Asbestos induces doxorubicin resistance in MM98 mesothelioma cells via HIF-1α.

Chiara Riganti[1,2], Sophie Doublier[1,2], Elisabetta Aldieri[1,2], Sara Orecchia[3], Pier Giacomo Betta[3], Elena Gazzano[1,4], Dario Ghigo[1,4], Amalia Bosia[1,2].

[1]Department of Genetics, Biology and Biochemistry, University of Torino; [2]Research Center on Experimental Medicine (CeRMS), Via Santena 5/bis, 10126 Torino, Italy; [3]Pathology Unit, Department of Oncology, Azienda Sanitaria Ospedaliera, Via Venezia 16, I-15100 Alessandria, Italy; [4]Interdepartmental Center "G. Scansetti" for Studies on Asbestos and Other Toxic Particulates, Università di Torino, Italy.

Corresponding author: Dario Ghigo, Dipartimento di Genetica, Biologia e Biochimica (Sezione di Biochimica), Via Santena, 5/bis, 10126 Torino, Italy. Phone: +39-011-6705849. Fax : +39-011-6705845. E-mail: *dario.ghigo@unito.it*

Short title: asbestos and drug resistance in mesothelioma.

ABSTRACT

Human malignant mesothelioma (HMM), which is strongly related to asbestos exposure, exhibits high resistance to many anticancer drugs. Asbestos fibers deposition in the lung may cause hypoxia and iron chelation at the fibers surface. Hypoxia-inducible factor-1α (HIF-1α), which is upregulated by a decreased availability of oxygen and iron, controls the expression of membrane transporters, such as P-glycoprotein (Pgp), which actively extrude the anticancer drugs. This study has been aimed to assess whether asbestos may play a role in the induction of doxorubicin resistance in HMM cells through the activation of HIF-1α and an increased expression of Pgp.

After a 24 h incubation with crocidolite asbestos, or with the iron chelator dexrazoxane or under hypoxia, HMM cells were tested for HIF-1α activation, Pgp expression, accumulation of doxorubicin and sensitivity to its toxic effect.

Crocidolite, dexrazoxane and hypoxia caused HIF-1α activation, Pgp overexpression and increased resistance to doxorubicin accumulation and toxicity. These effects were prevented by the coincubation with the cell-permeating iron salt ferric nitrilotriacetate (FeNTA), which caused an increase of intracellular iron bioavailability, checked as increased activity of the iron regulatory protein-1 (IRP-1). Crocidolite, dexrazoxane and hypoxia induce doxorubicin resistance in HMM cells by increasing HIF-1α activity, through an iron-sensitive mechanism.

Keywords: asbestos, doxorubicin resistance, hypoxia-inducible factor-1α, iron, mesothelioma, P-glycoprotein.

INTRODUCTION

Human malignant mesothelioma (HMM) is an aggressive tumor of the serosal cavities, which is strongly related to the exposure to asbestos fibers [1]. It has a poor prognosis, due to its resistance to many anticancer drugs [2] and to the difficult delivery of chemotherapeutic agents into the pleural tissue [3]. In several *in vitro* models of mesothelioma, it has been reported that the multidrug resistance (MDR) is caused by the overexpression of membrane transporters, such as P-

2

glycoprotein (Pgp) and MDR-associated proteins (MRPs), which actively extrude the drugs, lowering their intracellular concentration and activity [4, 5]. Pgp gene has an hypoxia responsive enhancer in the promoter and is upregulated by the transcription factor hypoxia-inducible factor-1α (HIF-1α) [6]. HIF-1α is composed of two subunits: β, which is constitutively expressed, and α, which is rapidly degraded under normal conditions but becomes stable when oxygen or iron supply decreases, thus leading to a net increase of HIF-1α [7, 8]. HIF-1α is constitutively high in the hypoxic areas of tumors; moreover many growth factors and cytokines increase HIF-1α synthesis under normoxic conditions [9]. When active, HIF-1α upregulates several genes involved in processes such as cellular growth, glucose and iron metabolism, pH control, angiogenesis, matrix remodelling, drug resistance [10]. Since HIF-1α promotes cellular proliferation, inhibition of apoptosis, invasion and MDR, its expression in tumors is related to poor prognosis [11]: thus, different therapeutic approaches have been attempted to reduce HIF-1α expression [10, 12]. In lung most cell types, including bronchial and alveolar epithelium, smooth muscle and vascular endothelium, overexpress HIF-1α under hypoxic conditions [13]. High levels of HIF-1α have been described in mesothelioma biopsies of patients, whereas mesothelial cells contain low amount of HIF-1α [14].

Asbestos may elicit both proliferation and apoptosis in mesothelial cells, thus representing a complete carcinogen [15]. Furthermore, crocidolite asbestos has been reported to act as an iron chelator and alter the intracellular availability of iron [16, 17]. In this study we investigated whether: 1) crocidolite asbestos may play a role in inducing doxorubicin resistance, which is observed in HMM cells, 2) such an effect may be mimicked by hypoxia and iron chelation, 3) the drug resistance eventually induced by asbestos, hypoxia and iron chelation is mediated by HIF-1α activation and Pgp overexpression, and 4) iron supply to the cells may prevent these effects.

MATERIALS AND METHODS

3

Materials. Foetal bovine serum and HAM'S F-12 medium were supplied by BioWhittaker (Verviers, Belgium); plasticware for cell culture was from Falcon (Becton Dickinson, Bedford, MA); MG132 and 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1) were from Calbiochem (La Jolla, CA). Electrophoresis reagents were obtained from Biorad Laboratories (Hercules, CA); the proteins content of cell monolayers and cell lysates was assessed with the BCA kit from Pierce (Rockford, IL). Dexrazoxane (ICRF-187) was purchased from Chiron (Amsterdam, The Netherlands). When not otherwise specified, the other reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Stock solutions of 3 mM ferric nitrilotriacetate (FeNTA) were prepared by mixing 1:1 6 mM nitrilotriacetic acid (NTA) in 1 N NaOH and 6 mM ferric chloride in 1 N HCl; the pH was adjusted at neutrality with NaOH [18].

Cells. MM98 cells were HMM cells established from the pleural effusion of a male patient with histologically confirmed malignant mesothelioma; the mesothelial origin of the isolated cells was confirmed by positive immunostaining [19, 20]. Pgp overexpression and doxorubicin resistance of MM98 cells was described previously [20]. Cells were cultured in HAM'S F-12 medium (containing 3 μM $FeSO_4$), supplemented with 10% foetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamine and maintained in a humidified atmosphere at 37°C, 20% O_2, 5% CO_2. When cultured under hypoxia, cells were maintained in a humidified atmosphere at 37°C, 3% O_2, 5% CO_2 for 24 h, in an appropriate Heracell incubator (Heraeus, Hanau, Germany), which can decrease the O_2 injection by increasing the N_2 flow injection. The O_2 tension was monitored throughout the incubation time by an O_2 sensor incorporated in the incubator, which automatically varies the N_2 flow injection in order to maintain a stable 3% O_2 level.

Cell seeding density may influence the activation of HIF-1α and the resistance to doxorubicin, as previously described [21]. In preliminary seeding density-dependence experiments we observed that a decreasing cellular confluence was accompanied by the decreased expression of HIF-1α and Pgp protein and the increased doxorubicin accumulation both in normoxic and hypoxic cells (data not shown). For these reasons we took care to perform all the experimental procedures reported in this

work with a constant high cellular density (90% cellular confluence, corresponding to 1×10^6 cells/ml).

Asbestos fibers. UICC (*Union International Contre le Cancer*) crocidolite fibers were sonicated (Labsonic sonicator, 100 W, 10 s) before incubation with cell cultures, in order to dissociate fiber bundles and allow their better suspension and diffusion in the culture medium.

Electrophoretic mobility shift assay (EMSA). Cells were plated in 60-mm diameter dishes at confluence and all procedures for nuclear protein extraction were performed at 4°C using ice-cold reagents, as described [22]. The probe containing the HIF-1α oligonucleotide consensus sequence was labeled with $[\gamma\text{-}^{32}P]$ATP (3,000 Ci/mmol, 250 µCi; Amersham International, Bucks, UK), using T4 polynucleotide kinase (Roche, Basel, Switzerland). The sequence of oligonucleotide was: 5'-TCTGTACGTGACCACACTCACCTC-3'; 3'-AGACATGCACTGGTGTGAGTGGAG-5' (Santa Cruz Biotechnology, Santa Cruz, CA). 10 µg of extracts were incubated for 20 min with 20,000 cpm of ^{32}P-labeled double-stranded oligonucleotide at 4°C. In supershift assay, nuclear extracts were pre-incubated for 30 min at room temperature with 2 µl of anti-HIF-1α (Santa Cruz Biotechnology); then the reaction mixture containing the ^{32}P-labeled double-stranded oligonucleotide was added. The DNA-protein complex was separated on a not-denaturating 4% polyacrilamide gel in TBE buffer (0.4 M Tris, 0.45 M Boric Acid, 0.5 M EDTA, pH 8.0). After electrophoresis, the gel was dried and autoradiographed by exposure to X-ray film for 24 h.

IRP-1 binding activity. To measure the iron regulatory protein-1 (IRP-1) activity, taken as an index of intracellular iron [8], the probe containing the Iron Responsive Element (IRE) sequence from ferritin mRNA was labeled with $[\gamma\text{-}^{32}P]$ATP via T4 polynucleotide kinase. The sequence of oligonucleotide was: 5'- GUUCUUGCUUCAACAGUGUUUGAACGGAAC - 3'. 10 µg of cytosolic lysate proteins were incubated for 20 min with 20,000 cpm of ^{32}P-labeled oligonucleotide at 4°C and subjected to EMSA as described above. In competition assays, an excess of unlabelled

(cold) IRP-1 oligonucleotide was added in the EMSA reaction mixture, then samples were processed as previously reported.

Western blot analysis. Western blot detection of Pgp and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed as previously described [20]. To detect thioredoxin and glutathione reductase, we used the following antibodies: anti-thioredoxin-1/2 (from rabbit, diluted 1: 250 in PBS-BSA 1%, Santa Cruz Biotechnology); anti-glutathione reductase (from goat, diluted 1: 200 in PBS-BSA 1%, Santa Cruz Biotechnology).

Doxorubicin accumulation. Cells were grown in 60-mm diameter Petri dishes, incubated for 24 h in fresh medium containing 5 μM doxorubicin, washed twice in ice-cold PBS and detached with trypsin/EDTA (0.05/0.02% v/v). Intracellular doxorubicin accumulation was measured as described elsewhere [23].

AnnexinV/propidium iodide (PI) assay. MM98 cells were incubated for 24 h in the experimental conditions described under Results, then they were washed twice with fresh PBS and incubated for 10 min at room temperature in 1 ml of binding buffer (100 mM Hepes/NaOH, pH 7.5, 140 mM NaCl, 25 mM $CaCl_2$) containing 10 μM annexin V/fluorescein isothiocyanate conjugate (FITC) or 2.5 μM PI. The cells suspensions were washed three times with fresh PBS and rinsed with 1 ml of binding buffer. An aliquot of cells suspension was used for the cell count, using the toluidine blue staining. In each assay 0.5×10^6 cells were employed. The fluorescence of each sample was recorded using a Perkin-Elmer LS-5 spectrofluorimeter (Perkin Elmer, Shelton, CT). Excitation and emission wavelengths were 488 and 530 nm (for annexin V/FITC) and 536 and 617 nm (for PI), respectively. FeNTA, dexrazoxane, hypoxia, YC-1 and verapamil did not exert any change in cellular viability versus control cells in the absence of doxorubicin, whereas 15% of cells were positive for annexin V and PI when incubated with crocidolite asbestos (data not shown). To assess the cytotoxicity of doxorubicin, MM98 cells were incubated in the experimental conditions indicated under Results, in a medium containing 5 μM doxorubicin. To evaluate the doxorubicin-dependent apoptosis, the fluorescence obtained under each experimental condition in the absence of

6

doxorubicin was taken as a blank and was subtracted from the fluorescence obtained under the same experimental conditions in the presence of doxorubicin. Results were expressed as fluorescence mU/10^6 cells.

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

RESULTS

Crocidolite asbestos enhances doxorubicin resistance in MM98 cells and Pgp expression, similarly to dexrazoxane and hypoxia.

MM98 cells, which exhibit per se high Pgp expression and doxorubicin resistance [20], were incubated for 24 h in a medium containing 5 μM doxorubicin, in the absence or presence of crocidolite asbestos, dexrazoxane, a well-known iron chelator [24], FeNTA, a cell-permeating compound which increases the intracellular iron [18], or under hypoxia (3% O_2). The presence of crocidolite asbestos significantly lowered the intracellular accumulation of doxorubicin (Fig 1, A) and clearly enhanced the expression of Pgp (Fig 1, B), if compared to control. Both dexrazoxane and hypoxia exerted the same effects. On the other hand, FeNTA reverted completely the effects of each experimental condition, increasing the cell content of the drug and decreasing the level of Pgp protein (Fig 1). When used alone, FeNTA increased the doxorubicin accumulation above the control levels (Fig 1, A). The doxorubicin-induced cell death in MM98 cells was due to apoptosis: indeed, dexrazoxane, crocidolite and hypoxia significantly reduced the number of MM98 cells positive for both Annexin V and PI (Fig 1, A). On the opposite, FeNTA per se significantly increased the cytotoxicity of doxorubicin and prevented the effects of dexrazoxane, crocidolite and hypoxia (Fig 1, A).

Crocidolite asbestos and dexrazoxane increase IRP-1 activity in MM98 cells. IRP-1 activity is strictly dependent on intracellular iron labile pool, and its increased binding to the IRE sequence of

ferritin mRNA is considered a sensitive index of decreased intracellular iron level [25], more useful than the measurement of the total amount of iron with other tools in providing information about the actual iron bioavailability. Resting MM98 cells exhibited a detectable IRP-1 binding activity (Fig 2), which was decreased by FeNTA and enhanced by dexrazoxane. Interestingly, also crocidolite asbestos increased IRP-1 binding activity when compared to control. FeNTA reverted the effect of both dexrazoxane and crocidolite (Fig 2). MM98 cells incubated under hypoxic conditions (3% O_2) for 24 h did not exhibit a different IRP-1 binding activity versus control cells: again, the addition of FeNTA reduced IRP-1 binding activity elicited by hypoxia (Fig 2).

Crocidolite asbestos, dexrazoxane and hypoxia induce HIF-1α activation in MM98 cells.

The expression of Pgp gene is strictly regulated by transcription factor HIF-1α [6], whose activity is very sensitive to a decrease of intracellular iron and to hypoxia [8]. A basal nuclear translocation of HIF-1α was detectable in control MM98 cells: crocidolite asbestos, dexrazoxane and hypoxia (3% O_2) induced a marked increase of HIF-1α level in nuclear extracts (Fig. 3). To the contrary, FeNTA repressed HIF-1α activity, both basal and elicited by crocidolite, dexrazoxane and hypoxia (Fig 3).

The activation of HIF-1α increases Pgp expression and doxorubicin resistance in MM98 cells.

To verify whether the increase of HIF-1α was responsible for Pgp overexpression and doxorubicin resistance, we measured these parameters in the presence of 5 μM YC-1, a specific inhibitor of HIF-1α [26]. In preliminary experiments we had observed that the addition of 5 μM YC-1 for 24 h efficiently reduced the HIF-1α nuclear translocation under any experimental condition (data not shown). This concentration of YC-1 was chosen for the subsequent experiments. YC-1 prevented the Pgp induction in all experimental conditions (Fig 4, A), increased per se the intracellular doxorubicin accumulation and cytotoxicity, and abolished the effect of crocidolite, dexrazoxane and hypoxia (Fig 4, B). Interestingly the effects of YC-1 on the content and cytotoxicity of doxorubicin were similar to those evoked by FeNTA, which reduced HIF-1α activity (Fig 3). The different expression of Pgp was crucial in regulating the doxorubicin accumulation in MM98 cells: to correlate the doxorubicin resistance with the induction of Pgp in our experimental model, we

8

measured the intracellular accumulation and the cytotoxic effect of the drug after a 24 h incubation with dexrazoxane, crocidolite and hypoxia, alone or together with FeNTA, in the presence of 50 μM verapamil, a well-known Pgp inhibitor [27]. Verapamil lowered the Pgp activity and increased the doxorubicin accumulation and toxicity under each experimental condition (Fig 5). Similarly to YC-1, verapamil mimicked the effect of FeNTA, which has been shown to reduce the Pgp expression in MM98 cells (Fig 1).

The effects of hypoxia on HIF-1α activation and doxorubicin resistance are reverted by a subsequent normoxia.

MM98 cells were cultured for 24 h in hypoxic conditions, then they were let grow at 20% O_2 for 1, 3, 6 and 24 h. After each time point, HIF-1α nuclear translocation, Pgp expression, doxorubicin accumulation and cytotoxicity were assessed: hypoxia induced a marked nuclear translocation of HIF-1α, increased the Pgp expression and significantly reduced the doxorubicin accumulation and cytotoxicity (Fig 6). After a 3 h incubation in normoxic conditions, the amounts of HIF-1α and Pgp were markedly reduced and returned to the baseline levels after 6 h of normoxia (Fig 6, A and B). After this time also the accumulation and the toxicity of doxorubicin were superimposable to those observed under constant normoxic conditions (Fig 6, C).

Crocidolite and FeNTA regulate the levels of HIF-1α by affecting its proteasomal degradation

To clarify the mechanisms of crocidolite-dependent HIF-1α induction in MM98 cells, we assessed the expression of thioredoxin, which is known to regulate HIF-1α protein synthesis [28, 29]. Thioredoxin was constitutively expressed in MM98 cells, being unchanged under each experimental condition (Fig 7). Also an increased oxidative stress, due to the decrease of antioxidant enzymes, such as glutathione reductase, may activate HIF-1α [30]: indeed crocidolite fibers decreased the amount of glutathione reductase in MM98 cells (Fig 7). However, when FeNTA was added together with crocidolite, the decrease of glutathione reductase was not reverted (Fig 7), while the HIF-1α activation was blunted (Fig 3). Thus the effect of crocidolite, dexrarozane, hypoxia and FeNTA on HIF-1α was not related to changes of thioredoxin and glutathione reductase in MM98 cells.

To assess whether FeNTA may repress the activation of HIF-1α by increasing its proteasomal degradation, we incubated MM98 cells with the proteasome inhibitor MG132 for 24 h. MG132 alone increased the basal HIF-1α translocation to the nucleus and completely prevented the FeNTA-induced decrease of the HIF-1α activation observed in the presence of crocidolite (Fig 8). In parallel, MG132 increased the expression of Pgp (Fig 9, A) and decreased the intracellular accumulation and toxicity of doxorubicin (Fig 9, B), under each experimental condition.

DISCUSSION

HMM exhibits a constitutive resistance to many common anticancer drugs [31], mainly due to the overexpression of membrane transporters, such as Pgp and MRPs [5]. The efficacy of doxorubicin, as well as platinum-derived compounds and antifolate drugs - which are commonly used in mesothelioma therapy [2] - is often reduced in mesothelioma cells. Indeed these chemotherapeutic agents are poorly deliverable in pleural tissue [3]. Moreover they are all substrates of Pgp and MRPs [2]. In a previous study we observed that MM98 cells have a prominent constitutive Pgp expression and are poorly sensitive to doxorubicin [20]. Since Pgp gene has an hypoxia responsive enhancer in the promoter and is upregulated by HIF-1α [6], we investigated whether HIF-1α could play a role in doxorubicin resistance in MM98 cells and whether asbestos, which is the major pathogenetic agent of mesothelioma, may play a role in the induction of drug resistance via HIF-1α modulation.

Crocidolite asbestos significantly lowered the intracellular accumulation and the pro-apoptotic effect of doxorubicin, and in parallel enhanced the expression of Pgp. As far as the mechanism is concerned, it is known that: 1) crocidolite asbestos alters intracellular iron availability, impairing several metabolic pathways involved in survival or damage repair [17, 32]; 2) asbestos fibers bind iron from solutions [17] and adsorb iron from intracellular ferritin [3, 16] crocidolite induces nitric oxide synthesis in murine alveolar macrophages by decreasing iron bioavailability: this effect is inhibited by iron supplementation and enhanced by the iron chelator deferoxamine [33]. To

investigate whether iron availability may affect drug resistance in MM98 cells, we manipulated intracellular iron by using FeNTA, an iron salt which permeates the cell membrane increasing intracellular iron [18], and dexrazoxane, a potent and specific iron chelator [24]. Similarly to asbestos, dexrazoxane and hypoxia made MM98 cells more resistant to doxorubicin, and in parallel increased the expression of Pgp. FeNTA reverted such effects, suggesting that crocidolite and dexrazoxane are likely to increase doxorubicin resistance by decreasing the intracellular iron availability. Notably, when used alone FeNTA increased the doxorubicin accumulation and cytotoxicity above the controls. Indeed the basal levels of HIF-1α and Pgp become undetectable in the presence of FeNTA, owing to the accelerated degradation of HIF-1α: thus FeNTA-treated MM98 cells may accumulate more intracellular doxorubicin than the control cells and decrease their constitutive resistance to the drug. A sensitive marker of the intracellular iron availability, particularly of the labile iron pool, is the activation of IRP-1 [8]. IRP-1 is an RNA binding protein which regulates the translation of several mRNA in response to cellular iron [25]. In MM98 cells FeNTA decreased and crocidolite and dexrazoxane increased IRP-1 binding to ferritin mRNA: these effects strongly suggest that FeNTA increases and crocidolite and dexrazoxane decrease the labile pool of intracellular iron. Crocidolite and dexrazoxane lost their ability to activate IRP-1 when incubated together with FeNTA, similarly to what we observed for the doxorubicin accumulation. Hypoxia per se did not change significantly IRP-1 activity, as it is not expected to affect the intracellular iron pool of MM98 cells. Since increased bioavailability of iron can revert the effect of hypoxia, this suggests that both iron and oxygen levels modulate drug resistance by interacting at the same target, which could be HIF-1α. HIF-1α is known to be activated by a decrease of intracellular iron availability [7, 8] and by hypoxia [6]. In its turn, activated HIF-1α enhances Pgp expression and plays a central role in inducing drug resistance in tumors [6, 10].

A basal level of HIF-1α nuclear translocation was detected in resting MM98 cells, and this could be responsible for the constitutive expression of Pgp observed in the same cells. Furthermore, Pgp and HIF-1α appeared to be modulated in the same way. Indeed, HIF-1α translocation to the nucleus was

significantly reduced by FeNTA and increased by crocidolite and dexrazoxane. The effect of crocidolite and dexrazoxane on HIF-1α activity was abolished by FeNTA. This result suggests that crocidolite asbestos and dexrazoxane regulate both HIF-1α activation and Pgp levels by decreasing the intracellular iron availability. When HIF-1α activation was blunted by the specific HIF-1α inhibitor YC-1, the Pgp expression was low and the doxorubicin accumulation and toxicity were high under any experimental condition. Similarly, when the Pgp activity was inhibited by verapamil, none of the stimuli reduced the content and the cytotoxic effect of doxorubicin. YC-1 and verapamil mimicked the effects of FeNTA, which reduced the HIF-1α activity and Pgp expression in MM98 cells. On the basis of these results, we hypothesized that crocidolite, dexrazoxane, hypoxia and FeNTA exert their effects on doxorubicin efficacy by affecting the activity of HIF-1α. HIF-1α in turn regulates the expression of Pgp, which represents a crucial factor for doxorubicin accumulation in MM98 cells.

Interestingly, all the effects of hypoxia were reverted by a subsequent exposure to normoxia: after a 24 h incubation under hypoxic conditions, MM98 cells showed an increased expression of HIF-1α and Pgp and accumulated less doxorubicin; however, when cells were let grow in normoxic conditions after the incubation under hypoxia, HIF-1α and Pgp decreased and the doxorubicin content and toxicity increased as a function of time. The different rate of degradation of HIF-1α under hypoxic and normoxic conditions may explain both the acquisition of a drug-resistant phenotype in hypoxia and the reversion of the resistance observed during the subsequent normoxia. Acting as an iron chelator, crocidolite may directly activate HIF-1α in cells: oxygen and iron decrease the stability of HIF-1α by promoting its hydroxylation and the subsequent proteasomal degradation [7]. Furthermore crocidolite may increase the HIF-1α levels with several different mechanisms: in mesothelial cells lines crocidolite asbestos induces thioredoxin proteins mRNA transcription [34]. Thioredoxins regulate the rate of HIF-1α synthesis [29]. In MM98 cells none of the agents which modulated the activity of HIF-1α had any effect on the levels of thioredoxin.

Therefore, the increase of HIF-1α activity observed after dexrazoxane, crocidolite or hypoxia was not mediated by thioredoxin overexpression.

HIF-1α can be activated in cells subjected to an oxidative stress [30]. Crocidolite may induce a strong oxidative stress in mesothelioma cells in several different ways [1]: indeed asbestos fibers contain iron [17], which may generate reactive oxygen species via a Fenton-like reaction [32]. Moreover, following the phagocytosis of asbestos fibers, macrophages may produce ROS by activating NADPH oxidase [32]. Asbestos may evoke an oxidative stress also with further mechanisms, such as the inhibition of the pentose phosphate pathway [35], the decrease of several antioxidant enzymes (glutathione peroxidase, glutathione reductase and catalase) [36, 37] and the enhanced leakage of reduced glutathione [38]. The asbestos-induced oxidative stress may directly damage DNA, impair the DNA repairing systems and activate some redox-sensitive transcription factors, such as NF-kB [1] and HIF-1α [30]. In MM98 cells crocidolite markedly reduced the amount of glutathione reductase. The increase of HIF-1α nuclear translocation elicited by crocidolite might be a consequence of the oxidative stress caused by the decrease of glutathione reductase. However, when FeNTA was added together with crocidolite, the decrease of glutathione reductase was not reverted, while under the same experimental condition the crocidolite-induced increase of HIF-1α activity was prevented. This suggests that glutathione reductase decrease and HIF-1α increase are not related in crocidolite-treated cells. We cannot exclude that at least part of the crocidolite effect is mediated by an oxidative stress, but our results with the proteasome inhibitor MG132 suggest that the proteasomal degradation is the critical step which regulates the levels of HIF-1α in mesothelioma cells. Indeed MG132 enhanced HIF-1α stability, Pgp expression and doxorubicin resistance in MM98 cells, and completely reverted the effects of FeNTA.

In summary, our results suggest that the exposure of MM98 cells to crocidolite asbestos induces the activation of HIF-1α, which is accompanied by the increased expression of Pgp and in parallel by increased resistance to doxorubicin accumulation and toxicity. Crocidolite is likely to activate HIF-1α by decreasing the iron availability in the cell, as it can be inferred by the ability of the iron

chelator dexrazoxane to elicit analogous effects and by the reverting action of the cell-permeant iron salt FeNTA. We may suppose that crocidolite, beside increasing cellular proliferation and inhibiting apoptosis in mesothelial cells [15], may play a role in the induction of MDR in MM98 cells. Also hypoxia promptly induces HIF-1α in MM98 cells. Such an activation might mimic the *in vivo* situation: indeed HIF-1α is often high in patients affected by malignant mesothelioma [14]. Moreover our data suggest that the increase of intracellular iron content may decrease doxorubicin resistance even in presence of crocidolite fibers or hypoxia. Although our results are obtained in an *in vitro* model, they may open a new therapeutic strategy to revert doxorubicin resistance in malignant mesothelioma.

REFERENCES

1. Manning CB, Vallyathan V, Mossman BT. Diseases caused by asbestos: mechanisms of injury and disease development. *Int Immunopharmacol* 2002; 2: 191-200.

2. Tomek S, Emri S, Krejcy K, Manegold C. Chemotherapy for malignant pleural mesothelioma: past results and recent developments. *Br J Cancer* 2003; 88: 167-74.

3. Lu C, Perez-Soler R, Piperdi B, Walsh GL, Swisher SG, Smythe WR, Shin HJ, Ro JY, Feng L, Truong M, Yalamanchili A, Lopez-Berestein G, Hong WK, Khokhar AR, Shin DM. Phase II study of a liposome-entrapped cisplatin analog (L-NDDP) administered intrapleurally and pathologic response rates in patients with malignant pleural mesothelioma. *J Clin Oncol* 2005; 23: 3495-3501.

4. Ogretmen B, Bahadori HR, McMauley MD, Boylan A, Green MR, Safa AR. Co-ordinated over-expression of the MRP and γ-glutamylcysteine synthetase genes, but not MDR1, correlates with doxorubicin resistance in human malignant mesothelioma cell lines. *Int J Cancer* 1998; 75: 757-761.

5. Soini Y, Jarvinen K, Kaarteenaho-Wiik R, Kinnula V. The expression of P-glycoprotein and multidrug resistance proteins 1 and 2 (MRP1 and MRP2) in human malignant mesothelioma. *Ann Oncol* 2001; 12: 1239-1245.

6. Comerford KM., Wallace TJ, Karhausen J, Louis NA, Montalto MC, Colgan SP. Hypoxia-inducible Factor-1-dependent Regulation of the Multidrug Resistance (*MDR1*) Gene. *Cancer Res* 2002; 62: 3387-3394.

7. Brahimi-Horn C, Mazure N, Pouyssegur J. Signalling via the hypoxia-inducible factor-1a requires multiple posttranslational modifications. *Cell Signal* 2005; 17: 1-9.

8. Kang GS, Li Q, Chen H, Costa M. Effect of metal ions on HIF-1α and Fe homeostasis in human A549 cells. *Mutat Res* 2006; 610: 48-55.

9. Haddad JJ, Harb HL. Cytokines and the regulation of hypoxia-inducible factor (HIF)-1a. *Int Immunopharmacol* 2005; 5: 461-483.

10. O'Donnell JL, Joyce MR, Shannon AM, Harmey J, Geraghty J, Bouchier-Hayes D. Oncological implications of hypoxia inducible factor-1a (HIF-1a) expression. *Cancer Treat Rev* 2006; 32: 407-416.

11. Quintero M, Mackenzie N, Brennan PA Hypoxia-inducible factor 1 (HIF-1) in cancer. *Eur J Surg Oncol* 2004; 30: 465-468.

12. Powis G, Kirkpatrick L. Hypoxia inducible factor-1A as a cancer drug target. *Mol Cancer Ther* 2004; 3: 647-54.

13. Yu AY, Frid MG, Shimoda LA, Wiener CM, Stenmark K, Semenza GL. Temporal, spatial, and oxygen-regulated expression of hypoxiainducible factor-1 in the lung. *Am J Physiol* 1998; 275: L818-L826.

14. Klabatsa A, Sheaff MT, Steele JPC, Evans MT, Rudd RM, Fennell DA. Expression and prognostic significance of hypoxia-inducible factor 1α (HIF-1α) in malignant pleural mesothelioma (MPM). *Lung Cancer* 2006; 51: 53-59.

15. Leard LE, Broaddus VC. Mesothelial cell proliferation and apoptosis. *Respirology* 2004; 9: 292-299.

16. Fubini B; Barcelo` F, Otero-Arean C. Ferritin adsorption on amosite fibers: possible implications in the formation and toxicity of asbestos bodies. *J Toxicol Environ Health* 1990; 29**:** 307–316.

17. Hardy JA, Aust AE. Iron in asbestos chemistry and carcinogenicity. *Chem Rev* 1995; 95**:** 97-118.

18. Byrd TF, Horwitz MA.Chloroquine inhibits the intracellular multiplication of *Legionella pneumophila* by limiting the availability of iron: a potential new mechanism for the therapeutic effect of chloroquine against intracellular pathogens. *J Clin Invest* 1991; 88: 351-357.

19. Orecchia S, Schillaci F, Salvio M, Libener R, Betta PG. Aberrant E cadherin and g-catenin expression in malignant mesothelioma and its diagnostic and biological relevance. *Lung Cancer* 2004; 45: S37-S43.

20. Riganti C, Orecchia S, Pescarmona GP, Betta PG, Ghigo D, Bosia A. Statins revert doxorubicin resistance via nitric oxide in malignant mesothelioma. *Int J Cancer* 2006; 119: 17-27.

21. Fang Y, Sullivan R, Graham CH. Confluence-dependent resistance to doxorubicin in human MDA-MB-231 breast carcinoma cells requires hypoxia-inducible factor-1 activity. *Exp Cell Res* 2007; 13: 867-877.

22. Aldieri E, Atragene D, Bergandi L, Riganti C, Costamagna C, Bosia A, Ghigo D. Artemisinin inhibits inducible nitric oxide synthase and nuclear factor NF-kB activation. *FEBS Lett* 2003; 552: 141-144.

23. Riganti C, Miraglia E, Viarisio D, Costamagna C, Pescarmona GP, Ghigo D, Bosia A. Nitric oxide reverts the resistance to doxorubicin in human colon cancer cells by inhibiting the drug efflux. *Cancer Res* 2005; 65: 516-525.

24. Buss JL, Hasinoff BB. The one-ring open hydrolysis product intermediates of the cardioprotective agent ICRF-187 (dexrazoxane) displace iron from iron-anthracycline complexes. *Agents Actions* 1993; 40: 86-95.

25. Constable A, Quick S, Gray NK, Hentze MW. Modulation of the RNA-binding activity of a regulatory protein by iron in vitro: switching between enzymatic and genetic function? *Proc Natl Acad Sci USA* 1992; 89: 4554-4558.

26. Yeo EJ, Chun YS, Cho YS, Kim J, Lee JC, Kim MS, Park JW. YC-1: A Potential Anticancer Drug Targeting Hypoxia-Inducible Factor 1. *J Natl Cancer Inst* 2003; 95: 516-525.

27. Biscardi M, Teodori E, Caporale R, Budriesi R, Balestri F, Scappini B, Gavazzi S, Grossi A. Multidrug reverting activity toward leukemia cells in a group of new verapamil analogues with low cardiovascular activity. *Leuk Res* 2006; 30: 1-8.

28. Csiki I, Yanagisawa K, Haruki N, Nadaf S, Morrow JD, Johnson DH, Carbone DP. Thioredoxin-1 Modulates Transcription of Cyclooxygenase-2 via Hypoxia-Inducible Factor-1A in Non–Small Cell Lung Cancer. *Cancer Res* 2006; 66:143-150.

29. Zhou J, Damdimopoulos AE, Spyrou G, Brune B. Thioredoxin 1 and Thioredoxin 2 Have Opposed Regulatory Functions on Hypoxia-inducible Factor-1α. *J Biol Chem* 2007; 282: 7482-7490.

30. Haddad JJ, Harb HL. γ-Glutamyl-l-cysteinyl-glycine (glutathione; GSH) and GSH-related enzymes in the regulation of pro- and anti-inflammatory cytokines: a signaling transcriptional scenario for redox(y) immunologic sensor(s)? *Mol Immunol* 2005; 42: 987-1014.

31. Catalano A, Rodilossi S, Rippo MR, Caprari P, Procopio A. Induction of Stem Cell Factor/c-Kit/Slug signal transduction in multidrug-resistant malignant mesothelioma cells. *J Biol Chem* 2004; 279: 46706-46714.

32. Kamp DW, Weitzman SA. The molecular basis of asbestos lung injury. *Thorax* 1999; 54**:** 638-652.

33. Aldieri E, Ghigo D, Tomatis M, Prandi L, Fenoglio I, Costamagna C, Pescarmona GP, Bosia A, Fubini B. Iron inhibits the nitric oxide synthesis elicited by asbestos in murin macrophages. *Free Rad Biol Med* 2001; 31: 412-417.

34. Nymark P, Lindholm PM, Korpela MV, Lahti L, Ruosaari S, Kaski S, Hollmén J, Anttila S, Kinnula VK, Knuutila S. Gene expression profiles in asbestos-exposed epithelial and mesothelial lung cell lines. *BMC Genomics* 2007, 8: 62-76.

35. Riganti C, Aldieri E, Bergandi L, Fenoglio I, Costamagna C, Fubini B, Bosia A, Ghigo D. Crocidolite asbestos inhibits pentose phosphate oxidative pathway and glucose 6-phosphate dehydrogenase activity in human lung epithelial cells. *Free Rad Biol Med* 2002; 32: 938-949.

36. Afaq F, Abidi P, Matin R, Rahman Q. Activation of alveolar macrophages and peripheral red blood cells in rats exposed to fibers/particles. *Toxicol Lett* 1998; 99: 175-182.

37. Tatrai E, Brozik M, Drahos A, Kováçiková Z, Six E, Csík M, Dám A. The effect of stone-wool on rat lungs and on the primary culture of rat alveolar macrophages and type II pneumocytes. *J Appl Toxicol* 2006; 26: 16-24.

38. Golladay SA, Park SH, Aust AE. Efflux of reduced glutathione after exposure of human lung epithelial cells to crocidolite asbestos. *Environ Health Perspect* 1997; 105: 1273-1277.

FIGURE LEGENDS

Figure 1. Effects of crocidolite asbestos, dexrazoxane, hypoxia and FeNTA on intracellular doxorubicin accumulation, cell survival and Pgp expression in MM98 cells. Cells were incubated for 24 h in the absence (*CTRL*) or presence of FeNTA (*FeN*, 60 µM), dexrazoxane (*DXR*, 100 µM), crocidolite fibers (*CRO*, 25 µg/cm^2), alone or in different combinations. Some samples were incubated under hypoxic conditions (*HYP*), i.e. under 3% (instead of 20%) O_2, alone or with FeNTA (*FeN*). **A.** Intracellular doxorubicin accumulation and annexin/PI assay. MM98 cells were incubated in the experimental conditions indicated above in the presence of 5 µM doxorubicin, then they were detached and lysed in ethanol/HCl: the intracellular content of the drug was measured as indicated under Materials and Methods. In parallel an aliquot of MM98 cells incubated in the same experimental conditions was used for the quantitative measurement of annexin V/PI fluorescence (see Materials and Methods). Measurements were performed in triplicate and data are presented as means $\pm$ SE (n = 4). vs. *CTRL*: * $p < 0.001$; vs. *DXR* or *CRO* or *HYP* respectively: $^{\circ}$ $p < 0.05$. In preliminary experiments of dose-dependence we had observed that 60 µM FeNTA and 10 µM dexrazoxane were the minimal doses able to increase and decrease, respectively, in a significant way the intracellular doxorubicin accumulation versus the respective control (data not shown). We then chose for this and subsequent experiments the concentrations of 60 µM FeNTA and 100 µM dexrazoxane (the latter in order to be sure of chelating the whole iron released from FeNTA). At these concentrations both compounds did not exert per se any cytotoxic effect on MM98 cells (data not shown). The culture medium used for MM98 cells contained approximately 3 µM $FeSO_4$. **B.** Pgp was immunoprecipitated from lysates of MM98 cells and then detected by Western blotting (as described under Materials and Methods). Expression of GAPDH, used as the product of an housekeeping gene, was detected in the same cell lysates as a control of equal loading. The figure is representative of three experiments with similar results.

Figure 1

A

□ doxo ▨ annexin ■ PI

B

Pgp (170 kDa)

GAPDH (37 kDa)

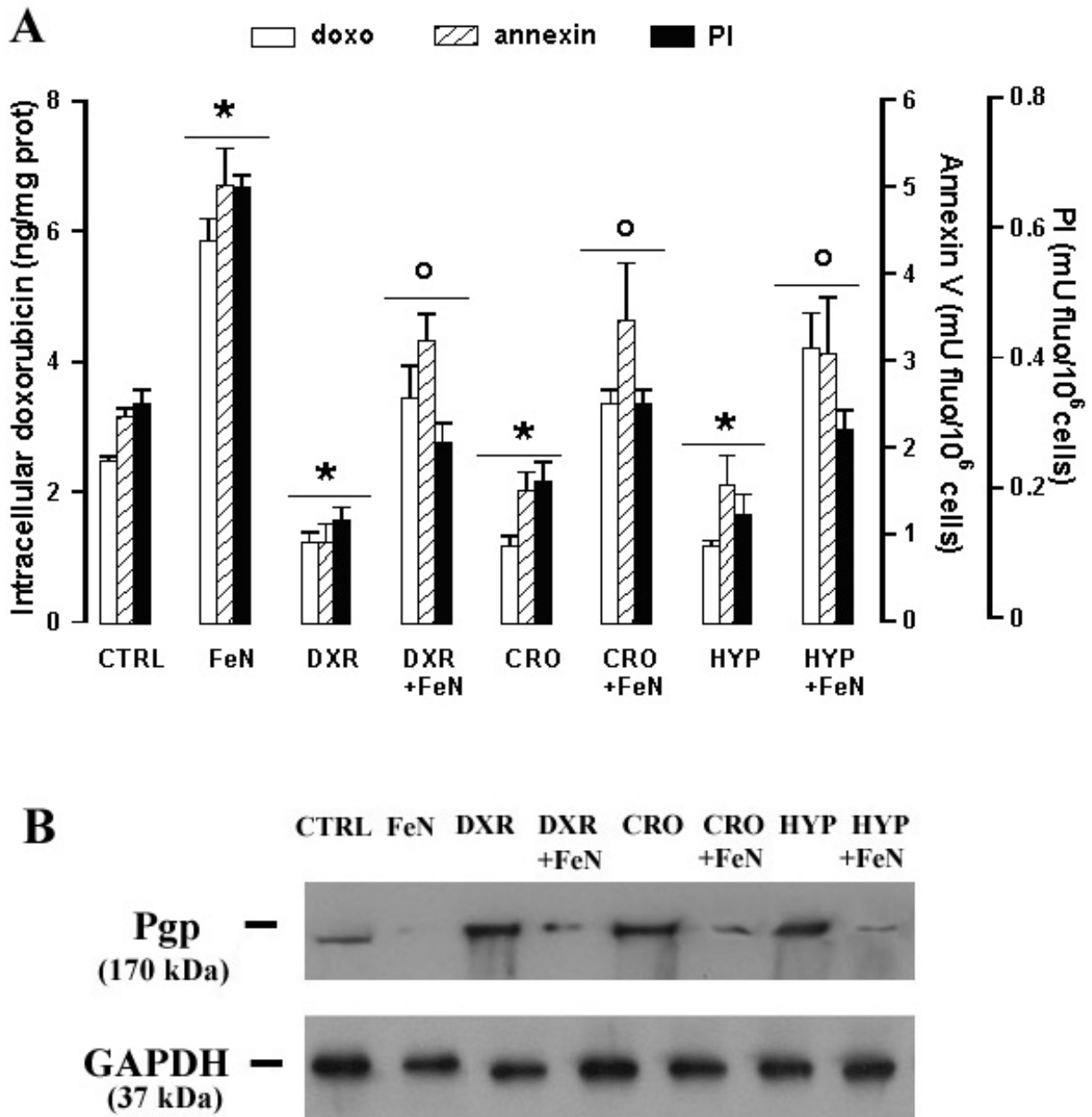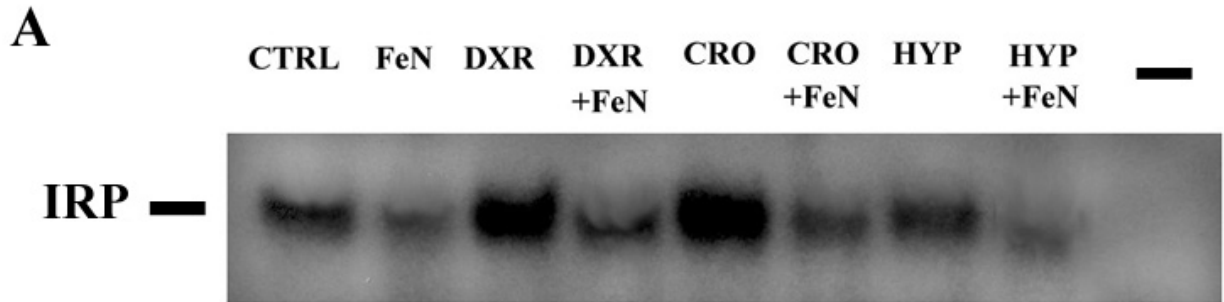CTRL FeN DXR DXR+FeN CRO CRO+FeN HYP HYP+FeN

Figure 2. Effects of crocidolite asbestos, dexrazoxane, hypoxia and FeNTA on IRP-1 binding

activity. Cells were incubated for 24 h in the absence (*CTRL*) or presence of FeNTA (*FeN*, 60 μM),

dexrazoxane (*DXR*, 100 μM), crocidolite asbestos fibers (*CRO*, 25 μg/cm^2), alone or in different

combinations. Some samples were incubated under hypoxic conditions (*HYP*), i.e. under an

atmosphere containing 3% (instead of 20%) O_2, alone or with FeNTA (*FeN*, 60 µM). **A**. Cytosolic

extracts were subjected to EMSA as indicated under the Materials and Methods section. This figure

is representative of three experiments with similar results. In each experiment one lane (-) was

loaded with bidistilled water in place of cellular extracts. **B.** The relative band intensity, calculated

by ImageJ Software (http://rsb.info.nih.gov/ij/) and expressed as arbitrary units, is presented as

mean $\pm$ SE (n =3). vs. *CTRL*: * $p < 0.05$; vs. *DXR* or *CRO* or *HYP* respectively: $^{\circ}$ $p < 0.05$.

Figure 2

A

B
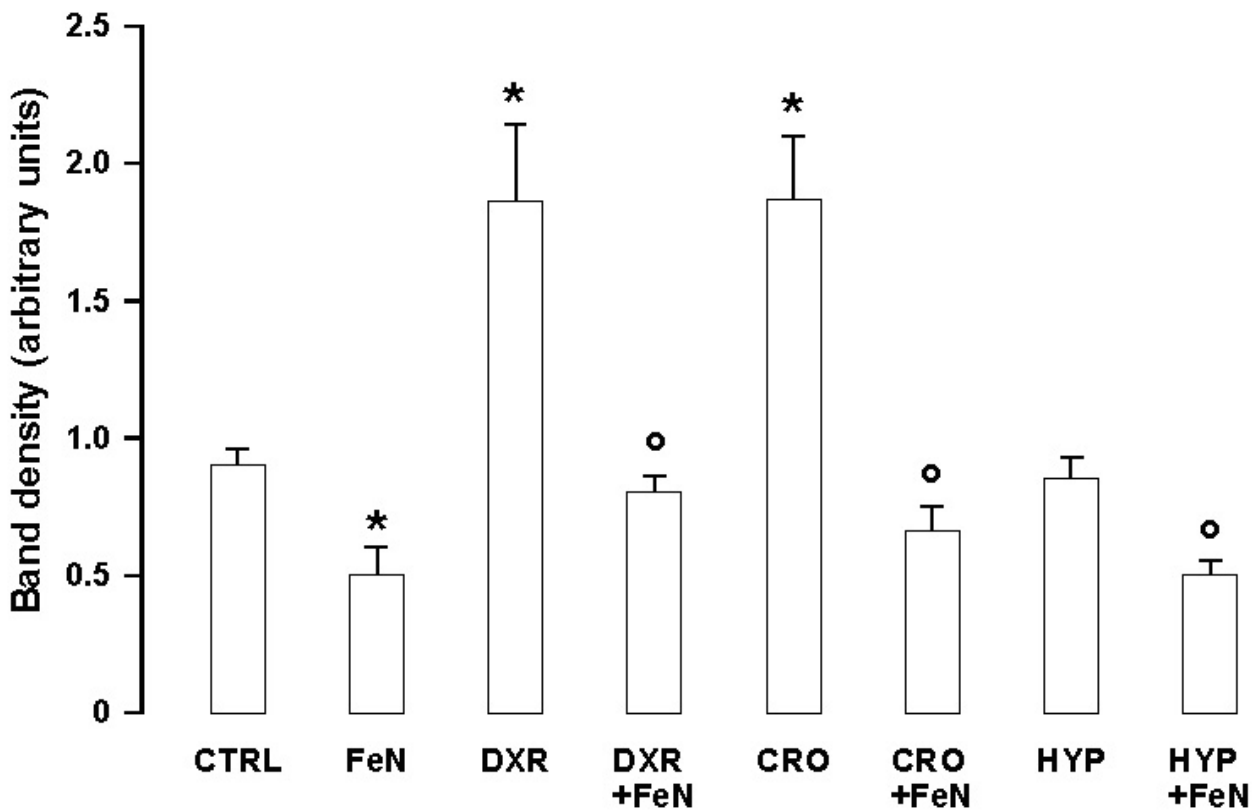
Figure 3. Effects of crocidolite asbestos, dexrazoxane, hypoxia and FeNTA on HIF-1α nuclear

translocation in MM98 cells. Cells were incubated for 24 h in the absence (*CTRL*) or presence of

FeNTA (*FeN*, 60 μM), dexrazoxane (*DXR*, 100 μM), crocidolite fibers (*CRO*, 25 μg/cm^2), alone

and in different combinations. Some samples were incubated under hypoxic conditions (*HYP*), i.e.

under an atmosphere containing 3% (instead of 20%) O_2, in the absence or presence of FeNTA

(*FeN*, 60 µM). EMSA detection of HIF-1α activation was performed on nuclear extracts as reported under Materials and Methods. This figure is representative of three experiments with similar results. In each experiment one lane (-) was loaded with bidistilled water in place of cellular extracts. In a further experimental point (*Anti HIF-1α HYP*), a supershift assay was performed on MM98 cells previously incubated for 24 h under 3% O_2, to assess the specificity of the HIF-1α binding (see Materials and Methods for details).

Figure 3
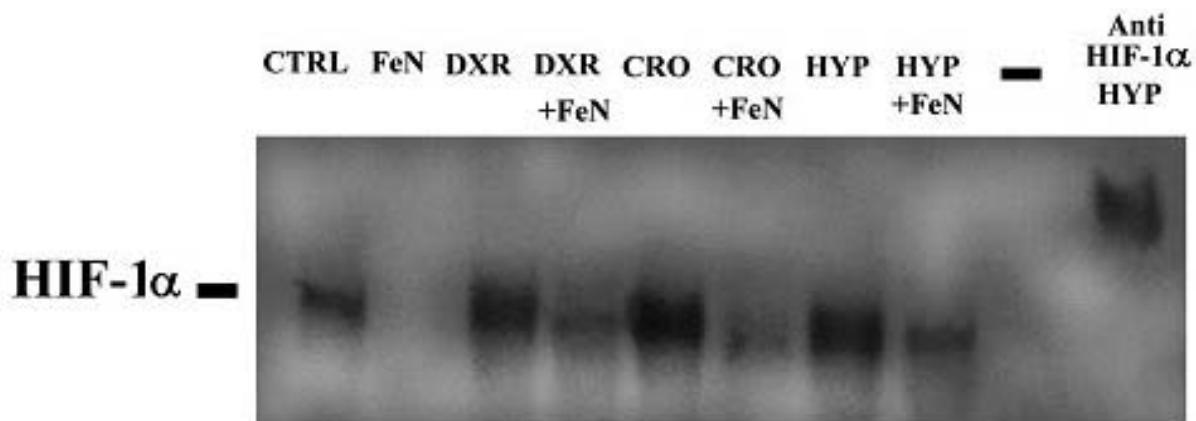
Figure 4. Effects of crocidolite asbestos, dexrazoxane, hypoxia and FeNTA on Pgp expression and doxorubicin efficacy, in the presence of YC-1. MM98 cells were incubated for 24 h in the absence (*CTRL*) or presence of FeNTA (*FeN*, 60 µM), dexrazoxane (*DXR*, 100 µM), crocidolite fibers (*CRO*, 25 µg/cm^2), alone or in different combinations. Some samples were incubated under hypoxic conditions (*HYP*), i.e. under 3% (instead of 20%) O_2, alone or with FeNTA (*FeN*). YC-1 (5 µM for 24 h) was added in all the experimental conditions (*YC-1*). **A.** Cells were lysed and Pgp was immunoprecipitated from lysates of MM98 cells and detected by Western blotting (as described under Materials and Methods). MM98 cells incubated under hypoxic conditions (3% O_2 for 24 h, *HYP*) in the absence of YC-1 were used as a positive control of HIF-1α activation. The expression of GAPDH, used as the product of an housekeeping gene, was detected in the same cell lysates as a

control of equal loading. The figure is representative of three experiments with similar results. **B.** Intracellular doxorubicin accumulation and annexin V/PI assays were performed on MM98 cells incubated as reported above in the presence of YC-1 (5 µM for 24 h, *YC-1*). The results obtained in the presence of YC-1 were compared to the control cells (*CTRL*) incubated without YC-1. Cells were cultured in a medium containing 5 µM doxorubicin, then they were detached and lysed in ethanol/HCl: the intracellular content of the drug was measured as indicated under Materials and Methods. In parallel an aliquot of MM98 cells incubated in the same experimental conditions was used for the quantitative measurement of annexin V/PI fluorescence (see Materials and Methods). Measurements were performed in triplicate and data are presented as means $\pm$ SE (n = 3). vs. *CTRL*: * $p < 0.001$.
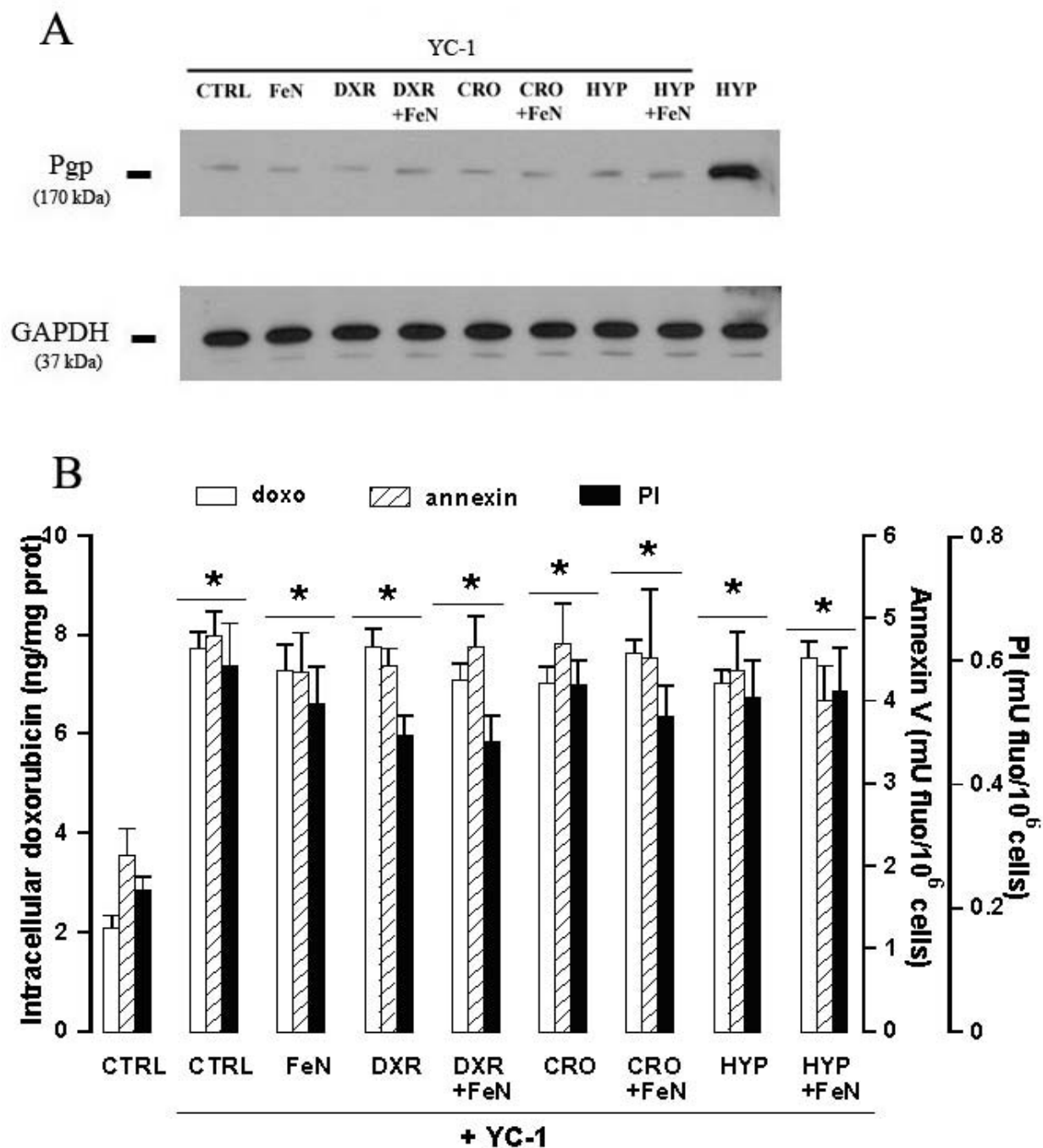
Figure 4

A

B

Figure 5. Effects of crocidolite asbestos, dexrazoxane, hypoxia and FeNTA on Pgp expression and doxorubicin efficacy, in the presence of verapamil. MM98 cells were incubated for 24 h in the absence (*CTRL*) or presence of FeNTA (*FeN*, 60 μM), dexrazoxane (*DXR*, 100 μM), crocidolite fibers (*CRO*, 25 μg/cm^2), alone or in different combinations. Some samples were incubated under hypoxic conditions (*HYP*), i.e. under 3% (instead of 20%) O_2, alone or with FeNTA (*FeN*). In each

experimental condition verapamil (50 μM, 24 h, *VER*) was added, then cells were assayed for the

intracellular doxorubicin content and the annexin V/PI fluorescence, as described in Materials and

Methods. The results obtained in the presence of verapamil were compared to the control cells

(*CTRL*) incubated without verapamil. Measurements were performed in triplicate and data are

presented as means $\pm$ SE (n = 3). vs. *CTRL*: * p < 0.001. Preliminary dose-dependence experiments

showed that 50 μM verapamil was the minimal dose able to increase significantly intracellular

doxorubicin versus the respective control (data not shown).
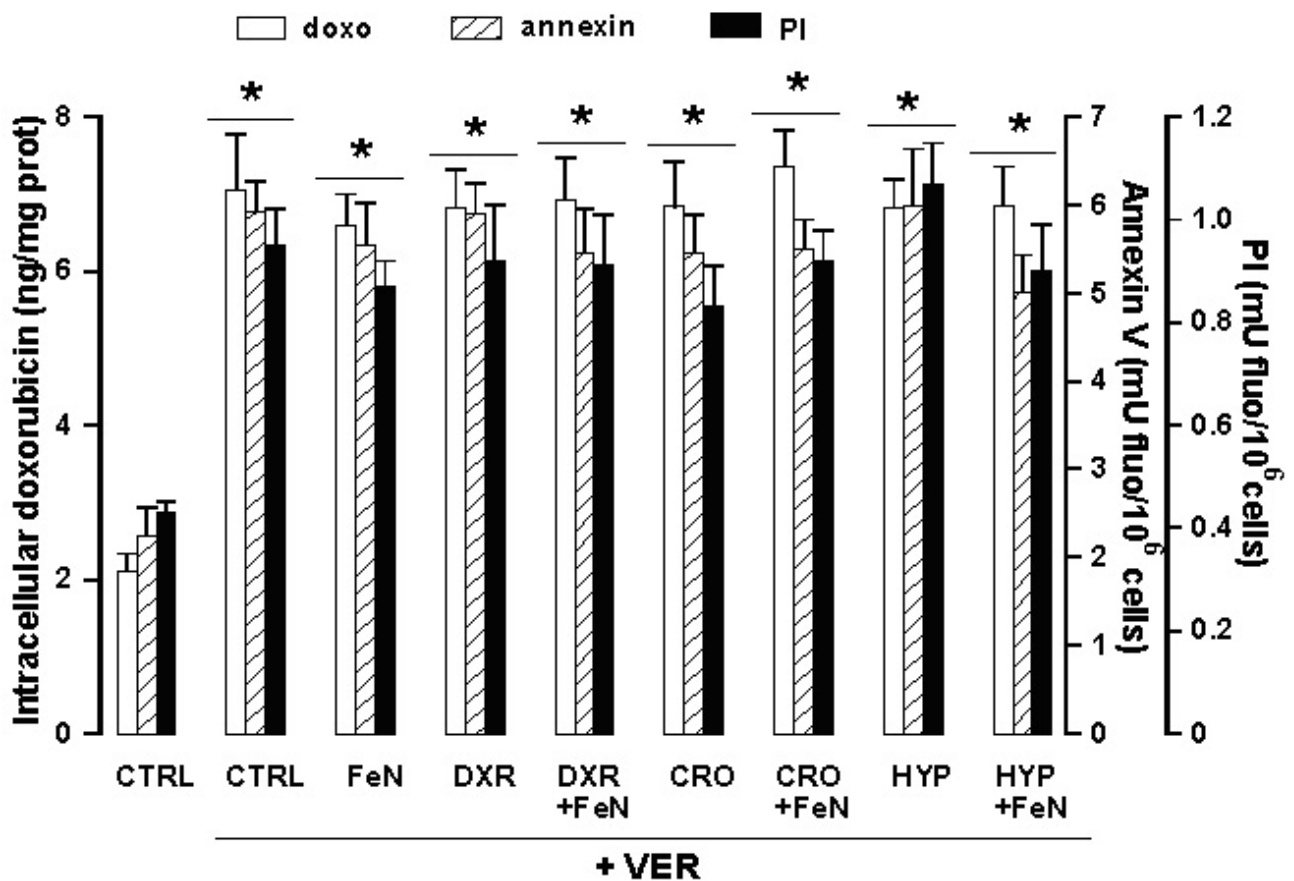
Figure 5

Figure 6. Effects of hypoxia followed by re-exposure to normoxia on HIF-1α activation, Pgp

expression and doxorubicin accumulation and cytotoxicity. MM98 cells were incubated for 24 h

under normoxic (20% O_2, *CTRL*) or hypoxic (3% O_2, *HYP*) conditions. Some samples, after 24 h in

hypoxia, were cultured under normoxic conditions (+*NORM*) for 1, 3, 6 and 24 h. After each time

point, cells were subjected to the following investigations. **A.** Cells were lysed and 10 µg of nuclear

extracts were used for EMSA detection as reported under Materials and Methods. In each

experiment one lane (-) was loaded with bidistilled water in place of cellular extracts. In a further

experimental point (*Anti HIF-1α HYP*), a supershift assay was performed on MM98 cells previously

incubated for 24 h under 3% O_2, to assess the specificity of the HIF-1α binding (see Materials and

Methods for details). **B.** Pgp was immunoprecipitated from the lysates of MM98 cells and then

detected by Western blotting (as described under Materials and Methods). Expression of GAPDH,

used as the product of an housekeeping gene, was detected in the same cell lysates as a control of

equal loading. The figure is representative of three experiments with similar results. **C.** MM98 cells

were incubated in the experimental conditions indicated above in the presence of 5 µM doxorubicin,

then they were detached and lysed in ethanol/HCl: the intracellular content of the drug was

measured as indicated under Materials and Methods. In parallel an aliquot of cells was used for the

quantitative measurement of annexin V/PI fluorescence (as reported under Materials and Methods).

Measurements were performed in triplicate and data are presented as means $\pm$ SE (n = 3). vs. *CTRL*:

* $p < 0.005$; vs. *HYP*: ° $p < 0.05$.

Figure 6

A

HIF-1α

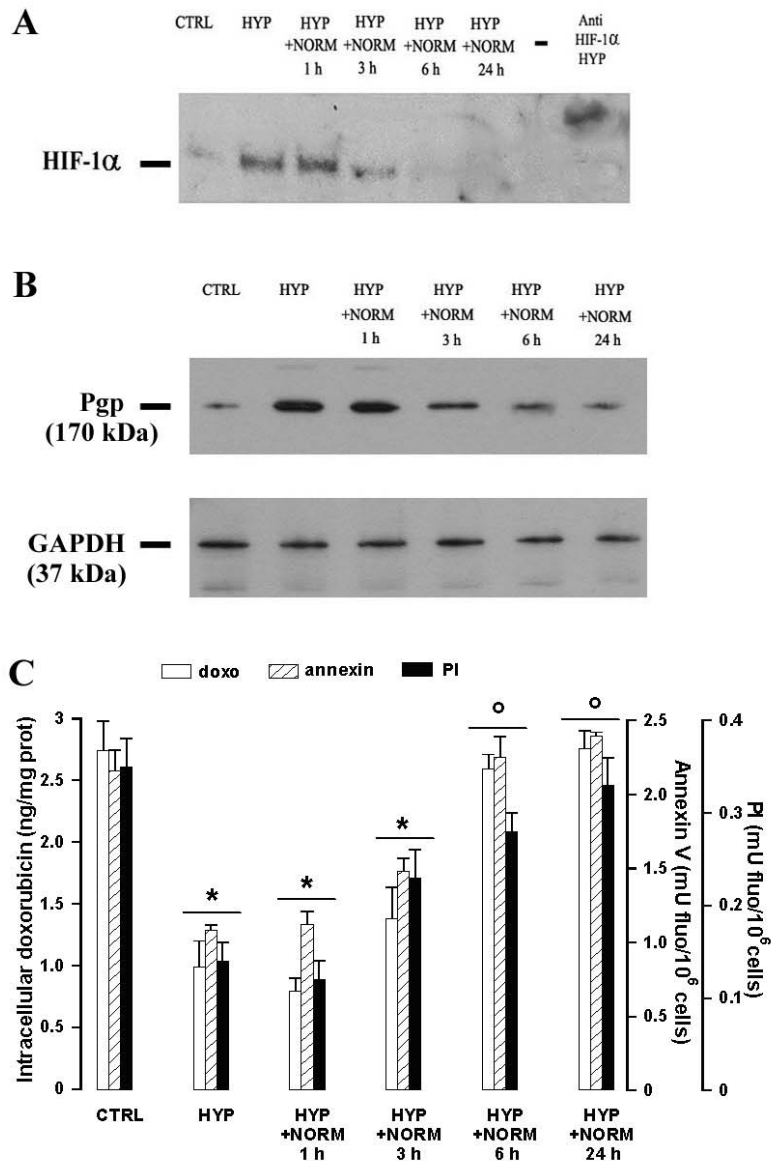**B**

Pgp (170 kDa)

GAPDH (37 kDa)

C

Figure 7. Effects of crocidolite asbestos, dexrazoxane, hypoxia and FeNTA on thioredoxin and glutathione reductase in MM98 cells. Cells were incubated for 24 h in the absence (*CTRL*) or presence of FeNTA (*FeN*, 60 μM), dexrazoxane (*DXR*, 100 μM), crocidolite fibers (*CRO*, 25 μg/cm^2), alone and in different combinations. Some samples were incubated under hypoxic conditions (*HYP*), i.e. under an atmosphere containing 3% (instead of 20%) O_2, in the absence or presence of FeNTA (*FeN*, 60 μM). Then cells were lysed and the detection of thioredoxin (*TRX*) and glutathione reductase (*GRX*) was performed as reported under the Materials and Methods

section. The expression of GAPDH, used as the product of an housekeeping gene, was detected in the same cell lysates as a control of equal loading. The figure is representative of three experiments with similar results.
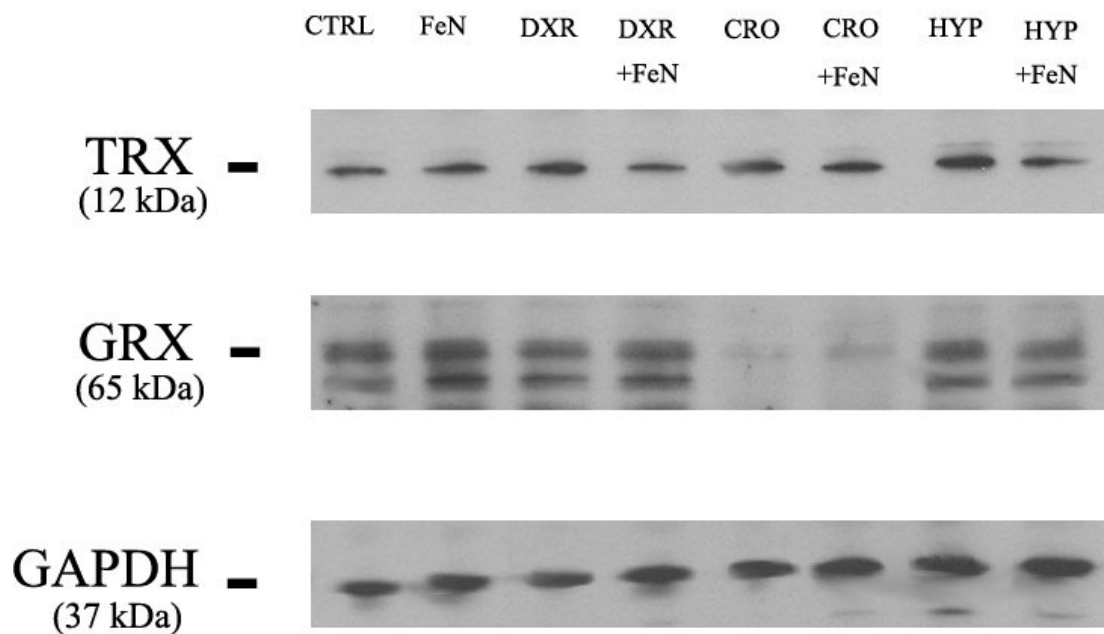
Figure 7

Figure 8. Effects of crocidolite asbestos, FeNTA and MG132 on HIF-1α nuclear translocation in MM98 cells. Cells were incubated for 24 h in the absence (*CTRL*) or presence of FeNTA (*FeN*, 60 μM), MG132 (*MG*, 10 μM), crocidolite fibers (*CRO*, 25 μg/cm^2), alone or in different combinations. EMSA detection of HIF-1α activation was performed on nuclear extracts as described under Materials and Methods. This figure is representative of three experiments with similar results. In each experiment one lane (-) was loaded with bidistilled water in place of cellular extracts. In the experimental points marked with *Anti HIF-1α MG*, a supershift assay was performed on MM98 cells previously incubated for 24 h with 10 μM MG132, to assess the specificity of the HIF-1α binding (as indicated in Materials and Methods).
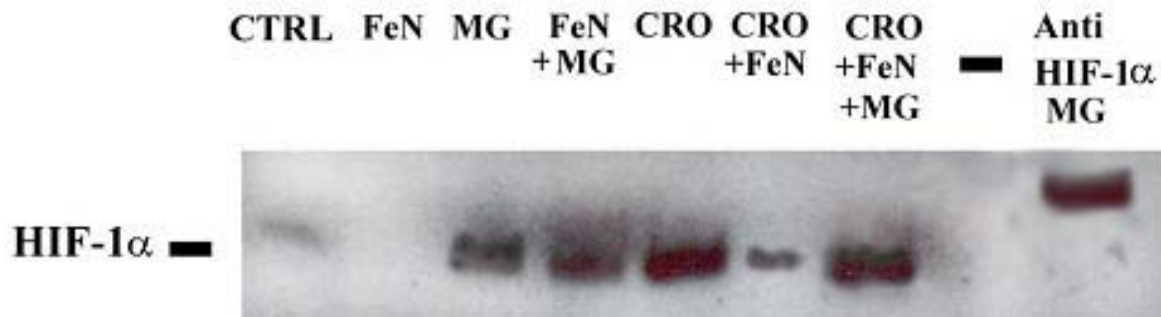
Figure 8

Figure 9. Effects of crocidolite asbestos, FeNTA and MG132 on Pgp expression and intracellular doxorubicin accumulation and toxicity in MM98 cells. Cells were incubated for 24 h in the absence (*CTRL*) or presence of FeNTA (*FeN*, 60 μM), MG132 (*MG*, 10 μM), crocidolite fibers (*CRO*, 25 μg/cm^2), alone or in different combinations. **A.** Pgp was immunoprecipitated from lysates of MM98 cells and then detected by Western blotting (see Materials and Methods). Expression of GAPDH, used as the product of an housekeeping gene, was detected in the same cell lysates as a control of equal loading. The figure is representative of three experiments, with similar results. **B.** Intracellular doxorubicin accumulation and annexin V/PI assay. MM98 cells were incubated under the different experimental conditions indicated above in the presence of 5 μM doxorubicin, then detached and lysed in ethanol/HCl: the intracellular accumulation of the drug was measured as reported under Materials and Methods. Another aliquot of MM98 cells incubated in the same experimental conditions was assayed for the Annexin V/PI positivity (see Materials and Methods). Measurements were performed in triplicate and data are presented as means $\pm$ SE (n = 3). vs.*CTRL*: * $p < 0.001$; vs *FeN*: ° $p < 0.001$; vs *CRO*: ✧ $p < 0.05$.

Figure 9

A

B