Acquired drug resistance was found to be suppressed in the doxorubicin-resistant breast cancer cell line MCF7/Dx after pre-treatment with GSNO (nitrosoglutathione). 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, to our knowledge, the first time that histone glutathionylation has been reported. Formation of the potential NO donor dinitrosyl–diglutathionyl–iron complex, bound to GSTP1-1 (glutathione transferase 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 a 14-fold higher amount of GSTP1-1 and increased glutathione concentration compared with 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 glutathione levels and increased expression of GSTP1-1. Owing to 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: drug resistance, glutathione, glutathione transferase

INTRODUCTION

One of the most common problems encountered during the treatment of tumours is the acquired resistance which eventually arises after a successful initial period of chemotherapy with drugs such as doxorubicin and cisplatin. Recent reports of NO (nitric oxide) 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 tumour cells, but different mechanisms have been proposed to explain this. It has been suggested that resistance to doxorubicin, owing to an increased efflux of the 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 in cells overexpressing a multidrug-resistance protein [2]. Other studies suggest that an increased rate of detoxification through Phase II enzymes [3], combined with high cellular glutathione content, could account for the effect [4]. NO 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 lifetime in the cell, but is stabilized and transported by NO donors such as the DNDGIC (dinitrosyl–diglutathionyl–iron complex) or GSNO (nitrosoglutathione). 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 signalling cascades, apoptosis, ion channels, redox systems and haemoproteins. We have previously shown that human GSTP1-1 (glutathione transferase P1-1) strongly binds DNDGIC in vitro and in vivo while maintaining its well-known detoxifying activity towards dangerous compounds [9,10]. A very high affinity for DNDGIC was also found for other GST classes (GSTM, GSTA and GSTT), 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 co-ordinated defence strategy, together with other glutathione-dependent enzymes, cytochrome P450s (Phase I enzymes) and some membrane transporters (Phase III), such as MRP1 (multidrug-resistance protein 1) and MRP2, to remove glutathione conjugates from the cell [13]. Among the human cytosolic GSTs, the Pi class enzyme GSTP1-1 is believed to be an important factor in tumour drug resistance, and overexpression of GSTP1-1 has been reported for a number of different human malignancies [3]. However, GSTP1-1 has also been proposed as a possible tumour marker for certain types of cancer (e.g. prostate cancer), where the lack of expression of this enzyme is actually an unfavourable prognostic factor [14]. In the present 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, which is the first time, to our knowledge, 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 tumour cell lines and in vitro treatments

The drug-sensitive human breast cancer cell line MCF7 and its derivative MDR (multidrug resistant) variant MCF7/Dx were provided by 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). MCF7 and MCF7/Dx cell lines were grown as monolayers in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 1-glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 μg/ml) at 37°C in a humidified 5% CO2 atmosphere. The 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.

For the treatments, exponentially growing MCF7 and MCF7/Dx cells were seeded at a density of 4 × 104/cm2 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 (at http://www.BiochemJ.org/bj/440/bj4400175add.htm). Thereafter, cells were treated with 0.5 mM GSNO, prepared as described previously [9], for a period ranging from 1 to 4 h. In both cell lines, the doxorubicin (Adriblastina) effect on cell viability was evaluated after 24 h treatment with 5 μM doxorubicin, with or without GSNO pre-treatment (see Supplementary Figure S2 at http://www.BiochemJ.org/bj/440/bj4400175add.htm). For microscopy analyses, cells were grown on coverslips.

HPLC determination of glutathione

The cells, either untreated or treated for up to 4 h, were sonicated with three pulses for 2 s in 0.1 ml of 10 mM Tris/HCl, pH 7.4 at room temperature (25°C) using a Vibra Cell Sonicator (Sonic and Materials). The HPLC determination of GSH and GSSG was performed as reported previously [15]. Briefly, for glutathione-free determinations, 100 μl of 12% sulfosalicylic acid was added to 50 μl of cell lysate, and glutathione content in the acid-soluble fraction was determined. The protein pellet was dissolved in 150 μl of 0.1 M NaOH, and protein-bound glutathione was determined. For GSSG determination, the cells were sonicated as described above at 4°C in the presence of 5 mM N-ethylmaleimide; 100 μl of 12% sulfosalicylic acid was added to 50 μl homogenates, and GSSG content in the acid-soluble fraction was determined. Total protein concentration was evaluated using the Lowry [15a] assay with BSA (Sigma–Aldrich) as control.

EPR detection of DNDGIC complex in intact cells

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

Determination of GSTP1-1 activity

After GSNO treatment and EPR analysis, MCF7 and MCF7/Dx cells were lysed by sonication as described above 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–visible spectrophotometer (Varian), at 25°C with 1-chloro-2,4-dinitrobenzene as co-substrate, monitoring product formation at 340 nm (ε = 9600 M−1·cm−1) [17]. Total protein concentration was evaluated using the Lowry [15a] assay with BSA as control.

Western blot analysis of GST isoenzymes and glutathionylated proteins

Cytosolic proteins (30 μg), previously extracted by lysis, as described above, of either MCF7 or MCF7/Dx cells, untreated or treated with 0.5 mM GSNO for a period ranging from 1 to 4 h, were resolved on SDS/PAGE (12% gels) under non-reducing conditions and transferred on to a nitrocellulose membrane (Bio-Rad). Western blotting was carried out according to the method of Towbin et al. [18] using polyclonal rabbit anti-GSTP1-1, anti-GSTA1-1, anti-GSTM2-2 (Calbiochem) and anti-GSTT1-1 (prepared in our laboratory) as primary antibodies and an antirabbit antibody conjugated to horseradish peroxidase as secondary antibody (Bio-Rad), with enhanced chemiluminescence detection (ECL Plus; GE Healthcare). Western blotting of glutathionylated proteins was carried out using a monoclonal anti-glutathione antibody (Virogen). For protein loading controls, the membrane was hybridized with monoclonal mouse anti-actin antibody (Abcam) and anti-mouse antibody conjugated to horseradish peroxidase (Sigma) with enhanced chemiluminescence detection (ECL Plus).

Immunoprecipitation

MCF7 and MCF7/Dx cells untreated or after 4 h treatment with 0.5 mM GSNO were lysed and total proteins (4 mg) were subjected to immunoprecipitation with a monoclonal anti-glutathione antibody as described previously [19]. Immunoprecipitated proteins were separated by SDS/PAGE (12% gels) under non-reducing conditions and subjected to Western blot analysis using either anti-glutathione (Virogen) or monoclonal anti-H3 antibodies (Abcam). The same samples were also immunoprecipitated with the anti-H3 antibody and analysed by Western blotting using either anti-glutathione, anti-H3 or anti-GSTP1-1 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 PI (propidium iodide) in the presence of 0.1 mg/ml RNAase, and apoptotic/necrotic cells were counted. At least 300 cells per sample were observed using CLSM (confocal laser scanning microscopy). All these reagents were purchased from Sigma. Samples were analysed using the flow cytometer FACS CaliburSM (Becton Dickinson) to evaluate the effect of doxorubicin and GSNO treatments on the cell cycle by analysing the DNA content after PI staining.

Doxorubicin accumulation assay

Doxorubicin accumulation was determined as following: (i) by analysing the intracellular distribution of the drug in living cells by CLSM, taking advantage of the intrinsic fluorescence of doxorubicin; or (ii) by flow cytometry according to methods described previously [20,21]. In detail, MCF7 and MCF7/Dx cells
were pre-treated with 0.5 mM GSNO for 1 or 3 h, and then 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 using CLSM or trypsinized, washed and resuspended in PBS and the amount of cellular doxorubicin was estimated by the flow cytometer FACS-Scan (BD Biosciences).

**RESULTS**

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

Treatment of MCF7/Dx cells with GSNO for up to 4 h revealed enhanced glutathionylation of proteins as detected by Western blotting analysis. Figure 1(A) shows a number of bands, with molecular masses ranging from 60 kDa down to 6 kDa reacting with the anti-glutathione antibody, with a more intense staining in the MCF7/Dx cell extracts and reaching a maximum level after 3 h of treatment. A more accurate analysis of this modification was carried out by HPLC analysis of the same samples (Figure 1B). Through a Swiss-Prot sequence search we identified the proteins corresponding to GSTP1-1 activity and expression, and the HPLC determination of GSH and GSSG, were estimated by Student’s t test and were considered to be statistically significant at \( P < 0.05 \).

Identification of glutathionylated proteins by immunoprecipitation and microsequencing analysis

We attempted to identify the glutathionylated proteins, observed by Western blotting in Figure 1(A), by immunoprecipitation of the MCF7/Dx cell lysate treated with 0.5 mM GSNO for 4 h with an anti-glutathione antibody. SDS/PAGE of the co-precipitated proteins with anti-glutathione antibody, stained with Coomassie Blue, showed many bands, in a broad range of molecular masses (Figure 2A), confirming that a number of proteins were subjected to glutathionylation. Owing to the low amount of sample we were only able to identify the three most abundant proteins, which ranged between 6 kDa and 19 kDa. These three bands, as indicated in Figure 2(A), were excised from the PVDF membrane and sequenced at the N-terminus through automated Edman degradation to give the following results. The upper band, with a molecular mass of approximately 12 kDa, showed two overlapping sequences as reported in Figure 2(A). A more accurate analysis of this modification was carried out by HPLC analysis of the same samples (Figure 1B). Through a Swiss-Prot sequence search we identified the proteins corresponding to GSTP1-1 activity and expression, and the HPLC determination of GSH and GSSG, were estimated by Student’s t test and were considered to be statistically significant at \( P < 0.05 \).
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 h. Ctrl, control. (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 h. Error bars represent S.D. for at least three separate 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 h) (upper panels), in comparison with MCF7 cell lines untreated or treated with 0.5 mM GSNO (3 h) (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 h was immunoprecipitated (IP) using anti-glutathione antibody and subjected to SDS/PAGE under non-reducing conditions. The gel was stained with Coomassie Blue and three bands corresponding to molecular masses 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 (WB) analysis with either anti-glutathione or anti-H3 antibodies of immunoprecipitates of MCF7 and MCF7/Dx cells, before and after exposure to 0.5 mM GSNO for 4 h, obtained by anti-H3 antibody. (C) Western blot analysis with either anti-glutathione or anti-H3 antibodies of immunoprecipitates of MCF7 and MCF7/Dx cells, before and after exposure to 0.5 mM GSNO for 4 h, obtained by anti-H3 antibody. (D) Western blot analysis with anti GSTP1-1 antibody of immunoprecipitates of MCF7 and MCF7/Dx cells, before and after exposure to 0.5 mM GSNO for 4 h, obtained by anti-H3 antibody. Purified recombinant GSTP1-1 was used as protein control (c).
GSNO treatment increased doxorubicin accumulation in the nucleus and reverted the drug resistance in MCF7/Dx cells

In drug-sensitive cells, doxorubicin appeared localized mainly in the nuclei, whereas 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), whereas 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 cells, as compared with untreated MCF7/Dx cells (Figure 3B). After 24 h of doxorubicin treatment, approximately 75% of drug-sensitive MCF7 cells showed morphological features of apoptotic/necrotic cells, whereas cell death was almost absent in drug-resistant MCF7/Dx cells (Figure 3C). Pre-treatment of MCF7/Dx cells with 0.5 mM GSNO for 1 h was able to revert the doxorubicin resistance of MCF7/Dx cells, leading to a 30-fold increase in the number of apoptotic/necrotic cells (approximately 65% of cells), a level comparable with that observed in drug-sensitive MCF7 cells (Figure 3C). It was obvious that neither doxorubicin nor GSNO alone (results not shown) were able to trigger cell death of drug-resistant cells, whereas their combined action was effective in abolishing 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 h doxorubicin treatment caused a dramatic block in G2/M-phase (Figure 4A), whereas in the drug-resistant MCF7/Dx cells, neither doxorubicin nor 1 h/3 h 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 h, we observed that approximately 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 was also confirmed using a cell viability assay (see Supplementary Figure S2). These results again demonstrate the synergy of NO and doxorubicin in the reversal of drug resistance in the MCF7/Dx cells.

GSNO treatment of both MCF7 and MCF7/Dx cells causes formation and binding of DNDGIC to GSTP1-1

In the cytosol, NO spontaneously forms a DNDGIC, which binds with very high affinity to intracellular GSTs [9–12,23]. EPR spectroscopy 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 GSTP1-1 [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 h of incubation (Figure 5B). Treatment with GSNO gave identical spectra for drug-sensitive MCF7 cells (results not shown), confirming 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 GSTP1-1 in either MCF7 or MCF7/Dx cells, as shown by Western blotting analysis (Figures 5C and 5D).

**MCF7/Dx cells overexpressed GSTP1-1 and had increased amounts of glutathione compared with MCF7 cells**

The presence of GSTP1-1 in the sensitive and resistant MCF7 cell line was analysed by Western blotting and enzyme activity measurements. There was little GSTP1-1 expression (as observed with Western blotting) and very low enzyme activity in the sensitive cell line, in contrast with the resistant line, which shows a marked band of GSTP1-1 protein and a 14-fold increase in specific activity (Figure 6A). The HPLC analysis of glutathione indicated that all forms (reduced, total and free) were significantly increased in the MCF7/Dx cells (Figure 6B), whereas there was no change in the glutathione bound to proteins, and GSSG was decreased (Figure 6C) in comparison with MCF7 cells. However, it should be noted that, in the MCF7/Dx cells after GSNO treatment, there was an increase of GSSG as compared with GSH (see Supplementary Figure S3 at http://www.BiochemJ.org/bj/440/bj4400175add.htm), indicative of nitrosative stress. We also probed both cell lines using immunoblotting for the presence of GSTM1-1, GSTA1-1 and GSTT1-1 without any positive results in any case (results not shown).

**DISCUSSION**

In the present study we show that it is possible to circumvent the acquired drug resistance of the breast tumour cell line MCF7/Dx through treatment with GSNO. A general enhancement of protein glutathionylation was observed after GSNO treatment of MCF7/Dx cells in comparison with MCF7 cells (Figure 1A).
Figure 5  GSNO treatment of MCF7/Dx caused DNDGIC binding to GSTP1-1 and partial inhibition of enzymatic activity

(A) EPR spectra of intact MCF7/Dx cells: (a) after 3 h exposure to 0.5 mM GSNO; and (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 4 h. (C) Western blotting analysis of both MCF7 and MCF7/Dx cells with human anti-GSTP1-1 antibody during GSNO treatment. (D) Densitometric analysis of Western blotting of MCF7/Dx cells. Error bars represent S.D. for at least three separate experiments. Ctrl, control.

Figure 6  Characterization of MCF7 and MCF7/Dx cells for GSTP1-1 expression and activity and for glutathione content

(A) Enzymatic activity and expression level (by Western blotting analysis, shown in the inset) of GSTP1-1 in MCF7/Dx and in MCF7 cells. (B) HPLC determination of the reduced, total (Tot) and free glutathione (G) content in the above cell lines. Free glutathione is defined as the fraction of glutathione not bound to proteins with respect to total glutathione and includes both GSH and GSSG. (C) HPLC determination of the glutathione bound to proteins (GS-Pro) and GSSG in both cell lines. Error bars represent S.D. for at least three separate experiments (*P < 0.05).

This result was confirmed by HPLC analysis of glutathionylated proteins (Figure 1B) and by CLSM of the same MCF7/Dx cells (Figure 1C), which also showed 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 separate proteins all belonging to the histone family (Figure 2). It is well known that these proteins (H3, H4, H2B and H2A) form an octamer core around which DNA can wind for packaging into the nucleosomes that regulate transcription [24]. Post-translational modifications of histones, such as methylation, ubiquitination, phosphorylation and acetylation, have been reported previously [25–27], 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-glutathione and anti-H3 antibodies (Figure 2). For histone H3, a possible target of glutathionylation might be its hyperactive Cys110, but this remains to be demonstrated.
The cytosolic distribution of doxorubicin in the MCF7/Dx cells, as compared with the nuclear localization of this drug in MCF7 cells (Figure 3A), may be explained by the fact that resistant tumour cells can avoid the cytotoxicity of this anticancer drug by overexpression of integral membrane transporters such as P-glycoprotein and MDR-associated proteins with the resultant 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 (Figures 3A and 3B). It has been suggested that NO could inhibit the efflux by blocking the P-glycoprotein 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 NO 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 with that of MCF7 cells when treated under identical conditions (Figures 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 (Figures 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 that, in both MCF7 and MCF7/Dx cells, upon GSNO treatment, DNDGIC formed and bound to GSTP1-1, which was associated with partial enzyme inhibition (Figures 5A and 5B). In the cytosol, NO spontaneously forms DNDGIC, 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 and enhanced glutathionylation) cannot be ascribed to differences in GSNO uptake and metabolism.

We have analysed the content of glutathione and GSTP1-1 in both sensitive and resistant cell lines. A strong decrease in enzymatic activity [29,30] and lack of GSTP1-1 expression in MCF7 cells, in comparison with MCF7/Dx cells, have already been reported and attributed to cytosine methylation of the GSTP1 promoter, mediated in part by MBDP2 protein bound to this region [31]. Our results from the present study showed a significant increase of glutathione and GSTP1-1 (Figures 6A and 6B) in the MCF7/Dx cells in comparison with the sensitive cell lines, thus confirming the importance of the antioxidant and detoxifying defence system mediated by glutathione [13] in the acquired resistance of certain tumour cells.

A number of glutathionylated proteins have been reported in the literature, suggesting an important role for this post-translational modification in terms of signalling and redox regulation [8,32]. Our results from the present study show that, in MCF7/Dx cells, which contain substantial amounts of glutathione and GSTP1-1, both increased glutathionylation and DNDGIC formation occurs, whereas only DNDGIC formation occurs in the MCF7 cells. Interestingly, the 4-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 GSTP1-1 is the covalent attachment of glutathione to a wide variety of substrates, and this enzyme can therefore be considered a likely catalyst of this glutathionylation. Recently, such a role for GSTP1-1 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 the present study (the glutathionylation of histones) poses several questions. First, is there any change in the function of histones upon their modification by glutathione? If so, this could influence the expression of genes involved in doxorubicin resistance/sensitivity. Secondly, the formation of a complex between GSTP1-1, H3 and glutathione, as suggested by immunoprecipitation studies using anti-H3 antibodies (Figures 2C and 2D), requires further studies to clarify the presence of GSTP1-1 in the nucleus. A similar complex involving GSTP1-1 has been already reported for the activation of the antioxidant enzyme 1-Cys peroxiredoxin, which requires glutathionylation mediated by GSTP1-1 heterodimer formation [34]. Preliminary experiments using GSTP1-1 antibodies (results not shown) suggest the presence of a nuclear GSTP1-1 in accordance with previous reports describing the presence of a perinuclear GST [35], or suggesting a specific transport system for nuclear transfer of GSTP1-1 as an extreme attempt of cellular defence against antitumour 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 tumour cells overexpressing human GSTP1-1 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.

**AUTHOR CONTRIBUTION**

Anastasia De Luca conducted the experiments and helped with manuscript preparation. Noemi Moroni performed in vitro cell line experiments. Annalucia Seralino and Pasquale Pierimarchi performed cell cycle analysis and CLSM experiments. Alessandra Primavera contributed to experiments and helped with manuscript preparation. Jenz Pedersen performed EPR experiments. Raffaele Petruzzelli carried out sequence analysis of glutathionylated proteins. Anna Pastore carried out HPLC determination of the various forms of glutathione. Maria Grazia Farrace performed immunoprecipitation assays. Gabriella Moroni contributed to cell line experiments. Giorgio Federici contributed to study design. Paola Sinibaldi Vallebona contributed to study design and data interpretation. Mario Lo Bello contributed to experimental design and co-ordination, and wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY ONLINE DATA

Treatment of doxorubicin-resistant MCF7/Dx cells with nitric oxide causes histone glutathionylation and reversal of drug resistance

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EXPERIMENTAL

Evaluation of cytotoxicity and/or cytostatic effect of GSNO on MCF7 and MCF7/DX

The cytotoxic effect of GSNO on MCF7 and MCF7/DX was assessed by incubating 100,000 cells/well with different GSNO concentrations, ranging from 0.125 to 0.5 mM. After 6, 24, 48 and 72 h of incubation, cells were washed twice with PBS, detached with trypsin and the cellular survival was evaluated by counting them in the presence of Trypan Blue. Error bars represent the S.D. for at least three different experiments.

Cell survival assay

Cell survival was evaluated using the Trypan Blue dye exclusion method after 24 h and 48 h of 5 μM doxorubicin treatment on MCF7 and the MDR variant MCF7/Dx, the latter in the absence or presence of 1 h and 3 h 0.5 mM GSNO pre-treatment. For the determination of doxorubicin cytotoxicity, cells were seeded at a density of 5.2 × 10^4/cm^2 (to obtain a 70% confluent monolayer) on to 25 cm^2 plates and allowed to adhere for 24 h before treatments.

Figure S1 Effect of dose/response of GSNO on MCF7 and MCF7/Dx cell proliferation

GSNO was used at 0.125 to 0.5 mM as indicated. Ctrl, control.

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Figure S2  Effect of 5 μM doxorubicin treatment on cell viability of MCF7 and MCF7/Dx with or without GSNO pre-treatment

CTR, control.

Figure S3  Relative ratios of GSH to GSSG in both MCF7 and MCF7/Dx cells after GSNO treatment

The amount of both reduced and oxidized glutathione was measured by HPLC analysis as reported in the Experimental section of the main text. Ctrl, control.