Molecular Pharmacology Fast Forward. Published on May 7, 2008 as doi:10.1124/mol.108.045286
This article has not been copyedited and formatted. The final version may differ from this version.

MOL manuscript # 45286

Activation of NF-kB pathway by simvastatin and RhoA silencing increases doxorubicin cytotoxicity in human colon cancer HT29 cells

Chiara Riganti, Sophie Doublier, Costanzo Costamagna, Elisabetta Aldieri, Gianpiero Pescarmona, Dario Ghigo, Amalia Bosia

Department of Genetics, Biology and Biochemistry, University of Torino, and Research Center on Experimental Medicine CeRMS Via Santena, 5/bis, 10126, Torino, Italy (C.R., S.D., C.C., E.A., G.P., D.G., A.B.).

Molecular Pharmacology Fast Forward. Published on May 7, 2008 as DOI: 10.1124/mol.108.045286
This article has not been copyedited and formatted. The final version may differ from this version.

MOL manuscript # 45286

Running title: RhoA/NF-kB modulate doxorubicin efficacy

Corresponding author: Chiara Riganti, Dipartimento di Genetica, Biologia e Biochimica (Sezione di Biochimica), Via Santena, 5/bis, 10126 Turin, Italy. Phone: 39-11-670-5851; Fax: 39-11-670-5845; E-mail: chiara.riganti@unito.it

Number of text pages: 32

Number of tables: 0

Number of figures: 8

Number of References: 38

Number of words in the Abstract: 250

Number of words in the Introduction: 541

Number of words in the Discussion: 2004

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; NF-kB, nuclear factor-kappa B; IkB, inhibitor of kappa B; IKK, IkB kinase; NO, nitric oxide; NOS, nitric oxide synthase; Pgp, P-glycoprotein; MRP3, multidrug resistance-related protein 3; ABC, ATP-binding cassette; siRNA, small interfering RNA; SNAP, S-nitroso-acetylpenicillamine.

Molecular Pharmacology Fast Forward. Published on May 7, 2008 as DOI: 10.1124/mol.108.045286
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

Doxorubicin efficacy in cancer therapy is hampered by the dose-dependent side effects, which may be overcome by reducing the drug's dose and increasing its efficacy. In the present work we suggest that the activation of the nuclear factor-kappa B (NF-kB) pathway and of nitric oxide (NO) synthase increases the doxorubicin efficacy in human colon cancer HT29 cells. To induce NF-kB, we took into account the effect of doxorubicin itself and of the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor simvastatin; as NF-kB inhibitors, we chose the sesquiterpene lactones parthenolide and artemisinin. Simvastatin increased the NF-kB activity and NO synthesis, elicited the tyrosine nitration of the multidrug resistance-related protein 3, and enhanced the doxorubicin intracellular accumulation and cytotoxicity. Simvastatin potentiated the effect of doxorubicin on the NF-kB pathway and the inducible NO synthase expression. The effects of simvastatin were due to the inhibition of the small G-protein RhoA and of its effector Rho kinase. Parthenolide and artemisinin prevented all the statin effects, by inducing RhoA/Rho kinase activation. On the opposite they did not reduce the NF-kB translocation and doxorubicin intracellular content when RhoA was silenced by small interfering RNA (siRNA). Interestingly, RhoA siRNA was sufficient to increase NF-kB translocation, NO synthase activity, doxorubicin accumulation and cytotoxicity also in non-stimulated cells. Our results suggest that artemisinin, a widely used antimalarial drug, may impair the response to doxorubicin in colon cancer cells; on the contrary, simvastatin and RhoA siRNA may represent future therapeutic approaches to improve doxorubicin efficacy, reducing the risk of doxorubicin-dependent adverse effects.

Molecular Pharmacology Fast Forward. Published on May 7, 2008 as DOI: 10.1124/mol.108.045286
This article has not been copyedited and formatted. The final version may differ from this version.

INTRODUCTION

Anthracyclines, such as doxorubicin, epirubicin and their derivatives, are drugs widely used in the treatment of solid and haematological cancers. However their efficacy is limited owing to their dose-dependent cardiotoxicity, an important adverse effect which impairs the patients outcome and survival (Bonadonna and Monfardini, 1969). Moreover, resistance to these drugs commonly arises in the cancer cells due to genetic and epigenetic alterations that affect drug sensitivity (Gottesman et al, 2002). We have previously shown that statins revert doxorubicin resistance in different cancer cell lines (Riganti et al, 2005; Riganti et al, 2006). Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which catalyses the rate-limiting step in cholesterol synthesis. Therefore, statins dramatically decrease both cholesterol and isoprenoid intermediates, impairing the isoprenylation and activity of different enzymes, such as the small G proteins families Ras and Rho (Dulak and Jòzkowicz, 2005). Rho GTPases play a key role in the regulation of tumor growth, migration and sensitivity to anticancer drugs (Fritz and Kaina, 2006). Some of these functions are dependent on the activity of the transcription factor nuclear factor-kappa B (NF-kB) (Fritz and Kaina, 2006). NF-kB is composed by protein dimers, such as the heterodimer p50/p65, and regulates the expression of genes involved in inflammation, cellular proliferation and apoptosis (Greten and Karin, 2004). In resting cells, members of the inhibitory IkB family proteins bind directly to NF-kB dimer in the cytoplasm, preventing its nuclear localization. NF-kB is free to translocate and bind to DNA on the target genes when IkBα is phosphorylated by the IkB kinase (IKK) complex, ubiquitinated and degraded by S26 proteasome (Greten and Karin, 2004). Statins, by inhibiting RhoA and its effector Rho kinase, can activate the IKK/NF-kB pathway (Kraynack et al, 2002; Rattan et al, 2003). Also doxorubicin can induce NF-kB translocation in cancer cells, via different mechanisms (Lin et al, 2007; Yu et al, 2008). By activating NF-kB, statins and doxorubicin may enhance the transcription of the inducible NO synthase (iNOS) (Greten and Karin, 2004), one of the three NOS isoforms, which catalyze the conversion of L-arginine to L-

citrulline and nitric oxide (NO) with a 1:1 stoichiometry (Nathan and Xie, 1994). NO is a signalling molecule involved in the control of cellular growth, differentiation and apoptosis (Greten and Karin, 2004). We have previously suggested that NO is implicated in the doxorubicin cytotoxicity in HT29 cells (Riganti et al, 2005) and reverts doxorubicin resistance, via the tyrosine nitration of P-glycoprotein (PgP) and multidrug resistance-related protein 3 (MRP3), two ATP-binding cassette (ABC) transporters which recognize doxorubicin as a substrate. Such a nitration reduces the drug efflux in doxorubicin-resistant cells (Riganti et al, 2005; Riganti et al, 2006).

Aim of this work has been to investigate whether statins, as well as RhoA silencing, may improve the cytotoxic effect of doxorubicin, via the enhanced activation of NF-kB/iNOS pathway on human colon cancer HT29 cells. In order to reduce the NF-kB activity we used parthenolide and artemisinin, two natural sesquiterpene lactones. The former is a well-known NF-kB inhibitor with anti-inflammatory and anti-septic properties (Wong and Menendez, 1999; Yip et al, 2004). The latter is an efficient antimalarial drug (Mohanty et al, 2006), and modulates the immune system response by inhibiting the cytokine-induced NF-kB translocation (Aldieri et al, 2003; Li et al, 2006).

Molecular Pharmacology Fast Forward. Published on May 7, 2008 as DOI: 10.1124/mol.108.045286
This article has not been copyedited and formatted. The final version may differ from this version.

MATERIAL AND METHODS

Material. Foetal bovine serum (FBS) and RPMI 1640 medium were supplied by BioWhittaker (Verviers, Belgium); plasticware for cell culture was from Falcon (Becton Dickinson, Bedford, MA). Simvastatin was purchased 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 Sigma Chemical Co (St. Louis, MO). When not otherwise specified, all the other reagents were purchased from Sigma Chemical Co.

Cells. Human colon cancer cells (HT29 cell line) were cultured in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine and maintained in a humidified atmosphere at 37°C and 5% CO_2.

Electrophoretic mobility shift assay (EMSA). Cells were plated in 60-mm diameter dishes at confluence and 10 µg of nuclear proteins extracts were used to detect NF-kB translocation as described (Riganti et al, 2006).

Western blot analysis. Western blot detection of IkBα, phospho(Ser 32)-IkBα, IKKα/β, phospho(Ser176-180)-IKKα/β, glyceraldehyde 3-phosphate dehydrogenase, MRP3 and nitrated MRP3, neuronal NOS (nNOS), inducible NOS (iNOS) or endothelial NOS (eNOS) antibodies was performed as previously reported (Riganti et al, 2005; Riganti et al, 2006). In addition 30 µg of nuclear proteins obtained as described (Riganti et al, 2006) were probed with anti-p50 (from mouse, diluted 1: 250 in PBS-BSA 1%, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-p65 (from rabbit, diluted 1: 500 in PBS-BSA 1%, Santa Cruz Biotechnology) antibodies.

IKK activity assay. IKK activity was measured as previously described (Riganti et al, 2006).

Measurement of NOS activity. Cells grown at confluence on 35-mm diameter Petri dishes were detached by trypsin/EDTA, re-suspended in 0.3 ml of Hepes/EDTA/DTT buffer (20 mM Hepes, 0.5 mM EDTA, 1 mM DTT, pH 7.2) and sonicated. NOS activity was measured on 100 µg of cell lysates with the Ultrasensitive Colorimetric Assay for Nitric Oxide Synthase kit (Oxford Biomedical Research, Oxford, MI). Results were expressed as nmol nitrite/min/mg cell protein.

Molecular Pharmacology Fast Forward. Published on May 7, 2008 as DOI: 10.1124/mol.108.045286
This article has not been copyedited and formatted. The final version may differ from this version.

Doxorubicin accumulation. Intracellular doxorubicin accumulation was measured as described elsewhere (Riganti et al, 2005), using a Perkin-Elmer LS-5 spectrofluorimeter (Perkin Elmer, Shelton, CT). Excitation and emission wavelengths were 475 and 553 nm. Fluorescence was converted in ng doxorubicin/mg cell proteins, using a calibration curve prepared previously.

Extracellular lactate dehydrogenase (LDH) activity. After a 6 h incubation under different experimental conditions in presence of 5 μM doxorubicin, LDH activity was measured in the extracellular medium and in the cell lysate, as previously described (Riganti et al, 2006; Beutler, 1971), to check the cytotoxicity of doxorubicin. Absorbance at 340 nm was measured for 10 min with a Lambda 3 spectrophotometer (Perkin Elmer). Both intracellular and extracellular enzyme activity was expressed as μmol NADH oxidized/min/dish, then extracellular LDH activity was calculated as percentage of the total LDH activity in the dish.

Trypan blue staining. After a 6 h incubation under the experimental conditions reported in the Results, cell monolayers were washed, detached with trypsin/EDTA and re-suspended in 1 ml of PBS. 10 μl of 20% (w/v) trypan blue were added to each sample. After a 1 min incubation at room temperature, 10 μl of each cellular suspension were analyzed in a Burker chamber under a light microscope and the trypan blue-positive cells were counted as percentage of dead cells on a total number of 200 cells.

Annexin V/propidium iodide (PI) assay. Cells were incubated for 6 h in the experimental conditions described under the Results section, then they were washed twice with fresh PBS, detached with the Cell Dissociation Solution (Sigma Chemical Co) and incubated for 10 min at room temperature in 1 ml of binding buffer (100 mM Hepes, 140 mM NaCl, 25 mM $CaCl_2$, pH 7.5) containing 10 μM annexin V-fluorescein isothiocyanate conjugate (FITC) and 2.5 μM PI. The cells suspensions were washed three times with fresh PBS and rinsed with 1 ml of binding buffer. The fluorescence of each sample was recorded using a FACSCalibur system (Becton Dickinson, San Jose, CA). For each analysis 10,000 events were collected; the green fluorescence (for Annexin V-FITC) was detected using a 530 nm band pass filter, while the red

Molecular Pharmacology Fast Forward. Published on May 7, 2008 as DOI: 10.1124/mol.108.045286
This article has not been copyedited and formatted. The final version may differ from this version.

fluorescence (for PI) was detected with a 640 nm longpass filter. The percentage of cells positive for annexin V-FITC, PI or both was calculated by the Cell Quest software (Becton Dickinson).

RhoA-GTP binding assay. RhoA-GTP binding activity was measured using the G-LISA™ RhoA Activation Assay Biochem Kit™ (Cytoskeleton Inc, Denver, CO). Cells were cultured in 35-mm diameter Petri dishes, washed with PBS, lysed in 0.2 ml lysis buffer of the kit and centrifuged at 13,000 x g for 5 min. 100 µl of supernatants were used for measurement of protein content and Rho-GTP binding, according to the manufacturer's instructions. Absorbance was read at 450 nm, using a Packard EL340 microplate reader (Bio-Tek Instruments, Winooski, VT). For each set of experiments, a titration curve was prepared, using serial dilution of the Rho-GTP positive control of the kit. Data were expressed as mU absorbance/mg cell proteins.

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, as previously reported (Riganti et al, 2006).

RhoA siRNA transfection. 30,000 cells/ml were plated in 35-mm diameter Petri dishes and cultured in RPMI-1640 medium containing 10% FBS. After 24 h cells were washed with 2 ml siRNA Transfection Medium (Santa Cruz Biotechnology) and incubated for 6 h with 1 ml siRNA transfection medium, containing 5 µl of siRNA Transfection Reagent (Santa Cruz Biotechnology) and 50 pmol of RhoA siRNA (Santa Cruz Biotechnology). 1 ml of RPMI-1640 medium containing 1% penicillin/streptomycin and 20% FBS was added for 24 h. Subsequently, cells were washed and grown for 72 h in RPMI-1640 medium with 1% penicillin/streptomycin and 10% FBS. Cellular toxicity was assessed by measuring the extracellular LDH release after 24, 48 and 72 h from transfection. To verify the siRNA efficacy, cells were lysed and the expression of RhoA protein was analysed by Western blotting using an anti-RhoA antibody (diluted 1: 250 in PBS-BSA 1%, Santa Cruz Biotechnology). To assess the siRNA specificity for RhoA, we checked the expression of GAPDH, a product of an housekeeping gene in both transfected and untransfected cells. The specificity of the silencing technique was further verified

Molecular Pharmacology Fast Forward. Published on May 7, 2008 as DOI: 10.1124/mol.108.045286
This article has not been copyedited and formatted. The final version may differ from this version.

incubating in each set of experiments an aliquot of HT29 cells with 50 pmol of Control siRNA-A (Santa Cruz Biotechnology),a non-targeting 20-25 nucleotides siRNA designed as a negative control, instead of RhoA siRNA: nor the RhoA neither GAPDH expressions, analysed by Western blotting, were modified (data not shown).

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.

Molecular Pharmacology Fast Forward. Published on May 7, 2008 as DOI: 10.1124/mol.108.045286
This article has not been copyedited and formatted. The final version may differ from this version.

RESULTS

Simvastatin and doxorubicin induce activation of the NF-kB pathway in HT29 cells, which is prevented by parthenolide and artemisinin.

HT29 cells showed undetectable levels of nuclear NF-kB, which was increased by simvastatin in a dose-dependent way (Figure 1A). The statin effect was clear after 6 and 24 h, whereas lower incubation time periods were not sufficient to induce a clear translocation of NF-kB. All the following experimental work was performed with 10 μM simvastatin for 6 h, which was as effective as TNF-α, a potent inducer of NF-kB activation in HT29 cells (Figure 1A). Parthenolide and artemisinin, which per se did not affect NF-kB nuclear levels, completely abolished the effect of the statin on NF-kB translocation (Figure 1B). Western blotting experiments on nuclear extracts confirmed the EMSA results: simvastatin, as well as TNF-α, elicited the p50/p65 dimer translocation into the nucleus, while parthenolide and artemisinin prevented it (Figure 1C). Interestingly, doxorubicin alone increased the NF-kB activity and the p50/p65 nuclear translocation; these drug effects were enhanced by simvastatin (Figure 1B and 1C).

The cytosolic level of IkBα was clearly detectable, while phospho(Ser 32)-IkBα protein was absent in the cytosolic extracts of HT29 cells resting or incubated with parthenolide or artemisinin (Figure 2A). On the contrary, the incubation with simvastatin decreased IkBα and increased phospho(Ser 32)-IkBα protein: these effects were reverted by the addition of the sesquiterpene lactones (Figure 2A). Total IKKα/β was not modified under any experimental condition and phospho-IKKα/β was absent in control HT29 cells and in the presence of parthenolide and artemisinin (Figure 2A). Simvastatin augmented the amount of phospho-IKKα/β, while a very low amount of phospho-IKKα/β was present when the statin was co-incubated with either parthenolide or artemisinin (Figure 2A).

To assess the kinase activity of IKK complex, we incubated the immunoprecipitated IKK protein, obtained from HT29 cells, with an excess of the substrate IkBα, obtained from HMM

Molecular Pharmacology Fast Forward. Published on May 7, 2008 as DOI: 10.1124/mol.108.045286
This article has not been copyedited and formatted. The final version may differ from this version.

cells, which are particularly rich of IkBα protein (Aldieri et al, 2004). During the assay we also added the proteasome inhibitor MG132, to prevent the degradation of phosphorylated IkBα protein by the residual proteasome eventually still present in the lysates. In this way, no change of IkBα protein was observed during such in vitro kinase assay (Figure 2B). Simvastatin significantly increased the IKK kinase activity (Figure 2B), whereas the IKK kinase activity was undetectable in control cells as well as in cells incubated with parthenolide or artemisinin (Figure 2B). Doxorubicin alone was able to reduce the IkBα amount, induce a prompt phosphorylation on IkBα and IKK and increase the activity of IKK; again, the doxorubicin effects were potentiated when the drug was co-incubated with simvastatin (Figure 2A and 2B). In summary, simvastatin, doxorubicin and TNF-α exerted the same effects on IkBα and IKK levels and activity, and doxorubicin and simvastatin together exerted a more potent effect on the IKK/IkBα pathway (Figure 2A and 2B).

Simvastatin increases NOS activity and doxorubicin efficacy in HT29 cells, parthenolide and artemisinin abolish the statin effect.

A low basal level of NOS activity was detectable in HT29 cells (Figure 3A). Doxorubicin greatly increased the NO synthesis (Figure 3A), the LDH release, the percentage of trypan blue-positive cells (Fig 3B) and the number of cells stained with annexin V-FITC and PI (Figure 3C). Simvastatin further enhanced the NOS activity and increased the intracellular doxorubicin accumulation in HT29 cells (Figure 3A), as well as the drug cytotoxicity, measured as LDH release and trypan blue staining (Figure 3B). Interestingly, statin reduced the percentage of the cells positive for annexin V-FITC alone, whereas it markedly increased the percentage of HT29 cells positive for PI alone and for both annexin V-FITC and PI (Figure 3C). The effect of doxorubicin and simvastatin on NO synthesis were likely due to an overexpression of the iNOS isoform: indeed iNOS was absent in untreated HT29 cells, each drug elicited a clear increase of iNOS expression, which was even greater when simvastatin and doxorubicin were incubated together (see supplemental data, Figure S1). A low basal amount of the eNOS and nNOS

Molecular Pharmacology Fast Forward. Published on May 7, 2008 as DOI: 10.1124/mol.108.045286
This article has not been copyedited and formatted. The final version may differ from this version.

isoforms was detectable in HT29 cells and did not change under any experimental condition (see supplemental data, Figure S1).

Parthenolide and artemisinin did not modify the NO synthesis in doxorubicin-treated HT29 cells and slightly protected the cells from doxorubicin accumulation and toxicity (Figure 3A and B). The two sesquiterpene drugs did not significantly modify the doxorubicin-induced apoptosis in HT29 cells (Figure 3C). According to their ability to block the NF-kB translocation, they inhibited the statin-induced increase of NOS activity, doxorubicin accumulation, LDH release, cell death and apoptosis (Figure 3A, 3B and 3C). The sesquiterpene lactones did not changed significantly the release of LDH and the staining with trypan blue or annexin V-FITC/PI in the absence of doxorubicin (data not shown).

Simvastatin, parthenolide and artemisinin modulate the tyrosine nitration of MRP3 protein.

HT29 cells express small amounts of MRP3 protein; no tyrosine nitration was detected in cells resting or incubated with parthenolide and artemisinin (Figure 4). On the contrary, simvastatin increased the levels of nitro-MRP3, an effect mimicked by the NO donor S-nitroso-acetylpenicillamine (SN) and inhibited by the co-incubation with parthenolide and artemisinin (Figure 4). Also doxorubicin slightly induced the tyrosine nitration of MRP3, and this effect was greatly enhanced by simvastatin. The total amount of MRP3 did not change under each experimental condition (Figure 4). No nitration of Pgp was observed in HT29 cells, as previously reported (Riganti et al, 2005).

Sesquiterpene lactones increase, while simvastatin decreases, RhoA and Rho kinase activity in HT29 cells.

Statins impair the isoprenylation of the Rho proteins as well as their ability to bind GTP and activate their effector, i.e. Rho kinase (Laufs and Liao, 2000). In HT29 cells simvastatin significantly reduced the level of RhoA-GTP and the activity of Rho kinase (Figure 5). Such statin effect was completely prevented by the co-incubation with the product of HMGCoA reductase, mevalonic acid, which per se increased both RhoA-GTP and Rho kinase activity

Molecular Pharmacology Fast Forward. Published on May 7, 2008 as DOI: 10.1124/mol.108.045286
This article has not been copyedited and formatted. The final version may differ from this version.

(Figure 5). Interestingly, parthenolide and artemisinin significantly augmented both RhoA-GTP and Rho kinase activity, and, less potently than mevalonic acid, were able to restore the activity of RhoA and Rho kinase when co-incubated with simvastatin (Figure 5). On the opposite, doxorubicin did not affect significantly RhoA and Rho kinase activity and did not modify the effect of simvastatin (Figure 5).

RhoA silencing with siRNA induces NF-kB activation and prevents the effects of parthenolide and artemisinin in HT29 cells.

RhoA was efficiently silenced in HT29 cells 72 hours after their transfection with 50 pmol of specific siRNA (Figure 6A). Cell viability, assessed as the LDH release in the supernatant, was not significantly different from non-transfected cells at 24, 48 and 72 h after the transfection (data not shown). The expression of GAPDH, the product of an housekeeping gene unrelated to RhoA protein, was not changed by the transfection procedure (Figure 6A). In RhoA-silenced cells, the NF-kB activity was intrinsically high (Figure 6B) and the dimer p50/p65 was detectable in the nucleus (Figure 6C). IkBα/IKK status varied accordingly: the IkBα level was lower than in non-transfected cells (Figure 7A), while the phospho-IkBα amount (Figure 7A), the phosphorylation of IKKα/β (Figure 7A) and the kinase activity of IKK complex (Figure 7B) were higher. Such a response was similar to that observed in non-transfected HT29 cells incubated with simvastatin and TNF-α (Figure 6 and 7). On the other hand, the presence of simvastatin did not modify the NF-kB signalling pathway in RhoA-silenced cells (Figure 6 and 7). When RhoA protein was knocked down, the addition of parthenolide and artemisinin to simvastatin did not affect the p50/p65 NF-kB dimer translocation (Figure 6B and 6C), the IkBα/phospho-IkBα levels, the IKKα/β phosphorylation and the IKK activity (Figure 7A and 7B).

HT29 cells become more sensitive to doxorubicin with RhoA siRNA.

RhoA siRNA augmented NOS activity at an extent similar to that obtained incubating non-transfected HT29 cells with simvastatin (Figure 7C). Again, when RhoA was silenced, NO

Molecular Pharmacology Fast Forward. Published on May 7, 2008 as DOI: 10.1124/mol.108.045286
This article has not been copyedited and formatted. The final version may differ from this version.

production was not further modified by simvastatin, alone or together with parthenolide and artemisinin (Figure 7C). Finally, RhoA siRNA greatly enhanced the doxorubicin accumulation (Figure 7C) and the drug-induced LDH release (Figure 8A), trypan blue staining (Figure 8A) and apoptosis (Figure 8B) under each experimental condition, and made HT29 cells insensitive to the effects of parthenolide and artemisinin (Figure 7C and 8).

Molecular Pharmacology Fast Forward. Published on May 7, 2008 as DOI: 10.1124/mol.108.045286
This article has not been copyedited and formatted. The final version may differ from this version.

DISCUSSION

As for many other chemotherapeutic agents, the clinical use of doxorubicin is hampered by several side effects, including cardiotoxicity (Bast et al, 2007), which is the major limitation to doxorubicin use and impairs the clinical response and the survival of patients (Bryant et al, 2007). To limit the myocardial damage, less cardiotoxic doxorubicin derivatives (Batist et al, 2001) or the co-administration with cardioprotectors, such as dexrazoxane, coenzyme Q10 (Batist et al, 2001) and flavonoids (Bast et al, 2007), have been proposed, without obtaining satisfying results. Different protocols of administration, i.e. continuous infusion vs bolus infusion, have been compared, but none of them significantly reduces adverse effects (Bryant et al, 2007). Rendering cancer cells more sensitive to doxorubicin would be an efficient approach to diminish the overall dose of anthracyclines infused and to increase the ratio between therapeutic effect and toxicity.

The present study has been focused on human colon cancer HT29 cells, which express low basal levels of Pgp and MRP3 and may be considered a model of doxorubicin-sensitive cells (Riganti et al, 2005). In human mesothelioma cells, which overexpress both Pgp and MRP3, the doxorubicin resistance has been corrected by inducing NF-kB activation and NO synthesis (Riganti et al, 2006). So far, we now investigated whether the doxorubicin efficacy could be enhanced with a similar mechanism in sensitive cancer cells.

NF-kB family comprehends many dimeric transcription factors, i.e. p50/p65 dimer, and controls several genes involved in the response to cellular stress, cellular survival and proliferation (Greten and Karin, 2004). The inhibitor protein IkBα sequesters NF-kB in the cytoplasm of resting cells, but different stimuli, such as inflammatory cytokines, bacterial lipopolysaccharide, oxidative stress or drugs like statins and doxorubicin, promote IkBα phosphorylation and ubiquitinylation, inducing the nuclear translocation of NF-kB. IkB-kinase (IKK) complex is responsible for the phosphorylation of IkBα on serine 32 and 36. Like other kinases, IKK becomes fully active when phosphorylated (Greten and Karin, 2004). Untreated HT29 cells did

Molecular Pharmacology Fast Forward. Published on May 7, 2008 as DOI: 10.1124/mol.108.045286
This article has not been copyedited and formatted. The final version may differ from this version.

not exhibit any basal NF-kB activation: NF-kB binding on DNA was absent, IkBα was abundant, while IKKα/β was nor phosphorylated neither active as a kinase. On the other hand, simvastatin elicited a clear translocation of p50/p65 NF-kB dimer into the nucleus, reduced the level of IkBα protein and enhanced IKKα/β phosphorylation and kinase activity. The effect of statin on NF-kB was dose-dependent and similar to that evoked by TNF-α, a potent inducer of NF-kB pathway. TNF-α promptly activated NF-kB after 15 minutes, but the degree of NF-kB translocation by TNF-α decreased after longer incubation times (data not shown). On the opposite, simvastatin elicited a slower and more sustained activation of NF-kB: a clear nuclear translocation was detectable only after 6 h and still remained high at 24 h. The different intracellular signalling pathways of TNF-α and simvastatin may account for this temporal discrepancy.

Sesquiterpene lactones have been widely used as NF-kB inhibitors, although the exact mechanism is not completely clear (Dirsch et al, 2000; Matsuda et al, 2000). We chose two of them, parthenolide and artemisinin, to modulate the NF-kB pathway in HT29 cells. Parthenolide is a natural potent NF-kB inhibitor (Wong and Menendez, 1999; Yip et al, 2004), with anti-inflammatory and anti-septic properties (Yip et al, 2004). Artemisinin, the active component of *Artemisia annua*, has a relevant clinical importance, because it is the most worldwide used antimalarial drug (Mohanty et al, 2004). Artemisinin has pleiotropic effects (Golenser et al, 2006) and has been shown to inhibit the cytokine-induced NF-kB activation in different cell lines (Aldieri et al, 2003; Li et al, 2006). Artemisinin, as well as parthenolide, did not exert any effect in resting HT29 cells, where NF-kB levels were nearly undetectable. On the contrary they completely blunted the activation of NF-kB pathway elicited by simvastatin. By inhibiting NF-kB, parthenolide and artemisinin may exert pro-apoptotic and anti-proliferative effects in some *in vitro* models (Posner et al, 2004; Kim et al, 2005). However, in our experimental models, they were not cytotoxic under the experimental conditions chosen. Also doxorubicin has been

described as an inducer of NF-kB (Lin et al, 2007; Yu et al, 2008): this observation has been confirmed in HT29 cells in the present work. Interestingly, when simvastatin was added together with doxorubicin, the activation of NF-kB was stronger than that caused by either drug alone. At the light of these results, we hypothesize that simvastatin may enhance the effect of doxorubicin, acting with a common mechanism.

The expression of the inducible form of NOS, which controls different cellular functions, such as growth, differentiation and apoptosis (Greten and Karin, 2004), is often increased by agents activating the NF-kB pathway, including statins (Kraynack et al, 2002; Rattan et al, 2003; Kotamraju et al, 2007) and doxorubicin (Riganti et al, 2005; Liu et al, 2006). HT29 cells exhibited a detectable amount of nNOS and eNOS, which may explain the basal NO production observed in untreated cells. Nor doxorubicin neither simvastatin altered the expression of these constitutive NOS isoforms. On the opposite, they both induced iNOS when used alone, and when used together they had a synergistic effect on the expression of iNOS and on the synthesis of NO, which is a cytotoxic agent for HT29 cells (Riganti et al, 2005). The iNOS overexpression is likely to play a critical role in the ability of simvastatin to enhance the doxorubicin-induced cell death: indeed doxorubicin increased the LDH release, reduced the cellular viability and promoted the apoptosis, as shown by the high percentage of HT29 cells positive for annexin V-FITC. Simvastatin potentiated the induction of NOS activity and cytotoxicity elicited by doxorubicin. The FACS analysis of apoptosis revealed that the statin markedly increased the percentage of HT29 cells positive for PI and for both annexin V-FITC and PI, whereas it reduced the percentage of the cells positive for annexin V-FITC alone. We may hypothesize that doxorubicin per se favours the entry of HT29 cells into apoptosis, but the addition of simvastatin accelerates the effect of doxorubicin and increases the number of cells in advanced stage of apoptosis or necrosis. The statin's action on doxorubicin efficacy is likely to be dependent on NF-kB activation; indeed it was completely abolished in the presence of parthenolide and artemisinin.

Molecular Pharmacology Fast Forward. Published on May 7, 2008 as DOI: 10.1124/mol.108.045286
This article has not been copyedited and formatted. The final version may differ from this version.

Beside being a cytotoxic agent, NO may modulate the activity of different enzymes and membrane proteins, through cysteine S-nitrosylation and/or tyrosine nitration (Foster et al, 2003). Pgp and MRP3, two ABC membrane transporters, which actively extrude several chemotherapeutic drugs, may be target of NO: as a consequence of the tyrosine nitration of these proteins, the efflux of doxorubicin is inhibited (Riganti et al, 2005; Riganti et al, 2006). HT29 colon cells display a very low basal level of MRP3 and Pgp proteins (Riganti et al, 2005); however, simvastatin, which enhanced NOS activity, elicited a detectable tyrosine nitration of MRP3 pump. Also doxorubicin slightly induced the nitration of MRP3, due to the iNOS induction exerted by the drug. However, when simvastatin was added to doxorubicin, the MRP3 nitration was enhanced. This result is in keeping with the more pronounced nuclear translocation of NF-kB, with the greater expression of iNOS and with the higher synthesis of NO. Such an effect increased the doxorubicin accumulation and cytotoxicity in HT29 cells. On the contrary, by inhibiting NF-kB translocation and NO synthesis, artemisinin and parthenolide reduced the MRP3 nitration and the intracellular doxorubicin accumulation induced by the statin. Taken together our results suggest the molecular mechanism by which simvastatin, artemisinin and parthenolide may modulate the efficacy of doxorubicin in HT29 colon cancer cells. This effect was not cell type-specific, since it has been observed also in human myelogenous leukemic K562 cells (data not shown).

The effect of sesquiterpenes on multidrug resistance is still matter of debate. Some natural terpenes are direct inhibitors of Pgp (Molnàr et al, 2006). Derivatives of artemisinin reduce Pgp functions in multidrug resistant K562/adr cells, with an IC_{50} of 115 μM, but this effect is evident only at high doses (Reungpatthanapong et al, 2002). The experimental evidence obtained in HT29 cells suggests that sesquiterpene lactones-induced effects are mediated by NF-kB inhibition. However, we cannot exclude that they exert other effects independent on NF-kB: indeed in the present study parthenolide and artemisinin alone slightly reduced the intracellular

doxorubicin accumulation and LDH release, when no change in NF-kB levels and NOS activity was detectable. We are presently investigating the possible mechanism of such an effect.

By inhibiting HMGcoA reductase, statins lower the intracellular levels of isoprenoids, and limit the isoprenylation of several proteins, such as Rho and Ras (Dulak and Jòzkowicz, 2005). By this way, statins efficiently block the activity of RhoA-GTP and prevent the activation of the downstream effectors, such as the serine-threonine Rho kinases 1 and 2 (Fritz and Kaina, 2006). Here we observed that simvastatin, which activated NF-kB translocation, reduced RhoA-GTP binding and Rho kinase activity, while artemisinin and parthenolide, which inhibited NF-kB activation, increased RhoA/Rho kinase activity. Interestingly, parthenolide and artemisinin had an effect even when simvastatin was present: they restored Rho-GTP binding and Rho kinase activity to the control level and inhibited the statin-induced NF-kB translocation. It has been already reported that statins exert a positive or negative modulation on NF-kB through the involvement of RhoA, but the exact mechanism has not yet been clarified (Kraynack et al, 2002; Rattan et al, 2003; Ahn et al, 2007). To our knowledge, this is the first evidence that sesquiterpene lactones may affect NF-kB through the modulation of RhoA. On the other hand, doxorubicin did not affect RhoA and Rho kinase activity, thereby it is conceivable that doxorubicin activates NF-kB in a RhoA-independent way. Several mechanisms have been proposed to explain the doxorubicin effect on NF-kB in other cell types, such as the generation of reactive oxygen species (Wang et al, 2002; Lin et al, 2007) and the activation of the phosphoinositide 3-kinase/Akt pathway (Yu et al, 2008): we are currently investigating in our laboratory whether these pathways are implicated in the doxorubicin-evoked activation of NF-kB in HT29 cells. Our data suggest that simvastatin and doxorubicin, although by a different mechanism, induce the nuclear translocation of NF-kB and the increase of NO synthesis in HT29 cells. By inhibiting RhoA, simvastatin may potentiate the doxorubicin-induced NF-kB activation and NO production, enhancing the cellular death mediated by the anthracycline drug.

Molecular Pharmacology Fast Forward. Published on May 7, 2008 as DOI: 10.1124/mol.108.045286
This article has not been copyedited and formatted. The final version may differ from this version.

The experiments with RhoA specific siRNA confirmed the central role played by RhoA in controlling NF-kB translocation and doxorubicin accumulation. The efficient silencing of RhoA did not affect HT29 cells viability and induced an intense activation of NF-kB pathway and NOS activity. Moreover, doxorubicin accumulation and its cytotoxic and pro-apoptotic effects were potentiated, similarly to what we observed in HT29 cells incubated with simvastatin. So far, our study shows that the silencing of RhoA increases the efficacy of doxorubicin in cancer cells. Indeed statins have been related to the correction of doxorubicin resistance in MDR human cancer cells lines (Schmidmaier et al, 2004; Riganti et al, 2006). However, the direct targeting of RhoA by siRNA appears to be a more specific tool than statins. In silenced HT29 cells, parthenolide and artemisinin were devoid of any effect: this result further confirms that the inhibitory effect of the sesquiterpene lactones on NF-kB nuclear translocation, NO synthesis and doxorubicin accumulation is mediated by RhoA and Rho kinase. The mechanism by which parthenolide and artemisinin may affect RhoA activity is currently under investigation in our laboratory.

In summary, our results suggest that simvastatin enhances the doxorubicin-induced NF-kB and iNOS activation in HT29 colon cancer cells by inhibiting RhoA. To the contrary, artemisinin makes cells more resistant to doxorubicin by increasing the RhoA activity. So far, the widely used antimalarial drug artemisinin may impair the patients' response to a concomitant antitumoral chemotherapy. On the other hand, our study suggests that simvastatin, a drug commonly used in the treatment of hypercholesterolemia, and the RhoA silencing with siRNA, which has been safely applied *in vivo* (Pillè et al, 2005; Pillè et al, 2006), may improve the doxorubicin efficacy in colon cancer, allowing to reduce the overall dose of doxorubicin and to increase the ratio between therapeutic benefits and adverse effects.

Molecular Pharmacology Fast Forward. Published on May 7, 2008 as DOI: 10.1124/mol.108.045286
This article has not been copyedited and formatted. The final version may differ from this version.

REFERENCES

Ahn KS, Sethi G and Aggarwal BB (2007) Simvastatin potentiates TNF-α-induced apoptosis through the down-regulation of NF-kB-dependent antiapoptotic gene products: role of IkB-α kinase and TGF-β-activated kinase-1. *J Immunol*, 178:2507-2516.

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

Aldieri E, Orecchia S, Ghigo D, Bergandi L, Riganti C, Fubini B, Betta PG and Bosia A (2004) Simian virus 40 infection down-regulates the expression of nitric oxide synthase in human mesothelial cells. *Cancer Res* 64:4082-4084.

Bast A., Kaiserova H, den Hartog GJM, Haenen GRMM and van der Vijgh VJF (2007) Protectors against doxorubicin-induced cardiotoxicity: Flavonoids. *Cell Biol Toxicol* 23:39-47.

Batist G, Ramakriskan G, Rao CS, Chandrasekharan A, Gutheil J, Guthrie T, Shah P, Khojasteh A, Nair MK, Hoelzer K, Tkaczuk K, Park YC and Lee LW (2001) Reduced cardiotoxicity and preserved antitumour efficacy of liposome-encapsulated doxorubicin and cyclophosphamide compared with conventional doxorubicin and cyclophosphamide in a randomised multicentre trial of metastatic breast cancer. *J Clin Oncol* 19:1444-1454.

Beutler E. (1971) *In Red cell metabolism. A manual of biochemical methods.* Grune & Stratton, New York.

Bonadonna G and Monfardini S (1969) Cardiac toxicity of daunorubicin. *Lancet* 1:837.

Bryant, J, Picot J, Baxter L, Levitt G, Sullivan I and Clegg A (2007) Clinical and cost-effectiveness of cardioprotection against the toxic effects of anthracyclines given to children with cancer: a systematic review. *British J Cancer* 96:226-230.

Dirsch VM, Stuppner H, Ellmerer-Mueller EP and Vollmar AM (2000) Structural Requirements of Sesquiterpene Lactones to Inhibit LPS-Induced Nitric Oxide Synthesis in RAW 264.7 Macrophages. *Bioorg Med Chem* 8:2747-2753.

Dulak J and Józkowicz A (2005) Anti-Angiogenic and Anti-Inflammatory Effects of Statins: Relevance to Anti-Cancer Therapy. *Curr Cancer Drug Targets* 5:579-594.

Foster MW, McMahon TJ and Stamler JS (2003) S-nitrosylation in health and disease. *Trends Mol Med* 9:160-167.

Fritz G and Kaina B (2006) Rho GTPases: Promising Cellular Targets for Novel Anticancer Drugs. *Curr Cancer Drug Targets* 6:1-14.

Golenser J, Waknine JH, Krugliak M, Hunt NH and Grau GE (2006) Current perspectives on the mechanism of action of artemisinins. *Int J Parasitol* 36:1427-1441.

Gottesman MM, Fojo T and Bates SE (2002) Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer* 2:48-58.

Greten FR and Karin M (2004) The IKK/NF-kB activation pathway-a target for prevention and treatment of cancer. *Cancer Lett* 206:193-199.

Kim JH, Liu L, Lee SO, Kim YT, You KR and Kim DG (2005) Susceptibility of Cholangiocarcinoma Cells to Parthenolide-Induced Apoptosis. *Cancer Res* 65:6312-6320.

Kraynack NC, Corey DA, Elmer HL and Kelley TJ (2002) Mechanism of NOS2 regulation by RhoGTPase signaling in airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 283:L604-L611.

Kotamraju S, Williams C and Kalyanaraman B (2007) Statin-Induced Breast Cancer Cell Death: Role of Inducible Nitric Oxide and Arginase-Dependent Pathways. *Cancer Res* 67:7386-7394.

Laufs U and Liao JK (2000) Targeting Rho in cardiovascular disease. *Circ Res* 87:526-528.

Li W, Dong Y, Tu Y and Lin Z (2006) Dihydroarteannuin ameliorates lupus symptom of BXSB mice by inhibiting production of TNF-alpha and blocking the signalling pathway NF-kappa B translocation. *Int Immunopharmacol* 6:1243-1250.

Lin X, Li Q, Wang YL, Ju YW, Chi ZQ, Wang MW and Liu JG (2007) Morphine inhibits doxorubicin-induced reactive oxygen species generation and nuclear factor κB transcriptional activation in neuroblastoma SH-SY5Y cells. *Biochem J* 406:215-221.

Liu B, Li H, Qu H and Sun B (2006) Nitric Oxide Synthase Expressions in ADR-induced Cardiomyopathy in Rats. *J Biochem Mol Biol* 39:759-765.

Matsuda H, Kagerura T, Toguchida I, Ueda H, Morikawa T and Yoshikawa M (2000) Inhibitory effects of sesquiterpenes from bay leaf on nitric oxide production in lipopolysaccharide-activated macrophages:structure requirement and role of heat shock protein induction. *Life Sci* 66:2151-2157.

Mohanty S, Patel DK, Pati SS and Mishra SK (2006) Adjuvant therapy in cerebral malaria. *Indian J Med Res* 124:245-260.

Molnár J, Gyémánt N, Tanaka M, Hohmann J, Bergmann-Leitner E, Molnár P, Deli J, Didiziapetris R and Ferreira MJ (2006) Inhibition of Multidrug Resistance of Cancer Cells by Natural Diterpenes, Triterpenes and Carotenoids. *Curr Pharm Des* 12:287-331.

Nathan C and Xie Q-W (1994) Regulation of biosynthesis of nitric oxide. *J Biol Chem* 269:13725-13728.

Pillè JY, Denoyelle C, Varet J, Bertrand JR, Soria J, Opolon P, Lu H, Pritchard LL, Vannier J, Malvy C, Soria C and Li H (2005) Anti-RhoA and Anti-RhoC siRNAs Inhibit the Proliferation and Invasiveness of MDA-MB-231 Breast Cancer Cells in Vitro and in Vivo. *Mol Ther* 11:267-274.

Pillè JY, Li H, Blot E, Bertrand JR, Pritchard LL, Opolon P, Maksimenko A, Lu H, Vannier JP, Soria J, Malvy C and Soria C (2006) Intravenous delivery of anti-RhoA small interfering RNA loaded in nanoparticles of chitosan in mice: safety and efficacy in xenografted aggressive breast cancer. *Hum Gene Ther* 17:1019-1026.

Posner GH, McRiner, Paik IH, Sur S, Borstnik K, Xie S, Shapiro TA, Alagbala A and Foster B (2004) Anticancer and Antimalarial Efficacy and Safety of Artemisinin-Derived Trioxane Dimers in Rodents. *J Med Chem* 47:1299-1301.

Rattan RS, Giri AK and Singh I (2003) RhoA negatively regulates cytokine-mediated inducible nitric oxide synthase expression in brain-derived transformed cell lines: negative regulation of IKKα. *Free Radic Biol Med* 35:1037-1050.

Reungpatthanaphong P and Mankhetkhorn S (2002) Modulation of Multidrug Resistance by Artemisinin, Artesunate and Dihydroartemisinin in K562/adr and GLC4/adr Resistant Cell Lines. *Biol Pharm Bull* 25:1555-1561.

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

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

Schmidmaier R, Baumann P, Simsek M, Dayyani F, Emmerich B and Meinhardt G (2004) The HMG-CoA reductase inhibitor simvastatin overcomes cell adhesion mediated drug resistance (CAM-DR) in multiple myeloma by geranylgeranylation of Rho protein and activation of Rho kinase. *Blood* 104:1825-1832.

Wang S, Kotamraju S, Konorev E, Kalivendi S, Joseph J and Kalyanaraman B (2002) Activation of nuclear factor-kB during doxorubicin-induced apoptosis in endothelial cells and myocytes is pro-apoptotic : the role of hydrogen peroxide. *Biochem J* 367:729-740.

Wong HR and Menendez IY (1999) Sesquiterpene Lactones Inhibit Inducible Nitric Oxide Synthase Gene Expression in Cultured Rat Aortic Smooth Muscle Cells. *Biochem Biophys Res Commun* 262:375–380.

Yip KH, Zheng MH, Feng HT, Steer JH, Joyce DA and Xu J (2004) Sesquiterpene Lactone Parthenolide Blocks Lipopolysaccharide-Induced Osteolysis Through the Suppression of NF-kB Activity. *J Bone Miner Res* 19: 1905-1916.

Yu HG, Ai YW, Yu LL, Zhou XD, Liu J, Li JH, Xu XM, Liu S, Chen J, Liu F, Qi YL, Deng Q, Cao J, Liu SQ, Luo HS and Yu JP (2008) Phosphoinositide 3-kinase/Akt pathway plays an important role in chemoresistance of gastric cancer cells against etoposide and doxorubicin induced cell death. *Int J Cancer* 122:433-443.

Molecular Pharmacology Fast Forward. Published on May 7, 2008 as DOI: 10.1124/mol.108.045286
This article has not been copyedited and formatted. The final version may differ from this version.

FOOTNOTES

Financial support: This work has been supported with grants from Fondazione Internazionale Ricerche Medicina Sperimentale (FIRMS), Compagnia di San Paolo, Regione Piemonte (Ricerca Sanitaria Finalizzata CIPE A201 2004/2005 e 2006) and Ministero dell'Università e della Ricerca.

Sophie Doublier is recipient of a Research Fellowship funded by the Fondazione Internazionale Ricerche Medicina Sperimentale (FIRMS), Torino, Italy.

The first two authors contributed equally to this work.

Requests for reprints: Chiara Riganti, Dipartimento di Genetica, Biologia e Biochimica (Sezione di Biochimica), Via Santena, 5/bis, 10126 Turin, Italy. Phone: 39-11-670-5851; Fax: 39-11-670-5845; E-mail: chiara.riganti@unito.it

Molecular Pharmacology Fast Forward. Published on May 7, 2008 as DOI: 10.1124/mol.108.045286
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE LEGENDS

Figure 1. Effects of simvastatin, doxorubicin, parthenolide and artemisinin on the NF-kB signalling pathway in HT29 cells. **A.** Cells were incubated for different time periods (*sim t* = 0, 1, 3, 6, 24 h) and with different concentrations (*sim c* = 1, 10, 50 μM) of simvastatin (*SIM*), then NF-kB translocation was detected on nuclear extracts as described under Materials and Methods. TNF-α (10 ng/ml for 15 min, *TNF*) was used as a positive control of NF-kB translocation. 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 point marked *COLD OLIGO SIM* a competition assay was performed in cells incubated with simvastatin (10 μM for 6 h) together with an excess of cold oligonucleotide, to asses the specificity of NF-kB binding.

B. Cells were incubated for 6 h in the absence (*CTRL*) or presence of parthenolide (10 μM, *PART*), artemisinin (10 μM, *ART*), simvastatin (10 μM, *SIM*), doxorubicin (5 μM, *DOX*), alone or in different combinations, or with TNF-α (10 ng/ml for 15 min, *TNF*). EMSA detection of NF-kB was performed on nuclear extracts (see 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 point marked with "*COLD OLIGO SIM"* a competition assay was performed in cells incubated with simvastatin (10 μM for 6 h) together with an excess of cold oligonucleotide. **C.** Cells were incubated in the experimental conditions indicated above and Western blotting detection of p65 and p50 was performed on nuclear cellular extracts (see Materials and Methods for details). The figure is representative of three experiments with similar results.

Figure 2. Effects of simvastatin, doxorubicin, parthenolide and artemisinin on IkBα protein, IKKα/β phosphorylation and IKK activity. HT29 cells were incubated for 6 h in the absence

Molecular Pharmacology Fast Forward. Published on May 7, 2008 as DOI: 10.1124/mol.108.045286
This article has not been copyedited and formatted. The final version may differ from this version.

(*CTRL*) or presence of parthenolide (10 μM, *PART*), artemisinin (10 μM, *ART*), simvastatin (10 μM, *SIM*), doxorubicin (5 μM, *DOX*), alone or in different combinations. TNF-α (10 ng/ml for 15 min, *TNF*) was used as a positive control of NF-kB translocation. Subsequently, the following investigations were performed.

A. Western blot detection of IkBα, phospho-IkBα (pIkBα), IKKα/β and phospho-IKKα/β (pIKK) proteins. Whole cell lysates were analysed by western blotting with anti-IkBα, anti-phospho(Ser32)-IkBα, anti-IKKα/β, anti-phospho(Ser176-180)-IKKα/β antibodies. An anti-GAPDH antibody was used as a control of equal protein loading. The figure is representative of three experiments with similar results. **B.** IKK activity in the cell lysate. The figure is representative of three experiments with similar results.

Figure 3. Effects of simvastatin, doxorubicin, parthenolide and artemisinin on NOS activity, doxorubicin accumulation, toxicity, viability and apoptosis. HT29 cells were incubated for 6 h in the absence (*CTRL*) or presence (+ *doxo*) of 5 μM doxorubicin, alone (*CTRL + doxo*) or together with parthenolide (10 μM, *PART + doxo*), artemisinin (10 μM, *ART + doxo*), simvastatin (10 μM, *SIM + doxo*), in different combinations. Subsequently cells were subjected to the following investigations.

A. NOS activity (*open bars*) and doxorubicin accumulation (*hatched bars)* were measured as described (see Materials and Methods). The measurements were performed in duplicate and data are presented as means $\pm$ SE (n = 3). The significance of CTRL versus each experimental condition marked with "+ *doxo*" was $p < 0.05$ for NOS activity and $p < 0.001$ for intracellular doxorubicin content. Vs CTRL + *doxo*: * $p < 0.05$; ** $p < 0.001$. Vs SIM: $^{\circ}$ $p < 0.05$; $^{\circ\circ}$ $p < 0.001$.

B. LDH release (*open bars*) and trypan blue staining (*hatched bars)* were performed as reported under the Materials and Methods section. The measurements were performed in duplicate and

Molecular Pharmacology Fast Forward. Published on May 7, 2008 as DOI: 10.1124/mol.108.045286
This article has not been copyedited and formatted. The final version may differ from this version.

data are presented as means ± SE (n = 3).The significance of CTRL vs each experimental condition marked with "+ *doxo*" was $p < 0.005$ for both LDH release and trypan blue staining. Vs CTRL + *doxo*: * $p < 0.05$. Vs SIM: $^{\circ}$ $p < 0.05$.

C. The staining of cells with annexin V-FITC (*open bars*), PI (*hatched bars)* or both (*solid bars*) was detected by FACS analysis, as described in the Materials and Methods section. The measurements were performed in duplicate and data are presented as means ± SE (n = 3). The significance of CTRL versus each experimental condition marked with "+ *doxo*" was always $p < 0.002$. Vs CTRL + *doxo*: * $p < 0.05$; ** $p < 0.001$. Vs SIM: $^{\circ}$ $p < 0.05$; $^{\circ\circ}$ $p < 0.001$.

Figure 4. Effects of simvastatin, doxorubicin, parthenolide and artemisinin on MRP3 nitration**.** HT29 cells were incubated for 6 h in the absence (*CTRL*) or presence of parthenolide (10 µM, *PART*), artemisinin (10 µM, *ART*), simvastatin (10 µM, *SIM*), doxorubicin (5 µM, *DOX*), alone or in different combinations, and with the NO donor S-nitroso-acetylpenicillamine (100 µM, *SN*). Cells were lysed and the whole cellular lysate was immunoprecipitated with an anti-nitrotyrosine polyclonal antibody or with an anti-MRP3 antibody. The immunoprecipitated proteins were subjected to Western blotting, using an anti-MRP3 antibody. The figure is representative of three experiments with similar results.

Figure 5. Effects of simvastatin, doxorubicin, parthenolide and artemisinin on RhoA and RhoA kinase activities. HT29 cells were incubated for 6 h in the absence (*CTRL*) or presence of parthenolide (10 µM, *PART*), artemisinin (10 µM, *ART*), simvastatin (10 µM, *SIM*), doxorubicin (5 µM, *DOX*), alone or in different combinations. In some experimental points mevalonic acid (100 µM, *MA*) was added. Subsequently, the cells were lysed and subjected to ELISA assay: the amount of RhoA-GTP (*open bars*) and the Rho kinase activity (*hatched bars*) were measured in

Molecular Pharmacology Fast Forward. Published on May 7, 2008 as DOI: 10.1124/mol.108.045286
This article has not been copyedited and formatted. The final version may differ from this version.

duplicate, as described under Materials and Methods. Data are presented as means ± SE (n = 3). Vs CTRL: * $p < 0.05$; ** $p < 0.001$. Vs SIM: ° $p < 0.05$; °° $p < 0.001$.

Figure 6. Effects of simvastatin, parthenolide and artemisinin on the NF-kB nuclear translocation in RhoA-silenced HT29 cells. Control and RhoA-silenced (*siRNA*) HT29 cells were incubated for 6 h in the absence (*CTRL*) or presence of simvastatin (10 μM, *SIM*), alone or in combination with parthenolide (10 μM, *PART*) or artemisinin (10 μM, *ART*). The following investigations were performed.

A. Western blotting experiments were performed on whole cells lysates using an anti-RhoA and an anti-GAPDH antibody, as described under the Materials and Methods section. The figure is representative of three experiments with similar results. **B.** EMSA detection of NF-kB was performed on nuclear extracts as indicated in the Materials and Methods section. TNF-α (10 ng/ml for 15 min, *TNF*) was used as a positive control of NF-kB translocation. In each experiment one lane was loaded with bidistilled water (-) in place of cellular extracts. In the experimental point marked with "*COLD OLIGO SIM"* a competition assay was performed in cells incubated with simvastatin (10 μM for 6 h) together with an excess of cold oligonucleotide. **C.** Nuclear extracts were checked for p65 and p50 expression by Western blotting (see Materials and Methods). TNF-α (10 ng/ml for 15 min, *TNF*) was used as a positive control of NF-kB translocation. The figure is representative of three experiments with similar results.

Figure 7. Effects of simvastatin, parthenolide and artemisinin on IkBα/IKK pathway, NOS activity and doxorubicin accumulation in RhoA-silenced HT29 cells. Control and RhoA-silenced (*siRNA*) HT29 cells were incubated for 6 h in the absence (*CTRL*) or presence of simvastatin (10 μM, *SIM*), alone or in combination with parthenolide (10 μM, *PART*) or artemisinin (10 μM,

Molecular Pharmacology Fast Forward. Published on May 7, 2008 as DOI: 10.1124/mol.108.045286
This article has not been copyedited and formatted. The final version may differ from this version.

ART). TNF-α (10 ng/ml for 15 min, *TNF*) was used as a positive control of NF-kB translocation. Cells were lysed and subjected to the following investigations.

A. The Western blotting detection of IkBα, phospho-IkBα (pIkBα), IKKα/β and phospho-IKKα/β (pIKK) was performed on whole cell lysates using anti-IkBα, anti-phospho(Ser32)-IkBα, anti-IKKα/β, anti-phospho(Ser176-180)-IKKα/β. The anti-GAPDH antibody was used as a control of equal protein loading. The figure is representative of three experiments with similar results.

B. Measurement of IKK activity. The figure is representative of three experiments with similar results.

C. NOS activity in cell lysates (*open bars*) and doxorubicin accumulation (*hatched bars*) were measured as described (see Materials and Methods). 5 μM doxorubicin was present in each experimental condition throughout the incubation time (*CTRL* = doxorubicin alone). In the absence of doxorubicin the NOS activity was 1.16 $\pm$ 0.05 nnmol nitrite/min/mg cell proteins (vs CTRL: $p < 0.05$); the intracellular doxorubicin content was 0.070 $\pm$ 0.002 ng/mg cell proteins (vs CTRL: $p < 0.001$). The measurements were performed in duplicate and data are presented as means $\pm$ SE (n = 3). Vs CTRL: * $p < 0.05$; ** $p < 0.005$. Vs SIM: $^{\circ}$ $p < 0.001$.

Figure 8. Effects of simvastatin, parthenolide and artemisinin on cytotoxicity, viability and apoptosis in RhoA-silenced HT29 cells. Control and RhoA-silenced (*siRNA*) HT29 cells were incubated for 6 h in the absence (*CTRL*) or presence of simvastatin (10 μM, *SIM*), alone or in combination with parthenolide (10 μM, *PART*) or artemisinin (10 μM, *ART*). 5 μM doxorubicin was present in each experimental condition throughout the incubation time (*CTRL* = doxorubicin alone), then cells were subjected to the following investigations.

A. LDH release (*open bars*) and trypan blue staining (*hatched bars)* were performed as reported under Materials and Methods. The measurements were performed in duplicate and data are

Molecular Pharmacology Fast Forward. Published on May 7, 2008 as DOI: 10.1124/mol.108.045286
This article has not been copyedited and formatted. The final version may differ from this version.

presented as means ± SE (n = 3). In the absence of doxorubicin the LDH release was 2.11 ± 0.23 % (vs CTRL: $p < 0.005$); the percentage of trypan blue-positive cells was 3.00 ± 0.58 % (vs CTRL: $p < 0.001$). Vs CTRL: $^{*}p < 0.05$; $^{**}p < 0.005$. Vs SIM: $^{\circ}p < 0.001$.

B. The staining of cells with annexin V-FITC (*open bars*), PI (*hatched bars)* or both (*solid bars*) was detected by FACS analysis, as described in the Materials and Methods section. The measurements were performed in duplicate and data are presented as means ± SE (n = 3). In the absence of doxorubicin the percentage of annexin V-FITC-positive cells was 1.42 ± 0.09 % (vs CTRL: $p < 0.001$); the percentage of PI-positive cells was 2.04 ± 0.03 % (vs CTRL: $p < 0.002$); the percentage of annexin V-FITC- and PI-positive cells was 1.98 ± 0.18 % (vs CTRL: $p < 0.001$). Vs CTRL: $^{*}p < 0.05$; $^{**}p < 0.005$. Vs SIM: $^{\circ}p < 0.05$.

Figure 1

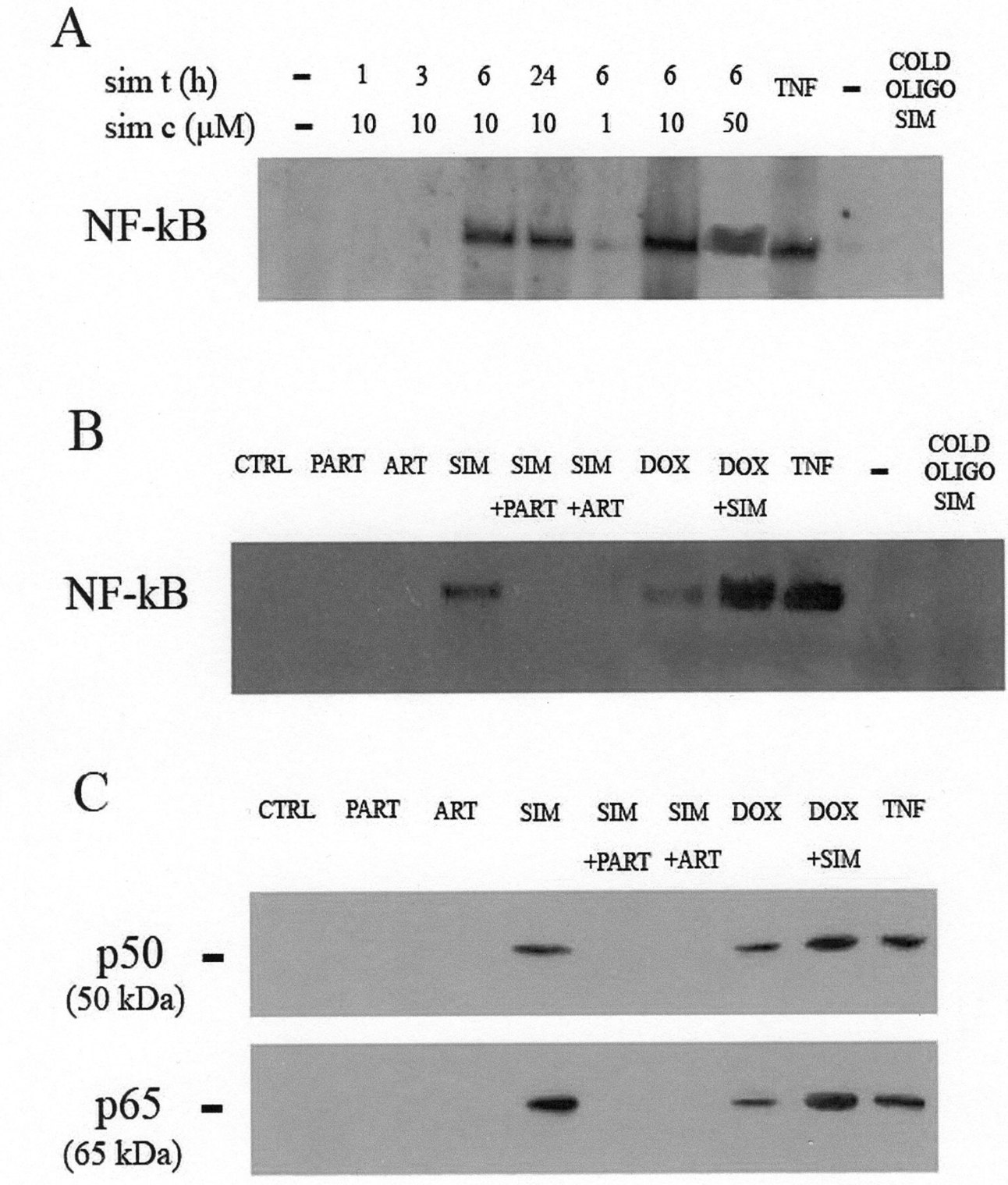

A
sim t (h) - 1 3 6 24 6 6 6 TNF - COLD OLIGO SIM
sim c (μM) - 10 10 10 10 1 10 50
NF-kB
B
CTRL PART ART SIM SIM +PART SIM +ART DOX DOX +SIM TNF - COLD OLIGO SIM
NF-kB
C
CTRL PART ART SIM SIM +PART SIM +ART DOX DOX +SIM TNF
p50 -
(50 kDa)
p65 -
(65 kDa)

Figure 2

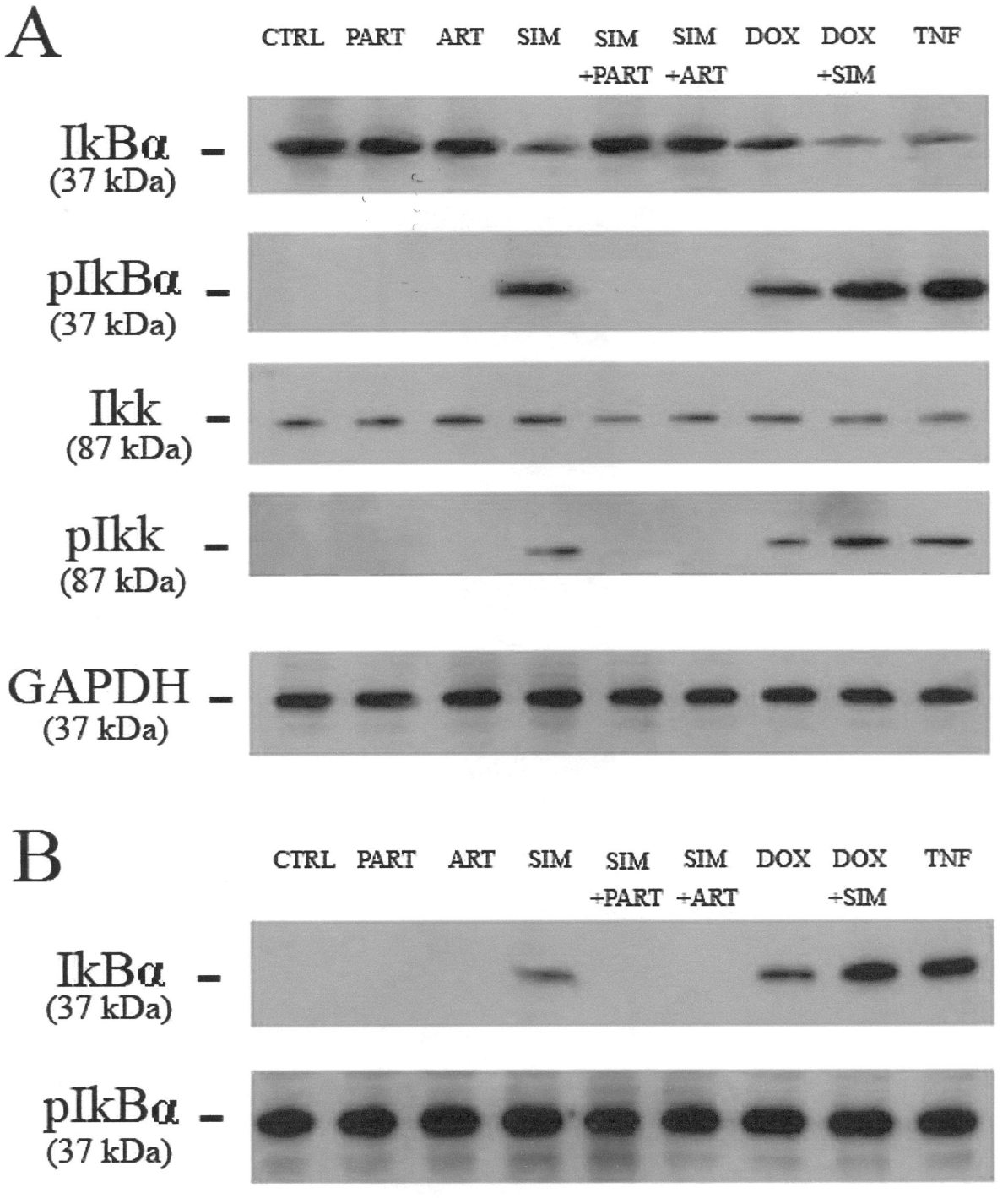
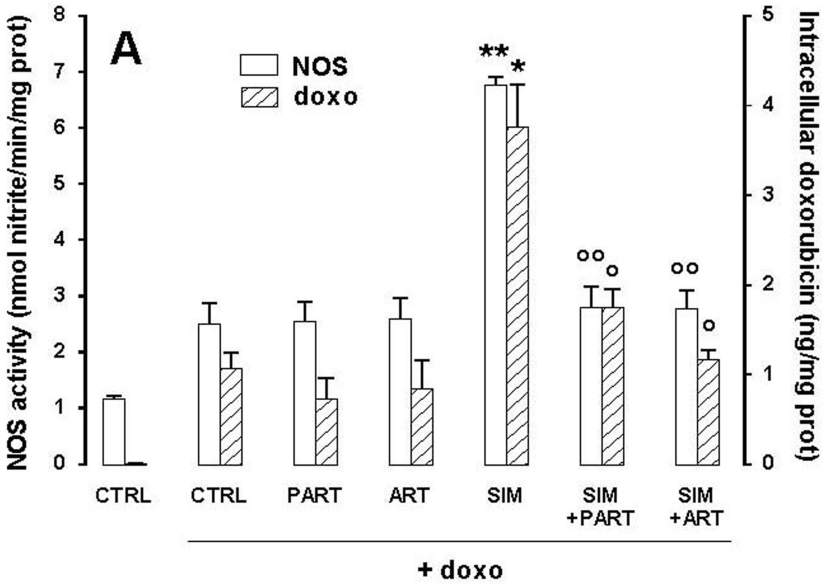
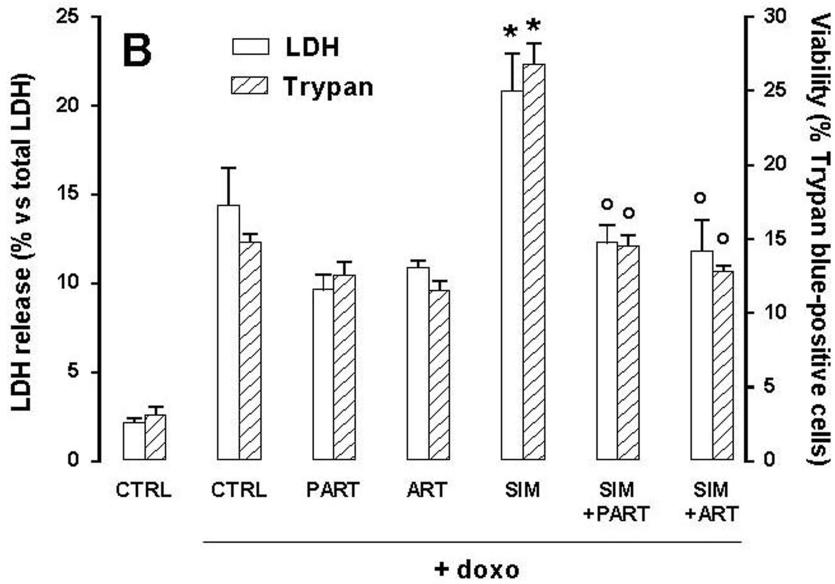

A
CTRL
PART
ART
SIM
SIM +PART
SIM +ART
DOX
DOX +SIM
TNF
IkBα (37 kDa)
pIkBα (37 kDa)
Ikk (87 kDa)
pIkk (87 kDa)
GAPDH (37 kDa)
B
CTRL
PART
ART
SIM
SIM +PART
SIM +ART
DOX
DOX +SIM
TNF
IkBα (37 kDa)
pIkBα (37 kDa)

Figure 3


A
NOS
doxo
NOS activity (nmol nitrite/min/mg prot)
Intracellular doxorubicin (ng/mg prot)
CTRL
CTRL
PART
ART
SIM
SIM +PART
SIM +ART
+ doxo


B
LDH
Trypan
LDH release (% vs total LDH)
Viability (% Trypan blue-positive cells)
CTRL
CTRL
PART
ART
SIM
SIM +PART
SIM +ART
+ doxo

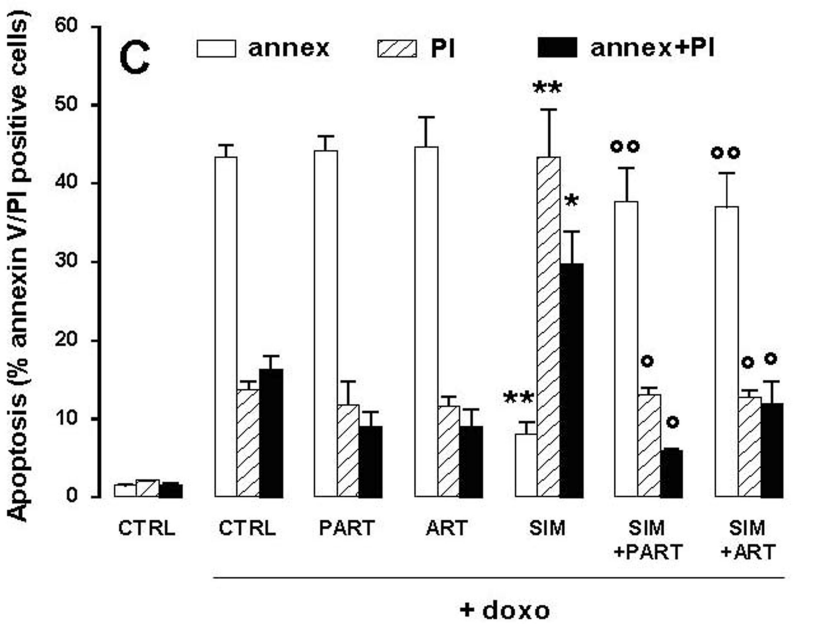

C
annex
PI
annex+PI
Apoptosis (% annexin V/PI positive cells)
CTRL
CTRL
PART
ART
SIM
SIM +PART
SIM +ART
+ doxo

Figure 4

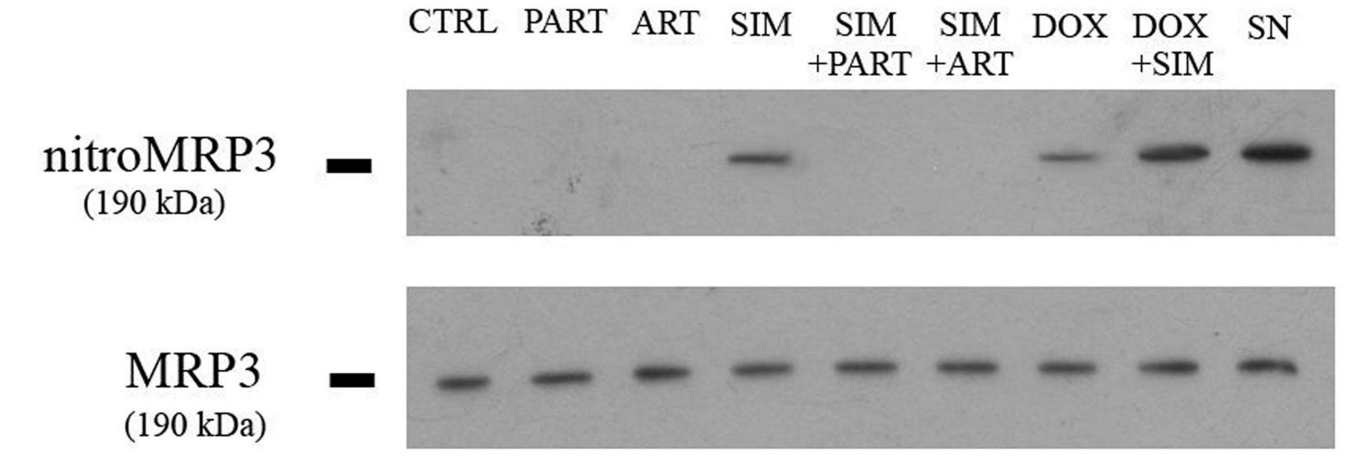

CTRL
PART
ART
SIM
SIM +PART
SIM +ART
DOX
DOX +SIM
SN
nitroMRP3
(190 kDa)
MRP3
(190 kDa)

Figure 5

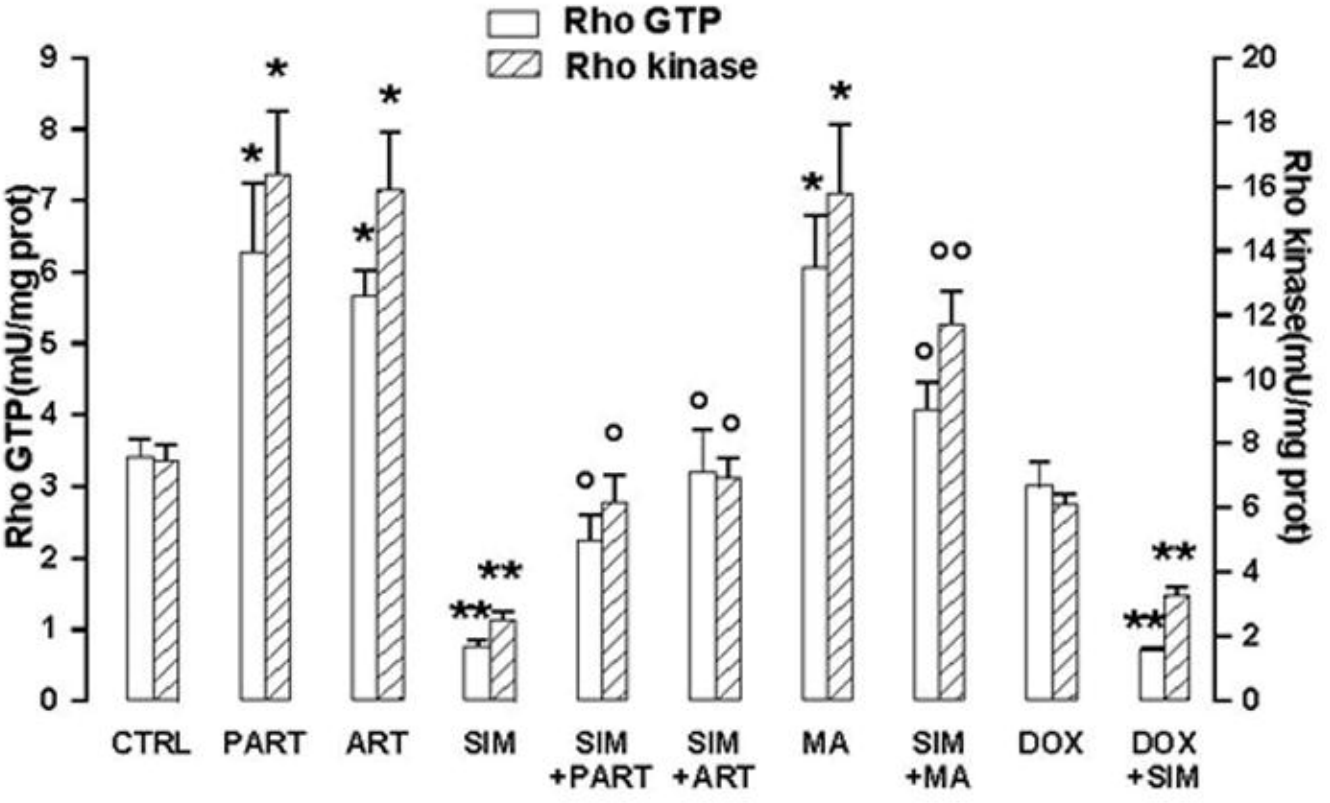

Rho GTP
Rho kinase
Rho GTP(mU/mg prot)
Rho kinase(mU/mg prot)
0
1
2
3
4
5
6
7
8
9
0
2
4
6
8
10
12
14
16
18
20
CTRL
PART
ART
SIM
SIM +PART
SIM +ART
MA
SIM +MA
DOX
DOX +SIM

Figure 6

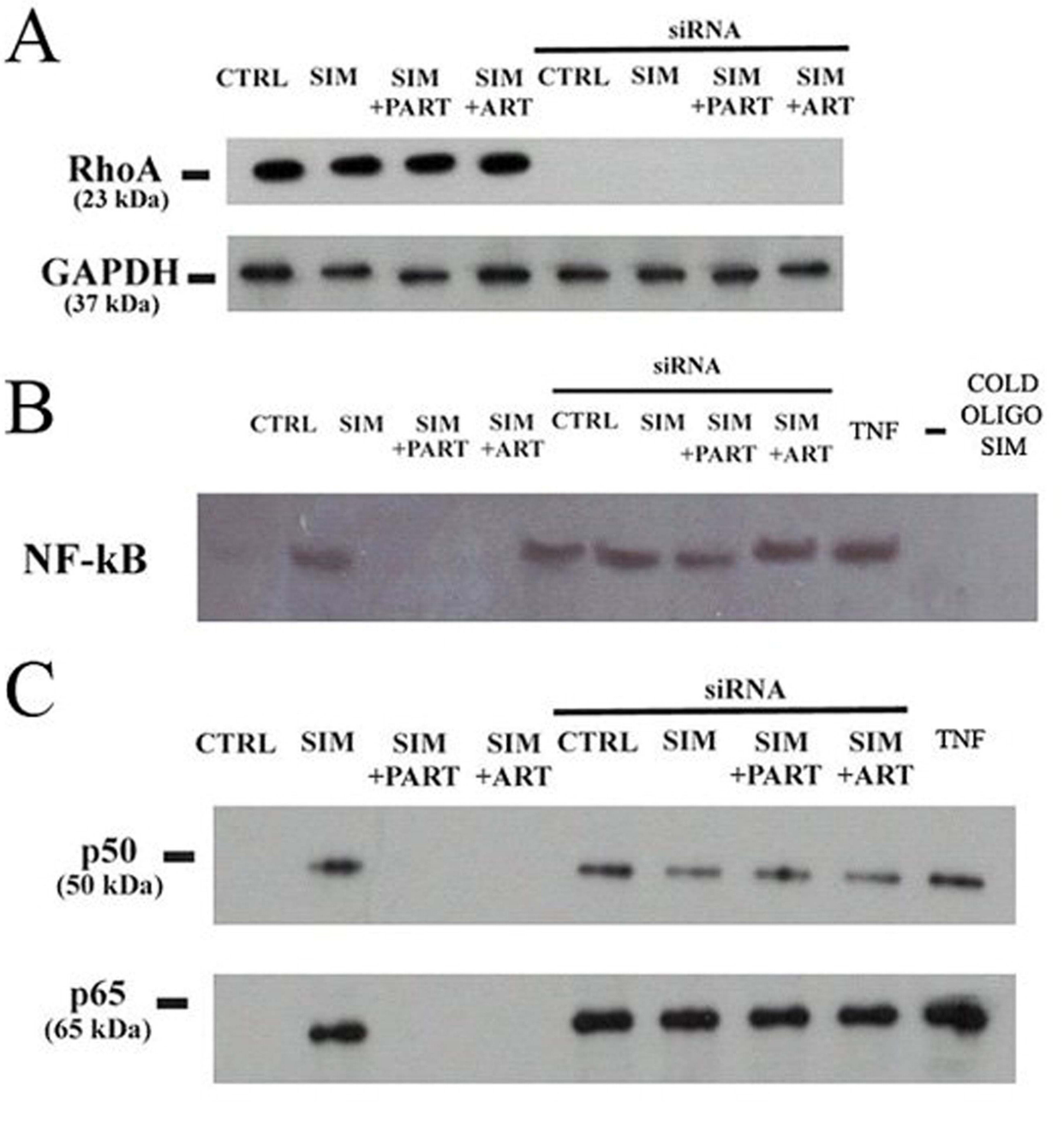

A
siRNA
CTRL
SIM
SIM +PART
SIM +ART
CTRL
SIM
SIM +PART
SIM +ART
RhoA
(23 kDa)
GAPDH
(37 kDa)
B
siRNA
CTRL
SIM
SIM +PART
SIM +ART
CTRL
SIM
SIM +PART
SIM +ART
TNF
COLD OLIGO SIM
NF-kB
C
siRNA
CTRL
SIM
SIM +PART
SIM +ART
CTRL
SIM
SIM +PART
SIM +ART
TNF
p50
(50 kDa)
p65
(65 kDa)

Figure 7

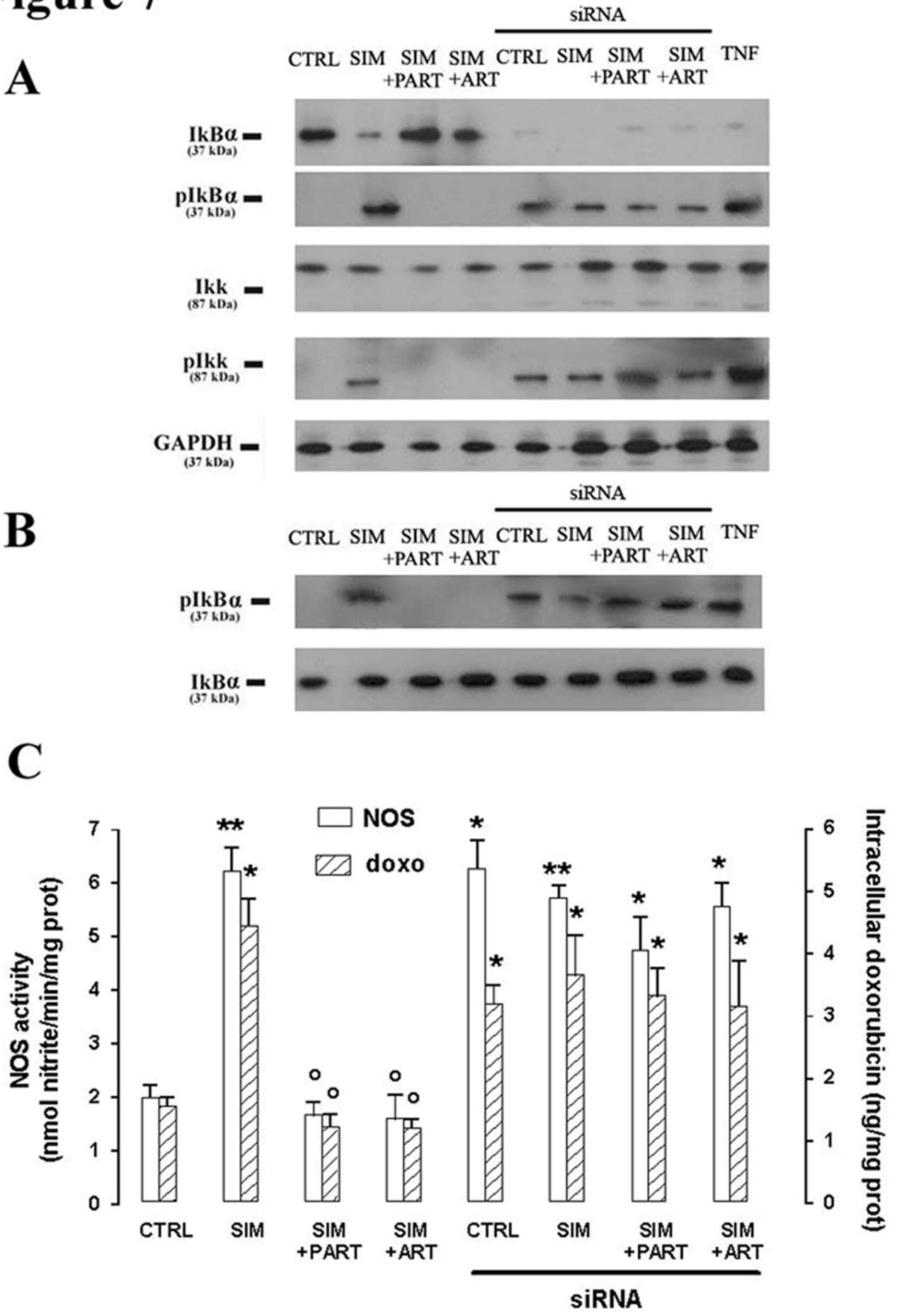
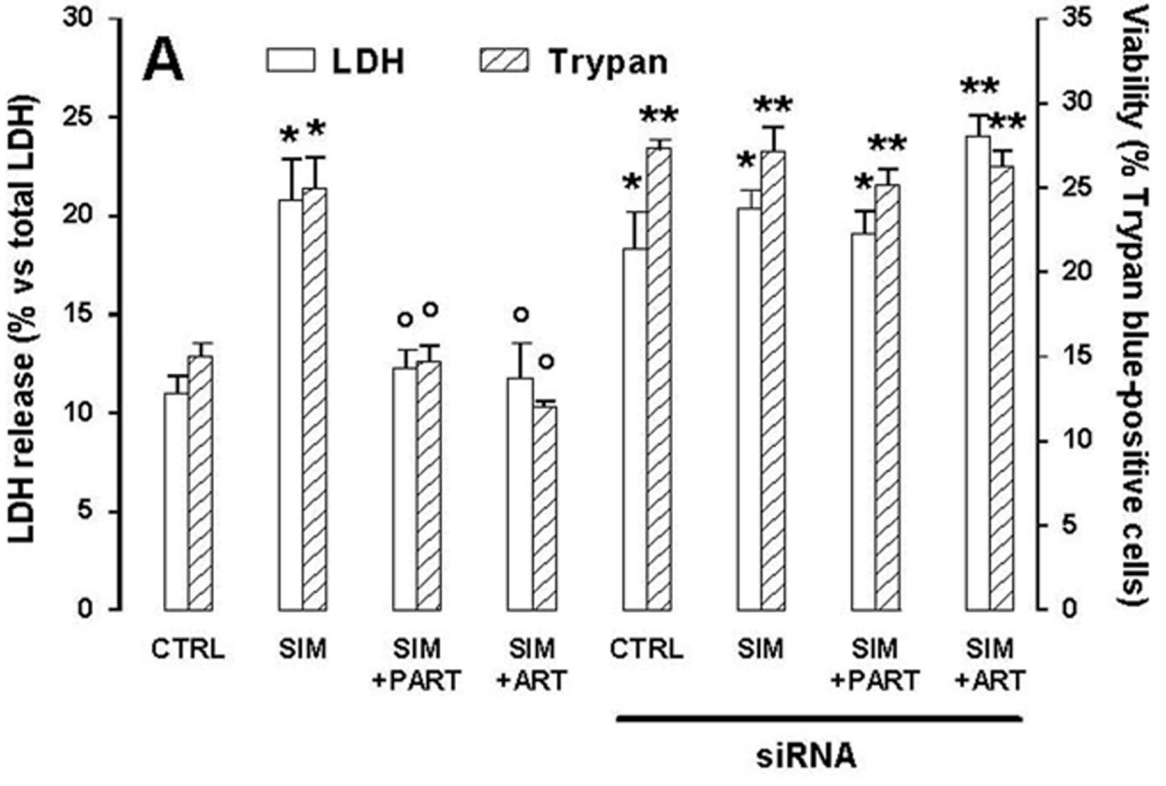
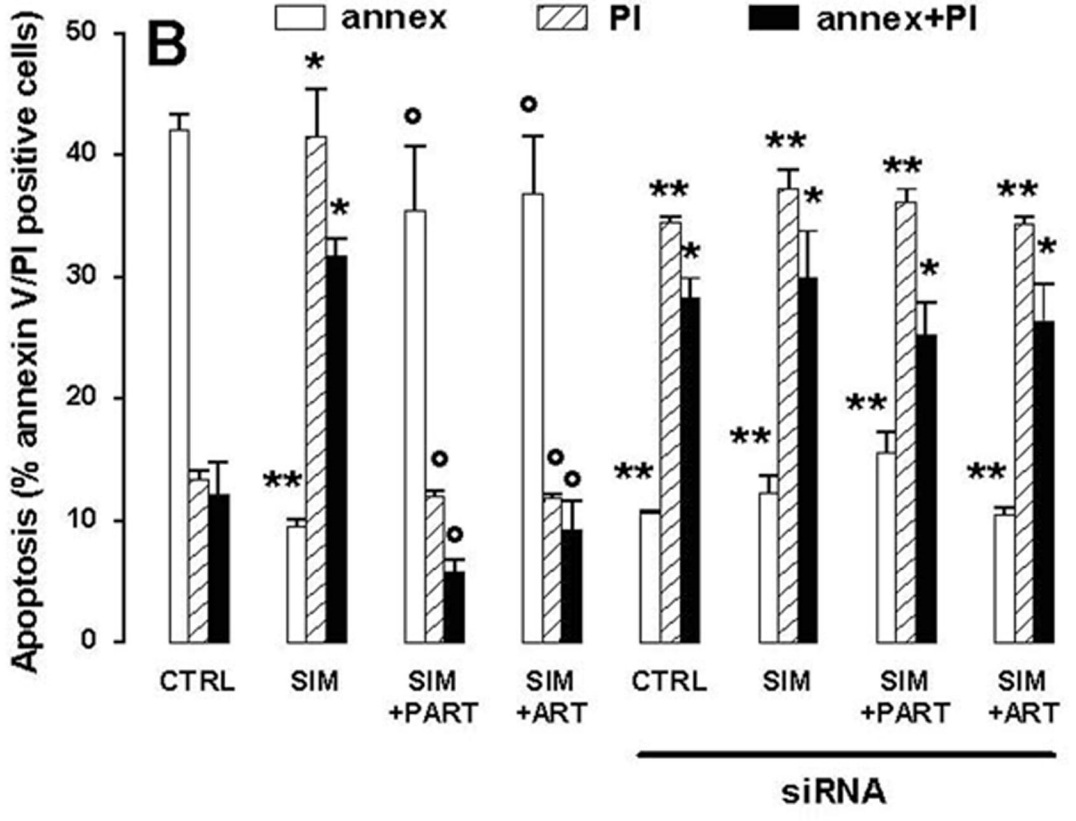

A
siRNA
CTRL SIM SIM +PART SIM +ART CTRL SIM SIM +PART SIM +ART TNF
IkBα (37 kDa)
pIkBα (37 kDa)
Ikk (87 kDa)
pIkk (87 kDa)
GAPDH (37 kDa)
B
siRNA
CTRL SIM SIM +PART SIM +ART CTRL SIM SIM +PART SIM +ART TNF
pIkBα (37 kDa)
IkBα (37 kDa)
C
NOS
doxo
NOS activity (nmol nitrite/min/mg prot)
Intracellular doxorubicin (ng/mg prot)
0 1 2 3 4 5 6 7
CTRL SIM SIM +PART SIM +ART CTRL SIM SIM +PART SIM +ART
siRNA

Figure 8


A
LDH
Trypan
LDH release (% vs total LDH)
Viability (% Trypan blue-positive cells)
0
5
10
15
20
25
30
35
CTRL
SIM
SIM +PART
SIM +ART
CTRL
SIM
SIM +PART
SIM +ART
siRNA


B
annex
PI
annex+PI
Apoptosis (% annexin V/PI positive cells)
0
10
20
30
40
50
CTRL
SIM
SIM +PART
SIM +ART
CTRL
SIM
SIM +PART
SIM +ART
siRNA