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ORIGINAL ARTICLE

Glucose uptake inhibitor sensitizes cancer cells to daunorubicin and overcomes drug resistance in hypoxia

Xianhua Cao · Lanyan Fang · Seth Gibbs · Ying Huang · Zunyan Dai · Ping Wen · Xincheng Zheng · Wolfgang Sadee · Duxin Sun

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Abstract
Purpose A high-rate glycolysis is a fundamental property of solid tumors and is associated with an over-expression of glucose transporters and glycolytic enzymes. We hypothesize that over-expression of glucose transporters in tumors prevents apoptosis, promotes cancer cell survival, and confers drug resistance. Inhibition of glucose transporter will preferentially sensitize the anticancer effects of chemotherapeutic drugs to overcome drug resistance in hypoxia.
Methods Glucose transporter expressions were detected in cancer tissues and NCI 60 cancer cells with immunostaining and DNA microarray. Glucose uptake was measured with ^{3}H-2-deoxy-glucose. Cytotoxicity of daunorubicin (DNR) in combination of glucose inhibitor was detected by MTS assay under hypoxic condition. Early stage apoptosis was monitored with Annexin V-FITC staining.
Results Immunostaining showed that GLUT1 was significantly increased in hypoxic regions of the human colon and breast tumors. The expression profiles of all glucose transporters in NCI 60 cancer cells exhibited distinct expression patterns. Phloretin exhibited more than 60% glucose uptake inhibition. Hypoxia conferred two to fivefold higher drug resistance in SW620 and K562 to DNR. Inhibition of glucose uptake by phloretin sensitized cancer cells to DNR for its anticancer activity and apoptosis to overcome drug resistance only under hypoxia.
Conclusion Cancer cells heavily rely on glucose transporters for glucose uptake to facilitate a high-rate glycolysis under hypoxia for their survival and drug resistance. Combination of glucose transporter inhibitors and chemotherapeutic drugs may provide a preferential novel therapeutic strategy to overcome drug resistance in hypoxia.

Keywords Glucose transporter · Glycolysis inhibitor · Combination · Drug resistance · Hypoxia

X. Cao · L. Fang · S. Gibbs · D. Sun (✉)
Division of Pharmaceutics, School of Pharmacy,
The Ohio State University, 500 W. 12th Ave,
Columbus, OH 43210, USA
e-mail: sun.176@osu.edu

Y. Huang · Z. Dai · W. Sadee
Department of Pharmacology, The Ohio State University,
Columbus, OH 43210, USA

P. Wen · X. Zheng
Department of Pathology, The Ohio State University,
Columbus, OH 43210, USA

Introduction

A high-rate glycolysis has been consistently observed in solid tumors for several decades, a phenomenon known as the Warburg effect [1, 2]. Cancer cells often switch glucose metabolism from tricaboxylic acid (TCA) cycle to anaerobic glycolysis for ATP production [3, 4]. However, anaerobic glycolysis only produces two molecules of ATP, while TCA produces 38 molecules of ATP from each glucose molecule. Thus, complete loss of TCA cycle for ATP production would result in a 19-fold increase in glucose consumption in cancer cell for the energy needs. Indeed, these characteristics are associated with increased metastasis and poor survival in cancer patients [5, 6] and has been clinically applied to tumor imaging with Positron Emission Tomography (PET) [7, 8]. At present, the

majority (~95%) of all clinical PET applications use 2-[^{18}F] fluoro-2-deoxy-D-glucose (FDG) as a standard tracer with overall sensitivity and specificity of 85% in various cancers including colorectal, breast, non-small cell lung, head and neck cancers, melanoma, and lymphoma [7, 8].

However, the underlying biochemical and molecular mechanisms and signaling pathways remained unclear until recently. The high rates of glycolysis in cancer can be contributed to either injury of mitochondria respiration (or mitochondria malfunction) and/or hypoxic stimuli [9, 10]. Mitochondria malfunction induces a high rate of glycolysis even under normoxia. Contributing factors for mitochondrial malfunction include mutations of mitochondria DNA, malfunction of the electron transfer chain, and changes in expression of genes involved in energy metabolism [11].

Alternatively, a hypoxic environment also induces a high rate of glycolysis in tumors. Although tumors have a high rate of blood vessel formation to deliver nutrients and oxygen for proliferation, angiogenesis is irregular and does not keep pace with tumor growth. Therefore, hypoxia is present, at least focally, in most solid tumors. Under hypoxia, cancer cells undergo adaptive changes to survive and proliferate via the regulation of hypoxia-inducible factor 1α (HIF-1α), which has been shown to be over-expressed in many cancers such as colon, breast, gastric, lung, skin, ovarian, pancreatic, prostate, and renal cancers [12]. HIF-1α-regulated genes include glucose transporters [13], glycolytic enzymes [12], VEGF, EGF, and IGF [14], nitric oxide synthase (NOS) [15, 16], and erythropoietin [17]. These gene products are essential for high-glycolytic rates, angiogenesis, immortalization, vasodilation, and pH regulation in solid tumors [18, 19].

The over-expression of glucose transporters to accommodate high rates of glycolysis in tumors suggests that the transporters and glycolysis are potential therapeutic targets in anticancer therapy. The glucose transporter family has 25 members in the human genome, which consists of 11 sodium-dependent glucose cotransporters (SLC5A1-11, or SGTL1-6, SMIT, NIS, SMVT, CHT, and AIT) and 14 sodium-independent facilitative glucose transporters (SLC2A1-14 or GLUT1-14) [20, 21]. Although different types of tumors have distinct expression profiles of GLUTs, the appearance of GLUT1 in tumors seems to be the common feature. The expression levels of GLUT1 have been linked to tumor stage, survival rate, and accuracy of PET. For example, in colorectal cancers, GLUT1 over-expression is correlated with the cancer progression, the depth of invasion, lymph node and hepatic metastasis, and the survival rate [22]. In addition, hexokinases (HKs) are also over-expressed in various stages of malignant tumors, which catalyze the first step of glycolysis from glucose to glucose-6-phosphate (G-6-P) [23]. Increased HK II levels were shown in thyroid [24], breast [25], and metastatic liver cancer [26]. PET with FDG uptake correlates with both GLUT1 and HK activity in various tumor models [27, 28].

In the present study, we hypothesize that over-expression of glucose transporters and a high rate of glycolysis in tumors prevents apoptosis, promotes cancer cell survival in the hostile hypoxic environment, and confers drug resistance in cancer therapy. Inhibition of glucose transporters and glycolysis will sensitize cancer cells to chemotherapeutic agents only under hypoxia. We first detected GLUT1 expression in primary colon and breast cancer tissues from patients and screened for the expression of all glucose transporters in NCI 60 cancer cells. Then phloretin, a glucose transporter inhibitor, was combined with daunorubicin (DNR) under both normoxic and hypoxic conditions to test cytotoxicity and apoptosis in both drug-sensitive and resistant cancer cells. We report that glucose transporters have distinct expression profiles in NCI 60 cancer cell lines and GLUT1 expression is significantly increased in the hypoxic areas of colon cancer and breast cancer tissues. Hypoxia conferred drug resistance in cancer cell lines SW620 and K562. Blocking glucose uptake and glycolysis under hypoxia sensitized both cancer cells to DNR. Furthermore, glycolysis inhibition in hypoxia overcame the multi-drug resistance (MDR)-mediated drug resistance in drug-resistant cancer cells (K562/Dox) and induced synergistic apoptosis when combined with DNR.

Materials and methods

Cell cultures and reagents

The NCI 60 cancer cell lines were cultured in 10% fetal bovine serum (FBS) RPMI-1640 or 10% FBS DMEM media at 37°C and 5% CO_2. Hypoxic conditions were maintained with 1% O_2, 5% CO_2, and 94% N_2 in a modular incubator chamber (Billupus-Rottemberg Inc., Del Mar, CA, USA). Drug-sensitive leukemia cells K562 and drug-resistant leukemia cells K562/Dox (a gift from J. P. Marie, Institut National de la Santé et de la Recherche Médicale, E9912, University of Paris 6, Paris, France) were cultured in RPMI 1640 supplemented with 10% FBS, 1% non-essential amino acid and 1% Penicillin (100 units/ml)/Streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The culture media was changed every 2–3 days.

Before each experiment, K562/Dox cells were treated with 0.1 μM doxorubicin at least for 1 week and then cultured for 10 days without doxorubicin exposure. It was assured that P-glycoprotein (P-gp) expression level was similar in every experiment. Samples of colon cancer and breast cancer tissue were obtained from paraffin-embedded tissue provided by the James Cancer Hospital in Ohio State University.

Expression of glucose transporters using DNA microarray

Oligonucleotide microarrays were spotted with 70-mer oligonucleotide as described [29]. Each probe was printed four times per array and the median was used to enhance precision of the measurements and to minimize the effect of single outlier points. Expression of each gene was assessed by the ratio of expression level in the samples against a pooled control from 12 of the NCI 60 cell lines [30, 31]. Total RNA (12.5 μg) was used for cDNA synthesis and then labeled with Cy5 or Cy3 (control) by amino-allyl coupling. The samples were then mixed, and the labeled cDNA was resuspended in 20 μl HEPES buffer (25 mM, pH 7.0) containing 1 μl of tRNA, 1.5 μl of polyA$^+$ RNA, and 0.45 μl of 10% SDS. The mixture was hybridized to the slides for 16 h at 65°C. Slides were washed, dried and scanned in an Affymetrix 428 scanner to detect Cy3 and Cy5 fluorescence. Background subtraction and calculation of medians of pixel measurements per spot were carried out using GenePix Software 3.0 (Foster City, CA, USA). Spots were filtered out if they had both red and green intensity less than 250 units after subtraction of the background. The fluorescence signal was normalized by statistical analysis using the statistical software package R (www.r-project.org). The plot of $M = \log_2 R/G$ vs. $A = \log_2 \sqrt{R \times G}$ showed dependence of the log ratio M on overall spot intensity A. Therefore, an intensity-dependent normalization method was preferred over a global method. Location and scale normalization methods were used to correct intensity and dye bias.

Total RNA isolation and cDNA synthesis

Total cellular RNA was extracted and purified using the TRIzol reagent (Invitrogen, UK). First-strand cDNA was transcribed from 3 μg of total RNA using random hexamers and SSII reverse transcriptase at 42°C for 50 min. After termination of the reaction, RNAse H was added and incubated for 20 min at 37°C before proceeding to amplification of the target cDNA.

Immumonostaining

Paraffin embedded tissue was sectioned at 4 μ and placed on positively charged slides. Slides with specimens were then placed in a 60°C oven for 1 h, cooled, and deparaffinized and rehydrated through xylenes and graded ethanol solutions to water. All slides were quenched for 5 min in a 3% hydrogen peroxide solution in water to block for endogenous peroxidase. Tissues were antigen retrieved using citrate buffer in a vegetable steamer. The primary antibody, anti-GLUT1 (goat anti-human GLUT1, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at a dilution of 1:200. Slides were then placed on a Dako Autostainer immunostaining system (Carpinteria, CA, USA). The detection system used was a labeled streptavidin-biotin complex. This method is based on the consecutive application of (1) a primary antibody against the antigen to be localized; (2) biotinylated linking secondary antibody against primary antibody; (3) peroxidase conjugated streptavidin to bind to biotin; and (4) enzyme substrate chromogen (DAB) for detection. Tissues were avidin and biotin blocked prior to the application of the biotinylated secondary antibody. Slides were then counterstained in Richard Allen hematoxylin, dehyrated through graded ethanol solutions and coverlipped. The staining was examined under microscope.

Glucose uptake assay

Cells were seeded and cultured in a 6-well plate to reach nearly confluent monolayer. The media was replaced by serum free RPMI 1640 for 2 h and cells were rapidly washed with KRP buffer (130 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.3 mM $MgSO_4$, 10 mM Na_2HPO_4, pH 7.4) at 37°C. Measurements of 2-deoxyglucose (2-DG) uptake were initiated by addition of 500 μl KRP buffer containing 0.5 μCi 2-deoxy-^{3}H-glucose and incubated for 5 min at 37°C. The cells were rapidly washed three times with 1 ml of ice-cold KRP buffer containing 20 mM glucose and 0.5 mM phloretin to quench the glucose uptake. Cell-associated radioactivity was determined by lysing cells with 0.05% SDS KRP buffer, followed by liquid scintillation counting. Total cellular protein concentration was measured by the BCA protein assay method (PIERCE) to normalize the glucose uptake.

MTS assay

A total of 2,000–5,000 cells were cultured in 96-well plate for 24 h. Glucose transporter inhibitors (phlore-

tin, 1–100 μM) and chemotherapeutic drugs (DNR, 0.1–10 μM) were added to the cell culture. The cells were maintained in either normoxia (5% CO_2 and 95% air) or hypoxic chamber (1% O_2, 5% CO_2, and 94% N_2). After 3 days, MTS (tetrazolium [3-(4,5–dimethythiazol-2-yl)]-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (2 mg/ml) and phenazine methosulfate (PMS, 25 μM) were added directly to the cell culture and incubated for 2 h at 37°C. The absorbance of formazan (the metabolite of MTS by viable cells) was measured at 490 nm to quantify the number of surviving cells.

Flow cytometry

The assay was performed on a Becton–Dickinson fluorescence activated cell sorting (FACS) calibur (San Jose, CA, USA) equipped with an ultraviolet argon laser (excitation at 488 nm, emission at 530/30, and 570/30 nm band-pass filters). Analysis was stopped after acquisition of 30,000 cells. Log fluorescence was collected and displayed as single parameter histograms.

Annexin-V apoptosis assay

Cells were seeded in chamber slides, grown overnight until 50–80% confluent, and were treated with different combinations of drug and inhibitors. Annexin V staining was performed using 5 μl of Annexin V-FITC and 10 μl of propidium iodide (PI) provided by apoptosis detection kit (BD Pharmingen, San Diego, CA, USA) at room temperature for 15 min in the dark. Then the cells were washed with PBS buffer and fixed in slides with 2% formaldehyde. The slides were immediately viewed with fluorescence microscopy.

Results

GLUT1 expression is significantly increased in primary colon cancer and breast cancer tissues

To confirm the over-expression of GLUT1 in human primary cancers, immunostaining of GLUT1 was performed in specimens of human colon cancer and breast cancer for comparison with adjacent normal tissues. Cancer tissues from four colon and five breast cancer patients were used. Representative sections from one colon and one breast cancer patient are shown (Fig. 1). In normal colon, weak staining of GLUT1 was detected in red blood cells (Fig. 1a1) and lymphocytes (Fig. 1a3), but not in epithelial cells (Fig. 1a1–a3). In colon cancer tissues, however, extensive GLUT1 staining was seen in cancer cells (Fig. 1b1–b3). Interestingly, cells with strong GLUT1 staining localized in the infiltrating fronds of malignant cells (Fig. 1b2), which may indicate the migration direction of these cancer cells. Similarly, in normal breast tissue, only red blood cells were slightly positive for GLUT1 staining (Fig. 1c3), while all other cells in normal breast tissue were GLUT1 negative. However, malignant breast cells showed extensive GLUT1 staining, especially in the hypoxic regions with necrosis (Fig. 1d1–d3).

Various glucose transporters are expressed in different NCI-60 cancer cells

The expression levels of all glucose transporters were determined in NCI-60 cancer cell lines with oligonucleotide DNA microarray. Total cellular RNA was first extracted from these cell lines, reverse transcribed to cDNA, and then labeled with Cy5 (Cy3 for control cells). The expression levels of glucose transporters in each cell line were determined by comparison with pooled control RNA and calculated as fold changes. Figure 2a shows cluster analysis of expression for all the glucose transporters by Cluster and Tree View programs [31–33]. The red and green colors indicated high- or low-expression levels, respectively. GLUT1 (SLC2A1) was expressed 1.5–3.5-fold higher in cancer cells such as colorectal carcinoma cells (HCC-2998, SW-620), non-small cell lung cancer cells (HOP-92, NCI-H460), breast cancer cells (MDA-MB-231), and renal cancer cells (RXF-393) than in the control cell pool. Levels of GLUT3 (SLC2A3) expression were 1.5–7-fold higher in most of the NCI 60 cancer cells compared to control, including colon cancer cells (HCT-15, SW-620), leukemia cells (K562, SR), and non-small cell lung cancer cells (NCI-H23, NCI-H322, NCI-H460). GLUT8 (SLC2A8) and SLC5A3 showed similar expression patterns to GLUT1; while SLC5A1 (SGLT1), SLC5A7, and SLC2A11 (GLUT11) showed similar patterns with each other; and SLC2A3 (GLUT3), SLC5A5, and SLC5A2 (SGLT2) showed similar expression patterns with each other. The similar expression patterns may indicate that the groups of transporters are similarly regulated in these cancer cells. As shown in Fig. 2b, GLUT1 (SLC2A1) expression was the highest in SW-620 cells and SLC2A3 (GLUT3), SLC2A8 (GLUT8), and SLC5A3 also showed higher expression levels than other transporters.

To confirm that phloretin can inhibit glucose uptake, we performed glucose transport study in SW620 cells. 3H-2-deoxyglucose (3H-2-DG) was used as a marker. The glucose transporter inhibitor phloretin (1–50 μM),

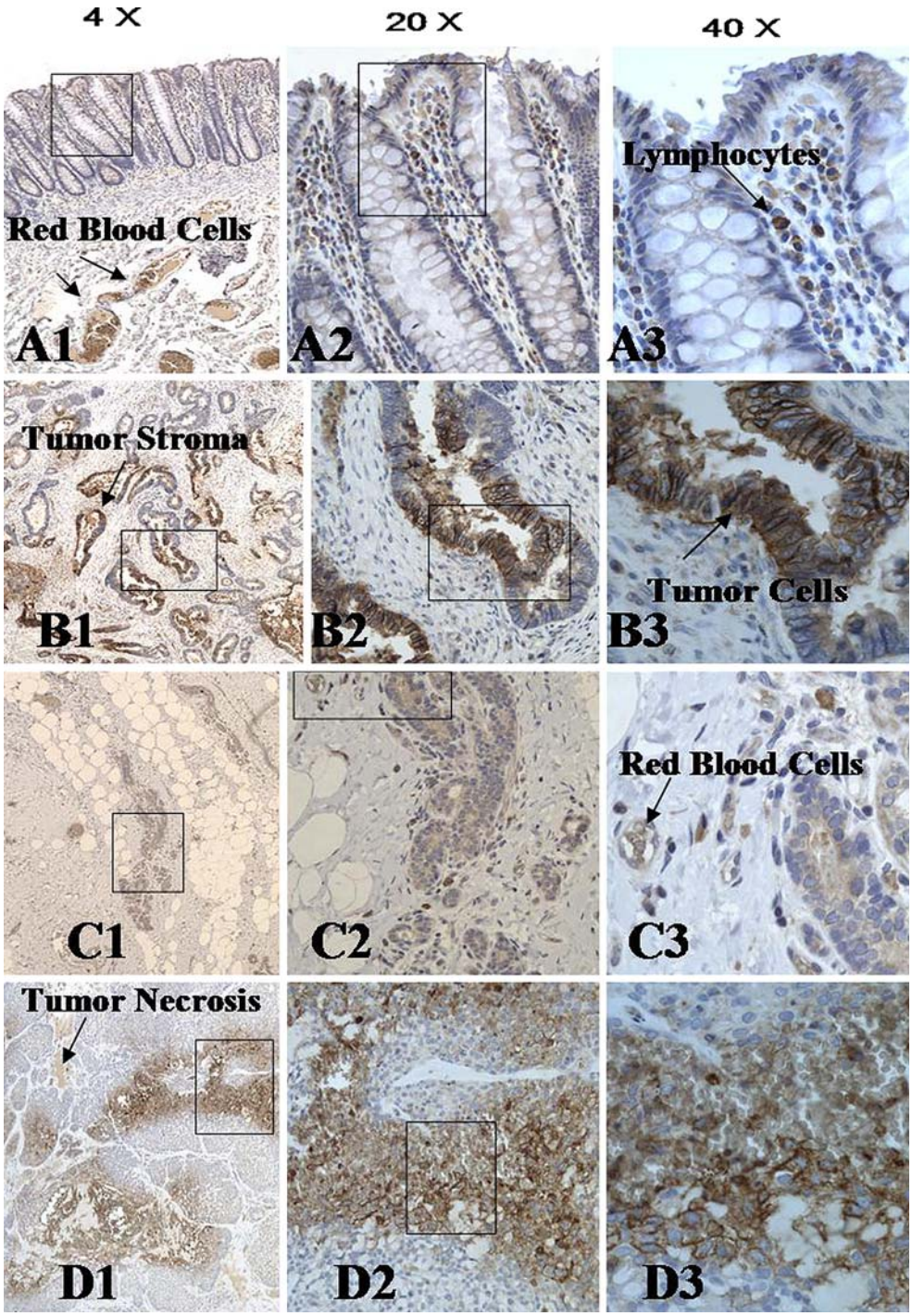

Fig. 1 GLUT1 is over-expressed in colon and breast cancer tissues. Goat anti-human GLUT1 antibody was used as the antibody (1:200 dilutions) in immunostaining of tissues. **a1–a3** Normal colon tissues at ×4, ×20, and ×40 magnification, respectively. **b1–b3** Colon cancer tissues at ×4, ×20, and ×40 magnification, respectively. **c1–c3** Normal breast tissues at ×4, ×20, and ×40 magnification, respectively. **d1–d3** Breast cancer tissues at ×4, ×20, and ×40 magnification, respectively

significantly blocked glucose transporter for glucose uptake by more than 50% (Fig. 2c). 2-DG was used as a control in this study. Unlike phloretin, 2-DG is a substrate of glucose transporters, and thus it only competitively inhibits glucose uptake. Due to the high-Km of glucose transporters (millimolar range) for 2-DG, a higher concentration of 2-DG (more than 1 mM) was required to show glucose uptake inhibition in SW620 cells.

Hypoxia conferred drug resistance can be reversed by inhibition of glucose uptake in drug-sensitive SW620 and K562 cancer cells

To confirm that hypoxia confers drug resistance, we tested the cytotoxicity (MTS assay) of DNR against drug-sensitive cells (colon cancer cells SW620 and leukemia K562). As shown in Fig. 3a, b, hypoxic conditions significantly reduced the drug sensitivity

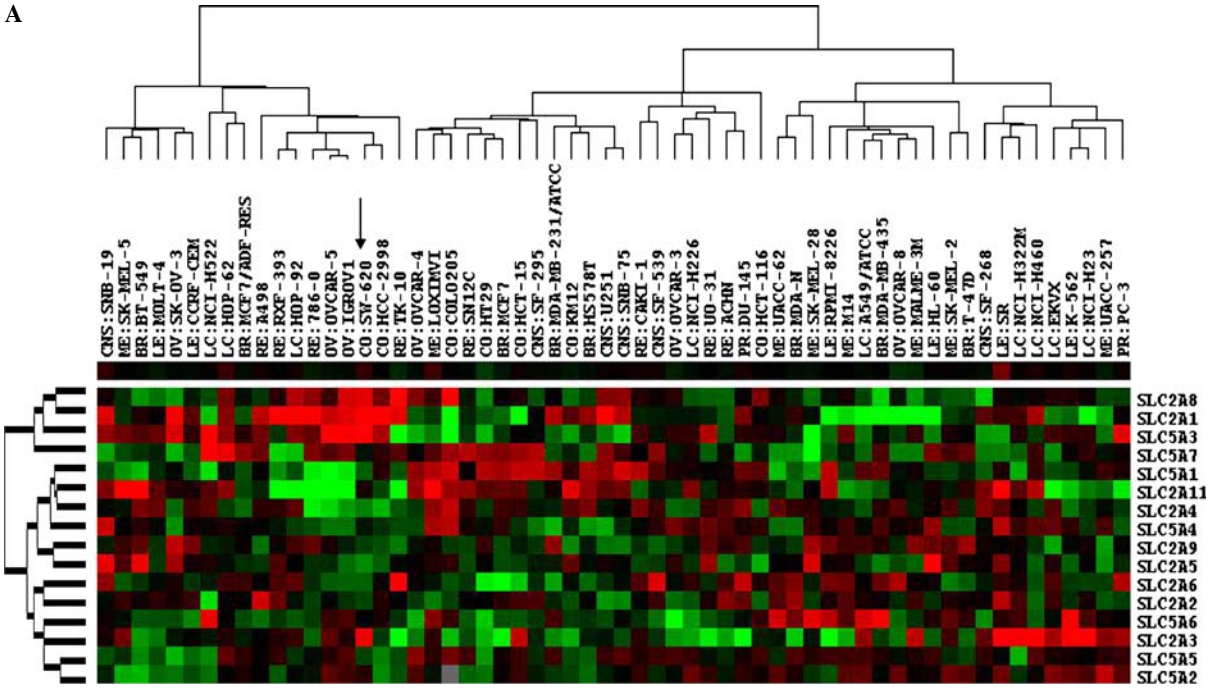

Fig. 2 Expression profiles of all glucose transporters in NCI 60 cancer cell lines. **a** Cluster analysis of the expression profiles of all glucose transporters in NCI 60 cancer cell lines. *Red color* indicates high-expression levels, while *green color* indicates low-expression levels. SLC2A1-11: GLUT1-11; SLC5A1: SGTL1; SLC5A2: SGTL2; SLC5A3: SMIT; SLC5A4: SGTL3; SLC5A5: NIS; SLC5A6: SMVT; SLC5A7: CHT. **b** Glucose transporter expression in the colon cancer cell line SW620. Fold expression difference was calculated by comparison of SW 620 to pooled control RNA from 12 cells lines. GLUT1 (SLC2A1), GLUT3 (SLC2A3), GLUT8 (SLC2A8), and SMIT (SLC5A3) were expressed 3.5-, 2.2-, 1.7-, and 2.0-fold higher compared to pool control cell lines, respectively. **c** Inhibitor of glucose transporter blocked 3H-2-deoxyglucose (*3H-2-DG*) uptake in SW620. Cells were incubated with 2-DG and indicated inhibitor at KRP buffer for 5 min at 37°C. Cell-associated radioactivity results were normalized to per microgram total proteins. *Columns* and *points*, means from three replicates; *bars*, SD

($p < 0.05$). The IC_{50} of DNR against SW620 and K562 under hypoxia increased by 2–5-fold compared to normoxic conditions (IC50 = 50 nM, 44 nM in hypoxia vs. IC50 = 11 and 21 nM in normoxia in SW620 and K562, respectively) (Table 1). Under hypoxia, the glucose transporter inhibitor phloretin (50 μM) significantly enhanced DNR's cytotoxic effects in both SW620 and K562 cells ($p < 0.05$, Fig. 3c, d), and overcame the hypoxia conferred drug resistance. In contrast, phloretin did not show any effect on cytotoxicity of DNR under normoxic conditions (Table 1). This suggests tumor selectivity for glucose transport inhibitors under hypoxia. Although phloretin did not exhibit any cell killing effect at concentration less than 50 μM, higher concentration of phloretin (1 mM) completely blocked glucose uptake and inhibited cell growth in both drug sensitive and drug-resistant cancer cells (SW620, K562, and K562/Dox, data not shown).

Inhibition of glucose transporters synergistically enhances daunorubicin cytotoxicity and induces apoptosis in drug-resistant cancer cells

To study the role of glucose transporters in cancer cell survival and drug resistance, we tested the glucose transporter inhibitor for cell survival in the drug-resistant cancer cells K562/Dox. K562/Dox was induced to exhibit drug resistance by treatment of low concentration of doxorubicin. To confirm the drug resistance of K562/Dox, we studied the cytotoxicity of DNR by MTS assay and explored the resistance mechanisms. DNR showed a much higher IC_{50} (>5 μM) against the drug-resistant K562/Dox cells than against the drug-sensitive K562 cells (21 nM) (Fig. 4a). These data strongly suggest that K562/dox cells are resistant to chemotherapy.

We then looked for the possible mechanisms of resistance that may be responsible for the drug resis-

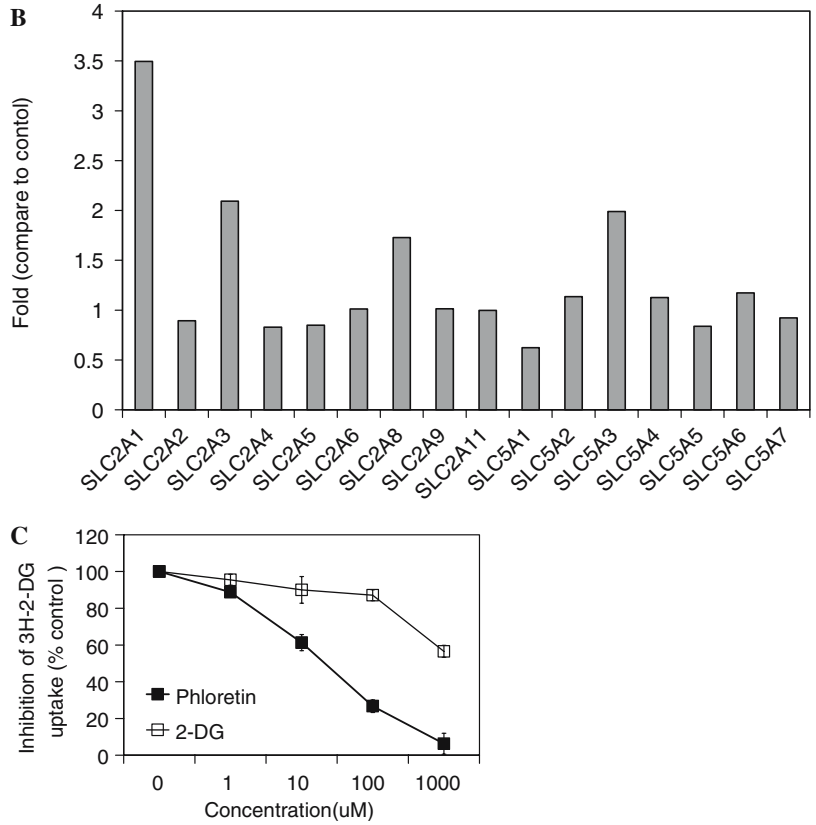

Fig. 2 continued

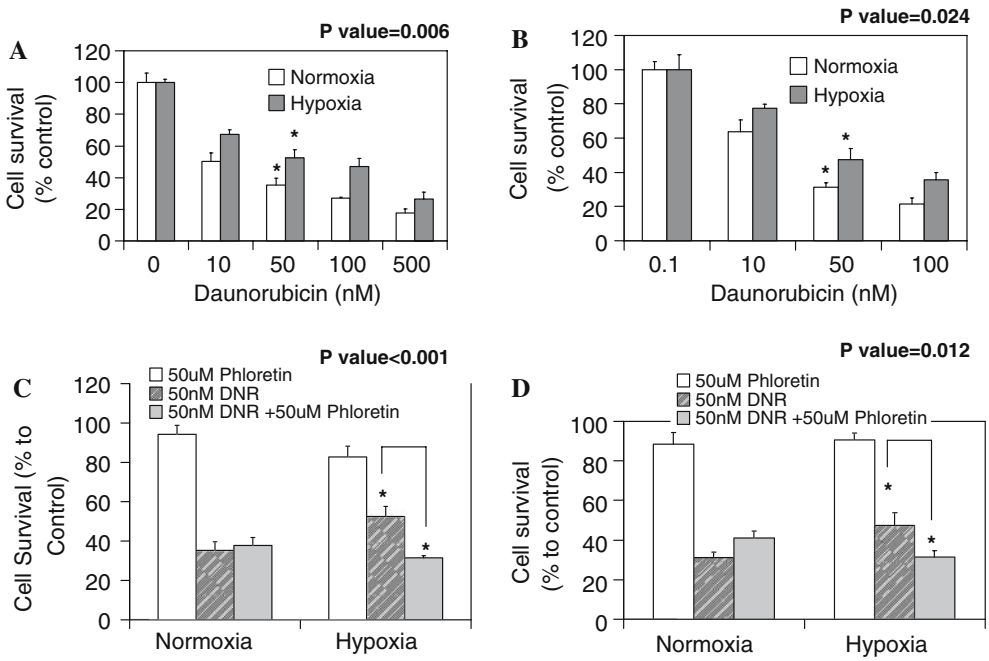

Fig. 3 Hypoxia-induced drug resistance is reversed by inhibition of glucose uptake in SW620 and K562. Cells were treated with the indicated concentration of DNR and/or phloretin for 72 h at 37°C under either normoxia or hypoxia. Cytotoxicity of different treatments was measured by cell survival rate through MTS assay. **a** Hypoxia-induced drug resistance against DNR in SW620. **b** Hypoxia-induced drug resistance against DNR in K562. **c** Glucose transporter inhibitor enhanced anticancer effect of DNR in SW620 under hypoxia. **d** Glucose transporter inhibitor enhanced anticancer effect of DNR in K562 under hypoxia. **p*-values were calculated by *t*-test

Table 1 IC50 (nM) of daunorubicin (*DNR*) under normoxia and hypoxia

IC50 (nM)	SW620		K562	
	Normoxia	Hypoxia	Normoxia	Hypoxia
DNR only	11.38 ± 2.77	50.44 ± 16.2	21.5 ± 3.8	43.6 ± 5.7
Phloretin only	>300,000	>300,000	>300,000	>300,000
DNR + 50 μM phloretin	14.15 ± 3.87	24.88 ± 3.5	35.3 ± 4.6	20.4 ± 1.6

tance of K562/DOX. One of which is the over-expression of MDR1, an ABC transporter with the gene product of P-gP. Real-time PCR confirmed that P-gp is over-expressed in K562/Dox by more than 700-fold compared to its drug-sensitive parent cell line K562 (Fig. 4b). Due to the P-gp over-expression in K562/Dox, P-gp exports anticancer drugs such as DNR out of the cells, which decreases intracellular drug concentrations, and thus confers drug resistance. In this case, the P-gp inhibitor cyclosporine A (CsA) can be used to block the drug efflux property of P-gp which then increases intracellular drug concentration, as confirmed by the flow cytometry (FACS) (Fig. 4c).

To test our hypothesis that glucose transporter inhibitors may sensitize drug-resistant cancer cells to chemotherapeutic compounds, we tested the cytotoxicity of DNR in combination with the glucose transporter inhibitor (phloretin) in K562/Dox by MTS assays. Fifty micromolar phloretin significantly enhanced anticancer activity of DNR in K562/Dox under hypoxia ($p = 0.025$) (Fig. 4d). For instance, DNR showed 40–50% cell killing effects at 1 μM, while a combination of DNR and phloretin showed more than 70% cell killing effects under hypoxia. However, either phloretin alone or combination with DNR under normoxia did not show any significant cell killing effect or enhanced effect.

Under the same conditions, phloretin also enhanced DNR-induced apoptosis in the drug-resistant K562/Dox cells as measured by Annexin V staining (Fig. 5a),

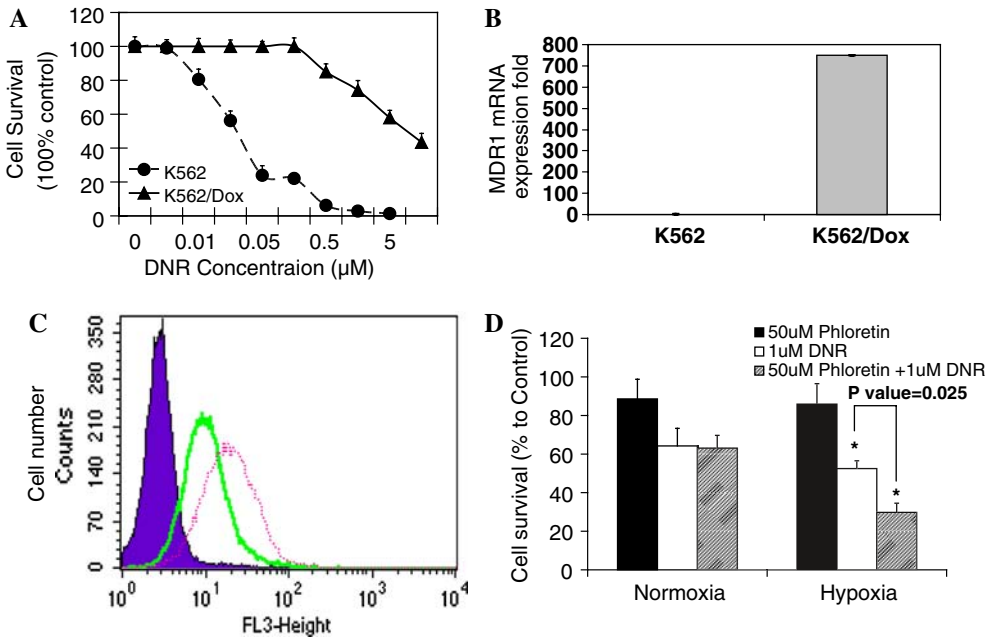

Fig. 4 Inhibition of glucose transporter overcomes drug resistance in P-gp over-expressed drug-resistant cells K562/Dox. **a** Drug resistance of K562/Dox against DNR over DNR-sensitive K562 cells. **b** MDR1 and P-gp expression level in K562 and K562/Dox cells. Bar graph was the relative MDR mRNA level normalized to beta-actin by real-time PCR. **c** DNR intracellular accumulation in K562/Dox in the presence (*red line*) or the absence (*green line*) of CsA. Differently treated Cells were incubated in 37°C for 30 min, washed and analyzed by flow cytometry. **d** Glucose transporter inhibitor enhanced daunorubincin anticancer activity (MTS assay) against drug-resistant K562/Dox cells under hypoxia. *Columns* and *points*, means from three replicates; *bars*, SD; **p*-values were calculated by *t*-test

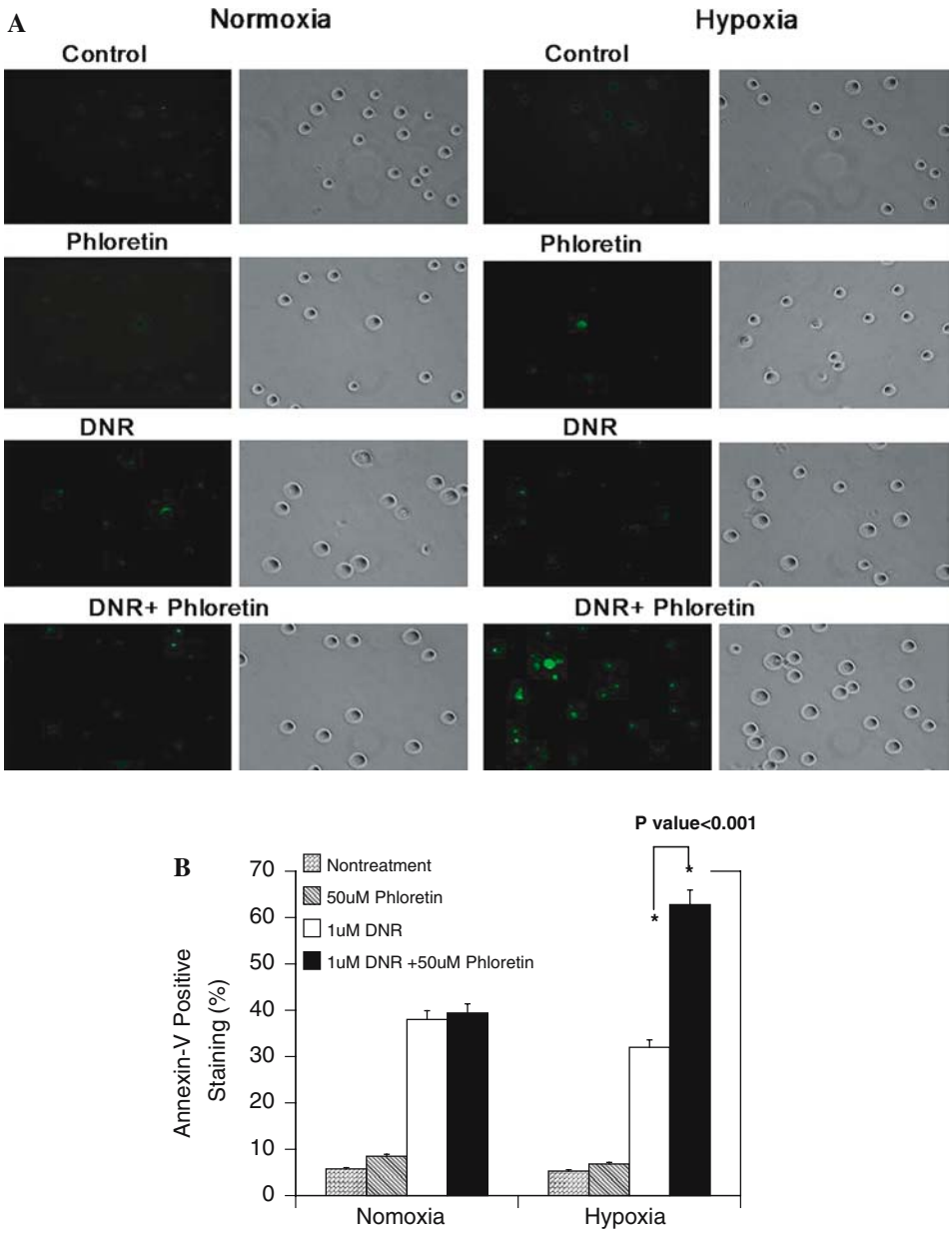

Fig. 5 Phloretin enhances DNR-induced apoptosis in drug-resistant K562/Dox under hypoxia. One micromolar DNR was used alone or combined with 50 μM phloretin to induce apoptosis in K562/Dox. Annexin-V-FITC staining was used to detect the early stage apoptotic cells. **a** Apoptotic cells were observed by fluorescence microscope. *Green cells* indicate the early stage apoptosis due to the exposure of PS to the cell surface (*left panels*). *Righ panel* showed the same cells under light phase-contrast microscopy. **b** Statistically counted Annexin-V positive staining cell numbers under different treatments. *Columns*, means from three replicates; *bars*, SD; **p*-values were calculated by *t*-test

while phloretin alone at 50 μM did not significantly induce apoptosis. In contrast to DNR alone (1 μM) induced apoptosis by 32%, combination of phloretin and DNR significantly increased apoptotic rate to 63% under hypoxia ($p < 0.001$, Fig. 5b). However, phloretin did not show any significant enhancement for the DNR induced apoptosis under normoxia. These data suggest that drug-resistant cancer cells rely heavily on glucose uptake and glycolysis rate for their survival along with efflux protein activities especially under hypoxia. Blocking glucose transporters and subsequent glycolysis may overcome drug resistance under hypoxia. The preferential effect of glucose inhibitor provides tumor selectivity under hypoxia.

Discussion

Most solid tumors show prolonged hypoxic characteristics. However, cancer cells can adapt to hypoxia for survival and growth, while normal tissues undergo apoptosis or necrosis [12]. To preserve the homeostatic levels of high-energy phosphates and prevent cell injury, glucose transporters are upregulated to increase the supply of glucose for the cancer cell's energy needs through a higher rate of anaerobic glycolysis under a hypoxic environment. Indeed, the glycolysis rate and metabolism alteration are associated with increased metastasis and poor survival in cancer patients, and hypoxia in tumors causes resistance to radiation ther-

apy and a wide variety of chemotherapeutic agents [34, 35]. For example, the IC_{50}'s of cisplatin, oxaliplatin, gemcitabine, etopophos, bleomycin, mitomycin C, irinotecan, and paclitaxel are increased by 5–100-fold under hypoxia against human embryonal carcinomas [36]. Our data also showed that hypoxia confers 2–5-fold higher resistance to DNR in the drug-sensitive SW620 and K562 cell lines. Thus, in vitro assay under normoxia might overestimate the anticancer drug activity, and the hypoxia induced drug resistance should not be neglected in a solid tumor model.

Multidrug resistance is a simultaneous development of resistance to a variety of anticancer drugs in cancer therapy. Strategies for reversing drug resistance have been explored based on over-expression of ABC transporters and oncogenic alterations of apoptotic and metabolism pathways [37]. However, limited success has been achieved. Huang laboratory reported that a high-rate glycolysis, which is induced by mitochondrial defects in the respiration deficient cells, C6F (derived from HL-60), confers drug resistance to chemotherapeutic compounds including arabinofuranosylcytosine (ara-C), doxorubicin, taxol, and vincristine [11]. These cells are sensitive to glycolysis inhibition by a hexokinase II inhibitor (3-BrPA). The activity of 3-BrPA against drug resistant cancer cells is via the dephosphorylation of BAD and translocation of BAX to the mitochondria, which then causes cytochrome C to be released for apoptosis. Our data showed that cancer cells rely heavily on glucose (and subsquent glycolysis) for their survival under hypoxia. In the P-gp over-expressed drug resistant cell line K562/Dox, although hypoxia did not further enhance the drug resistance (Fig. 4d), the blocking of glucose transporters still synergistically increased DNR-induced cytotoxicity and apoptosis under hypoxia (Figs. 4d, 5b). It suggests that both the hypoxia-induced drug resistance and the MDR-mediated resistance are energy dependent under hypoxic conditions. However, the glucose transporter inhibitor did not show any effect on the cytotoxicity and apoptosis of DNR under normoxic conditions. The preferential effect of the glucose uptake inhibitor may provide tumor selectivity in an in vivo model.

In summary, we demonstrated that GLUT1 was over-expressed in primary colon and breast cancer tissues, especially in hypoxic regions. The expression profiles of all glucose transporters in the NCI 60 cells exhibited distinct expression patterns in various cancer cells, in which GLUT1 was highly expressed in colon cancer cells (SW 620), non-small cell lung carcinoma (HOP-92) and renal carcinoma (RXF 393). Hypoxia conferred drug resistance in cancer cells, while inhibition of glucose transporters sensitized cancer cells to chemotherapeutic drugs and overcome drug resistances only under hypoxia. These data suggest that cancer cells under hypoxia rely heavily on glucose transporters for their survival through the uptake of glucose and subsequent glycolysis. Inhibition of glucose transporters may preferentially sensitizes cancer cells to chemotherapeutic agents under hypoxia. Blocking the function of glucose transporters may provide a novel therapeutic strategy for overcoming drug resistance in hypoxia.

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