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TREATMENT OF DOXORUBICIN RESISTANT MCF7/Dx CELLS WITH NITRIC OXIDE CAUSES HISTONE GLUTATHIONYLATION AND REVERSAL OF DRUG RESISTANCE

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Short title: Nitric oxide and histone glutathionylation

SYNOPSIS

Acquired drug resistance was found to be suppressed in doxorubicin resistant breast cancer cells (MCF7/Dx cells) after a pre-treatment with nitrosoglutathione (GSNO). The effect was accompanied by enhanced protein glutathionylation and accumulation of doxorubicin in the nucleus. Among the glutathionylated proteins we identified three members of the histone family; this is the first time that histone glutathionylation has been reported. Formation of the potential nitric oxide donor dinitrosyl diglutathionyl iron complex, bound to glutathione transferase (GST P1-1), was observed in both MCF7/Dx cells and drug-sensitive MCF7 cells, to a similar extent. In contrast, histone glutathionylation was found to be markedly increased in the resistant MCF7/Dx cells, which also showed 14-fold higher amount of GST P1-1 and increased glutathione concentration compared to MCF7 cells. These results suggest that the increased cytotoxic effect of combined doxorubicin and GSNO treatment involves the glutathionylation of histones, through a mechanism that requires high GSH levels and increased expression of GST P1-1. Because of the critical role of histones in the regulation of gene expression, the implication of this finding may go beyond the phenomenon of doxorubicin resistance.

Key Words: nitric oxide, glutathione, GST P1-1, drug resistance, histones, tumor cells

Abbreviations used: CLSM, Confocal laser scanning microscopy; DNDGIC, dinitrosyl diglutathionyl iron complex; EDTA, ethylenediaminetetraacetic acid; EPR, electronic paramagnetic resonance; GSH, glutathione; GSNO, nitrosoglutathione; GSSG, oxidized glutathione; GST, glutathione transferase; MCF7, doxorubicin sensitive human breast cancer cell line; MCF7/Dx, doxorubicin resistant human breast cancer cell line; PgP, P-glycoprotein; PI, propidium iodide; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

INTRODUCTION

One of the most common problems encountered during the treatment of tumors is the acquired resistance which eventually arises after a successful initial period of chemotherapy with drugs such as doxorubicin and cisplatin. Recent reports of nitric oxide (NO) effects on drug resistant cell lines suggest it may be possible to overcome resistance through administration of NO donors and it is generally agreed that NO at low concentrations can exert at least a cytostatic effect on these tumor cells, but different mechanisms have been proposed to explain this. It has been suggested that resistance to doxorubicin, due to an increased efflux of drug through ATP dependent transporters, can be reversed by NO production [1], or alternatively that inhibition of cell proliferation occurs through NO-mediated iron release together with glutathione (GSH), in cells overexpressing a multidrug resistance protein [2]. Others studies suggest that an increased rate of detoxification through Phase II enzymes [3] combined with high cellular GSH contents could account for the effect [4]. Nitric oxide affects a number of important biological processes including the regulation of blood pressure, the relaxation of smooth muscle [5] and the modulation of cell proliferation [6]. NO has a very short life time in the cell but is stabilized and transported by NO-donors such as the dinitrosyl diglutathionyl iron complex (DNDGIC) or nitrosoglutathione (GSNO). This latter NO-donor can readily modify protein thiol groups to form S-nitrosylated [7] or S-glutathionylated proteins [8], which in turn may modulate different proteins involved in signaling cascades, apoptosis, ion channels, redox systems and hemoproteins. We have previously shown that human glutathione transferase (GST) P1-1 strongly binds DNDGIC *in vitro* and *in vivo* whilst maintaining its well known detoxifying activity towards dangerous compounds [9, 10]. A very high affinity for DNDGIC was also found for other GST classes (Mu, Alpha and Theta), suggesting a common mechanism by which the more recently evolved GSTs may act as intracellular NO carriers or scavengers [11, 12].

GSTs [EC 2.5.1.18] are a superfamily of multifunctional enzymes involved in a coordinated defense strategy, together with other GSH-dependent enzymes, cytochrome P450s (Phase I enzymes) and some membrane transporters (Phase III) such as MRP1, and MRP2, to remove GSH conjugates from the cell [13]. Among the human cytosolic GSTs, the Pi class enzyme (GST P1-1) is believed to be an important factor in tumor drug resistance and over-expression of GST P1-1 has been reported for a number of different human malignancies [3]. However, GST P1-1 has also been proposed as a possible tumor marker for certain types of cancer (e.g. prostate cancer), where the lack of expression of this enzyme actually is an unfavourable prognostic factor [14]. In this paper we report that GSNO treatment of breast cancer cells resistant to doxorubicin can reverse the acquired drug resistance in a time dependent manner. As a result of the treatment several histones were found to be glutathionylated, the first time this type of histone modification has ever been reported. Because of the critical role of these nuclear proteins in the regulation of gene expression, this modification could increase the exposure of potential nucleic acid binding sites to doxorubicin.

EXPERIMENTAL

Human tumor cell lines and in vitro treatments

The drug-sensitive human breast cancer cell line MCF7 and its derivative MDR variant MCF7/Dx were kindly provided from Dr. Gabriella Zupi (Regina Elena Cancer Institute, Rome, Italy). These cell lines were cryo-preserved in our laboratory and tested for mycoplasma contamination prior to each experiment using Hoechst 33258 fluorescence dye (Invitrogen, S. Giuliano Milanese (MI), Italy). MCF7 and MCF7/Dx cell lines were grown as monolayers in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, L-glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 µg/ml) at 37°C in a humidified 5% CO₂ atmosphere. MCF7/Dx cell line was grown in the presence of 10 µM doxorubicin and cultured for 4 weeks in

drug-free medium prior to use. Cells were serially passaged after being detached from culture flasks with 0.05% trypsin and 0.002% EDTA solution. All media and supplements for cell cultures were obtained from Hyclone (Labs Inc. Logan, UT).

For the treatments, exponentially growing MCF7 and MCF7/Dx cells were seeded at a density of $4 \times 10^4/\text{cm}^2$ and maintained at 37°C, in a humidified atmosphere with 5% CO₂ for 24 h before treatments. The effect of dose/response of GSNO on MCF7 and MCF7/Dx cell viability was evaluated and is shown in Supplementary Figure S1. Thereafter, cells were treated with 0.5 mM GSNO, prepared as previously described [9], for a period ranging from 1 to 4 hours. In both cell lines, doxorubicin (Adriplastina, Pfizer Italia S.r.l., Borgo San Michele (LT), Italy) effect on cell viability was evaluated after 24 hours treatment with 5 µM doxorubicin, with or without GSNO pre-treatment (Supplementary Figure S2). For microscopy analyses, cells were grown on cover slips.

HPLC determination of GSH

The cells, either untreated or treated for up to four hours, were sonicated three times for 2 sec in 0.1 ml of 10 mM Tris-HCl, pH 7.4. The HPLC determination of various forms of GSH was performed as previously reported [15]. Briefly for free-GSH determinations, 100 µl of 12% sulfosalicylic acid were added to 50 µl of cells lysate, and GSH content on the acid-soluble fraction was determined. The protein pellet was dissolved in 150 µl of 0.1 N NaOH, and protein bound-GSH (GS-Pro) determined. For GSSG determination, the cells were sonicated in the presence of 5 mM N-ethyl maleimide; 100 µl of 12% sulfosalicylic acid was added to 50 µl homogenates, and GSSG content on the acid-soluble fraction was determined. Total protein concentration was evaluated by the Lowry assay using bovine serum albumin (Sigma-Aldrich, St. Louis, MO) as control.

EPR Detection of Dinitrosyl Diglutathionyl Iron Complex in Intact Cells

EPR spectra were recorded using 60 µl samples of either MCF7 or MCF7/Dx pellet, before and after exposure to 0.5 mM GSNO for up 4 hours, in flat glass capillaries (inner cross-section 5×0.3 mm) to optimize instrument sensitivity as previously described [16]. All measurements were made as previously reported [10].

Determination of GST P1-1 activity

After GSNO treatment and EPR analysis, MCF7 and MCF7/Dx cells were lysed by sonication and the resulting supernatant was assayed for GST activity and protein concentration. The enzymatic activities were determined spectrophotometrically, using a double beam Cary Win 4000 UV-Vis spectrophotometer (Varian, Leini, Torino Italy), at 25°C with 1-chloro-2,4-dinitrobenzene as cosubstrate following the product formation at 340 nm, $\epsilon = 9600 \text{ M}^{-1}\text{cm}^{-1}$ [17]. Total protein concentration was evaluated by the Lowry assay using bovine serum albumin as control.

Western blot analysis of GSTs isoenzymes and glutathionylated proteins

30 µg of cytosolic proteins, previously extracted by lysis of either MCF7 or MCF7/Dx cells, untreated or treated with 0.5 mM GSNO for a period ranging from 1 to 4 hours, were resolved on 12% SDS-polyacrylamide gels under non reducing conditions and transferred onto a nitrocellulose membrane (BIO-RAD, Hercules, CA). Western blotting was carried out according to the method of Towbin et al. [18] using polyclonal rabbit anti GST P1-1, anti A1-1, anti M2-2 (Calbiochem Darmstadt, Germany) and anti T1-1 (prepared in our lab) as primary antibodies and an antirabbit antibody conjugated to horseradish peroxidase as secondary antibody (BIO-RAD), with enhanced chemiluminescence detection (ECL plus, GE-Healthcare Life Sciences, Limão, São Paulo - SP). Western blotting of glutathionylated proteins was carried out using a monoclonal anti GS-proteins

antibody (Virogen Corporation, Watertown, MA). For protein loading control, the membrane was hybridized with monoclonal mouse anti actin antibody (Abcam, Cambridge, UK) and anti mouse antibody conjugated to horseradish peroxidase (Sigma) with enhanced chemiluminescence detection (ECL plus).

Immunoprecipitation

MCF7 and MCF/Dx cells untreated or after 4 hours treatment with 0.5 mM GSNO, were lysed and total proteins (4 mg) were subjected to immunoprecipitation with a monoclonal anti GSH antibody as previously described [19]. Immunoprecipitated proteins were separated by 12% SDS-PAGE under non reducing conditions and subjected to western blot using either anti GSH (Virogen) or a monoclonal anti H3 antibodies (Abcam). The same samples were also immunoprecipitated with the anti H3 antibody and analyzed by western blotting using either anti GSH or anti H3 or anti GST P1-1 antibodies.

Cytotoxicity assay and cell cycle analysis

To evaluate the doxorubicin cytotoxicity, after treatment MCF7 and MCF7/Dx cells were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and nuclei were stained with 2 µg/ml propidium iodide (PI) in the presence of 0.1 mg/ml RNAase and apoptotic/necrotic cells were counted. At least 300 cells per sample were observed using a confocal laser scanning microscope (CLSM). All these reagents were purchased from Sigma. Samples were analyzed using the flow cytometer FACSCalibur™ (Becton Dickinson, Detroit, MI) to evaluate the effect of doxorubicin and GSNO treatments on the cell cycle analyzing the DNA content after PI staining.

Doxorubicin accumulation assay

Doxorubicin accumulation was determined as following: i) analyzing the intracellular distribution of the drug in living cells by confocal laser scanning microscopy, taking advantage of doxorubicin intrinsic fluorescence; ii) by flow cytometry according to a previously described method [20, 21]. In details, MCF7 and MCF/Dx cells were pretreated with 0.5 mM GSNO for 1 h or 3 h, and then were incubated with 5 µM doxorubicin at 37°C for 1 h. Controls consisted of cells treated with doxorubicin without GSNO pre-treatment and of untreated cells. After incubation, cells were observed under the CLSM or trypsinized, washed and resuspended in PBS and the amount of cellular doxorubicin was estimated by the flow cytometer FACS-Scan (BD Biosciences, San Jose, CA).

Confocal laser scanning microscopy analysis of glutathionylated proteins

The intracellular distribution of glutathionylated proteins in MCF7 and MCF7/Dx cells was evaluated on untreated controls and 0.5 mM GSNO-treated cells, by immunofluorescence staining using the mouse monoclonal antibody against the GSH bound to proteins as primary antibody. After 1 h and 3 h treatment, cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.2% Triton X-100 (Sigma) and indirect immunofluorescence staining was carried out. The primary antibody was detected with the secondary Alexa Fluor 488-conjugated anti mouse IgG (Molecular Probes, Invitrogen Corporation, Grand Island, NY). Samples were observed with a LEICA TCS SP5 confocal laser scanning microscope (Leica Microsystems, Bannockburn, IL).

Sequencing of the glutathionylated proteins

MCF7/Dx cells treated with 0.5 mM GSNO for 4 hours were lysed and proteins (4 mg) were immunoprecipitated using anti GSH antibody. Glutathionylated proteins obtained by immunoprecipitation were separated by 12% SDS-PAGE under non reducing conditions, electrotransferred onto a polyvinylidene difluoride membrane (Immobilon P, Millipore Corporation, Billerica, MA) and stained with Blue Coomassie, as reported elsewhere [22]. Bands were cut and

sequenced with an Applied Biosystem model 473A pulsed liquid sequencer with an on-line PTH-amino acid analyzer.

Statistical analysis

Differences between MCF7 and MCF7/Dx, regarding to GST P1-1 activity and expression, and the HPLC determination of the various forms of GSH was estimated by a Student's *t* test and were considered to be statistically significant at $p < 0.05$.

RESULTS

The nitrosative stress significantly enhanced the glutathionylation of proteins only in MCF7/Dx cells

Treatment of MCF7/Dx cells with GSNO for up to four hours revealed an enhanced glutathionylation of proteins as detected by western blotting analysis. Figure 1A showed a number of bands, with molecular masses ranging from 60 kDa down to 6 kDa, reacting with the anti-GSH antibody, with a more intense staining in the MCF7/Dx cell extracts and reaching a maximum level after 3 hours treatment. A more accurate analysis of this modification was carried out by HPLC analysis of the same samples (Figure 1B). There was a basal level of glutathionylation (about 10 nmol of GSH/mg protein) which remained substantially unchanged over the time of treatment in the sensitive cells, while it was dramatically increased in the resistant cells, reaching a maximum of up to 43 nmol of GSH/mg protein after 3 hours of GSNO treatment. Confocal Laser Scanning Microscopy (CLSM) imaging further confirmed this result showing an increased amount of glutathionylated proteins in the resistant cells after 3 hours treatment with 0.5 mM GSNO, in comparison with either the untreated MCF7/Dx cells or the MCF7 cells as control (Figure 1C). It is remarkable that in the untreated MCF7/Dx cells the glutathionylated proteins appeared mainly located within the cytosol rather than in the nucleus. GSNO treatment was able to induce an increase in glutathionylated proteins not only in the cytoplasm but also at nuclear level.

Identification of glutathionylated proteins by immunoprecipitation and microsequencing analysis

We attempted to identify the glutathionylated proteins, observed by western blotting (Figure 1A), by immunoprecipitation of MCF7/Dx cells lysate treated with 0.5 mM GSNO for 4 hours with an anti GSH antibody. SDS-PAGE of the co-precipitated proteins with anti GSH, stained with Blue Coomassie, showed many bands, in a broad range of molecular weight (Figure 2A) confirming that a number of proteins were subjected to glutathionylation. Due to low amount of sample we were only able to identify the three most abundant proteins which ranged between 6 kDa and 19 kDa. Three bands, as indicated in Figure 2A were excised from the polyvinylidene difluoride membrane and sequenced at the N-terminus through automated Edman degradation to give the following results. The upper band, with a molecular weight of about 12 kDa, showed two overlapping sequences as reported in Figure 2A. Through a Swiss-Prot sequence search we identified the proteins corresponding to these bands as histones H3 and H2B, respectively. The middle band, with a molecular weight of 11 kDa, was identified in a similar way and corresponded to the H2B type 2D protein. The lower band, with a molecular weight of 9.5 kDa, gave no sequence, probably because the N-terminus is acetylated, but should correspond to the H4 protein, as reported elsewhere [23]. Thus all identified proteins were nuclear proteins belonging to the family of histones, which are already known to be crucial targets of important modifications (methylation, acetylation, or phosphorylation). It should be noted that these three bands, as revealed on the gel, were large fragments of the native proteins since the molecular weight of the entire proteins for H3, H2B and H2B type 2D are: 15.3 kDa, 13.8 kDa and 18.0 kDa, respectively. To validate these results we subjected MCF7 and MCF7/Dx cells, untreated or after 4 hours treatment with 0.5 mM GSNO, to immunoprecipitation with anti GSH antibody and further western blotting analysis either with anti

GSH antibody or with anti H3 antibody obtaining two bands of glutathionylated proteins (Figure 2B). The upper and more intense band which corresponds to the histone H3 showed an increase of glutathionylation upon GSNO treatment in both sensitive and resistant cell lines as compared to the control. The lower and weaker band should correspond to the H2B type 2D since it is absent from the same samples when analyzed with anti H3 antibody (Figure 2B). To exclude any artifact we did the opposite experiment by immunoprecipitation of same samples with anti H3 antibodies and further western blotting analysis using either anti GSH or anti H3 antibodies (Figure 2C), or anti GST P1-1 antibody (Figure 2D), respectively. The results shown in Figure 2C suggested that only the untreated or GSNO-treated MCF7/Dx cell lines possess a glutathionylated albeit weak H3 band consistent with the increase of glutathionylation observed already in these drug resistant cells. The absence of GST P1-1 in the untreated or GSNO-treated MCF7 cells (Figure 2D) was expected since this drug sensitive cell line does not express GST P1-1 (see also Figure 4). Conversely, a strong band corresponding to GST P1-1 (Figure 2D) was observed in both the untreated or treated MCF7/Dx cell lines, suggesting the formation of a complex between histone H3, GST P1-1 and GSH. The specificity of this immunoprecipitate was tested using PARP antibodies as negative control (results not shown).

GSNO treatment increased doxorubicin accumulation in the nucleus and reverted the drug resistance in MCF7/Dx cells

In drug sensitive cells doxorubicin appeared mainly located in the nuclei while in drug resistant cells it was located throughout the cytoplasm with the nuclei being almost completely negative for the doxorubicin fluorescent signal (Figure 3A, control). Exposure of drug resistant cells to GSNO caused doxorubicin accumulation in the nuclei in a time dependent manner (Figure 3A, lower panels), while no significant differences were observed in drug sensitive cells (Figure 3A, upper panels). Cytofluorimetric analysis confirmed that GSNO pre-treatment increased the amount of cellular doxorubicin in MCF7/Dx, as compared to untreated MCF7/Dx cells (Figure 3B). After 24 hours of doxorubicin treatment, about 75% of drug sensitive MCF7 cells showed morphological features of apoptotic/necrotic cells, while cell death was almost absent in drug resistant MCF7/Dx (Figure 3C). Pre-treatment of MCF7/Dx with 0.5 mM GSNO for 1 hour was able to revert doxorubicin resistance of MCF7/Dx cells, leading to a 30-fold increase in the number of apoptotic/necrotic cells (about 65% of cells), a level comparable to that observed in drug sensitive MCF7 cells (Figure 3C). It was obvious that neither doxorubicin nor GSNO alone (data not shown) were able to trigger cell death of drug resistant cells whereas their combined action was effective to abolish drug resistance completely (Figure 3D). Cytofluorimetric analysis of the same cell lines shed more light on this phenomenon (Figure 4). In the drug sensitive cells 24 hours doxorubicin treatment caused a dramatic block in G2/M phase (Figure 4A), while in the drug resistant MCF7/Dx cells neither 24 hours doxorubicin nor 1 hour/3 hours GSNO treatment alone were able to block the cell cycle in G2/M phase (Figure 4B). However, when these cells were pretreated with GSNO (1-3 h) and then exposed to doxorubicin for 24 hours we observed that about 80% of the cells were in G2/M phase (Figure 4B). The ability of GSNO pretreatment to restore the sensitivity to doxorubicin of the drug resistant MCF7/Dx cells has also been confirmed by cell viability assay (Supplementary Figure S2). These results demonstrate again the synergy of nitric oxide and doxorubicin in the reversal of drug resistance in the MCF7/Dx cells.

GSNO treatment of both MCF7 and MCF7/Dx cells caused formation and binding of DNDGIC to GST P1-1

In the cytosol nitric oxide spontaneously forms a dinitrosyl diglutathionyl iron complex (DNDGIC), which binds with very high affinity, to intracellular GSTs [9-12, 23]. Electron paramagnetic resonance spectroscopy (EPR) measurements of MCF7/Dx cells exposed to GSNO for different times showed the formation of intracellular DNDGIC (Figure 5A); the spectra showed signal line shapes and positions typical for the complex bound to GST P1-1 [9, 10]. It is known that

only one of the two active sites in this enzyme binds DNDGIC with very high affinity, resulting in the loss of half of the GST activity [9-11]. Formation of DNDGIC-GST could therefore be verified through the gradual inhibition of GST proportionally to the concentration of DNDGIC, reaching maximum inactivation after 3 hours of incubation (Figure 5B). Treatment with GSNO gave identical spectra for drug sensitive MCF7 cells (data not shown), confirmed that the two cell lines produced similar amounts of DNDGIC and thus were exposed to the same levels of intracellular NO. On the other hand, exposure to GSNO alone was not able to induce changes in the expression of GST P1-1, neither in MCF7 nor in MCF7/Dx cells, as shown by western blotting (Figure 5C and 5D).

MCF7/Dx cells overexpressed GST P1-1 and had increased amounts of GSH compared with MCF7 cells

The presence of GST P1-1 in the sensitive and resistant MCF7 cell line was analyzed by western blotting and enzyme activity measurements. There was little GST P1-1 expression (as observed with western blotting) and very low enzyme activity in the sensitive cell line, in contrast to the resistant line which shows a marked band of GST P1-1 protein and a 14 fold increase in specific activity (Figure 6A and Inset). The HPLC analysis of GSH indicated that all forms of GSH (reduced, total and free) were significantly increased in the MCF7/Dx cells (Figure 6B) whilst there was no change in the GSH bound to proteins and the oxidized form of GSH (GSSG) was decreased (Figure 6C) in comparison with MCF7 cells. However, it should be noted that in the MCF7/Dx cells, after the GSNO treatment, there was an increase of oxidized GSH (GSSG) as compared with the reduced GSH (see Supplementary Figure S3) indicative of a nitrosative stress. We have also probed both cell lines, by western blotting, for the presence of GST M1-1, GST A1-1 and GST T1-1 without any positive results in any case (data not shown).

DISCUSSION

In this paper we show that it is possible to circumvent the acquired drug resistance of the breast tumor cell line MCF7/Dx through treatment with nitrosoglutathione. A general enhancement of protein glutathionylation was observed after GSNO treatment of MCF7/Dx cells in comparison to MCF7 cells (Figure 1A). This result was confirmed by HPLC analysis of glutathionylated proteins (Figure 1B) and by CLSM of the same MCF7/Dx cells (Figure 1C) showing also an increased distribution of glutathionylated proteins at the nuclear level. Perhaps the most surprising finding is that among the glutathionylated proteins we identified at least three different proteins all belonging to the family of histones (Figure 2). It is well known that these proteins (H3, H4, H2B, H2A) form an octamer core around which DNA can wind for packaging into the nucleosomes that regulate the transcription [24]. Posttranslational modifications of histones such as methylation, ubiquitination, phosphorylation and acetylation [25-27] have been already reported, but to our knowledge this is the first report of the glutathionylation of histones. We have further validated this modification of histone H3 by immunoprecipitation studies using anti GSH and anti H3 antibodies (Figure 2). For histone H3 a possible target of glutathionylation might be its hyperactive Cys 110, but this remains to be demonstrated.

The cytosolic distribution of doxorubicin in the MCF7/Dx cells, as compared to the nuclear localization of this drug in MCF7 cells (Figure 3A), may be explained by the fact that resistant tumor cells can avoid the cytotoxicity of this anticancer drug by overexpression of integral membrane transporters such as P-glycoprotein (PgP) and MDR-associated proteins with the resulting transport of drug out of the cells [1, 2]. After GSNO treatment there was an increased accumulation of doxorubicin in the nucleus of the resistant cells (Figure 3A and 3B). It has been suggested that nitric oxide could inhibit the efflux by blocking PgP pump via tyrosine nitration [28], however, we cannot exclude the possibility that the nuclear and cytoplasmic doxorubicin levels may

depend on different mechanisms. Our results demonstrate that nitric oxide plays an important role in reversing the acquired resistance of the MCF7/Dx cells and its combined action with doxorubicin is sufficient to induce cell death in these drug resistant cells to an extent comparable to that of MCF7 cells, when treated under identical conditions (Figure 3C and 3D). Cytofluorimetric analysis showed that doxorubicin caused a block of the cell cycle in G2/M phase both in MCF7 cells and in MCF7/Dx cells treated with GSNO (Figure 4A and 4B). All results are in agreement with a mechanism in which cytotoxicity is due exclusively to doxorubicin whereas NO is responsible mainly for the reversal of resistance.

We found in both MCF7 and MCF7/Dx cells, upon GSNO treatment, DNDGIC formation and binding to GST P1-1, along with partial enzyme inhibition (Figure 5A and 5B). In the cytosol nitric oxide spontaneously forms a dinitrosyl diglutathionyl iron complex, which binds with very high affinity to intracellular GSTs leading to a decrease in GST activity [9-11]. Surprisingly, the results showed identical levels of NO in resistant and sensitive cells, which means that the downstream effects (reversal of resistance, enhanced glutathionylation) cannot be ascribed to differences in GSNO uptake and metabolism.

We have analyzed the content of GSH and GST P1-1 enzyme in both sensitive and resistant cell lines. A strong decrease in enzymatic activity [29, 30] and lack of GST P1-1 expression in MCF7 cells, in comparison to MCF7/Dx cells, were already reported and attributed to cytosine methylation of the *GSTP1* promoter, mediated in part by MBDP 2 protein bound to this region [31]. Our data showed a significant increase of glutathione and GST P1-1 (Figure 6A and Figure 6B) in the MCF7/Dx cells in comparison with the sensitive cell lines, thus confirming the importance of the antioxidant and detoxifying defense system mediated by GSH [13] in the acquired resistance of certain tumor cells.

A number of glutathionylated proteins have been reported in the literature suggesting an important role for this post-translational modification in terms of signaling and redox regulation [8, 32]. Our data show that in MCF7/Dx cells, which contain substantial amounts of GSH and GST P1-1, both increased glutathionylation and DNDGIC formation occurs, whereas only DNDGIC formation occurs in the MCF7 cells. Interestingly, the four-fold increase of glutathionylation of the same proteins is highly suggestive of a cell response mechanism under enzymatic control. The classic reaction carried out by GST P1-1 is the covalent attachment of GSH to a wide variety of substrates, and this enzyme can therefore be considered a likely catalyst of this glutathionylation. Very recently such a role for GST P1-1 during oxidative or nitrosative stress has been proposed by Townsend et al. [33] and our results are fully consistent with their suggestions.

The main finding of this study (the glutathionylation of histones) poses several questions. First, is there any change in the function of histones upon their modification by GSH? If so, this could influence the expression of genes involved in doxorubicin resistance/sensitivity. Second, the formation of complex between GST P1-1, H3 and GSH, as suggested by immunoprecipitation studies using anti H3 antibodies (Figure 2C and 2D), requires further studies to clarify the presence of GST P1-1 into the nucleus. A similar complex involving GST P1-1 has been already reported for the activation of the antioxidant enzyme 1-Cys peroxiredoxin which requires glutathionylation mediated by GST P1-1 heterodimer formation [34]. Preliminary experiments using CLSM with GST P1-1 antibodies (data not shown) suggest the presence of a nuclear GST P1-1 in accordance with previous reports describing the presence of a perinuclear GST [35], or suggesting a specific transport system for nuclear transfer of GST P1-1 as an extreme attempt of cellular defense against antitumor drugs [36]. Because of the critical role of these nuclear proteins in the regulation of gene expression, this modification could increase the exposure of potential nucleic acid binding sites to doxorubicin.

On the basis of these results we propose that the GSNO mediated glutathionylation of histones in tumor cells overexpressing human GST P1-1 could be considered a possible epigenetic gene regulatory mechanism. We are currently pursuing studies in this direction to unravel the molecular mechanisms of this phenomenon.

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FIGURE LEGENDS

Figure 1. Enhanced protein glutathionylation after GSNO treatment.

(A) Glutathione bound to proteins was detected by western blot analysis in both MCF7 and MCF7/Dx cell lines before and after exposure to 0.5 mM GSNO for up to 4 hours. (B) Determination of glutathionylated proteins by HPLC analysis in both MCF7 and MCF7/Dx cell lines before and after exposure to 0.5 mM GSNO for up to 4 hours. Error bars represent standard deviation of at least three different experiments. (C) CLSM representative images showing the intracellular distribution of glutathionylated proteins in MCF7/Dx cells untreated or treated with 0.5 mM GSNO (3 hours) (upper panels), in comparison with MCF7 cell lines untreated or treated with 0.5 mM GSNO (3 hours) (lower panels).

Figure 2. Identification of glutathionylated proteins by immunoprecipitation.

(A) Protein extract of MCF7/Dx cells treated with 0.5 mM GSNO for 4 hours was immunoprecipitated using anti GSH antibody and subjected to SDS-PAGE under non reducing conditions. The gel was stained with Blue Coomassie and three bands corresponding to molecular weights between 19 kDa and 6 kDa were excised from the gel and sequenced at the N-terminus by automated Edman degradation. The results are shown for the first two bands. The lower band could not be sequenced. (B) Western blot analysis with either anti GSH or anti H3 antibodies of immunoprecipitates of MCF7 and MCF7/Dx cells, before and after exposure to 0.5 mM GSNO for 4 hours, obtained by anti GSH antibody. (C) Western blot analysis with either anti GSH or anti H3 antibodies of immunoprecipitates of MCF7 and MCF7/Dx cells, before and after exposure to 0.5 mM GSNO for 4 hours, obtained by anti H3 antibody. (D) Western blot analysis with anti GST P1-1 antibody of immunoprecipitates of MCF7 and MCF7/Dx cells, before and after exposure to 0.5 mM GSNO for 4 hours, obtained by anti H3 antibody. Purified recombinant GST P1-1 was used as (c) protein control.

Figure 3. GSNO treatment increased doxorubicin accumulation in the nucleus and reversed the drug resistance in MCF7/Dx cells.

(A) CLSM analysis of doxorubicin intracellular distribution in MCF7 and MCF7/Dx cells, in the absence or in the presence of 0.5 mM GSNO (after 1 hour and 3 hours, respectively). Merged images of differential interference contrast (*grey*) and doxorubicin inherent fluorescence (*red*) are shown. (B) Cytofluorimetric evaluation of the amount of cellular doxorubicin in MCF7/Dx after GSNO treatment, as compared to untreated MCF7/Dx and MCF7 cells. (C) CLSM representative images of drug sensitive MCF7 and drug resistant MCF7/Dx cells treated 24 hours with 5 μ M doxorubicin in the absence or in the presence of 0.5 mM GSNO (1 hour pre-treatment). Merged images of differential interference contrast (*grey*) and nuclei staining with PI fluorescent dye (*red*) are shown. In the insets, details of apoptotic/necrotic cell are reported. (D) Doxorubicin cytotoxicity in drug sensitive MCF7 and drug resistant MCF7/Dx cells, in the absence or in the presence of GSNO treatment, evaluated by counting the number of apoptotic/necrotic cells, scored for morphological features such as hypercondensed and fragmented nuclei. At least 300 cells per sample were observed by CLSM and results are given as percentage of apoptotic/necrotic cells. Results showed in this figure are representative of at least 3 independent experiments.

Figure 4. GSNO treatment reversed resistance of MCF7/Dx cells.

Cell cycle analysis by flow cytometry, in drug sensitive MCF7 (A) and drug resistant MCF7/Dx (B) cells, treated for 24 h with 5 μ M doxorubicin in the absence or in the presence of 0.5 mM GSNO (1 hour and 3 hours pre-treatment), as compared with either untreated controls or cells treated with

GSNO alone (panel B, bottom). Results showed in this figure are representative of at least 3 independent experiments.

Figure 5. GSNO treatment of MCF7/Dx caused DNDGIC binding to GST P1-1 and partial inhibition of enzymatic activity.

(A) EPR Spectra of intact MCF7/Dx cells; a: after 3 hours exposure to 0.5 mM GSNO; b: before exposure to GSNO as control; (B) Time course of the DNDGIC formation (▲) and of GST enzymatic activity (■) in MCF7/Dx after exposure to GSNO for 3 hours. (C) Western blotting analysis of both MCF7 and MCF7/Dx cells with human antibody GST P1-1, during the GSNO treatment. (D) Densitometric analysis of western blotting of MCF7/Dx cells. Error bars represent standard deviation of at least three different experiments.

Figure 6. Characterization of MCF7 and MCF7/Dx cells for GST P1-1 expression and activity and for glutathione content.

(A) Enzymatic activity and expression level (by western blotting) of GST P1-1 in MCF7/Dx and in MCF7 cells. (B) HPLC determination of the reduced, total and free GSH content in the above cell lines. Free GSH is defined as the fraction of GSH not bound to proteins with respect to total GSH and includes both the reduced and the oxidized forms of GSH. (C) HPLC determination of the GSH bound to proteins and the oxidized form of GSH (GSSG), in both cell lines. Error bars represent standard deviation of at least three different experiments.

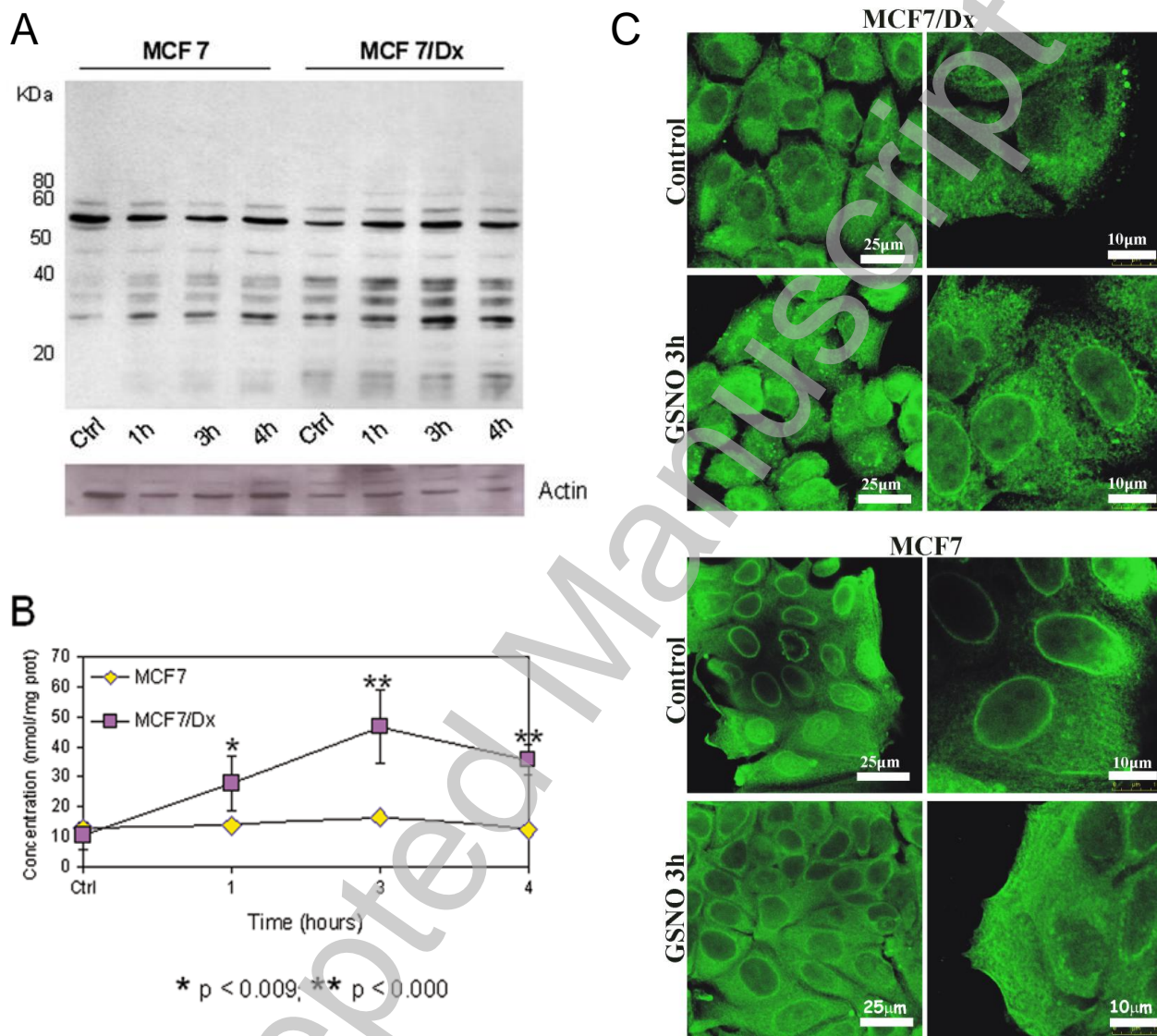


Figure 1

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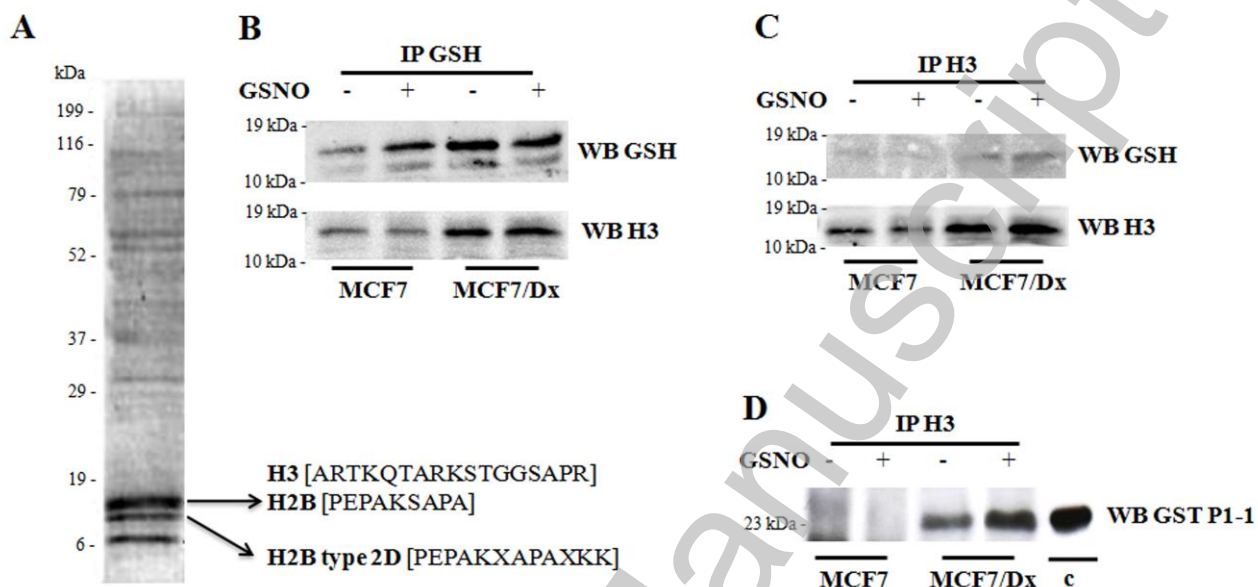


Figure 2

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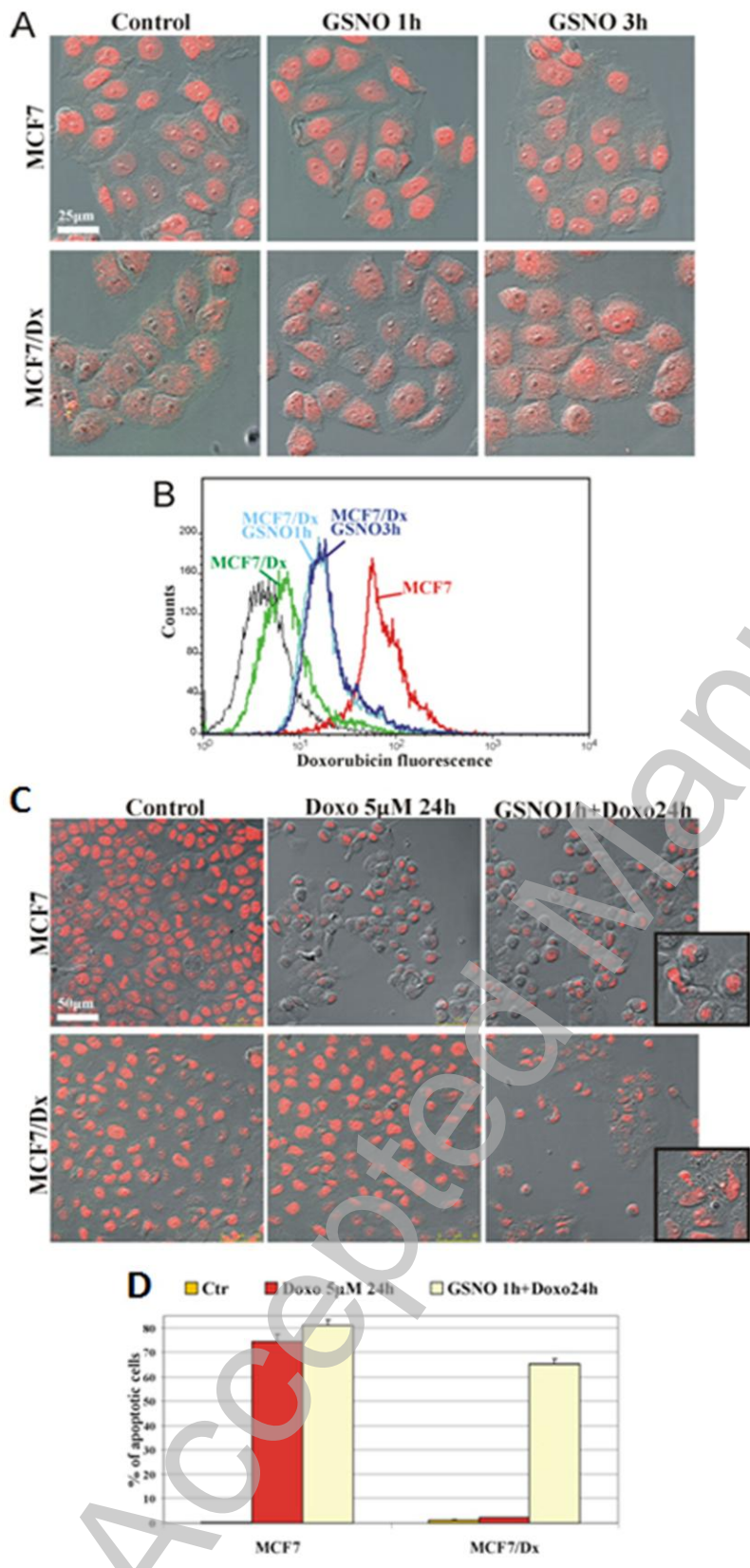


Figure 3

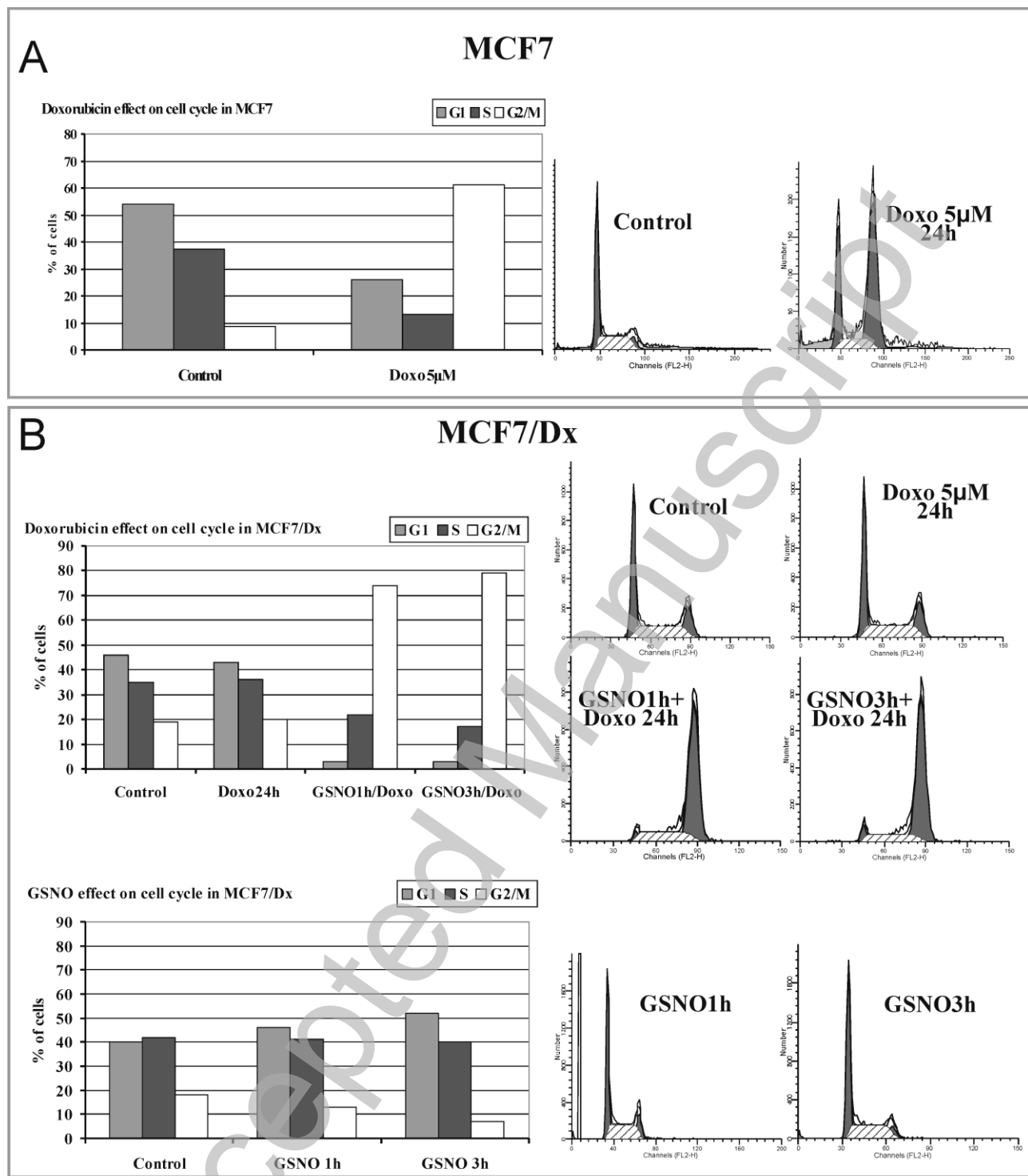


Figure 4

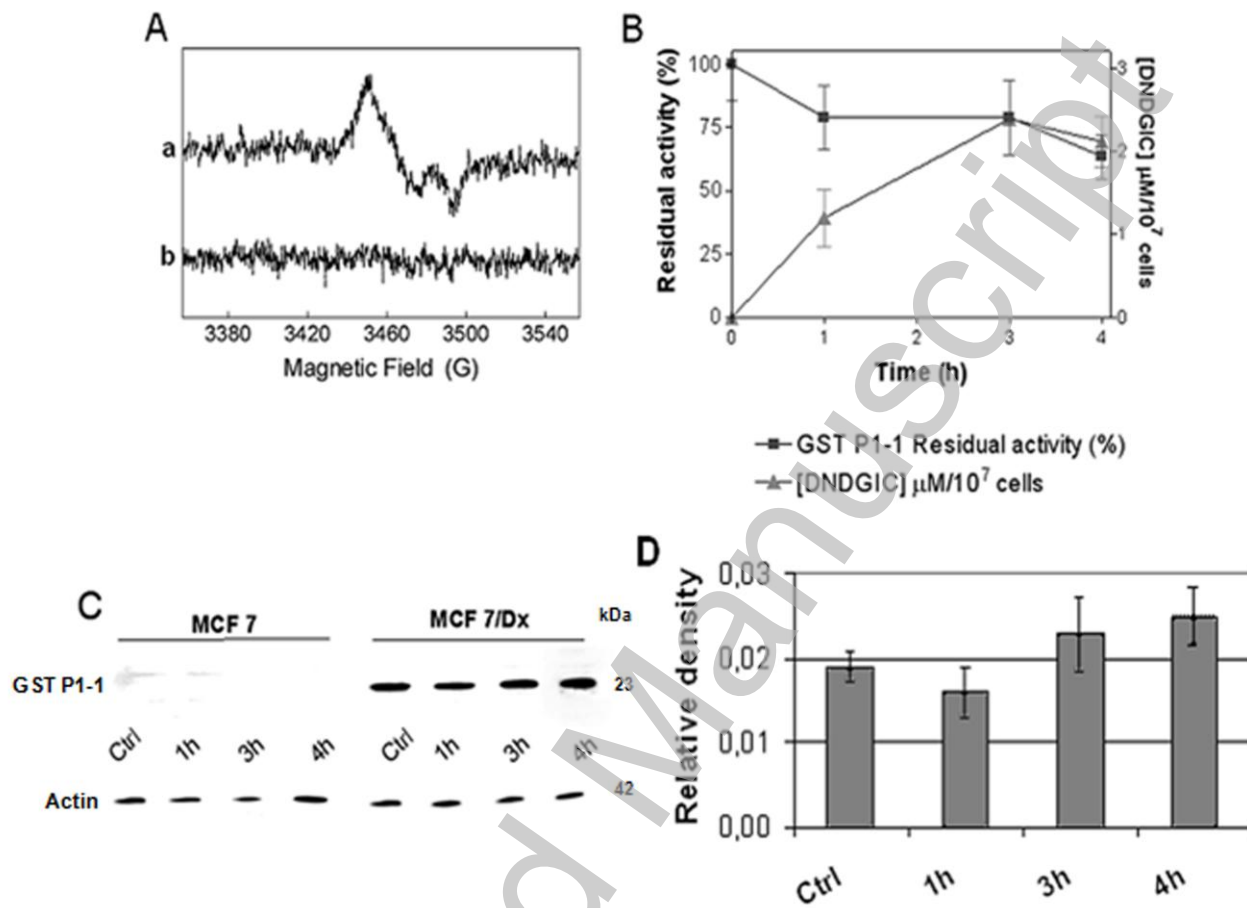


Figure 5

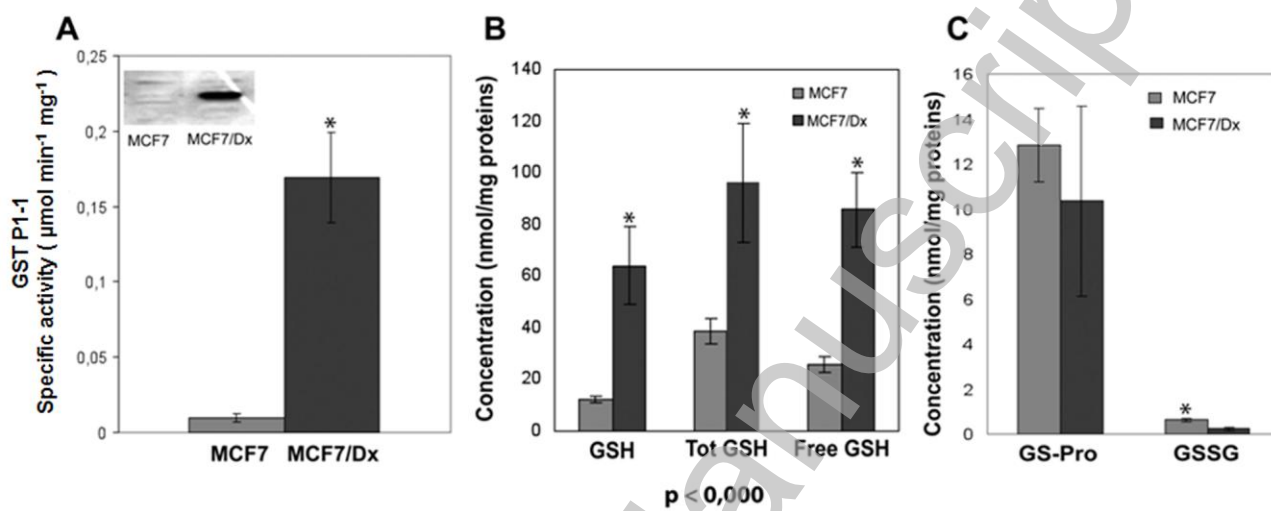


Figure 6