

# Nitric Oxide Reverts the Resistance to Doxorubicin in Human Colon Cancer Cells by Inhibiting the Drug Efflux

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## Abstract

**Multidrug resistance (MDR) is a phenomenon by which cancer cells evade the cytotoxic effects of chemotherapeutic agents. It may occur through different mechanisms, but it often correlates with the overexpression of integral membrane transporters, such as P-glycoprotein (Pgp) and MDR-associated proteins (MRPs), with resulting decrease of drug accumulation and cellular death. Doxorubicin is a substrate of Pgp; it has been suggested that its ability to induce synthesis of nitric oxide (NO) could explain, at least in part, its cytotoxic effects. Culturing the human epithelial colon cell line HT29 in the presence of doxorubicin, we obtained a doxorubicin-resistant (HT29-dx) cell population: these cells accumulated less intracellular doxorubicin, were less sensitive to the cytotoxic effects of doxorubicin and cisplatin, overexpressed Pgp and MRP3, and exhibited a lower NO production (both under basal conditions and after doxorubicin stimulation). The resistance to doxorubicin could be reversed when HT29-dx cells were incubated with inducers of NO synthesis (cytokines mix, atorvastatin). Some NO donors increased the drug accumulation in HT29-dx cells in a guarosine-3':5'-cyclic monophosphate-independent way; this effect was associated with a marked reduction of doxorubicin efflux rate in HT29 and HT29-dx cells, and tyrosine nitration in the MRP3 protein. Our results suggest that onset of MDR and impairment of NO synthesis are related; this finding could point to a new strategy to reverse doxorubicin resistance in human cancer. (Cancer Res 2005; 65(2): 516–25)**

## Introduction

Multidrug resistance (MDR) is a phenomenon by which tumor cells that have been exposed to a cytotoxic agent develop cross-resistance to a range of structurally and functionally unrelated compounds (1). MDR is one of the major obstacles to the successful pharmacologic treatment of tumors (2). Many different mechanisms have been suggested to explain the development of an MDR phenotype in cancer cells, such as a change in the drug's specific target, the reduced uptake or increased efflux of a drug, the reduced capacity to enter apoptosis, an increase in the ability to repair DNA damage, a different compartmentalization, and an increased rate of detoxification of the drug (1). In cancer cell models, one of the most studied of these mechanisms has been the overexpression of several energy-dependent drug efflux pumps that belong to the ATP-binding cassette family of transporters, such as the P-glycoprotein (Pgp) and

the MDR-associated proteins (MRPs). The overexpression of these integral membrane proteins causes tumor cells to become resistant to a variety of anticancer drugs (anthracyclines, Vinca alkaloids, epipodophyllotoxins, taxanes, actinomycin-D, mitoxantrone, etc.; ref. 1) and seems to be related to high proliferative activity and negative prognostic value (3). Evidence that drug efflux pumps, especially Pgp, play a significant role in clinical drug resistance has stimulated the introduction of various Pgp inhibitors into the clinical arena (1, 4).

Doxorubicin is commonly used in the therapy of solid tumor; its efficacy, however, is often reduced because it is a substrate for both Pgp and MRPs. Remarkably, doxorubicin induces the production of nitric oxide (NO; refs. 5, 6), a small signaling molecule that regulates many physiologic and pathologic processes (7). NO is synthesized by three NO synthase (NOS; EC 1.14.13.39) isoforms, which favor the conversion of L-arginine to L-citrulline and NO with a 1:1 stoichiometry (8); in oxygenated living systems, NO is rapidly converted into nitrite and nitrate (9). NO plays an important role in cell growth and differentiation and in apoptosis (10, 11). It has been postulated that doxorubicin could also exert its therapeutic effect via a NO-dependent mechanism (5). We hypothesized that doxorubicin-sensitive and doxorubicin-resistant cells could show a different capacity to produce NO and that this feature might contribute to drug resistance. Therefore, we induced human colon cancer HT29 cells, a cell type very susceptible to develop a MDR phenotype (12), to become doxorubicin-resistant. Quite surprisingly, we observed very different rates of NO production in sensitive and resistant cells. We then asked the question whether a reduced synthesis of NO could be implicated in the onset of MDR and if the restoration of NO production could reverse it.

## Materials and Methods

**Materials.** Fetal bovine serum and RPMI 1640 were supplied by BioWhittaker (Verviers, Belgium); plasticware for cell culture was from Falcon (Becton Dickinson, Bedford, MA); the cationic exchange resin Dowex AG50WX-8, *N*-(1-naphthylethylenediamine) dihydrochloride and sulfanilamide were from Aldrich (Milan, Italy); L-[2,3,4,5-<sup>3</sup>H]arginine monohydrochloride (62 Ci/mmol) was obtained from Amersham International (Bucks, United Kingdom). Recombinant human tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and recombinant human interferon- $\gamma$  (IFN- $\gamma$ ) were from R&D Systems (Minneapolis, MN), recombinant human interleukin-1 $\beta$  (IL-1 $\beta$ ) was purchased from Promega Corporation (Madison, WI). Atorvastatin, calcium salt, and 8-bromoguanosine-3':5'-cyclic monophosphorothioate, Rp-isomer (Rp-8-Br-cGMPs) were obtained from Calbiochem-Novabiochem Corporation (La Jolla, CA). Electrophoresis reagents were obtained from Bio-Rad Laboratories (Hercules, CA), PCR-primers and Superscript II One-Step Reverse Transcriptase-PCR (RT-PCR) System with Platinum Taq DNA Polymerase were from Life Technologies (San Giuliano Milanese, Italy). The proteins content of cell monolayers and cell lysates was assessed with the bicinchoninic acid kit from Pierce (Rockford, IL). Doxorubicin hydrochloride and all other reagents not specified here were purchased from Sigma Chemical Co. (St. Louis, MO).

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**Cell Cultures.** Human colon cancer cells (HT29, provided by Istituto Zooprofilattico Sperimentale "Bruno Umbertini", Brescia, Italy) were cultured in RPMI 1640, supplemented with 10% fetal bovine serum, in a 5% carbon dioxide atmosphere at 37°C. A subpopulation of HT29 cells, after 20 to 25 passages in RPMI 1640 + 10% fetal bovine serum supplemented with 68 nmol/L doxorubicin, exhibited a significantly lower intracellular level of drug after a doxorubicin bolus (4 µmol/L), and parallelly became more resistant to the drug's toxic effects (see RESULTS); these cells were named HT29-dx, and subsequently cultured in RPMI 1640 containing 34 nmol/L doxorubicin ("maintenance" dose).

**Extracellular Lactate Dehydrogenase Activity.** Aliquots of extracellular culture medium, after a 24 to 48 hours' incubation in different experimental conditions, were collected for the measurement of lactate dehydrogenase (LDH) activity using a Lambda 3 spectrophotometer (Perkin-Elmer, Shelton, CT), as previously described (13). The LDH activity, used as a sensitive index of the drug's cytotoxic effects (14), was expressed as micromoles of NADH oxidized per minute per milligram of cell protein.

**Drug Accumulation and Efflux.** Cells were grown in 60 mm diameter Petri dishes. Before every test, cells were washed and cultured in fresh medium without doxorubicin for 24 hours. They were then incubated in PBS containing 4 µmol/L doxorubicin for different time periods (as indicated in RESULTS), washed twice in ice-cold PBS and detached with trypsin/EDTA (0.05/0.02% v/v). Cells were centrifuged for 30 seconds at 13,000 rpm (4°C) and resuspended in 1 mL of a 1:1 mixture of ethanol/0.3 N HCl. Fifty microliters of cell suspension were sonicated on crushed ice with two 10-second bursts (Labsonic sonicator, 100 W) and used for measurement of cellular proteins; the remaining part was checked for the doxorubicin content using a Perkin-Elmer LS-5 spectrofluorimeter (Perkin-Elmer). Excitation and emission wavelengths were 475 and 553 nm, respectively. A blank was prepared in the absence of cells in every set of experiments and its fluorescence was subtracted from that obtained in the presence of cells. Fluorescence was converted in nanograms of doxorubicin per milligram of cellular protein, using a calibration curve prepared previously. To measure the drug efflux, we incubated the cell monolayers with 4 µmol/L doxorubicin for 10 minutes; cells were washed twice with PBS, then incubated in fresh PBS: 1 mL of supernatant was acquired at different times (as indicated in RESULTS) and checked for doxorubicin-associated fluorescence as described above.

In order to calculate the kinetics of the drug efflux, we incubated the cell monolayers with 1 to 250 µmol/L doxorubicin for 10 minutes; cells were then washed with PBS, resuspended in 1 mL of ethanol/0.3 N HCl and analyzed for the intracellular drug content as described above. In parallel, other dishes, after incubation with doxorubicin under the same experimental conditions, were washed with PBS, left for a further 10 minutes in PBS at 37°C, then washed again, resuspended and tested for intracellular doxorubicin. The difference between the two types of cellular samples under each experimental condition was expressed as nanograms of doxorubicin extruded per minute per milligram of cellular protein.  $V_{max}$  and  $K_m$  of the drug efflux were estimated using the Enzfitter software (Biosoft Corporation, Cambridge, United Kingdom).

**Nitrite Production.** Confluent cell monolayers in 35 mm diameter Petri dishes were incubated in fresh medium for 24 hours under the experimental conditions indicated in RESULTS. Then nitrite production was measured by adding 0.15 mL of cell culture medium to 0.15 mL of Griess reagent (15) in a 96-well plate, and after a 10-minute incubation at 37°C in the dark, absorbance was measured at 540 nm with a Packard EL340 microplate reader (Bio-Tek Instruments, Winooski, VT). A blank was prepared for each experimental condition in the absence of cells, and its absorbance was subtracted from that obtained in the presence of cells. Nitrite concentration was expressed as nanomoles of nitrite per 24 h/mg cell protein.

**Measurement of Nitric Oxide Synthase Activity.** Cells grown at confluence on 35 mm diameter Petri dishes, after incubation under the experimental conditions described in RESULTS, were detached by trypsin/EDTA, washed with PBS, resuspended in 0.3 mL of Hepes/EDTA/DTT buffer [at 20, 0.5, and 1 mmol/L, respectively (pH 7.2)], and then sonicated on crushed ice with two 10-second bursts. In each test tube, the following reagents were added to 100 µL lysate at the following final concentrations:

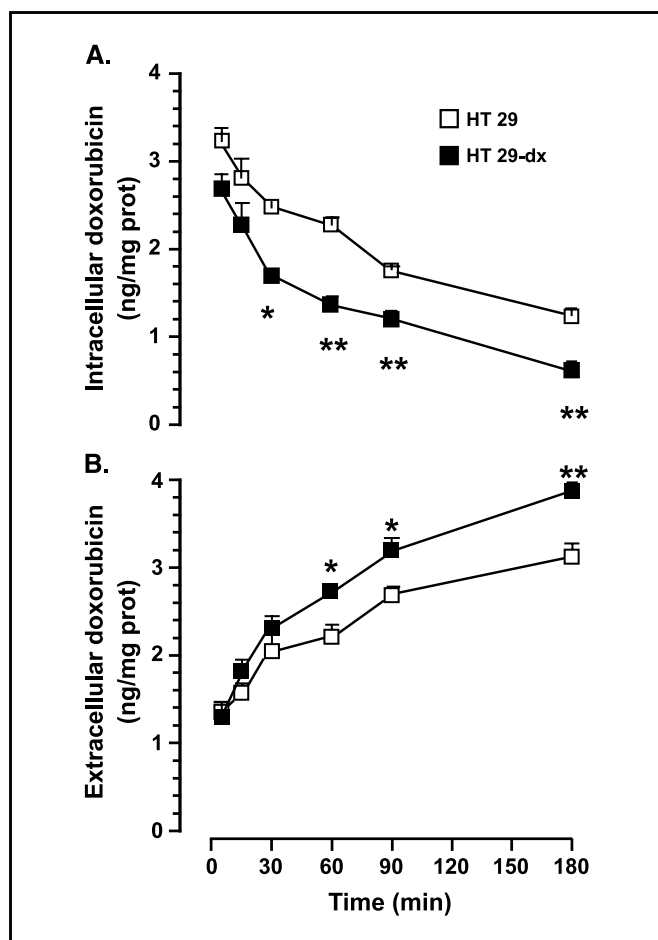
2 mmol/L NADPH, 2.5 µCi L-[<sup>3</sup>H] arginine (0.4 µmol/L), 100 µmol/L tetrahydrobiopterin, 1.5 mmol/L CaCl<sub>2</sub> (15). After a 15-minute incubation at 37°C, the reaction was stopped by adding 2 mL Hepes-Na/EDTA buffer [at 20 and 2 mmol/L, respectively (pH 6)]; the whole reaction mixture was applied to 2 mL columns of Dowex AG50WX-8 (Na<sup>+</sup> form) and eluted with 4 mL of water. The radioactivity corresponding to [<sup>3</sup>H]citrulline content in 6.1 mL eluate was measured by liquid scintillation counting. Citrulline synthesis was expressed as picomoles of citrulline per minute per milligram of cell protein.

**Western Blot Analysis.** Cells were directly solubilized in the lysis buffer (25 mmol/L Hepes, 135 mmol/L NaCl, 1% NP40, 5 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L ZnCl<sub>2</sub>, 50 mmol/L NaF, 10% glycerol), supplemented with protease inhibitor cocktail set III (100 mmol/L AEBSF, 80 µmol/L aprotinin, 5 mmol/L bestatin, 1.5 mmol/L E-64, 2 mmol/L leupeptin, and 1 mmol/L pepstatin; Calbiochem-Novabiochem), 2 mmol/L phenylmethylsulfonyl fluoride and 1 mmol/L sodium orthovanadate. From the whole cell lysates, Pgp, MRP1, MRP2, and MRP3 were immunoprecipitated overnight with the rabbit polyclonal antibodies (diluted 1:250, Santa Cruz, Santa Cruz, CA) anti-human Pgp (H-241), anti-human MRP1 (C-20), anti-human MRP2 (H-17) or anti-human MRP3 (H-16). Immunoprecipitated proteins were separated by SDS-PAGE (8%), transferred to polyvinylidene difluoride membrane sheets (Immobilon-P, Millipore, Bedford, MA) and probed with the same antibodies (diluted 1:500 in PBS-bovine serum albumin 1%); after a 1-hour incubation, the membrane was washed with PBS-Tween 0.1% and subjected for 1 hour to a peroxidase-conjugated anti-rabbit or anti-goat IgG (Amersham International, diluted 1:1,000 in PBS-Tween with blocker nonfat dry milk 5%, Bio-Rad). The membrane was washed again with PBS-Tween and proteins were detected by enhanced chemiluminescence (Bio-Rad).

To assess the presence of nitrated proteins, the whole cell extract was subjected to overnight immunoprecipitation using a rabbit polyclonal anti-nitrotyrosine antibody (diluted 1:1,000 in blocker nonfat dry milk 1%, Upstate, Lake Placid, NY). Immunoprecipitated proteins were separated by SDS-PAGE (8%), transferred to polyvinylidene difluoride membrane sheets and probed, respectively, with anti-Pgp and anti-MRP3 antibodies (diluted 1:500 in PBS-BSA 1%), as previously described.

For Western blot analysis of NOS isoforms, cells were solubilized in the lysis buffer, supplemented with protease inhibitor cocktail set III, 2 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L sodium orthovanadate; cell lysates, containing equal amounts of proteins (30 µg), were directly electrophoresed in a 8% polyacrylamide gel, transferred to polyvinylidene difluoride membrane sheets and probed with anti-human neuronal NOS (nNOS; mouse monoclonal, diluted 1:1000 in PBS-BSA 1%, Transduction Laboratories, Lexington, KY), anti-human inducible NOS (iNOS; mouse monoclonal, diluted 1:500 in PBS-BSA 1%, Transduction Laboratories) or anti-human endothelial NOS (eNOS; mouse monoclonal, diluted 1:500 in PBS-BSA 1%, Transduction Laboratories) antibodies; after a 1-hour incubation, the membrane was washed with PBS-Tween 0.1% and subjected to a peroxidase-conjugated anti-mouse IgG (from sheep; Amersham International, diluted 1:1,000 in PBS-Tween with blocker nonfat dry milk 5%) for 1 hour. The membrane was washed again with PBS-Tween and proteins were detected by enhanced chemiluminescence, as described above.

**RT-PCR.** Total RNA was obtained by the guanidium thiocyanate-phenol-chloroform method (16). Total RNA (30 ng) was reverse-transcribed into cDNA with the Superscript II One-Step RT-PCR System with Platinum Taq DNA Polymerase (cycling conditions: 1 cycle 50°C for 30 minutes, 1 cycle 94°C for 2 minutes). cDNA products were determined by PCR amplification, carried out in a total volume of 50 µL, according to the manufacturer's recommendations. The RT-PCR efficiency was controlled by amplifying a β-actin fragment, used as a housekeeping gene. Primers for nNOS (0.3 µmol/L) were: 5'-TTGGG-GGCCTGGGATTCTGG-3', 5'-GTTGGCATGGGGAGTGAGC-3' (456 bp); primers for iNOS (0.3 µmol/L) were: 5'-TCCGAGCAAACAGCACATTCA-3', 5'-GGTGTGGGGTGTGGTGATGT-3' (462 bp); primers for eNOS (0.6 µmol/L) were: 5'-CCAGCTAGCCAAAGTCACCAT-3', 5'-GTCTCGGAGCCATACAGGATT-3' (672 bp); primers for β-actin (0.5 µmol/L) were: 5'-GGTC-ATCTTCTCGCGGTGGCCTGGGGT-3', 5'-CCCCAGGCCAGGGCGGTGAT-3' (230 bp). PCR amplification for nNOS was: 1 cycle of denaturation at 95°C for 2 minutes, 35 cycles of denaturation at 95°C for 2 minutes, annealing at 60°C for 30 seconds, elongation at 72°C for 2 minutes, and 1 cycle



**Figure 1.** Intracellular accumulation (A) and extracellular efflux (B) of doxorubicin in cultures of HT29 (open symbols) and HT29-dx (solid symbols) cells. A, cells were incubated with 4  $\mu\text{mol/L}$  doxorubicin at different time periods in PBS, then aliquots of cell extracts were analyzed for doxorubicin-associated fluorescence, as described in MATERIALS AND METHODS. B, cells were incubated with 4  $\mu\text{mol/L}$  doxorubicin for 10 minutes, then washed and incubated in PBS; aliquots of supernatant were checked at different times for doxorubicin-associated fluorescence, as described in MATERIALS AND METHODS. Measurements were done in duplicate and data are presented as mean  $\pm$  SE ( $n = 3$ ). HT29-dx versus the corresponding experimental point in HT29 cells: \*,  $P < 0.01$ , \*\*,  $P < 0.001$ .

of extension at 72°C for 10 minutes; for iNOS: 1 cycle of denaturation at 95°C for 2 minutes, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 1 minute, elongation at 72°C for 30 seconds, and 1 cycle of extension at 72°C for 10 minutes; for eNOS: 1 cycle of denaturation at 95°C for 2 minutes, 35 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 2 minutes, elongation at 72°C for 2 minutes, and 1 cycle of extension at 72°C for 10 minutes; for  $\beta$ -actin: 1 cycle of denaturation at 94°C for 3 minutes, 35 cycles of denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute, elongation at 72°C for 1 minute, and 1 cycle of extension at 72°C for 7 minutes. Samples were electrophoresed in 1.5% agarose gels containing ethidium bromide in Tris-acetate/EDTA buffer to visualize the PCR products.

**Statistical Analysis.** All data in text and figures are provided as mean  $\pm$  SE. The results were analyzed by a one-way ANOVA and Tukey's test.  $P < 0.05$  was considered significant.

## Results

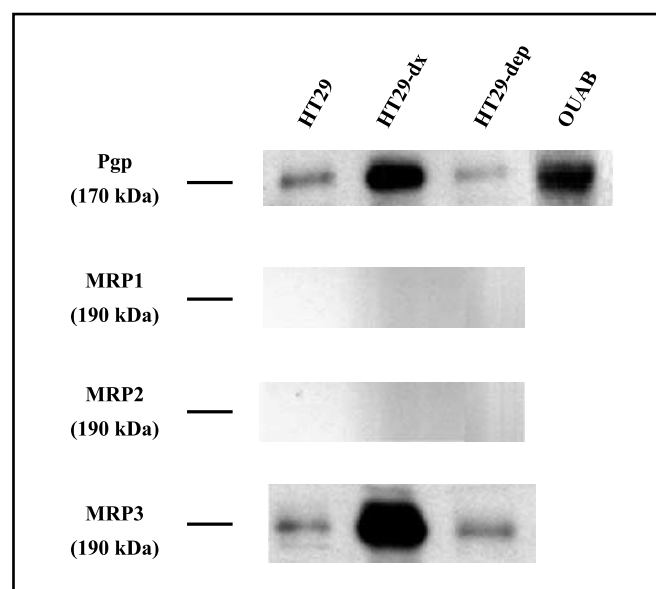
**HT29-dx Cells Accumulate Less Doxorubicin Than Control HT29 Cells and Are More Resistant to the Drug's Cytotoxic Effects.** After incubation with doxorubicin, the cellular accumula-

tion of the drug was lower in HT29-dx than in HT29 cells at all incubation times; specifically, at 3 hours, the intracellular doxorubicin in HT29-dx cells was approximately 50% of that measured in HT29 cells (Fig. 1A). Subsequently, all cell samples were loaded for 10 minutes with doxorubicin, washed, and incubated for 10 to 180 minutes in PBS. A progressive drug efflux was detectable, much faster in HT29-dx than in HT29 cells (Fig. 1B).

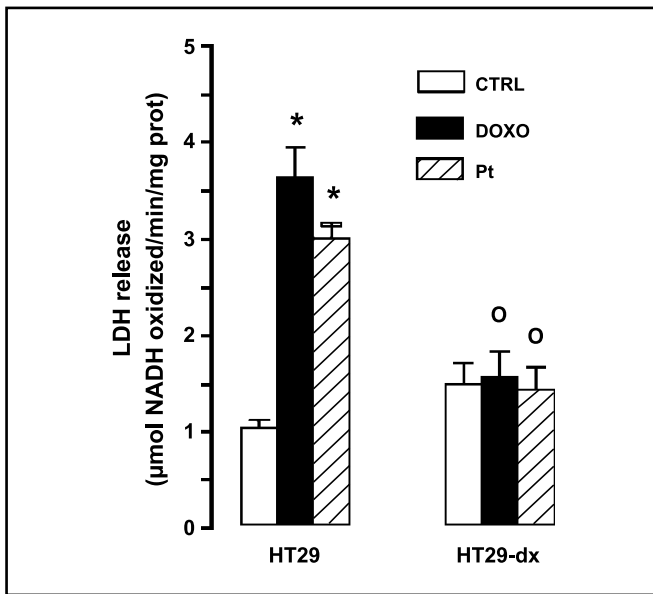
We then investigated the expression of the drug efflux pumps, Pgp and MRP1-3, which have been implicated in doxorubicin resistance (17, 18): an overexpression of Pgp and MRP3 proteins was detectable in HT29-dx cells in comparison with HT29 cells, whereas MRP1 and MRP2 were absent in both drug-sensitive and resistant cells (Fig. 2). The content of Pgp in HT29-dx cells was similar to that observed after a 48-hour incubation of HT29 cells with 0.2  $\mu\text{mol/L}$  ouabain, a potent Pgp inducer (19) (Fig. 2). When we withdrew the maintenance dose of doxorubicin (34 nmol/L) in the culture medium of an aliquot of HT29-dx cells (which we named HT29-dep) for 7 days, the expression of both Pgp and MRP3 returned similar to that observed in HT29 cells (Fig. 2).

We then measured the release of LDH activity in the extracellular medium after a 24-hour incubation as an index of the drug cytotoxic effects. This activity was not significantly different between untreated HT29 and HT29-dx cells (Fig. 3). In the presence of doxorubicin, extracellular LDH activity increased significantly in the supernatant of HT29 cells, but not in that of HT29-dx cultures. The two cell populations also behaved in a similar way when incubated with cisplatin for 48 hours, in place of doxorubicin for 24 hours (Fig. 3).

**Doxorubicin Fails to Induce Nitric Oxide Production in HT29-dx Cells.** Nitrite accumulation in the extracellular medium is an index of the actual production of NO by whole cultured cells, whereas NOS activity in the cell lysate indicates the total amount of active enzyme and its maximal activity in the presence of saturating concentration of substrate. After a 24-hour incubation



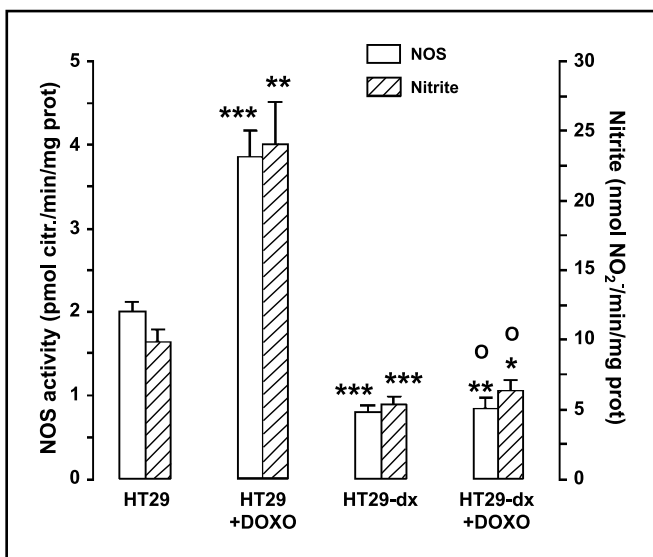
**Figure 2.** Western blot detection after immunoprecipitation of Pgp, MRP1, MRP2, MRP3 in HT29 cells, HT29-dx cells, and in a subpopulation of HT29-dx cells (HT29-dep) cultured for 7 days in a medium deprived of the maintenance dose of doxorubicin. Ouabain (OUAB, 0.2  $\mu\text{mol/L}$  for 48 hours), a potent inducer of Pgp, was used as a positive control. The results shown here are representative of three similar experiments.



**Figure 3.** Effects of doxorubicin and cisplatin on the release of LDH in the supernatant of HT29 and HT29-dx cells. Cells were incubated in the absence (CTRL) or presence of doxorubicin (DOXO, 5 µmol/L, 24 hours) or cisplatin (Pt, 100 µmol/L, 48 hours), then LDH activity was measured in the extracellular medium, as described in MATERIALS AND METHODS. Measurements were done in duplicate and data are presented as mean ± SE ( $n = 4$ ). Versus respective CTRL. \*,  $P < 0.001$ ; HT29-dx versus the same experimental condition in HT29 cells: O,  $P < 0.001$ .

with doxorubicin, NOS activity and nitrite production were significantly increased in HT29 but not in HT29-dx cells (Fig. 4). NOS activity and nitrite production were also significantly lower in HT29-dx than in HT29 cells under basal conditions (Fig. 4).

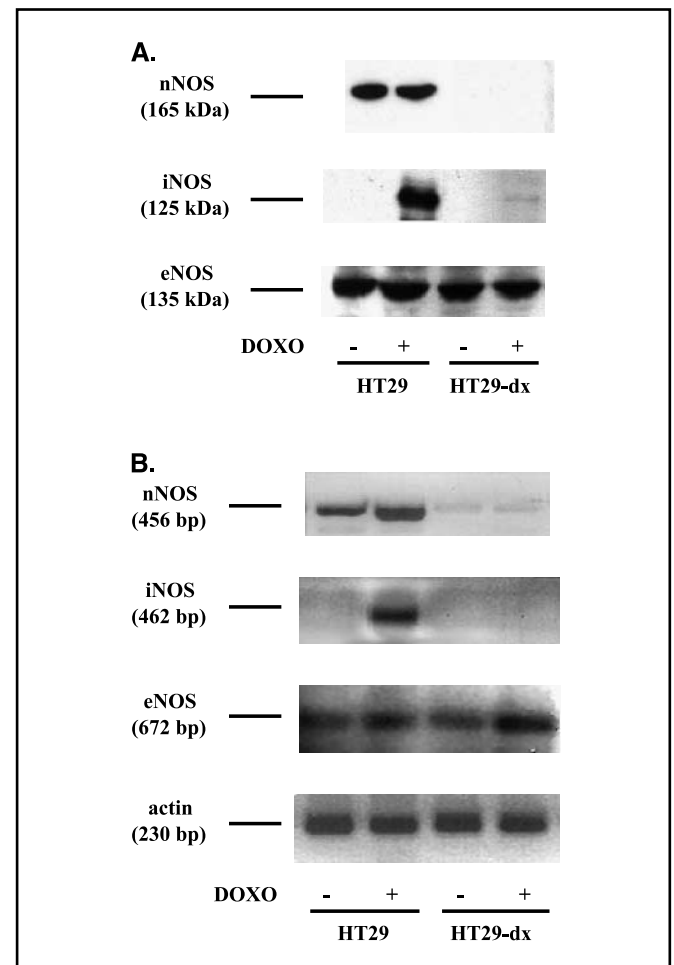
Western blotting and RT-PCR experiments showed that resting HT29 cells express two NOS isoforms (eNOS and nNOS), whereas



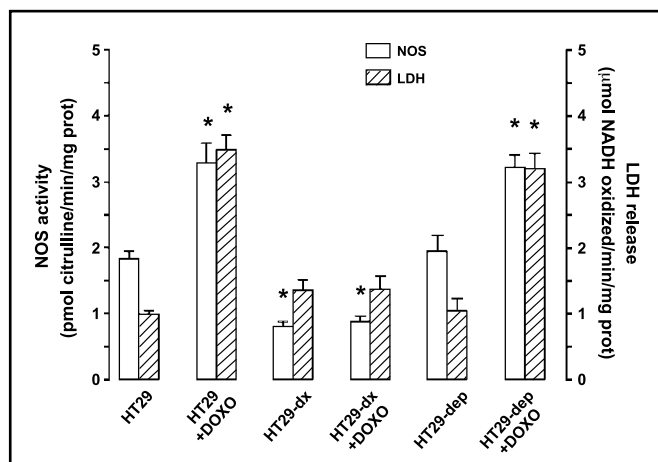
**Figure 4.** NOS activity (open columns) and nitrite levels (hatched columns) in HT29 and HT29-dx cells. After a 24-hour incubation in the absence and presence of doxorubicin (DOXO, 5 µmol/L), NOS activity was measured in cell lysates, and extracellular medium was checked for nitrite concentration (see MATERIALS AND METHODS). Measurements were done in duplicate and data are presented as mean ± SE ( $n = 6$ ). Versus HT29: \*,  $P < 0.05$ ; \*\*,  $P < 0.002$ ; \*\*\*,  $P < 0.001$ ; versus HT29 + DOXO: O,  $P < 0.001$ .

iNOS protein was not detectable, and the corresponding mRNA transcript was very low: following a 24-hour incubation with doxorubicin, the expression of eNOS and nNOS did not change, whereas that of iNOS (both protein and mRNA) clearly increased (Fig. 5). In HT29-dx cells, the NOS expression pattern was quite different: although eNOS content was the same as that found in HT29, nNOS was absent, both in resting and doxorubicin-stimulated cells; whereas, after the exposure to doxorubicin, the increase of iNOS protein and mRNA was absent (Fig. 5).

NOS activity could be fully restored in HT29-dx cells when doxorubicin was withdrawn from the culture medium (Fig. 6). After 7 days of suspension, both basal and doxorubicin-induced NOS activities of the HT29-dep cells were similar to those measured in the original HT29 cells. At the same time, after 7 days of suspension, HT29-dep cells recovered the same susceptibility to doxorubicin (Fig. 6). In line with these results, the cellular accumulation of the drug after a 3-hour incubation, which was  $0.608 \pm 0.015$  ng/mg cell protein ( $n = 3$ ) in HT29-dx cells, rose to  $1.221 \pm 0.09$  ng/mg cell protein ( $n = 3$ ,  $P < 0.001$ ) in HT29-dep



**Figure 5.** Western blotting detection of the NOS isoforms nNOS, iNOS, and eNOS (A) and RT-PCR (B) for NOS isoforms and β-actin in HT29 and HT29-dx cells. After a 24-hour incubation in the absence (-) or presence (+) of doxorubicin (DOXO, 5 µmol/L), cells were lysed and Western blottings and RT-PCR were done as described in MATERIALS AND METHODS. These figures are representative of three similar experiments for each technique.



**Figure 6.** NOS activity (open columns) and LDH release (hatched columns) in HT29 and HT29-dx cells. Before performing these experiments, a subpopulation of HT29-dx cells (HT29-dep) was cultured for 7 days in a medium deprived of the maintenance dose of doxorubicin. After a further 24-hour incubation of HT29, HT29-dx, and HT29-dep cells in the absence and presence of doxorubicin (DOXO, 5 μmol/L), NOS activity was measured in cell lysates, and extracellular medium was checked for LDH release (see MATERIALS AND METHODS). Measurements were done in duplicate and data are presented as mean ± SE ( $n = 3$ ). Versus HT29: \*,  $P < 0.001$ .

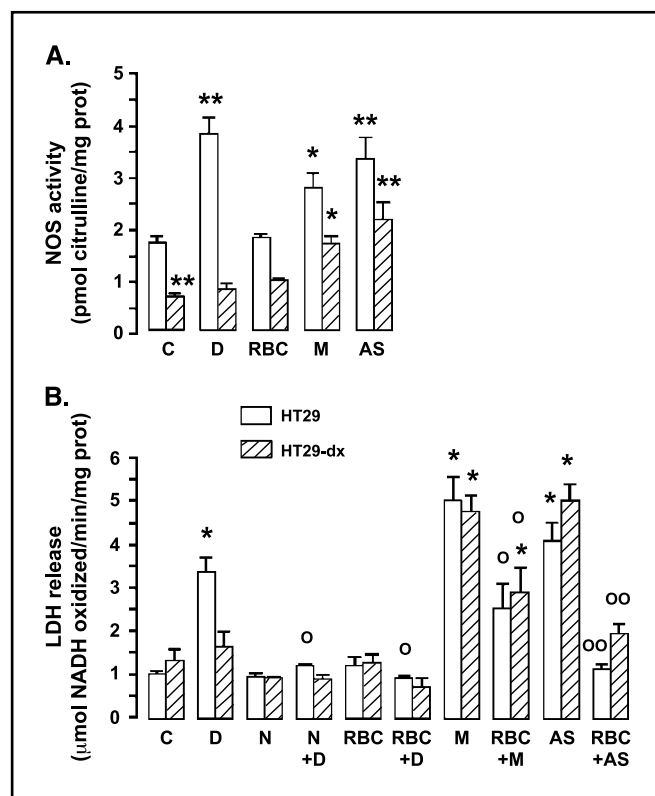
cells, a similar level to that found in the original HT29 cells ( $1.233 \pm 0.05$  ng/mg cell protein,  $n = 3$ ).

**Increased Endogenous Nitric Oxide Production Elicits a Cytotoxic Effect in HT29-dx Cells.** Because iNOS is inducible by inflammatory cytokines in a variety of tissues, including human colon cells (20), we incubated HT29 and HT29-dx cells for 24 hours with a mix of IFN- $\gamma$ , TNF- $\alpha$ , and IL-1 $\beta$ : this treatment induced a significant increase of both NOS activity and extracellular LDH in both cell populations (Fig. 7). It is known that several statins, used clinically as anti-cholesterolemic agents, could induce NO synthesis in different human tissues (21, 22). In previous experiments done in our laboratory on HT29 cells, atorvastatin strongly increased NO synthesis, in a dose-dependent and time-dependent manner (data not shown). We then incubated our cells with 25 μmol/L atorvastatin for 24 h: this dose induced NOS activity and increased LDH release significantly in both HT29 and HT29-dx cells (Fig. 7). Both cytokines mix and atorvastatin increased, under the same experimental conditions, the expression of iNOS (mRNA and protein) in HT29 and HT29-dx cells (data not shown). The cytotoxic effects of cytokines mix, atorvastatin, and doxorubicin were mediated by NO synthesis. Indeed, in the presence of the NOS inhibitor *N*<sup>G</sup>-monomethyl-L-arginine (L-NMMA) or of packed human erythrocytes (a reservoir of the potent NO scavenger oxyhemoglobin, devoid per se of effects on NOS, as shown in Fig. 7A), the cells exhibited a significant reduction of LDH release after incubation with doxorubicin. The presence of RBC inhibited also the release of LDH elicited by the cytokines mix or atorvastatin.

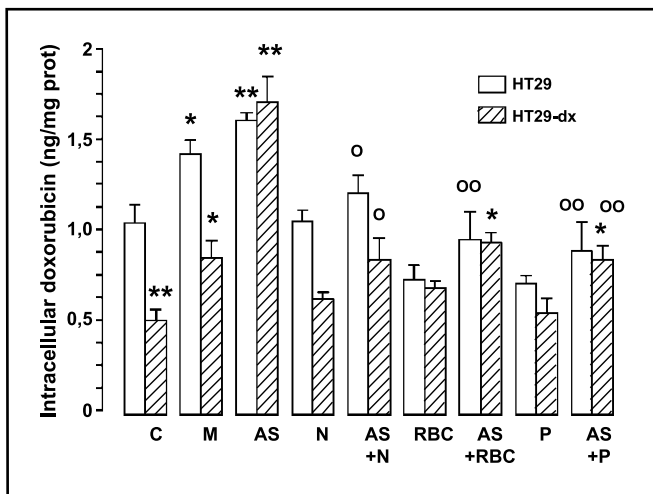
**Restoration of Nitric Oxide Production in HT29-dx Cells Reverses Doxorubicin-Resistance.** In order to investigate whether NO could be implicated not only as a mediator of the drug-induced cytotoxicity, but also as a modulator of intracellular drug accumulation, we incubated HT29 and HT29-dx cells with doxorubicin for 24 hours in the absence or presence of two NOS inducers, a NOS inhibitor and two NO scavengers (Fig. 8). Cytokines and atorvastatin potentiated the intracellular accumulation of the drug in both HT29 and HT29-dx cells. The coincubation of atorvastatin with L-NMMA or with the NO scavengers, i.e., packed erythrocytes

or 2-phenyl-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide (PTIO), significantly decreased the accumulation of intracellular doxorubicin in comparison with atorvastatin alone. No change of the drug content was seen with L-NMMA, erythrocytes or PTIO alone in comparison with control.

The effect of atorvastatin was reversible, and the changes in doxorubicin accumulation were concordant with the variations of NOS activity (Fig. 9): after a 24-hour incubation with atorvastatin, we observed an increase of both doxorubicin accumulation and NOS activity in HT29-dx cells; when we washed the cells after this period and reincubated them in fresh medium without atorvastatin for 48 hours, these two parameters returned to baseline. When we attempted a second stimulation with atorvastatin, both intracellular doxorubicin accumulation and NOS activity increased again (Fig. 9). The same behavior was observed in doxorubicin-sensitive cells (data not shown). These experiments gave us further evidence of a link between NO production and the reversion of drug resistance. We then supposed that the incubation of cells with a NO donor could increase doxorubicin accumulation. All the NO donors we chose, i.e., *S*-nitrosoglutathione, *S*-nitrosopenicillamine (SNAP), and sodium nitroprusside, increased the doxorubicin accumulation in a time-dependent and dose-dependent manner (data not shown).

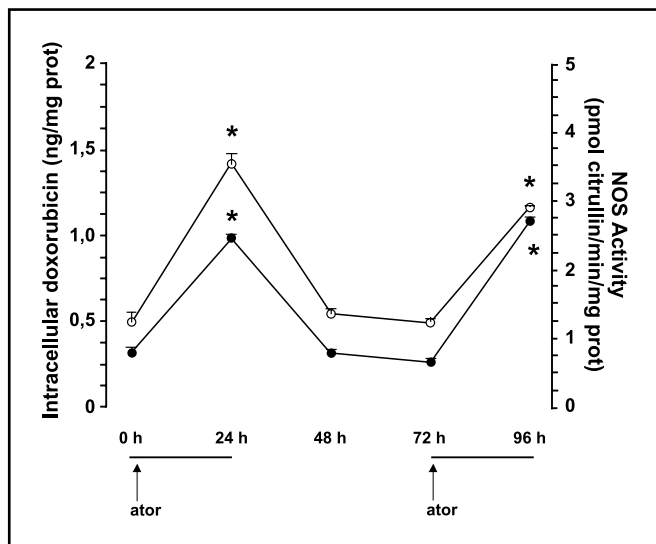


**Figure 7.** NOS activity (A) and LDH release (B) in HT29 (open columns) and HT29-dx (hatched columns) cells, in the presence of stimuli affecting NOS activity or NO levels. Cells were incubated for 24 hours in the absence (C) or presence of doxorubicin (D, 5 μmol/L), a cytokines mix (M: IFN- $\gamma$  5 ng/mL, TNF- $\alpha$  10 ng/mL, IL-1 $\beta$  0.5 ng/mL), atorvastatin (AS, 25 μmol/L); in some experimental sets, we added the NOS inhibitor L-NMMA (N, 1 mmol/L) or packed RBC (RBC, 10 μL/mL), as NO scavengers. NOS activity and LDH release were then measured as described in MATERIALS AND METHODS; measurements were done in duplicate and data are presented as mean ± SE ( $n = 3$ ). Versus control (C): \*,  $P < 0.01$ ; \*\*,  $P < 0.001$ ; versus doxorubicin or mix or atorvastatin alone: ○,  $P < 0.05$ ; ○○,  $P < 0.001$ .

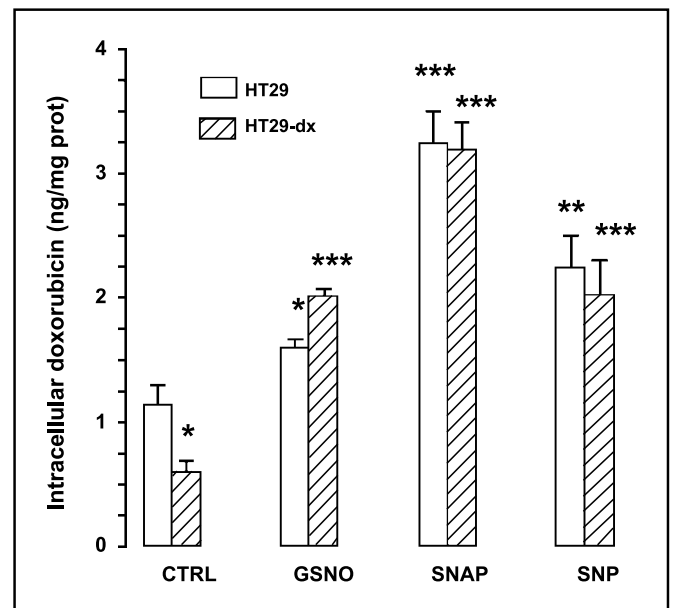


**Figure 8.** Effect of NOS induction, NOS inhibition and NO scavenging on intracellular doxorubicin accumulation. HT29 (open columns) and HT29-dx (hatched columns) cells were incubated in the absence (C) or presence of a cytokines mix (M: IFN- $\gamma$ , TNF- $\alpha$ , and IL-1 $\beta$  at 5, 10, and 0.5 ng/mL, respectively) or atorvastatin (AS, 25  $\mu$ mol/L), alone or together with L-NMMA (N, 1 mmol/L) or packed RBC (RBC, 10  $\mu$ L/mL) or PTIO (P, 100  $\mu$ mol/L). After 24 hours, the cells were washed and incubated for a further 3 hours in fresh medium containing 4  $\mu$ mol/L doxorubicin. The intracellular content of doxorubicin was measured as described in MATERIALS AND METHODS. Measurements were done in triplicate and data are presented as mean  $\pm$  SE ( $n = 5$ ). Versus HT29 (C): \*,  $P < 0.05$ ; \*\*,  $P < 0.001$ ; versus HT29-dx (C):  $\circ$ ,  $P < 0.05$ ;  $\circ\circ$ ,  $P < 0.001$ .

The effect of each compound was maximal at 100  $\mu$ mol/L for 3 hours (Fig. 10). The same effects that exogenous (NO donors) or endogenous (atorvastatin stimulation) NO had on doxorubicin resistance in HT29-dx cells were observed in two other doxorubicin-resistant cell populations, obtained from the human lung epithelial



**Figure 9.** Reversibility of atorvastatin's effect on doxorubicin accumulation and NOS activity in HT29-dx cells. Cells were incubated with atorvastatin (ator, 25  $\mu$ mol/L, 24 hours), then washed and detached; an aliquot of cells were lysed and checked for NOS activity and doxorubicin content (see MATERIALS AND METHODS). Another aliquot of atorvastatin-treated cells were cultured in atorvastatin-free medium for 48 hours, then incubated again with atorvastatin (25  $\mu$ mol/L, 24 hours). Throughout this time, NOS activity ( $\bullet$ ) and intracellular doxorubicin ( $\circ$ ) were measured every 24 hours. Each measurement ( $n = 3$ ) was done in duplicate and data are presented as mean  $\pm$  SE. Versus time 0 hour: \*,  $P < 0.001$ .

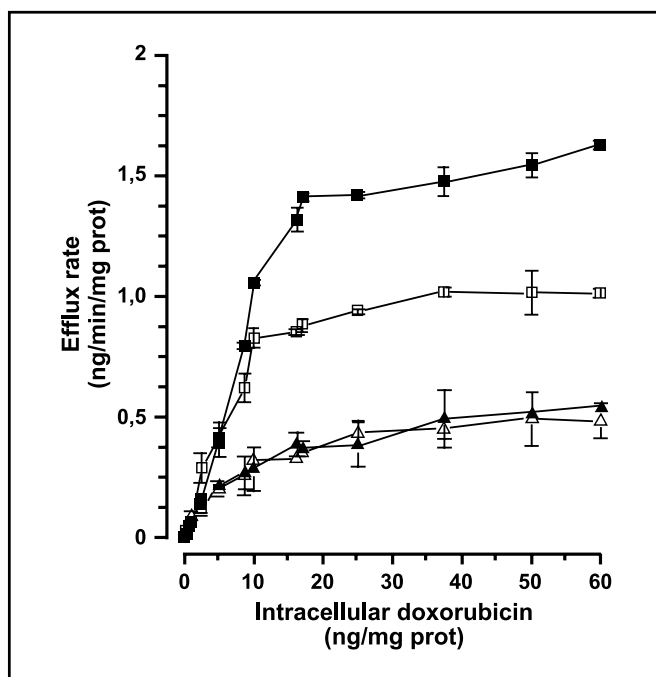


**Figure 10.** Effect of different NO donors on intracellular doxorubicin accumulation. HT29 (open columns) and HT29-dx (hatched columns) cells were incubated for 3 hours with doxorubicin (4  $\mu$ mol/L), in the absence (CTRL) or presence of one of the following NO donors (100  $\mu$ mol/L): S-nitrosoglutathione (GSNO), S-nitrosopenicillamine (SNAP), sodium nitroprusside (SNP). At the end of this incubation time, cells were washed, detached and their doxorubicin contents were analyzed as previously described. Measurements were done in duplicate and data are presented as mean  $\pm$  SE ( $n = 3$ ). Versus HT29 cells (CTRL): \*,  $P < 0.02$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

cells A549 (A549-dx) and from the human myelogenous leukemic cells K562 (K562-dx; data not shown).

**The  $V_{max}$  of Doxorubicin Efflux Is Lowered by the Exposure to the Nitric Oxide Donor SNAP.** In order to clarify the mechanism by which NO affects doxorubicin accumulation, we studied the kinetics of the drug efflux. After incubating the cells in the absence or presence of the NO donor SNAP, we loaded them with different amounts of doxorubicin, and measured the subsequent efflux of the drug in 10 minutes (Fig. 11). The efflux of doxorubicin showed a higher  $V_{max}$  in HT29-dx cells than in HT29 cells ( $1.66 \pm 0.02$  versus  $0.98 \pm 0.01$  ng/min/mg protein), whereas the affinity for the substrate did not change significantly ( $K_m$ ,  $7.90 \pm 1.54$  versus  $6.00 \pm 0.3$  ng/mg protein). When cells were preincubated with SNAP, the  $V_{max}$  of the efflux decreased dramatically ( $0.50 \pm 0.07$  in HT29-dx cells and  $0.43 \pm 0.04$  ng/min/mg protein in HT29 cells, respectively), with no modification in  $K_m$  ( $6.50 \pm 1.57$  versus  $5.58 \pm 1.12$  ng/mg protein). Our data thus suggest that NO donors could affect doxorubicin efflux acting as uncompetitive inhibitors both in sensitive and resistant cells.

**Nitric Oxide Increases Doxorubicin Accumulation in a cGMP-Independent Way.** NO may stimulate cGMP synthesis via the activation of a soluble guanylate cyclase; the increase of intracellular cGMP can lead to the activation of a cGMP-dependent protein kinase (PKG), that can mediate several effects of NO, such as smooth muscle relaxation, platelet aggregation, cell growth, and differentiation (23). To check whether the NO-induced reversion of drug resistance was mediated by a cGMP increase and PKG activation, we measured the accumulation of doxorubicin in the absence or presence of 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ), a selective inhibitor of soluble guanylate cyclase, 8-Br-cGMP, a more stable cGMP analogue, and Rp-8-Br-cGMPs, a PKG inhibitor; these



**Figure 11.** The NO donor SNAP modifies the kinetics of doxorubicin efflux. HT29 (□) and HT29-dx (■) cells were loaded in PBS buffer for 10 minutes at 37°C with different amounts of doxorubicin (1-250 μmol/L), then washed: one aliquot of each sample was checked immediately for the content of drug (time 0), whereas a second aliquot was further incubated in fresh PBS for another 10 minutes, washed and tested for the drug content as previously described (for details see MATERIALS AND METHODS). The rate of doxorubicin efflux was calculated from the difference between the drug concentration at time 0 and at 10 minutes, respectively. The same procedure was repeated after having previously incubated HT29 (△) and HT29-dx (▲) cells for 3 hours with 100 μmol/L SNAP. Measurements ( $n = 3$ ) were done in duplicate and data are presented (mean ± SE) as the rate of efflux versus the intracellular drug concentration at time 0.

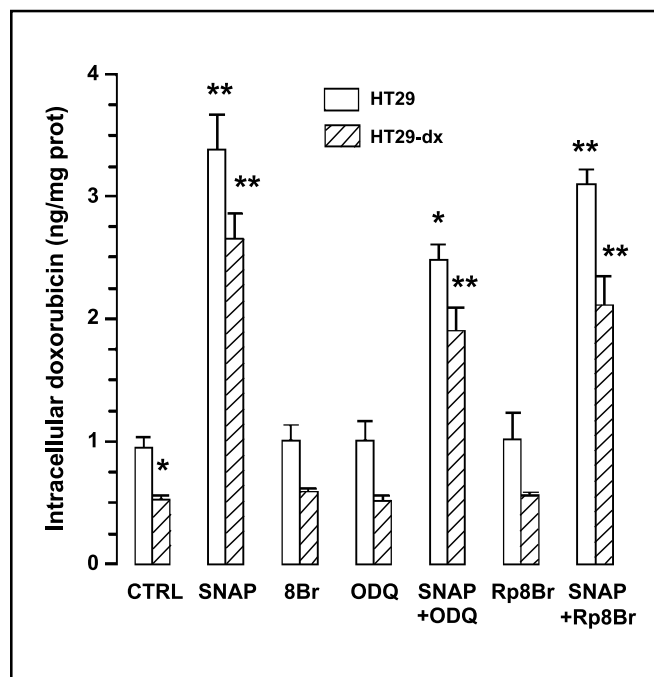
agents were incubated in different combinations with doxorubicin (Fig. 12). The effect of SNAP on doxorubicin accumulation was neither inhibited by ODQ or Rp-8-Br-cGMPS, nor could it be mimicked by 8-Br-cGMP.

#### SNAP Induces Tyrosine Nitration in the MRP3 Transporter.

To check whether NO could affect doxorubicin accumulation through the nitration of tyrosine residues in the drug efflux pumps (previously observed to be overexpressed in HT29-dx cells), we incubated HT29, HT29-dx, and HT29-dep cells for 3 hours with SNAP. The whole cellular lysate was then immunoprecipitated with a specific anti-nitrotyrosine antibody and the immunoprecipitated proteins were subjected to Western blotting, using anti-Pgp or anti-MRP3 antibodies (Fig. 13): with this procedure, the presence of nitrotyrosine residues were detectable in MRP3 (mainly in HT29-dx cells, where MRP3 was more expressed, as shown in Fig. 2), but not in Pgp protein.

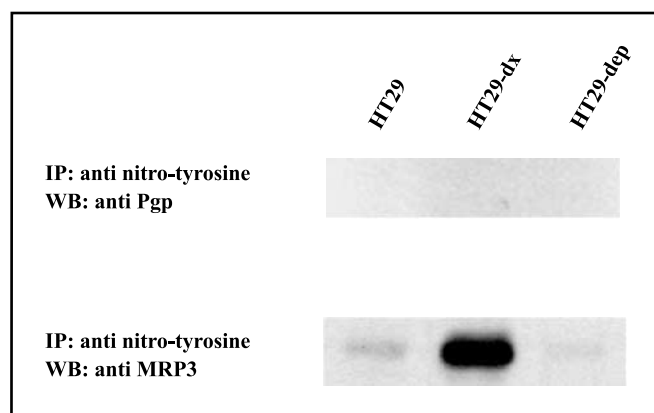
## Discussion

Chemotherapy, one of the most efficient therapeutic approaches to solid and hematologic cancers, has led to improved remission and survival in many malignancies. Innate or acquired cross-resistance to different antineoplastic agents occurs frequently, and may alter the prognosis of some common cancers, such as colon, lung, breast cancers, and certain types of adult leukemias. MDR is regarded as a multifactorial process (2). However, several studies



**Figure 12.** Role of cGMP pathway in NO-mediated doxorubicin accumulation. HT29 (open columns) and HT29-dx (hatched columns) cells were incubated for 3 hours with doxorubicin (4 μmol/L), in the absence (CTRL) or presence of SNAP (100 μmol/L), 8-Br-cGMP (8Br, 1 mmol/L), ODQ (ODQ, 100 μmol/L) and Rp-8-Br-cGMPS (Rp8Br, 100 μmol/L). The intracellular doxorubicin accumulation was measured as described in MATERIALS AND METHODS. Measurements were done in duplicate and data are presented as mean ± SE ( $n = 3$ ). Versus HT29: \*,  $P < 0.005$ ; \*\*,  $P < 0.001$ .

have indicated that a cardinal mechanism of resistance could be a major extrusion rate of antineoplastic drugs. Indeed, the decreased cellular retention of several of these toxic drugs is one of the most peculiar features of MDR. The discovery that the ATP-binding cassette family transporters recognize many antineoplastic agents as



**Figure 13.** Western blot detection of nitrated drug efflux pumps. HT29, HT29-dx, and HT29-dep (HT29-dx cells cultured for 7 days in a medium without doxorubicin) cells were incubated for 3 hours with 100 μmol/L SNAP. Afterwards, cells were lysed and the whole cellular lysate was immunoprecipitated (IP) with an anti-nitrotyrosine polyclonal antibody. The immunoprecipitated proteins were subjected to Western blotting (WB), using an anti-Pgp or an anti-MRP3 antibody (see MATERIALS AND METHODS). The experiment is representative of three similar experiments.

their substrates, has led to the development of compounds that try to reverse MDR with different mechanisms (1, 4).

Cultured tumor cells, when selected for resistance to an anti-neoplastic agent, often acquire cross-resistance to others. In this study, we used a cell line derived from human colon cancer, one of the most frequent solid tumors that show MDR. From these cells we obtained a model of doxorubicin-resistant cells (HT29-dx) through previous experimental protocols (24, 25). Doxorubicin is a spontaneously fluorescent compound: we used this characteristic to measure the different abilities of HT29 and HT29-dx cells to accumulate the antineoplastic agent during 3 hours of exposure. During this time, the intracellular levels of doxorubicin progressively decreased. At the same time, we observed a progressive efflux of the drug in the extracellular medium when we used cells that had been preloaded with doxorubicin. By the end of the first hour, the accumulation of the drug was significantly lower, and its efflux higher, in HT29-dx cells when compared with HT29 cells. We then asked the question as to whether some of the pumps implicated in MDR showed differential levels of expression in these two cell populations. Pgp is well known as the main extrusion pump for hydrophobic drugs (such as anthracyclines, Vinca alkaloids, epipodophyllotoxins, taxanes, actinomycin D, mitoxantrone; ref. 26) and its level of expression is high, both in normal and transformed intestinal tissue (12). Other members of the ATP-binding cassette transporter family, which may contribute to doxorubicin resistance (27), are also commonly found in intestinal mucosa (26, 28). HT29 cells did not express detectable levels of Pgp and MRP1-3, whereas HT29-dx cells expressed both Pgp and MRP3, but not MRP1 and MRP2. In other experimental works (29, 30), no correlation has been found between MRP3 expression and protection from doxorubicin's cytotoxicity. This discrepancy could be due to different experimental conditions. In our study, to test the ability of resistant cells to activate the drug efflux, we used a short incubation time (3 hours), whereas Kool and coworkers (29, 30) did not use the HT29 cell line and exposed different doxorubicin-resistant and cisplatin-resistant cell lines to the drug for up to 6 days. To correlate accumulation data and the onset of a doxorubicin-resistance, we measured the release of LDH in the extracellular medium, as a sensitive index of cytotoxicity (14); the 24-hour incubation with doxorubicin increased significantly extracellular LDH in HT29 but not in HT29-dx cultures. According to the development of a MDR phenotype, HT29-dx cells also showed resistance to the cytotoxic effects of another antineoplastic drug, structurally unrelated to doxorubicin. Indeed, after a 48-hour incubation with cisplatin, the LDH release was substantially unmodified in HT29-dx cells, but was significantly increased in HT29 cells. This suggests that HT29-dx cells also exhibit a resistance to the cytotoxic effects of cisplatin, a finding consistent with the overexpression of MRP3, a transporter that in some models has been related to cisplatin resistance (1).

NO has been shown to inhibit glycolysis (31) and respiration (32), to suppress DNA synthesis and cell cycle progression (33), and to induce apoptosis (34). Doxorubicin evokes a significant increase of NO synthesis in murine breast cancer EMT-6 cells (5), mouse macrophages (35), and rat cardiac H9c2 cells (6). NO can act as a cytotoxic and proapoptotic agent (11), and doxorubicin has also been shown to inhibit tumorigenesis via a NO-dependent mechanism (5). Therefore, conceptually, a reduced ability to produce NO may be a way to elude the doxorubicin action in resistant cells. Our results show that human colon carcinoma cells synthesize NO in response to doxorubicin: this effect is accompanied by an increase

of intracellular NOS activity and iNOS expression. Conversely, in another colorectal cancer cell line (DLD-1), doxorubicin did not increase nitrite in the culture medium, and inhibited the synthesis of NO induced by IFN- $\gamma$ /IL-1 $\beta$  (36): such a difference can be explained by the use of different cell types. This hypothesis is supported by the observation that, under the same experimental conditions we used to study the effects of doxorubicin on NO synthesis, Jung and colleagues (36) did not observe a cytotoxic effect of the drug, in spite of a concentration of doxorubicin that was 4-fold higher than in our experiments. In HT29-dx cells, differently than in HT-29, doxorubicin did not elicit any increase of NO synthesis and iNOS expression. Interestingly, even before drug stimulation, NO synthesis and NOS activity were significantly lower in HT29-dx than in HT29 cells: this could be attributable to the disappearance of nNOS expression observed in HT29-dx cells. The reason for this different pattern of nNOS expression in sensitive and resistant cells is currently under investigation in our laboratory.

The HT29-dx phenotype was not permanent, as cells had to be cultured continuously in the presence of a low dose of doxorubicin for the drug resistance to be maintained. Removal of the drug pressure led to the recovery of the previous sensitive phenotype: indeed, HT29-dx cells deprived for 7 days of the maintenance dose (HT29-dep cells) showed levels of Pgp and MRP3 expression and of NOS activity superimposable to those of HT29 cells. The ability of an acute doxorubicin exposure to increase intracellular drug accumulation, NOS activity, and LDH release was completely restored as well. This is not surprising, as HT29 cells exhibit reversal of drug resistance when the drug is withdrawn (24, 37, 38). This pharmacologic adaptation, by which tumor cells can modulate some signaling pathways depending on environmental conditions, is an intriguing mechanism, which poses itself as an alternative to the classical clonal selection (39), and may underlie, at least partly, MDR *in vivo*. To our knowledge, no study has exhaustively investigated the relative contributions to MDR of these two adaptative mechanisms.

As NOS activity and LDH release seemed to be directly related in HT29-dx cells, we hypothesized that NO could be responsible, at least partly, for the doxorubicin-induced cellular damage, and that decreased NO synthesis could be linked to the appearance of an MDR phenotype. If our hypothesis held true, the induction of an increased NO synthesis in HT29-dx cells should promote cell death. To obtain a high stable production of NO, we used well-known agents inducing iNOS, such as a mix of IFN- $\gamma$ , TNF- $\alpha$ , and IL-1 $\beta$ . Several statins also increase NO synthesis in human (21, 22) and murine cells (40, 41). By inhibiting the 3-hydroxy-3-methylglutaryl CoA (HMGCoA) reductase, statins prevent the isoprenylation of small GTPases such as Rho proteins, playing an important role in the regulation of iNOS expression (42). In many experimental models, different statins have induced apoptosis (43, 44) and potentiated the cell death induced by some chemotherapeutic agents (45, 46), but a correlation between statin-induced apoptosis and NO has not yet been reported. Atorvastatin, a lipophilic HMGCoA reductase inhibitor, increased the synthesis of NO in HT29 and HT29-dx cells in a dose-dependent and time-dependent manner, as well as the release of LDH in the extracellular medium. To correlate the cytotoxic effect with the production of NO, we incubated the cells with doxorubicin, cytokines, and atorvastatin in the presence of the arginine analogue L-NMMA, a competitive NOS inhibitor, or of RBC, potent NO scavengers because of the presence



of oxyhemoglobin. When NOS activity was inhibited or NO was removed, we observed a significant decrease of the cellular damage, providing further evidence of the role of NO in resistance and apoptosis of HT29 cells.

Doxorubicin did not increase NO synthesis in HT29-dx cells: this characteristic of HT29-dx cells could be explained with their capacity to extrude doxorubicin more rapidly, thus reducing the intracellular amount of drug and consequently its ability to induce iNOS. What this did not explain was why HT29-dx cells also exhibited a basal NOS activity significantly lower than that observed in HT29 cells. We therefore wondered if we could explain the direct correlation we observed between NO synthesis and drug accumulation in another way, i.e., whether it was the reduced NOS activity in HT29-dx cells responsible, at least partly, for the onset of drug resistance. With this goal in mind, we wanted to know if increasing the endogenous NO synthesis or exposing the cells to NO donor compounds could modulate the intracellular accumulation of doxorubicin. A 24-hour incubation with cytokines or atorvastatin potentiated the doxorubicin accumulation in HT29 and HT29-dx cells. Statins have been reported to be competitive inhibitors of Pgp activity (47): in our experiments, however, the effect of atorvastatin was NO-dependent (as well as the effect of cytokines, not shown), because it was reversed by L-NMMA and NO scavengers. Furthermore, the actions of atorvastatin on both doxorubicin accumulation and NOS activity were reversible, and these two events happened in parallel during the experiment, lending further support to the hypothesis that they were related. Finally, when the cells were exposed to exogenous NO by incubation with three different NO donors, the accumulation of doxorubicin markedly increased both in sensitive and resistant cells.

Taken as a whole, these data suggest that NO inhibits the efflux of doxorubicin, thus decreasing the resistance of the cell to the drug. This hypothesis was confirmed by kinetic data. Doxorubicin transport is a saturable process: no variation of  $K_m$  was observed between sensitive and resistant cells, although significant differences in  $V_{max}$  occurred. HT29-dx cells exhibited a nearly double  $V_{max}$  than HT29 cells, suggesting the presence of higher amounts of transporter molecules in the membrane of resistant cells. SNAP drastically reduced the  $V_{max}$  in both the cell populations, without affecting  $K_m$ . This suggests that NO reduces the number of functionally active transporters, perhaps by

altering the proper conformation of the protein(s) at a site crucial for drug transport.

Because soluble guanylate cyclase is an important target of NO, and PKG plays a key role in different NO-mediated cellular processes (23), we investigated if cGMP and PKG could mediate the action of NO on drug transport. Neither activators nor inhibitors of the cGMP/PKG pathway appreciably changed doxorubicin accumulation. On the other hand, NO may influence the conformation and activity of enzymes and transporters via direct cysteine S-nitrosylation and tyrosine nitration (48, 49). Such modifications involving membrane proteins have been proposed as a novel signaling transduction mechanism (48). We investigated if Pgp or MRP3, two glycoproteins involved in MDR which we found overexpressed in HT29-dx cells, were nitratable by the NO donor SNAP. This occurred with MRP3 but not with Pgp. This is not surprising, if we consider that compared with Pgp, MRP3 contains a higher number of tyrosine residues potentially susceptible to nitration (17 versus 42). The greater nitration observed in HT29-dx cells could be explained by the higher expression of MRP3 in this population when compared with HT29 and HT29-dep cells. However, the low nitration detectable in HT29 cells may explain why they exhibited increased doxorubicin accumulation when stimulated by exogenous and endogenous NO.

To our knowledge, this is the first work that shows a relationship between NO and antineoplastic drug resistance. We also observed that NO donors increase drug accumulation in two other doxorubicin-resistant cell populations, obtained from the human lung epithelial cells A549 and the human myelogenous leukemic cells K562. Our work suggests the possibility that doxorubicin resistance could be reverted by using well-known drugs, such as atorvastatin and NO donors. Although our data were obtained *in vitro*, they point to novel possibilities as to their use *in vivo* for the clinical correction of MDR.

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