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Silybin counteracts doxorubicin resistance by inhibiting GLUT1 expression

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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 a synergistic effect in LoVo DOX cells. Although many unanswered questions still remain about the molecular mechanism of silybin, these data suggest that targeting GLUTs may be a good strategy to restore doxorubicin sensitivity and elude drug resistance.

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 diasteroisomers A and B, is the major component of silymarin, flavonolignan mixture extracted from *Silybum marianum* (L.) Gaertner fruits, also containing isosilybin (as diasteroisomers 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

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

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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 h 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 (Fig. 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 toward 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 μM, 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 (Fig. 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 (Fig. 2). These results were corroborated by cytometric analysis that revealed a reduced mitochondrial potential in doxorubicin-resistant cells (~0.4 fold as compared with wild type cells, Fig. 2B) that is not correlated with a lower mitochondrial mass (Fig. 2C).

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 Fig. 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 glucose and lactate transporters (respectively GLUT1 and MCT4) were further confirmed by western blotting that clearly evidences a significant overexpression of both these proteins in resistant cells (Fig. 3B–C).

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 μM) 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 (Fig. 4). Moreover, the combined treatment with doxorubicin and silybin showed a synergistic effect in LoVo-DOX cells (Fig. 5A–B), as confirmed by isobologram analysis (according to [27,28]) and by a Combination Index (CI, according to [29,30]) ranging between 0.41 and 0.81. The data suggest that the up-regulation of

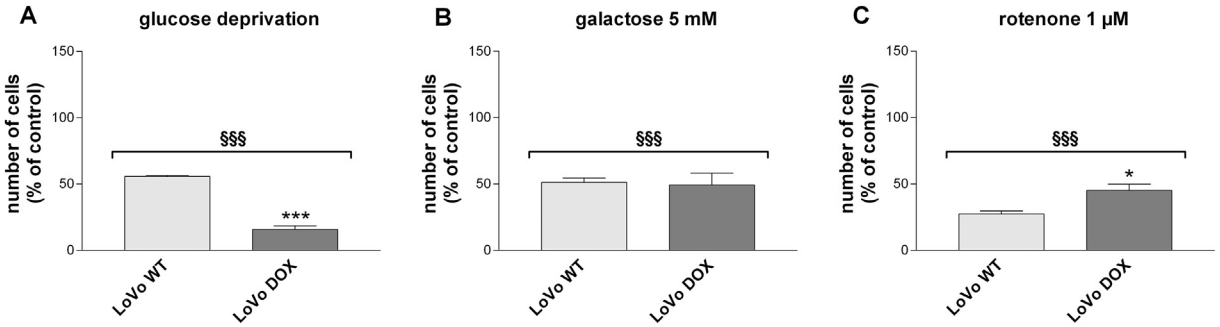

Fig. 1. LoVo cells resistant to doxorubicin mainly depend from glucose for their survival. Effect of glucose deprivation (A), 5 mM galactose (B) and 1 μM rotenone (C) on cell viability of colon adenocarcinoma cancer cells sensitive (LoVo WT) and resistant to doxorubicin (LoVo DOX). Data are expressed as percentage of cell number compared to the respective control. Data are the mean ± SEM of 3–4 independent cultures. ***p < 0.001, *p < 0.05; resistant cells *vs* WT cells. §§§p < 0.001; treatment *vs* control.