

Activation of Nuclear Factor- κ B Pathway by Simvastatin and RhoA Silencing Increases Doxorubicin Cytotoxicity in Human Colon Cancer HT29 Cells^[S]

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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- κ B (NF- κ B) pathway and of nitric-oxide (NO) synthase increases the doxorubicin efficacy in human colon cancer HT29 cells. To induce NF- κ B, we took into account the effect of doxorubicin itself and of the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor simvastatin; as NF- κ B inhibitors, we chose the sesquiterpene lactones parthenolide and artemisinin. Simvastatin increased the NF- κ B 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- κ B pathway and the inducible NO syn-

these 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 of the statin effects by inducing RhoA/Rho kinase activation. On the other hand, they did not reduce the NF- κ B translocation and doxorubicin intracellular content when RhoA was silenced by small interfering RNA (siRNA). It is interesting that RhoA siRNA was sufficient to increase NF- κ B 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.

Anthracyclines, such as doxorubicin, epirubicin, and their derivatives, are drugs widely used in the treatment of solid and hematological cancers. However, their efficacy is limited because of their dose-dependent cardiotoxicity, an important adverse effect which impairs patient 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 shown previously that statins revert doxorubicin resistance in different cancer cell lines (Riganti et al., 2005, 2006). Statins are competitive inhibitors of HMG-CoA reductase, which catalyzes 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-protein families Ras and Rho (Dulak and Jozkowicz, 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- κ B (NF- κ B) (Fritz and Kaina, 2006).

NF- κ B is composed of protein dimers, such as the het-

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ABBREVIATIONS: NF- κ B, nuclear factor- κ B; I κ B, inhibitor of κ B; IKK, I κ B kinase; NOS, nitric-oxide synthase; PgP, P-glycoprotein; MRP3, multidrug resistance-related protein 3; siRNA, small interfering RNA; iNOS, inducible nitric-oxide synthase; eNOS, endothelial nitric-oxide synthase; FBS, fetal bovine serum; EMSA, electrophoretic mobility shift assay; PBS, phosphate-buffered saline; BSA, bovine serum albumin; LDH, lactate dehydrogenase; PI, propidium iodide; FITC, fluorescein isothiocyanate; TNF- α , tumor necrosis factor- α ; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FACS, fluorescence-activated cell sorting; MG132, *N*-benzoyloxycarbonyl (Z)-Leu-Leu-leucinal.

erodimer 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 I κ B family proteins bind directly to NF- κ B dimer in the cytoplasm, preventing its nuclear localization. NF- κ B is free to translocate and bind to DNA on the target genes when I κ B α is phosphorylated by the I κ B 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- κ B pathway (Kraynack et al., 2002; Rattan et al., 2003). In addition, doxorubicin can induce NF- κ B translocation in cancer cells via different mechanisms (Lin et al., 2007; Yu et al., 2008). By activating NF- κ B, statins and doxorubicin may enhance the transcription of the inducible nitric-oxide (NO) synthase (iNOS) (Greten and Karin, 2004), one of the three NOS isoforms that catalyze the conversion of L-arginine to L-citrulline and NO with a 1:1 stoichiometry (Nathan and Xie, 1994). NO is a signaling molecule involved in the control of cellular growth, differentiation, and apoptosis (Greten and Karin, 2004). We have suggested previously 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 transporters that recognize doxorubicin as a substrate. Such a nitration reduces the drug efflux in doxorubicin-resistant cells (Riganti et al., 2005, 2006).

The aim of this work was to investigate whether statins and RhoA silencing may improve the cytotoxic effect of doxorubicin via the enhanced activation of NF- κ B/iNOS pathway on human colon cancer HT29 cells. To reduce the NF- κ B activity, we used parthenolide and artemisinin, two natural sesquiterpene lactones. The former is a well known NF- κ B inhibitor with anti-inflammatory and antiseptic 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- κ B translocation (Aldieri et al., 2003; Li et al., 2006).

Materials and Methods

Materials. Fetal bovine serum (FBS) and RPMI 1640 medium were supplied by Lonza Verviers SPRL (Verviers, Belgium); Falcon plastic ware was used for cell culture (BD Biosciences Discovery Labware, Bedford, MA). Simvastatin was purchased from Calbiochem (La Jolla, CA). Electrophoresis reagents were obtained from Bio-Rad Laboratories (Hercules, CA); the protein content of cell monolayers and cell lysates was assessed with the BCA kit from Sigma Chemical (St. Louis, MO). When not otherwise specified, all other reagents were purchased from Sigma.

Cells. Human colon cancer cells (HT29 cell line) were cultured in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% L-glutamine and maintained in a humidified atmosphere at 37°C and 5% CO₂.

Electrophoretic Mobility Shift Assay. Cells were plated in 60-mm diameter dishes at confluence, and 10 μ g of nuclear proteins extracts was used to detect NF- κ B translocation as described previously (Riganti et al., 2006).

Western Blot Analysis. Western blot detection of I κ B α , phospho(Ser32)-I κ B α , IKK α / β , phospho(Ser176-180)-IKK α / β , glyceraldehyde 3-phosphate dehydrogenase, MRP3 and nitrated MRP3, neuronal NOS (nNOS), iNOS, or endothelial NOS (eNOS) antibodies

was performed as reported previously (Riganti et al., 2005, 2006). In addition, 30 μ g of nuclear proteins obtained as described previously (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 described previously (Riganti et al., 2006).

Measurement of NOS Activity. Cells grown at confluence on 35-mm diameter Petri dishes were detached by trypsin/EDTA, resuspended in 0.3 ml of HEPES/EDTA/dithiothreitol buffer (20 mM HEPES, 0.5 mM EDTA, and 1 mM dithiothreitol, pH 7.2), and sonicated. NOS activity was measured in 100 μ g of cell lysates with the Ultrasensitive Colorimetric Assay for Nitric Oxide Synthase kit (Oxford Biomedical Research, Oxford, MI). Results were expressed as nanomoles of nitrite per minute per milligram of cell protein.

Doxorubicin Accumulation. Intracellular doxorubicin accumulation was measured as described elsewhere (Riganti et al., 2005) using a spectrofluorimeter (LS-5; PerkinElmer Life and Analytical Sciences, Waltham, MA). Excitation and emission wavelengths were 475 and 553 nm, respectively. Fluorescence was converted in nanograms of doxorubicin per milligram of cell proteins using a calibration curve prepared previously.

Extracellular Lactate Dehydrogenase Activity. After a 6-h incubation under different experimental conditions in the presence of 5 μ M doxorubicin, lactate dehydrogenase (LDH) activity was measured in the extracellular medium and in the cell lysate as described previously (Beutler, 1971; Riganti et al., 2006) to check the cytotoxicity of doxorubicin. Absorbance at 340 nm was measured for 10 min with a Lambda 3 spectrophotometer (PerkinElmer Life and Analytical Sciences). Both intracellular and extracellular enzyme activity was expressed as micromoles of NADH oxidized per minute per dish, and then extracellular LDH activity was calculated as a percentage of the total LDH activity in the dish.

Trypan Blue Staining. After a 6-h incubation under the experimental conditions reported under *Results*, cell monolayers were washed, detached with trypsin/EDTA, and resuspended in 1 ml of PBS. Ten microliters of 20% (w/v) trypan blue were added to each sample. After a 1-min incubation at room temperature, 10 μ l of each cellular suspension was analyzed in a Burkholder chamber under a light microscope, and the Trypan blue-positive cells were counted as the percentage of dead cells from a total of 200 cells.

Annexin V/Propidium Iodide Assay. Cells were incubated for 6 h in the experimental conditions described under *Results*, and then they were washed twice with fresh PBS, detached with the Cell Dissociation Solution (Sigma), and incubated for 10 min at room temperature in 1 ml of binding buffer (100 mM HEPES, 140 mM NaCl, and 25 mM CaCl₂, pH 7.5) containing 10 μ M annexin V-fluorescein isothiocyanate conjugate (FITC) and 2.5 μ M propidium iodide (PI). The cell 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 (BD Biosciences). For each analysis, 10,000 events were collected; the green fluorescence (for Annexin V-FITC) was detected using a 530 nm bandpass filter, whereas the red fluorescence (for PI) was detected with a 640 nm long-pass filter. The percentage of cells positive for annexin V-FITC, PI, or both was calculated by the Cell Quest software (BD Biosciences).

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 of lysis buffer of the kit, and centrifuged at 13,000g for 5 min. Supernatants (100 μ l) were used for the 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 milliunits of absorbance per milligram of 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 reported previously (Riganti et al., 2006).

RhoA siRNA Transfection. Cells (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 of siRNA Transfection Medium (Santa Cruz Biotechnology) and incubated for 6 h with 1 ml of siRNA transfection medium containing 5 μ l of siRNA Transfection Reagent (Santa Cruz Biotechnology) and 50 pmol of RhoA siRNA (Santa Cruz Biotechnology). One milliliter of RPMI 1640 medium containing 1% penicillin/streptomycin and 20% FBS was added for 24 h. Thereafter, 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 analyzed 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 a housekeeping gene in both transfected and untransfected cells. The specificity of the silencing technique was further verified by incubating in each set of experiments an aliquot of HT29 cells with 50 pmol of control siRNA-A (Santa Cruz Biotechnology), a nontargeting 20- to 25-nucleotide siRNA designed as a negative control, instead of RhoA siRNA; neither the RhoA nor GAPDH expression, analyzed by Western blotting, was modified (data not shown).

Statistical Analysis. All data in text and figures are provided as means \pm S.E. The results were analyzed by a one-way analysis of variance and Tukey's test. $p < 0.05$ was considered significant.

Results

Simvastatin and Doxorubicin Induce Activation of the NF- κ B Pathway in HT29 Cells, Which Is Prevented by Parthenolide and Artemisinin. HT29 cells showed undetectable levels of nuclear NF- κ B, which was increased by simvastatin in a dose-dependent way (Fig. 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- κ B. All of the following experimental work was performed with 10 μ M simvastatin for 6 h, which was as effective as TNF- α , a potent inducer of NF- κ B activation in HT29 cells (Fig. 1A).

Parthenolide and artemisinin, which per se did not affect NF- κ B nuclear levels, completely abolished the effect of the statin on NF- κ B translocation (Fig. 1B). Western blotting experiments on nuclear extracts confirmed the electrophoretic mobility shift assay (EMSA) results: simvastatin and TNF- α elicited the p50/p65 dimer translocation into the nucleus, whereas parthenolide and artemisinin prevented it (Fig. 1C). It is interesting that doxorubicin alone increased the NF- κ B activity and the p50/p65 nuclear translocation; these drug effects were enhanced by simvastatin (Fig. 1, B and C).

The cytosolic level of I κ B α was clearly detectable, whereas phospho(Ser32)-I κ B α protein was absent in the cytosolic extracts of HT29 cells resting or incubated with parthenolide or artemisinin (Fig. 2A). On the contrary, the incubation with simvastatin decreased I κ B α and increased phospho(Ser32)-I κ B α protein; these effects were reverted by the addition of the sesquiterpene lactones (Fig. 2A). Total IKK α / β was

not modified under any experimental condition, and phospho-IKK α / β was absent in control HT29 cells and in the presence of parthenolide and artemisinin (Fig. 2A). Simvastatin augmented the amount of phospho-IKK α / β , whereas a very low amount of phospho-IKK α / β was present when the statin was coincubated with either parthenolide or artemisinin (Fig. 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 I κ B α , obtained from HMM cells, which are particularly rich in I κ B α protein (Aldieri et al., 2004). During the assay, we also added the pro-

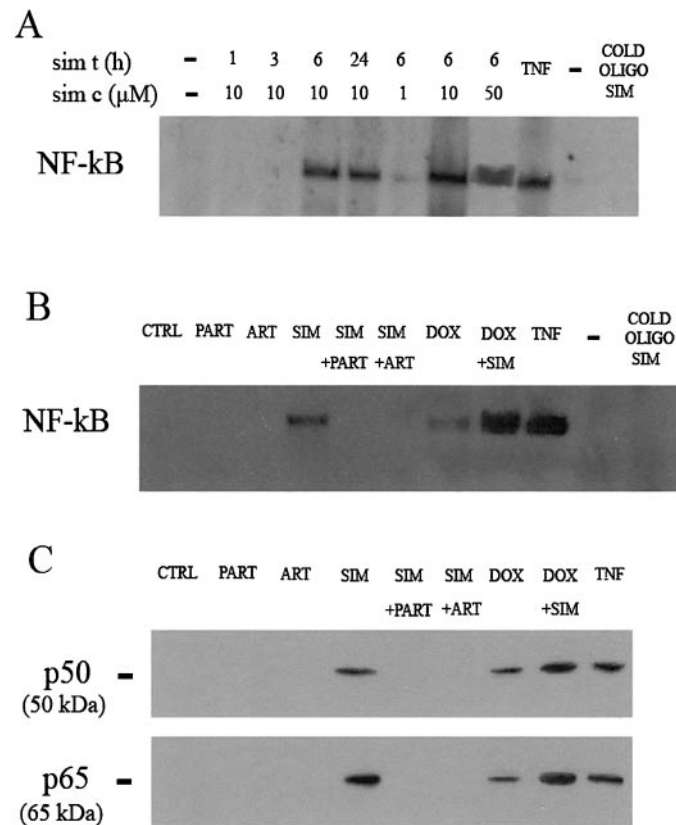


Fig. 1. Effects of simvastatin, doxorubicin, parthenolide and artemisinin on the NF- κ B signaling pathway in HT29 cells. A, cells were incubated for different time periods (sim t = -, 1, 3, 6, and 24 h) and with different concentrations (sim c = 1, 10, and 50 μ M) of simvastatin (SIM), and then NF- κ B translocation was detected on nuclear extracts as described under *Materials and Methods*. TNF- α (10 ng/ml for 15 min) was used as a positive control of NF- κ B translocation. This figure is representative of three experiments with similar results. In each experiment, one lane was loaded with double-distilled water (-) in place of cellular extracts. In the experimental point COLD OLIGO SIM, a competition assay was performed in cells incubated with simvastatin (10 μ M for 6 h) together with an excess of unlabeled oligonucleotide, to assess the specificity of NF- κ B binding. B, cells were incubated for 6 h in the absence (CTRL) or presence of parthenolide (10 μ M, PART), artemisinin (10 μ M, ART), simvastatin (10 μ M, SIM), doxorubicin (5 μ M, DOX), alone or in different combinations, or with TNF- α (10 ng/ml for 15 min, TNF). EMSA detection of NF- κ B 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 double-distilled water (-) in place of cellular extracts. In the experimental point COLD OLIGO SIM, a competition assay was performed in cells incubated with simvastatin (10 μ M for 6 h) together with an excess of unlabeled oligonucleotide. C, cells were incubated in the experimental conditions indicated above, and Western blotting detection of p65 and p50 was performed on nuclear extracts (see *Materials and Methods* for details). The figure is representative of three experiments with similar results.

teasome inhibitor MG132 to prevent the degradation of phosphorylated I κ B α protein by the residual proteasome eventually still present in the lysates. In this way, no change of I κ B α protein was observed during such in vitro kinase assay (Fig. 2B). Simvastatin significantly increased the IKK kinase activity (Fig. 2B), whereas the IKK kinase activity was undetectable in control cells and in cells incubated with parthenolide or artemisinin (Fig. 2B). Doxorubicin alone was able to reduce the I κ B α amount, induce a prompt phosphorylation on I κ B α and IKK, and increase the activity of IKK; again, the doxorubicin effects were potentiated when the drug was coincubated with simvastatin (Fig. 2, A and B). In summary, simvastatin, doxorubicin, and TNF- α exerted the same effects on I κ B α and IKK levels and activity, and doxorubicin and simvastatin together exerted a more potent effect on the IKK/I κ B α pathway (Fig. 2, A and B).

Simvastatin Increases NOS Activity and Doxorubicin Efficacy in HT29 cells, and Parthenolide and Artemisinin Abolish the Statin Effect. A low basal level of NOS activity was detectable in HT29 cells (Fig. 3A). Doxorubicin greatly increased the NO synthesis (Fig. 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 (Fig. 3C). Simvastatin further enhanced the NOS activity and increased the intracellular doxorubicin accumulation in HT29 cells (Fig. 3A) and the drug cytotoxicity, measured as LDH release and Trypan blue staining (Fig. 3B). It is interesting that 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 (Fig. 3C). The effects of doxorubicin and simvastatin on NO synthesis were probably due to an overexpression of the iNOS isoform: indeed, iNOS was absent in untreated HT29 cells, because each drug elicited a clear increase of iNOS expression, which was even greater when simvastatin and doxorubicin were incubated together (see Supplemental Data, Fig. S1). A low basal amount of the eNOS and nNOS isoforms was detectable in HT29 cells and did not change under any experimental condition (see Supplemental Data, Fig. 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 (Fig. 3, A and B). The two sesquiterpene drugs did not significantly modify the doxorubicin-induced apoptosis in HT29 cells (Fig. 3C). According to their ability to block the NF- κ B translocation, they inhibited the statin-induced increase of NOS activity, doxorubicin accumulation, LDH release, cell death, and apoptosis (Fig. 3, A–C). The sesquiterpene lactones did not change 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 (Fig. 4). On the contrary, simvastatin increased the levels of nitro-MRP3, an effect mimicked by the NO donor *S*-nitroso-acetylpenicillamine and inhibited by the coincubation with parthenolide and artemisinin (Fig. 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 (Fig. 4). No nitration of PgP was observed in HT29 cells as reported previously (Riganti et al., 2005).

Sesquiterpene Lactones Increase, whereas Simvastatin Decreases RhoA and Rho Kinase Activity in HT29 Cells. Statins impair the isoprenylation of the Rho proteins and 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 (Fig. 5). Such statin effect was completely prevented by the coincubation with the product of HMG-CoA reductase, mevalonic acid, which per se increased both RhoA-GTP and Rho kinase activity (Fig. 5). It is interesting that 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 coincubated with simvastatin (Fig. 5). On the other hand, doxorubicin did not affect significantly RhoA and Rho kinase activity and did not modify the effect of simvastatin (Fig. 5).

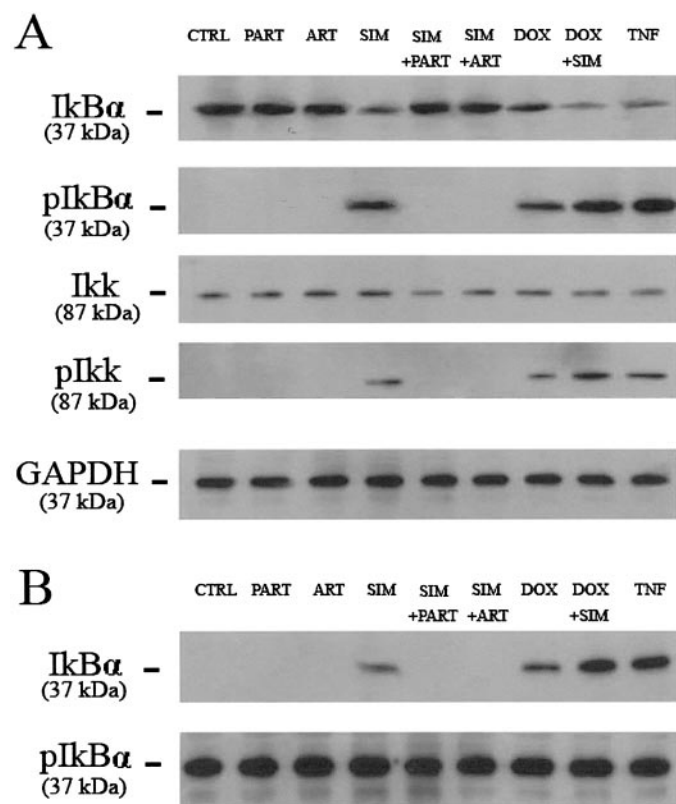


Fig. 2. Effects of simvastatin, doxorubicin, parthenolide, and artemisinin on I κ B α protein, IKK α / β phosphorylation, and IKK activity. HT29 cells were incubated for 6 h in the absence (CTRL) or presence of parthenolide (10 μ M, PART), artemisinin (10 μ M, ART), simvastatin (10 μ M, SIM), and doxorubicin (5 μ M, DOX), alone or in different combinations. TNF- α (10 ng/ml for 15 min, TNF) was used as a positive control of NF- κ B translocation. Thereafter, the following investigations were performed. A, Western blot detection of I κ B α , phospho-I κ B α (pI κ B α), IKK α / β , and phospho-IKK α / β (pIKK) proteins. Whole-cell lysates were analyzed by Western blotting with anti-I κ B α , anti-phospho(Ser32)-I κ B α , anti-IKK α / β , and anti-phospho(Ser176-180)-IKK α / 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.

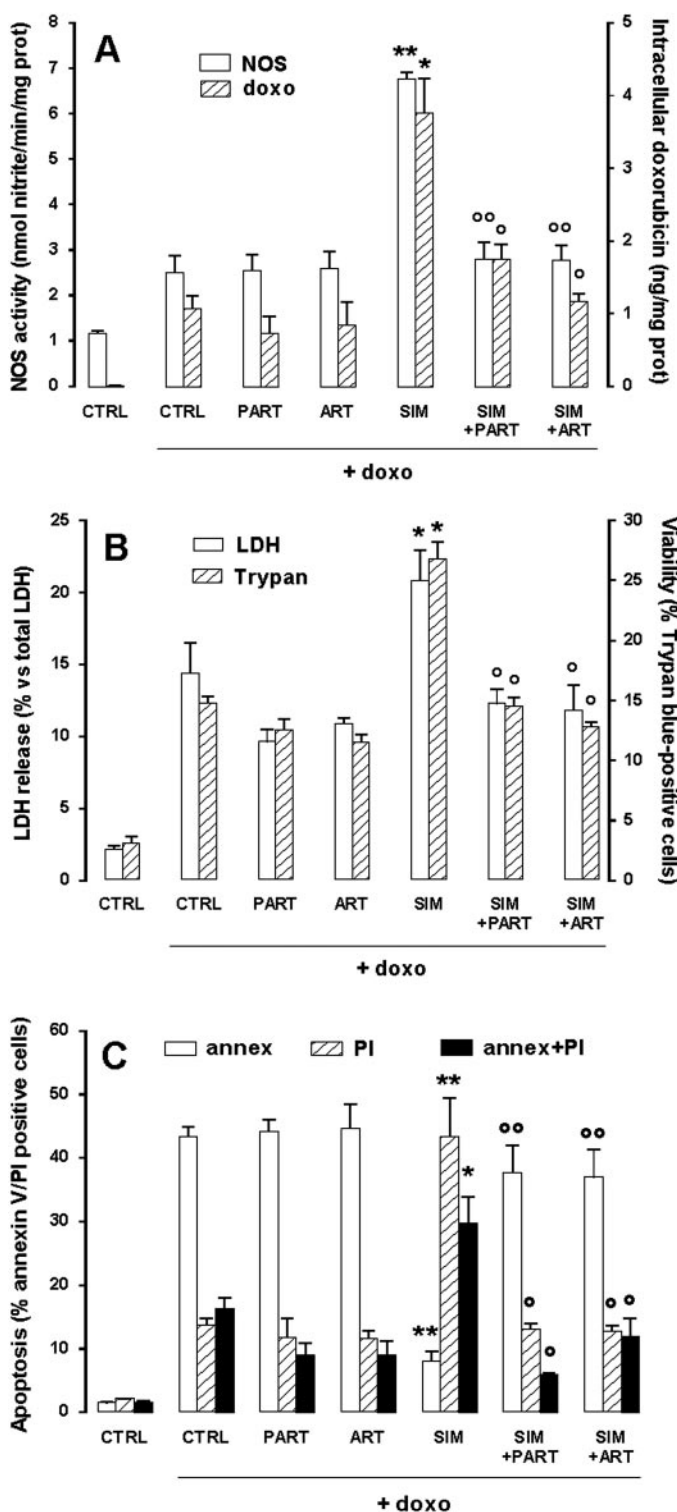


Fig. 3. Effects of simvastatin, doxorubicin, parthenolide, and artemisinin on NOS activity, doxorubicin accumulation, toxicity, viability, and apoptosis. HT29 cells were incubated for 6 h in the absence (CTRL) or presence (+ doxo) of 5 μ M doxorubicin, alone (CTRL + doxo) or together with parthenolide (10 μ M, PART + doxo), artemisinin (10 μ M, ART + doxo), and simvastatin (10 μ M, SIM + doxo) in different combinations. Subsequently cells were subjected to the following investigations. A, NOS activity (□) and doxorubicin accumulation (▨) were measured as described previously (see *Materials and Methods*). The measurements were performed in duplicate, and data are presented as means \pm S.E. ($n = 3$). 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. Versus CTRL + doxo: *, $p < 0.05$; **, $p < 0.001$.

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

HT29 Cells Become More Sensitive to Doxorubicin with RhoA siRNA. RhoA siRNA augmented NOS activity at an extent similar to that obtained incubating nontransfected HT29 cells with simvastatin (Fig. 7C). Again, when RhoA was silenced, NO production was not further modified by simvastatin, alone or together with parthenolide and artemisinin (Fig. 7C). Finally, RhoA siRNA greatly enhanced the doxorubicin accumulation (Fig. 7C) and the drug-induced LDH release (Fig. 8A), Trypan blue staining (Fig. 8A), and apoptosis (Fig. 8B) under each experimental condition and made HT29 cells insensitive to the effects of parthenolide and artemisinin (Figs. 7C and 8).

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 coadministration with cardioprotectors, such as dexrazoxane, coenzyme Q10 (Batist et al., 2001), and flavonoids (Bast et al., 2007), has been proposed without obtaining satisfying results. Different pro-

Versus SIM: °, $p < 0.05$; °°, $p < 0.001$. B, LDH release (□) and Trypan blue staining (▨) were performed as reported under *Materials and Methods*. The measurements were performed in duplicate and data are presented as means \pm S.E. ($n = 3$). The significance of CTRL versus each experimental condition marked with “+ doxo” was $p < 0.005$ for both LDH release and Trypan blue staining. Versus CTRL + doxo: *, $p < 0.05$. Versus SIM: °, $p < 0.05$. C, the staining of cells with annexin V-FITC (□), PI (▨), or both (■) was detected by FACS analysis, as described under *Materials and Methods*. The measurements were performed in duplicate, and data are presented as means \pm S.E. ($n = 3$). The significance of CTRL versus each experimental condition marked with “+ doxo” was always $p < 0.002$. Versus CTRL + doxo: *, $p < 0.05$; **, $p < 0.001$. Versus SIM: °, $p < 0.05$; °°, $p < 0.001$.

ocols of administration (i.e., continuous infusion versus 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 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- κ B activation and NO synthesis (Riganti et al., 2006). So far, we have investigated whether the doxorubicin efficacy could be enhanced with a similar mechanism in sensitive cancer cells.

The NF- κ B 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 I κ B α sequesters NF- κ B in the cytoplasm of resting

cells, but different stimuli, such as inflammatory cytokines, bacterial lipopolysaccharide, oxidative stress, or drugs like statins and doxorubicin promote I κ B α phosphorylation and ubiquitinylation, inducing the nuclear translocation of NF- κ B. IKK complex is responsible for the phosphorylation of I κ B α on serines 32 and 36. Like other kinases, IKK becomes fully active when phosphorylated (Greten and Karin, 2004). Untreated HT29 cells did not exhibit any basal NF- κ B activation; NF- κ B binding on DNA was absent, I κ B α was abundant, whereas IKK α / β was neither phosphorylated nor active as a kinase. On the other hand, simvastatin elicited a clear translocation of p50/p65 NF- κ B dimer into the nucleus, reduced the level of I κ B α protein, and enhanced IKK α / β phosphorylation and kinase activity. The effect of statin on NF- κ B was dose-dependent and similar to that evoked by TNF- α , a potent inducer of NF- κ B pathway. TNF- α promptly activated NF- κ B after 15 min, but the degree of NF- κ B translocation by TNF- α decreased after longer incubation times (data not shown). On the other hand, simvastatin elicited a slower and more sustained activation of NF- κ B. A clear nuclear translocation was detectable only after 6 h and still remained high

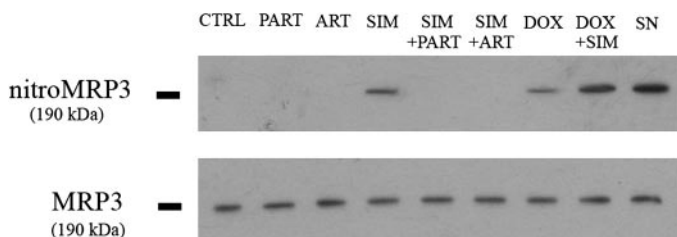


Fig. 4. Effects of simvastatin, doxorubicin, parthenolide, and artemisinin on MRP3 nitration. HT29 cells were incubated for 6 h in the absence (CTRL) or presence of parthenolide (10 μ M, PART), artemisinin (10 μ M, ART), simvastatin (10 μ M, SIM), and doxorubicin (5 μ M, DOX), alone or in different combinations, and with the NO donor *S*-nitroso-acetylpenicillamine (100 μ M, SN). Cells were lysed, and the whole cellular lysate was immunoprecipitated with an antinitrotyrosine 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.

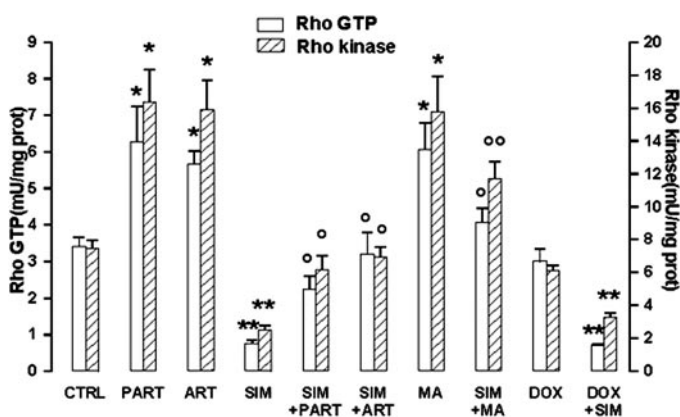


Fig. 5. Effects of simvastatin, doxorubicin, parthenolide, and artemisinin on RhoA and RhoA kinase activities. HT29 cells were incubated for 6 h in the absence (CTRL) or presence of parthenolide (10 μ M, PART), artemisinin (10 μ M, ART), simvastatin (10 μ M, SIM), and doxorubicin (5 μ M, DOX), alone or in different combinations. In some experimental points mevalonic acid (100 μ M, MA) was added. Thereafter, the cells were lysed and subjected to enzyme-linked immunosorbent assay. The amount of RhoA-GTP (□) and the Rho kinase activity (▨) were measured in duplicate, as described under *Materials and Methods*. Data are presented as means \pm S.E. ($n = 3$). Versus CTRL: *, $p < 0.05$; **, $p < 0.001$. Versus SIM: °, $p < 0.05$; °°, $p < 0.001$.

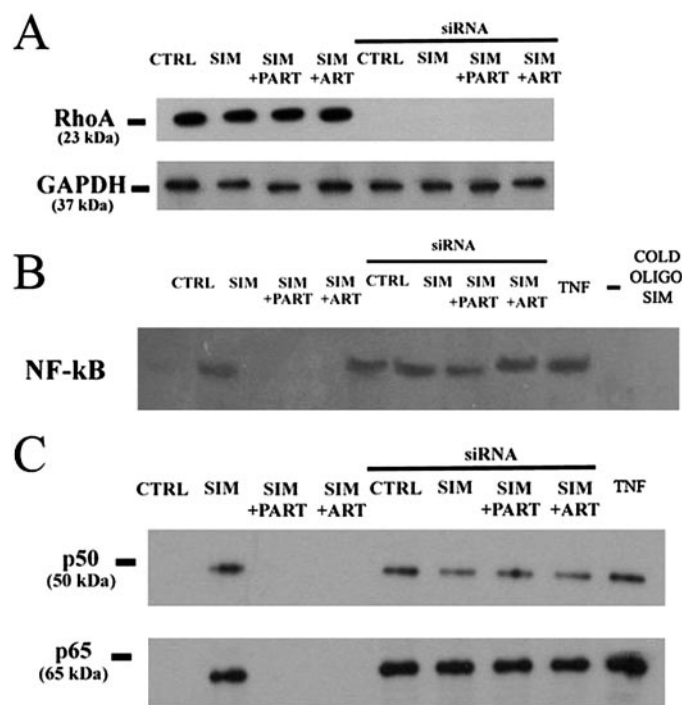


Fig. 6. Effects of simvastatin, parthenolide, and artemisinin on the NF- κ B nuclear translocation in RhoA-silenced HT29 cells. Control and RhoA-silenced (siRNA) HT29 cells were incubated for 6 h in the absence (CTRL) or presence of simvastatin (10 μ M, SIM), alone or in combination with parthenolide (10 μ M, PART) or artemisinin (10 μ M, 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 *Materials and Methods*. The figure is representative of three experiments with similar results. B, EMSA detection of NF- κ B was performed on nuclear extracts as indicated under *Materials and Methods*. TNF- α (10 ng/ml for 15 min, TNF) was used as a positive control of NF- κ B translocation. In each experiment, one lane was loaded with double-distilled water (–) in place of cellular extracts. In the experimental point COLD OLIGO SIM, a competition assay was performed in cells incubated with simvastatin (10 μ M for 6 h) together with an excess of unlabeled 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- κ B translocation. The figure is representative of three experiments with similar results.

at 24 h. The different intracellular signaling pathways of TNF- α and simvastatin may account for this temporal discrepancy.

Sesquiterpene lactones have been widely used as NF- κ B 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- κ B pathway in HT29 cells. Parthenolide is a natural

potent NF- κ B inhibitor (Wong and Menendez, 1999; Yip et al., 2004) with anti-inflammatory and antiseptic properties (Yip et al., 2004). Artemisinin, the active component of *Artemisia annua*, has a relevant clinical importance, because it is the most used antimalarial drug worldwide (Mohanty et al., 2006). Artemisinin has pleiotropic effects (Golenser et al., 2006) and has been shown to inhibit the cytokine-induced NF- κ B activation in different cell lines (Aldieri et al., 2003; Li et al., 2006). Artemisinin and parthenolide did not exert any effect in resting HT29 cells, whereas NF- κ B levels were

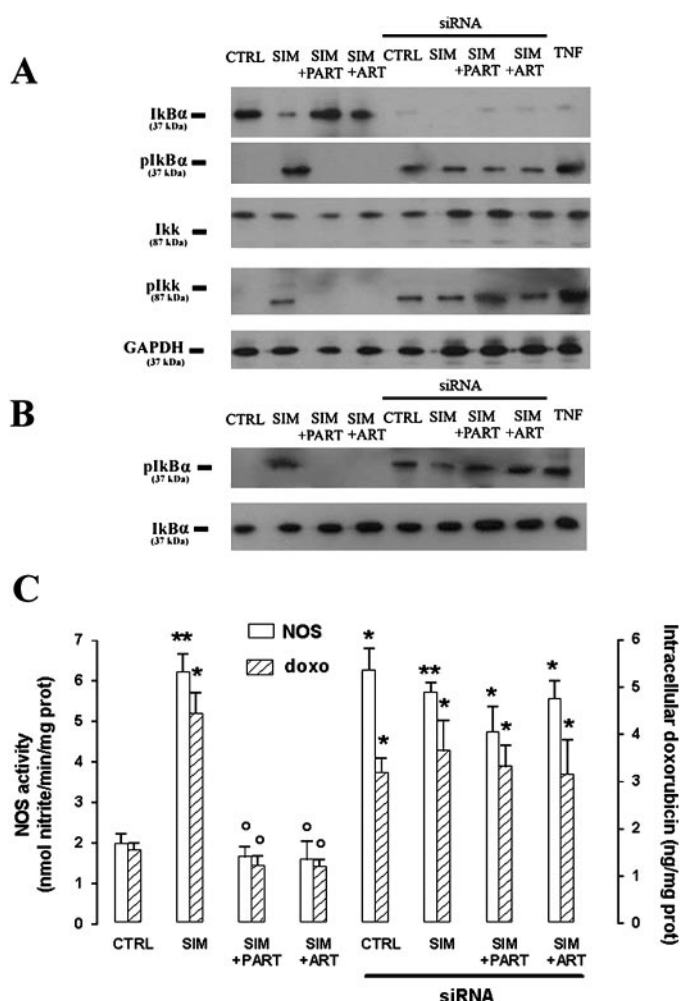


Fig. 7. Effects of simvastatin, parthenolide, and artemisinin on I κ B α /IKK pathway, NOS activity, and doxorubicin accumulation in RhoA-silenced HT29 cells. Control and RhoA-silenced (siRNA) HT29 cells were incubated for 6 h in the absence (CTRL) or presence of simvastatin (10 μ M, SIM), alone or in combination with parthenolide (10 μ M, PART) or artemisinin (10 μ M, ART). TNF- α (10 ng/ml for 15 min, TNF) was used as a positive control of NF- κ B translocation. Cells were lysed and subjected to the following investigations. A, the Western blotting detection of I κ B α , phospho-I κ B α (pI κ B α), IKK α / β , and phospho-IKK α / β (pIKK) was performed on whole-cell lysates using anti-I κ B α , anti-phospho(Ser32)-I κ B α , anti-IKK α / β , and anti-phospho(Ser176-180)-IKK α / 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 (\square) and doxorubicin accumulation (\blacksquare) were measured as described previously (see *Materials and Methods*). Doxorubicin (5 μ M) was present in each experimental condition throughout the incubation time (CTRL = doxorubicin alone). In the absence of doxorubicin, the NOS activity was 1.16 ± 0.05 nmol nitrite/min/mg cell proteins (versus CTRL: $p < 0.05$); the intracellular doxorubicin content was 0.070 ± 0.002 ng/mg cell protein (versus CTRL: $p < 0.001$). The measurements were performed in duplicate, and data are presented as means \pm S.E. ($n = 3$). Versus CTRL: *, $p < 0.05$; **, $p < 0.005$. Versus SIM: $^{\circ}$, $p < 0.001$.

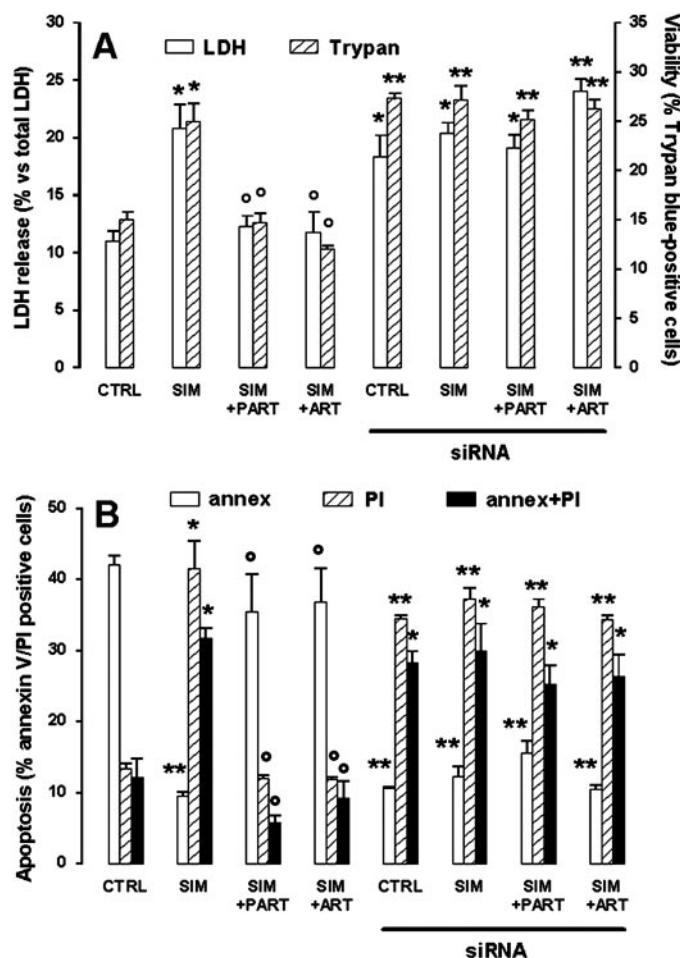


Fig. 8. Effects of simvastatin, parthenolide, and artemisinin on cytotoxicity, viability, and apoptosis in RhoA-silenced HT29 cells. Control and RhoA-silenced (siRNA) HT29 cells were incubated for 6 h in the absence (CTRL) or presence of simvastatin (10 μ M, SIM) alone or in combination with parthenolide (10 μ M, PART) or artemisinin (10 μ M, ART). Doxorubicin (5 μ M) was present in each experimental condition throughout the incubation time (CTRL = doxorubicin alone), and then cells were subjected to the following investigations. A, LDH release (\square) and Trypan blue staining (\blacksquare) were performed as reported under *Materials and Methods*. The measurements were performed in duplicate and data are presented as means \pm S.E. ($n = 3$). In the absence of doxorubicin the LDH release was $2.11 \pm 0.23\%$ (versus CTRL: $p < 0.005$); the percentage of Trypan blue-positive cells was $3.00 \pm 0.58\%$ (versus CTRL: $p < 0.001$). Versus CTRL: *, $p < 0.05$; **, $p < 0.005$. Versus SIM: $^{\circ}$, $p < 0.001$. B, the staining of cells with annexin V-FITC (\square), PI (\blacksquare), or both (\blacksquare) was detected by FACS analysis, as described under *Materials and Methods*. The measurements were performed in duplicate and data are presented as means \pm S.E. ($n = 3$). In the absence of doxorubicin the percentage of annexin V-FITC-positive cells was $1.42 \pm 0.09\%$ (versus CTRL: $p < 0.001$); the percentage of PI-positive cells was $2.04 \pm 0.03\%$ (versus CTRL: $p < 0.002$); the percentage of annexin V-FITC- and PI-positive cells was $1.98 \pm 0.18\%$ (versus CTRL: $p < 0.001$). Versus CTRL: *, $p < 0.05$; **, $p < 0.005$. Versus SIM: $^{\circ}$, $p < 0.05$.

nearly undetectable. On the contrary, they completely blunted the activation of NF- κ B pathway elicited by simvastatin. By inhibiting NF- κ B, parthenolide and artemisinin may exert proapoptotic and antiproliferative 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- κ B (Lin et al., 2007; Yu et al., 2008). This observation has been confirmed in HT29 cells in the present work. It is interesting that when simvastatin was added together with doxorubicin, the activation of NF- κ B was stronger than that caused by either drug alone. In 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- κ B 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. Neither doxorubicin nor simvastatin altered the expression of these constitutive NOS isoforms. On the other hand, 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 hypothesize that doxorubicin *per se* favors the entry of HT29 cells into apoptosis, but the addition of simvastatin accelerates the effect of doxorubicin and increases the number of cells in an advanced stage of apoptosis or necrosis. The statin's action on doxorubicin efficacy is likely to be dependent on NF- κ B activation; indeed, it was completely abolished in the presence of parthenolide and artemisinin.

Besides 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 ATP-binding cassette membrane transporters, which actively extrude several chemotherapeutic drugs, may be targets of NO. As a consequence of the tyrosine nitration of these proteins, the efflux of doxorubicin is inhibited (Riganti et al., 2005, 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- κ B, 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- κ B 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, because it has been observed also in human myelogenous leukemic K562 cells (data not shown).

The effect of sesquiterpenes on multidrug resistance is still a matter of debate. Some natural terpenes are direct inhibitors of PgP (Molnár et al., 2006). Derivatives of artemisinin reduce PgP functions in multidrug-resistant K562/adr cells, with an IC₅₀ value of 115 μ M, but this effect is evident only at high doses (Reungpatthanaphong et al., 2002). The experimental evidence obtained in HT29 cells suggests that sesquiterpene lactone-induced effects are mediated by NF- κ B inhibition. However, we cannot exclude that they exert other effects independent of NF- κ B; indeed, in the present study, parthenolide and artemisinin alone slightly reduced the intracellular doxorubicin accumulation and LDH release, when no change in NF- κ B levels and NOS activity was detectable. We are presently investigating the possible mechanism of such an effect.

By inhibiting HMG-CoA reductase, statins lower the intracellular levels of isoprenoids and limit the isoprenylation of several proteins, such as Rho and Ras (Dulak and Jozkowicz, 2005). In 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- κ B translocation, reduced RhoA-GTP binding and Rho kinase activity, whereas artemisinin and parthenolide, which inhibited NF- κ B activation, increased RhoA/Rho kinase activity. It is interesting that 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- κ B translocation. It has been reported already that statins exert a positive or negative modulation on NF- κ B 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- κ B through the modulation of RhoA. On the other hand, doxorubicin did not affect RhoA and Rho kinase activity. Therefore, it is conceivable that doxorubicin activates NF- κ B in a RhoA-independent way. Several mechanisms have been proposed to explain the doxorubicin effect on NF- κ B 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- κ B in HT29 cells. Our data suggest that simvastatin and doxorubicin, although by a different mechanism, induce the nuclear translocation of NF- κ B and the increase of NO synthesis in HT29 cells. By inhibiting RhoA, simvastatin

may potentiate the doxorubicin-induced NF- κ B activation and NO production, enhancing the cellular death mediated by the anthracycline drug.

The experiments with RhoA-specific siRNA confirmed the central role played by RhoA in controlling NF- κ B translocation and doxorubicin accumulation. The efficient silencing of RhoA did not affect HT29 cell viability and induced an intense activation of NF- κ B pathway and NOS activity. Moreover, doxorubicin accumulation and its cytotoxic and proapoptotic 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 seems 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- κ B 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- κ B and iNOS activation in HT29 colon cancer cells by inhibiting RhoA. On the contrary, artemisinin makes cells more resistant to doxorubicin by increasing the RhoA activity. So far, the widely used antimalarial drug artemisinin may impair patient 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 (Pillé et al., 2005, 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.

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