Human malignant mesothelioma (HMM) is resistant to many anti-cancer drugs, including doxorubicin. Mevastatin and simvastatin, 2 inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase, potentiated the intracellular accumulation and the cytotoxicity of doxorubicin in HMM cells constitutively expressing P-glycoprotein and multidrug resistance-associated protein 3. This effect of statins was nitric oxide (NO)-dependent, since it was reverted by either an NO synthase inhibitor or an NO scavenging system. The NO synthase up-regulation in HMM and other cells is known to be associated with the activation of the transcription factor NF-xB; in HMM cells statins increased the NF-xB translocation into the nucleus, decreased the level of the NF-xB inhibitor IKBz and increased the phosphorylation/activation of IKB kinase (IKKz). IKKz is under the negative control exerted by RhoA in its prenylated (active) form: incubation of HMM cells with statins lowered the amount of active RhoA and the level of Rho-associated kinase activity. All statins’ effects were reverted by mevalonic acid, thus suggesting that they were mediated by the inhibition of HMGCoA reductase and were likely to be subsequent to the reduced availability of precursor molecules for RhoA prenylation. Both the Rho kinase inhibitor Y27632 and the RhoA inhibitor toxin B (from Clostridium difficile) mimicked the statins’ effects, enhancing doxorubicin accumulation, NO synthesis and IKB degradation and decreasing the amount of IKBz in HMM cells. Simvastatin, Y27632 and toxin B elicited tyrosine nitration in the P-glycoprotein, thus providing a likely mechanism by which NO reverts the doxorubicin resistance in HMM cells.

Key words: statins; mesothelioma; doxorubicin; nitric oxide; drug resistance

Malignant mesothelioma is a peculiar tumor of the serosal cavities, which is characterized by a strong correlation with exposure to asbestos fibers and by a very poor prognosis. Human malignant mesothelioma (HMM) often exhibits in vivo and in vitro resistance to many anticancer drugs (multidrug resistance, MDR), such as doxorubicin and cisplatin. In most cases this resistance is caused by an active extrusion of drugs, caused by the overexpression of ATP-binding cassette (ABC) transporters, such as P-glycoprotein (Pgp) and MDR-associated proteins (MRPs).1–6

Nitric oxide (NO) is a small signalling molecule involved in different cellular functions, including growth, differentiation and apoptosis.7 It is synthesized by 3 NO synthase (NOS; EC 1.14.13.39) isoforms, which favor the conversion of l-arginine to l-citrulline and NO with a 1:1 stoichiometry.8,9 In many cell types a great variety of stimuli, such as bacterial lipopolysaccharide, cytokines, oxidative stress,10–12 crocrodile asbtox11,12–14 stimulate the production of high amounts of NO via the activation of the nuclear factor-xB (NF-xB) transcription factor,13 which subsequently increases the expression of inducible NOS (iNOS). We have previously shown that in a doxorubicin-resistant human colon cancer cell population (HT29-dx) the drug resistance could be reverted by NO, both released by donor compounds and endogenously produced via the induction of iNOS by atorvastatin: the exposure of HT29-dx cells to NO was associated with the nitration of MRP3 protein and reduced drug efflux.15 By inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase, system 3- the prenylation of small GTPases such as the Rho proteins, thus affecting several G protein-mediated cellular functions. In the present work we have investigated the ability of statins to revert the resistance to doxorubicin in constitutively drug resistant HMM primary cells and the molecular mechanism of this reversion.

Materials and methods

Fetal 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); the cationic exchange resin Dowex AG50WX-8, n-(1-naphthyl)-ethylenediamine dihydrochloride and sulfanilamide were from Aldrich (Milan, Italy); L-[2,3,4,5-3H]arginine monohydrochloride (62 Ci/mmol) was obtained from Amersham International (Bucks, UK); mevastatin, simvastatin, Y27632, MG132, GGTI-286 and FTase Inhibitor III were from Calbiochem (La Jolla, CA). Electrophoresis reagents were obtained from Biorad Laboratories (Hercules, CA); the protein content of cell monolayers and cell lysates was assessed with the BCA kit from Pierce (Rockford, IL). When not otherwise specified, the other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Cells

Human malignant mesothelioma (HMM) cells, referred to as MM98, OC99 and GF99, respectively, were established from the pleural effusion of 3 patients with histologically confirmed malignant mesothelioma; the mesothelial origin of the isolated cells was confirmed by positive immunostaining as previously described.18 Cells were cultured in HAM’S F-12 medium supplemented with 10% foetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamine and maintained in a humidified atmosphere at 37°C and 5% CO2. Since MM98, OC99 and GF99 cells showed the same behavior under each experimental condition, the results were grouped as a whole. Human colon doxorubicin-sensitive cancer cells (HT29) and doxorubicin-resistant cancer cells (HT29-dx) were obtained as previously described.19 The NIH mouse glial cell line was a gift from Dr. Marco Righi (CNR Institute of Neuroscience, Section of Cellular and Molecular Pharmacology, Milan, Italy).

Doxorubicin accumulation

Intracellular doxorubicin accumulation was measured as described elsewhere.16 Cells were grown in 60-mm diameter Petri dishes, incubated for 24 hr in fresh medium containing 4 μM doxorubicin.

Abbreviations: ABC, ATP-binding cassette; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; HMGCoA, 3-hydroxy-3-methylglutaryl coenzyme A; HMM, human malignant mesothelioma; IKKz, IKB kinase z; iNOS, inducible NOS; LDH, lactate dehydrogenase; MDR, multidrug resistance; MRP, MDR-associated protein; NF-xB, nuclear factor-xB; NO, nitric oxide; NOS, nitric oxide synthase; Pgp, P-glycoprotein; Rock, Rho-dependent kinase; SNAP, S-nitrosothiol; SOD, superoxide dismutase; WST-1, tetrazolium.)

Correspondence to: Dipartimento di Genetica, Biologia e Biochimica - Sezione di Biochimica, Via Santena 5/8, 10126 Torino, Italy. Fax: +39-011-6705845. E-mail: dario.ghiigo@unito.it

Received 10 August 2005; Accepted after revision 20 December 2005 DOI 10.1002/ijc.21832

Published online 31 January 2006 in Wiley InterScience (www.interscience.wiley.com).
orubicin (as indicated in Results), washed twice in ice-cold PBS and detached with trypsin/EDTA (0.05/0.02%, v/v). Cells were centrifuged for 30 sec at 13,000g (4°C) and resuspended in 1 ml of a 1:1 mixture of ethanol/0.3 N HCl. The amount of cell proteins was measured in 50 μl of suspension; the remaining part was checked for the doxorubicin-associated fluorescence using a Perkin-Elmer LS-5 spectrofluorimeter (Perkin Elmer, Shelton, CT). Excitation and emission wavelengths were 475 and 553 nm, respectively. A blank was prepared in the absence of cells in each set of experiments and its fluorescence was subtracted from that measured in the samples. Fluorescence was converted in ng doxorubicin/mg cell proteins, using a calibration curve prepared previously.

**Extracellular lactate dehydrogenase activity**

After a 24 hr incubation under different experimental conditions, the extracellular medium was centrifuged at 12,000g for 15 min to pellet cellular debris, whereas cells were washed with fresh medium, detached with trypsin/EDTA, washed with PBS, resuspended at 1 × 10^6 cells/ml in 0.2 ml of 82.3 mM triethanolamine phosphate hydrochloride (TRAP, pH 7.6) and sonicated on ice with two 10 sec bursts. Lactate dehydrogenase activity was measured in the extracellular medium and in the cell lysate, as previously described, in order to check the cytotoxic effect of doxorubicin.20 Hundred microliters of supernatant from extracellular medium or 10 μl of cell lysate were incubated at 37°C with 82.3 mM TRAP (pH 7.6) and 5 mM NADH (final volume, 1 ml). The reaction was started by adding 20 mM pyruvic acid and was followed for 10 min, measuring absorbance at 340 nm with a Lambda 3 spectrophotometer (Perkin Elmer). The reaction kinetics was linear throughout the time of measurement. Both intracellular and extracellular enzyme activity was expressed in μmol NADH oxidized/min/dish, and the extracellular LDH activity was calculated as a percentage of the total LDH activity in the dish.

**Trypan blue cell staining assay**

After a 24 hr incubation under different experimental conditions, cell monolayers in 35-mm diameter Petri dishes were washed with PBS, detached with trypsin/EDTA and resuspended in 1 ml of PBS. Ten microliters of 20% (w/v) Trypan Blue (Sigma Chemical Co.) was added to each sample. After a 1 min incubation at room temperature, 10 μl of each cellular suspension was analyzed under a light microscope, and the trypan blue-positive cells were counted as percentage of dead cells on a total number of 200 cells.

**Nitrite production**

Confluent cell monolayers in 35-mm diameter Petri dishes were incubated in fresh medium for 24 hr under the experimental conditions indicated in Results. Then nitrite production was measured by adding 0.15 ml of cell culture medium to 0.15 ml of Griess reagent21 in a 96-well plate, and after a 10 min incubation at 37°C in the dark, absorbance was measured at 540 nm with a Packard 1503 spectrofluorimeter (Perkin Elmer, Shelton, CT). Excitation and emission wavelengths were 520 and 550 nm, respectively. A blank was prepared for each experimental condition in the absence of cells, and its absorbance was subtracted from that measured in the samples. Nitrite concentration was expressed in nmol nitrite/mg cell proteins.

**Measurement of NOS activity**

Cells grown at confluence on 35-mm diameter Petri dishes, after incubation under the experimental conditions described in Results, were detached by trypsin/EDTA, washed with PBS, resuspended in 0.3 ml of Hepes/EDTA/dithiothreitol (DTT) buffer (20 mM Hepes, 0.5 mM EDTA, 1 mM DTT, pH 7.2) and then sonicated on crushed ice with two 10 sec bursts. In each assay tube, the following reagents were added to 100 μl of lysate at the following final concentrations: 2 mM NADPH, 2.5 μCi [3H]arginine (0.4 μM), 100 μM tetrahydrobiopterin and 1.5 mM CaCl_2. After a 15 min incubation at 37°C, the reaction was stopped by adding 2 ml HesperoNa/EDTA buffer (20 mM Hespero sodium salt, 2 mM EDTA, pH 6); the whole reaction mixture was applied to 2 ml columns of Dowex AG50WX-8 (Na⁺ form) and eluted with 4 ml of water. The radioactivity corresponding to [3H]citrulline content in 6.1 ml eluate was measured by liquid scintillation counting. Citrulline synthesis was expressed in pmol citrulline/mg cell proteins.

**Electrophoretic mobility shift assay**

Cells were plated in 100-mm diameter dishes at confluence and all the procedures for nuclear protein extraction were performed at 4°C using ice-cold reagents, as described. Cells were mechanically scraped in PBS, washed and resuspended (1 × 10^6 cells/0.5 ml) in lysis buffer A (15 mM KCl, 10 mM Hepes, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM DTT, 10 μg/ml aprotinin, 2 μg/ml leupeptin, 0.1% NP-40, pH 7.6). This suspension was incubated for 10 min on ice with occasional vortexing, and centrifuged for 30 sec at 13,000g to pellet nuclei, which were rinsed with 0.2 ml of wash buffer B (2 M KCl, 25 mM Hepes, 0.1 mM EDTA, 1 mM PMSF, 1 mM DTT, 10 μg/ml aprotinin, 2 μg/ml leupeptin, pH 7.6) and incubated at 4°C for 20 min. Then an equal volume of buffer C (25 mM Hepes, 0.1 mM EDTA, 20% glycerol, pH 7.6) was added, the mix was centrifuged at 20,000g and the supernatant stored at −80°C until used for electrophoretic mobility shift assay (EMSA). The probe containing the transcription factor binding sequence was synthesized using [γ-^32P]ATP (Amersham International) (3,000 Ci/mmol, 250 μCi/ml), using T4 polynucleotide kinase (Roche, Basel, Switzerland). The sequence of oligonucleotides was determined by the binding site underlined: 5'-AGTT-GAGGGGACTTTCCCCAGG-3' (Promega Corporation, Madison, WI). Ten micrograms of extracts were incubated for 20 min with 20,000 cpm of [γ-^32P]labeled double-stranded oligonucleotide at 4°C in a reaction mixture containing the following: 2 μl of 10 μg/ml BSA, 2 μl of buffer D (100 mM KCl, 20 mM Hepes, 0.5 mM EDTA, 2 mM DTT, 0.1 mM PMSF, 20% glycerol, 0.25% NP-40, pH 7.6), 4 μl of buffer E (300 mM KCl, 100 mM Hepes, 10 mM DTT, 100 μM PMSF, 20% Ficoll, pH 7.6) and 2 μg of poly(dI-dC) (Roche). The final volume of the mix was brought to 25 μl with water. In competition assays, a 100X amount of unlabelled (cold) NF-κB oligonucleotide was added. The DNA–protein complex was separated on a nondenaturing 4% polyacrylamide gel in TBE buffer (pH 8.0). After electrophoresis, the gel was dried and autoradiographed by exposure to X-ray film for 48 hr.

**Western blot analysis**

Cells were directly solubilized in the lysis buffer—25 mM Heps, 135 mM NaCl, 1% Nonidet P-40 (NP40), 5 mM EDTA, 1 mM EGTA, 1 mM ZnCl₂, 50 mM sodium fluoride (NaF) and 10% glycerol—supplemented with protease inhibitor cocktail set III (100 mM AEBSF, 80 μg aprotinin, 5 mM bestatin, 1.5 mM E-64, 2 mM leupeptin and 1 mM pepstatin; Calbiochem). 2 mM PMSF and 1 mM sodium orthovanadate. Whole-cell extracts containing 30 μg of proteins were separated by SDS-PAGE, transferred to PVDF membrane sheets (Immobilon-P, Millipore, Bedford, MA) and probed with the following antibodies: anti-IkBα (from rabbit, diluted 1:500 in PBS-BSA 1%, Santa Cruz Biotechnology, Santa Cruz, CA), anti-IKKα (from rabbit, diluted 1:500 in PBS-BSA 1%, Santa Cruz Biotechnology), anti- phosphoSer(180)-IKKα (from rabbit, diluted 1:250 in PBS-BSA 1%, Cell Signaling Technology, Beverly, MA), anti-RoCK1 (from rabbit, diluted 1:500 in PBS-BSA 1%, Santa Cruz Biotechnology), anti-RoCK2 (from rabbit, diluted 1:500 in PBS-BSA 1%, Santa Cruz Biotechnology), anti-glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH, from rabbit, diluted 1:500 in PBS-BSA 1%, Santa Cruz Biotechnology). After a 1 hr incubation, the membrane was washed with PBS-Tween 0.1% and subjected for 1 hr to a peroxidase-conjugated anti-rabbit antibody (Amersham International, diluted 1:1,000 in PBS-Tween with Blocker Not-Fat Dry Milk 5%, Biorad, Hercules, CA). The membrane was washed again with PBS-Tween, and proteins...
were detected by enhanced chemiluminescence (Immun-Star, Bio-rad). Pgp, MRP1, MRP2, MRP3 proteins were immunoprecipitated overnight, from the whole cellular lysates, with the rabbit polyclonal anti-Pgp (H-241) antibody (diluted 1:250, Santa Cruz Biotechnology) and the goat polyclonal anti-MRP1 (C-20), anti-MRP2 (H-17) and anti-MRP3 (H-16) antibodies (diluted 1:250, Santa Cruz Biotechnology). Immunoprecipitated proteins were separated by SDS-PAGE (8%), transferred to PVDF membrane sheets and probed with the same antibodies (diluted 1:500 in PBS-BSA 1%).

To assess the presence of nitrated Pgp and MRP3, the whole cellular extract was subjected to immunoprecipitation overnight using a rabbit polyclonal anti-nitrotyrosine antibody (1%; Upstate, Lake Placid, NY; diluted 1:1,000 in Blocker Not-Fat Dry Milk, Biorad). Proteins were resolved by SDS-PAGE (8%), transferred to PVDF membrane sheets and probed with anti-Pgp and anti-MRP3 antibodies (diluted 1:250 in PBS-BSA 1%), as above described. In each Western blot experiment the equal loading of proteins was checked by measuring the level of GAPDH, used as the product of a housekeeping gene.

RhoA-GTP and Ras-GTP pull-down

Biochemical assay for activity of RhoA and Ras was performed as described. Cells were lysed in MLB buffer (125 mM Tris-HCl, pH 7.4, 750 mM NaCl, 1% NP40, 10% glycerol, 50 mM MgCl₂, 5 mM EDTA, 25 mM NaF, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin and 1 mM PMSF) and centrifuged at 13,000 g for 10 min at 4°C. An aliquot of supernatant was taken out for determination of protein content. Another aliquot of the same lysate was directly probed with an anti-RhoA antibody (1:250, in PBS-BSA 1%, Santa Cruz Biotechnology) and an anti-Ras antibody (1:250, in PBS-milk 5%, Upstate), to measure total RhoA and Ras protein. Further, 30 μg of the supernatant was incubated for 45 min at 4°C with agarose-glutathione beads coupled with a fusion protein containing glutathione S-transferase and the Rho-binding domain of the Rho effector protein rhoetkin (Upstate) or with the Ras Assay Reagent (Raf-1 RBD, agarose conjugate; Upstate). The beads were then washed 3 times in MLB buffer and harvested by the addition of 2× Laemmli buffer (125 mM Tris, 4% w/v SDS, 20% v/v glycerol and 1% β-mercaptoethanol). The RhoA and Ras activity was analyzed resolving the samples by 12% SDS-PAGE and Western blotting using an anti-RhoA or an anti-Ras antibody to detect GDP-bound activated RhoA and Ras, respectively.

Rho kinase activity assay

Rho kinase activity was measured using the CycLex Rho Kinase Assay Kit (CycLex Co., Nagano, Japan), a single site binding immunoassay. Cells were cultured in 35-mm diameter Petri dishes, washed with ice-cold PBS and lysed in 0.2 ml lysis buffer (50 mM Tris-HCl, pH 8.0, 0.1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 2 mM NaF, 2 mM sodium orthovanadate, 0.5 μg/ml leupeptin, 1 μg/ml pepstatin, 0.2 mM PMSF and 10 μM β-mercaptoethanol). Samples were sonicated on crushed ice with two 10 sec bursts and centrifuged at 13,000g for 5 min at 4°C. Supernatants were treated following manufacturer’s instructions and the protein content was measured. Briefly, samples were diluted 1:4 in the kinase buffer provided with the kit, containing 20 mM ATP, and incubated for 60 min at 30°C in 96-wells plates, precoated with the recombinant C-terminus of the myosin binding subunit (MBS) of myosin phosphatase. Wells were washed 5 times with 2% Tween-20, and 100 μl of the horseradish peroxidase-conjugated anti-phosphoThr(96)-MBS antibody were added. After a 60 min incubation at room temperature, samples were washed again, and 100 μl of the chromogenic substrate tetra-methylbenzidine were added. After a 15 min incubation at room temperature, the reaction was stopped with 100 μl of 0.5 N H₂SO₄ and absorbance was read at 450 nm, using a Packard EL314 microplate reader (Bio-Tek Instruments). For each set of experiments, a titration curve was prepared, using serial dilution of recombinant Rock2 (MBL Inc, Woburn, MA) in kinase buffer. Data were expressed as mU absorbance/mg cell proteins.

IKK activity assay

IKK activity was measured as previously described. Cells were washed with ice-cold PBS and solubilized in 0.5 ml of lysis buffer (50 mM Hepes, 150 mM NaCl, 2 mM MgCl₂, 1 mM EDTA, 0.1% NP40, 100 μM NaF, 10 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 1 mM PMSF and 250 μM DTT). Samples were centrifuged at 13,000g for 15 min and the supernatant was used for the assay and cell protein quantification. To purify the IKK complex, equal amounts of the whole lysate (0.5 mg cell proteins/test) were immunoprecipitated with an anti-IKKα antibody (from rabbit, diluted 1:200 in PBS-BSA 1%, Santa Cruz Biotechnology) for 90 min at 4°C. Samples were centrifuged (13,000g for 15 min) and washed 3 times with kinase buffer (20 mM Hepes, 20 mM β-glycerolphosphate, 1 mM MnCl₂, 5 mM MgCl₂, 2 mM NaF and 250 μM DTT). 0.1 μg of the immunoprecipitated proteins were incubated with 1 mM ATP in the presence of the pan-IκBα inhibitor MG132 (10 μM); to provide the reaction mix with IkBα protein, the substrate of IKK, 30 μg of total cellular lysate of SV40-positive HMM cells, obtained under nonnondenaturing conditions, were added. SV40-positive HMM cells were chosen, since they were previously shown to exhibit a very high basal amount of IkBα. Reaction was carried over at 30°C for 30 min and stopped with 30 μl of Laemmli buffer. Finally, samples were subjected to electrophoresis in a 12% SDS-PAGE, transferred to PVDF membrane sheets and probed with an anti-IκBα antibody (from rabbit, diluted 1:250 in PBS-BSA 1%, Santa Cruz Biotechnology) and an anti-phosphoSer(32)-IkBα antibody (from mouse, diluted 1:250 in PBS-BSA 1%, Santa Cruz Biotechnology), respectively.

Statistical analysis

All data in text and figures are provided as means ± SE. The results were analyzed by a one-way analysis of variance (ANOVA) and Tukey’s test. p < 0.05 was considered significant.

Results

HMM cells express Pgp and MRP3 and are resistant to doxorubicin

We examined the expression of Pgp, MRP1, MRP2 and MRP3 in HMM cells, compared with the doxorubicin-sensitive human colon cancer HT29 cells and the doxorubicin-resistant HT29-dx cells. HMM cells exhibited a prominent expression of Pgp, as well as HT29-dx cells, but a weak expression of MRP3, similar to HT29 cells (Fig 1a). MRP1 and MRP2 were absent in HMM cells (data not shown), as well as in HT29 and HT29-dx cells. After a 24 hr incubation with doxorubicin, drug accumulation in HMM was significantly lower than that observed in both HT29 and HT29-dx cells (Fig 1b). So far, HMM cells appeared to be a model of intrinsically doxorubicin-resistant cells.

Statins potentiate the intracellular accumulation and the cytotoxicity of doxorubicin via an increased NO production in HMM cells

We have previously observed that an inhibitor of HMGCoa reductase, atorvastatin, reverts doxorubicin resistance and potentiates toxicity in HT29-dx cells, through the increase of iNOS activity and NO production. After a 24 hr incubation of HMM cells with doxorubicin, in the absence or presence of the HMGCoa reductase inhibitors mevastatin or simvastatin, we measured the intracellular accumulation of doxorubicin and its cytotoxic effect (determined as the release of LDH activity) (Fig. 2). Doxorubicin (4 μM, 24 hr) increased the release of LDH in the extracellular medium (calculated as percentage of the total LDH activity in each dish) from 8.3% ± 1.54% (control) to 18.55% ± 1.22% (n = 4; p < 0.005). Both statins significantly increased the intracellular content of the drug and the level of extracellular LDH activity.
Data on HMM cells are presented as mean ± SE of the measurements on the 3 cell populations (MM98, OC99 and GF99), in each of which the measurements were performed in triplicate. (Fig. 2). After the same incubation time, the statins induced a significant increase of intracellular NOS activity and extracellular nitrite produced in the presence of statins (Fig. 3). A superimposable pattern of cytotoxicity was observed under all experimental conditions. The NOS inhibitor L-NMMA (1 mM, NM) or with packed human erythrocytes (RBC), used as a NO scavenger, were added. Data are presented as means ± SE of the measurements on the 3 cell populations (MM98, OC99 and GF99), in each of which the measurements were performed in triplicate. vs. CTRL: *p < 0.05, **p < 0.001. vs. the corresponding statin (MVS or SIM) alone: †p < 0.05, ‡p < 0.001.

FIGURE 2 – Release of LDH in the extracellular culture medium (open bars) and intracellular accumulation of doxorubicin (hatched bars) in HMM (MM98, OC99 and GF99) cells. Cells were incubated with 4 μM doxorubicin for 24 hr in the absence (CTRL) or presence of mevastatin (100 μM, MVS), simvastatin (10 μM, SIM) and mevalonic acid (100 μM, MA), in different combinations; in some experimental conditions, the NOS inhibitor L-NMMA (1 mM, NM) or packed red blood cells (10 μl/ml, RBC), used as NO scavengers, were added. Data are presented as means ± SE of the measurements on the 3 cell populations (MM98, OC99 and GF99), in each of which the measurements were performed in triplicate. vs. CTRL: *p < 0.05, **p < 0.001. vs. the corresponding statin (MVS or SIM) alone: †p < 0.05, ‡p < 0.001.

HT29, HT29-dx and HMM cells and then detected by Western blotting (see Materials and Methods). Each lane is representative of 3 experiments per cell type, with similar results. (b) HT29, HT29-dx and HMM cells were incubated for 24 hr in culture medium containing 4 μM doxorubicin, then detached and lysed in ethanol/HCl: the intracellular content of the drug was measured as described under Material and Methods. Measurements on HT29 and HT29-dx cells were performed in triplicate and data are presented as means ± SE (n = 3). Data on HMM cells are presented as mean ± SE of the measurements on the 3 cell populations (MM98, OC99 and GF99), in each of which the measurements were performed in triplicate. vs. HT29 cells, *p < 0.001. vs. HT29-dx cells, †p < 0.005.

FIGURE 1 – Expression of P-glycoprotein (PgP) and MDR-related protein 3 (MRP3), and doxorubicin accumulation in HT29, HT29-dx and HMM (MM98, OC99 and GF99) cells. (a) PgP, MRP3 and glycer-aldehyde 3-phosphate dehydrogenase (GAPDH, used to check the equal protein loading) were immunoprecipitated from lysates of HT29, HT29-dx and HMM cells and then detected by Western blotting (see Materials and Methods). Each lane is representative of 3 experiments per cell type, with similar results. (b) HT29, HT29-dx and HMM cells were incubated for 24 hr in culture medium containing 4 μM doxorubicin, then detached and lysed in ethanol/HCl: the intracellular content of the drug was measured as described under Material and Methods. Measurements on HT29 and HT29-dx cells were performed in triplicate and data are presented as means ± SE (n = 3). Data on HMM cells are presented as mean ± SE of the measurements on the 3 cell populations (MM98, OC99 and GF99), in each of which the measurements were performed in triplicate. vs. HT29 cells, *p < 0.001. vs. HT29-dx cells, †p < 0.005.

Statins increase NF-κB translocation into the nucleus and decrease the level of IκBα in HMM cells

Mevastatin and simvastatin induced nuclear translocation of NF-κB (Fig. 4a) and elicited a decrease of the intracellular amount of the NF-κB inhibitor IκBα (Fig. 4b). Mevalonic acid reverted both effects (Fig. 4).

Statins elicit a down-regulation of active RhoA and RhoA-associated kinase activity, and an increase of IκKz phosphorylation/activation in HMM cells

Since RhoA can bind GTP only when prenylated, the GTP-bound RhoA can be taken as an index of RhoA prenylation and activation. The incubation with mevastatin and simvastatin caused a decrease of the amount of GTP-bound RhoA: such an effect was abolished by mevalonic acid (Fig. 5a). Toxin B from Clostridium difficile, which catalyzes the monoglycosylation and subsequent inactivation of Rho proteins, significantly reduced the amount of GTP-bound RhoA, without affecting cell viability (extracellular LDH vs. total LDH: 6.6% ± 1.1%, n = 4). The level of total RhoA did not change significantly under any experimental
Mevastatin, simvastatin and toxin B, Y27632,29,30 markedly reduced the Rho kinase activity in HMM cells (Fig. 5b). Similar to toxin B, Y27632 per se (20 μM) did not affect the cell viability (extracellular LDH vs. total LDH: 8.6% ± 0.9%, n = 4). When mevalonic acid was incubated together with statins, the Rho kinase activity was partially restored (Fig. 5b). It is possible that the incomplete reversion of Rho kinase activity, measured “in vitro” on cells supplemented with mevalonic acid, is due to the dilution of active enzyme molecules with inactive ones accumulated during the incubation period in the presence of statins. The expression of Rho kinase in the whole cellular lysate was checked by using anti-Rock1 and anti-Rock2 antibodies: none of the experimental conditions described in Figure 5 significantly changed the protein expression in HMM cells (data not shown).
creased phosphorylation of IKK

...Y27632 and toxin B enhance doxorubicin accumulation and cytotoxicity via increased NO synthesis in HMM cells

Since a relationship between Rho kinase and IKKα/IkBα/NF-kB pathway has already been hypothesized in other cell types, we investigated whether in HMM cells the RhoA/Rho kinase inhibition could lead to an IKKα-mediated nuclear translocation of NF-kB. A central event in IKKα activation is its serine/threonine phosphorylation. Indeed, statins induced an increase in the amount of phospho(Ser180)-IKKα in HMM cells, which was reverted by mevalonic acid (Fig. 6a). No change of total IKKα was observed under any experimental condition (Fig. 6a). When activated, IKKα phosphorylates IkBα, leading to its ubiquitination and degradation and allowing NF-kB translocation to the nucleus. The IKK activity in HMM cells was measured as the ability of the immunoprecipitated IKK complex to promote phosphorylation of IkBα. Simvastatin and mevastatin increased the IKK-mediated production of phospho-IkBα from IkBα (Fig. 6b), an effect which was completely reverted by mevalonic acid.

Y27632 and toxin B enhance doxorubicin accumulation and cytotoxicity via increased NO synthesis in HMM cells

FIGURE 6 – Effect of statins on IKKα phosphorylation and IKK activity. HMM (MM98, OC99 and GP99) cells were incubated for 24 hr in fresh medium only (CTRL) or with mevastatin (100 μM, MVS) and simvastatin (10 μM, SIM) in the absence or presence of mevalonic acid (100 μM, MA). Subsequently, the cells were subjected to the following investigations. (a) Western blot detection of phospho-IKKα (pIKKα) and IKKα. Whole cell lysates were analyzed by Western blotting with an anti-phospho(Ser180)-IKKα antibody, an anti-IKKα antibody and an anti-GAPDH antibody (as described under Materials and Methods). The picture is representative of 3 experiments (1/cell type) with similar results. (b) IKK activity. Measurement of IKK activity is described under Materials and Methods. The figure is representative of 3 experiments (1/cell type) with similar results.

The NF-kB pathway plays a role in statin-induced NO synthesis and doxorubicin accumulation in HMM cells

To verify the hypothesis that statins, toxin B and Y27632 induce NO synthesis and doxorubicin accumulation in HMM cells by activating the NF-kB signaling pathway, we measured their effect on extracellular nitrite, intracellular NOS activity and doxorubicin accumulation in the presence of parthenolide, a known inhibitor of IKKα′s activation. Parthenolide significantly inhibited both NO synthesis and doxorubicin accumulation induced by simvastatin, mevastatin, toxin B and Y27632 (Fig. 10).

Simvastatin, Y27632 and toxin B induce Pgp tyrosine nitration in HMM cells

We have previously shown that the NO donor S-nitrosothiolamine (SNAP) can nitrate tyrosine residues in the MRP3 protein, thus reducing the drug efflux in doxorubicin-resistant cells. In HMM cells a 24 hr incubation with simvastatin, Y27632, toxin B and SNAP elicited a nitration of tyrosine residues in Pgp but not

FIGURE 7 – Effect of Y27632 and toxin B of C. difficile on IKKα phosphorylation. After a 24 hr incubation with fresh medium (CTRL) or with mevastatin (100 μM, MVS), simvastatin (10 μM, SIM), Y27632 (2 μM, Y276) and toxin B (0.1 ng/ml, TOX B), cells (MM98, OC99 and GP99) were lysed and cellular lysates were subjected to Western blotting with anti-phospho(Ser180)-IKKα, anti-IKKα, anti-IkBα and anti-GAPDH antibodies (see Materials and Methods). The figure is representative of 3 experiments (1/cell type) with similar results.
in MRP3 (Fig. 11). SNAP increased both the LDH release and the drug accumulation induced by doxorubicin (Fig. 12).

Discussion

The treatment of HMM is still largely ineffective\(^3\)\(^7\),\(^3\)\(^8\): the surgical resection, which is possible only in few cases diagnosed in very early stages, is associated to high morbidity and relapse rates, and HMM exhibits strong \textit{in vitro} and \textit{in vivo} resistance to many common anticancer drugs.\(^3\) HMM is considered an intrinsically resistant tumor,\(^4\) but no general agreement exists about the molecular mechanisms: MDR in this tumor has been attributed to overexpression of ABC transporters\(^4\)–\(^6\) and \(g\)-glutamylcysteine synthetase\(^3\)\(^9\) or to other mechanisms.\(^4\)\(^0\),\(^4\)\(^1\) ABC transporters, such as Pgp, MRP1 and MRP2,\(^4\)\(^0\),\(^4\)\(^1\) actively extrude several anticancer drugs, including doxorubicin, which has been one of the chemotherapeutic agents most used in HMM therapy.\(^3\)\(^4\) Thus, both single agent and combinatory therapy obtain a successful response in not more than 20% of patients.\(^3\)\(^8\)\(^4\)\(^2\)

We have previously shown that doxorubicin-sensitive and doxorubicin-resistant human colon cancer cells exhibit a different capacity to produce NO\(^1\)\(^6\) and that doxorubicin resistance is reverted by inducers of NO production, such as cytokines and atorvastatin, or NO donors.\(^1\)\(^6\) Starting from these observations, we focused our study on the role of NO in the resistance of HMM cells to doxorubicin.

We first analyzed which drug membrane transporters were present in HMM cells, comparing their expression with that of doxorubicin-sensitive HT29 cells and doxorubicin-resistant HT29-dx cells.\(^1\)\(^6\) HMM cells exhibited a significant expression of Pgp, in the absence of any previous exposure to doxorubicin. MRP1 and MRP2 were absent in HMM cells, and a low amount of MRP3 was detectable. The expression of Pgp and MRPs 1–3 was not substantially modified by a 24 hr incubation with mevastatin (100 \(\mu\)M).

We have previously shown that doxorubicin-sensitive and doxorubicin-resistant human colon cancer cells exhibit a different capacity to produce NO\(^1\)\(^6\) and that doxorubicin resistance is reverted by inducers of NO production, such as cytokines and atorvastatin, or NO donors.\(^1\)\(^6\) Starting from these observations, we focused our study on the role of NO in the resistance of HMM cells to doxorubicin.

We first analyzed which drug membrane transporters were present in HMM cells, comparing their expression with that of doxorubicin-sensitive HT29 cells and doxorubicin-resistant HT29-dx cells.\(^1\)\(^6\) HMM cells exhibited a significant expression of Pgp, in the absence of any previous exposure to doxorubicin. MRP1 and MRP2 were absent in HMM cells, and a low amount of MRP3 was detectable. The expression of Pgp and MRPs 1–3 was not substantially modified by a 24 hr incubation with mevastatin (100 \(\mu\)M),
simvastatin (10 μM), mevalonic acid (100 μM) and doxorubicin (4 μM), alone or in different combinations (data not shown).

The HMM cells we used accumulated significantly lower amounts of drug in comparison to both HT29 cells and HT29-dx cells. This observation suggested that HMM cells could represent a useful model of cells constitutively resistant to doxorubicin. Since we had previously observed that the HMGCoA reductase inhibitor atorvastatin reverts doxorubicin resistance in HT29-dx cells,16 we exposed HMM cells to other 2 lipophilic statins, mevastatin and simvastatin. A 24 hr incubation of HMM cells with these compounds caused augmented doxorubicin accumulation, potentiation of the doxorubicin-induced cellular death, enhanced nitrite production and NOS activity. Both statins did not induce per se a cytotoxic effect, investigated by measuring both LDH release and trypan blue cell staining. The absence of their proapoptotic effect in HMM cells was confirmed by further experiments showing that neither simvastatin nor mevastatin induced the release of cytochrome c from mitochondria to the cytosol, an effect which was, instead, clearly detectable when the cells were incubated with the well known apoptotic stimulus camptothecin (data not shown).

In different in vitro models statins have been demonstrated to cause apoptosis13,14 or to cooperate with chemotherapeutic agents in inducing cellular death.15,16 Our results suggest that such effects may be related to the known ability of statins to elicit NO synthesis. It has been observed indeed that, by inhibiting HMGCoA reductase, statins prevent prenylation of small GTPases such as Rho proteins, thus removing the inhibition exerted by these G proteins on the iNOS expression.12,14
Our results show that, in the presence of mevalonic acid, the product of HMGCoA reductase, mevastatin and simvastatin did not increase nitrite accumulation or NOS activity and did not potentiate the doxorubicin accumulation and the drug-induced release of LDH, thus confirming that the statins’ effect was mediated by the blockade of HMGCoA reductase. The effect of statins on doxorubicin accumulation and cytotoxicity was NO-dependent, since it was reverted in the presence of an NO inhibitor or an NO scavenging system. This rules out the hypothesis that, in our experimental conditions, statins might increase the uptake of doxorubicin by operating as competitive inhibitors of the uptake of Pgp.47

NF-κB includes a family of proteins, assembled in dimeric transcription factors, controlling a large number of genes in response to various cellular stresses.53 In resting cells, the nuclear localization of NF-κB is hindered by the binding of the inhibitory protein IκBα, which sequesters NF-κB in the cytoplasm. Inflammatory cytokines, bacterial lipopolysaccharide or oxidative stress result in phosphorylation, ubiquitinylation and proteasomal degradation of IκBα, followed by NF-κB nuclear translocation (for review see refs. 11,48). The relationship between statins, NF-κB pathway and iNOS expression is still a matter of debate and has led to different results.49–51 We have previously observed that the NOS up-regulation in HMM cells is associated with the activation of the transcription factor NF-κB.11 With EMSA experiments in HMM cells, we observed that both statins induced nuclear translocation of NF-κB. In an inverse fashion, statins reduced the amount of the NF-κB inhibitor IκBα, probably because of its ubiquitinylation and degradation. Again, these statins’ effects were reverted by mevalonic acid. IκKα protein acts as a part of the IKK complex and is responsible for the phosphorylation of IκBα on serine 176 and 180.33 Similar to many other kinases, the IKK complex is fully active when phosphorylated: a critical step in the activation is the phosphorylation on serine 176 and 180 of IκKα protein.33 In resting HMM cells, where no nuclear translocation of NF-κB was observed, IκKα appeared only slightly phosphorylated. Mevastatin and simvastatin increased the amount of phospho(Ser 180)-IκKα, an effect inhibited by the coinubcation with mevalonic acid. None of these agents modified the amount of total IκKα protein, suggesting that statins modulate IκKα activity rather than its expression; indeed simvastatin increased the IKK activity in HMM cells, promoting the phosphorylation of IκBα on serine 32.

It has been reported that lovastatin, by inhibiting RhoA prenylation, stimulates IκKα activity and increases NF-κB nuclear translocation and iNOS expression in cytokine-stimulated C6 glial cells.32 Other experimental works have suggested that RhoA protein may exert a positive or negative modulation on NF-κB.31,52,53 A regulation of RhoA activity may be postulated also in HMM cells; both mevastatin and simvastatin reduced the amount of prenylated GTP-bound RhoA, while mevalonic acid, providing cells with a source of additional isoprenoid groups, restored the levels of prenylated RhoA.

Rho proteins are involved in different cellular events, such as cytoskeleton organization, control of cells’ volume and morphology, proliferation and motility.54–56 Interesting downstream effectors of RhoA are the Rho-dependent kinases Rok1 and Rok2, 2 serine/threonine kinases activated only when RhoA is prenylated. Rho kinases play an important role in some cellular crucial events, such as transformation, tumor invasion and chemokinesis.28; for these reasons, specific Rho kinase inhibitors have been developed and of one them, Y27632, has been proposed as a potential anti-cancer and/or antiinflammatory drug.39,55 Different bacterial toxins are able to modulate G proteins: toxin B from C. difficile is considered to be a specific inhibitor of GTP binding to RhoA.26,27 Toxin B and statins lowered the amount of active RhoA protein in HMM cells and the level of Rho kinase activity. Also in this case, mevalonic acid partially reverted the statins’ effects. As a further evidence that RhoA inhibition is related to NF-κB activation in human mesothelioma, both Y27632 and toxin B augmented IκKα phosphorylation and diminished the amount of IκBα.

A wide range of doses of Y27632 has been used in in vitro and in vivo experimental works.57–59 In our experimental conditions, 2 μM Y27632 was sufficient to increase nitrite production, NOS activity and doxorubicin accumulation in HMM cells. Simvastatin and Y27632 have already been used to revert cellular adhesion-mediated drug resistance in multiple myeloma46; in these cells the resistance was independent from the overexpression of Pgp or other membrane transporters. The reverting effect of statins, Y27632 and toxin B in HMM appeared to be NO-mediated, since the enhanced doxorubicin accumulation and drug-induced cell death were abolished in the presence of an NO scavenger. The NF-κB inhibitor parthenolide significantly inhibited both NO synthesis and doxorubicin accumulation induced by simvastatin, mevastatin, toxin B and Y27632, supporting the hypothesis that these compounds induce such effects by activating the NF-κB signaling pathway in HMM cells. NO may modulate the activity of different enzymes and membrane proteins, via cysteine S-nitrosylation and/or tyrosine nitration.55 In HT29-dx cells the NO donor SNAP promoted tyrosine nitration on the MRPs protein, which could result in a decreased doxorubicin efflux rate.56 Interestingly, agents inducing NO synthesis in HMM cells (simvastatin, Y27632 and toxin B) elicited a pronounced nitration of Pgp protein. No nitration was detected in the MRP3 protein, probably because of its low expression in HMM cells. It is conceivable that NO may revert doxorubicin resistance in human mesothelioma via a negative modulation of an ABC transporter, similar to what has been observed in human colon cancer cells, although in HMM the protein target of NO is different. This hypothesis is strengthened by the observation that SNAP increased both the LDH release and the drug accumulation induced by doxorubicin in HMM cells.

Our results suggest a correlation between NF-κB activation and the reversion of doxorubicin resistance. On the contrary, in certain tumors a constitutive NF-κB activation seems to be related to chemoresistance and increased cellular survival.57,58 In some experimental models NF-κB is constitutively high,56 in other cases it is activated after treatment with a chemotherapeutic agent that can also promote Pgp overexpression,25,27; a reduction of NF-κB translocation has been associated to reduced drug-mediated Pgp overexpression59 and to enhanced apoptosis.60,61 Instead, other works reported that activation of NF-κB is necessary to promote apoptosis induced by chemotherapeutic agents56 or to repress mdrl promoter activity.61 It is generally acknowledged that the relationship between NF-κB and chemoresistance is highly cell-specific and depends upon the basal level of NF-κB and the drugs investigated.59 In HMM cells, where NF-κB is constitutively low and Pgp is high, independently from the exposure to any drugs, NF-κB activation seems to be aimed to revert chemoresistance.

To our knowledge, this is the first report showing a correlation between RhoA/Rho kinase inhibition, NO production and reversion of doxorubicin-resistance. Furthermore, we were able to revert doxorubicin resistance in cultured HMM cells by the help of drugs that are widely used in clinical practice, such as mevastatin and simvastatin, or compounds proposed for clinical trials, as the Rho kinase inhibitor Y27632. Our results could suggest new clinical strategies aimed to improve doxorubicin efficacy in the treatment of human malignant mesothelioma.

References

46. Arlt A, Vorndamm J, Uhnrich M, Fuchs UR, Kalthoff H, Schmidt WE, Schafar H. Inhibition of NFκB sensitizes human pan-
creatic carcinoma cells to apoptosis induced by etoposide (VP16) and doxorubicin. Oncogene 2001;20:859–68.