Pharmacological Impairment of *S*-Nitrosoglutathione or Thioredoxin Reductases Augments Protein *S*-Nitrosation in Human Hepatocarcinoma Cells

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Abstract. *Background/Aim: S-Nitrosoglutathione reductase (GSNOR) and thioredoxin enzyme systems participate in cellular defence against nitrosative stress. Pharmacological interventions against these enzyme systems might represent valuable strategies to impair S-nitrosothiol (SNO) homeostasis in tumour cells. Materials and Methods: Human HepG2 cells were pre-treated with mithramycin A or auranofin and exposed to S-nitroso-L-cysteine. GSNOR mRNA levels were analyzed by quantitative real-time reverse transcriptase-polymerase chain reaction and S-nitrosated proteins were detected and purified using the biotin-switch approach. Proteins were identified using electrospray ionization tandem mass spectrometry. Results: Mithramycin interfered with GSNOR induction resulting in an increased cellular sensitivity to protein S-nitrosation. Moreover, the thioredoxin reductase inhibitor auranofin also increased cellular susceptibility to S-nitrosoprotein formation. The impairment of these two cellular defense systems against nitrosative stress resulted in different sets of S-nitrosated proteins, as revealed by the proteomics approach. Conclusion: Our results suggest that pharmacological intervention with mithramycin or auranofin may constitute promising tools for altering SNO homeostasis in tumour cells.*

Nitric oxide (NO) is a simple, diatomic molecule that is synthesized *in vivo* and is responsible for a range of physiological processes such as vascular homeostasis, neurotransmission and host defense mechanisms (1, 2). As a result, conditions of NO excess or deficiency are currently believed to be responsible to some degree for a variety of biological derangements. Many actions of NO are thought to be mediated by means of stimulation of soluble guanylyl cyclase (sGC), which results in the production of the second messenger cyclic GMP. There is burgeoning evidence, however, that the sGC-cGMP pathway represents only a part of the alternative biochemical pathways through which NO can trigger or modulate cell signalling. Of these pathways, the best known is that of thiol nitrosation, often referred to as *S*-nitrosylation, a post-translational protein modification that could play a critical role in health and disease (3). *S*-Nitrosation/denitrosation of proteins is regulated by diverse physiological stimuli in different cell types and *in vitro* systems (4). It has been proposed that disruption or deregulation of *S*-nitrosothiol (SNO) signalling leads to impairment of cellular function and disease (3). Accumulating evidence indicates the existence of a nitrosative stress-response that helps in maintaining NO/SNO homeostasis. *S*-Nitrosoglutathione (GSNO) reductase (GSNOR) is a highly conserved enzyme that appears to be responsible for the major GSNO-metabolizing activity in eukaryotes (5). GSNOR has been shown to be essential in vascular homeostasis and for survival during endotoxic challenge, underlining the important role that GSNOR plays in the cellular defence against nitrosative stress (6). In this regard, we have previously shown that treatment of human hepatocytes with L-nitrosocysteine (CSNO) causes a rapid increase in *S*-nitrosoprotein content, which returns later to basal levels due to an increase in GSNOR activity (7, 8). This increased GSNOR activity in CSNO-treated hepatocytes appeared to be related to higher mRNA levels of *ADH5*, the gene encoding for human alcohol dehydrogenase 5/GSNOR, in these cells (8). Furthermore, we have recently shown in cholestatic rats that the hepatoprotective effect of inhibiting

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NO synthesis was related to the recovery of hepatic GSNOR/ADH5 expression facilitating SNO homeostasis (9).

Sp1 is a strong transcriptional activator of the human GSNOR/*ADH5* promoter (10). This transcription factor is important to the transcription of cellular genes that contain GC boxes in their promoters (11). Therefore, Sp1 inhibitors such as mithramycin A may have profound consequences in the maintenance of SNO cellular homeostasis. Mithramycin A, also known as aureolic acid and plicamycin, is an aureolic acid-type polyketide produced by various soil bacteria of the genus *Streptomyces* (12). This antineoplasic antibiotic has been utilized clinically to treat Paget's disease and testicular carcinoma (13, 14).

Other enzymes might also influence SNO and *S*-nitrosoprotein levels. In this regard, the thioredoxin/thioredoxin reductase system has been described to cleave GSNO (15) and *S*-nitrosoproteins (16). Furthermore, a recent study suggests that the thioredoxin system may constitute a specific enzymatic mechanism of regulating basal and stimulus-induced protein denitrosation in distinct cellular compartments (17).

The present study aimed to explore the effects of mithramycin A, or the thioredoxin reductase inhibitor auranofin on the sensitivity to nitrosative stress in human hepatoma cells.

Materials and Methods

Materials. Mithramycin A and auranofin were obtained from Sigma-Aldrich (Madrid, Spain) and Biomol International (Plymouth Meeting, PA, USA), respectively. *S*-Nitrosocysteine was synthesized as described elsewhere (18) by incubation of L-cysteine with acidified sodium nitrite and quantification by absorbance at 334 nm using a molar absorption coefficient of 0.74 mM^{-1} cm^{-1}. All other reagents were from Sigma-Aldrich unless otherwise stated.

HepG2 cell culture and sample preparation. Cells (150,000 cells/cm^2) were cultured at 37˚C and 5% CO_2 in minimal essential medium (MEM) containing 2.2 g/l HCO_3Na, 100 mM sodium pyruvate, 0.292 g/l glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin and 10% fetal calf serum. HepG2 cells were treated during 30 min with the indicated concentrations of *S*-nitroso-L-cysteine (CSNO). Mithramycin A (1 μM) or auranofin (2 μM) were administered 1 h before CSNO. After the different treatments, cells were washed with phosphate-buffered saline, scraped and resuspended in non-denaturing lysis solution (50 mM Tris–HCl, pH 7.4, 300 mM NaCl, 5 mM EDTA, 0.1 mM neocuproine, 1% Triton X-100 and 1 mM phenylmethanesulphonylfluoride plus aprotinin and leupeptin), incubated on ice for 15 min, and centrifuged at 10,000 × g, 4˚C for 15 min. Supernatant was collected and protein was quantified with Bradford reagent (Bio-Rad, Hercules, CA, USA).

Detection of protein S-nitrosation by the Biotin Switch Method. The procedure was performed as previously described (19). Briefly, cell lysates were incubated with 20 mM methyl methanethiosulfonate (Sigma) followed by acetone precipitation. Precipitates were centrifuged and resuspended in HENS buffer (250 mM HEPES pH 7.7, 1 mM EDTA, 0. 1 mM neocuproine, and 1% SDS) and then incubated with 1 mM ascorbic acid and 4 mM *N*-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide (Biotin-HPDP; Pierce, Rockford, IL, USA) for 1 h. Because biotin-HPDP is cleavable under reducing conditions, prepared samples were loaded onto sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels without dithiothreitol. All steps preceding SDS-PAGE were carried out in the dark. Biotinylated samples were then detected by Western blot analysis using a primary monoclonal anti-biotin antibody (Sigma), a secondary antibody coupled to horseradish peroxidase (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and the ECL Advance detection system (Amersham Biosciences, Uppsala, Sweden).

Analysis of GSNOR/ADH5 mRNA by quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA from hepatocytes was extracted using Trizol reagent according to the manufacturer's recommendations (Life Technologies Inc., Carlsbad, CA, USA). RNA was precipitated using ice-cold isopropanol, washed with 75% ethanol and resuspended in RNAse-free water (Sigma). RNA was treated with 1 IU/μg RNA DNAse I (Promega, Madison, WI, USA) at 37˚C for 30 min. DNAse I was then degraded at 65˚C for 10 min. The integrity of RNA was verified following separation by electrophoresis on 0.8% agarose gel containing 0.5% (v/v) ethidium bromide. The expression of mRNA for *ADH5* was examined by quantitative real-time RT-PCR using the LightCycler thermal cycler system (Roche Diagnostics, Indianapolis, USA). RT-PCR was performed in one step, using the QuantiTect SYBR Green RT-PCR kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's protocol. The ADH5 gene-specific primers, 5'-ATGGTGCTGCTGTGAACACT-3' (forward) and 5'-GGAAGCACCAGCCACTTTAC-3' (reverse) were designed using Primer3 software, v.0.4.0 (SourceForge open source software, Mountain View, Ca, USA). First-strand cDNA synthesis was performed with 50 ng of total RNA diluted in reaction mixture including Omniscript and Sensiscript Reverse Transcriptase and *ADH5* primers. The RT conditions were 20 min at 55˚C and 15 min at 95˚C. The amplification protocol consisted of 50 cycles of incubation after initial denaturation at 95˚C for 15 s, 60˚C for 15 s and 72˚C for 30 s. The melting conditions were fixed at 65˚C. Results were normalized to that of a housekeeping gene, *RPL13A*, 5'-CCTGGAGGAGAAGAGGAAAGAG A-3' (forward), and 5'-TTGAGGACCTCTGTGTATTTGTCAA-3' (reverse). To verify amplification specificity, *ADH5* (113 bp) and *RPL13A* (126 bp) PCR products were subjected to a melting curve analysis to confirm production of a single product and analyzed by 1.5% agarose gel electrophoresis containing ethidium bromide. Quantitation of relative expression was determined by the $2^{-\Delta(\Delta CT)}$ method (20).

Purification of S-nitrosoproteins. Cell lysates (3-4 mg protein) were biotinylated as described above. Biotin–HPDP was removed by acetone precipitation and centrifugation, and the pellet was resuspended in HENS buffer as above. Two volumes of neutralization buffer (20 mM HEPES pH 7.7, 100 mM NaCl, 1 mM EDTA and 0.5% Triton X-100) were added, and EZview™ Red streptavidin–agarose (Sigma) was added to purify biotinylated proteins. Biotinylated proteins were incubated with the resin for 1 h at room temperature, washed 5 times with neutralization buffer adjusted to 600 mM NaCl, and then incubated with elution buffer (20 mM HEPES pH 7.7, 100 mM NaCl, 1 mM EDTA and 100 mM 2-mercaptoethanol) for 20 min at 37˚C with gentle agitation to recover the bound protein. Supernatants were collected and proteins were separated in 10% SDS-PAGE gels, which

were stained with Sypro Ruby protein stain (Biorad). Gel bands were excised using a robotic workstation (Investigator™ Propic™, Genomics Solutions, Ann Harbor, MI, USA) and were trypsin-digested using a robotic digestion system (ProGest™; Genomic Solutions).

Protein identification. For the electrospray ionization tandem mass spectrometry (ESI-MS/MS) analysis, microcapillary reversed-phase liquid chromatography (LC) was performed with a CapLC™ (Waters, Milford, MA, USA) capillary system. Reversed-phase separation of tryptic digests were performed with an Atlantis, C18, 3 μm, 75 μm × 10 cm Nano Ease™ fused silica capillary column (Waters) equilibrated in 5% acetonitrile with 0.2% formic acid. After injection of sample (18 μl) the column was washed during 5 min with the same buffer and the peptides were eluted using a linear gradient of 5-50% acetonitrile in 30 min at a constant flow rate of 0.2 μl/min. The column was coupled online to a Q-TOF Micro (Waters) using a PicoTip nanospray ionization source (Waters). The heated capillary temperature was 80˚C and the spray voltage was 1.8-2.2 kV. MS/MS data were collected in an automated data-dependent mode. The three most intense ions in each survey scan were sequentially fragmented by collision-induced dissociation (CID) using an isolation width of 2.5 and a relative collision energy of 35%. Data processing was performed with MassLynx 4 and ProteinLynx Global Server 2 (Waters).

Statistical analysis. Results are expressed as means with their corresponding standard errors. Comparisons were made using ANOVA with least significant difference (LSD) test. Statistical significance was set at $p \leq 0.05$.

Results

We first explored the effects of mithramycin A on the capability of hepatoma cells to respond to alteration of SNO homeostasis. To that end, HepG2 cells were preincubated with 1 μM mithramycin A, and then were exposed to different doses of *S*-nitroso-L-cysteine (CSNO). This physiological nitrosothiol is readily taken up by cells *via* the amino acid transporter system L (21), and can transfer the bioactivity of the *S*-nitroso functional group from the extracellular to the intracellular space through its participation in transnitrosation reactions. As shown in Table I, the treatment of HepG2 cells with CSNO augmented *GSNOR/ADH5* mRNA levels in a dose-dependent manner. Notably, the pre-treatment with mithramycin impaired the induction of *GSNOR/ADH5* expression by CSNO in HepG2 cells.

We next explored whether this effect of mithramycin A on *GSNOR/ADH5* expression altered the levels of *S*-nitrosoproteins in these cells. Therefore, the biotin-switch method, which labels *S*-nitrosoproteins with an affinity tag to facilitate their detection (19), was used to explore changes in the pattern of *S*-nitrosoproteins in HepG2 cells after CSNO treatment. As shown in Figure 1, treatment of HepG2 cells with CSNO resulted in increased levels of *S*-nitrosoproteins, as detected by the biotin-switch method. Notably, those cells that were pre-treated with mithramycin A exhibited a higher increase in their *S*-nitrosoprotein content after CSNO exposure than did their corresponding controls (Figure 1).

Table I. *Relative GSNOR/ADH5 expression in HepG2 cells pre-treated or not with mithramycin A (MIT) and exposed to S-nitroso-L-cysteine.*

Treatment[a]	–MIT	+MIT
None	89.8±5.9	76.3±3.6
0.5 mM CSNO	95.3±4.6	79±0.5*
2.0 mM CSNO	125.1±4.7	89.9±12.1*

[a]ADH5 mRNA levels were determined by real-time quantitative RT-PCR in HepG2 cells treated during 30 min with the indicated concentrations of S-nitroso-L-cysteine (CSNO). Mithramycin A (MIT) was administered (1 μM) 1 h before CSNO. *$p<0.05$, comparing with the respective control without mithramycin A. Data are the mean±SE of three separate experiments.

We next decided to examine whether the impairment of other important cellular enzymatic defense against nitrosative stress, such as the thioredoxin/ thioredoxin reductase system, could also affect *S*-nitrosoprotein levels in HepG2 cells. Therefore, cells were pre-treated with the organogold compound auranofin, a highly specific inhibitor of thioredoxin reductase, and cellular lysates were subjected to the biotin-switch method. As shown in Figure 2, the pre-treatment of HepG2 cells with auranofin augmented the *S*-nitrosoprotein content in cells exposed to CSNO. However, this increment was not as severe as that observed with mithramycin.

To further explore the negative effects of mithramycin and auranofin in the preservation of cellular SNO homeostasis, we decided to identify the *S*-nitrosoproteins in HepG2 cells exposed to CSNO after pre-treatment with mithramycin or with auranofin. Therefore, cell lysates were subjected to the biotin-switch assay and the resulting biotinylated proteins were purified by immobilization in streptavidin-agarose and elution with 2-mercaptoethanol. As the biotin is incorporated *via* a disulfide bond and elution is carried out in reducing conditions, endogenously biotinylated proteins are not purified. Eluted proteins were then separated by SDS-PAGE, and the protein bands were excised from the gel, trypsin-digested and the corresponding proteins identified by LC-MS/MS analysis. Tables II and III list the *S*-nitrosoproteins identified in HepG2 cells exposed to CSNO after pre-treatment with mithramycin A and auranofin, respectively. As shown in Table II, 26 proteins involved in metabolism, antioxidant defense, detoxification, signalling and cellular homeosatasis were identified in hepatoma cells pre-treated with mithramycin A and exposed to CSNO. On the other hand, 11 proteins were identified when HepG2 cells were exposed to CSNO after pre-treatment with auranofin (Table III). All of the *S*-nitrosoproteins purified from cells pre-treated with auranofin were also purified when HepG2 were pre-treated with mithramycin A. Proteins with different subcellular locations, including membrane (4%), cytosol (65%), endoplasmic reticulum (ER) (19%), mitochondria (8%)

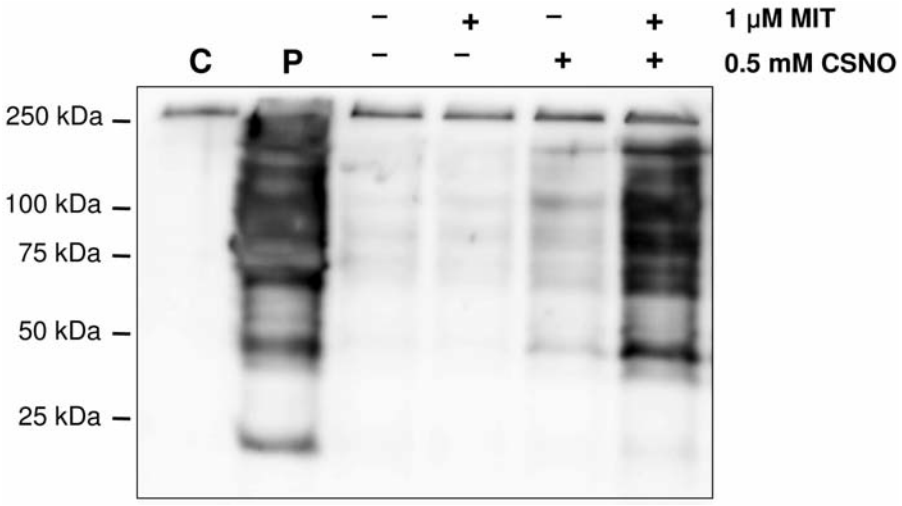

Figure 1. *Detection of S-nitrosated proteins in HepG2 cells pre-treated with mithramycin A. Lysates from HepG2 cells pretreated or not with 1 μM mithramycin A (MIT) 1 h before treatment with the indicated dose of S-nitroso-L-cysteine (CSNO) were subjected to the biotin-switch assay to detect S-nitrosoproteins. P indicates a positive control obtained by preincubating a control cell lysate with 200 μM S-nitrosoglutathione. The image is representative of three different experiments.*

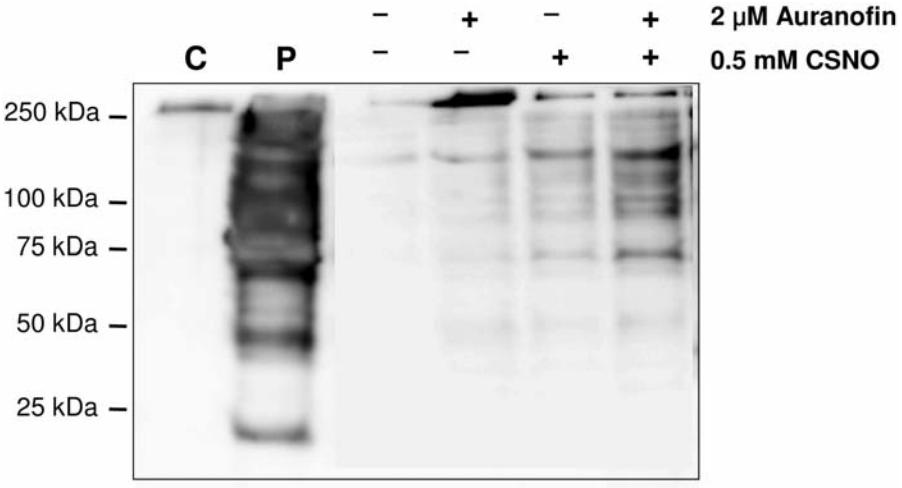

Figure 2. *Detection of S-nitrosated proteins in HepG2 cells pre-treated with auranofin. Lysates from HepG2 cells pretreated or not with 1 μM auranofin 1 h before treatment with the indicated dose of S-nitroso-L-cysteine (CSNO) were subjected to the biotin-switch assay to detect S-nitrosoproteins. Figure includes the control lanes (C and P) from Figure 1 for comparison of S-nitrosoprotein levels. The image is representative of three different experiments.*

and nucleus (4%), were identified in cells pre-treated with mithramycin A. However, those proteins identified in cells pre-treated with auranofin were predominantly of cytosolic origin.

Discussion

Protein modifications induced by NO were initially detected only on protein-bound transition metals, such as the haem Fe^{+2} of guanylate cyclase (1). However, research over the past decade has shown that the principal target of cellular NO is the thiol group of cysteine residues of peptides and proteins, and that such post-translational modification can positively or negatively regulate various signalling pathways, proteins and metabolic processes (3, 22). The role of NO in the modulation of cell survival in tumour cells is controversial and may depend on the concentration and localization of NO, as well as the timing of an apoptotic stimulus, the cell type and cellular redox state (23). Thus, post-translational modifications induced by NO may be of significance in carcinogenesis and cancer chemotherapy. Although specific enzymes that exclusively

Table II. *S-Nitrosated proteins identified by LC-MS/MS analysis in HepG2 cells pre-treated with mithramycin A and exposed to S–nitroso-L-cysteine*.

Protein name	NCBI accession number	Number of sequenced peptides	Sequence coverage (%)	Subcellular location
Cytoskeleton proteins				
Actin cytoplasmic 1	P60709	3	23	Cyt
Tubulin beta-5 chain	P07437	4	13	Cyt
Metabolic enzymes				
Alpha-enolase	P06733	6	23	Cyt
Dihydroxyacetone kinase	Q3LXA3	2	4	Cyt
Fructose-bisphosphate aldolase A	P04075	2	18	Cyt
Glutamate dehydrogenase 1	P00367	2	3	Mit
Glyceraldehyde-3-phosphate dehydrogenase	P04406	3	9	Cyt, Nucl
Pyruvate kinase isozymes M1/M2	P14618	1	8	Cyt
Triosephosphate isomerase	P60174	1	11	Cyt
Molecular chaperones				
60 kDa heat-shock protein	P10809	11	36	Mit
78 kDa glucose-regulated protein precursor	P11021	11	31	ER
Endoplasmin precursor (GRP94)	P14625	4	20	ER
Heat-shock protein HSP 90-beta	P08238	11	25	Cyt
Peptidyl-prolyl *cis-trans* isomerase A	P62937	2	20	Cyt
Peptidyl-prolyl *cis-trans* isomerase B	P23284	1	15	ER
Protein disulfide isomerase precursor	P07237	4	21	ER, Membr
Protein disulfide-isomerase A4 precursor	P13667	5	19	ER
Antioxidant and detoxification enzymes				
Peroxirredoxin 1	Q06830	2	16	Cyt
Peroxirredoxin 6	P30041	2	20	Cyt
Thioredoxin	P10599	1	12	Cyt
UDP-glucose 6-dehydrogenase	O60701	2	9	Cyt
Signalling proteins				
Leucocyte immunoglobulin-like receptor subf B member 1	Q8NHL6	3	14	Membr
Elongation factor 1-alpha 1	P68104	2	10	Cyt
14-3-3 protein beta/alpha	P31946	1	14	Cyt
Other proteins				
Annexin A5	P08758	1	9	Cyt
Nucleolin	P19338	1	3	Nucl

Table III. *S-Nitrosated proteins identified by LC-MS/MS analysis in HepG2 cells pre-treated with auranofin and exposed to S-nitroso-L-cysteine*.

Protein name	NCBI accession number	Number of sequenced peptides	Sequence coverage (%)	Subcellular location
Cytoskeleton proteins				
Tubulin beta-5 chain	P07437	2	9	Cyt
Metabolic enzymes				
Triosephosphate isomerase	P60174	1	11	Cyt
Molecular chaperones				
Heat-shock protein HSP 90-beta	P08238	1	10	Cyt
Protein disulfide isomerase precursor	P07237	1	6	ER, Membr
Peptidyl-prolyl *cis-trans* isomerase A	P62937	1	26	Cyt
Antioxidant and detoxification enzymes				
Peroxirredoxin-6	P30041	1	21	Cyt
Signalling proteins				
14-3-3 protein epsilon	P62258	1	11	Cyt
14-3-3 protein zeta/delta	P63104	2	13	Cyt
14-3-3 protein theta	P27348	2	9	Cyt
Elongation factor 1 alpha 1	P68104	1	2	Cyt

mediate *S*-nitrosation/de-*S*-nitrosation have not been characterized, GSNOR and thioredoxin reductase have been identified as important mediators that participate in the cellular maintenance of SNO homeostasis (5, 17). The alteration of these enzymatic systems may constitute a rational approach to investigate *S*-nitrosation targets in specific cell types (22).

In the present study, we have shown that interference with GSNOR or thioredoxin reductase using mithramycin A and auranofin caused enhanced sensitivity to nitrosative stress in human hepatoma cells. This augmented susceptibility was shown by the augmented levels of cellular *S*-nitrosoproteins after treatment of HepG2 cells with the *S*-nitrosothiol CSNO. Therefore, pharmacological intervention with mithramycin A or auranofin may constitute a promising tool to manipulate SNO homeostasis in tumour cells. The identification of the *S*-nitrosated proteins also provided significant data about the molecular alterations in the sensitized HepG2 cells. It is interesting to note that all of the *S*-nitrosoproteins identified in cells sensitized with auranofin were also identified in cells sensitized with mithramycin A. The impairment of GSNOR induction with mithramycin not only resulted in a higher cell susceptibility to CSNO, but also in *S*-nitrosoproteins of diverse subcellular locations, including membrane, cytosol, ER, mitochondria and nucleus. However, the proteins identified in cells pre-treated with auranofin were largely of cytosolic origin. This finding may be explained by the different mechanisms through which GSNOR and thioredoxin reductase participate in the maintenance of cellular SNO homeostasis. Impairment in GSNOR alters the dynamic equilibrium between intracellular GSNO and *S*-nitrosoproteins, favouring *S*-nitrosoprotein formation *via* transnitrosation with GSNO. These transnitrosation reactions are known to be favoured in organelles such as mitochondria and ER, which are known to have comparatively more appropriate environments promoting *S*-nitrosation and stability of *S*-nitrosoproteins (24). On the other hand, in the case of thioredoxin reductase impairment, the effects may be more related to the direct protein de-*S*-nitrosation activity of this enzyme (17). Also in this regard, although auranofin effectively inhibits mitochondrial thioredoxin reductase (25), the treatment of cells with this compound may primarily influence the denitrosation of cytosolic proteins that are substrates for cytosolic thioredoxin reductase.

Many of the *S*-nitrosoproteins identifed in HepG2 cells sensitized with mithramycin A and with auranofin corresponded to previously reported *S*-nitrosated proteins during hepatotoxin- and CSNO-induced cell death in human primary hepatocytes (7, 8). Our proteomic analysis of *S*-nitrosated proteins in HepG2 cells revealed important enzymes responsible for energy production and metabolism, as well as molecular chaperones and proteins involved in the structural integrity of the cells. The enhanced susceptibility to nitrosative stress in human hepatoma cells treated with mithramycin A resulted in the modification of key components of the cellular chaperone machinery, such as GRP94, GRP78, Hsp90, Hsp60, and protein disulfide isomerase. The *S*-nitrosation of these proteins may have profound effects in the preservation of cellular homeostasis and may render tumour cells more susceptible to chemotherapy. For instance, glucose-regulated proteins GRP94 and GRP78/BiP, are members of the ER chaperone family, originally discovered as proteins inducible by glucose starvation (26). Several studies have shown GRP94 and GRP78/BiP play a protective role in maintaining cell viability and are involved in the resistance against chemotherapy in cancer (27, 28).

Significantly, one of the *S*-nitrosoproteins identified in the HepG2 cells sensitized with mithramycin A was the classic glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Recent studies have highlighted the role of this protein as a mediator for cell death. *S*-Nitrosation of GAPDH facilitates its binding with Siah1, an E3 ubiquitin ligase, and the GAPDH-Siah protein complex translocates to the nucleus, dependent on the nuclear localization signal of Siah1, degrading Siah1 substrates in the nucleus, which results in cytotoxicity (29).

Several members of the 14-3-3 family of proteins were also identified as *S*-nitrosoproteins in HepG2 cells. The 14-3-3 proteins function as molecular scaffolds by modulating the conformation of their binding partners. Through the functional modulation of a wide range of binding partners, 14-3-3 proteins are involved in many processes, including apoptotic signaling, cell growth signaling and tumor suppression (30). The 14-3-3 proteins are tightly integrated into the pathways that often become dysregulated in cancer, including hepatocellular carcinoma (31). Our finding that several 14-3-3-proteins are targets for *S*-nitrosation in human hepatoma cells underlines the significance of the impairment of SNO homeostasis in these tumour cells.

In summary, our study demonstrates that pharmacological intervention with mithramycin A, and auranofin represent valuable strategies to impair SNO homeostasis in tumour cells. The subsequent alteration in the metabolism of *S*-nitrosoproteins, targeting proteins that are key factors in maintaining cell viability and that are involved in the resistance against chemotherapy, may be of practical benefit in the design of combined therapies for cancer treatment.

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