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Modulation of doxorubicin resistance by the glucose 6-phosphate dehydrogenase activity.

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SYNOPSIS

How anti-neoplastic agents induce multidrug resistance (MDR) in cancer cells and the role of glutathione (GSH) in the activation of pumps such as the multidrug resistance-associated proteins (MRPs) are still open questions. In this paper we illustrate that a doxorubicin-resistant human colon cancer cell line (HT29-DX), exhibiting decreased doxorubicin accumulation and increased intracellular GSH content and MRP1 and MRP2 expression in comparison to doxorubicin-sensitive HT29 cells, shows increased activity of the pentose phosphate pathway (PPP) and of glucose 6-phosphate dehydrogenase (G6PD). We observed the onset of MDR in HT29 cells overexpressing G6PD, accompanied by an increase of GSH; the G6PD inhibitors dehydroepiandrosterone (DHEA) and 6-aminonicotinamide (6-AN) reversed the increase of G6PD and GSH and inhibited MDR both in HT29-DX cells and in HT29 cells overexpressing G6PD. In our opinion, these results suggest that the activation of the PPP and an increased activity of G6PD are necessary to some multidrug resistant cells to keep high the GSH content, which is in turn necessary to extrude anticancer drugs out of the cell. We think that our data provide a new further mechanism for GSH increase and its effects on MDR acquisition.

KEYWORDS: multidrug resistance, doxorubicin, glucose 6-phosphate dehydrogenase, pentose phosphate pathway, glutathione.

FOOTNOTES

Abbreviations: 6-AN, 6-aminonicotinamide; DHEA, dehydroepiandrosterone; DTNB, 5,5'-dithiobis-2-nitrobenzoic acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; G6PD, glucose 6-phosphate dehydrogenase; γ -GCS, γ -glutamylcysteine synthase; GSH, glutathione; GSSG, glutathione disulfide; GST, glutathione S-transferase; LDH, lactate dehydrogenase; MDR, multidrug resistance; MRPs, multidrug resistance-associated proteins; 6PGD, 6-phosphogluconate dehydrogenase; Pgp, glycoprotein P; PPP, pentose phosphate pathway; RLIP76, Ral-interacting protein 76.

INTRODUCTION

The resistance to anticancer drugs, such as the anthracycline doxorubicin, is still a major cause of chemotherapy failure in cancer patients [1, 2]. The mechanisms involved are complex and multifactorial and may depend on an inadequate drug exposure and/or to genetic and epigenetic alterations in the cancer cell itself [3]. On the other hand, the amount of doxorubicin which can be administered is limited by its dose-dependent cardiotoxicity [4]. Tumor cells that develop drug resistance generally exhibit a cross-resistance to a large number of compounds that are chemically and structurally unrelated; this acquired multidrug resistance (MDR) is partly determined by the overexpression of efflux pumps, such as P-glycoprotein and multidrug resistance-associated proteins (MRPs) [1]. These efflux transporters show a different substrate selectivity. In particular, some MRPs are involved in the transport of glutathione- (GSH), glucuronate- or sulphate-conjugates of organic anions that arise from detoxification reactions by phase II conjugating enzymes [5], but MRPs also export GSH, and the co-transport with GSH is required for the MRP-mediated extrusion of chemotherapeutic drugs [6]. Furthermore, both carcinogenesis and MDR phenotype are frequently associated with an increased oxidative stress and activation of the cellular redox metabolism [7]. GSH is an ubiquitous tripeptide that represents the most abundant cellular thiol: owing to its reactivity and high intracellular concentration, GSH functions as an effective detoxification system that plays a pivotal role in cancer [8] and MDR development [9].

The pentose phosphate pathway (PPP) is the most important pathway for cellular GSH recycling. In the PPP oxidative phase, glucose 6-phosphate is irreversibly converted into ribulose 5-phosphate and CO₂, leading to the synthesis of NADPH, a redox cofactor for many antioxidant enzymes. Glucose 6-phosphate dehydrogenase (G6PD) catalyzes the first and rate-limiting step of the pathway, and is mainly regulated by the NADPH/NADP⁺ ratio. The NADPH produced is used by glutathione reductase to reduce glutathione disulfide (GSSG) to GSH.

Aim of the present study has been to clarify the role of PPP, and in particular of its key enzyme G6PD, in MDR. After having confirmed that doxorubicin-resistant human colon cancer cells (HT29-DX) overexpress MRP1 and MRP2, accumulate less doxorubicin and show an increased content of GSH when compared to doxorubicin-sensitive human colon cancer cells (HT29), we investigated whether the increase of intracellular GSH or G6PD may be sufficient to induce the onset of MDR phenotype in sensitive HT29 cells, and whether the G6PD inhibition may increase the cytotoxic effect of doxorubicin in drug-resistant cells.

EXPERIMENTAL

Materials. Plasticware for cell culture was from Falcon (Becton Dickinson, Bedford, MA); [$1\text{-}^{14}\text{C}$]glucose and [$6\text{-}^{14}\text{C}$]glucose were from Dupont-New England Nuclear (Boston, MA); the protein content of cells was assessed with the BCA kit. When not otherwise specified, reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Cells. Human epithelial lung adenocarcinoma cells (A549 cells), colon doxorubicin-sensitive cancer cells (HT29 cells) (both provided by Istituto Zooprofilattico Sperimentale “Bruno Ubertini”, Brescia, Italy) and doxorubicin-resistant cancer cells (HT29-DX cells), obtained as previously described [10], were cultured in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and maintained in a humidified atmosphere at 37°C and 5% CO₂. HT29-DX cells were cultured in RPMI 1640 containing 34 nMol/L doxorubicin (“maintenance” dose). Depending on the number of cells required for specific experiments, cells were plated on either 100-mm, 60-mm or 35-mm Petri dishes and cultured until 70-80% confluence approximately.

Doxorubicin accumulation. Intracellular doxorubicin accumulation was measured as described elsewhere [10] by using a Perkin-Elmer LS-5 fluorimeter (Perkin Elmer, Shelton, CT). Fluorescence was converted in nmol of doxorubicin per mg of cellular proteins, using a calibration curve.

Measurement of extracellular LDH activity. The cytotoxic effect of doxorubicin was measured as leakage of lactate dehydrogenase (LDH) activity into the extracellular medium [11], after 24 h of incubation, by using a Packard EL340 microplate reader (Bio-Tek Instruments, Winooski, VT). Both intracellular and extracellular enzyme activities were expressed as μmol of NADH oxidized/min/dish, then extracellular LDH activity (LDH out) was calculated as percentage of the total (intracellular + extracellular) LDH activity (LDH tot) in the dish.

Detection of apoptosis. Induction of apoptosis due to the cytotoxic effect of doxorubicin was evaluated using the annexin V-FITC apoptosis detection KIT, as previously described [12]. The fluorescence level of annexin V was recorded using a FACS Calibur system; the percentage of cells positive for annexin V was calculated by the Cell Quest software. Results were expressed as average percentage values obtained from triplicate experiments versus controls.

Measurement of PPP activity. The metabolic fluxes through the PPP and the tricarboxylic acid cycle were measured, as previously described [11], by using a closed experimental system to trap the $^{14}\text{CO}_2$ developed from [^{14}C]glucose in 1 h, in the absence or presence of an oxidative stress (menadione). The PPP metabolic flux (expressed as pmol CO₂/h/mg cell proteins) was obtained by subtracting the amount of CO₂ developed from [$6\text{-}^{14}\text{C}$]glucose from the CO₂ released from [$1\text{-}^{14}\text{C}$]glucose. The extent of [$6\text{-}^{14}\text{C}$]glucose metabolism, an index of the tricarboxylic acid cycle alone, did not change significantly in the different experimental conditions (data not shown).

Measurement of glutathione. The cells were washed with PBS, and 600 μl of HCl 0.01 N were added to each cell monolayer. After gentle scraping, the cells were frozen/thawed twice and the proteins were precipitated by adding 120 μl of 5-sulfosalicylic acid 6.5% to 480 μl of cell lysate. Each sample was placed in ice for 1 h and centrifuged for 15 min at 13,000 x g (4°C). The content of GSH was measured, as previously described [13], by using a Packard microplate reader EL340. Results were expressed as nmol of GSH/mg cellular proteins.

Measurement of G6PD and 6-phosphogluconate dehydrogenase (6PGD) activities. Enzymatic activities were measured by using a Packard microplate reader EL340 as previously described [11]. A first measurement is performed by adding to the assay system a saturating amount of both 6-phosphogluconate and glucose 6-phosphate; in this way the rate of NADP⁺ reduction is the result of both G6PD and 6PGD activities. A second assay is performed with 6-phosphogluconate only as a substrate; this procedure allows us to measure 6PGD activity alone. G6PD activity is obtained by subtracting the rate of the second assay from the rate of the first one [11]. Enzymatic activity was expressed as μmol of NADP⁺ reduced/min/mg of cell proteins.

Western blotting. Whole-cell extracts were directly solubilized in cold sample buffer (for G6PD; 63 mMol/L Tris, 2% SDS and 10% glycerol, pH 6.8) or SDS lysis buffer (for MRP1, MRP2 and RLIP76; 50 mMol/L Tris, 10 mMol/L EDTA, 1% SDS, pH 8.1) and supplemented with the protease inhibitor

cocktail set III (100 mMol/L AEBSF, 80 μ Mol/L aprotinin, 5 mMol/L bestatin, 1.5 mMol/L E-64, 2 mMol/L leupeptin, and 1 mMol/L pepstatin; Calbiochem-Novabiochem Corporation, San Diego, CA), 2 mMol/L phenylmethylsulfonyl fluoride and 1 mMol/L sodium orthovanadate. Samples were sonicated on ice with two 10-s bursts and incubated for 20 min at 4°C in agitation. Proteins were separated by 12% or 7% SDS-PAGE and probed with antibodies diluted in TBS-Tween 0.1% with Blocker Not-Fat Dry Milk 5%. Primary antibodies were against G6PD (from rabbit, diluted 1:250; Santa Cruz Biotechnology, Santa Cruz, CA), MRP1 (from mouse, diluted 1:50; Abcam, Cambridge, UK), MRP2 (from mouse, diluted 1:50; Abcam), RLIP76 (from mouse, dilute 1:50; Abcam) and GAPDH (glyceraldehyde 3-phosphate dehydrogenase, used as an housekeeping gene) (from rabbit, diluted 1:500; Santa Cruz Biotechnology). Blots were finally probed with horseradish peroxidase-conjugated anti-rabbit (from goat, Bio-Rad; diluted 1:4000) or anti-mouse (from goat, Bio-Rad; diluted 1:4000) antibodies. Proteins were detected by enhanced chemiluminescence (Perkin Elmer). Densitometric analysis of the bands was performed using *Image J* software; the relative quantitation of each sample was performed comparing each band with GAPDH.

Quantitative Real Time Polymerase Chain Reaction (RT-PCR). Total RNA was obtained by the guanidinium thiocyanate-phenol-chloroform method [14]. Three μ g of total RNA were reversely transcribed into cDNA and quantitative Real Time-PCR was carried out using IQ™ SYBR Green Supermix (Bio-Rad), according to the manufacturer's instructions, for the quantitation of G6PD, MRP1, MRP2, RLIP76 and GAPDH (used as an housekeeping gene). RT-PCR primers used were illustrated in Table I. PCR amplification was 1 cycle of denaturation at 95°C for 3 min, 45 cycles of denaturation at 94°C for 30 s, annealing for 30 s, synthesis at 72°C for 30 s (G6PD, GAPDH) or 1 min (MRP1, MRP2, RLIP76). The relative quantitation of each sample was performed comparing each PCR gene product with the GAPDH product, using the Bio-Rad Software Gene Expression Quantitation (Bio-Rad).

Immunofluorescence staining. 0.5 x 10⁶ cells were grown on sterile glass coverslips, rinsed with PBS, fixed with 4% w/v paraformaldehyde (diluted in PBS), washed three times with PBS and probed with primary antibodies against MRP1 and MRP2 (from mouse, diluted 1:50 in PBS + FBS 1%; Abcam) and TRITC-conjugated anti-mouse antibodies (from goat; diluted 1:400 in PBS + FBS 1%). Then, cells were washed with PBS and incubated with 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI, diluted 1:20000). Fluorescently labeled cells were washed three times with PBS and once with water, and then the slides were mounted with 4 μ L of Gel Mount Aqueous Mounting and examined with a Leica DC100 fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany). For each experimental point, a minimum of 5 microscopic fields were examined.

DNA constructs, retroviral production and cell infection. To obtain the G6PD construct, total cDNA from HT29 cell lines was amplified using specific primers flanking the G6PD coding region. Primers were as follows: G6PD forward 5'-TGAGGCGGGTCCGCTCA-3' and G6PD reverse 5'-TGCCCAGGGCTCAGAGCTT-3'. PCR products were cloned into PCRII vector using the TA-cloning system (Invitrogen) and sequenced. For retroviral production, G6PD cDNA was then cloned into the retroviral vector Pallino [15]. G6PD construct was digested to obtain a *HindIII-XhoI* fragment, and cloned into the *HindIII-XhoI* sites of Pallino. G6PD retroviruses were obtained by co-transfection of Pallino expression vector containing G6PD with pMD2VSV-G plasmid into GP-293 packaging cell line (Invitrogen). The original GP-293 cell line was created by stably transfecting the *gag/pol* genes into HEK-293 cells and selecting for the *Streptoalloteichus hindustanus* bleomycin gene. Empty vector-Pallino retroviruses were generated as control. Retroviruses released in culture medium were collected 24 h after transfection. 500 μ l of filtered (pore size 0.45 μ m) supernatants from 293GP cells were supplemented with 8 μ g/ml polybrene and added to 1 x 10⁵ target cells. After a 12 h incubation, 1 ml of complete medium was added and the cells were cultured for further 2 days. The percentages of transfected cells were analyzed for GFP expression on a FACSCalibur flow cytometer (Becton Dickinson) (data not shown). The Cell Quest software (Becton Dickinson) was used for the data acquisition and analysis.

Pgp silencing. 150,000 cells were treated for 6 h with 1 mL siRNA transfection medium, containing 5 μ L siRNA transfection reagent and 50 pmol Pgp siRNA (Santa Cruz Biotechnology). In each set of

experiments, one dish was treated with 50 pmol of Control siRNA-A (Santa Cruz Biotechnology), a non-targeting 20- to 25-nucleotide siRNA designed as a negative control, instead of Pgp siRNA. After 72 h the silencing efficacy was verified by Western blotting for Pgp protein. Cell toxicity was assessed by monitoring the extracellular release of LDH and was superimposable to the one observed in untreated cells (data not shown).

Statistical analysis. All data in text and figures are provided as means \pm SEM. The results were analyzed by a one-way Analysis of Variance (ANOVA) and Tukey's test. $p < 0.05$ was considered significant.

RESULTS

HT29-DX cells are a good model of acquired doxorubicin-resistant cells: they accumulate less doxorubicin than their normal counterpart HT29 cells and are subsequently less sensitive to its cytotoxic effects [10]. We have investigated the expression of three MRP drug efflux pumps (MRP1, MRP2 and RLIP76) which have been implicated in GSH-associated drug resistance [9]. In keeping with the data from the literature obtained in other cell types, HT29-DX cells showed a significantly increased expression (in terms of both mRNA and protein) of MRP1 and MRP2 when compared to their sensitive control (**figure 1**). On the other hand, RLIP76 expression was similar in both cellular subpopulations (**supplemental data**).

The basal level of GSH in HT29-DX cells was significantly higher than in HT29 cells (**figure 2, first dataset**), whereas on the opposite the accumulation of doxorubicin was significantly lower than in controls (**figure 2, second dataset**). In the presence of doxorubicin 5 $\mu\text{Mol/L}$, HT29 cells showed a significant decrease of GSH, as expected due to the well known ability of doxorubicin to elicit an oxidative stress [16], an effect which was not observed in HT29-DX cells (**figure 2, second dataset**).

A way to increase the intracellular level of GSH is to maintain glutathione in the reduced form by activating the PPP flux via the activation of G6PD. HT29-DX cells showed a significantly higher basal activity of the PPP versus the control (**figure 3**). When the PPP activity was stimulated by the presence of menadione, a compound which exerts an oxidative stress by generating superoxide anion through its redox cycling and by forming a conjugate with GSH [17], a very significant increase of the metabolic pathway was observed in both cell cultures (**figure 3**). The menadione-stimulated PPP activity is an useful index of maximal activation of the pathway and therefore of its antioxidant potential. HT29 and HT29-DX cells were also incubated in presence of the non-competitive G6PD inhibitor dehydroepiandrosterone (DHEA) [18-20] or the competitive inhibitor 6-aminonicotinamide (6-AN) [21, 22] for 24 h before the measurement. Both compounds were able to inhibit significantly the PPP activity, both basal and menadione-induced, in HT29-DX and HT29 cells (**figure 3**). G6PD is the key regulatory enzyme of the PPP flux. HT29-DX cells showed a significantly increased basal expression of G6PD (mRNA and protein) when compared to their sensitive counterpart (**figure 4A**).

After a 24 h incubation with either DHEA or 6-AN, the expression of G6PD was not significantly modified in both cell culture types when compared to their controls (data not shown). Resistant cells showed a significantly increased enzymatic activity in comparison with HT29 cells (**figure 4B**). A 24 h incubation of cells with DHEA or 6-AN inhibited the G6PD activity in HT29-DX cells, and also in HT29 cells but only at the higher concentration. This inhibition was quite specific, since the activity of another oxidoreductase of the same pathway, 6PGD, was not modified in comparison with controls (**figure 4B**). The different degree of inhibition of PPP and G6PD by DHEA and 6-AN can be explained with the different conditions of measurement of these two events. PPP flux is strictly related to the *in vivo* G6PD activity, and is measured at the intracellular concentrations of reagents, which are low and not saturating, so that the G6PD rate is normally far from V_{max} . Instead, the G6PD activity is measured in lysed cells in the presence of saturating concentrations of reagents, then the enzyme rate matches the V_{max} .

HT29 and HT29-DX cells were incubated for 24 h in the absence or presence of DHEA or 6-AN, then 5 $\mu\text{Mol/L}$ doxorubicin was added during the last 3 h of incubation. As previously described (**figure 2, second dataset**), the basal drug accumulation in HT29-DX cells was significantly lower than the one observed in HT29 cells (**figure 5A**). DHEA and 6-AN induced a significant increase of intracellular doxorubicin in resistant HT29-DX cells compared to HT29 cells; a significant increase was observed also in sensitive HT29 cells incubated with DHEA, but only at the highest concentration with 6-AN (**figure 5A**). To clarify the eventual role of Pgp in the accumulation of doxorubicin, HT29-DX cells were silenced for 72 h for Pgp [23] and subsequently were incubated with 5 $\mu\text{Mol/L}$ doxorubicin for 24 h, in the presence or absence of DHEA (100, 250 and 500 $\mu\text{Mol/L}$). In Pgp-silenced HT29-DX cells, DHEA treatment did not significantly modify the accumulation of doxorubicin versus the Pgp-containing cells (**supplemental data**). LDH is a soluble cytosolic enzyme that is released into the culture medium following loss of membrane integrity, and it represents a sensitive index of cytotoxicity.

Furthermore, the cell damage induced by doxorubicin was also investigated in terms of apoptosis as fluorescence of cell-associated annexin V. As previously described [10], after a 24 h incubation doxorubicin caused in sensitive cells an increase of LDH release into the extracellular medium and of the percentage of cells positive for annexin V, while it did not induce any modification in HT29-DX cells (**figures 5A** and **5B**). DHEA and 6-AN were able to increase the concentration-dependent cytotoxic effect of doxorubicin, observed after a 24 h incubation, only in HT29-DX cells (**figure 5A**), while in the absence of doxorubicin they did not modify the LDH release (data not shown). On the other hand, apoptosis induced by doxorubicin was detectable after a 24 h incubation with DHEA or 6-AN in both cell types: in the absence of doxorubicin the percentage of annexin V-positive cells was significantly higher only in control HT29 cells (**figure 5B**).

The PPP flux is very important to provide the cell with the NADPH necessary to keep GSH in the reduced form. We had previously observed that the intracellular content of GSH, in agreement with the PPP activity, shows a significantly higher level in HT29-DX cells than in the sensitive cells. After a 24 h incubation of cells with DHEA or 6-AN, a significant decrease of GSH was observed in both cell types (**figure 5C**).

We tried to induce a doxorubicin resistance in HT29 cells by enhancing their GSH intracellular levels. After a 3 h incubation of HT29 cells with GSH 30 mMol/L, the intracellular GSH in HT29 cells was significantly increased, overcoming the level of GSH in HT29-DX cells in the absence of exogenous doxorubicin (**figure 2**). In GSH-enriched HT29 cells doxorubicin caused a slight decrease of GSH, whose content was still significantly higher than in control cells. HT29-DX cells did not show changes of the GSH level after GSH loading. The enrichment of GSH caused a significant decrease of the accumulation of intracellular doxorubicin in HT29 cells, mimicking the behavior of HT29-DX cells. This effect was lacking in A549 cells, a human epithelial pulmonary cancer cell line that accumulates doxorubicin but is not able to uptake extracellular glutathione [24] (**figure 2**). As expected, no difference in doxorubicin accumulation was observed in HT29-DX cells, in keeping with the lack of response to the GSH loading.

Finally, to demonstrate a correlation between MDR and cellular GSH content we investigated whether MDR could be induced in HT29 cells by overexpression of the *G6PD* gene. Interestingly, HT29 cells transfected with G6PD showed a significant increase in the expression of G6PD mRNA, protein (**figure 6A**) and enzymatic activity (**figure 6B**), while HT29 cells infected with the retrovirus without G6PD showed a behavior similar to control cells. The enhanced enzymatic activity was significantly reduced with DHEA or 6-AN (**figure 6B**). Moreover, the up-regulation of G6PD induced a significant increase of intracellular GSH levels in HT29 cells, similar to the one observed in non-transfected HT29-DX cells (**figure 7A**). GSH decreased significantly when cells were incubated for 24 h with DHEA or 6-AN (**figure 7A**). After a 3 h incubation with 5 μ Mol/L doxorubicin the *G6PD*-transfected HT29 cells showed a drug-resistant phenotype, assessed as reduced accumulation of intracellular drug, which was significantly reversed when cells were incubated with DHEA or 6-AN (**figure 7B**). However, although *G6PD*-transfected HT29 cells showed similar cellular GSH levels when compared to resistant cells, they accumulated more doxorubicin than HT29-DX cells, thus suggesting that the GSH content is not likely to be the only cause of drug-resistance, but other molecular mechanisms are involved. On the other hand, when we measured the extracellular levels of GSH and the intracellular/extracellular levels of oxidized glutathione we did not observe any significant change (data not shown): this means, in our opinion, that glutathione interacts in a not yet understood way with the drug and/or a transporter. On the other hand, no difference in the expression of MRP1, MRP2 or RLIP76 proteins (data not shown) was shown between HT29 cells transfected with G6PD or with empty virus only, suggesting that the modified expression of transporters is not the main responsible for the MDR.

DISCUSSION

Doxorubicin is commonly used in the therapy of solid tumors. As for many other chemotherapeutic agents, the clinical use of doxorubicin is hampered by several side effects, including MDR and cardiotoxicity [25], which impair the clinical response and the survival of patients [26]. Making cancer cells more sensitive to doxorubicin would be an efficient approach to decrease the overall dose of anthracyclines infused and to increase the ratio between therapeutic effect and toxicity. In the present study, we used a cell line derived from human colon cancer, one of the most frequent solid tumors that show MDR: from these cells we obtained a model of doxorubicin-resistant cells (HT29-DX) as previously described [10].

MDR is regarded as a multifactorial process [1], but several studies indicate that a cardinal mechanism of resistance is an increased rate of removal of the drug out of the cell [2]. Several transporters such as MRP1, MRP2 and RLIP76 require GSH for the transport activity [27-29]. Our results, showing that the G6PD transfection does not modify the MRP1, MRP2 and RLIP76 (data not shown) expression in HT29 cells, suggest that the different expression of redox-modulated transporters is not responsible for MDR in these cells.

Our results show that HT29-DX cells, in comparison to their drug-sensitive counterpart (HT29 cells), have a higher level of intracellular GSH, confirming previous data obtained in other MDR tumor cells [26, 30]. Cellular thiols could inactivate chemotherapeutics before they reach their target, and enhance DNA repair by providing a reducing environment [31], but it is well known that GSH may also be implicated in the glutathionation of chemotherapeutic agents or as a cofactor in the transport of unconjugated drugs [9].

High cellular GSH concentrations may sometimes be related to an increased expression of γ -GCS, whose increased level has been observed in MDR tumors and cell lines. Exposure of cells to a specific inhibitor of γ -GCS, buthionine sulfoximine, decreased MDR for doxorubicin and vincristine [32], by reducing the cellular GSH levels [33], whereas the exposure of these cells to GSH ethyl ester increased the GSH levels and restored the resistance to these drugs [33].

An alternative way, less investigated, to increase the level of GSH, is to activate the PPP flux via the activation of G6PD. The PPP flux is one of the metabolic pathways most sensitive to a cell oxidative stress, via the G6PD activation, and leads to the synthesis of NADPH, necessary to keep GSH in the reduced form [34]. Menadione-stimulated PPP activity is a useful index of the maximal activation of the pathway and therefore of its antioxidant potential. Several studies show that doxorubicin is able to induce oxidant species production [35]: it promotes the oxidation of GSH to GSSG inducing production of free radicals and H_2O_2 [36, 37]. We observed that an acute administration of doxorubicin in HT29 cells induces a significant decrease of GSH level and an increased G6PD activation (data not shown), but a prolonged exposure to low concentrations of doxorubicin produces a persistent oxidative stress that could lead to enhanced content of GSH and increased G6PD expression and activity. We observed that HT29-DX cells continuously exposed to doxorubicin show a significantly higher (basal and menadione-stimulated) activity of the PPP when compared to HT29 cells. Since the PPP flux is regulated by the activity of G6PD, this metabolic pathway can be considered a sensitive index of the actual G6PD activity in a whole cell. Our results show also that HT29-DX cells exhibit a significantly increased G6PD expression, in terms of both mRNA and protein, and a significantly higher G6PD enzymatic activity if compared to sensitive cells. Although substantial evidences have been provided that oxidative stress plays a role in MDR, a lot of controversial data are still present, probably due to the different cell types investigated. A decreased G6PD activity and PPP flux were observed in adriamycin-resistant MCF-7 cells [38]. An increased G6PD activity in resistant cancer cells, but no correlation between GSH level or G6PD activity and drug resistance, was detected by Yu and colleagues [39]. Anthracycline-resistant HL60/AR cells showed down-regulation of cellular GSH [40], while human embryonic kidney (HEK293) sensitive and resistant cells exhibited similar levels of intracellular GSH [39]. On the contrary, several studies demonstrated an elevated PPP cycle, G6PD activity and cellular GSH in human MDR cells [30, 42-44].

In this study, for the first time to our knowledge, we used the non-competitive G6PD inhibitor DHEA and the competitive inhibitor 6-AN to down-regulate PPP and to see their effect on the MDR phenotype. The inhibition of PPP flux and G6PD by DHEA and 6-AN was more evident in HT29-DX cells, probably because these metabolic activities were higher than in HT29 cells.

The lower ability to activate PPP in response to an oxidative stress was likely responsible for a parallel consumption of GSH, which was more evident in HT29-DX cells. DHEA and 6-AN induced in HT29-DX cells a significant decrease of GSH, reaching a level similar to the one found in sensitive cells. Doxorubicin sensitivity is known to increase in parallel with GSH depletion [45]. The increase of drug accumulation and cytotoxic effect (evaluated as leakage of LDH activity and amount of annexin V-positive cells) suggests that the MDR phenotype was reversed by DHEA and 6-AN. The treatment with DHEA did not change significantly the accumulation of doxorubicin in Pgp-silenced HT29-DX cells, thus suggesting that Pgp is not implicated in the GSH-dependent cellular efflux of doxorubicin.

To confirm our hypothesis of a linkage between GSH and drug resistance, we induced a MDR phenotype in sensitive HT29 cells by enhancing their intracellular GSH levels or by inducing overexpression of G6PD. In GSH-enriched HT29 cells we observed a decreased accumulation of doxorubicin. Moreover, a human epithelial pulmonary cancer cell line (A549) unable to uptake extracellular glutathione [24] did not show MDR acquisition. HT29 cells transfected with the *G6PD* gene showed elevated expression of G6PD mRNA, protein and enzymatic activity, a significant decrease of intracellular doxorubicin and a significant increase of intracellular GSH. Moreover, both DHEA and 6-AN incubation decreased significantly the G6PD enzymatic activity and GSH levels reverting the induction of resistance phenotype in *G6PD*-transduced HT29 cells, thus confirming the involvement of G6PD in the onset of doxorubicin-resistance.

On the basis of our data we suggest that the improving effects of G6PD transfection or GSH addition on the ability of HT29 cells to extrude cellular GSH-conjugates is not due to a modified expression of MRP1, MRP2 or RLIP76, but rather to the increased availability of intracellular GSH, which then activates one or more of these transporters. The higher content of MRP1 and MRP2 in HT29-DX cells, although not playing a primary role, may be however important in enhancing the drug extrusion.

In summary, in agreement with other data previously reported in literature about the role of redox metabolism in MDR [7], our results confirm that the activation of intracellular PPP may be one of the causes of acquired MDR phenotype, suggesting a further mechanism for GSH increase and its effects on MDR, i.e. the hyperactivity of the PPP and of its rate-limiting enzyme G6PD. Finding new inhibitors of G6PD, more active at lower concentrations than those used for DHEA and 6-AN, could allow to reduce the overall dose of doxorubicin and to increase the ratio between therapeutic benefits and side effects. Moreover, now our intention is to better understand the molecular mechanism leading to altered cellular redox metabolism in HT29-DX cells: the knowledge of this molecular pathway could allow to improve chemotherapy preventing the up-modulation of G6PD and/or protein pumps such as MRPs in cancer cells.

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The authors declare that they have no conflict of interest.

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FIGURE LEGENDS

Table I. Primers sequence.

Primers used for quantitative RT-PCR and annealing temperature.

Figure 1. MRP1 and MRP2 expression.

Relative expression of MRP1 and MRP2 mRNA and proteins, checked by quantitative RT-PCR and western blotting in HT29 and HT29-DX cells. Upper panels: means \pm SEM obtained in $n = 3$ different experiments. Vs HT29 sensitive cells: MRP1: * $p < 0.0001$; MRP2: * $p < 0.0001$. Lower panels: each figure is representative of three experiments giving similar results.

Figure 2. GSH levels and doxorubicin accumulation.

HT29, HT29-DX and A549 cells were incubated for 3 h in the absence (CTRL) or presence of doxorubicin 5 $\mu\text{Mol/L}$ (DOXO), with or without GSH 30 mMol/L (GSH) ($n = 4$), and then intracellular GSH and doxorubicin accumulation were measured as indicated in Experimental procedures. GSH: Vs HT29 CTRL: * $p < 0.05$, ** $p < 0.0001$. DOXO: Vs HT29 DOXO: ° $p < 0.05$, °° $p < 0.001$.

Figure 3. DHEA and 6-AN effects on PPP activity.

Effect of either DHEA or 6-AN on basal and menadione-stimulated PPP activity in HT29 and HT29-DX cells. Cells were incubated for 24 h in the absence (CTRL) or presence of either DHEA (100, 250 or 500 $\mu\text{Mol/L}$) or 6-AN (100, 250 or 500 $\mu\text{Mol/L}$), and PPP was measured after 1 h further of incubation in the absence or presence (MEN) of menadione 100 $\mu\text{Mol/L}$, as indicated in Experimental procedures ($n = 5$). In the tests with menadione the concentrations of DHEA and 6-AN were 100 $\mu\text{Mol/L}$. Vs HT29 CTRL: * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$; Vs HT29-DX CTRL: ° $p < 0.05$, °° $p < 0.0001$; Vs HT29 + MEN: # $p < 0.0001$; Vs HT29-DX + MEN: § $p < 0.0001$.

Figure 4. G6PD expression and activity.

(A) Relative expression of G6PD mRNA and protein, evaluated by quantitative RT-PCR and Western blotting, respectively, in HT29 and HT29-DX cells. Upper panel: means \pm SEM obtained in $n = 3$ different experiments. Vs HT29 cells: * $p < 0.02$. Lower panel: the figure is representative of three experiments giving similar results. Vs HT29 cells: * $p < 0.002$. (B) Effect of either DHEA or 6-AN on G6PD activity and 6PGD activity in HT29 and HT29-DX cells. Cells were incubated for 24 h in the absence (CTRL) or presence of either DHEA (100, 250 or 500 $\mu\text{Mol/L}$) or 6-AN (100, 250 or 500 $\mu\text{Mol/L}$) ($n = 3$). Vs HT29 CTRL: * $p < 0.05$, ** $p < 0.0001$; Vs HT29-DX CTRL: ° $p < 0.001$, °° $p < 0.0001$.

Figure 5. DHEA and 6-AN effects on doxorubicin accumulation, cytotoxicity and on intracellular GSH levels.

Effect of either DHEA or 6-AN on doxorubicin accumulation, LDH release into the extracellular medium (A) and induction of apoptosis (B) in HT29 and HT29-DX cells. For doxorubicin accumulation, cells were incubated for 24 h in the absence (CTRL) or presence of either DHEA (100, 250 or 500 $\mu\text{Mol/L}$) or 6-AN (100, 250 or 500 $\mu\text{Mol/L}$), then (excepted CTRL) in the last 3 h of incubation doxorubicin 5 $\mu\text{Mol/L}$ was added ($n = 5$); for LDH release and apoptosis, cells (excepted CTRL) were incubated for 24 h with doxorubicin 5 $\mu\text{Mol/L}$ in the presence of either DHEA (100, 250 or 500 $\mu\text{Mol/L}$) or 6-AN (100, 250 or 500 $\mu\text{Mol/L}$) ($n = 5$). (A) DOXO: Vs HT29 DOXO * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$; Vs HT29-DX DOXO: ° $p < 0.05$, °° $p < 0.001$, °°° $p < 0.0001$. LDH: Vs HT29 CTRL § $p < 0.05$; Vs HT29-DX DOXO # $p < 0.05$, ## $p < 0.001$, ### $p < 0.0001$. (B) Results were expressed as relative values obtained compared with HT29 cells (assumed as value = 1) ($n = 3$). Vs HT29 CTRL: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$; Vs HT29 + doxo: § $p < 0.05$, §§ $p < 0.005$; Vs HT29-DX + doxo: # $p < 0.05$, ## $p < 0.01$. (C) Cells were incubated for 24 h in the absence (CTRL) or presence of either

DHEA (100, 250 or 500 $\mu\text{Mol/L}$) or 6-AN (100, 250 or 500 $\mu\text{Mol/L}$) ($n = 5$). Vs HT29 CTRL: * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$; Vs HT29-DX CTRL: $^{\circ} p < 0.05$, $^{\circ\circ} p < 0.001$, $^{\circ\circ\circ} p < 0.0001$.

Figure 6. G6PD expression and activity in HT29-transfected cells.

(A) Relative expression of G6PD mRNA and protein in HT29, HT29-DX, HT29 + empty virus and HT29 + virus containing the *G6PD* gene. Upper panel: means \pm SEM obtained in $n = 3$ different experiments. Vs HT29: * $p < 0.02$, ** $p < 0.0001$; Vs HT29-DX: $^{\circ} p < 0.0001$; Vs HT29 + empty virus: $\S p < 0.0001$. Lower panel: the figure is representative of three experiments giving similar results. Vs HT29: * $p < 0.002$; Vs HT29 + empty virus: $\S p < 0.002$. (B) Basal G6PD activity and 6PGD activity and effect of either DHEA or 6-AN on enzymatic activities in HT29, HT29-DX, HT29 + empty virus and HT29 + virus containing the *G6PD* gene. Cells were incubated for 24 h in the absence (CTRL) or presence of either DHEA (100, 250 or 500 $\mu\text{Mol/L}$) or 6-AN (100, 250 or 500 $\mu\text{Mol/L}$) ($n = 3$). Vs HT29: * $p < 0.0001$; Vs HT29-DX: $^{\circ} p < 0.0001$. Vs HT29 + empty virus CTRL: * $p < 0.05$, ** $p < 0.0001$; Vs HT29 + G6PD-virus CTRL: $^{\circ} p < 0.0001$.

Figure 7. GSH levels and doxorubicin accumulation in HT29-transfected cells.

(A) Intracellular GSH levels and effect of either DHEA or 6-AN in HT29 + empty virus and HT29 + virus containing the *G6PD* gene. Cells were incubated for 24 h in the absence (CTRL) or presence of either DHEA (100, 250 or 500 $\mu\text{Mol/L}$) or 6-AN (100, 250 or 500 $\mu\text{Mol/L}$) ($n = 3$). Values in HT29 and HT29-DX cells were reported for a comparison. Vs HT29 + empty virus CTRL: * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$; Vs HT29 + G6PD-virus CTRL: $^{\circ} p < 0.005$, $^{\circ\circ} p < 0.001$, $^{\circ\circ\circ} p < 0.0001$. (B) Cells were incubated in the absence (CTRL) or presence of DHEA (100, 250 or 500 $\mu\text{Mol/L}$) or 6-AN (100, 250 or 500 $\mu\text{Mol/L}$) for 24 h; during the last 3 h of incubation doxorubicin 5 $\mu\text{Mol/L}$ was added ($n = 3$). Values in HT29 and HT29-DX cells were reported for a comparison. Vs HT29 + empty virus CTRL: * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$; Vs HT29 + G6PD-virus CTRL: $^{\circ} p < 0.0001$.

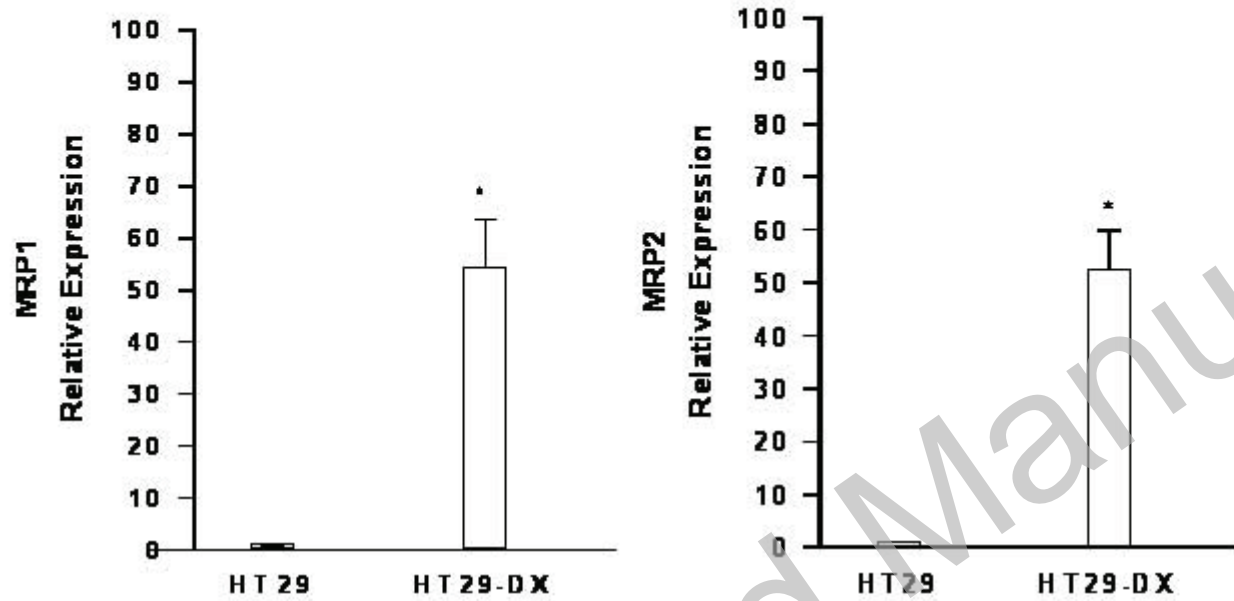
Table I. Primers sequence.

Gene	Primer forward (sequence 5'-3')	Primer reverse (sequence 5'-3')	Annealing T	PCR Product
G6PD	TGCCTTCATCAGTCGGATACAC	TGGTGGGGTAGATCTTCTTCTGG	60°C	83 bp
MRP1	TCTGGTCAGCCCAACTCTCT	CCTGTGATCCACCAGAAGGT	60°C	360 bp
MRP2	TGCTTCCTGGGGATAATCAG	CACGATAACTGGCAAACCT	56°C	211 bp
RLIP76	CAGCCATTTGGGAAAAAGAA	GCAGAGGTGAGGGGTACAAA	56°C	165 bp
GAPDH	CCCTTCATTGACCTCAACTACATG	TGGGATTTCCATTGATGACAAGC	60°C	205 bp

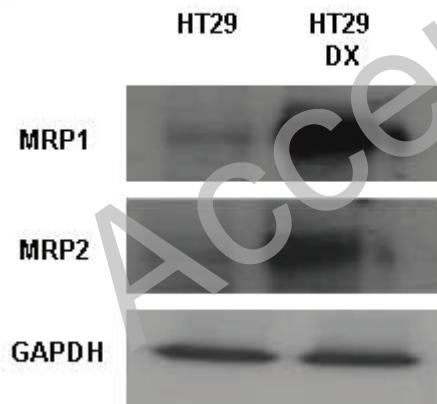
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Figure 1

mRNA

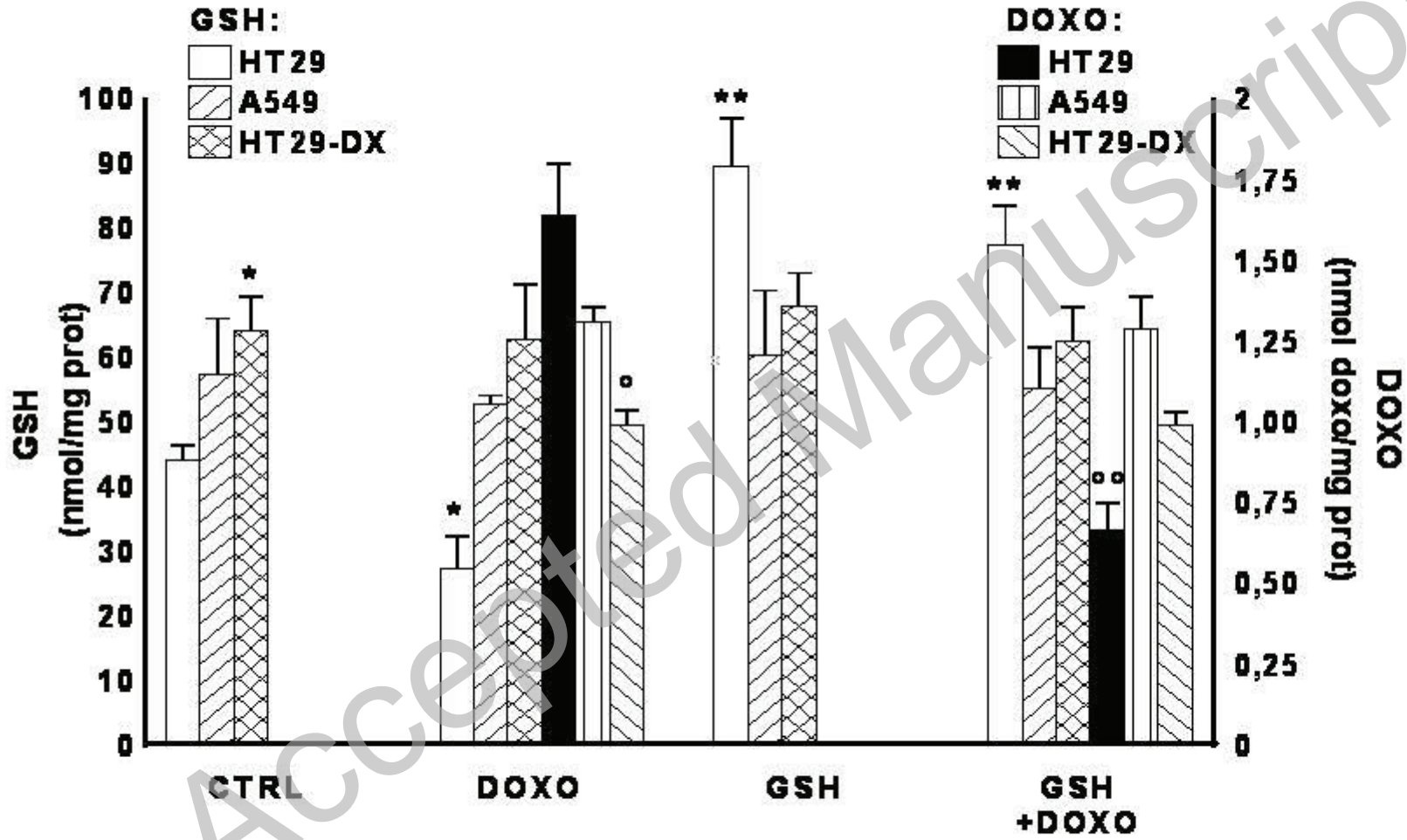


protein



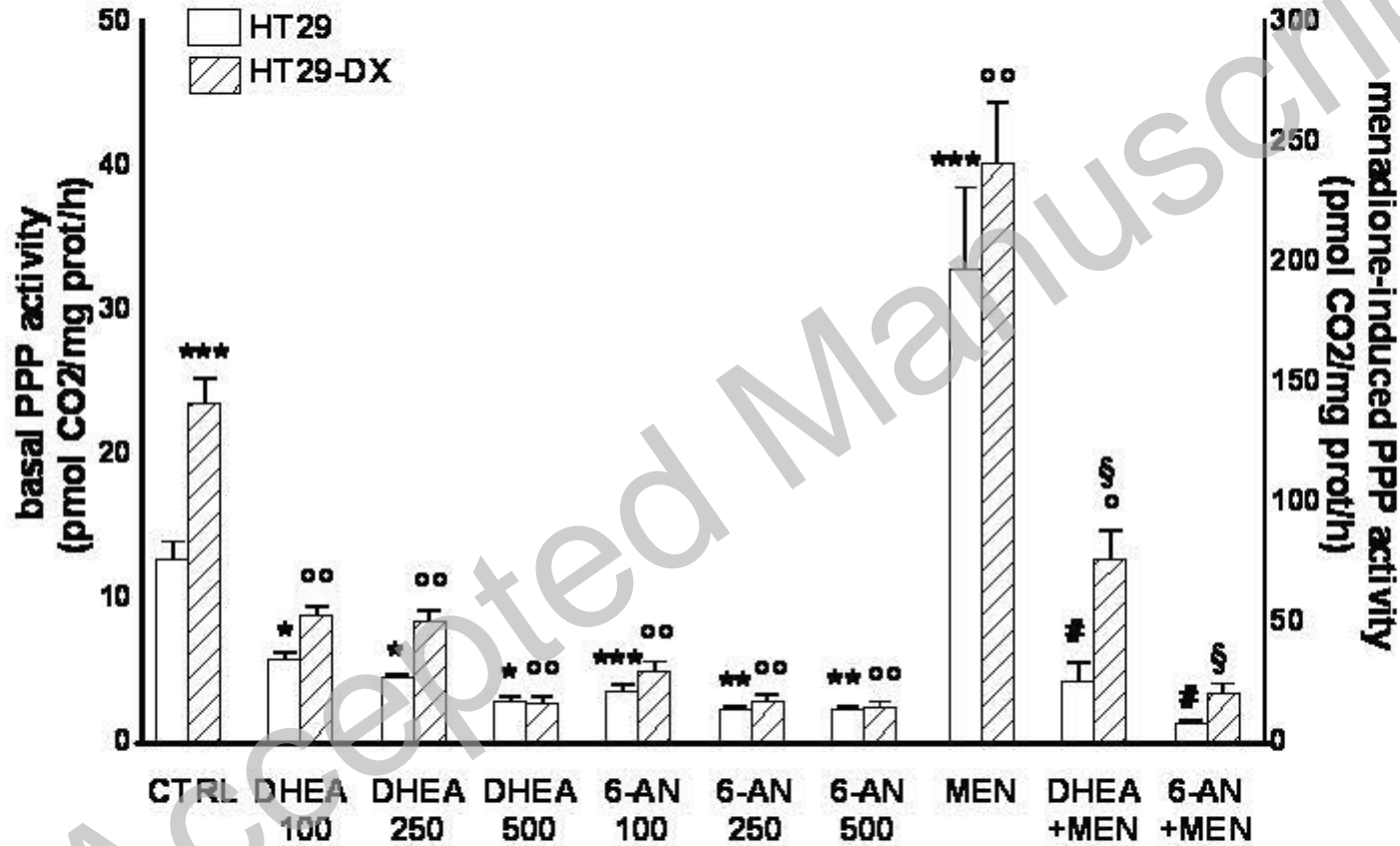
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Figure 2



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20102016

Figure 3

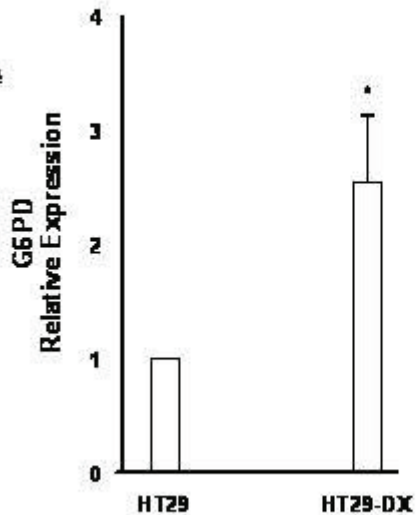


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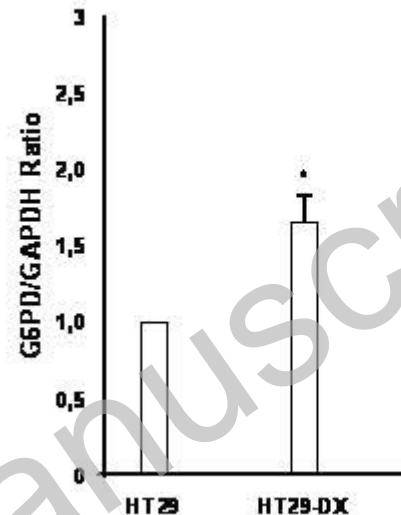
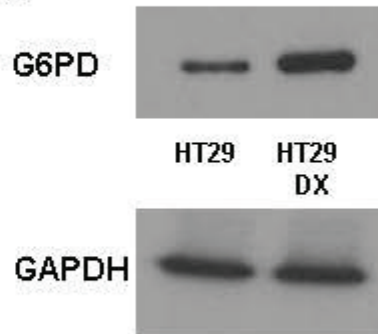
Figure 4

A)

mRNA



protein



B)

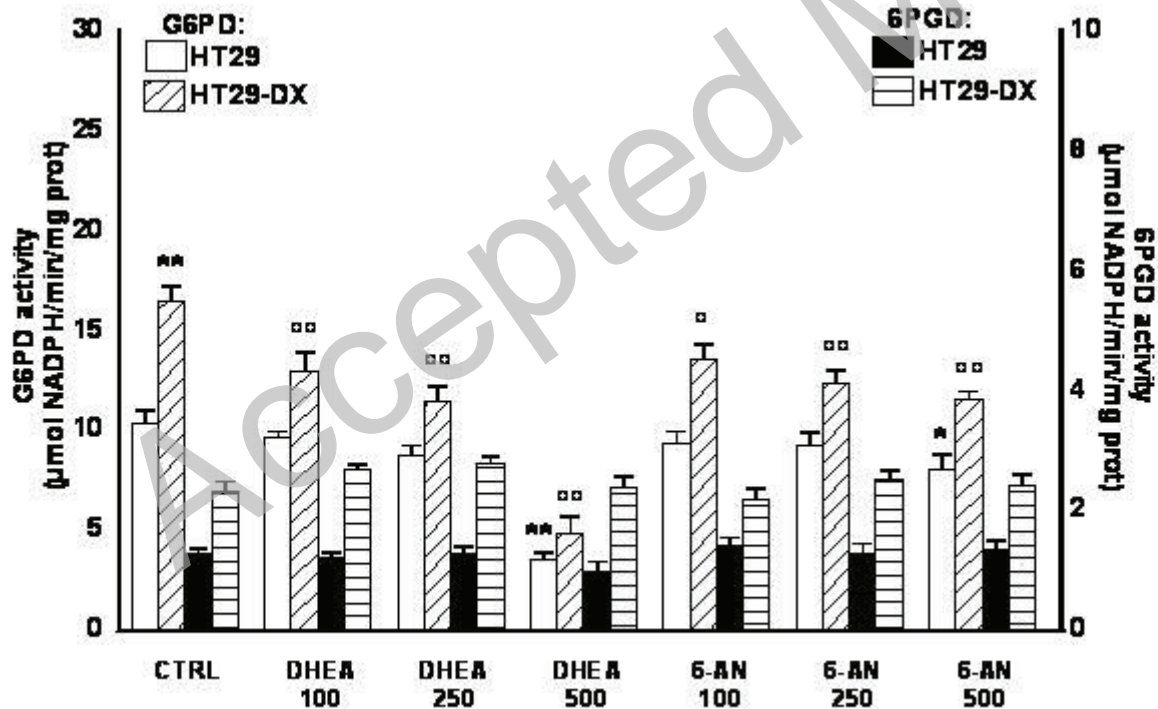


Figure 5

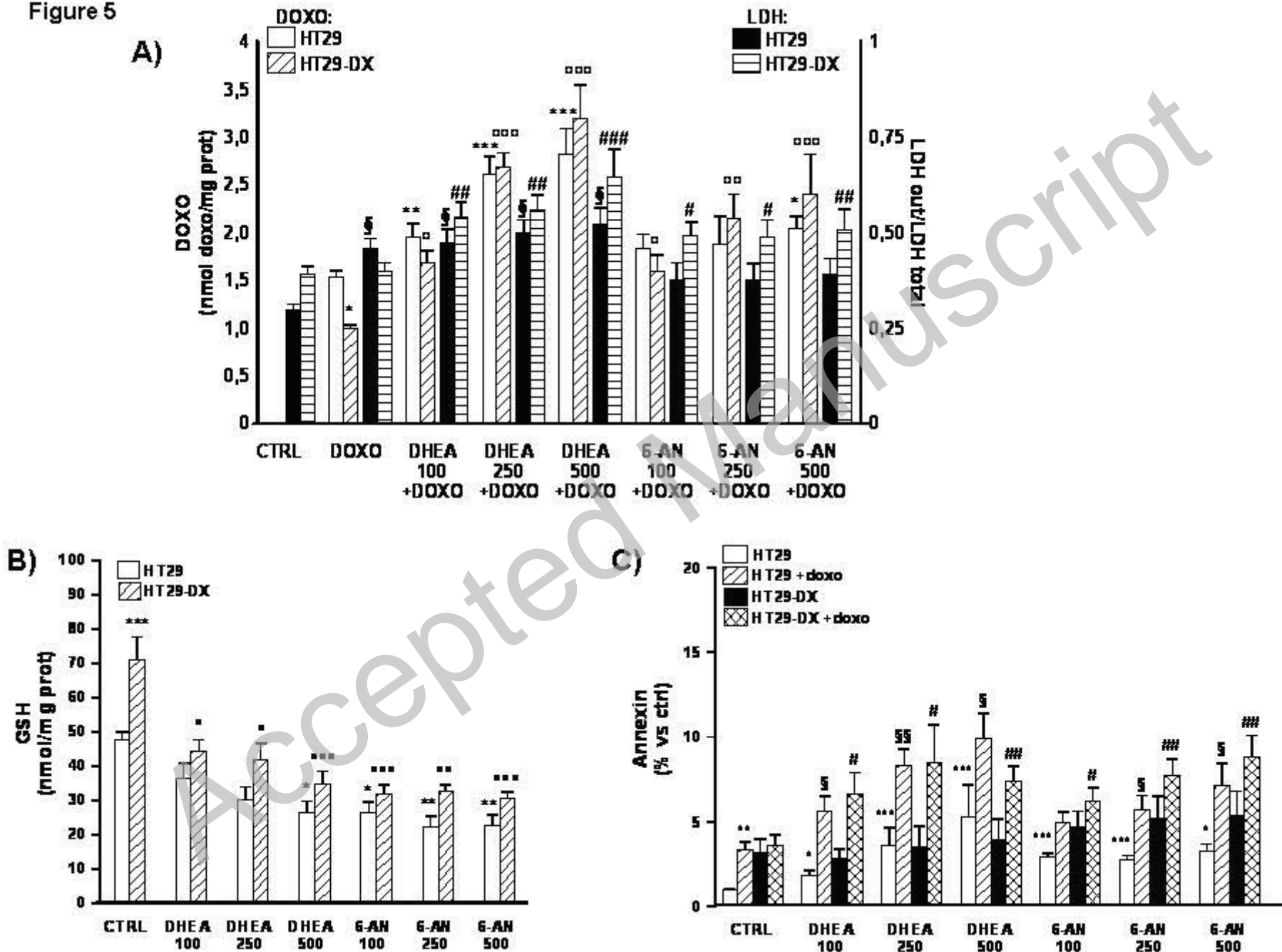


Figure 6

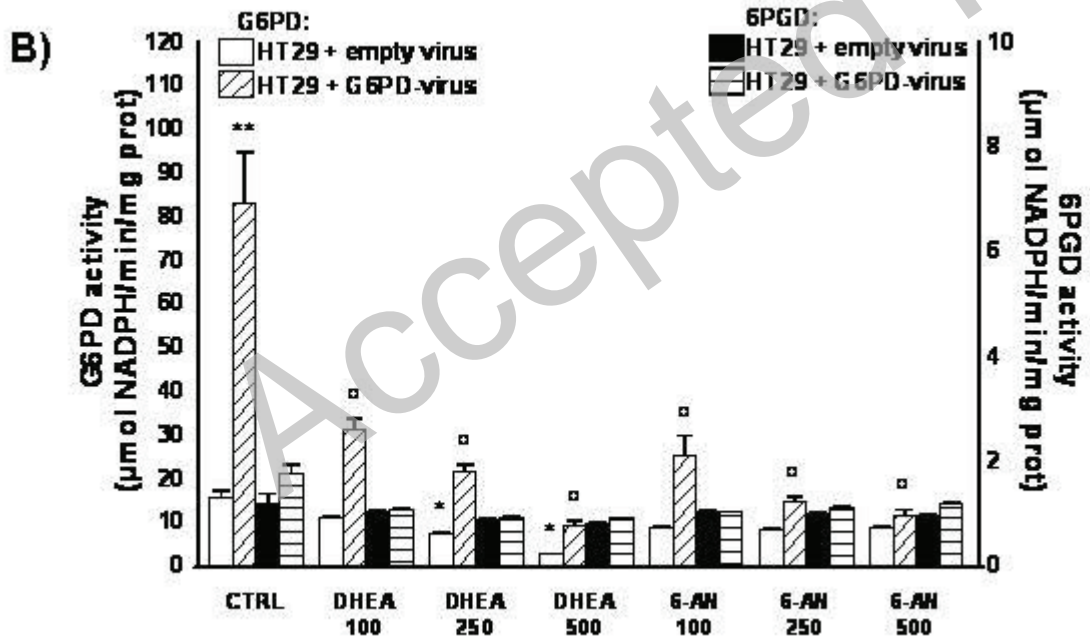
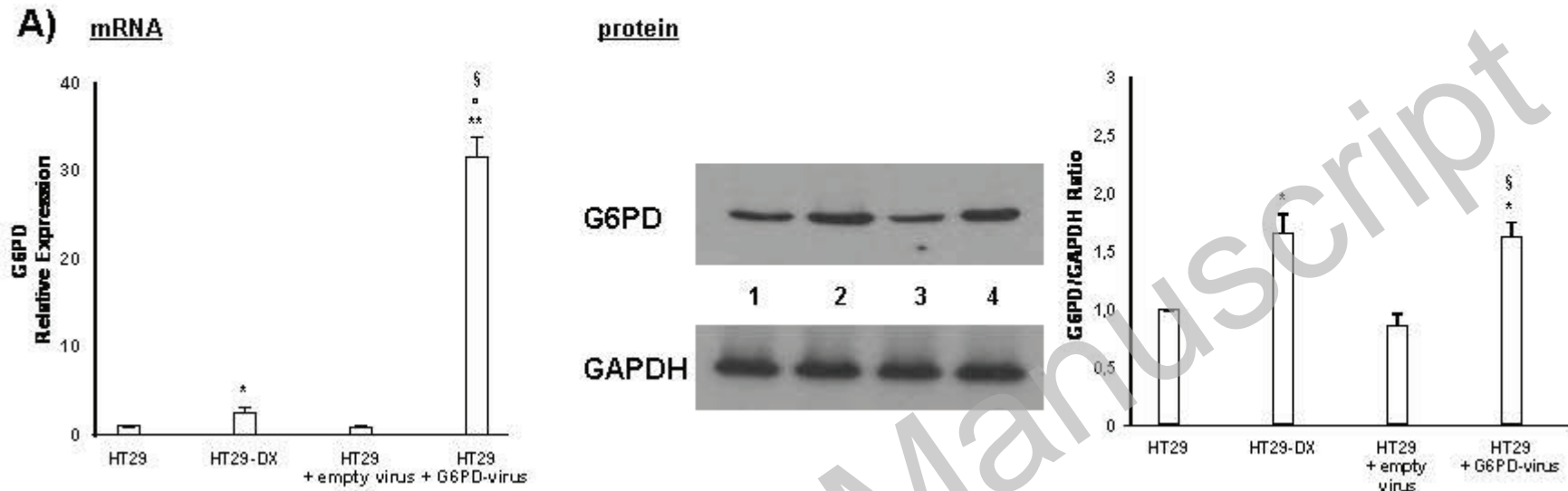


Figure 7

