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SILYBIN COUNTERACTS DOXORUBICIN RESISTANCE BY INHIBITING GLUT1 EXPRESSION

Daniela Catanzaro^a, Daniela Gabbia^a, Marco Biagi^b, Eugenio Ragazzi^a, Monica Montopoli^{a,*}, Maria Carrara^a

^aDepartment of Pharmaceutical and Pharmacological Sciences, University of Padova, 35131 Padova, Italy. daniela.catanzaro@unipd.it; daniela.gabbia@unipd.it; eugenio.ragazzi@unipd.it; maria.carrara@unipd.it; monica.montopoli@unipd.it

^bDepartment of Physical Sciences, Earth and Environment, University of Siena, Italy. biagi4@unisi.it

*corresponding author - monica.montopoli@unipd.it

ABSTRACT

Despite significant advances in the diagnosis and treatment of cancer, the development of drug resistance still remains one of the principal causes that hampers the effectiveness of the therapy. Emerging evidences support the idea that the dysregulated metabolism could be related to drug resistance. The major goal of this study was to target cancer metabolic pathways using new pharmacological approaches coming from natural sources in order to possibly prevent or overcome this phenomenon. Firstly, the metabolic profile of human colorectal adenocarcinoma cells sensitive (LoVo WT) and resistant to doxorubicin (LoVo DOX) was delineated demonstrating that resistant cells remodel their metabolism toward a glycolytic phenotype. In particular it was observed that doxorubicin-resistant cancer cells exhibit an increased dependency from glucose for their survival, associated with overexpression of the glycolytic pathway. Moreover, both GLUT1 mRNA and protein expression significantly increased in LoVo DOX cells. Given the results about the metabolic profile, silybin, modulator of GLUTs, was selected as potential candidate to overcome doxorubicin resistance and, intriguingly, data revealed not only that silybin is more active in resistant cells than in wild type cells, but also that the combined treatment with doxorubicin and silybin presents an additive effect in LoVo DOX cells. Although many unanswered questions still remain about the molecular mechanism of silybin, these data strongly support that targeting GLUT1 may be a good strategy to restore doxorubicin sensitivity and elude drug resistance.

Keywords: Silybin, cancer chemosensitization, doxorubicin, drug resistance

ABBREVIATIONS

GLUTs: glucose transporters; OXPHOS: oxidative phosphorylation; PFKM: 6-phosphofructokinase; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; PGK1: phosphoglycerate kinase 1; LDHA: L-lactate dehydrogenase A.

1. INTRODUCTION

The use of natural products as therapeutic agents has a long history and, to date, numerous types of bioactive substances continue to be isolated and characterized [1-4]. Nowadays, more than half of chemotherapeutic agents derive from natural sources including plants, animals, marine organism

and microbes [5,6] and a number of promising molecules are already in clinical trials. Even if not all the isolated molecules have been proven to be eligible for treating cancer, they often represent an irreplaceable source of novel structures useful to develop new drugs [7]. The anticancer activity of phytochemicals is generally attributed to their capability to induce apoptosis or cell cycle arrest [8-10].

Silybin, as diastereoisomers A and B, is the major component of silymarin, flavonolignan mixture extracted from *Silybum marianum* (L.) Gaertner fruits, also containing isosilybin (as diastereoisomers A and B), silidianin and silicristin. This extract has been used in the treatment of liver diseases for over two millennia, but in the last decades its role as anticancer agent has emerged [11,12]. Silybin activity was traditionally attributed to its antioxidant effects, but it is now well established that its antiproliferative properties are due to more complex molecular mechanisms. Among others, its capability to regulate cancer metabolism has recently emerged, by inhibiting glucose uptake through competitive interactions with GLUTs [13].

Despite the advances in the diagnosis and treatment of cancer, the development of drug resistance still remains one of the major causes that hampers the effectiveness of the therapy. It is thus evident that new anticancer strategies are needed and, in this scenario, naturally-derived compounds become a potentially effective source to better investigate. It is known that drug resistance is a multifactorial phenomenon whose molecular mechanism is still not completely understood [14]. Current studies support the idea that, among others, drug resistance might be correlated with the dysregulation of cancer cell metabolism [15]. Cancer metabolism is an emerging hallmark that only recently has been deeply explored to elucidate which biochemical processes are involved in the growth and reproduction of cancer cells, in the maintenance of their cellular structures, and in their response to environmental alterations. In contrast to normal cells, cancer cell metabolism appears to be adapted to facilitate the uptake and the incorporation of nutrients [16]. Indeed, several altered oncogenes and tumor suppressor genes directly control and activate metabolic pathways that maintain and enhance an efficient wiring between the glycolysis, the oxidative phosphorylation (OXPHOS), the pentose phosphate pathway and the glutamine metabolism, that allow for both NADPH production and acetyl-CoA flux to the cytosol for lipid synthesis [17].

In our previous works, we demonstrated the involvement of energetic metabolism in the onset of drug resistance revealing that cisplatin-resistant ovarian cancer cells underpin profound metabolic changes as compared with their sensitive counterpart [18,19]. In this study we investigated the hypothesis that doxorubicin-resistant cells might present a similarly altered phenotype that could be effectively targeted to restore drug sensitivity. With this aim, we used LoVo colorectal adenocarcinoma cells sensitive and resistant to doxorubicin as *in vitro* model and we found that resistant cells rewire their metabolism toward the glycolytic pathway.

Recent studies demonstrated that some natural compounds, among their other activities, are able to counteract drug resistance, being active both in sensitive and resistant cells and restoring drug

efficacy if associated with the traditional chemotherapeutic agent [20-23]. Against this backdrop, we identified silybin, a modulator of glucose transporters (GLUTs), as a good candidate for a combinatory therapy with doxorubicin to enhance drug efficacy and possibly overcome drug resistance.

2. RESULTS

2.1 Cell viability after metabolic stresses

To understand which metabolic pathway was preferentially exploited by resistant cells to produce ATP, cell viability was measured after exposure to three different experimental tools causing metabolic stress. The increase of anaerobic glycolysis, even in the presence of oxygen (Warburg effect), is the first observation indicating the alteration of energetic metabolism used by tumor cells as a strategy to adapt and grow independently from the availability of the substrate [24]. Thus, LoVo sensitive (wild type: LoVo WT) and resistant to doxorubicin (LoVo-DOX) were incubated for 24 hours in a glucose-free medium and its effect was tested by trypan blue exclusion assay. As expected, glucose deprivation reduced cell viability of both cell lines (**Figure 1A**). Of note, the percentage of viable cells, with respect to control, was 50% in wild type cells, while was around 15% in doxorubicin-resistant cells, suggesting a higher dependency from glucose for their survival. To verify the hypothesis of a metabolic switch from OXPHOS towards glycolysis, two different metabolic tools causing mitochondrial stress were used. Cancer cells were therefore incubated in a glucose-free/galactose medium to force ATP production through mitochondrial OXPHOS [25], or in medium added with rotenone 1 μ M, to block the mitochondrial respiratory chain by inhibiting the NADH-CoQ oxidoreductase [26]. Of note, cell viability of resistant cells, as compared to wild-type cells, is higher after rotenone treatment and equal after galactose exposure (**Figure 1B-C**). These results suggest that resistant cells only partially rely on mitochondria for energy production.

2.2 Mitochondrial functionality and morphology

Data about LoVo mitochondrial asset were further confirmed by labeling cells with Mitotracker Orange, a potential-dependent mitochondrial probe that gives information about mitochondrial morphology and functionality. Representative images, acquired by Zeiss confocal microscope, evidence a very different mitochondrial organization between wild type and doxorubicin-resistant cells. While LoVo WT present a well organized mitochondrial network with tubular structure, LoVo-DOX display a drastic fall of mitochondrial potential associated with a complete lack of mitochondrial network structure (**Figure 2**).

2.3 mRNA levels and protein expression of glycolytic enzymes

To shed some light on the metabolic alterations previously observed in cancer resistant cells, the relative mRNA expression of some key enzymes involved in the glycolytic flux was determined by qRT-PCR. As shown in **Figure 3A**, LoVo-DOX resistant cells present higher mRNA levels of some glycolytic enzymes with respect to LoVo WT, confirming an increased dependency from this

metabolic pathway for their survival. Data about the glucose transporter GLUT1 was further confirmed by western blotting that clearly evidences a significant overexpression of GLUT1 in resistant cells (**Figure 3B**).

2.4 Cell viability after silybin treatment

Given the data about the metabolic profiling of LoVo sensitive and resistant to doxorubicin, silybin, a modulator of glucose transporters, was tested on LoVo cell viability. The effect of silybin (5-10-50 μ M) was evaluated after 24-48-72h of exposure by MTT test. Of note, silybin resulted more cytotoxic in doxorubicin-resistant cells with respect to sensitive ones (**Figure 4**). Moreover, the combined treatment with doxorubicin and silybin, showed an additive effect in LoVo-DOX cells (**Figure 5**), suggesting that the up-regulation of GLUTs could be a targetable mechanism of resistance to doxorubicin. The experiment was performed also after 24-48 hours of treatment (data not shown), but the additive effect was observed only after 72 hours (**Figure 5**).

2.5 GLUT1 protein expression after silybin treatment

To verify the molecular mechanism responsible of silybin antiproliferative effect, the protein expression of GLUT1 was measured after treatment with silybin. As shown in **Figure 6**, silybin 10 μ M reduced GLUT1 expression both in sensitive and doxorubicin-resistant cells, while silybin 50 μ M resulted effective only in resistant cells. These data indicate the ability of silybin to modulate glucose transporters, a mechanism which was selectively cytotoxic in resistant cells.

3. MATERIAL AND METHODS

3.1 CELL LINES

LoVo human colorectal adenocarcinoma cells derived from metastatic supraclavicular region (kind gift of Dr. Giuseppe Toffoli, CRO Aviano National Cancer Institute, Italy), were grown in Ham's F12 medium supplemented with 10% fetal bovine serum (FBS), 2mM glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin, in humidified condition at 5% CO₂ and 37°C. Doxorubicin-resistant cells (LoVo-DOX) were obtained from wild type cells by repeated exposure to doxorubicin and were maintained in culture with 100 ng/ml doxorubicin.

All reagents for cell culture were from Cambrex-Lonza (Basel, Switzerland) and FBS from Gibco, Invitrogen (Carlsbad, CA, USA).

3.2 CELL VIABILITY ASSAYS

3.2.1 Trypan blue exclusion assay

12-multiwells plates were seeded with a constant number of cells and, following overnight incubation, were exposed to different treatments according to experimental protocols. After treatments, cells were washed, detached with 0.25% trypsin-0.2% EDTA and suspended in trypan blue (Sigma-Aldrich, St Louis, MO, USA) at 1:1 ratio in medium solution [27]. Cells were counted using a chamber Burkert hemocytometer.

3.2.2 MTT test

96-multiwells plates were seeded with a constant number of cells and, following overnight incubation, were exposed to different treatments according to experimental protocols. After 24-48-72 hours of treatment, 20 μ L/well of a 5 mg/mL MTT solution (Sigma-Aldrich) was added and cells incubated for 4 hrs at 37°C. The formazan crystals were dissolved by adding 200 μ L of acidic isopropanol and the absorbance (Abs) was measured at 570 nm using a Victor3X multilabel plate counter (Wallac Instruments, Turku, Finland).

3.3 CONFOCAL MICROSCOPE

Cells were seeded at approximately 30% of confluence on glass cover slips and incubated overnight. After 24h, cells were incubated for 30 min at 37°C with 25 nM Mitotracker Orange (Molecular Probes, Invitrogen, Carlsbad, CA, USA) and, after washing, were fixed with 4% formaldehyde (Sigma-Aldrich) for 15 min. The coverslips were mounted on glass slides by using Mowiol 40-88 (Sigma-Aldrich) added with μ M of DAPI (Sigma-Aldrich). Cells were imaged using a laser scanner microscope (Zeiss LSM 800, 60X magnification). A volumetric reconstruction was then obtained and analyzed using the software ImageJ.

3.4 QUANTITATIVE REAL-TIME PCR (qRT-PCR)

Total mRNA was isolated with TRIzol (Life Technologies, Carlsbad, CA, USA) as previously described by Chomczynski P and Sacchi N [28] and measured with a NanoDrop Spectrophotometer (Thermo Scientific, Waltham, MA, USA). The relative expression of each gene was determined by quantitative real-time PCR (Eco™ Illumina, Real-Time PCR system, San Diego, CA, USA) using One Step SYBR PrimeScript RT-PCR Kit (Takara Bio, Inc., Otsu, Shiga, Japan) and the primers designed as follow: GLUT1: F atgggctctcgaactggg R ccgcagtacacaccgatgat; PFKM: F gccatcagccttgacaga R ctccaaaagtcgcatcactg; GAPDH: F ctgactcaacagcgacacc R gtggtccaggggtcttactc; PGK1: F cagctgctgggtctgtcat, R gctggctcggctttaacc; LDHA: F tggcagccttttccttagaa R cgctccaataacacggttt. Melt-curve analysis was used to confirm the specificity of amplification and absence of primer dimers. All genes were normalized to β -actin designed as follow: F ccaaccgcgagaagatga R ccagaggcgtagaggatag. Expression levels of the indicated genes were calculated by the $\Delta\Delta C_t$ method using respectively the dedicated StepOne software or Eco™ Software v4.0.7.0.

3.5 WESTERN BLOT ASSAY (WB)

6-multiwells plates were seeded with a constant number of cells and, following overnight incubation, were exposed to 10-50 μ M silybin (Sigma-Aldrich, CAS: 22888-70-6). After 48 hours of treatment, cells were lysed with ice-cold lysis buffer supplemented with the protease inhibitor cocktails (Roche Molecular Biochemicals, Mannheim, Germany). The protein content was determined by Lowry procedure (Bio-rad DC Protein Assay, MA, USA). Equal amounts of protein (15 μ g) were loaded on a polyacrylamide gel and electrophoretically separated in running buffer. After electrophoresis, the proteins were blotted onto an Hybond-P PVDF membrane (Amersham Biosciences, Buckinghamshire, UK). After blocking with a 10% skim milk solution, the membrane was exposed to

the primary antibody anti-GLUT1 (1:2000; AbCam, Cambridge, UK) and, after washing, it was incubated with HRP-conjugated anti-rabbit secondary antibody (1:3500; PerkinElmer, MA, USA). The signal was visualized with an enhanced chemoluminescent kit (Amersham Biosciences) according to the manufacturer's instructions and analyzed by Molecular Imager VersaDoc MP 4000 (Bio-Rad, Hercules, CA, USA). GLUT1 was normalized to β -actin (1:7000; AbCam, Cambridge, UK).

3.6 STATISTICAL ANALYSES

All data were analyzed with GraphPad software and are expressed as mean \pm SEM. One sample *t*-tests was used to analyze results expressed as ratio of control (qRT-PCR and WB), while unpaired Student's *t*-tests was used to analyze all the other results. Significance was considered at $p < 0.05$.

4. DISCUSSION

To date, cancer is the second commonest cause of human death after cardiovascular diseases [29]. Current treatments include radiotherapy, chemotherapy and surgery, but, despite the advances of the last decades, the development of drug resistance still remains one of the major causes that hampers therapy effectiveness. It is thus evident that new anticancer strategies are needed and, in this scenario, naturally-derived compounds become a potentially effective source to better investigate.

It is now well established that cancer cells are characterized by aberrant proliferation supported, among others, by metabolic adaptations that, enhancing nutrients uptake and incorporation, satisfy their energy demand and biosynthesis requirements for rapid cell growth and division [16,30]. Metabolic reprogramming, with genomic instability, inflammation and evasion from the immune system, is therefore believed one of the hallmarks of cancer [30]. Several studies already verified that dysregulated metabolism might be involved also in the onset of drug resistance [15,18,19]. In this work we demonstrated that human colorectal adenocarcinoma cells resistant to doxorubicin remodel their energetic metabolism toward a glycolytic phenotype. With this assumption, the study aimed to identify natural compounds that, targeting this specific pathway, might overcome drug resistance.

Cancer cell metabolic switch from OXPHOS to glycolysis was firstly observed by Otto Warburg in the first half of the 20th century [31]. He showed that tumor cells exhibit high rates of glucose uptake and lactate production even in the presence of oxygen, postulating this metabolic rewiring as a consequence of mitochondrial injuries leading to increased aerobic fermentation, critical event for the origin of cancer [24]. Consistent with this view, in this study we demonstrate that colorectal adenocarcinoma cancer cells resistant to doxorubicin (LoVo-DOX) underpin a profound remodeling of their mitochondrial asset, with loss of mitochondrial potential associated with network disorganization. In line with a compensatory activation of glycolysis in the presence of mitochondrial defects, LoVo-DOX also show a metabolic shift from OXPHOS toward the glycolytic pathway as demonstrated by significant increased level of GLUT1 mRNA and protein expression correlated with a higher dependency from glucose for their survival.

Cancer's reliance on glucose, as primary source of carbons for energy production, is not a news in oncologic knowledge. The higher glucose uptake and metabolism is well documented in several types of tumors as revealed by a number of *in vitro* and *in vivo* studies [32-34]. The increased demand of glucose is essential not only to support ATP production, but overall to divert glucose intermediates to the synthesis of building blocks and precursors of DNA, fatty acids and redox regulators useful to enhance cancer growth and proliferation [35-37]. Glucose deprivation has been frequently used in oncologic studies to reduce cell proliferation and/or to force the apoptotic cascade [38,39]. In line with this evidence, we observed that both sensitive and resistant LoVo cells are affected by glucose deprivation, but intriguingly this effect is significantly more evident in LoVo-DOX cells where cell viability reduction is around 85% as compared to wild type cells where the percentage of viable cells is 50%.

In the last years several compounds have been developed to target proteins and enzymes involved in glucose transport and metabolism. 2-deoxyglucose (2-DG), scientifically recognized as GLUTs inhibitor, was proven to be effective in sensitizing human osteosarcoma and non-small cell lung cancers to adriamycin and paclitaxel [40], but unfortunately its use in clinical trials was suspended because of its toxicity [41]. Drug-correlated toxicity still remains one of the major limit of chemotherapy and the use of natural compounds with a toxicological profiles safer than synthetic molecules, might be an attractive source to overcome this issue.

Silybin is the principal bioactive component of the extract from seeds of *Silybum marianum*, a popular dietary supplement commonly used as adjuvant in the treatment of hepatitis, cirrhosis and liver damages [42-44]. Recent *in vitro* and *in vivo* studies also revealed the ability of silybin to reduce tumor formation and growth [11,12]. The activity of silybin, either alone or in combination with other chemotherapeutic compounds, was evaluated in human cancer patients, demonstrating to be well tolerated and highly available [45-48]. Its effectiveness is further proved by its ongoing on clinical Phase I [46] and Phase II (ClinicalTrials.gov Identifier: NCT00487721) trials for prostate cancer. Moreover, a number of toxicological tests proved the safety of silybin bringing out negligible side effects after human administration [46,48].

Being glucose uptake the first-limiting step of the glycolytic flux, silybin, recently proven to be a modulator of glucose transporters (GLUTs) [13,49], appeared to be a good candidate to target the over-expressed glucose metabolism of LoVo-DOX cells. As confirmation of silybin ability to interact with glucose transporters, we observed, both in sensitive and in resistant LoVo cells, a reduction of GLUT1 protein expression after silybin treatment. Interestingly, this effect resulted selectively cytotoxic in resistant cells corroborating their higher dependency from glycolysis to survive. Moreover data revealed that silybin, when associated with doxorubicin, potentiates its efficacy in resistant cells. Of note, the association of 1 μ M doxorubicin and silybin is already cytotoxic at the lowest concentration, opening up new perspectives for a future combinatory therapy.

These data strongly support our first hypothesis that targeting GLUTs may be a good strategy to restore doxorubicin sensitivity and elude drug resistance. Even if many unanswered questions still remain about the molecular mechanism of silybin, our data suggest further investigation.

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8. FIGURES

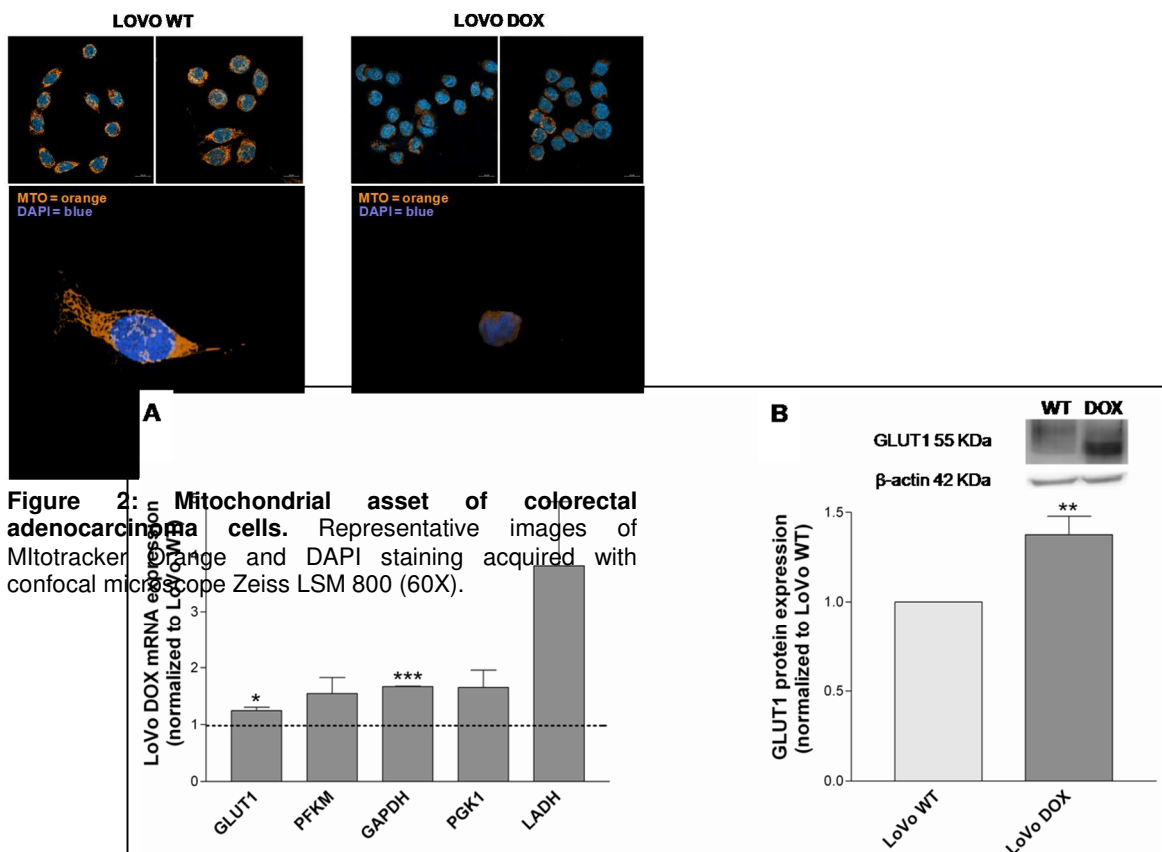
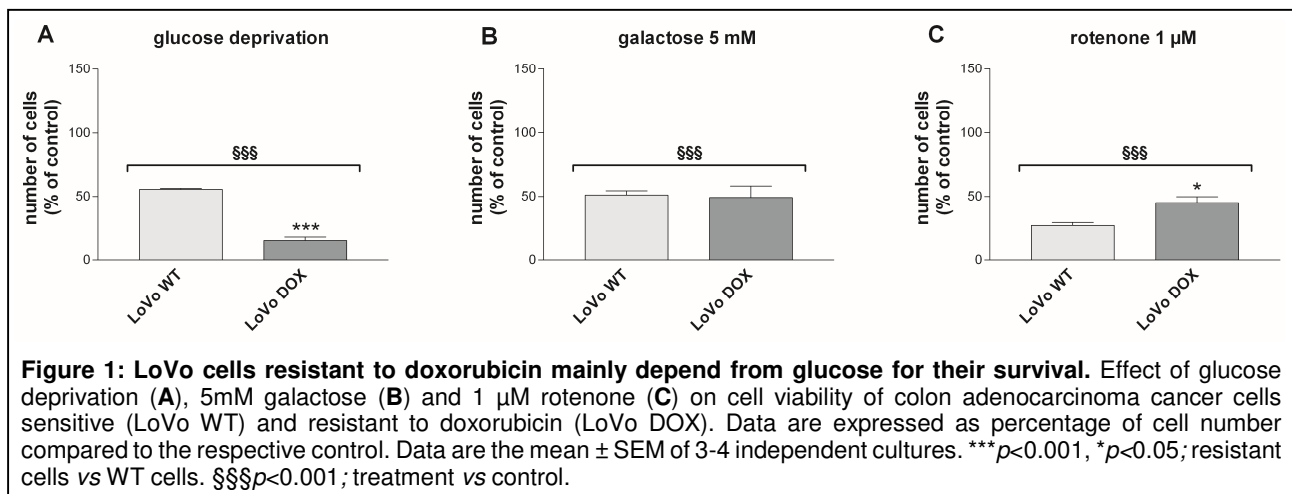


Figure 3: Doxorubicin-resistant cells over-express glycolytic proteins/enzymes. (A) mRNA levels of key proteins/enzymes involved in the glycolytic flux. All genes were normalized to β-actin. Data are the mean of 3-4 independent experiment. (B) GLUT1 protein expression measured by western blot. GLUT1 was normalized to β-actin. Data are the mean ± SEM of 8 independent experiments. **p<0.001, *p<0.01, *p<0.05; resistant cells vs WT cells.

