preincubated with epalrestat than in cells exposed only to imatinib (Fig. 4A). This suggests that treatment with epalrestat can increase intracellular stress levels due to imatinib exposure. Furthermore, we used CFSE staining to determine whether the decrease in cell proliferation was due to intracellular stress levels that increased after treatment with epalrestat. CFSE fluorescence intensity is reduced by half following each cell division. Similar to the decrease in intracellular oxidative stress shown in Fig. 4A, imatinib treatment of K562 cells preincubated with epalrestat resulted in a remarkable increase in CFSE fluorescence compared to that of imatinib alone, thereby demonstrating suppressed cell proliferation (Fig. 4B). These results suggest that AKR1B1/10, whose expression increases with TKI exposure, helps cells attenuate intracellular stress and that AKR1B1/10 inhibition can increase stress levels in CML cells.

3.5. Inhibition of AKR1B1/10 can enhance the effect of TKIs on CML cells

We investigated whether the anticancer effect of TKIs could be enhanced by the preincubation with AKR1B1/10 inhibitors in K562 cells. We first confirmed that epalrestat alone can induce only marginal cell death in K562 cells (Fig. 5A). Next, we observed that exposure of the cells to imatinib following their 72 h preincubation with epalrestat caused significantly enhanced cell death in a time- and imatinib concentration-dependent manner (Fig. 5B). A similar time-dependent phenomenon was observed when the other TKIs nilotinib and bosutinib were used (Fig. 5C). These results suggest

that inhibition of AKR1B1/10, which is believed to exacerbate the stress from TKI exposure, enhances the anticancer effect of TKIs.

Because epalrestat is a drug with a relatively high inhibitory capacity toward AKR1B1, similar studies were conducted using HCCFA, an AKR1B10-specific inhibitor that we synthesized independently (IC₅₀ for AKR1B1 and AKR1B10: 277 nM and 3.5 nM, respectively).¹⁹ K562 cells were preincubated with HCCFA for 72 h before continuous exposure to imatinib. We found that co-exposure to HCCFA markedly enhanced sensitivity of the cells to imatinib (Fig. 6). Since HCCFA has a high inhibitory capacity against AKR1B10, we concluded that AKR1B10 may contribute significantly to attenuating TKI-induced intracellular stress in CML cells.

As depicted in Fig. 7, TKI generates intracellular stress via glucose accumulation in CML cells, leading to enhanced transcription of AKR1B1/10, which helps cell survival by avoiding stress. Therefore, suppressing these enzymes using specific inhibitors can increase intracellular stress and lead to cell death.

4. Discussion

Imatinib has been reported to maintain a trough blood concentration of ~1200 ng/mL when 400 mg, a normal clinical dose, is administered once a day.³² Moreover, imatinib exposure decreases the mRNA expression of glycolytic enzymes, such as hexokinase and lactate dehydrogenase, probably in a hypoxia-inducible factor 1 α -dependent manner in CML cells.^{14,33} We also previously revealed that the glycometabolism in CML cells was reprogrammed from glycolysis to mitochondrial oxidative phosphorylation by continuous imatinib exposure.¹⁴ In the present study, the estimated imatinib concentration was equivalent to the clinical blood concentration when adjusted for the effects of the protein-bound (89%–96%).³⁴ Herein, we clarified that an equal dose of imatinib induced glucose accumulation in CML K562 cells by glycolytic suppression which was also supported by a previous report.³⁵ Since excessive glucose accumulation in cells generally results in carbonyl stress,³⁶ we first speculated that such stress increased during TKI exposure. However, continuous TKI exposure of CML cells caused minimal death. Therefore, the carbonyl stress induced by glucose accumulation must have been attenuated. One of the key enzyme subfamilies to accomplish this goal is AKR1B, whose members have the ability to reduce a wide variety of carbonyl compounds in response to carbonyl stresses.^{22,37} We also confirmed AKR1B1/10 upregulation with TKI exposure, and this

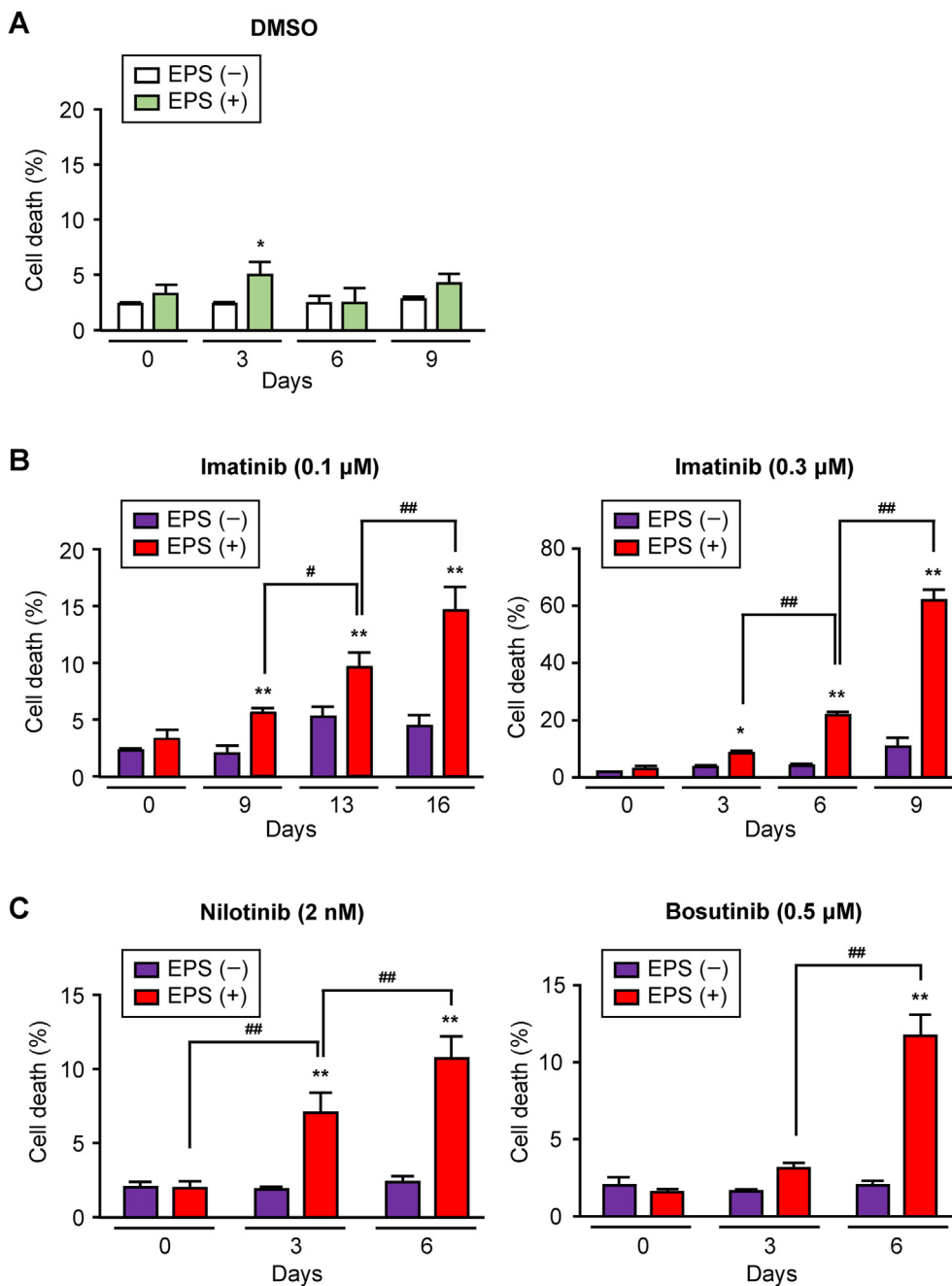


Fig. 5. Epalrestat (EPS) enhances anticancer effects of tyrosine kinase inhibitors (TKIs) in chronic myelogenous leukemia cells (A–C) K562 cells pretreated with (80 μM) or without EPS for 72 h were then treated with vehicle (DMSO) only (A), imatinib (B), nilotinib, or bosutinib (C) for the indicated days. Cell death was then evaluated. Data represent means \pm SD of three independent cell cultures. Two-way ANOVA was followed by Bonferroni's test. * $P < 0.05$, ** $P < 0.01$ compared with corresponding value from cells not treated with EPS. # $P < 0.05$, ## $P < 0.01$.

upregulation was dependent on glucose accumulation because culturing in low-glucose medium (0.2 g/L) severely diminished AKR1B1/10 upregulation with imatinib treatment. Considering that the fasting blood sugar level of a healthy adult is usually ~ 100 mg/dL,³⁸ the blood glucose concentration in humans is much higher than that in low-glucose medium; thus, glucose accumulation in CML cells of patients can occur due to TKI exposure. Based on these findings, it is possible that AKR1Bs help cancer cells avoid intracellular stress during CML treatment with TKIs in clinical practice, and it is presumed that AKR1Bs are involved in the acquisition of resistance to TKIs.

Excess intracellular glucose accumulation can produce highly reactive carbonyl compounds through non-enzymatic protein binding and self-oxidation.²³ These compounds cause functional impairment by irreversibly modifying intracellular proteins and DNA³⁹ and cause intracellular carbonyl stress by increasing cell oxidative stress.³⁶ The AKR enzyme superfamily reduces carbonyl groups in aliphatics, aldehydes, ketones, prostaglandins, and steroids to alcohol groups and plays a role in avoidance of intracellular carbonyl stress.¹⁷ This study also suggested that AKR1B1/10 attenuates the carbonyl stress caused by intracellular glucose accumulation due to TKI exposure. In addition, along with the increased

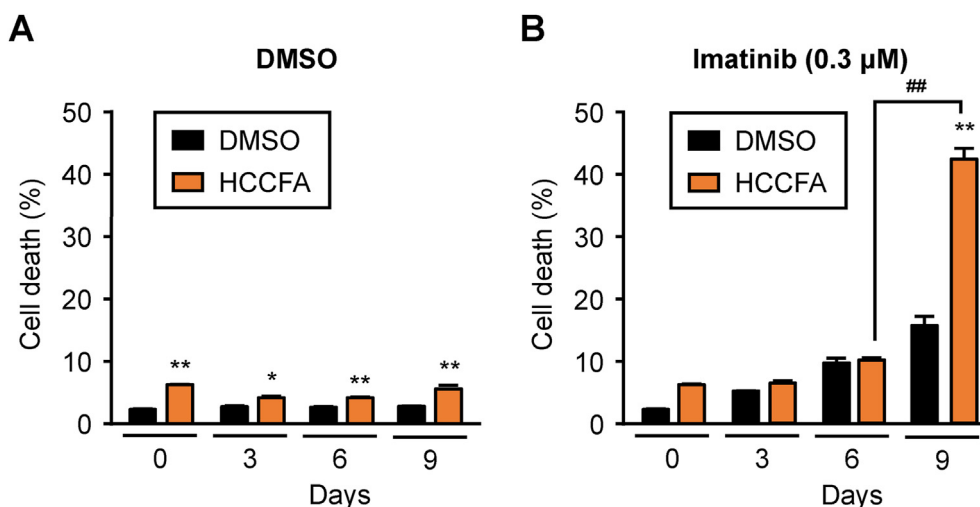


Fig. 6. AKR1B10 inhibition enhances anticancer effects of imatinib in chronic myelogenous leukemia cells (A, B) K562 cells pretreated with HCCFA, a selective inhibitor of AKR1B10 (10 μ M) for 72 h were then treated with vehicle (DMSO) only (A) or imatinib (B) for the indicated days. Cell death was then evaluated. Data represent means \pm SD of three independent cell cultures. Two-way ANOVA was followed by Bonferroni's test. * P < 0.05, ** P < 0.01 compared with corresponding value from cells not treated with HCCFA. ## P < 0.01.

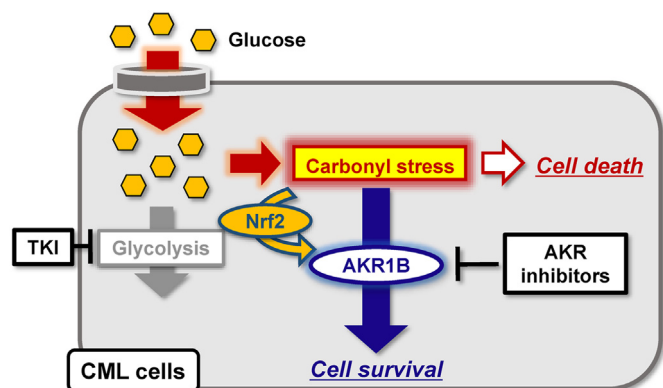


Fig. 7. Overview of the study findings. Exposure of chronic myelogenous leukemia (CML) cells to a tyrosine kinase inhibitor (TKI) generates intracellular stress via glucose accumulation. However, the enhanced stress of glucose accumulation increases AKR1B functions and is subsequently attenuated by these enzymes. Therefore, the inhibition of AKR1Bs using specific inhibitors contributes to the accumulation of intracellular stress and can lead to the death of CML cells.

expression of AKR1Bs upon exposure to imatinib in a glucose concentration-dependent manner, we identified increased nuclear translocation of the transcription factor Nrf2. Nrf2 is an important factor that is activated by highly reactive carbonyl compounds such as methylglyoxal, and it senses intracellular carbonyl stress to protect cells.²⁷ In the absence of oxidative stress, Nrf2 can bind to Kelch-like ECH-associated protein (Keap1), which prevents the nuclear translocation of Nrf2.⁴⁰ However, Keap1 undergoes molecular modification when exposed to intracellular and extracellular factors, such as electrophiles and reactive oxygen species, which leads to its dissociation from Nrf2.⁴¹ Nrf2 then translocates into the nucleus from the cytoplasm and becomes transcriptionally active.⁴¹ Based on our experiments, we believe that the stress generated by glucose accumulation in CML cells due to exposure to imatinib was transmitted to Keap1 to promote the nuclear translocation of Nrf2. AKR1Bs are antioxidant enzymes that are transcriptionally activated downstream of Nrf2.²⁴ When we consider that cancer cells are generally susceptible to oxidative stress, such a cell protection mechanism provides an avenue for cancer cells to proliferate and acquire drug resistance.

Epalrestat is used to treat diabetic peripheral neuropathy.⁴² The pathology is caused by sorbitol accumulation of several tissues in the polyol pathway via glucose, partially because the expression level of sorbitol dehydrogenase gene is lower in these tissues and some patients have polymorphic variation in this gene.^{43,44} Here, we found that co-administration of the AKR1B inhibitor, epalrestat, may be useful for the treatment of CML with TKIs, through inhibiting the generation of reactive carbonyl compounds, probably not the polyol pathway. The TKI concentration that was used in this study is almost the same as the concentration at which the proliferation of K562 cells begins to decrease.⁴⁵ Since imatinib, nilotinib, and bosutinib have inhibitory capacities against BCR-ABL of 260–678 nM, < 10–25 nM, and 41.6 nM, respectively,⁴⁶ we believe that there was a sufficient inhibitory effect against BCR-ABL. Moreover, the exposure of cells to a low concentration (0.1 μ M) of imatinib, which has no marked influence on cell proliferation, suppresses glycolysis in CML cells in a manner dependent on BCR-ABL.¹⁴ This suggests that the intracellular accumulation of glucose during TKI exposure, as identified in our study, is dependent on BCR-ABL, which led to the accumulation of carbonyl stress in the cells. Epalrestat has an IC_{50} of 0.021 and 0.33 μ M for AKR1B1 and AKR1B10, respectively,³¹ but even when using a compound such as HCCFA, which is more selective towards AKR1B10 (IC_{50} for AKR1B1 and AKR1B10: 277 nM and 3.5 nM, respectively),¹⁹ we observed a similar enhancement in the anticancer effect of TKIs in CML cells. AKR1B10 is an NADPH-dependent reductase that shows $\geq 70\%$ amino acid homology with AKR1B1 and has been reported to be highly expressed in various carcinomas, including liver, lung, pancreatic, and breast cancers.¹⁵ AKR1B10 is well-known to have a high capacity of reducing reactive carbonyl compounds such as methylglyoxal, glyceraldehyde, and 3-deoxyglucosone,²² which are non-enzymatically generated from glucose and harmful for cells.²³ AKR1B10 not only detoxifies and reduces reactive aldehydes, but it also promotes retinoic acid metabolism to reduce the amount of intracellular retinoic acid, suppresses cell differentiation, and eventually increases cell survival and proliferation potential.⁴⁷ AKR1B10, whose expression is elevated in CML cells by TKI exposure, promotes cell survival by relieving intracellular stress among other factors, and it is conceivable that its inhibition may have enhanced cell damage.

TKIs such as gefitinib and erlotinib suppress glycolysis in EGFR-TKI-sensitive lung cancer cells in addition to CML cells.⁴⁸ Cytotoxic

anticancer agents such as cisplatin also exhibit an inhibitory effect on glycolysis in breast cancer cells,⁴⁹ and we surmise that the suppression of glycolysis and subsequent accumulation of glucose in cells widely occur during treatments with anticancer agents. Considering that high AKR1B1/10 expression has been reported in many solid cancers (e.g., liver lung cancers) and this is closely related to the survival rate of cancer patients,¹⁵ we believe that it is clinically significant to inhibit these enzymes. We expect that focusing on AKR1B1/10 expression levels and co-administering suitable inhibitors against them in future cancer treatments will be a useful treatment strategy not only for CML, but also for cancers overexpressing these proteins and cancers in which these proteins are upregulated during anticancer treatments.

Declaration of competing interest

The authors declare no conflicts of interest.

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