Shikonin, vitamin K_3 and vitamin K_5 inhibit multiple glycolytic enzymes in MCF-7 cells

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Abstract. Glycolysis is the most important source of energy for the production of anabolic building blocks in cancer cells. Therefore, glycolytic enzymes are regarded as potential targets for cancer treatment. Previously, naphthaquinones, including shikonin, vitamin K_3 and vitamin K_5, have been proven to decrease the rate of glycolysis in cancer cells, which is partly due to suppressed pyruvate kinase activity. In the present study, enzymatic assays were performed using MCF-7 cell lysate in order to screen the profile of glycolytic enzymes in cancer cells inhibited by shikonin, vitamin K_3 and vitamin K_5, in addition to pyruvate kinase. Results revealed that hexokinase, phosphofructokinase-1, fructose bisphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase produced in the process of glycolysis were inhibited by shikonin, vitamin K_3 and vitamin K_5. The results indicated that shikonin, vitamin K_3 and vitamin K_5 are chemical inhibitors of glycolytic enzymes in cancer cells and have potential uses in translational medical applications.

Introduction

An increased rate of aerobic glycolysis followed by lactate fermentation, named the Warburg Effect, is a hallmark of cancer cells, and it assists cancer cells to survive despite an insufficient oxygen supply in the tumor mass due to a disordered blood vessel system (1). Glycolysis does not produce extensive energy and building blocks alone in order to support the survival and proliferation of cancer cells. However, intermediates of glycolysis involved in certain biochemical processes provide the nutrients for the rapid proliferation of cancer cells. For example, glucose-6-phosphate participates in the pentose phosphate pathway to generate NADPH, an important antioxidant defense product, in addition to ribose-5-phosphate and erythrose 4-phosphate to assist with the synthesis of fatty acids, nucleotides and aromatic amino acids in cells (2,3). Furthermore, glucose-6-phosphate supports the generation of glycogen (2). Dihydroxyacetone phosphate participates in triacylglyceride and phospholipid synthesis (3). Pyruvate participating in the citric acid cycle is responsible for the production of ATP, isoprenoids, cholesterol and fatty acids (2). As the end product of glycolysis, lactate produces an acidic intercellular microenvironment surrounding a tumor, which favors tumor invasion (4).

Previous studies have demonstrated that suppressing the expression and activity of glycolytic enzymes is efficient in reducing the glycolytic rate and inhibiting the development of cancer (1-3). Hypoxia-inducible factor (HIF), a transcriptional complex overexpressed and activated in cancer cells, induces the expression of glycolytic enzymes (5). Inhibiting HIF expression and activity reduced aerobic glycolysis in cancer cells, which may contribute to the inhibition of survival and growth of cancer cells (5). A number of HIF inhibitors have been under clinical and preclinical development for use in cancer treatment (6). In addition to the indirect inhibition of glycolysis by targeting HIF, direct inhibition of glycolysis was developed as novel anticancer treatment (1,2). RNA interference and chemical inhibitors of glucose and lactate transporters reduced the glycolysis rate by inducing the decrease of glycolysis precursors and the increase of glycolysis products (1,2,7,8). Reducing the expression of glycolytic enzymes using small interfering RNA and targeting associated microRNAs additionally downregulated the glycolytic pathway and suppressed the growth of various cancer types (7,8). Chemical inhibitors targeting glycolytic enzymes have been developed with promising translational applications in treating cancer (2). A number of these inhibitors which exhibit a potent efficiency in suppressing

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cancer have been used in clinical and pre-clinical trials, including hexokinase inhibitors 2-deoxyglucose, 3-bromopyruvate and lonidamine, phosphofructose kinase inhibitor 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one and lactate dehydrogenase (LDH) inhibitor FX11 (2).

Previously, it has been revealed that naphthaquinones including shikonin, vitamin K_3 and vitamin K_5 are efficient inhibitors of glycolysis (9,10). Inhibition of the rate of glycolysis is toxic to cancer cells (11). Pyruvate kinase M2 (PKM2) was demonstrated to be a target responsible for the inhibited glycolytic rate in previous studies (9-12). Replacing PKM2 with PKM1 partially reduced the death of cancer cells induced by naphthaquinones with increased pyruvate kinase activity (10). As the inhibition of glycolysis by naphthaquinones was only partially due to the suppressed activity of PKM2, it was hypothesized in the present study that shikonin, vitamin K_3 and vitamin K_5 may be able to interrupt the activity of other glycolytic enzymes in addition to PKM2. In the present study, the enzyme profile inhibited by shikonin, vitamin K_3 and vitamin K_5 in the pathway from glucose to lactate was screened in order to determine whether there were other targets involved in the inhibition of glycolysis in cancer cells aside from PKM2.

Materials and methods

Reagents. RPMI 1640, fetal calf serum, penicillin-streptomycin, trypsin and HEPES were purchased from Gibco, Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Mammalian protein extraction reagent containing protease inhibitor cocktail kit and the BCA protein assay kit were from Thermo Fisher Scientific, Inc. Shikonin was purchased from the Tokyo Chemistry Industry Co., Ltd. (Tokyo, Japan). Vitamin K_3 was purchased from Sigma-Aldrich, Merck KGaA (Darmstadt, Germany). Vitamin K_5 was purchased from Wako Pure Chemical Industries, Ltd. (Okasa, Japan). Triphosphopyridine nucleotide ($NADP^+$), nicotinamide adenine dinucleotide (NDA^+), nicotinamide adenine dinucleotide, reduced form (NADH), adenosine diphosphate (ADP) and phosphoenolpyruvic acid were purchased from Roche Diagnostics (Basel, Switzerland). Glucose, fructose-6-phosphate, glyceraldehyde-3-phosphate, 3-phosphoglycerate, 2-phosphoglycerate, adenosine monophosphate (AMP), adenosine triphosphate (ATP), dimethylsulfoxide (DMSO), fructose 1,6-bisphosphate, pyruvate, glucose-6-phosphate dehydrogenase, triose phosphate isomerase (TPI), α-glycerophosphate dehydrogenase, aldolase, glyceraldehyde phosphate dehydrogenase, enolase and LDH were from Sigma-Aldrich Merck KGaA. All enzymes used in the enzyme-coupled assays were quantified with their activity (unit). One unit enzyme converts 1 mol substrates into products in 1 min at the temperature and PH as indicated in the following protocols.

Cell cultures. Breast cancer cell line MCF-7 was purchased from the Type Culture Collection of the Chinese Academy of Sciences Cell Bank (Shanghai, China), and were maintained in RPMI 1640 containing 10% fetal calf serum and 100 U/ml penicillin-streptomycin. Cells were grown in a humidified CO_2 incubator at 37˚C, and subcultured with 0.25% trypsin containing 0.02% EDTA, as previously described (10).

Preparation of cell extract. MCF-7 cells were trypsinized and collected, and then were lysed using a mammalian protein extraction reagent containing protease inhibitor cocktail. Following centrifugation at 17,000 x g, 4˚C for 15 min, and the protein content in the supernatant was determined using a BCA protein assay kit according to the manufacturer's protocol.

Measurement of intracellular accumulation of shikonin. A total of $1x10^6$ MCF-7 cells were plated in each well of a 6-well plate. Subsequent to attachment, the cells were incubated with 1, 5, 10 and 25 μM shikonin or 1/1,000 (v/v) DMSO at 37˚C for 0, 1, 3 or 4 h. Cells were washed rapidly using ice-cold PBS three times, and intracellular shikonin was extracted using 500 μl ethanol. Cellular debris was spun at 17,000 x g at 4˚C for 5 min. The supernatant was then collected for high performance liquid chromatography (HPLC).

The HPLC experiment was conducted using a Series 1100 from Hewlett Packard (Palo Alto, CA, USA). An ODS Hypersil C18 column (5 μm, 125x4 mm; Hewlett Packard) was used at 30˚C. Solvent A [H_2O and methanol (95:5, v/v) with 0.1% trifluoroacetic acid], solvent B [H_2O and methanol (5:95, v/v) with 0.1% trifluoroacetic acid] were used, and 0.1 ml sample was loaded. The quantitative determination of shikonin was achieved by a gradient elution starting from a 50 to 100% solvent B in 15 min with a flow rate of 1 ml/min, detected at wavelength 515 nm, at 30˚C.

The intracellular concentration of shikonin was calculated based on the value of the measured shikonin content in one million cells, and converted to a micromolar value. The average cell volume is $6.024x10^{-6}$ μl, which is calculated on the basis of the diameter (measured microscopically) of >500 cells.

Hexokinase activity assay. Hexokinase activity of the cell extract was measured using a glucose-6-phosphate dehydrogenase coupled assay. A total of 200 ng/μl cell extract were incubated with naphthaquinones (shikonin, vitamin K_3 and vitamin K_5) at concentrations of 5, 10, 20, 25, 40 or100 μM for 1 h at 25˚C. The reaction solution contained 50 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 0.1 M glucose, 0.5 mM ATP, 0.2 mM $NADP^+$ and 1 unit glucose-6-phosphate dehydrogenase per ml reaction solution. Relative hexokinase activity was calculated at 25˚C by comparing the changes of absorbance at 340 nm between the vehicle controls (200 ng/μl cell extract treated with 0.1% (v/v) DMSO) and naphthaquinone incubated groups once cell extract added to the reaction solution, as previously described (13,14).

Phosphoglucose isomerase activity assay. Phosphoglucose isomerase activity of the cell extract was measured using a glucose-6-phosphate dehydrogenase coupled assay. A total of 200 ng/μl cell extract was incubated with 1 mM naphthaquinone (shikonin, vitamin K_3 and vitamin K_5) for 1 h at 25˚C. The reaction solution contained 50 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 0.2 mM $NADP^+$, 2 mM fructose-6-phosphate and 1 unit glucose-6-phosphate dehydrogenase per ml reaction solution. Relative phosphoglucose isomerase activity was calculated at 25˚C by comparing the change in absorbance at 340 nm between the vehicle controls [200 ng/μl cell extract treated with 1% (v/v) DMSO] and naphthaquinone incubated

groups once the cell extract was added to the reaction solution, as previously described (15).

Phosphofructokinase-1 (PFK-1) activity assay. PFK-1 activity of the cell extract was measured using a three-enzyme-coupled assay. A total of 200 ng/μl cell extract was incubated with naphthaquinones (shikonin, vitamin K_3 and vitamin K_5) with concentrations of 5, 20, 50, 100, 500 or 1,000 μM for 1 h at 25˚C. The reaction solution contained 50 mM HEPES (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, 5 mM Na_2HPO_4, 1 mM NH_4Cl, 0.1 mM AMP, 0.2 mM NADH, 5 mM fructose-6-phosphate, 2 mM ATP, 5 units TPI per ml reaction solution, 1 unit adolase per ml reaction solution and 1 unit glycerol-3-phosphate dehydrogenate per ml reaction solution. Relative PFK-1 activity was calculated at 25˚C by comparing the change in absorbance at 340 nm between vehicle controls [200 ng/μl cell extract treated with 1% (v/v) DMSO] and naphthaquinone incubated groups once the cell extract was added to the reaction solution, as previously described (13,16,17).

Fructose bisphosphate aldolase activity assay. Fructose bisphosphate aldolase activity of the cell extract was measured using a two-enzyme-coupled assay. A total of 200 ng/μl cell extract was incubated with naphthaquinones (shikonin, vitamin K_3 and vitamin K_5) with concentrations of 10, 100, 200, 500 or 1,000 μM for 1 h at 25˚C. The reaction solution contained 50 mM HEPES (pH 7.5), 1 mM fructose-1,6-biphosphate, 0.2 mM NADH, 1 unit glycerol-3-phosphate dehydrogenate per ml reaction solution and 5 units TPI per ml reaction solution. Relative fructose bisphosphate aldolase activity was calculated at 25˚C by comparing the change in absorbance at 340 nm between vehicle controls [200 ng/μl cell extract treated with 1% (v/v) DMSO] and naphthaquinone incubated groups once the cell extract was added to the reaction solution, as previously described (18).

TPI activity assay. TPI activity of the cell extract was measured using a glycerol-3-phosphate dehydrogenate coupled assay. A total of 200 ng/μl cell extract was incubated with naphthaquinone (shikonin, vitamin K_3 and vitamin K_5) with concentrations of 1 mM for 1 h at 25˚C. The reaction solution contained 50 mM HEPES (pH 7.5), 0.5 mM EDTA, 0.2 mM NADH, 1 mM glyceraldehyde-3-phosphate and 1 unit glycerol-3-phosphate dehydrogenate per ml reaction solution. Relative TPI activity was calculated at 25˚C by comparing the change in absorbance at 340 nm between vehicle controls [200 ng/μl cell extract treated with 1% (v/v) DMSO] and naphthaquinone incubated groups once the cell extract was added to the reaction solution, as previously described (19).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity assay. GAPDH activity of the cell extract was measured using a one-step assay from D-glyceraldehyde-3-phosphate to D-1,3-biphosphateglycerate. A total of 200 ng/μl cell extract was incubated with naphthaquinones (shikonin, vitamin K_3 and vitamin K_5) with concentrations of 10, 100, 200, 500 or 1,000 μM for 1 h at 25˚C. The reaction solution contained 50 mM HEPES (pH 7.5), 0.2 mM EDTA, 1 mM NAD^+, 50 mM Na_2HPO_4 and 1 mM glyceraldehyde-3-phosphate. Relative glyceraldehydes-3-phosphate dehydrogenase activity was calculated at 25˚C by comparing the change in absorbance at 340 nm between vehicle controls [200 ng/μl cell extract treated with 1% (v/v) DMSO] and naphthaquinone incubated groups once the cell extract was added to the reaction solution, as previously described (20).

Phosphoglycerate kinase (PGK) activity assay. PGK activity of the cell extract was measured using a GAPDH coupled assay. Total of 200 ng/μl cell extract was incubated using 1 mM naphthaquinone (shikonin, vitamin K_3 and vitamin K_5) for 1 h at 25˚C. The reaction solution contained 50 mM HEPES (pH 7.5), 6 mM $MgSO_4$, 0.2 mM EDTA, 2 mM ATP, 0.2 mM NADH, 2 mM 3-phosphoglycerate and 2 units glyceraldehydes-3-phosphate dehydrogenase per ml reaction solution. Relative PGK activity was calculated at 25˚C by comparing the change in absorbance at 340 nm between vehicle controls [200 ng/μl cell extract treated with 1% (v/v) DMSO] and naphthaquinone incubated groups once the cell extract was added to the reaction solution, as previously described (21).

Phosphoglycerate mutase (PGAM) activity assay. PGAM activity of the cell extract was measured using an enolase coupled assay. A total of 200 ng/μl cell extract was incubated with 1 mM naphthaquinones (shikonin, vitamin K_3 and vitamin K_5) for 1 h at 25˚C. The reaction solution contained 50 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 2 mM 3-phosphoglycerate and 0.2 unit enolase per ml reaction solution. Relative PGAM activity was calculated at 25˚C by comparing the change in absorbance at 240 nm between vehicle controls [200 ng/μl cell extract treated with 1% (v/v) DMSO] and naphthaquinone incubated groups once the cell extract was added to the reaction solution, as previously described (22).

Enolase activity assay. Enolase activity of the cell extract was measured using a one step assay from 2-phosphateglycerate to phosphoenolpyruvate. A total of 200 ng/μl cell extract was incubated with 1 mM naphthaquinones (shikonin, vitamin K_3 and vitamin K_5) for 1 h at 25˚C. The reaction solution contained 50 mM HEPES (pH 7.5), 1 mM $MgCl_2$, 50 mM KCl, 1 mM EDTA and 0.5 mM 2-phosphoglycerate. Relative enolase activity was calculated at 25˚C by comparing the change in absorbance at 240 nm between vehicle controls [200 ng/μl cell extract treated with 1% (v/v) DMSO] and naphthaquinone incubated groups once the cell extract was added to the reaction solution, as previously described (23).

Pyruvate kinase activity assay. Pyruvate kinase activity of the cell extract was measured using a LDH coupled assay. A total of 200 ng/μl cell extract was incubated with naphthaquinones (shikonin, vitamin K_3 and vitamin K_5) at concentrations of 5, 10, 20, 50, 100, 150, 200 or 1,000 μM for 1 h at 25˚C. The reaction solution contained 50 mM HEPES (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 0.2 mM NADH, 2 mM ADP, 2 mM phosphoenolpyruvate and 8 units LDH per ml reaction solution. Relative pyruvate kinase activity was calculated at 25˚C by comparing the change in absorbance at 340 nm between vehicle controls [200 ng/μl cell extract treated with 1% (v/v) DMSO] and naphthaquinones incubated groups after the cell extract was added to the reaction solution (9,10).

LDH activity assay. LDH activity of the cell extract was measured using a one step assay from pyruvate to lactate. A total of 200 ng/μl cell extract was incubated with 1 mM naphthaquinones (shikonin, vitamin K_3 and vitamin K_5) for 1 h at 25˚C. The reaction solution contained 50 mM HEPES (pH 7.5), 0.2 mM NADH and 2 mM pyruvate. Relative LDH activity was calculated at 25˚C by comparing the change in absorbance at 340 nm between vehicle controls [200 ng/μl cell extract treated with 1% (v/v) DMSO] and naphthaquinone incubated groups once the cell extract was added to the reaction solution, as previously described (9,10).

Statistical analysis. All data were expressed as the mean ± the standard deviation from at least three independent experiments and analyzed using a paired Student's t-test by Excel 2007 (Microsoft, Redmond, Washington, USA). $P<0.05$ was considered to indicate a statistically significant difference. The half maximal inhibitory concentration (IC_{50}) was analyzed using SigmaPlot 10.0 (Alfasoft, London, UK).

Results

Enzyme inhibition assays using cell lysate may mimic in vivo assays. In one previous study, it was revealed that although the IC_{50} of shikonin for recombinant monomeric and allosteric activated PKM2 were only 0.3 and 0.8 μM, respectively, the IC_{50} of shikonin for PKM2 *in vivo* is ~10 μM (10), ~30 fold higher (10). Thus, it is worth investigating whether intracellular shikonin may accumulate to a similarly high level. By incubating cells with 10 μM shikonin, it was revealed that steady-state shikonin accumulation may be reached ~1 h after incubation, and MCF-7 cells demonstrated a substantial capacity for the uptake of shikonin (Fig. 1A). While incubating the cells for 1 h, shikonin accumulated in the cells in a dose-dependent manner, and the intracellular concentration of shikonin was ~14 times higher compared with the extracellular concentration (Fig. 1B). No further experiments were conducted into why cancer cells are able to accumulate a high concentration of shikonin and why the inhibitory efficiency of shikonin on purified recombinant PKM2 and endogenous PKM2 differed. It may be that shikonin is sequestered by proteins including PKM2 and that the inhibitory effect of shikonin on PKM2 may be disrupted by shikonin binding proteins, PKM2 binding complex and PKM2 allosteric structure in cells (10,24,25). The inhibitory efficiency of shikonin on the activity of pyruvate kinase derived from cell lysate was investigated, and revealed that 12.2 μM shikonin was able to inhibit 50% of the activity of pyruvate kinase (Table I), which is similar to the IC_{50} of shikonin for PKM2 *in vivo*. The inhibitory effect of naphthaquinones on enzymes in cell lysate (Table I) is consistent with that using intracellular enzymes (10), and that cell lysate contains factors to retain allosteric and contextual conditions of enzymes (10,24,25). Thus, in order to study the influence of shikonin, vitamin K_3 and K_5 on the activity of endogenous glycolytic enzymes in the breast cancer cell line MCF-7 and their physiological effect, it is more appropriate to measure enzymatic activity in cell extracts as opposed to purified recombinant enzymes.

Shikonin, vitamin K_3 and vitamin K_5 inhibited hexokinase activity potently. Glucose is phosphorylated to form glucose-6-phosphate by hexokinase intracellularly, which is the first reaction of glycolysis (1-3). In addition to glycolysis, glucose-6-phosphate serves as the precursor of the pentose phosphate pathway, glycogenesis and the hexosamine biosynthetic pathway, which makes hexokinase an important regulator of anabolism and catabolism in cells (3,26,27). A number of classical inhibitors of hexokinase have been used in clinical trials to treat patients with cancer (28,29). In the present study, a glucose-6-phosphate dehydrogenase couple assay was performed in order to test whether the inhibition of glycolysis in MCF-7 cells induced by naphthaquinones was partially caused by the decrease of hexokinase activity. As presented in Fig. 2A-a, the increase of absorbance at 340 nm demonstrated the production of NADPH, and shikonin, vitamin K_3 and K_5 decreased the production of NADPH, compared with the vehicle control, revealing the inhibitory effect of naphthaquinones on the hexokinase activity of the cell extract. Once the inhibition of hexokinase activity using a series of concentrations of naphthaquinones was measured, it was revealed that the inhibition was dose-dependent (Fig. 2A-b), and the IC_{50} of shikonin, vitamin K_3 and K_5 on the hexokinase activity of MCF-7 cells were 9.7, 16.7 and 20.8 μM respectively (Table I), whilst 40 μM shikonin, 100 μM vitamin K_3 and 100 μM vitamin K5 inhibited >80% hexokinase activity (Fig. 2A-a and b). Shikonin was the most potent of the three compounds in inhibiting hexokinase, and vitamin K_3 was a significantly more potent hexokinase inhibitor compared with vitamin K_5, as the inhibition induced by 25 μM vitamin K_3 was significantly stronger compared with 25 μM vitamin K_5 ($P=0.044$; Fig. 2A-b). The results suggested that the three naphthaquinones were potent inhibitors of hexokinase in MCF-7 cells.

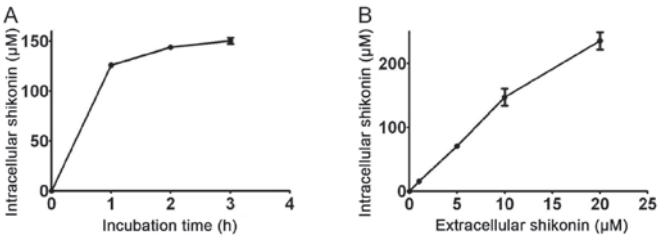

Figure 1. Intracellular accumulation of shikonin in (A) time and (B) extracellular dose dependent manners.

Shikonin, vitamin K_3 and vitamin K_5 inefficiently inhibit phosphoglucose isomerase activity. Phosphoglucose isomerase is a housekeeping enzyme that catalyzes the reversible reaction from glucose-6-phosphate to fructose-6-phosphate and serves an important function in glycolysis and glucogenesis (2). Phosphoglucose isomerase is secreted by tumor cells as a motility factor promoting the migration, invasion and metastasis of cancer, and its overexpression in cancer is associated with a poor prognosis (30,31). Inhibiting the activity of tumor derived phosphoglucose isomerase has potential applications in the treatment of cancer (2). In the present study, the ability of glycolysis inhibitors (shikonin, vitamin K_3 and K_5) to serve as inhibitors of phosphoglucose isomerase was tested. The activity of phosphoglucose isomerase was measured using a glucose-6-phosphate dehydrogenase couple assay. The change of absorbance at 340 nm revealed the reduction of $NADP^+$

Table I. Concentration of naphthaquiones to inhibit 50% of the activity of glycolytic enzymes or the activity of glycolytic enzymes in the presence of 1 mM naphthaqinones.

	IC_{50} (μM)[a]									
Naphthaquiones	Hexokinase	Phosphoglucose isomerase	Phosphofructo kinase-1	Fructose bisphosphate aldolase	Triose phosphate isomerase	Glyceraldehyde-3-phosphate dehydrogenase	Phospho glycerate kinase	Phospho glycerate mutase	Enolase	Pyruvate kinase	Lactate dehydrogenase
Shikonin	9.7±0.1[b]	96.8±5.4%, 1 mM[e]	17.2±0.7[b]	158.4±2.3[b]	n.d.[d]	1052.0±10.7[b]	89.5±3.7%, 1 mM[e]	n.d.[d]	n.d.[d]	12.2±0.1[b]	n.d.[d]
Vitamin K_3	16.7±0.2[b]	90.4±0.8%, 1 mM[e]	1,192.4±4.9[b]	n.d.[d]	n.d.[d]	82.1±0.6%, 1 mM[e]	71.6±2.3%, 1 mM[e]	n.d.[d]	n.d.[d]	70.5±5.4%, 1 mM[e]	n.d.[d]
Vitamin K_5	20.8±0.1[b]	86.6±2.6%, 1 mM[e]	35.9±0.5[b]	n.d.[d]	n.d.[d]	462.0±1.8[b]	63.3±0.8%, 1 mM[e]	n.d.[d]	n.d.[d]	73.6±1.4%, 1 mM[e]	n.d.[d]

[a]All values (mean ± standard deviation) were from three independent experiments; [b]Concentration of naphthaquiones to inhibit 50% of the activity of glycolytic enzymes; [c]The activity of glycolytic enzymes in the presence of 1 mM naphthaqinones, as the inhibition of enzyme activity was not potent enough to analyze the IC_{50}; [d]Not determined. Naphthaquinones did not inhibit enzymatic activity.

demonstrating the activity of phosphoglucose isomerase (Fig. 2B). While incubating MCF-7 cell lysate with shikonin, vitamin K_3 and vitamin K_5 with a concentration of 1 mM, the highest concentration shikonin, vitamin K_3 and vitamin K_5 could reach in the incubation solution, phosphoglucose isomerase activity decreased very little compared with DMSO treated cell lysate (Fig. 2B). Additionally, 4.2, 9.6 and 13.4% of phosphoglucose isomerase activity was inhibited by shikonin, vitamin K_3 and K_5, respectively (Table I). These results demonstrate that shikonin, vitamin K_3 and K_5 are not efficient inhibitors of phosphoglucose isomerase.

Shikonin, vitamin K_3 and vitamin K_5 inhibited PFK-1 activity efficiently. Another rate-limiting enzyme, PFK-1 phosphorylates fructose-6-phosphate into fructose-1,6-phosphate (1,3). Downregulation of PFK-1 may reduce the rate of aerobic glycolysis, biosynthesis, viability and anchorage-independent growth of cancer cells (32,33), suggesting that PFK-1 serves an important role in the metabolic reprogramming of cancer cells, and targeting PFK-1 activity has potential for uses in the development of novel cancer therapies. In order to study whether shikonin, vitamin K_3 and vitamin K_5 exert a negative effect on PFK-1 activity, a fructose bisphosphate aldolase, TPI and glycerol-3-phosphate dehydrogenate coupled assay was performed. PFK-1 activity was observed by the reduction of absorbance at 340 nm revealing the oxidation of NADH (Fig. 2C-a). The result revealed that shikonin, vitamin K_3 and K_5 reduced the rate of the reaction dose-dependently (Fig. 2C-b), with an IC_{50} of 17.2, 1192.4 and 35.9 μM respectively (Table I), indicating that shikonin may inhibit PFK-1 more potently compared with the other two naphthaquinones. Additionally, vitamin K_5 exhibited significantly more potent inhibition compared with vitamin K_3 at concentrations of 50, 500 and 1,000 μM (P=0.0093, P=0.0041 and P=0.021, respectively).

Shikonin is an efficient inhibitor of fructose bisphosphate aldolase. Fructose bisphosphate aldolase catalyzes the reversible conversion of fructose-1,6-bisphosphate to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate (2). In addition to its important role in glycolysis, fructose bisphophate aldolase is involved in other biological processes including signal transduction, vesicle trafficking and cell motility (34,35). Thus, altering the activity of fructose bisphophate aldolase in cancer cells has potential uses in translational medical studies. In the present study, TPI and glycerol-3-phosphate dehydrogenase coupled assays were used to measure the effect of shikonin, vitamin K_3 and K_5 on the activity of MCF-7 cell derived fructose bisphophate aldolase. Fructose bisphophate aldolase activity was revealed by the reduction of absorbance at 340 nm representing the oxidation of NADH (Fig. 2D-a). Results revealed that, compared with the vehicle control, shikonin inhibited the fructose bisphophate aldolase activity efficiently and dose-dependently with an IC_{50} of 158.4 μM, however vitamin K_3 and K_5 did not reduce the activity of fructose bisphophate aldolase even at their highest concentration (1 mM) in the incubation system (Fig. 2D-b; Table I).

Shikonin, vitamin K_3 and vitamin K_5 did not inhibit TPI activity. TPI is a homodimeric enzyme that catalyzes the

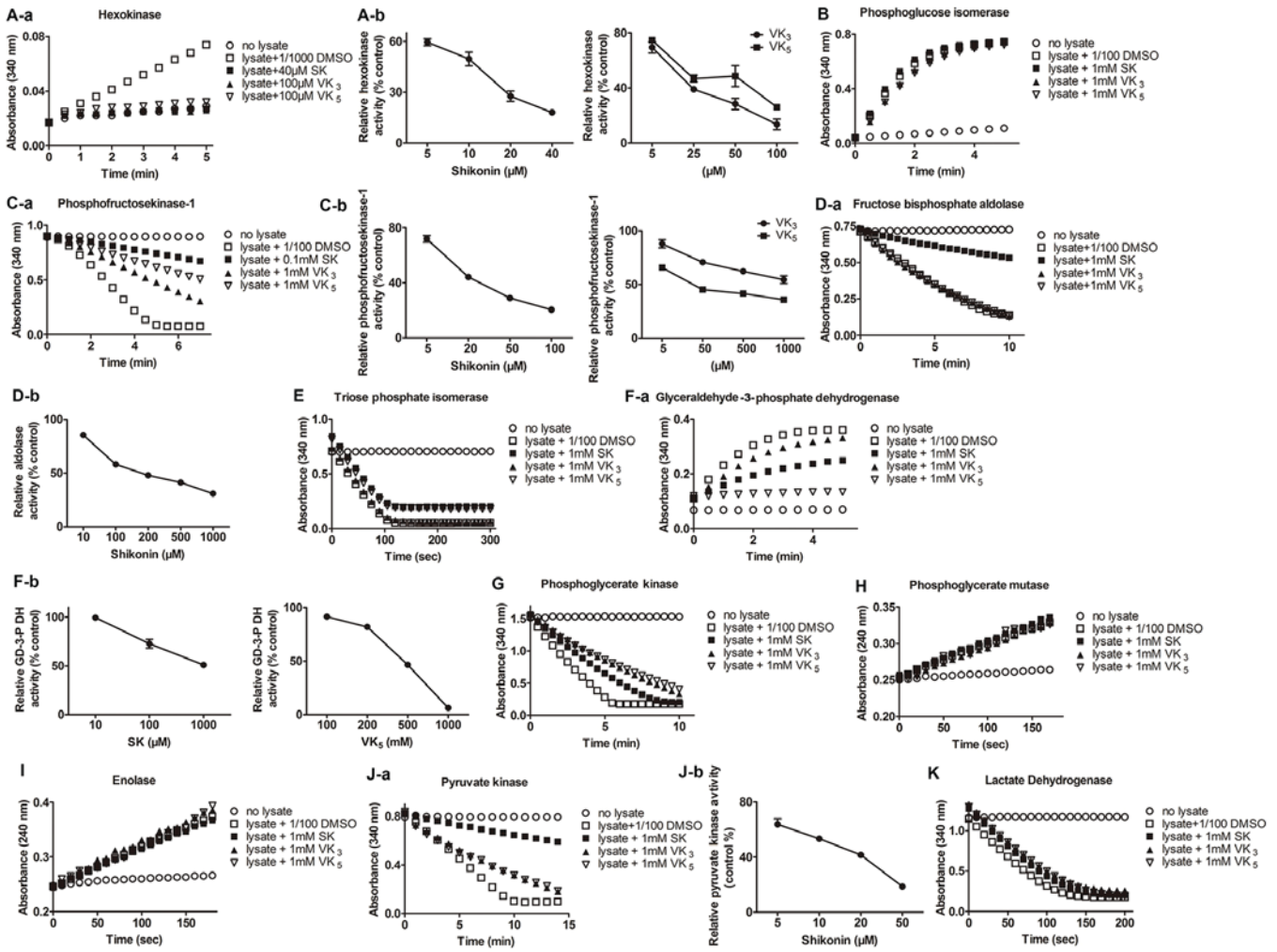

Figure 2. Activity of glycolytic enzymes in the absence or presence of naphthaquinones (SK, VK_3 and VK_5) in cell lysate of MCF-7. Activity of (A-a and b) hexokinase, (B) phosphoglucose isomerase, (C-a and b) phosphofructokinase-1, (D-a and b) fructose bisphophate aldolase, (E) triose phosphate isomerase, (F-a and b) glyceraldehyde-3-phosphate dehydrogenase, (G) phosphoglycerate kinase, (H) phosphoglycerate mutase, (I) enolase, (J-a and b) pyruvate kinase and (K) lactate dehydrogenase following treatment with SK, VK_3 and VK_5. SK, shikonin; VK_3, vitamin K_3; VK_5, vitamin K_5. $^*P<0.05$ and $^{**}P<0.01$. GD-3-P, glyceraldehydes-3-phosphate dehydrogenase.

reversible conversion from dihydroxyacetone phosphate into glyceraldehyde-3-phosphate (2). Glyceraldehyde-3-phosphate may complete glycolysis, whilst dihydroxyacetone phosphate participates in the pentose phosphate pathway, a key source of reduced NADPH and a cofactor for anabolic pathways in maintaining redox balance (3). Thus, as a regulator of distributed metabolites between the glycolysis and pentose phosphate pathway (36), TPI is a potential target to disrupt the metabolism of cancer cells. In the present study, the potential of shikonin, vitamin K_3 and K_5 to be inhibitors of TPI was tested using a glycerol-3-phosphate dehydrogenate coupled assay in which the TPI activity was demonstrated by the absorbance at 340 nm representing the oxidation of NADH (Fig. 2E). Results suggested that shikonin, vitamin K_3 and vitamin K_5 did not affect TPI activity even up to the soluble limit (1 mM) in the incubation system (Fig. 2E; Table I).

Shikonin and vitamin K_5 markedly inhibited the activity of GAPDH. GAPDH catalyzes the reversible conversion from glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate coupled with the production of NADH, an essential source of reducing power in cancer cells (1,2). GAPDH is regarded to be a housekeeping gene expressed in a majority of cells (2). However, its expression is upregulated in cancer cells, and the expression level is associated with cancer aggressiveness and drug resistance (36). Certain naphthalene derivatives have exhibited inhibitory effects on GAPDH activity (37). In the present study, the influence of shikonin, vitamin K_3 and vitamin K_5 on GAPDH activity in the human breast cell line MCF-7 was examined, defined by the velocity of the conversion from NAD+ to NADH catalyzed by GAPDH exhibited by the increase in absorbance at 340 nm (Fig. 2F-a). The results indicated that shikonin and vitamin K_5 were able to inhibit the activity of GAPDH with an IC_{50} of 1,052.0 and 462.0 μM, respectively, whilst vitamin K_3 at a high concentration had little inhibitory effect on GAPDH (Fig. 2F-a and b; Table I).

Shikonin, vitamin K_3 and vitamin K_5 inhibited the activity of PGK. PGK catalyzes the sixth step of glycolysis to reversibly convert 1,3-bisphosphoglycerate to 3-phosphoglycerate coupled with the generation of ATP from ADP (2). Human cells have two isoforms of PGK (2). An increase in the

expression of PGK1 has been demonstrated in metastatic cancer cells (38-41). The overexpression of PGK1 has been proven to promote invasion and metastasis in the colon (38), gastric (39) and prostate cancer (40), and radioresistance in astrocytoma (41). As PGK1 activity is not limited to glycolysis intracellularly but additionally in the microenviroment of tumor masses as a reductase, it is a potential target for manipulating cancer growth despite the fact that its role in the progression of different cancer types is inconsistent (40). In the present study, the effect of shikonin, vitamin K_3 and vitamin K_5 on regulating PGK activity in MCF-7 cells was measured using a glyceraldehyde-3-phosphate dehydrogenase coupled assay. PGK activity was exhibited by the decrease of absorbance at 340 nm representing the conversion of NADH to NAD+ (Fig. 2G). The result demonstrated that shikonin, vitamin K_3 and K_5 exerted a mild inhibitory effect on PGK activity and inhibited 10.5, 28.6 and 36.7% PGK activity, respectively, even at the soluble limit of 1 mM (Fig. 2G; Table I).

Shikonin, vitamin K_3 and vitamin K_5 did not inhibit the activity of PGAM. PGAM catalyzes the interconversion from 3-phosphoglycerate to 2-phosphoglycerate in glycolysis (2). It is a regulator of glycolysis and biosynthesis, as most of the glycolytic intermediates that are used as precursors for anabolism are upstream of this step (42). Thus, PGM is a promising therapeutic target for cancer. Aromatic compound MJE3 (43) and PGM1-004A (42) were potent inhibitors of PGAM, and were toxic to cancer cells. In the present study, the influence of aromatic compounds shikonin, vitamin K_3 and K_5 on PGAM were measured using enzymatic assays. The experiment was performed using an enolase coupled reaction to measure the production of phosphoenolpyruvate indicated by the absorbance at 240 nm (Fig. 2H). The result demonstrated that shikonin, vitamin K_3 and K_5 were unable to inhibit the activity of PGAM (Fig. 2H; Table I).

Shikonin, vitamin K_3 and vitamin K_5 did not inhibit the activity of enolase. Enolase catalyzes the interconversion of 2-phosphoglycerate to phosphoenolpyruvate (2). The expression of enolase is upregulated and associated with the invasion ability of cancer (44,45). Enolase is regarded to be a potential target for cancer treatment. An aromatic compound, ENOblock, was identified to exhibit potent inhibition of enolase activity and anticancer effects (46). In the present study, whether the glycolysis inhibitors shikonin, vitamin K_3 and K_5 affect the activity of enolase was examined. The assay was performed by detecting the production of phosphoenolpyruvate indicated by the increase of the absorbance at 240 nm (Fig. 2I). The result demonstrated that shikonin, vitamin K_3 and vitamin K_5 did not exert any inhibition on the activity of enolase, even at their soluble limits (Fig. 2I; Table I).

Shikonin notably inhibited the activity of pyruvate kinase derived from MCF-7 lysate. Pyruvate kinase catalyzes the last rate limiting step of glycolysis, converting phosphoenolpyruvate to pyruvate irreversibly (2). PKM2, an isoform of pyruvate kinase, is expressed specifically and ubiquitously in cancer cells (47,48). Interrupting its activity inhibits the glycolytic flux and growth of cancer cells (47). PKM2 has become highly focused on in the development of novel cancer therapy methods (1,3,11). A number of chemical inhibitors of PKM2 have been identified (9,10,12). In previous studies, shikonin, vitamin K_3 and K_5 were revealed to inhibit the activity of purified recombinant PKM2 potently, with an IC_{50} of 0.3, 150 and 28 μM respectively (9,10). However, it was additionally revealed that shikonin may inhibit 50% of PKM2 activity in MCF-7 cells with a concentration of up to 10 μM (10). The inconsistent effect on the inhibitory effect on PKM2 activity *in vitro* and *in vivo* may be caused by the different allosteric structures of PKM2 and the conjugation of naphthaquinones with the various components of cells (24,25). In the present study, a LDH coupled assay was applied in order to measure the inhibitory effect of shikonin, vitamin K_3 and K_5 on PKM2 in MCF-7 cell lysate. The result was indicated by the consumption of NADH with the absorbance at 340 nm (Fig. 2J-a). It was revealed that, compared with the vehicle control, shikonin exhibited a notable inhibitory efficiency with an IC_{50} of 12.2 μM, while vitamin K_3 and K_5 inhibited PKM2 in cell extract very little, reducing 29.5 and 26.4% of PKM2 activity, respectively, at their soluble limits (Fig. 2J-a and b; Table I).

Shikonin, vitamin K_3 and vitamin K_5 did not inhibit the activity of LDH. LDH converts the pyruvate to lactate reversibly (2). LDHA and LDHB are the two major isoforms of LDH in mammalian cells (2). LDHA preferably transforms pyruvate to lactate and LDHB favors the reverse conversion (49). Chemical inhibitors of LDHA have been proven to possess antitumor activity; and of these inhibitors, gossypol was used in clinical trials (50). In the present study, the activity of LDH in catalyzing the conversion from pyruvate to lactate in the presence of shikonin, vitamin K_3 or K_5 was measured, which was indicated by the consumption of NADH at an absorbance of 340 nm (Fig. 2K). The results demonstrated that shikonin, vitamin K_3 and vitamin K_5 were unable to inhibit the LDH activity of MCF-7 cell lysate, even at their soluble limits (Fig. 2K; Table I).

Discussion

Shikonin, vitamin K_3 and K_5 are glycolytic suppressors, and have been previously revealed to inhibit PKM2 in cancer cells (9,10). The present study firstly screened the inhibition profile of glycolytic enzymes of identified PKM2 inhibitors, shikonin, vitamin K_3 and K_5. The inhibitory effect of naphthaquinones on enzymes in cell lysate (Table I) is consistent with that using intracellular enzymes (10), and that cell lysate contains factors to retain allosteric and contextual conditions of enzymes (10,24,25). Thus, in order to study the influence of shikonin, vitamin K_3 and K_5 on the activity of endogenous glycolytic enzymes in the breast cancer cell line MCF-7 and their physiological effect, it is more appropriate to measure enzymatic activity in cell extracts as opposed to purified recombinant enzymes, thus using a systematic method of exploring inhibitors of biological enzymes.

Hexokinase II (HK II), an isoform of hexokinase, is specifically upregulated in cancer cells (27) and is mainly expressed in MCF-7 cells (51). The expression of HK II is associated with a poor prognosis in patients with cancer, and the survival and growth of cancer cells (27). Therefore, HK II is regarded to be an important target in cancer treatment (1-3). Chemicals

including 2-deoxyglucose, lonidamine, 3-bromopyruvate and imatinib that compete with the substrate of hexokinase (glucose) or inhibit the activity of HK II, exert a notable effect on treating cancer alone or in combination with traditional chemotherapy and radiotherapy (1,2). Imatinib has been approved for clinical usage (2). Lonidamine and 2-deoxyglucose have been used in clinical trial stages II/III and I/II respectively (1,2), while 3-bromopyruvate has been used in pre-clinical trials (1). A potent HK II inhibitor, 3-bromopyruvate, with a concentration of 25 μM, may inhibit ~40% of the hexokinase activity of MCF-7 cells (29), suggesting that an IC_{50} >25 μM higher compared with the IC_{50} of shikonin, vitamin K_3 and K_5. The results of the present study indicated that shikonin, vitamin K_3 and K_5 inhibited the hexokinase activity of MCF-7 cells more potently compared with 3-bromopyruvate.

PFK1 is an allosteric enzyme activated by fructose-2,6-phosphate. Decreased fructose-2,6-phosphate may reduce the rate of aerobic glycolysis, biosynthesis, viability and anchorage-independent growth of cancer cells indirectly due to allosterically downregulated PFK1 (32,33), suggesting that PFK1 serves an important role in the metabolic reprogramming of cancer cells, and targeting PFK1 activity has potential uses in the development of novel cancer therapies. A number of small chemical inhibitors were discovered to dissociate PFK1's tetrameric form and reduce its activity directly (52,53). Acetylsalicylic acid and salicylic acid, medicines proven to be efficient in preventing breast cancer in clinical trials (54), inhibited the PFK1 activity of MCF-7 cells, with an IC_{50} of ~10 mM (52). Resveratrol, naturally present in grapes, inhibited the glycolytic rate and growth of MCF-7 cells through decreasing PFK1 activity, with an IC_{50} of around 100 μM (53). Compared with the inhibitory effect of acetylsalicylic acid, salicylic acid and resveratrol on the PFK1 activity of MCF-7 cells, shikonin and vitamin K_5 were notably more efficient inhibitors.

Aldolase A, a muscle specific isoform, is highly expressed in cancer cells and the expression level is associated with the metastasis and poor prognosis of cancer (34,35), suggesting that it may be a potential target in treating cancer. A number of aromatic compounds were discovered to be efficient inhibitors of muscle specific aldolase, with a minimum IC_{50} of ~30 μM required to inhibit purified recombinant aldolase A (55). In the present study, it was revealed that 158.4 μM shikonin may suppress 50% of the aldolase activity of MCF-7 cell lysate (Table I). As cell lysate was used as opposed to purified enzyme in the inhibition assays, it is unreasonable to compare the inhibitory activity between shikonin and classical aldolase inhibitors. Taking into consideration the fact that the inhibitory activity was interrupted by complicated components in the cell lysate, shikonin exhibited potent efficiency in suppressing aldolase activity.

The sub-cellular location of GAPDH is associated with the apoptosis of cells (2). One previous study on the manipulation of the location of GAPDH to the nucleus or mitochondria in order to induce cell death shed light on the development of cancer therapies (56). Nevertheless, preventing GAPDH activity in glycolysis provides a different perspective in order to invent novel treatments of cancer (2). Given that GAPDH has a high affinity and activity toward NAD+, biochemists have synthesized naphthalene analogs with a similar chemical structure to NAD+ in order to compete with NAD+ in docking with the binging site (37). These naphthalene derivatives demonstrated a potent inhibition of GAPDH activity derived from trypanosome but not in human cells (37). In the present study, shikonin and vitamin K_5, two naphthalene derivatives, exhibited efficient inhibitory activity on GAPDH in human derived cells, which provides an indication of how to distinguish regulatory characteristics of GAPDH derived from human and trypanosome.

PKM2, the major pyruvate kinase isoform in MCF-7 cells, had been previously proven to be substantially inhibited by shikonin, vitamin K_3 and vitamin K_5 (9,10). However, the inhibitory efficiency was measured using assays applying purified recombinant PKM2. The results revealed that vitamin K_3 and vitamin K_5 exhibit mild but notable suppression of PKM2 activity in MCF-7 cell lysate with various components. Shikonin exhibited a similar inhibitory effect on PKM2 derived from cell lysate and living cells (10). Given that factors regulating the allosteric structure of PKM2 (12,47,48) and proteins binding PKM2 (24,25) to form complexes are reserved in cell lysate as intracellularly, using cell lysate in inhibition assays may mimic the inhibitory efficiency of shikonin in physiological condition as opposed to using purified recombinant PKM2.

In conclusion, shikonin, vitamin K_3 and vitamin K_5 may inhibit a number of glycolytic enzymes simultaneously, and the suppression of the glycolytic rate by shikonin, vitamin K_3 and vitamin K_5 was due to the reduced activity of certain enzymes. Among the inhibited enzymes, three rate-limiting enzymes (hexokinase, phosphofructokinase-1 and pyruvate kinase) were main targets of the naphthaquinones, which primarily contributed to the decreased consumption of glucose and the production of lactate in cancer cells.

Previously, the amount of glucose and lactate in culture media was measured in order to examine the inhibitory effect of shikonin, vitamin K_3 and vitamin K_5 on the glycolysis process. The IC_{50} of shikonin, vitamin K_3 and K_5 in inhibiting glucose consumption were 10, 50 and 105 μM, respectively, and the IC_{50} in inhibiting lactate production were 4, 30 and 45 μM, respectively (9,10). The IC_{50} of shikonin, vitamin K_3 and vitamin K_5 toward the reduction of glucose in culture media were inconsistent with those toward the increase of lactate, which indicates that the transporters that assist the intercellular flux of glucose and lactate (31) may be partially blocked by naphthaquinones. Suppression of glycolysis *in vivo* may be attributed partially to the inhibition of the activity of enzymes and partly to the reduced import and export of glucose and lactate (1). In the present study, vitamin K_5 inhibited glycolytic enzymes notably more potently compared with vitamin K_3 (Table I), but the previous study demonstrated a lower efficiency in inhibiting glycolysis (9), indicating that vitamin K_3 may be stronger in inhibiting transporters of glucose and lactate compared with vitamin K_5.

Overall, the present study revealed that naphthaquinones, shikonin, vitamin K_3 and K_5 inhibit aerobic glycolysis efficiently by reducing the activity of glycolytic enzymes in cancer cells, which provides evidence for the development of novel chemical drugs to treat cancer.

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Availability of data and materials

The datasets generated and analyzed in the present study are included in this published article.

Authors' contributions

JCh performed the experiments, analyzed the data and wrote the manuscript. XH conceived and designed the study, and supervised the whole project. JCu provided technical support.

Ethics and consent to participate

The present study was conducted on cell line MCF-7. There was no ethical issue in the present study.

Consent for publication

No human participant was involved in this study.

Competing interests

The authors declare that they have no competing interests.

References

1. Porporato PE, Dhup S, Dadhich RK, Copetti T and Sonveaux P: Anticancer targets in the glycolytic metabolism of tumors: A comprehensive review. Front Pharmacol 2: 49, 2011.
2. Pelicano H, Martin DS, Xu RH and Huang P: Glycolysis inhibition for anticancer treatment. Oncogene 25: 4633-4646, 2006.
3. Hamanaka RB and Chandel NS: Targeting glucose metabolism for cancer therapy. J Exp Med 209: 211-215, 2012.
4. Gatenby RA, Gawlinski ET, Gmitro AF, Kaylor B and Gillies RJ: Acid-mediated tumor invasion: A multidisciplinary study. Cancer Res 66: 5216-5223, 2006.
5. Courtnay R, Ngo DC, Malik N, Ververis K, Tortorella SM and Karagiannis TC: Cancer metabolism and the Warburg effect: The role of HIF-1 and PI3K. Mol Biol Rep 42: 841-851, 2015.
6. Poon E, Harris AL and Ashcroft M: Targeting the hypoxia-inducible factor (HIF) pathway in cancer. Expert Rev Mol Med 11: e26, 2009.
7. Lew CR and Tolan DR: Targeting of several glycolytic enzymes using rna interference reveals aldolase affects cancer cell proliferation through a non-glycolytic mechanism. J Biol Chem 287: 42554-42563, 2012.
8. Chan B, Manley J, Lee J and Singh SR: The emerging roles of microRNAs in cancer metabolism. Cancer Lett 356: 301-308, 2015.
9. Chen J, Jiang Z, Wang B, Wang Y and Hu X: Vitamin K(3) and K(5) are inhibitors of tumor pyruvate kinase M2. Cancer Lett 316: 204-210, 2012.
10. Chen J, Xie J, Jiang Z, Wang B, Wang Y and Hu X: Shikonin and its analogs inhibit cancer cell glycolysis by targeting tumor pyruvate kinase-M2. Oncogene 30: 4297-4306, 2011.
11. Christofk HR, Vander Heiden MG, Harris MH, Ramanathan A, Gerszten RE, Wei R, Fleming MD, Schreiber SL and Cantley LC: The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. Nature 452: 230-233, 2008.
12. Vander Heiden MG, Christofk HR, Schuman E, Subtelny AO, Sharfi H, Harlow EE, Xian J and Cantley LC: Identification of small molecule inhibitors of pyruvate kinase M2. Biochem Pharmacol 79: 1118-1124, 2010.
13. Rady P, Arany I, Bojan F and Kertai P: Activities of four glycolytic enzymes (HK, PFK, PK and LDH) and isozymic pattern of LDH in mouse lung tumor induced by urethan. J Cancer Res Clin Oncol 95: 287-289, 1979.
14. Boyland E, Goss GC and Williamsashman HG: The hexokinase activity of animal tumours. Biochem J 49: 321-325, 1951.
15. Gracy RW and Tilley BE: Phosphoglucose isomerase of human erythrocytes and cardiac tissue. Methods Enzymol 41: 392-400, 1975.
16. Vora S, Halper JP and Knowles DM: Alterations in the activity and isozymic profile of human phosphofructokinase during malignant transformation in vivo and in vitro: Transformation- and progression-linked discriminants of malignancy. Cancer Res 45: 2993-3001, 1985.
17. El-Bacha T, de Freitas MS and Sola-Penna M: Cellular distribution of phosphofructokinase activity and implications to metabolic regulation in human breast cancer. Mol Genet Metab 79: 294-299, 2003.
18. Pontremoli S, Melloni E, Salamino F, Sparatore B, Michetti M and Horecker BL: Changes in activity of fructose-1,6-bisphosphate aldolase in livers of fasted rabbits and accumulation of crossreacting immune material. Proc Natl Acad Sci USA 76: 6323-6325, 1979.
19. Rieder SV and Rose IA: The mechanism of the triosephosphate isomerase reaction. J Biol Chem 234: 1007-1010, 1959.
20. Brenneman FN and Volk WA: Glyceraldehyde phosphate dehydrogenase activity with triphosphopyridine nucleotide and with diphosphopyridine nucleotide. J Biol Chem 234: 2443-2447, 1959.
21. Yoshida A and Watanabe S: Human phosphoglycerate kinase. I. Crystallization and characterization of normal enzyme. J Biol Chem 247: 440-445, 1972.
22. Rose ZB and Dube S: Phosphoglycerate mutase. Kinetics and effects of salts on the mutase and bisphosphoglycerate phosphatase activities of the enzyme from chicken breast muscle. J Biol Chem 253: 8583-8592, 1978.
23. Wold F and Ballou CE: Studies on the enzyme enolase. II. Kinetic studies. J Biol Chem 227: 313-328, 1957.
24. Christofk HR, Vander Heiden MG, Wu N, Asara JM and Cantley LC: Pyruvate kinase M2 is a phosphotyrosine-binding protein. Nature 452: 181-186, 2008.
25. Mazurek S, Zwerschke W, Jansen-Durr P and Eigenbrodt E: Metabolic cooperation between different oncogenes during cell transformation: Interaction between activated ras and HPV-16 E7. Oncogene 20: 6891-6898, 2001.
26. Patra KC and Hay N: The pentose phosphate pathway and cancer. Trends Biochem Sci 39: 347-354, 2014.
27. Roberts DJ and Miyamoto S: Hexokinase II integrates energy metabolism and cellular protection: Akting on mitochondria and TORCing to autophagy. Cell Death Differ 22: 248-257, 2015.
28. Zhang D, Li J, Wang F, Hu J, Wang S and Sun Y: 2-Deoxy-D-glucose targeting of glucose metabolism in cancer cells as a potential therapy. Cancer Lett 355: 176-183, 2014.
29. Wu L, Xu J, Yuan W, Wu B, Wang H, Liu G, Wang X, Du J and Cai S: The reversal effects of 3-bromopyruvate on multidrug resistance in vitro and in vivo derived from human breast MCF-7/ADR cells. PLoS One 9: e112132, 2014.
30. Yanagawa T, Funasaka T, Tsutsumi S, Watanabe H and Raz A: Novel roles of the autocrine motility factor/phosphoglucose isomerase in tumor malignancy. Endocr Relat Cancer 11: 749-759, 2004.
31. Chiu CG, St-Pierre P, Nabi IR and Wiseman SM: Autocrine motility factor receptor: A clinical review. Expert Rev Anticancer Ther 8: 207-217, 2008.
32. Ros S and Schulze A: Balancing glycolytic flux: The role of 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatases in cancer metabolism. Cancer Metab 1: 8, 2013.
33. Phan LM, Yeung SC and Lee MH: Cancer metabolic reprogramming: Importance, main features and potentials for precise targeted anti-cancer therapies. Cancer Biol Med 11: 1-19, 2014.

34. Long F, Cai X, Luo W, Chen L and Li K: Role of aldolase A in osteosarcoma progression and metastasis: *In vitro* and *in vivo* evidence. Oncol Rep 32: 2031-2037, 2014.
35. Sun Y, Long JH and Zhou Y: Angiopoietin-like 4 promotes melanoma cell invasion and survival through aldolase A. Oncol Lett 8: 211-217, 2014.
36. Lincet H and Icard P: How do glycolytic enzymes favour cancer cell proliferation by nonmetabolic functions? Oncogene 34: 3751-3759, 2015.
37. Kennedy KJ, Bressi JC and Gelb MH: A disubstituted NAD+ analogue is a nanomolar inhibitor of trypanosomal glyceraldehyde-3-phosphate dehydrogenase. Bioorg Med Chem Lett 11: 95-98, 2001.
38. Ahmad SS, Glatzle J, Bajaeifer K, Bühler S, Lehmann T, Königsrainer I, Vollmer JP, Sipos B, Ahmad SS, Northoff H, *et al*: Phosphoglycerate kinase 1 as a promoter of metastasis in colon cancer. Int J Oncol 43: 586-590, 2013.
39. Zieker D, Konigsrainer I, Tritschler I, Löffler M, Beckert S, Traub F, Nieselt K, Bühler S, Weller M, Gaedcke J, *et al*: Phosphoglycerate kinase 1 a promoting enzyme for peritoneal dissemination in gastric cancer. Int J Cancer 126: 1513-1520, 2010.
40. Wang J, Ying G, Wang J, Jung Y, Lu J, Zhu J, Pienta KJ and Taichman RS: Characterization of phosphoglycerate kinase-1 expression of stromal cells derived from tumor microenvironment in prostate cancer progression. Cancer Res 70: 471-480, 2010.
41. Ding H, Cheng YJ, Yan H, Zhang R, Zhao JB, Qian CF, Zhang WB, Xiao H and Liu HY: Phosphoglycerate kinase 1 promotes radioresistance in U251 human glioma cells. Oncol Rep 31: 894-900, 2014.
42. Hitosugi T, Zhou L, Elf S, Fan J, Kang HB, Seo JH, Shan C, Dai Q, Zhang L, Xie J, *et al*: Phosphoglycerate mutase 1 coordinates glycolysis and biosynthesis to promote tumor growth. Cancer Cell 22: 585-600, 2012.
43. Evans MJ, Saghatelian A, Sorensen EJ and Cravatt BF: Target discovery in small-molecule cell-based screens by *in situ* proteome reactivity profiling. Nat Biotechnol 23: 1303-1307, 2005.
44. Song Y, Luo Q, Long H, Hu Z, Que T, Zhang X, Li Z, Wang G, Yi L, Liu Z, *et al*: Alpha-enolase as a potential cancer prognostic marker promotes cell growth, migration and invasion in glioma. Mol Cancer 13: 65, 2014.
45. Principe M, Ceruti P, Shih NY, Chattaragada MS, Rolla S, Conti L, Bestagno M, Zentilin L, Yang SH, Migliorini P, *et al*: Targeting of surface alpha-enolase inhibits the invasiveness of pancreatic cancer cells. Oncotarget 6: 11098-11113, 2015.
46. Jung DW, Kim WH, Park SH, Lee J, Kim J, Su D, Ha HH, Chang YT and Williams DR: A unique small molecule inhibitor of enolase clarifies its role in fundamental biological processes. ACS Chem Biol 8: 1271-1282, 2013.
47. Iqbal MA, Gupta V, Gopinath P, Mazurek S and Bamezai RN: Pyruvate kinase M2 and cancer: An updated assessment. FEBS Lett 588: 2685-2692, 2014.
48. Mazurek S, Boschek CB, Hugo F and Eigenbrodt E: Pyruvate kinase type M2 and its role in tumor growth and spreading. Semin Cancer Biol 15: 300-308, 2005.
49. Miao P, Sheng S, Sun X, Liu J and Huang G: Lactate dehydrogenase A in cancer: A promising target for diagnosis and therapy. IUBMB Life 65: 904-910, 2013.
50. Doherty JR and Cleveland JL: Targeting lactate metabolism for cancer therapeutics. J Clin Invest 123: 3685-3692, 2013.
51. Lu CL, Qin L, Liu HC, Candas D, Fan M and Li JJ: Tumor cells switch to mitochondrial oxidative phosphorylation under radiation via mTOR-mediated hexokinase II inhibition-a Warburg-reversing effect. PLoS One 10: e0121046, 2015.
52. Spitz GA, Furtado CM, Sola-Penna M and Zancan P: Acetylsalicylic acid and salicylic acid decrease tumor cell viability and glucose metabolism modulating 6-phosphofructo-1-kinase structure and activity. Biochem Pharmacol 77: 46-53, 2009.
53. Gomez LS, Zancan P, Marcondes MC, Ramos-Santos L, Meyer-Fernandes JR, Sola-Penna M and Da Silva D: Resveratrol decreases breast cancer cell viability and glucose metabolism by inhibiting 6-phosphofructo-1-kinase. Biochimie 95: 1336-1343, 2013.
54. Zhao YS, Zhu S, Li XW, Wang F, Hu FL, Li DD, Zhang WC and Li X: Association between NSAIDs use and breast cancer risk: A systematic review and meta-analysis. Breast Cancer Res Treat 117: 141-150, 2009.
55. Blonski C, De Moissac D, Périé J and Sygusch J: Inhibition of rabbit muscle aldolase by phosphorylated aromatic compounds. Biochem J 323: 71-77, 1997.
56. Colell A, Green DR and Ricci JE: Novel roles for GAPDH in cell death and carcinogenesis. Cell Death Differ 16: 1573-1581, 2009.