Glycometabolic adaptation mediates the insensitivity of drug-resistant K562/ADM leukaemia cells to adriamycin via the AKT-mTOR/c-Myc signalling pathway

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Abstract. In human leukaemia, resistance to chemotherapy leads to treatment ineffectiveness or failure. Previous studies have indicated that cancers with increased levels of aerobic glycolysis are insensitive to numerous forms of chemotherapy and respond poorly to radiotherapy. Whether glycolysis serves a key role in drug resistance of leukaemia cells remains unclear. The present study systematically investigated aerobic glycolytic alterations and regulation in K562/adriamycin (ADM) multidrug-resistant (MDR) and ADM-sensitive K562 leukaemia cells in normoxia, and the association between drug resistance and improper glycometabolism. The cell proliferating activity was assessed with an MTT colorimetric assay, glycolysis, including glucose consumption, lactate export and key-enzyme activity was determined by corresponding commercial testing kits. The expression levels of hexokinase-II (HK-II), lactate dehydrogenase A (LDHA), glucose transporter-4 (GLUT-4), AKT, p-AKT473/308, mammalian target of rapamycin (mTOR), p-mTOR, c-Myc and hypoxia-inducible factor-1α (HIF-1α) were analyzed by western blot or reverse transcription-quantitative polymerase chain reaction (RT-qPCR). K562/ADM cells exhibited increased glucose consumption and lactate accumulation, increased lactate dehydrogenase, hexokinase and pyruvate kinase activities, and reduced phosphofructokinase activity. In addition, K562/ADM cells expressed significantly more HK-II and GLUT-4. Notably, inhibition of glycolysis effectively killed sensitive and resistant leukaemia cells and potently restored the sensitivity of MDR cells to the anticancer agent ADM. The AKT serine/threonine kinase (AKT)/mechanistic target of rapamycin (mTOR) signalling pathway, a crucial regulator of glycometabolic homeostasis, mediated over-activation and upregulation of c-Myc expression levels in K562/ADM cells, which directly stimulated glucose consumption and enhanced glycolysis. In conclusion, the present study demonstrated that MDR leukaemia cells exhibit increased aerobic glycolytic activity and that this may be responsible for resistance to chemotherapeutics in leukaemia MDR cells via activation of the AKT-mTOR-c-Myc signalling pathway. Therefore, inhibition of aerobic glycolysis may be a potential therapeutic strategy to efficiently treat multidrug resistance in relapsed or refractory leukaemia and cancers.

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Introduction

Leukaemia is a malignant and proliferative disease originating from multipotent haemopoietic stem cells. Although treatment of leukaemia has greatly improved over the past decades, conventional combination chemotherapy remains ineffective for numerous patients. Most therapeutic failures are attributed to cellular resistance to anti-leukemic therapy. Various factors contribute to drug resistance, including alteration in drug transport, dysregulation of DNA replication and repair, and impaired apoptosis (1).

Recently, abnormal glycometabolism of cancer cells has become of focus. Previous studies supported the idea that enhanced glycolysis is associated with decreased sensitivity to various forms of tumour therapy; glycolytic cancers have been demonstrated to be highly refractory to chemo- and radiotherapies (2-4). Over 90 years ago, Warburg observed that cancer cells exhibited increased glycolysis despite the presence of ample oxygen, which was termed the 'Warburg effect', or aerobic glycolysis (5). Aerobic glycolysis inhibition has been demonstrated to increase drug sensitivity in certain cancer cells. Key proteins in the glycolytic pathway have been thoroughly investigated, including glucose transporters (GLUTs), HK, pyruvate kinase (PK) and -LDH (6-9). However, the effects of glycolytic metabolism on chemo-agent sensitivity, and the causal association between increased glycolytic activity and decreased sensitivity to anticancer agents in refractory tumours and leukemias, remains to be fully elucidated. It remains unknown whether targeting cancer

cell energy supply via the glycolytic pathway may serve as a potential therapeutic strategy to overcome MDR. How glycolytic metabolism affects cell processes, particularly anticancer agent efficiency, is not fully understood. The present study systematically investigated the glycolysis-metabolic status, and the association between drug sensitivity and aerobic glycolysis in sensitive and MDR leukaemia cells in normoxic conditions. Increased aerobic glycolysis was demonstrated to be present in leukaemia MDR cells, and inhibition of glycolysis potently sensitises MDR cells to the anticancer agent adriamycin (ADM), accompanied by overactivation of the AKT serine/threonine kinase (AKT)-mechanistic target of rapamycin (mTOR)-c-Myc pathway.

Materials and methods

Reagents and antibodies. The reagents used in this study included oxamate (Ox; Alfa-Aesar, Haverhill, MA, USA), 2-deoxyglucose (2-DG; Yuanye Bio-Technology Co., Ltd., Shanghai, China), ADM (Wanle Bio-Technology Co., Ltd., Hangzhou, China), glucose, lactate, HK, PK, and LDH assay kits (Jiancheng Bioengineering Institute, Nanjing, China) and a phosphofructokinase (PFK) assay kit (Kemin Industries Co., Ltd., Zhuhai, China). The following primary antibodies were used: Mouse polyclonal anti-β-actin (Zhongshan Jinqiao Bio-Technology Co., Ltd., Beijing, China), rabbit polyclonal anti-GLUT4, anti-HK-II, anti-phosphorylated (p)-AKT (Thr308), anti-mTOR, anti-p-mTOR (Ser2448) (ImmunoWay Biotechnology, Plano, TX USA); anti-AKT, anti-p-AKT (Ser473), anti-c-Myc (Cell Signaling Technology, Inc., Danvers, MA, USA), and rabbit polyclonal anti-LDHA (Hangzhou HuaAn Biotechnology Co., Ltd., Hangzhou, China).

Cell culture. The K562 human leukaemia cell line was purchased from the American Type Culture Collection (Manassas, VA, USA), and the K562/ADM ADM-resistant cell line was obtained from the Shanghai Jiaotong University School of Medicine (Shanghai, China). The cells were maintained in RPMI-1640 media (HyClone; GE Healthcare Life Sciences, Inc.) supplemented with 10% foetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and cultivated at 37˚C in a 5% CO_2 incubator. K562/ADM cells were stimulated with 5 mg/l ADM every 45 days to maintain increased drug resistance, and then were used after cultured 2 weeks without ADM.

In vitro cytotoxicity assay. For the cytotoxicity assay, a density $1x10^5$ cells/ml were plated into 96-well plates. Cells were subsequently incubated with various concentrations of Ox (0, 1, 2.5, 5, 10 and 20 mM) or 2-DG (0, 0.05, 0.25, 0.5, 1 and 2 mM) and ADM (0.01 mg/l for K562 cells, 0.8 mg/l for K562/ADM cells) for 48 h at 37˚C. A total of 10 μl MTT solution was then added to each well, followed by incubation for 4 h at 37˚C. 10% SDS was added to each of the wells, and incubated overnight at 37˚C to dissolve the formazan crystals. Finally, the absorbance values of each well at 570 nm were quantified using a PowerWave X Plate Reader (BioTek Instruments, Inc., Winooski, VT, USA). Each dose of the compound was tested in quadruplicate.

Measurement of glucose concentration and lactate production. Cells were seeded at a density of $1x10^5$ cells/ml. Culture media was collected at 48 h after treatment with various concentrations of Ox (0, 2.5 and 10 mM) or 2-DG (0, 0.5 and 2 mM) at 37˚C and stored at -80˚C until assayed. The glucose and lactate assay kits (Jiancheng Bioengineering Institute, Nanjing, China) were used to determine the concentrations of glucose and lactate in the culture media. Experiments were performed in triplicate.

Quantification of enzymatic activity. Cells were seeded at a density of $1x10^5$ cells/ml and incubated for 48 h at 37˚C and were centrifuged at 1,000 x *g* for 6 min at room temperature and the supernatant was collected. Then the supernatant was washed with PBS and lysed in ice-cold WIP tissue and cell lysis solution (Beijing Cellchip Biotechnology Co., Ltd. Beijing, China) for 5 min. The cell lysates were centrifuged at 12,000 x *g* for 15 min at 4˚C to collect the supernatant. Cell lysate was stored at -80˚C until assayed. HK, PFK, PK and LDH activity in cell lysates was determined using HK, PK, and LDH assay kits (Jiancheng Bioengineering Institute, Nanjing, China) and PFK assay kit (Kemin Industries Co., Ltd., Zhuhai, China). The protein concentrations in the cell lysates were quantified using a bicinchoninic acid (BCA) protein assay kit (Beyotime Biotechnology Co., Ltd., Shanghai, China). Enzymatic activity was normalized to the quantity of total protein. Experiments were performed in triplicate.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from the cells was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). cDNA was derived using the total RNA as a template by a PrimeScript RT reagent kit (Perfect Real Time) obtained from Takara Bio, Inc. (Otsu, Japan) according to the manufacturer's protocol. qPCR was conducted using the SYBR Premix Ex Taq™ II (Tli RNaseH Plus) kit (Takara Bio, Inc.). Gene expression levels were analysed using a Rotor-Gene 3000 qPCR amplifier (Corbett Co., Ltd., Australia). The thermocycling conditions were as follows: 10 sec at 95˚C; followed by 40 cycles of 95˚C for 5 sec, 60˚C for 30 sec. All samples were analyzed using β-actin gene expression as an internal control. The relative mRNA level of GLUT4, LDHA and hypoxia-inducible factor-1α (HIF-1α) was determined by the $2^{-\Delta\Delta Cq}$ method (10). The primers used for qPCR (Table I) were designed and synthesized by Takara Biotechnology Co., Ltd. (Dalian, China).

Western blotting. Treated cells were washed with PBS and lysed in ice-cold RIPA lysis buffer (Beijing Solarbio Science & Technology Co. Ltd., Beijing, China). The cell lysates were centrifuged at 12,000 x *g* for 15 min at 4˚C to collect the supernatant. A bicinchoninic acid (BCA) protein assay kit was used to determine the protein concentration. The proteins (30 μg) were separated using 10% SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). The membranes were blocked using 5% non-fat milk for 1 h, followed by overnight incubation with primary antibodies at 4˚C. The primary antibodies used were as follows: β-actin (1:700; cat. no. sc-1616-R), GLUT4 (1:1,000; cat. no. YT1930), HK-II (1:4,000; cat. no. YM0350), LDHA (1:1,000; cat. no. 1007-2), AKT (1:1,000; cat. no. 4691),

p-AKT (Ser473) (1:1,000; cat. no. 4060), p-AKT (Thr308) (1:500; cat. no. YP0007), mTOR (1:1,000; cat. no. YT2915), p-mTOR (Ser2448) (1:1,000; cat. no. YP0176) and c-Myc (1:500; cat. no. 13987). Subsequently, the membranes were washed with Tween-20 and PBS and then incubated again for 1 h at room temperature with horseradish peroxidase conjugated goat anti-rabbit (cat. no. ZB-2301) or goat anti-mouse (cat. no. ZB-2305) secondary antibody (1:10,000; Zhongshan Jinqiao Bio-Technology Co., Ltd.). Protein bands were visualized using enhanced chemiluminescence reagents (EMD Millipore). β-actin served as an internal control. Band intensities were determined using ImageJ software version 1.45S (National Institutes of Health, Bethesda, MD, USA; imagej.nih.gov/ij/).

Statistical analysis. Data are expressed as the mean ± standard deviation. Two-tailed Student's t-test was used to assess the difference between two groups. One way analysis of variance followed by Dunnett's multiple comparisons test was used to determine differences between groups. $P<0.05$ was considered to indicate a statistically significant difference. Statistical analysis was performed using SPSS software version 13.0 (SPSS, Inc., Chicago, IL, USA).

Results

K562/ADM leukaemia cells exhibit increased aerobic glycolytic activity. Our previous study demonstrated that K562/ADM cells acquired MDR, which was induced by ADM treatment of the K562 parental sensitive cell line and associated with expression levels of P-glycoprotein (P-gp) (11). In the present study, the K562 and K562/ADM leukaemic cell lines were used to assess the association between glycolytic activity and MDR. Metabolic flux in the two cell lines was assessed by glucose consumption and lactate export. As presented in Fig. 1A, glucose consumption in K562/ADM cells was increased compared with the sensitive control cells ($P<0.05$). A similar trend was observed in lactate export (Fig. 1B). The lactate export of K562/ADM cells was increased by 1.34-fold compared with K562 cells ($P<0.001$). Increases in glucose consumption and lactate accumulation indicated that the glycolytic pathway is highly active in ADM-resistant cells.

To investigate the mechanisms underlying these differences, the enzyme activities of HK (Fig. 1C), PFK (Fig. 1D), PK (Fig. 1E) and LDH (Fig. 1F) were compared between K562 and K562/ADM due to their key roles in glycolysis. ADM-resistant K562/ADM cells had increased HK, PK, and LDH activity compared with their treatment-sensitive counterparts, which may contribute to increased glycolysis. Notably, LDH activity was markedly increased; ~2-fold greater LDH activity was observed in K562/ADM cells compared with treatment-sensitive controls, which is consistent with its increased lactate export. PK activity was slightly increased, and the increase in HK activity was moderate. In addition, as presented in Fig. 1D, the activity levels of PFK, a critical driver of glycolytic flux, were decreased in K562/ADM cells, which supported the increased proliferation of K562 cells (data not shown). Thus, LDH and HK were regarded as two important targets for subsequent experiments.

Table I. Primer sequences.

Gene	Primer sequence
GLUT4	F: GCTGCGAATAAACAGGCAGGA
	R: CAGCACAGCAGTGATGACAGTGA
LDHA	F: CGTGCATTCCCGATTCCT
	R: CAACAGCACCAACCCCAAC
HIF-1α	F: TTGCTCATCAGTTGCCACTTCC
	R: AGCAATTCATCTGTGCTTTCATGTC
β-actin	F: TGGCACCCAGCACAATGAA
	R: CTAAGTCATAGTCCGCCTAGAAGCA

F, forward; R, reverse; GLUT4, glucose transporter type 4; LDHA, lactate dehydrogenase A; HIF-1α, hypoxia-inducible factor-1α.

The expression levels of key enzymes are additionally responsible for metabolic alterations; previous research has suggested that LDH and HK are associated with tumour drug resistance (4,12). Therefore, the expression levels of LDHA (Fig. 1G) and HK-II (Fig. 1H) were measured, which are isoforms of LDH and HK, respectively. No significant differences in LDHA expression levels were identified between K562 and K562/ADM cells; however, HK-II expression levels were significantly increased in K562/ADM cells compared with K562 cells ($P<0.01$). These data implied that K562/ADM cells have increased glycolysis compared with K562 cells, primarily due to increased HK-II expression levels and/or HK and LDH activity.

K562/ADM leukaemia cells have increased sensitivity to glycolytic inhibitors. MTT assays were performed to investigate the effects of Ox and 2-DG on cell viability in the two cell lines. Ox is an established inhibitor of LDHA with a potent inhibitory effect on glycolysis. LDHA catalyses the conversion of pyruvate to lactate, the last step in the glycolytic pathway. This is a key step that influences the quantity of pyruvates that enter glycolysis. Incubation of K562 and K562/ADM with Ox caused glucose consumption and lactate export to decrease, confirming its ability to block energy metabolism. No significant differences in glucose consumption inhibition were observed between K562 and K562/ADM cells (Fig. 2A). However, lactate export inhibition was increased in K562/ADM cells compared with K562 cells following the administration of Ox ($P<0.001$; Fig. 2B). Furthermore, as presented in Fig. 2C, dose-dependent cytotoxicity was revealed in the two cell lines, with ADM-resistant cells exhibiting increased sensitivity to the LDHA inhibitor compared with control cells. The inhibition rates of Ox at 2.5 and 10 mM were 0±1.93 and 19.60±2.09% (for K562 cells) and 5.25±2.52 and 28.91±2.97% (for K562/ADM cells), respectively. The increase in lactate export inhibition in K562/ADM cells following treatment with Ox paralleled its increased inhibition ratio, which revealed a significant association between drug resistance and enhanced lactate accumulation. The glycolytic inhibitor 2-DG was additionally used in this study, which is a compound known to inhibit the first phase

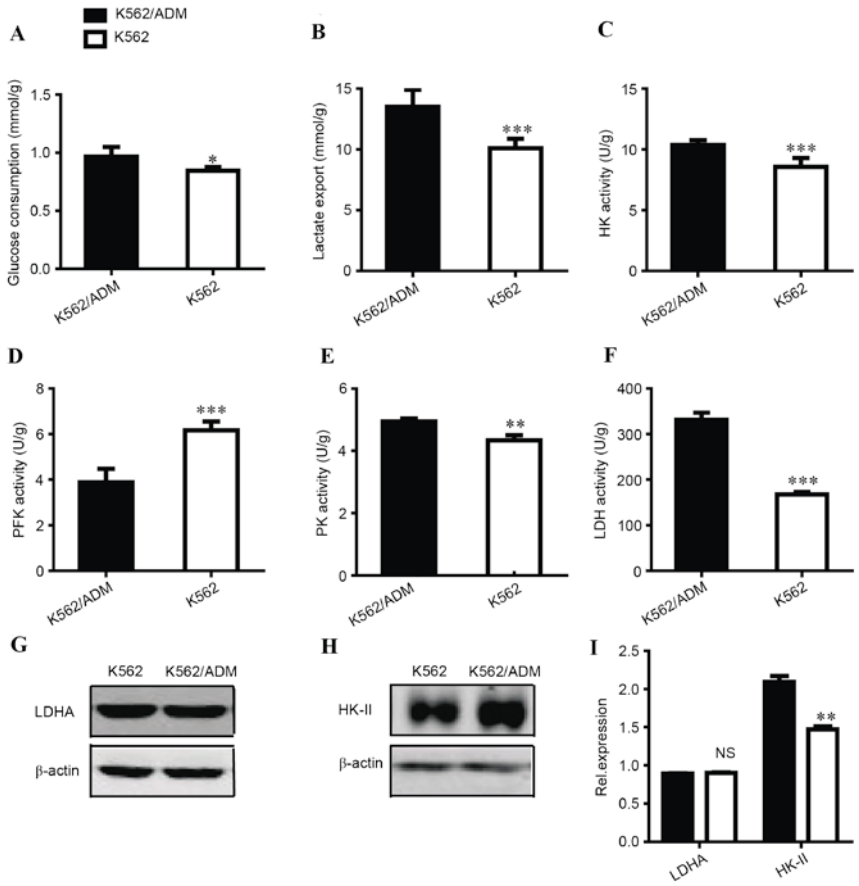

Figure 1. Comparison of the glycolytic status between K562 and K562/ADM cells. (A) Glucose consumption, (B) lactate export, (C) HK activity, (D) PFK activity, (E) PK activity and (F) LDH activity were measured. Western blot images of (G) LDHA and (H) HK-II protein expression levels. (I) Quantification of LDHA and HK-II relative protein expression levels. Data are presented as the mean ± standard deviation (n=3). *P<0.05, **P<0.01, and ***P<0.001. NS, not significant; ADM, adriamycin; HK, hexokinase; PFK, phosphofructokinase; PK, pyruvate kinase; LDHA, lactate dehydrogenase A.

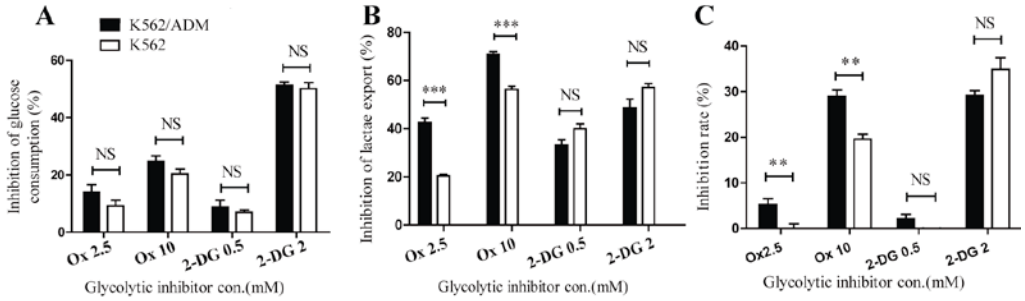

Figure 2. Effect of glycolytic inhibitor on cell viability and glycolysis in ADM-sensitive and -resistant leukaemia cells. Inhibition of (A) glucose consumption, (B) lactate export and (C) cell viability following treatment with 2.5 or 10 mM Ox, or 0.5 or 2 mM 2-DG for 48 h. All the values in non-treated cells were set at 100%. Data are presented as the mean ± standard deviation (n=3). **P<0.01, ***P<0.001. NS, not significant; ADM, adriamycin; Ox, oxamate; 2-DG, 2-deoxyglucose.

of glycolysis catalysed by HK. Following treatment with 2-DG, glycolytic flux was additionally negatively affected in both cell lines. Notably, glucose consumption (Fig. 2A) and lactate export inhibition (Fig. 2B) were similar between K562 and K562/ADM cells, and no significant differences were observed in cytotoxicity levels between the two cell lines (Fig. 2C), in contrast with the results with Ox treatment. The inhibition of 2-DG 0.5 mM to K562 cells was undetectable in the present study. These data provided further evidence to confirm the important role of lactic acid accumulation in drug-resistant leukaemia cells.

Inhibition of glycolysis effectively restores the sensitivity of K562/ADM cells to ADM treatment. The above observations suggested a novel strategy for effectively killing cancer cells and overcoming drug resistance. One approach is to inhibit glycolysis and thus decrease lactic acid production during chemotherapy. The cytotoxicity of ADM was markedly increased following treatment with Ox in K562 (Fig. 3A) and K562/ADM (Fig. 3B) cells, and 2-DG in K562 (Fig. 3C) and K562/ADM (Fig. 3D) cells. In K562/ADM cells, this inhibition efficacy was significant when Ox and 2-DG concentrations were >5 mM or >0.25 mM, respectively. In

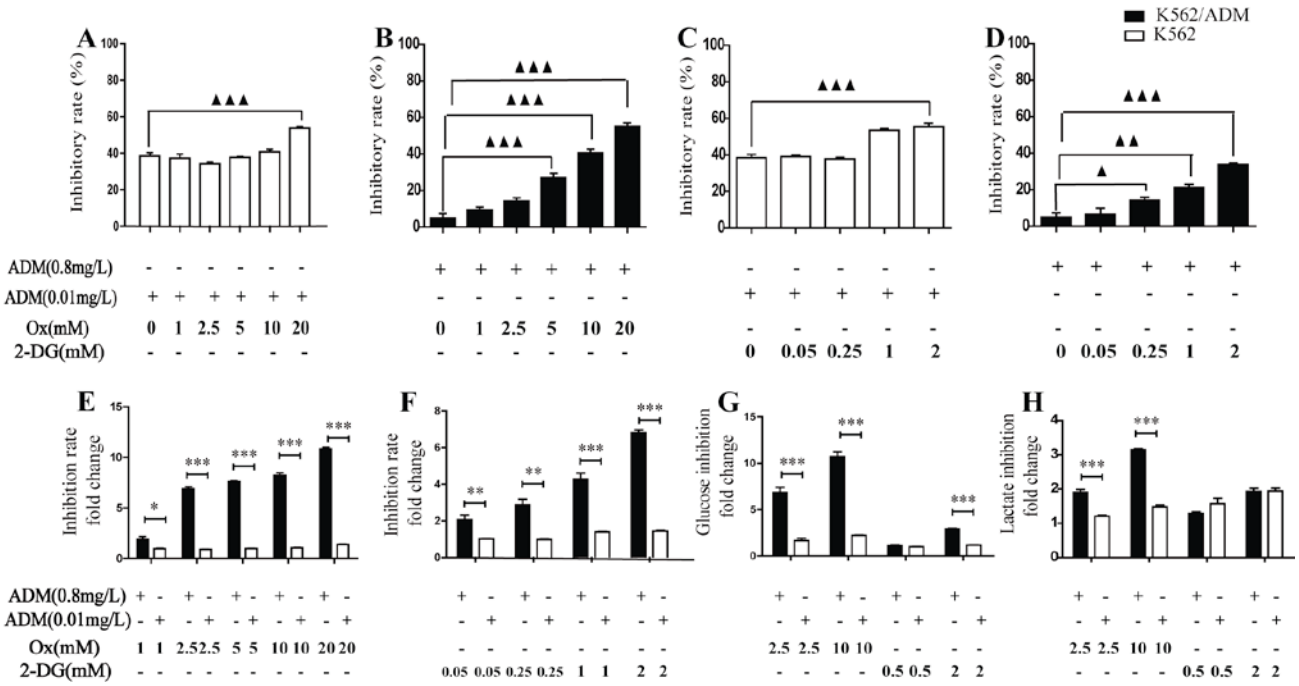

Figure 3. Cell viability and glycolysis effect of the combination of ADM treatment and glycolysis inhibitors in ADM-sensitive and -resistant leukaemia cells. Concentrations of ADM for -resistant and -sensitive cell lines varied depending on cellular toxicity. Inhibition of cell viability following Ox treatment in (A) K562 and (B) K562/ADM cells. Inhibition of cell viability following 2-DG treatment in (C) K562 and (D) K562/ADM cells. ▲P<0.05, ▲▲P<0.01 and ▲▲▲P<0.001. Fold increase in cell inhibitory rate following treatment with ADM plus (E) Ox or (F) 2-DG. Fold increase in (G) glucose consumption and (H) lactate export inhibition. Increased folds were value compared with ADM alone. *P<0.05, **P<0.01 and ***P<0.001. Cell indices in non-treated cells were set at 100%. Data are presented as the mean ± standard deviation (n=3). ADM, adriamycin; Ox, oxamate; 2-DG, 2-deoxyglucose.

K562 cells, significance was observed at 20 mM or 2 mM for Ox and 2-DG, respectively. Following treatment with Ox (Fig. 3E) and 2-DG (Fig. 3F), increased inhibition rates were observed in K562/ADM cells compared with K562 cells. These data indicated that while drug-resistant K562/ADM cells were less sensitive to chemotherapy compared with K562 cells, they demonstrated increased chemotherapeutic efficacy when combined with increasing concentrations of a glycolytic inhibitor. To further confirm the glycolysis inhibition effect and understand alterations in energy metabolism following combination treatment, glucose consumption and lactate production as glycolysis biochemical indicators were measured in the two cell lines. Following treatment with ADM plus the glycolysis inhibitor for 48 h, glucose consumption and lactate production were revealed to be decreased in the two cell types, compared with ADM treatment alone. In accordance with the varying effect on cell viability of combination treatment of K562 and K562/ADM cells, drug-resistant cells exhibited increased glycolysis inhibition efficacy compared with drug-sensitive cells (Fig. 3G and H). Following treatment with 10 mM Ox or 2 mM 2-DG, the glucose consumption was decreased by 10.71±1.01 (Ox) and 2.91±0.13 (2-DG) fold change for K562/ADM cells, and 2.23±0.04 (Ox) and 1.21±0.05 (2-DG) fold change for K562 cells; the lactate production was decreased by 3.15±0.06 (Ox) and 1.93±0.18 (2-DG) fold change for K562/ADM cells, 1.48±0.09 (Ox) and 1.94±0.17 (2-DG) fold change for K562 cells, respectively, relative to ADM treated alone.

These results demonstrated that inhibition of glycolysis caused glucose consumption and lactate production to decrease, leading to increased sensitivity to chemotherapy in leukaemia cells. Notably, the results additionally revealed that inhibition of glycolysis may condition K562/ADM cells to respond more efficiently to chemotherapy compared with K562 cells.

AKT-mTOR pathway over-activation and increased glycolysis are observed in K562/ADM cells. Previous studies indicated that certain factors are associated with the metabolic dysregulation of cancer cells, including c-Myc, the phosphatidylinositol-4,5-bisphosphate/AKT-mTOR signalling pathway, HIF-1α, AMP-activated protein kinase and p53 (5,13-17). The AKT/mTOR signalling pathway appears to be a crucial controller of metabolic homeostasis via regulation of the expression or translocation of metabolic genes associated with glycometabolism, including LDHA, HK-II and GLUTs (18). As dysfunction in these genes has previously been associated with an enhanced Warburg effect in K562/ADM cells, the present study compared the expression levels of AKT, p-AKT (Fig. 4A), mTOR, p-mTOR (Fig. 4B), HIF-1α, and c-Myc (Fig. 4C) in the K562/ADM and K562 cell lines. AKT, p-AKT (Thr308), mTOR, p-mTOR (Ser2448), and c-Myc protein expression levels were upregulated in K562/ADM cells compared with parental controls, whereas no significant differences were observed in HIF-1α mRNA (Fig. 4D) and p-AKT (Ser473) protein expression levels between the two cell types. Previous research has indicated that the AKT-mTOR-c-Myc signalling pathway has multiple roles in stimulating glucose consumption and metabolism by regulating GLUTs, HK-II and LDHA (19). Therefore, the present study investigated increased glycolytic activity in K562/ADM cells, which may be in part caused

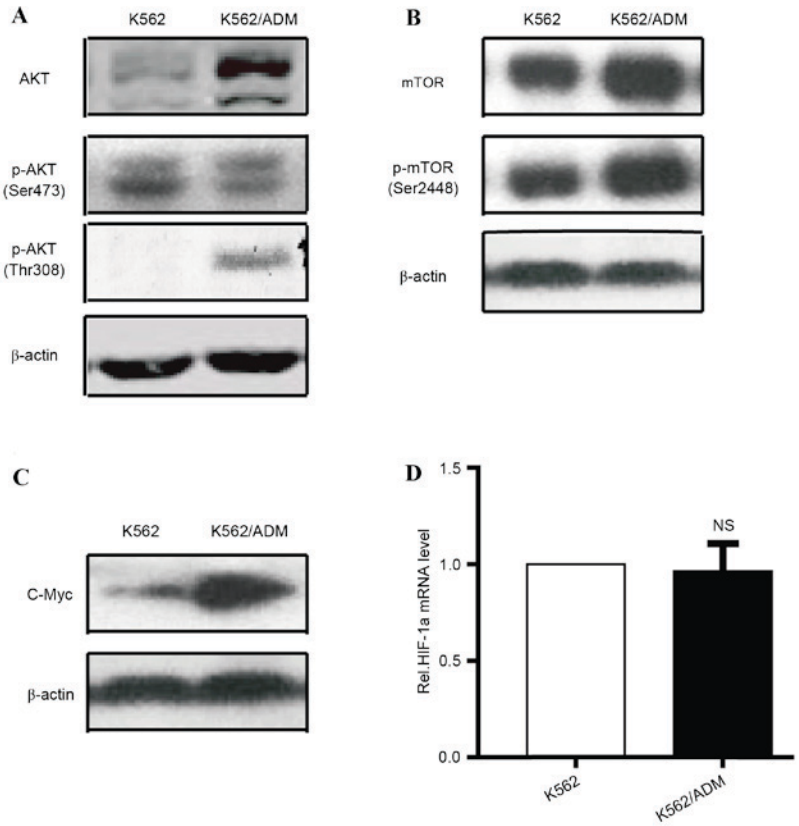

Figure 4. AKT-mTOR-c-Myc signalling pathway activation is increased in multidrug-resistant leukaemia cells. Representative western blot images of protein expression levels of (A) AKT and p-AKT, (B) mTOR and p-mTOR and (C) c-Myc. (D) Quantification of HIF-1α relative mRNA expression levels. Data are presented as the mean ± standard deviation. AKT, AKT serine/threonine kinase; p, phosphorylated; mTOR, mechanistic target of rapamycin; HIF-1α, hypoxia-inducible factor-1α; NS, not significant.

by increased key enzyme activity and/or overexpression, which was primarily attributable to over-activation of the AKT-mTOR-c-Myc signalling pathway.

GLUT4 is overexpressed in K562/ADM cells. AKT has previously been reported to be an important mediator of the glucose consumption signalling pathway via its effect on GLUT translocation and activation. Notably, as a substrate of the AKT-mTOR-c-Myc signalling pathway, GLUT4 was determined to have increased protein ($P<0.001$; Fig. 5A and B) and mRNA ($P<0.001$; Fig. 5B) expression levels in K562/ADM cells compared with K562 cells, further confirming the over-activity of the AKT-mTOR-c-Myc signalling pathway in ADM-resistant cells. Overexpression of GLUT4 may to contribute to increased glucose consumption, resulting in aerobic glycolysis.

Discussion

A previous study demonstrated that tumour cells have substantially different glycometabolism compared with healthy cells or tissues (7), and have increased dependency on the glycolytic pathway, rather than mitochondrial oxidative phosphorylation (5). Compared with healthy cells, cancer cells are characterised by increased glucose intake and lactic acid production, and cancer types with increased glycolysis levels are frequently insensitive to chemo- and radiotherapy. It is generally recognised that mitochondrial metabolic defects, aberrant expression levels and/or activity of glycolytic enzymes, and a hypoxic microenvironment are primary factors that contribute to the Warburg effect.

How glycolytic metabolism affects drug resistance in cancer and leukaemia cells remains to be fully elucidated. The present study used K562 and K562/ADM leukaemia cell lines to investigate the effects of glycolytic metabolism on MDR of leukaemia cells in normoxic conditions. Increased glucose consumption and lactate export suggested that in K562/ADM cells, the ADM-resistant MDR cell line exhibited an enhanced anaerobic metabolic phenotype compared with ADM-sensitive K562 cells. Therefore, the present study focused on the expression levels and activity of glycolytic enzymes or GLUTs. The detection of key glycolytic proteins indicated that K562/ADM cells exhibited increased HK-II and GLUT4 expression levels, and increased LDH, HK and PK activities, compared with ADM-sensitive cells. These data suggested that MDR required greater glycolytic metabolic adaptation, which may regulate the MDR phenotype of chemoresistant leukaemia cells.

The cells, which were heavily dependent on glycolysis in a normoxic environment, were potentially sensitive to glycolytic inhibition. Therefore, 2-DG (a HK inhibitor) and Ox (an LDHA inhibitor) were used as pharmacological tools to inhibit glycolysis by blocking the first and the last step of glycolysis, respectively. The present study demonstrated that Ox and 2-DG treatment reduced glucose consumption and lactate export in the two cell lines, and although glucose consumption inhibition did not differ between the two cell types, lactate export

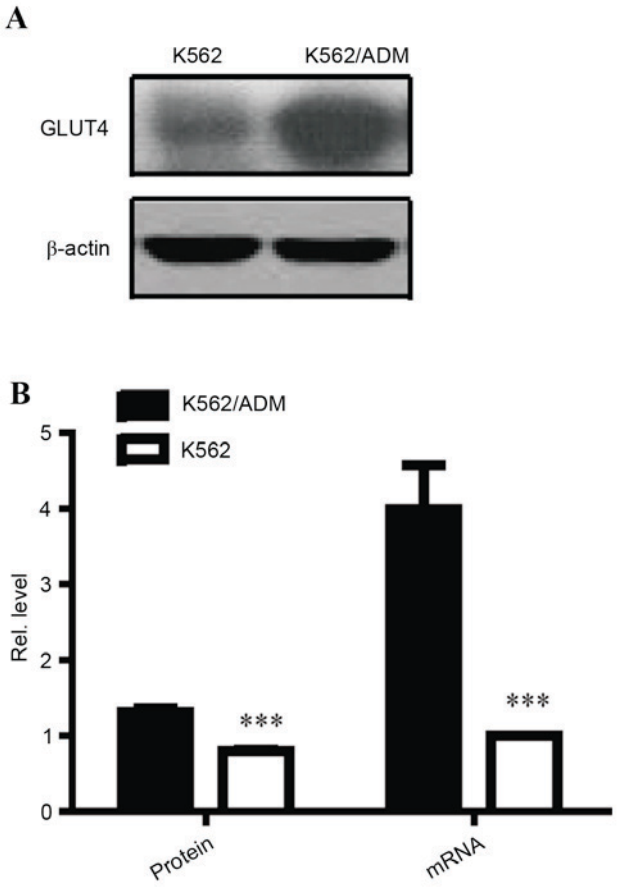

Figure 5. GLUT4 is overexpressed in K562/ADM cells. (A) Representative western blot images of GLUT4 protein expression levels. (B) Quantification of GLUT4 relative protein and mRNA expression levels. ***P<0.001 vs. K562/ADM. ADM, adriamycin; GLUT4, glucose transporter type 4.

inhibition was increased in K562/ADM cells treated with Ox, in contrast with the results of inhibition by 2-DG. Notably, Ox and 2-DG treatment inhibited proliferation in the two cell types, and inhibition of aerobic glycolysis and of LDHA activity by Ox were more effective in killing K562/ADM cells, which cells showed an MDR phenotype, whereas no significant inhibition of 2-DG was observed between the two cell lines (Fig. 2C). One logical conclusion is that inhibition of LDHA prevented the Warburg effect, and increased growth inhibition in K562/ADM cells was caused by increased inhibition of lactate production, which contributed to increased K562/ADM cell sensitivity to Ox. Therefore, the present study hypothesised that LDH has an important role in leukaemia drug resistance, and K562/ADM exhibited 'lactate addiction'. Further experiments demonstrated that glycolytic inhibition improved the therapeutic effect of ADM in the two cell lines and re-sensitised ADM-resistant cells, with Ox exhibiting stronger effects on chemotherapy than 2-DG. It is understood that ATP-binding cassette transporters of resistant cells require ATP as the energy source to pump anticancer drugs out of the cells to avoid their lethal effects (20). Our previous study revealed that drug-resistant K562/ADM cells overexpressed P-gp compared with K562 cells, in a manner closely associated with their drug-resistance (11,21). The results of the present study suggested that the quick ATP supply in K562/ADM cells may activate P-gp and maintain the drug efflux via glycolysis and depletion of ATP by glycolysis inhibition blocked pump function of P-gp. Furthermore, increased aerobic glycolysis caused an increase in lactate production, resulting in acidification of the intracellular microenvironment, which may additionally decrease drug absorption and efficiency by upregulating H^+-linked ATPases and transporters (7,22). In addition, enhanced glycolysis has been demonstrated to produce numerous intermediate metabolites, including nucleotides, lipids and proteins, which support the synthesis of macromolecules required for cell proliferation (23). Therefore, metabolic alterations may confer adaptive, proliferative, survival and drug-resistant advantages to K562/ADM cells.

It is not established why MDR cells favour glycolysis in normoxic conditions, or how glycolysis regulates MDR in resistant leukaemia cells. Previous studies have indicated that the AKT/mTOR signalling pathway is associated with a wide array of cellular processes, including cell proliferation, metabolism, cell cycle regulation and drug resistance (5,13,15,24). Recent findings have demonstrated that AKT may increase transcription of c-Myc and reduce degradation of c-Myc indirectly (25). Notably, mTOR regulates c-Myc-driven carcinogenesis (26,27), and c-Myc, a versatile transcription factor, directly triggers transcription of genes encoding glycolytic enzymes, including HK-II, LDHA, and GLUTs (28). The present study demonstrated significant increases in AKT, mTOR and c-Myc expression levels in K562/ADM cells compared with treatment-sensitive K562 cells, accompanied by enhancement of p-AKT (Thr308) and p-mTOR (Ser2448). Thr308 and Ser473 are the two critical phosphorylation sites of AKT; increased phosphorylation of Thr308 may acutely increase the enzymatic activity of AKT. These results suggested that the AKT/mTOR/c-Myc signalling pathway is involved in maintenance of the glycolysis-mediated MDR phenotype of drug-resistant leukaemia cells. It is possible that increased expression levels and enhanced phosphorylation of AKT led to over-activation of mTOR, which increased the transcription of c-Myc, sequentially facilitating the expression levels and activity of LDHA and HK-II to accelerate aerobic glycolysis activity in K562/ADM cells. In addition, significantly increased GLUT4 expression levels further confirmed the over-activation of the AKT/mTOR signalling pathway in K562/ADM cells, which may reinforce the ability of MDR cells to rapidly transport and consume glucose by glycolysis to generate ATP.

Numerous previous studies have suggested that hypoxia is an important factor contributing to the Warburg effect in cancer cells. Hypoxia activates HIF-1α, which regulates the transcription of a variety of glycolysis-associated target genes against hypoxia-induced injury. In hypoxic conditions, HIF-1α primarily regulates genes involved in glycolysis, whereas c-Myc regulates the same genes in normoxic conditions (29). In the present study, no significant differences were observed in HIF-1α expression levels between K562 and K562/ADM cells in normoxia; however, K562/ADM cells expressed significantly increased levels of c-Myc compared with K562 cells. Therefore, in normoxic conditions, adaptive glucose metabolic alterations in drug-resistant cells were potentially mediated by c-Myc rather than HIF-1α, and increased glycolysis in MDR leukaemia may not be due to the intracellular hypoxia. This requires clarification in future studies. According to these findings, it is assumed that during the process of acquiring

drug resistance, inherently increased glycolytic leukaemia cell populations are selected, or induced, due to proliferation or survival advantages, and may ultimately be responsible for MDR.

In conclusion, the results of the present study indicated that leukaemia MDR cells exhibit enhanced aerobic glycolytic activity in normoxic conditions, and the inhibition of glycolysis is more damaging to resistant leukaemia cells and potently reverses the resistance of MDR cells to anticancer agents. Increased glycolysis in MDR cells is potentially mediated by activation of the AKT-mTOR/c-Myc pathway. Glycolytic inhibition leading to depletion of ATP and acidification of the microenvironment, causing blockade of the ATP-dependent drug-efflux functions of P-gp, may be a potential strategy to reverse MDR. The present study indicated that a combination of glycolysis inhibitors represents a potential chemotherapeutic strategy to overcome MDR in relapsed/refractory leukaemia or cancer.

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