Chemosensitization of Cancer *In vitro* and *In vivo* by Nitric Oxide Signaling

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Abstract **Purpose:** Hypoxia contributes to drug resistance in solid cancers, and studies have revealed that low concentrations of nitric oxide (NO) mimetics attenuate hypoxia-induced drug resistance in tumor cells *in vitro*. Classic NO signaling involves activation of soluble guanylyl cyclase, generation of cyclic GMP (cGMP), and activation of cGMP-dependent protein kinase. Here, we determined whether chemosensitization by NO mimetics requires cGMP-dependent signaling and whether low concentrations of NO mimetics can chemosensitize tumors *in vivo*.

Experimental Design: Survival of human prostate and breast cancer cells was assessed by clonogenic assays following exposure to chemotherapeutic agents. The effect of NO mimetics on tumor chemosensitivity *in vivo* was determined using a mouse xenograft model of human prostate cancer. Drug efflux *in vitro* was assessed by measuring intracellular doxorubicin-associated fluorescence.

Results: Low concentrations of the NO mimetics glyceryl trinitrate (GTN) and isosorbide dinitrate attenuated hypoxia-induced resistance to doxorubicin and paclitaxel. Similar to hypoxia-induced drug resistance, inhibition of various components of the NO signaling pathway increased resistance to doxorubicin, whereas activation of the pathway with 8-bromo-cGMP attenuated hypoxia-induced resistance. Drug efflux was unaffected by hypoxia and inhibitors of drug efflux did not significantly attenuate hypoxia-induced chemoresistance. Compared with mice treated with doxorubicin alone, tumor growth was decreased in mice treated with doxorubicin and a transdermal GTN patch. The presence of GTN and GTN metabolites in plasma samples was confirmed by gas chromatography.

Conclusion: Tumor hypoxia induces resistance to anticancer drugs by interfering with endogenous NO signaling and reactivation of NO signaling represents a novel approach to enhance chemotherapy.

There is evidence that the tumor microenvironment plays a significant role in determining the sensitivity of cancer cells to chemotherapeutic agents. For example, glucose deprivation has been shown to induce chemoresistance in Chinese hamster cells and human colon and ovarian cell lines (1, 2). Similarly, changes in the pH of the tumor microenvironment lead to alterations in cell membrane permeability, which in turn can affect the ability of chemotherapeutic drugs to enter the cell (3, 4). There is also evidence that, independently of changes in pH, tumor hypoxia decreases the efficacy of chemotherapy against solid cancers (5). One explanation for the lack of cytotoxicity toward hypoxic tumor cells is that many conventional anticancer drugs require oxygen for maximal activity (5). However, regardless of the oxygen requirements for maximal drug action, studies have shown that transient exposure of cells to hypoxia alters their phenotype so that they increase their intrinsic resistance to chemotherapeutic agents (6, 7). Hypoxia inducible factor-1 is a transcriptional activator of many oxygen-regulated genes and has recently been implicated in hypoxia-induced resistance to chemotherapeutic agents (8). Although the nature of hypoxia-induced drug resistance is still poorly understood, it is likely that the participation of hypoxia inducible factor–dependent and –independent genes is required.

Our studies have revealed that the rapid acquisition of drug resistance resulting from exposure of various types of cancer cells to hypoxia can be attenuated by low concentrations of nitric oxide (NO) mimetics (9, 10). Matthews et al. (9) showed that the hypoxia-mediated acquisition of resistance to doxorubicin and 5-fluorouracil in human breast carcinoma and mouse melanoma cells could be significantly inhibited by administration of

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Received 7/25/06; revised 10/20/06; accepted 11/29/06.

Grant support: Canadian Institutes of Health Research grant MOP-57871 and U.S. Army Medical Research and Materiel Command grant DAMD1703-1-0070 (C.H. Graham); Heart and Stroke Foundation of Ontario grant T-5162 (B.M. Bennett); Canadian Institutes of Health Research/Canadian Prostate Cancer Research Initiative Doctoral Research Award (L.J. Frederiksen); Natural Sciences and Engineering Council of Canada Postgraduate Scholarship and Canadian Institutes of Health Research Canada Graduate Scholarship Doctoral Award (R. Sullivan).

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doi:10.1158/1078-0432.CCR-06-1807

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low concentrations of the NO mimetics glyceryl trinitrate (GTN) or diethylenetriamine-NO adduct. The fact that pharmacologic inhibition of NO production in cancer cells resulted in a drug resistance phenotype similar to that induced by hypoxia led us to propose that an important aspect of the mechanism of hypoxia-induced drug resistance in cancer cells is the suppression of endogenous NO production (9). More recently, studies have also revealed that low concentrations of NO mimetics are able to attenuate the acquisition of resistance to doxorubicin in human PC-3 and mouse TRAMP-C2 prostate cancer cells incubated in hypoxia (10) or drug resistance associated with spheroid culture of human MDA-MB-231 breast carcinoma cells (11).

Nitric oxide is a small gaseous molecule with key roles in numerous physiologic processes such as vasodilation, platelet adhesion, vascular remodeling, neurotransmission, immune response regulation, cell growth, and apoptosis. It is produced from L-arginine in a reaction catalyzed by the enzymes NO synthases (NOS), with oxygen as a required cofactor (12–14). Some studies have shown that hypoxia increases NOS expression (15, 16). However, our previous studies revealed no significant effect of hypoxia on the levels of the three known isoforms of NOS enzymes in MDA-MB-231 and PC-3 cells (9, 10). Whereas NO signaling is multifaceted, the fact that low concentrations of this molecule (<1 μmol/L) are able to inhibit hypoxia-induced drug resistance suggests that the mechanism responsible for this effect requires the interaction of NO with transition metals (17). Classic NO signaling involves the NO-mediated activation of the iron-containing enzyme soluble guanylyl cyclase (sGC), which in turn catalyzes the conversion of GTP into cyclic GMP (cGMP). The latter subsequently activates protein kinase G (PKG), leading to the phosphorylation of various target molecules that regulate cell function and gene expression.

Therefore, based on the evidence presented above indicating that NO may act as an important chemosensitizer of hypoxic tumor cells, the main objective of the present study was to determine whether NO interferes with hypoxia-induced drug resistance by activating a signaling pathway involving the production of cGMP. The study also determined whether activation of NO signaling results in chemosensitization of tumors *in vivo*.

Materials and Methods

Cells. Human PC-3 and DU-145 prostatic adenocarcinoma cells and human MDA-MB-231 breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA). PC-3 cells were maintained in monolayer culture in Kaighn's modification of Ham's F12 medium supplemented with 10% fetal bovine serum (Life Technologies Invitrogen Corporation, Burlington, Ontario, Canada), whereas DU-145 and MDA-MB-231 cells were cultured in RPMI 1640 (Life Technologies Invitrogen Corporation) supplemented with 5% fetal bovine serum.

Culture conditions. Cells were cultured in six-well plates and were at 70% to 80% confluence at the start of all experiments. The culture medium was changed on all groups before and following incubations in standard or hypoxic conditions as well as incubations in the presence of chemotherapeutic agents. For incubations in standard conditions (20% O_2), cells plated in six-well culture plates were placed in a Sanyo CO_2 incubator (5% CO_2 in air, 37°C; Esbe Scientific, Markham, Ontario, Canada), whereas for culture in hypoxia, cells were placed in airtight chambers (BellCo Biotechnology, Vineland, NJ) that were flushed with a gas mixture of 5% CO_2/95% N_2. Oxygen concentrations within these chambers were maintained for 24 h at 0.5% using Pro-Ox model 110 O_2 regulators (Biospherix, Redfield, NY).

To determine the effect of exogenous NO on hypoxia-induced drug resistance, randomly selected culture plates were incubated with various concentrations (0.1 nmol/L-1 μmol/L) of the NO mimetic agents GTN (Sabex, Boucherville, Quebec, Canada) or isosorbide dinitrate (ISDN; Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) administered at the beginning of the 24-h hypoxic exposure.

The expression of NOS-II and NOS-III has been shown in DU-145 cells (18), whereas all three NOS isoforms (I, II, and III) have been shown to be expressed by PC-3 and MDA-MB-231 cells (9, 10). To examine the effect of inhibition of endogenous NO production on drug resistance, cells were exposed to various concentrations of the NO synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA; 5 μmol/L-5 mmol/L; Calbiochem-Novobiochem, La Jolla, CA) or the inactive enantiomer D-NMMA in 20% O_2 for 12 h before doxorubicin exposure.

The involvement of the NO-cGMP-PKG signaling pathway in the regulation of chemosensitivity was assessed by clonogenic assays using DU-145 cells treated for 24 h as outlined below before doxorubicin exposure. To inhibit cGMP production, well-oxygenated (20% O_2) cells were incubated in the presence of the specific sGC inhibitor 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ, 50 nmol/L-10 μmol/L; Sigma-Aldrich Canada). To inhibit PKG activity, cells were incubated with the selective PKG inhibitor KT5823 (1-15 μmol/L) at 20% O_2. To stimulate PKG activity, cells were incubated with the nonhydrolyzable analogue of cGMP, 8-bromo-cGMP (8-Br-cGMP; 10-100 nmol/L), in 20% or 0.5% O_2.

Exposure to chemotherapeutic agents and clonogenic (colony formation) assay. Following exposure of cells to either 20% or 0.5% O_2, cultures were incubated with doxorubicin (0-100 μmol/L; Sigma-Aldrich Canada) or paclitaxel (50 μmol/L; Sigma-Aldrich Canada) for 1 h at 20% O_2 in a standard CO_2 incubator. No additional chemicals or inhibitors were present in the culture media during the 1-h incubation with chemotherapeutic agent. Cultures were then washed with drug-free PBS and cells were harvested with 0.075% trypsin-EDTA in PBS and counted with a hemocytometer. Cells were reseeded in six-well tissue culture plates at appropriate numbers to allow for ease of colony counting. Typically, 100 to 250 cells per well were plated for control (no chemotherapeutic) groups and 1,000 to 2,500 cells per well for the chemotherapeutic agent–treated groups. Survival of the tumor cells following exposure to chemotherapeutic agents was determined by a clonogenic assay as previously described (10). After 7 to 14 days, colonies were fixed with acetic acid-methanol (1:4) and stained with dilute crystal violet before counting.

To determine the potential role of ATP-binding cassette (ABC) drug efflux transporters, cells were incubated with doxorubicin (12.5 μmol/L) following exposure to either 20% or 0.5% O_2 as described above. Cells were then reseeded in medium containing either R(+)-verapamil (10-100 μmol/L; Sigma-Aldrich Canada) or reserpine (0.1-10 μmol/L; Sigma-Aldrich Canada). The medium was replaced 24 h later with regular medium and cells were cultured for 7 to 14 days before colonies were fixed, stained, and counted.

Doxorubicin efflux assay. Cells were exposed to either 20% or 0.5% O_2 for 24 h and incubated with doxorubicin (12.5 μmol/L) for 1 h in the dark under standard culture conditions. Cells were then washed with PBS and allowed to efflux in regular medium for 1.5 h at 37°C. Cells incubated at 4°C were included to control for background efflux. After efflux, cells were washed twice with ice-cold PBS and the intracellular doxorubicin-associated median fluorescence intensity was measured with a Beckman Coulter EPICS Altra HSS flow cytometer. Excitation was performed with an argon laser operating at 488 nm; the emitted fluorescence was collected at 575 ± 10 nm; and at least 10,000 events per sample were collected. Data are expressed relative to the median fluorescence intensity at $t = 0$ for cells exposed to 20% O_2 and reported as the mean median fluorescence intensity from three independent experiments.

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Growth of human PC-3 prostate tumors in nude mice. Studies involving mice were conducted in accordance with the guidelines of the Canadian Council on Animal Care. Six- to eight-week-old male NIH Swiss Nude mice (Taconic, Germantown, NY) were inoculated s.c. into the left hind flank with 2×10^6 PC-3 human prostate cancer cells in 0.2-mL PBS. The viability of the inoculated cells was 95% as determined by clonogenic assays on parallel samples. Therapeutic interventions were initiated when tumors reached a volume of 200 to 250 mm^3. In a pilot study, it was determined that biweekly i.p. injections of 4 mg/kg doxorubicin did not result in a complete inhibition of tumor growth. Thus, this protocol was used in subsequent experiments to determine whether low-dose GTN administration enhances doxorubicin therapy.

Mice were randomized into four treatment groups (10 to 12 mice per group): (*a*) Mice receiving doxorubicin + a square (0.25 cm^2) Minitran GTN transdermal patch (3M Pharmaceuticals, London, Ontario, Canada; 7.5 μg/h changed daily) placed on the back of the neck. Transdermal patches were covered with a thin layer of New Skin Liquid Bandage (Medtech, Jackson, WY) to prevent mice from removing them during treatment. (*b*) Mice receiving doxorubicin + transdermal placebo patch. (*c*) Mice receiving vehicle (PBS) + Minitran GTN transdermal patch. (*d*) Mice receiving vehicle (PBS) + transdermal placebo patch.

Mice were weighed and tumors were measured with digital calipers twice a week. Tumor volumes were calculated according to the formula [largest tumor diameter × (smallest tumor diameter)^2] × 0.5. Mice were sacrificed by pentobarbital overdose when they met the criteria for euthanasia. These criteria, established by the Canadian Council on Animal Care, included 20% weight loss, immobility, abnormal posture, withdrawal, restlessness, and sunken eyes.

Determination of plasma GTN and GTN metabolites in nude mice. Following pentobarbital overdose at the end of treatment course, blood (1 mL) was collected by cardiac puncture, placed into heparinized microcentrifuge tubes, and immediately centrifuged. Plasma was then removed and stored at −80°C until further analysis. Aliquots of plasma (250 μL) were assayed for GTN and the GTN metabolites glyceryl-1,2-dinitrate (1,2-GDN) and glyceryl-1,3-dinitrate (1,3-GDN) by megabore capillary column gas chromatography with electron capture detection as previously described (19).

Calculations and statistical analysis. Plating efficiency was calculated from the number of surviving colonies expressed as a proportion of the total number of cells seeded. Surviving fractions were determined by dividing the plating efficiency of drug-treated groups by the plating efficiency of their respective control groups. All data are presented as mean relative surviving fraction (all groups normalized to control) with SEs. Unless otherwise indicated, relative survival was calculated using the survival of cells incubated at 20% O_2 alone followed by a 1-h exposure to 12.5 μmol/L doxorubicin as a reference point. Statistical significance was determined by use of one-way-ANOVA followed by Fisher's post hoc analysis. Unpaired two-tailed Student's *t* test was used to determine significance when only two sets of data were compared. For the *in vivo* studies, the slopes of the linear portion of the tumor growth curve for each mouse were calculated using linear regression. Slopes of growth curves for individual tumors were determined using 4 to 13 tumor volume measurements (mean of 8 measurements) covering the linear component of the growth curve. For all groups, R^2 values for the slopes of the tumor growth curves ranged from 0.79 to 0.99, with a mean R^2 of 0.93 ± 0.05 (SD). Because the slopes did not assume a normal distribution, as determined by the Kolmogorow-Smirnov test, data were analyzed using the Kruskal-Wallis test followed by Dunn's comparison post hoc test to determine significant differences between individual groups. All statistical tests were two sided and differences were considered statistically significant at $P < 0.05$.

Results

Effect of hypoxia and NO mimetics on resistance to chemotherapeutic agents. We previously reported that hypoxia increased doxorubicin resistance in human PC-3 prostatic carcinoma and MDA-MB-231 breast carcinoma cells and that this effect of hypoxia could be prevented by low concentrations of NO mimetics (9, 10). Results of the present study revealed that incubation of human DU-145 prostatic adenocarcinoma cells in 0.5% O_2 for 24 h significantly increased their survival following exposure to various concentrations of doxorubicin (3.1-100 μmol/L) compared with cells incubated in 20% O_2 (Fig. 1A). This hypoxia-induced resistance to doxorubicin was significantly attenuated when GTN (0.1 nmol/L-1 μmol/L) or ISDN (0.1 nmol/L) were administered at the onset of the 24-h hypoxic exposure (Fig. 1B and C); however, the sensitivity of cells incubated in 20% O_2 was not affected by exposure to either of these NO mimetic agents (Fig. 1B and C). To determine whether the chemosensitizing effect of NO mimetics extends to another class of chemotherapeutic agents and to other cell types, DU-145 and PC-3 prostate cancer cells, as well as MDA-MB-231 breast carcinoma cells, were exposed to hypoxia in the presence or absence of low concentrations of GTN before a 1-h exposure to paclitaxel (50 μmol/L). Similar to the induction of doxorubicin resistance in DU-145 cells, hypoxia was able to significantly stimulate resistance to paclitaxel in these three cell lines (Fig. 2A-C); this effect of hypoxia on paclitaxel resistance was significantly attenuated by low concentrations of GTN (Fig. 2A-C).

Role of the NO-cGMP signaling pathway on tumor cell chemosensitivity. The involvement of NO signaling at the levels of NO generation and sGC and PKG activation was determined by the use of inhibitors and activators of this pathway, with DU-145 cells serving as a model system. Inhibition of NO synthesis with L-NMMA resulted in up to a 2.3-fold significant increase in resistance to 12.5 μmol/L doxorubicin (Fig. 3A). In contrast, the inactive stereoisomer D-NMMA did not significantly affect survival (Fig. 3B).

Inhibition of sGC activity with ODQ in cells incubated in 20% O_2 resulted in a dose-dependent increase in survival with a significant 50% increase at the highest dose (Fig. 4A). Similarly, inhibition of PKG with the selective blocker KT5823 revealed a dose-dependent increase in drug resistance with a significant 2-fold increase at 15 μmol/L (Fig. 4B). In contrast, the resistance to doxorubicin in cells incubated in 0.5% O_2 was significantly attenuated following administration of the non-hydrolyzable cGMP analogue 8-Br-cGMP in an inverse dose-dependent manner, with maximal attenuation observed at a concentration of 8-Br-cGMP of 10 nmol/L (Fig. 4C).

Effect of GTN as adjuvant to doxorubicin in PC-3 human prostate tumor xenografts. Our earlier study revealed that low concentrations of GTN are able to chemosensitize hypoxic PC-3 cell *in vitro* (10). Here, we chose to determine whether GTN enhances the *in vivo* antitumor effect of chemotherapeutic agents using a well-established PC-3 prostate tumor xenograft model. Injection of 2×10^6 human PC-3 prostate cancer cells into the left flank of male NIH Swiss nude mice resulted in tumors 200 to 250 mm^3 in volume 2 to 3 weeks later. Treatment with doxorubicin was therefore initiated at this time and continued for the following 5 weeks. Compared with treatment with doxorubicin alone, the combination of doxorubicin and continuous transdermal GTN delivery resulted in a 40% decrease in the median rate of tumor growth and a 55% decrease in the mean rate of tumor growth ($P < 0.01$; Fig. 5A and B). This experiment was terminated at week 5

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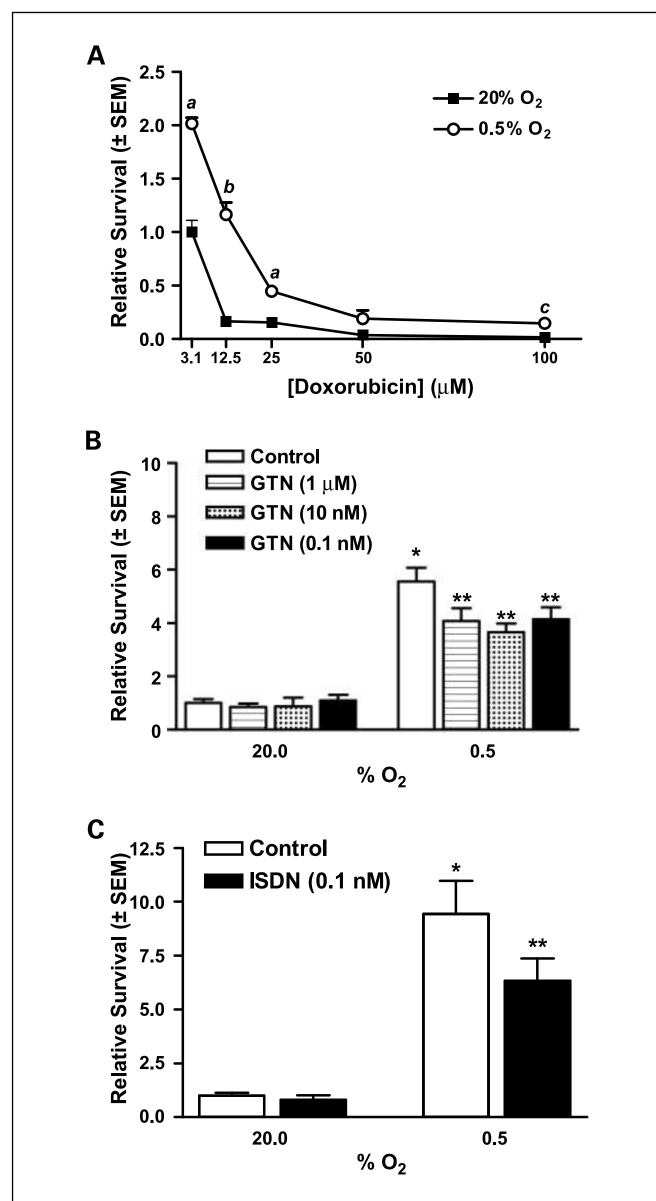

Fig. 1. Effect of GTN and ISDN on the hypoxia-induced resistance to doxorubicin. Compared with the survival of cells incubated in 20% O_2, the survival of DU-145 cells incubated for 24 h in 0.5% O_2 (*A*) was significantly increased (a, $P < 0.002$; b, $P < 0.001$; c, $P < 0.0005$) following a 1-h exposure to various concentrations of doxorubicin (expressed relative to cells incubated at 20% O_2 and treated with 3.125 μmol/L doxorubicin). Both GTN (*B*) and ISDN (*C*) attenuated the acquisition of hypoxia-induced resistance to 12.5 μmol/L doxorubicin following a 24-h incubation period in hypoxia. *, $P < 0.0001$, significant difference in survival compared with cells incubated in 20% O_2 at 12.5 μmol/L doxorubicin. **, $P < 0.01$, significant difference in survival compared with cells incubated in 0.5% O_2 alone. Representative results of pooled survival assays conducted in triplicate and repeated thrice are shown.

(measurement 10) of treatment when mice developed overt signs of toxicity (as defined in the Canadian Council on Animal Care Guidelines) due to doxorubicin treatment or excessive tumor burden. The time for development of signs of toxicity was not different between groups of mice treated with doxorubicin alone and mice treated with doxorubicin in combination with GTN.

Results of gas chromatographic analysis revealed the presence of GTN and metabolites in plasma of treated mice, indicating effective transdermal delivery of the drug. At the time of sampling, plasma levels of GTN, 1,2-GDN, and 1,3-GDN were 2.1 ± 2.0 (SD), 120 ± 43.7, and 93.3 ± 72.9 nmol/L, respectively ($N = 4$). Blood taken from mice treated with

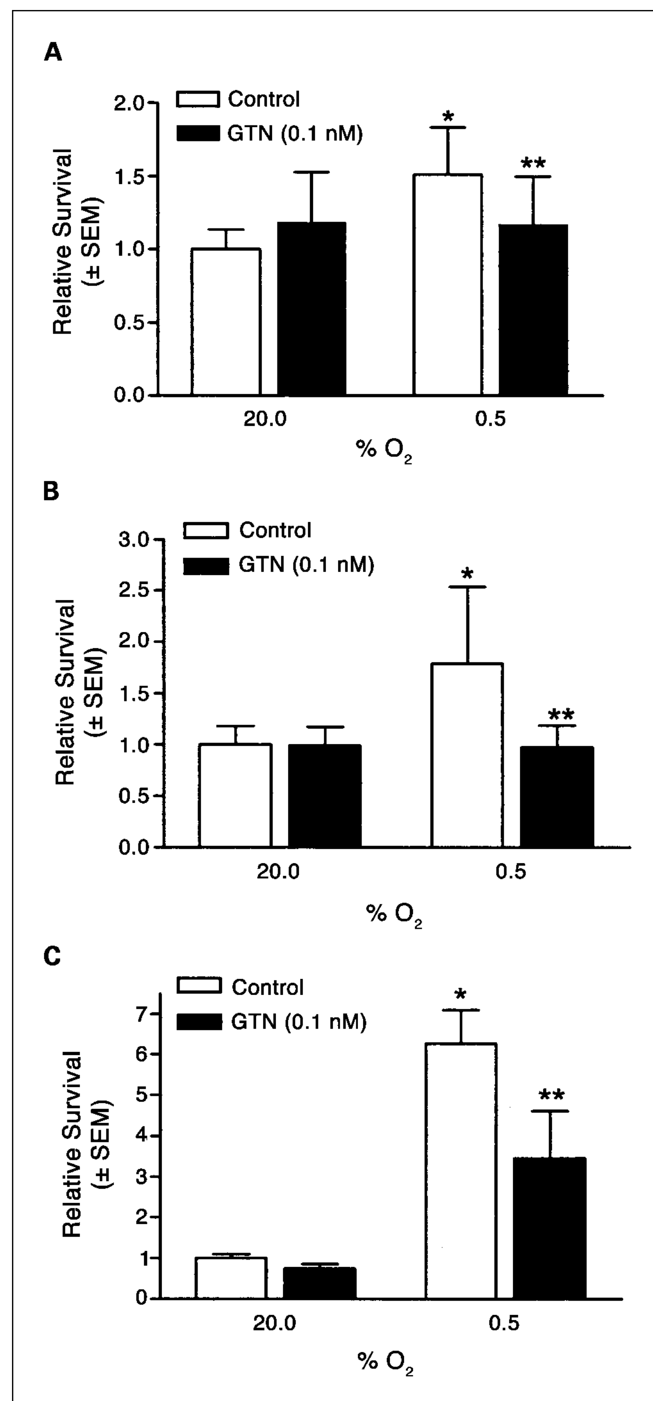

Fig. 2. Effect of GTN on the hypoxia-induced resistance to paclitaxel in various cell lines. Treatment of DU-145 (*A*), PC-3 (*B*) prostate cancer cells, and MDA-MB-231 breast carcinoma cells (*C*) with low concentrations of GTN attenuated the acquisition of hypoxia-induced resistance to paclitaxel (50 μmol/L) following a 24-h incubation in hypoxia. *, $P < 0.002$, significant difference in survival compared with cells incubated in 20% O_2. **, $P < 0.005$, significant difference in survival compared with cells incubated in 0.5% O_2 alone. Representative results of pooled survival assays conducted in triplicate and repeated at least thrice for each cell line are shown.

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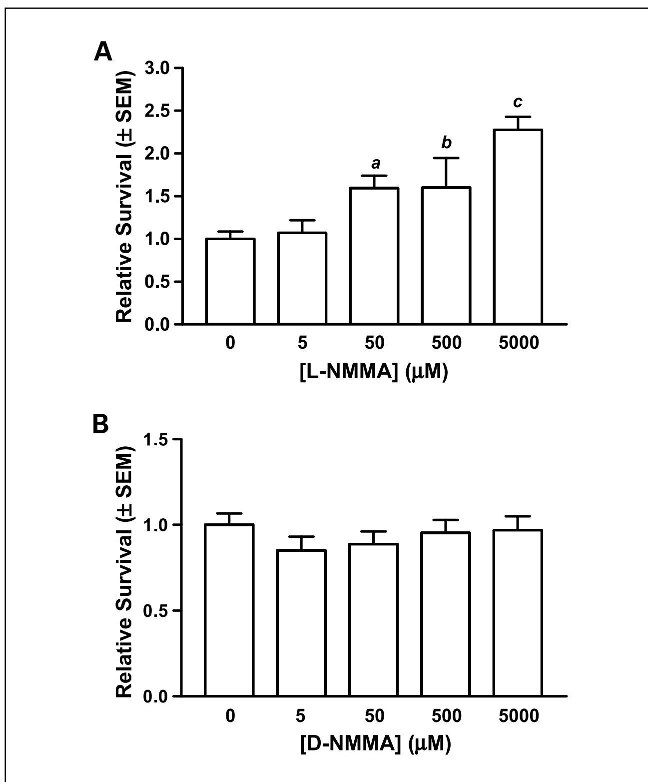

Fig. 3. Effect of NOS inhibition on resistance to doxorubicin in DU-145 cells incubated in 20% O_2. Inhibition of NO signaling with the NOS inhibitor L-NMMA significantly increased survival following a 1-h exposure to 12.5 μmol/L doxorubicin (*A*), whereas the inactive enantiomer D-NMMA did not significantly affect survival (*B*). a, $P < 0.04$; b, $P < 0.008$; c, $P < 0.0001$.

placebo patches did not have any detectable levels of GTN or GTN metabolites.

Role of the multidrug efflux transporters in hypoxia-induced drug resistance. Given that both doxorubicin and paclitaxel are substrates for members of the ABC drug efflux transporters (20), we addressed the possibility that decreased intracellular drug accumulation was the mechanism responsible for hypoxia-induced drug resistance in DU-145 and MDA-MB-231 cells *in vitro*. Fluorescence measurements taken immediately following drug treatment revealed that doxorubicin uptake in DU-145 cells previously exposed to hypoxia was comparable to that of cells maintained in standard culture conditions (Fig. 6A). Following a 90-min incubation, intracellular doxorubicin fluorescence was decreased, suggesting active drug efflux; however, pre-exposure of DU-145 cells to hypoxia did not significantly affect doxorubicin levels relative to cells maintained under standard culture conditions. Similar findings were obtained with MDA-MB-231 cells, although the relative levels of pump activity were lower compared with those observed in DU-145 cells (data not shown).

To further investigate the potential involvement of drug efflux in hypoxia-induced cell survival following exposure to chemotherapeutic agents, we performed clonogenic assays in which cells were treated with ABC transporter inhibitors after exposure to doxorubicin. Our previous study revealed that the increased resistance to doxorubicin after a 24-h exposure to hypoxia is transient and lost after a subsequent 12- to 24-h incubation in standard oxygen conditions (9). We hypothesized that if hypoxia-induced drug resistance is due to an increase in ABC transporter activity, then prevention of drug efflux by treatment with pump inhibitors during the initial 24 h following doxorubicin treatment should abolish the hypoxia-induced increase in survival. R(+)-verapamil is a well-established inhibitor of ABCB1 (originally identified as multidrug resistance protein or P-glycoprotein) and ABCC1 (multidrug resistance–associated protein 1; ref. 20), whereas reserpine has been shown to inhibit both ABCB1 (21) and ABCG2 (also known as mitoxantrone-resistance protein or

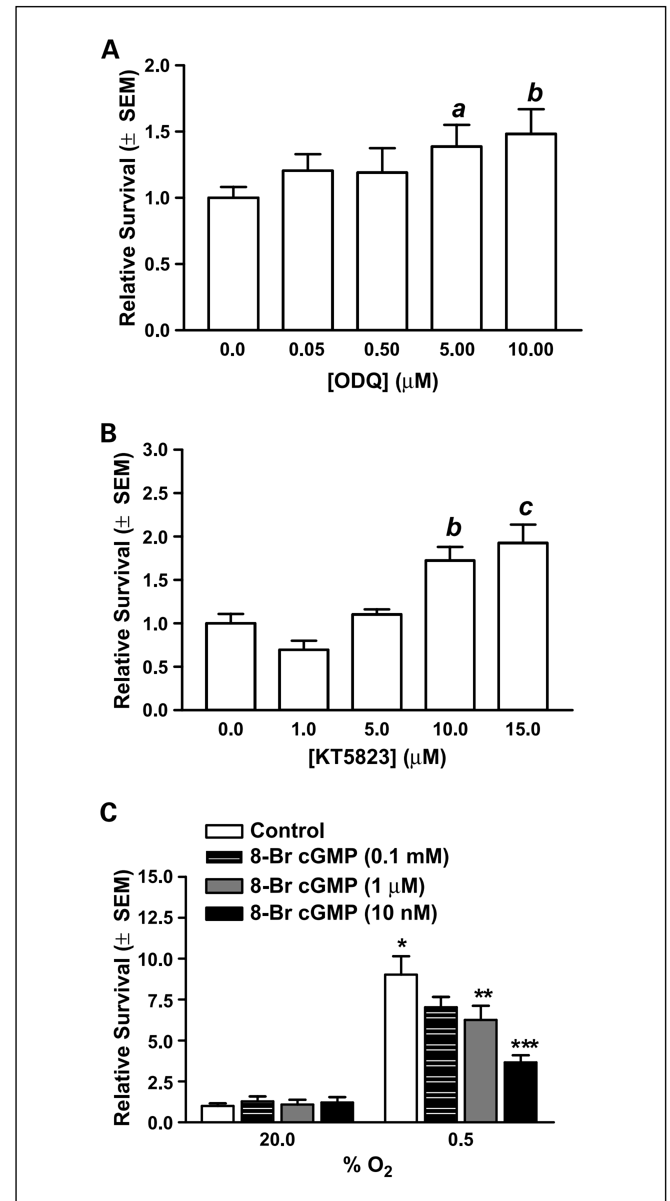

Fig. 4. Effect of cGMP signaling on doxorubicin resistance in DU-145. In cells incubated in 20% O_2, inhibition of cGMP production with the selective sGC blocker ODQ for 24 h (*A*) or inhibition of the cGMP-dependent protein kinase (PKG) with KT5823 (*B*) significantly increased survival after a subsequent 1-h exposure to 12.5 μmol/L doxorubicin (a, $P < 0.04$; b, $P < 0.008$; c, $P < 0.0001$). In contrast, in cells incubated in 0.5% O_2, activation of cGMP signaling with the nonhydrolyzable cGMP analogue 8-Br-cGMP resulted in attenuation of the hypoxia-induced resistance to doxorubicin (*C*). *, $P < 0.0001$, significant difference in survival compared with cells incubated in 20% O_2 at 12.5 μmol/L doxorubicin. **, $P < 0.005$; ***, $P < 0.001$, significant difference in survival compared with cells incubated in 0.5% O_2 alone.

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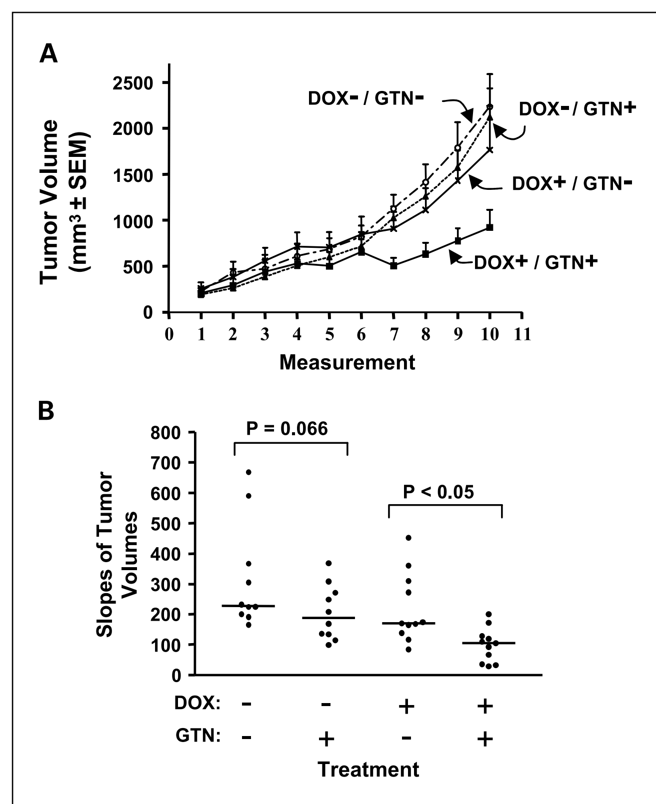

Fig. 5. *In vivo* chemosensitizing effect of GTN. *A,* average tumor volumes over the course of treatment. *B,* rates of tumor growth (i.e., slopes of the linear portion of the tumor growth curves) for individual mice, as well as the median slope values for each treatment group (*horizontal lines*). Mice treated twice a week with i.p. injections of doxorubicin and a GTN transdermal patch changed daily had significantly slower tumor growth compared with treatment with doxorubicin alone ($P < 0.05$). DOX, doxorubicin.

breast cancer resistance protein; ref. 22). Incubation of DU145 cells in increasing concentrations of verapamil or reserpine for 24 h following doxorubicin treatment resulted in a dose-dependent decrease in survival (Fig. 6B and C); however, the presence of these inhibitors did not prevent hypoxia from significantly increasing survival relative to cells maintained in 20% O_2. Similar observations were made using MDA-MB-231 cells with equivalent concentrations of verapamil and reserpine (data not shown).

Discussion

This study provides evidence that the NO signaling pathway involving cGMP production and subsequent activation of PKG is an important mediator of chemosensitivity in cancer cells and that low doses of NO mimetics such as nitroglycerin may have potential clinical applications as adjuvants to chemotherapy. Furthermore, the present findings indicate that hypoxia-induced drug resistance in tumor cells is at least partly due to inhibition of endogenous NO signaling. This concept is supported by the fact that low concentrations of NO mimetics were able to attenuate the hypoxia-induced drug resistance. The conversion of L-arginine into L-citrulline and NO requires molecular oxygen (12), and previous studies have shown that exposure of endothelial cells and macrophages to low levels of O_2 (1-3%) inhibits NO production by up to 90% (13, 14). Furthermore, our previous studies revealed that cGMP production is markedly decreased in MDA-MB-231 tumor cells incubated in hypoxia (0.5% O_2) for 24 h (23). Hypoxia has also been shown to increase arginase activity in macrophages (24), thus diverting

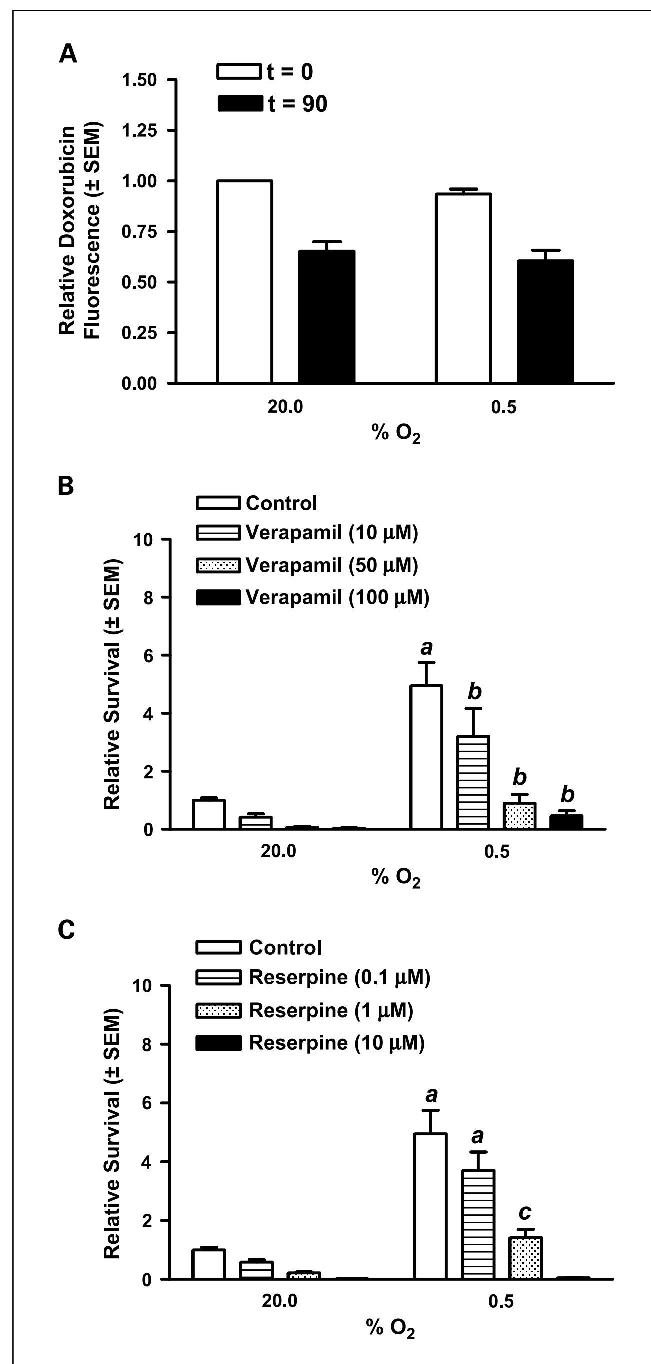

Fig. 6. Effect of verapamil and reserpine on the hypoxia-induced resistance to doxorubicin. Compared with cells incubated in 20% O_2, the cellular concentration of doxorubicin in DU-145 cells incubated for 24 h in 0.5% O_2 was not significantly different after a 1-h exposure to 12.5 µmol/L doxorubicin ($t = 0$; $P = 0.0637$) or following 90 min of efflux under standard culture conditions ($t = 90$; $P = 0.5316$; *A*). Treatment of DU-145 cells with R(+)-verapamil (*B*) or reserpine (*C*) for 24 h following a 1-h exposure to doxorubicin significantly decreased survival but did not abolish the hypoxia-induced resistance acquired by 24 h of incubation in 0.5% O_2. a, $P < 0.0001$; b, $P < 0.01$; c, $P < 0.001$, significant differences in survival compared with cells incubated in 20% O_2 in equivalent medium. Representative results of pooled survival assays conducted in sextuplicate and repeated thrice are shown.

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L-arginine metabolism away from the NO generation pathway and into the urea cycle. In the present study, further evidence in favor of a role for NO as a mediator of tumor cell chemosensitivity is provided by results showing that, in a manner similar to exposure to hypoxia, pharmacologic inhibition of endogenous NO production increased drug resistance in tumor cells.

Nitric oxide exerts its effects through various pathways including nitration, nitrosylation, DNA deamination, and activation of sGC; however, these can be separated into high versus low NO concentration effects (17). Most of the low concentration effects are attributable to NO binding to sGC with the subsequent production of cGMP and activation of PKG (17). The fact that, in the present study, low concentrations (<1 μmol/L) of NO mimetics were able to significantly chemosensitize hypoxic tumor cells suggests that this effect occurs via activation of sGC because, at such concentrations, it is the only known pathway to be activated (17). Most assays designed to measure NO production in cells are based on the assessment of accumulated levels of nitrate and nitrite. The levels of NO present in the cells used in our study have not been detectable using these standard assays,[4] thus indicating that they are lower than those required to produce nitrates or nitrites and, as such, can only exert their effects through the low-concentration NO-cGMP signaling pathway. The participation of the cGMP-PKG pathway in the chemosensitizing effect of NO was confirmed by results of experiments showing that the inhibitors of sGC and PKG activity, ODQ and KT5823, respectively, increased drug resistance in a dose-dependent manner. Moreover, direct activation of PKG with 8-Br-cGMP attenuated the acquisition of hypoxia-induced resistance to doxorubicin.

Interestingly, 8-Br-cGMP attenuated hypoxia-induced doxorubicin resistance in DU-145 cells in an inverse dose relationship. There are many potential reasons for this inverse dose effect. One possible explanation is that higher concentrations of 8-Br-cGMP may lead to the activation of protein kinase A (25). There is evidence that the cyclic AMP/protein kinase A cascade is able to inhibit cGMP-mediated responses such as neuronal migration (26). We have observed a similar inverse dose chemosensitizing effect with atrial natriuretic peptide, a well-characterized inducer of cGMP production (27). Further investigation is required to determine the full nature of this inverse dose effect of 8-Br-cGMP.

We previously reported that hypoxia induces resistance to doxorubicin in prostate and breast cancer cell lines and that this effect of hypoxia is attenuated by low concentrations of GTN (9, 10). In this study, we extend these initial observations to show that hypoxia induces resistance to other drugs in a variety of tumor cell types and that this effect of hypoxia can also be attenuated by low concentrations of the NO mimetic ISDN. Paclitaxel, one of the drugs used in the present study, is now considered among the most active chemotherapeutic agents for clinical breast cancer along with doxorubicin and cisplatin (28–31), and its use in the treatment of prostate cancer has increased over the last decade (32). Docetaxel, a closely related taxane, has recently been found to be the first chemotherapeutic agent to increase overall survival in hormone-refractory prostate cancer (33, 34). Similar to GTN, the organic nitrate ISDN is a prodrug that requires bioactivation through a poorly understood process before releasing its NO-like activity (19). However, unlike GTN, ISDN has good oral bioavailability and is denitrated slower by the liver than is GTN (35). We previously showed that diethylenetriamine-NO adduct, a compound that releases NO at physiologic pH and therefore does not require bioactivation, is also able to chemosensitize hypoxic tumor cells (9).

The cell lines used in our study are known to express various members of the ABC transporter family (36–38) that can actively transport the chemotherapeutic agents used in these experiments. Consistent with these reports, active efflux of doxorubicin was observed in DU-145 and MDA-MB-231 cells; however, we did not observe a difference in the level of drug efflux in cells following exposure to hypoxia. Furthermore, treatment with potent inhibitors of the ABC transporters did not prevent hypoxia-induced drug resistance. Together, these findings suggest that decreased intracellular accumulation of drug is not the mechanism leading to the hypoxia-induced resistance to doxorubicin and paclitaxel. Doxorubicin exerts its cytotoxic effects by inhibiting topoisomerase II and through its ability to intercalate in DNA, thereby promoting breaks in the genomic DNA and blocking DNA synthesis and transcription. In contrast, paclitaxel binds to β-tubulin and promotes actin-tubulin polymerization and stabilization of microtubules, thus preventing normal cell division and causing cell cycle arrest at the G_2-M phase. Given that hypoxia can promote resistance to drugs with such distinct mechanisms of action, it is unlikely that a single mechanism will account for reduced drug sensitivity; instead, the effect of hypoxia on drug resistance is likely to involve a multifactorial process. Additional work is required to identify the potential downstream targets of the NO-cGMP signaling pathway that contribute to hypoxia-induced drug resistance.

An important finding of this study was that transdermal delivery of low doses of GTN resulted in increased chemosensitivity of human prostate tumor xenografts. Previous studies revealed that daily i.p. injections with higher doses of ISDN and isosorbide 5-mononitrate (200 or 600 μg) were capable of inhibiting the growth of Lewis lung carcinomas implanted intramuscularly into mice (39). Our present study, however, represents the first report of a chemosensitizing effect of low doses of a NO mimetic agent *in vivo*. Although it is possible that the effects of GTN were, in part, due to modulation of blood flow, based on our accumulated *in vitro* results, we believe that a large extent of the tumor growth inhibition is due to a direct chemosensitizing effect of GTN on the tumor cells. We chose prostate cancer xenografts to test the chemoadjuvant effect of NO mimetics *in vivo* because human prostate cancers exhibit high levels of intrinsic chemoresistance. Indeed, the dose of doxorubicin used in our study (4 mg/kg biweekly) did not result in a significant inhibition of tumor growth when administered in the absence of GTN. Interestingly, our results showed that administration of GTN alone resulted in a small, although not significant ($P = 0.066$), decrease in the rate of tumor growth as compared with controls. It is possible that earlier administration of GTN when tumor burden is minimal may significantly decrease tumor growth even in the absence of conventional chemotherapy. In a recent study, we showed that incubation of mouse melanoma cells with very low concentrations of GTN before i.v. inoculation

[4] Frederiksen LJ, Sullivan R, Graham CH, unpublished observations.

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inhibits their hypoxia-induced metastatic ability (40). These findings suggest that, at low concentrations, NO possesses antitumor activity, which may be independent of its chemo-adjuvant properties.

A major drawback of many combination therapies is increased toxicity to the patient. In our study, mice treated with doxorubicin and the transdermal GTN patch did not exhibit increased signs of toxicity (i.e., cachexia, lack of grooming, agitation, etc.) compared with mice treated with doxorubicin alone. One possible explanation for this lack of increased toxicity by the combination therapy is that the NO mimetics may selectively chemosensitize hypoxic tumor cells and not the well-oxygenated, nonmalignant cells. Our *in vitro* studies showed that incubation in the presence of GTN increased the sensitivity to doxorubicin and paclitaxel in cells exposed to hypoxia but not in cells at 20% O_2. It has been reported that the biotransformation of GTN is increased under hypoxic conditions (19, 41). Therefore, GTN may represent an important and useful agent for selectively targeting the more malignant hypoxic cells.

In summary, the results of this study indicate that NO mimetics act through cGMP-dependent signaling to attenuate hypoxia-induced drug resistance. In addition, GTN can chemosensitize tumors *in vivo*. Therefore, intervention in one or more steps of the mechanism of hypoxia-induced chemoresistance may provide a novel approach to enhance therapeutic efficacy in cancer patients.

Acknowledgments

We thank Dr. Bruce Elliott (Department of Pathology and Molecular Medicine, Queen's University) for helping with the *in vivo* analysis of tumor growth; Diane Anderson (Department of Pharmacology and Toxicology, Queen's University) for analyzing plasma samples for GTN and GTN metabolites; Matthew Gordon (Cancer Research Laboratories, Queen's University) for conducting the flow cytometric analysis; and Geneviève Paré (Department of Anatomy and Cell Biology, Queen's University) for carefully reviewing the manuscript and for technical assistance.

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Clin Cancer Res 2007;13:2199-2206.

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