Therapeutic Potential of Nitric Oxide-Modified Drugs in Colon Cancer Cells

Marija Mojic, Sanja Mijatovic, Danijela Maksimovic-Ivanic, Djordje Miljkovic, Stanislava Stosic-Grujicic, Marija Stankovic, Katia Mangano, Salvatore Travali, Marco Donia, Paolo Fagone, Mai-Britt Zocca, Yousef Al-Abed, James A. McCubrey, and Ferdinando Nicoletti

Department of Immunology, Institute for Biological Research "Sinisa Stankovic" (M.M., S.M., D.M.-I., D.M., S.S.-G.) and Institute of Molecular Genetics and Genetic Engineering (M.S.), Belgrade University, Belgrade, Serbia (M.S.); Department of Biomedical Sciences, University of Catania, Catania, Italy (K.M., S.T., M.D., P.F.); OncoNOx Aps, Copenhagen, Denmark (M.-B.Z., F.N.); Laboratory of Medicinal Chemistry, North Shore Long Island Jewish Health System, Long Island, New York (Y.A.-A.); and Department of Microbiology and Immunology, East Carolina University, Greenville, North Carolina (J.A.M.)

Received January 19, 2012; accepted July 13, 2012

ABSTRACT

We have examined the influence of the nitric oxide (NO)-modified anti-inflammatory drug (S,R)-3-phenyl-4,5-dihydro-5-isoxasole acetic acid (VGX-1027) named GIT-27NO or the NO-modified antiviral drug saquinavir (Saq) named Saq-NO on two colon cancer cell lines, mouse CT26CL25 and human HCT116. The effects of the drugs on cell viability, apoptosis, proliferation, and metastatic potential were analyzed. The release of NO and oxygen and nitrogen species was also determined. The efficacy of the drugs was evaluated in vivo in BALB/c mice injected with CT26CL25 cells. Both agents suppressed the growth of colon cancer cells in vitro and reduced tumor volume in syngeneic BALB/c mice. However, their mechanisms of action were different because GIT-27NO released larger amounts of nitrite than Saq-NO in cell cultures and its antitumor action depended on the intracellular NO release inside the cells. On the contrary, Saq-NO released barely detectable amounts of NO and its antitumor action was NO-independent. In fact, cotreatment with an NO-peroxynitrite scavenger revealed that GIT-27NO but not Saq-NO acts through peroxynitrite-mediated cell destruction. At the cellular level, GIT-27NO prevalently induced proapoptotic signals followed by caspase-dependent apoptosis. In contrast, Saq-NO blocked cell proliferation, changed the adhesive, migratory, and invasive properties of the cells, and decreased metastatic potential in vivo. In conclusion, differences in NO release and oxidative stress generation between GIT-27NO and Saq-NO resulted in different mechanisms that caused cell death.

Introduction

Colorectal cancer remains the second most frequent cause of cancer-related death despite recent major prophylactic and therapeutic advancements that have reduced its mortality rate by 9% (Wolpin and Mayer, 2008; Giuliani et al., 2010; Labianca and Merelli, 2010). This severity of colorectal cancer is probably due to development of metastasis and chemoresistance of colorectal cancer cells (Gallagher and Kemeny, 2010; Zhang et al., 2012). Recent data revealed promising potential for drugs initially designed for treatment of inflammation and infection in therapy of cancers whose tumorigenesis is related to the above-mentioned processes.

It is known that nonsteroidal anti-inflammatory drugs (NSAIDs) reduce the incidence of colon cancer in clinical trials (Coimbra et al., 2009; Rothwell et al., 2010, 2011) and that attachment of a NO moiety to the parent NSAIDs reduced gastrotoxicity and increased their antitumor properties owing to an intrinsic feature of this reactive molecule to induce tumor cell death (Yeh et al., 2004; Gao et al., 2005; Rigas and Williams, 2008).

ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; NO, nitric oxide; VGX-1027, (S,R)-3-phenyl-4,5-dihydro-5-isoxasole acetic acid; ROS, reactive oxygen species; RNS, reactive nitrogen species; Saq, saquinavir; DETA NONOate, diethylenetriamine NONOate; MEG sulfate, (2-mercaptoethyl)-guanidine sulfate; SNAP, S-nitroso-N-acetyl-D,L-penicillamine; SIN-1 chloride, linsidomine; AnnV, annexin V-FITC; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; SNP, sodium nitroprusside; CV, crystal violet; DHR, dihydrorhodamine 123; PI, propidium iodide; p, phospho; PI3K, phosphatidylinositol 3-kinase.

(*S*,*R*)-3-Phenyl-4,5-dihydro-5-isoxasole acetic acid (VGX-1027) is an anti-inflammatory compound in early clinical development for the treatment of rheumatoid arthritis and type 1 diabetes. VGX-1027 has elicited immunomodulatory and anti-inflammatory effects mediated by down-regulation of interleukin-1β, tumor necrosis factor, nuclear factor-κB, and inducible nitric-oxide synthase expression (Stojanovic et al., 2007; Stosic-Grujicic et al., 2007). VGX-1027 does not possess antitumor properties. Covalent linkage of a NO moiety to VGX-1027 generated a novel drug, GIT-27NO, effective against numerous tumor cell lines (Maksimovic-Ivanic et al., 2008; Mijatovic et al., 2008, 2010; Donia et al., 2009). GIT-27NO liberates NO upon encountering the cell or cell-secreted products. Internalized NO leads to higher production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) influencing the activity of essential signaling pathways, transcription factors, and pro- and antiapoptotic molecule expression and, as a consequence, elicited caspase-dependent or -independent apoptosis (cell death by autophagy) (Maksimovic-Ivanic et al., 2008; Mijatovic et al., 2008, 2010; Donia et al., 2009).

An important structural and pharmacological difference with GIT-27NO from other NO-NSAIDs such as NO-aspirin whose antitumor properties depend on the original substance is that the NO carrier and NO itself are in direct linkage of the NO moiety to original VGX-1027 (Kashfi and Rigas, 2007). This is important because genotoxicity was observed with the linker used to generate NO-aspirin, which resulted in cessation of the clinical trials with this agent (NicOx press release, 2007, http://www.nicox.com/files/pdf/PR2007061800EN.pdf).

The antitumor properties of protease inhibitors designed for treatment of human immunodeficiency virus infection are well documented (Sgadari et al., 2003; Bernstein and Dennis, 2008; Chow et al., 2009). The prototypical protease inhibitor saquinavir (Saq) significantly lowered the incidence of human immunodeficiency virus-related cancers (Lebbé et al., 1998; Bower et al., 1999; Niehues et al., 1999; Sgadari et al., 2003). However, numerous side effects hamper the use of Saq and other protease inhibitors as antineoplastic drugs. A NO derivative of Saq was developed with the aim of improving its anticancer efficacy (Maksimovic-Ivanic et al., 2009). Compared with the parent drug Saq, Saq-NO exhibits lower in vitro and in vivo toxicity and increased anticancer action. In addition, the antiretroviral activity of Saq-NO was similar to that of the parent compound (Maksimovic-Ivanic et al., 2009; Canducci et al., 2011; Donia et al., 2011; Mijatovic et al., 2011). Although Saq-NO primarily exerted its anticancer action via inhibition of cell proliferation, apoptosis was also induced in some circumstances (Donia et al., 2011; Mijatovic et al., 2011). Moreover, Saq-NO efficiently sensitized tumor cells to tumor necrosis factor-related apoptosis-inducing ligand and chemotherapy, regardless of p53, P-glycoprotein, multidrug resistance-associated protein 1, or breast cancer resistance protein 1 expression (Rothweiler et al., 2010; Donia et al., 2011; Mijatovic et al., 2011). Unlike the parent compound Saq, Saq-NO transiently up-regulated Akt (Maksimovic-Ivanic et al., 2009; Mijatovic et al., 2011). Inhibition of Akt has been related to the toxic effects of Saq (Schütt et al., 2004; Gupta et al., 2005). Because Saq-NO releases minor amounts of NO, it is unlikely that its lower toxicity and enhanced antitumor action are immediate consequences of NO release (Maksimovic-Ivanic et al., 2009).

In this study, we compared the responsiveness of two metastatic colon cancer cell lines with those of GIT-27NO and Saq-NO. Both compounds strongly inhibited the growth of colon cancers in vitro and in vivo. Whereas GIT-27NO acted through NO-mediated caspase-dependent apoptosis, without affecting metastatic potential, Saq-NO converted cells into a nonproliferative phenotype and abrogated their metastatic potential.

Materials and Methods

Reagents and Cells. Saq-NO (OX1001) and GIT-27NO (OX27-NO) were obtained from OncoNOx (Copenhagen, Denmark). DETA NONOate, MEG sulfate, SNAP, and SIN-1 chloride were from Cayman Chemical (Ann Arbor, MI). Doxorubicin was from Sigma-Aldrich (Milan, Italy). For flow cytometry studies, annexin V (AnnV)-FITC was obtained from BD Pharmingen (San Diego, CA), and acridine orange was from Labo-Moderna (Paris, France). Moloney leukemia virus reverse transcriptase and random primers used for reverse transcription were from Fermentas (Vilnius, Lithuania). Real-time polymerase chain reaction (PCR) was performed with SYBR Green PCR Master Mix from Applied Biosystems (Carlsbad, CA). The pan-caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp was purchased from R&D Systems (Minneapolis, MN). Other reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

Murine (CT26CL25) and human (HCT116) colon cancer cell lines were obtained from American Type Culture Collection (Manassas, VA). Cells were routinely maintained in HEPES-buffered RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 0.01% sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol, and antibiotics (culture medium) at 37°C in a humidified atmosphere with 5% CO_2. Cells were collected with 0.25% trypsin-1 mM EDTA solution in PBS and seeded at a density of 10^4/well in 96-well plates or 2×10^5/well in six-well plates, unless otherwise indicated.

Animals. Six- to 8-week-old male BALB/c mice, weighing 25 to 28 g, were purchased from Harlan-Nossan (San Pietro al Natisone, Udine, Italy). The mice were kept under standard laboratory conditions (nonspecific pathogen-free) with free access to food and water. The animal studies were performed in accordance with local guidelines and approved by the institutional animal care and use committee (Fagone et al, 2012).

Cell Viability Evaluation. Cells were seeded in 96-well plates, incubated for 24 h in the presence of different concentrations of GIT-27NO, Saq-NO, VGX-1027, or conventional NO donors such as DETA NONOate, sodium nitroprusside (SNP), SIN-1 chloride, SNAP, or the peroxynitrite scavenger MEG sulfate, and viability was estimated using crystal violet (CV) staining for adherent cells as described previously (Flick and Gifford, 1984). The viability of treated cells is shown as a percentage of value obtained for untreated cultures that was arbitrarily set to 100%.

For evaluation of cell viability after the treatment in a three-dimensional environment, cells (5×10^3/well) were plated onto 96-well plates that were coated with a thin layer of Matrigel (10 mg/ml; BD Biosciences Discovery Labware, Bedford, MA) (Kleinman et al., 1982). Cultures were incubated for 72 h with IC_{30} doses of Saq $\pm$ NO and photographed by using a phase microscope.

Detection of Extracellular NO Release, Intracellular NO Accumulation, and ROS Production. After the cell culture treatment with GIT-27NO, Saq $\pm$ NO, DETA NONOate, SNP, SIN-1 chloride, or SNAP, extracellular nitrite accumulation was detected by the Griess reaction as described previously (Maksimovic-Ivanic et al., 2008). Intracellular NO accumulation was evaluated by the fluorescent dye DAF-FM diacetate (Invitrogen, Carlsbad, CA). ROS production was detected with redox-sensitive dihydrorhodamine 123 (DHR). Fluorescence intensity of DAF- and DHR-stained cultures were analyzed by a Chameleon multiplate reader (Hidex, Turku,

Finland) or FACSCalibur flow cytometer (BD, Heidelberg, Germany) with CellQuest Pro software (BD) as described previously (Maksimovic-Ivanic et al., 2008; Donia et al., 2011).

Flow Cytometry. After treatment of the cell cultures seeded in six-well plates with GIT-27NO, Saq-NO, VGX-1027, or Saq, cell cycle analysis, early apoptosis detection with annexin V-PI, caspase activation with ApoStat, and cell proliferation of carboxyfluorescein diacetate succinimidyl ester-stained cells were performed as described previously (Maksimovic-Ivanic et al., 2008, 2009). Cells were analyzed by a FACSCalibur flow cytometer with CellQuest Pro software.

Cell Matrix Adhesion Assay. Cells were pretreated for 24 h with either Saq or Saq-NO (18.7 μM for CT26CL25 cells and 9.4 μM for HCT116 cells), collected, and allowed to recover from trypsinization for 30 min at 37°C with 5% CO_2 before the adhesion assay was performed. Approximately 6×10^4 cells per well were seeded in a 96-well dish precoated with 20 μg/ml reconstituted basement membrane (Matrigel) at 4°C overnight. After a 1-h incubation at 37°C, nonadherent cells were washed with PBS, and the cells that remained attached were fixed with 4% paraformaldehyde and stained with 2% crystal violet solution. The dye was dissolved in 33% acidic acid, and absorbance was measured at 540 and at 670 nm for background.

Transmigration Assays. To quantitatively evaluate the effect of either Saq or Saq-NO on the in vitro migration and invasion potential of CT26CL25 cells, we used Transwells (membrane pore size, 8 μm; diameter, 6.4 mm; BD Biosciences Discovery Labware) assembled in 24-well plates. For invasion assays, the upper surfaces of the Transwell membranes were covered with a layer of Matrigel (500 μg/ml), whereas for migration assays membranes remained uncoated. At the end of the 24-h pretreatment with 18.7 or 9.4 μM concentrations of either Saq or Saq-NO for CT26CL25 and HCT116 cells, respectively, 2×10^5 cells were resuspended in 0.1% bovine serum albumin-RPMI 1640 medium and seeded in the upper chambers. As a chemoattractant for the cells, the lower chambers were filled up with 10% fetal calf serum-RPMI 1640 medium. The plates were incubated at 37°C with 5% CO_2 for 12 h for invasion and 18 h for the migration assay. After the incubation period, Transwell membranes were fixed in 4% paraformaldehyde-PBS and stained with Mayer's hematoxylin (BioOptica, Milan, Italy). The cells that failed to migrate/invade remained on the upper surface and were carefully removed with a cotton swab. Cells on the lower surface of the membranes were counted under a light microscope at 40× magnification using the new computer-assisted CAST Integrator System (Visiopharm, Hoersholm, Denmark). The average number of cells attached to the lower surface in 30 independent fields (200 μm × 200 μm) per membrane is presented. Each experiment was performed in triplicate.

RNA Isolation and Real-Time PCR. The potential of GIT-27NO treatment to modify expression levels of pro- and anti-apoptotic genes was evaluated using real-time PCR. The CT26CL25 cells were treated with 75 μM GIT-NO for 6 h when total RNA was isolated with TRI Reagent Solution (Ambion, Austin, TX) according to the manufacturer's instructions. Reverse transcription and PCR amplification were performed as described previously (Donia et al., 2011). Primer pairs were the following: Bax, 5′-TGAAGACAGGGGCCTTTTTG-3′ and 5′-AATTCGCCG-GAGACACTCG-3′; Bcl-2, 5′-TCGCAGAGATGTCCAGTCAG-3′ and 5′-CCTGAAGAGTTCCTCCACCA-3′; Bcl-xL, 5′-CGG-AGAGCGTTCAGTGATCT-3′ and 5′-TGCAATCCGACTCCC-AATA-3′, caspase 3, 5′-TCT GAC TGG AAA GCC GAA ACT-3′ and 5′-AGG GAC TGG ATG AAC CAC GAC-3′; caspase-8, 5′-TCA ACT TCC TAG ACT GCA ACC G-3′ and 5′-CTC AAT TCC AAC TCG CTC ACT T-3′ (Metabion, Martinsried, Germany); and β-actin, 5′-GGACCTGACAGACTACC-3′ and 5′-GGCATAGAGGTCTT-TACGG-3′ (Integrated DNA Technologies, Coralville, IA). The expression level of each gene was calculated using formula $2^{-(Cti-Cta)}$, where Cti is the cycle threshold value of the gene of interest and Cta was the cycle threshold value of β-actin. Expression levels are presented as fold increase of values obtained from untreated cultures that were arbitrarily set to 1.

JC-1 Staining. Cells (2.5×10^5/well) were treated with an IC_{50} dose of GIT-27NO for 48 h and then were trypsinized and stained with JC-1 dye (5 μg/ml; Biotium, Hayward, CA) for 20 min at 37°C. Finally, cells were washed, resuspended in PBS, and analyzed with the FACSCalibur flow cytometer.

Immunocytochemical Detection. The expression of p53 was assessed immunocytochemically as described previously (Maksimovic-Ivanic et al., 2009). The cells were cultivated on glass chamber slides (3×10^4 cells/well), and p53 expression was analyzed with specific antibodies against p53 (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Detection was performed with the rabbit ExtrAvidin Peroxidase staining kit according to the manufacturer's instructions (Sigma-Aldrich), using diaminobenzidine (R&D Systems) as a substrate. The cells were counterstained with Mayer's hematoxylin, and slides were mounted with Glycergel mounting medium (Dako Denmark A/S, Glostrup, Denmark).

Immunoblot Analysis. CT26CL25and HCT116 cells (10^6) were seeded in flasks (25 cm³) and were treated with an IC_{50} dose of Saq-NO and corresponding dose of Saq at the indicated time points. After incubation, cells were harvested in protein lysis buffer containing 62.5 mM Tris-HCl (pH 6.8 at 25°C), 2% (w/v) SDS, 10% glycerol, 50 mM dithiothreitol, and 0.01% (w/v) bromphenol blue and subjected to electrophoresis on 12% SDS-polyacrylamide gels or 10% SDS-polyacrylamide gels for detection of inducible nitric-oxide synthase. Electrotransfer to polyvinylidene difluoride membranes at 5 mA/cm² was performed with a semidry blotting system (Fastblot B43; Bio-Rad, Goettingen, Germany). The membranes were blocked with 5% (w/v) nonfat dry milk in PBS with 0.1% Tween 20, and blots were probed with specific antibodies to Akt, p-Akt, S6, pS6, p-53, p-p53 (all from Cell Signaling Technology, Danvers, MA), β-actin, and cyclin D3 (all from Santa Cruz Biotechnology, Inc.), followed by incubation with secondary antibody (ECL donkey anti-rabbit horseradish peroxidase-linked; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Bands were visualized using a chemiluminescence detection system (ECL; GE Healthcare).

Induction of Colon Cancer and Experimental Treatments. CT26CL25 cells (5×10^6) were injected subcutaneously into the flank of each mouse using a 0.6-mm needle. Tumor growth was observed two times a week and measured with a caliper (two perpendicular diameters), and the tumor volume (mm³) was calculated according to the formula: $a \times b^2 \times 0.52$, where a is the longest and b is the shortest diameter. When the tumors were palpable, approximately 12 days after induction, the animals were randomly allocated to five groups of 10 and were treated daily, respectively, with Saq, Saq-NO (10 mg/kg), vehicle (dimethyl sulfoxide 20%) for 15 consecutive days and doxorubicin (2 mg/kg) for 5 consecutive days as a positive control. In a different set of experiments, after tumor induction, animals were randomly allocated to four groups of 10 mice treated, respectively, with GIT-27NO (0.5 mg/mouse), doxorubicin, or vehicle. One group of mice was left untreated. Postrandomization analysis revealed no significant differences in tumor volumes at the beginning of the treatment among the different groups.

Induction of Lung Metastasis. For evaluation of the impact of Saq and Saq-NO on metastatic potential, CT26CL25 cells were treated with 37.5 μM Saq or Saq-NO for 24 h. At the end of cultivation, the cells were washed and resuspended in PBS, and 3×10^5 cells were inoculated into the tail vein of BALB/c mice. Animals were sacrificed 18 days after inoculation of cells, and lung metastases were analyzed. Numbers of lung nodules were counted in each mouse.

Results

NO-Modified Drugs Inhibited the Growth of Colon Cancer Cells In Vitro and In Vivo. To evaluate the impact of NO modification on activity of the parent drugs, human HCT116 and mouse colon CT26CL25 cell lines were exposed

to different concentration of drugs for 24 h, and cell viability was estimated with the CV test. As shown previously, treatment with the parent drug VGX-1027 did not affect the viability of either cell line (Fig. 1A, left). NO attachment to the parent compound created a potent anticancer agent. The viability of both human and mouse cell lines was significantly diminished upon the GIT-27NO treatment with IC_{50} values of approximately 75 μM (Fig. 1A, left). On the other hand, both original and NO-modified Saq reduced cell growth, but the IC_{50} dose for Saq-NO was significantly lower than the IC_{50} dose for Saq (18.8 versus 90.5 μM for HCT116 and 37.5

versus 111.3 μM for CT26CL25 cells) (Fig. 1A, right). It is evident that NO modification significantly improved the in vitro anticancer potential of the parent drugs.

We next investigated their potential in vivo using a syngeneic tumor model in BALB/c mice. Saq-NO was administered daily starting on day 14 after tumor inoculation. The tumor volumes were significantly inhibited ($p < 0.05$) from day 16 until day 23 of the study in the groups treated with Saq-NO compared with those in vehicle-treated mice (Fig. 1B). The potency of Saq-NO was similar to that observed with the positive control chemotherapeutic drug doxorubicin. In addition,
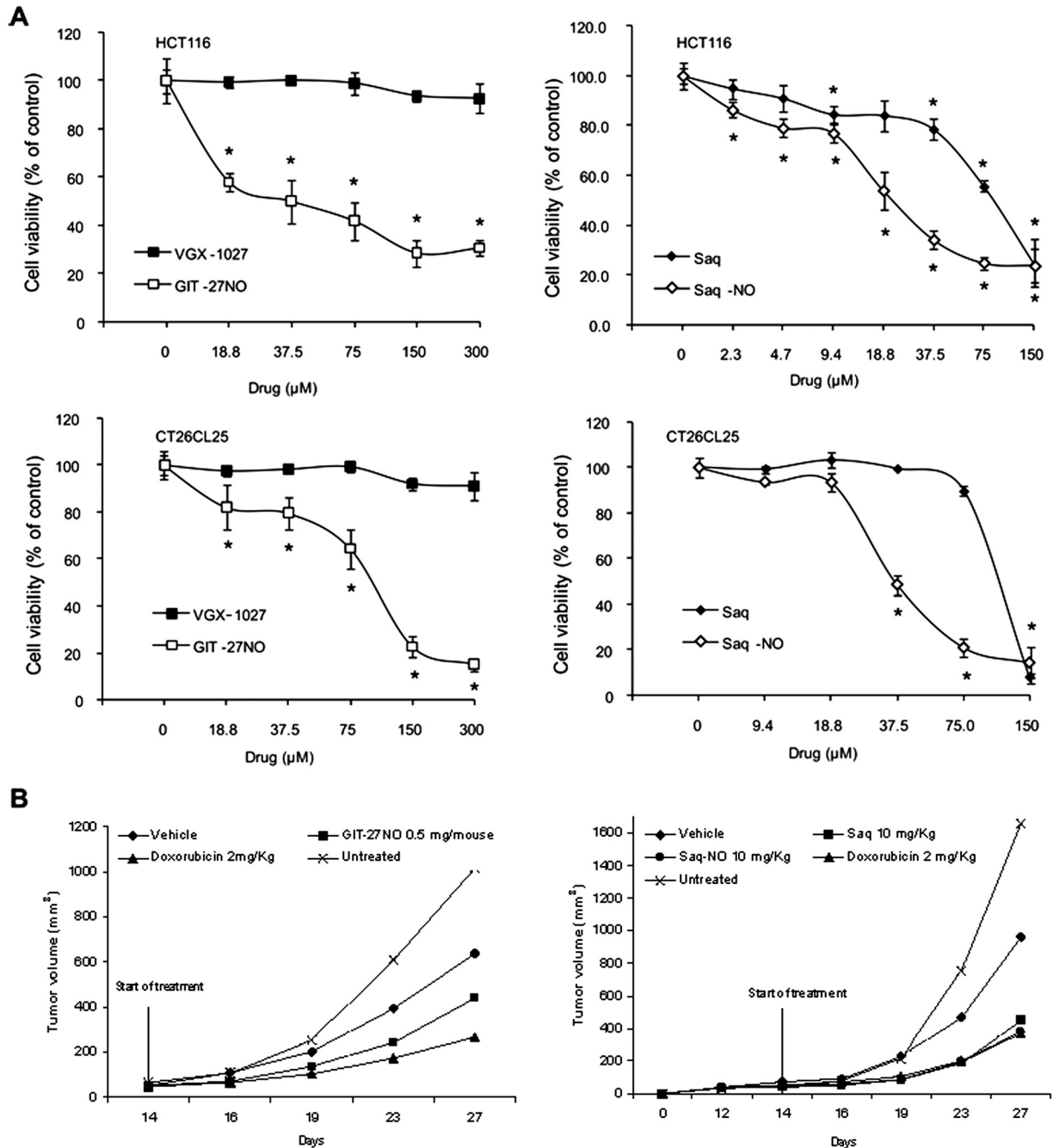

Fig. 1. NO-modified drugs decreased tumor cell growth. A, HCT116 and CT26CL25 cells (10^4 cells/well) were treated with a range of concentrations of the indicated drugs for 24 h, and then cell viability was determined by CV assay. The data are presented as the mean $\pm$ S.D. from a representative of three independent experiments. *, $p < 0.05$, refers to untreated cultures. B, tumors were induced by subcutaneous implantation of 5×10^6 CT26CL25 cells. Then after 14 days to allow the tumors to take in the mice, the mice were injected intraperitoneally with the drug daily for 14 days. Tumor volumes were measured two times per week until the end of the experiment.

the treatment with Saq also induced a significant reduction in the tumor volume (Fig. 1B).

The administration of GIT-27NO significantly reduced the growth of the murine colon cancer cell line CT26CL25 in BALB/c mice from day 23 until the end of the study (Fig. 1B). The positive control doxorubicin reduced tumor growth significantly from day 19 to day 27 after injection of colon cancer cells.

GIT-27NO, but Not Saq-NO, Induced Caspase-Dependent Apoptosis. To evaluate the cause of decreased tumor cell viability, CT26CL25 and HCT116 cells were treated with IC_{50} doses of NO-modified compounds and the same doses of corresponding original drugs, and cell cycle analysis was performed. Application of Saq did not affect cell cycle distribution significantly compared with that in the control (Fig. 2A, left). Treatment with Saq-NO for 24 h led to cell cycle arrest in the G_0/G_1 phase of the cell cycle without considerable accumulation of hypodiploid cells or a decreased percentage of cells in the S phase (Fig. 2A, left). In agreement with these results AnnV-PI double staining revealed the absence of early and late apoptosis (not shown). On the other hand, treatment of CT26CL25 cells with GIT-27NO for 24 h increased the percentage of cells in the sub-G compartment (Fig. 2A, right). Exposure of HCT116 cells to GIT-27NO also elevated the amount of apoptotic cells, but the effects were not as great as those observed with CT26CL25 cells (Fig. 2A, right). Increased percentages of both $AnnV^+PI^-$ and double positive ($AnnV^+PI^+$) CT26CL25 and HCT116 cells were detected after 24 and 48 h, respectively (Fig. 2B). Staining of CT26CL25 cells with ApoStat revealed significant activation of caspases after the treatment with an IC_{50} dose of GIT-27NO (Fig. 2C). In concordance with this result, enhanced gene expression of caspase 8 and of the effector caspase 3 was observed (Fig. 2D). Cotreatment of CT26CL25 cells with the
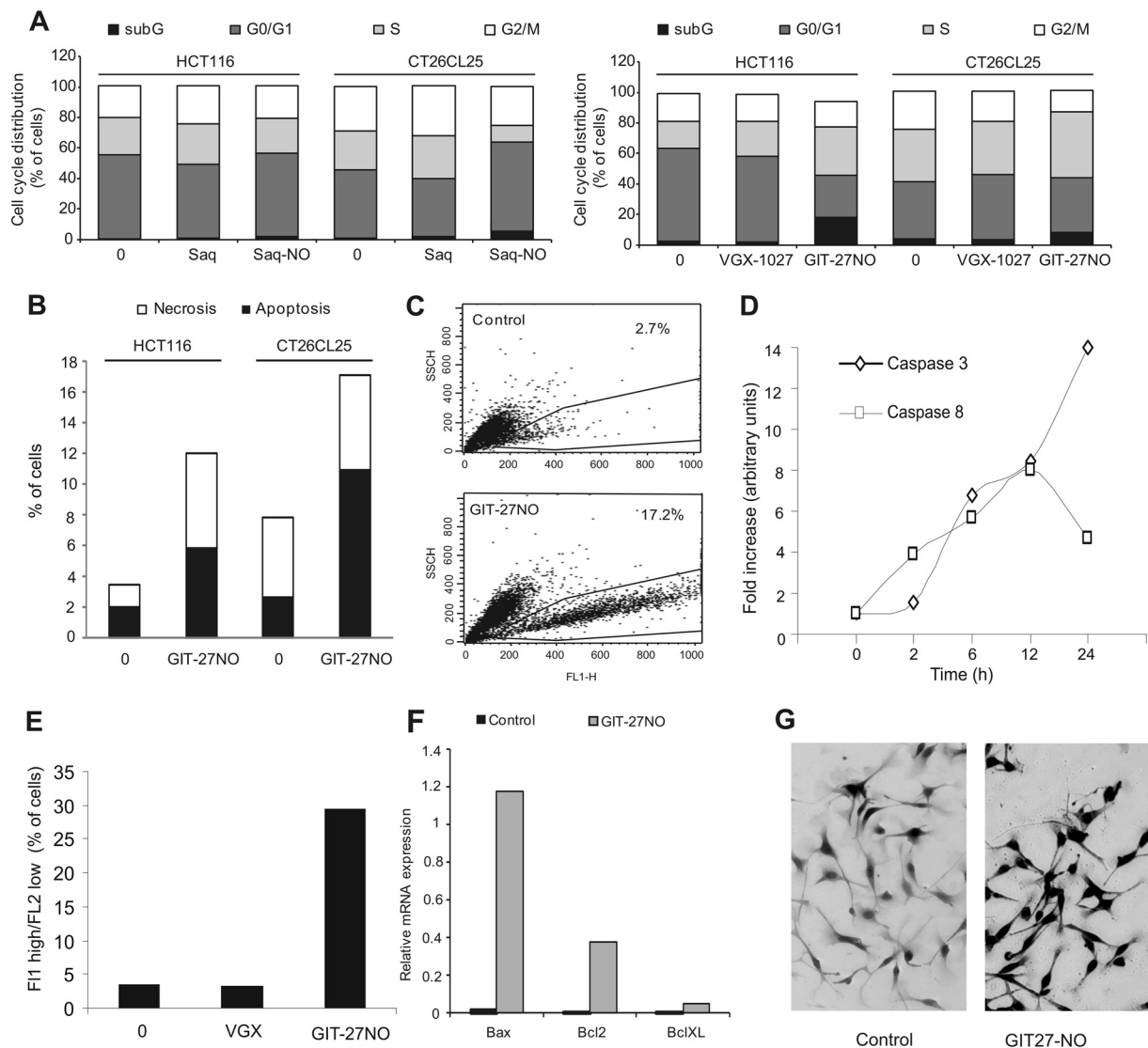

Fig. 2. GIT-27NO-induced caspase-dependent apoptosis. Cells were treated with an IC_{50} dose of the drugs and cell cycle analysis (A) and staining by AnnV-PI (B) and ApoStat (C) was performed by flow cytometry after 24 or 48 h for CT26CL25 and HCT116 cells, respectively. (SSCH, side scatter height). D, real-time PCR analyses for caspase 3 and caspase 8 were performed after 6 h of incubation in the presence of the drug, and data are presented as relative expression of mRNA. E, mitochondrial membrane depolarization of JC-1-stained CT26CL25 cells was determined by flow cytometry after 24 h. F, real-time PCR analyses for Bax, Bcl-2, and Bcl-X_L were performed as mentioned above. G. the presence of p53 in CT26CL25 cells after incubation for 24 h without (control) or with GIT-27NO was detected by immunocytochemical analysis and analyzed by light microscopy.

pan-caspase inhibitor Z-VAD (20 μM) and an IC$_{50}$ dose of GIT-27NO for 24 h partly reversed cell viability (47.4 $\pm$ 0.9% in GIT-27NO-treated cultures versus 69.5 $\pm$ 3.4% in co-treated cultures), thus confirming the contribution of caspase activation in tumoricidal action of the drug. Mitochondrial membrane depolarization determined by the fluorescent dye JC-1 indicated involvement of the mitochondrial pathway in apoptosis triggered by GIT-27NO in CT26CL25 cells but not in Bax-deficient HCT116 cells (Fig. 2E) (Van Geelen et al., 2004). This effect was accompanied by dominant expression of Bax in CT26CL25 cells. Because enhanced expression of cytoprotective Bcl family members Bcl-2 and Bcl-X$_L$ (Fig. 2F) was detected but was significantly less pronounced than expression of the proapoptotic Bax protein in the mouse colon line, the balance between pro- and antiapoptotic mediators was shifted toward the proapoptotic signal (Bax/Bcl-2 = 3.1; Bax/Bcl-XL = 24.9). Finally, remarkably elevated total p53 in cells exposed to GIT27-NO (Fig. 2G) is well synchronized with the dominant proapoptotic signal. In summary, GIT-27NO induced caspase-dependent apoptosis in both colon cancer cell lines, whereas the anticancer action of Saq-NO was not caused by the apoptotic process but rather resulted from inhibition of proliferation.

Saq-NO Suppressed Proliferation of Colon Cancer Cells. The lack of apoptosis and cell cycle arrest observed in both cell lines upon treatment with Saq-NO indicated that inhibition of proliferation could result in decreased viability. To prove this, the cells were stained with carboxyfluorescein diacetate succinimidyl ester and then treated with an IC$_{50}$ dose of Saq-NO or the same dose of original drug, and flow cytometric analysis was performed after 72 h. Whereas treatment of CT26CL25 cells with Saq exerted no effect on cellular proliferation, Saq-NO almost completely blocked the division of cells (11.9% of divided cells versus 86.6 and 90.1% in control and Saq-treated cells, respectively). Inhibition of HCT116 cell proliferation was less pronounced (50% of divided cells versus 90% and 70% in control and Saq-treated cells, respectively). We next examined whether this effect of Saq-NO required the continuous presence of the drug. For that purpose, CT26CL25 cells were pretreated with Saq-NO for 24 h, and then the drug was removed and cell viability was measured after an additional 24, 48, or 72 h of incubation (Fig. 3A). Exposure of CT26CL25 cells to Saq-NO irreversibly blocked the proliferative ability of the cells. To clarify the molecular background of this effect, the PI3K/Akt signaling pathway as a pivotal regulator of cell proliferation was examined. Whereas p-Akt levels were transiently up-regulated upon the treatment with NO modified Saq, in contrast to that with the original drug, the downstream target of mTOR, S6 kinase, was continuously inactivated through the decreased rate of phosphorylation on Ser240/244 (Fig. 3B). This phenomenon showed a similar pattern in both cell lines tested and is compatible with the previously observed decrease in cell dividing potential. Decreased expression of cyclin D3 revealed by Western blot in CT25CL26 cells indirectly confirmed the abolished activity of the downstream part of the PI3K/Akt pathway (Fig. 3C). In addition, Saq-NO remarkably elevated p53 protein expression without preventing its degradation as judged by the level of p53 phosphorylated on Ser20 (Fig. 3C). In contrast to the modified drug, Saq strongly induced phosphorylation of p53, which protected this molecule from murine double minute (mdm2) mediated degradation whereas total p53 expression was not significantly affected (Fig. 3C) (Mijatovic et al., 2010).

Saq-NO Significantly Reduced Metastatic Properties of Colon Cancer Cells In Vitro and In Vivo. To evaluate the antimetastatic potential of original and NO-modified Saq, we examined the growth of cells on reconstituted basement membrane (Matrigel) during 3 days in the presence of subtoxic doses of the drugs. CT26CL25 and HCT116 cells elicited different patterns of growth on Matrigel. Whereas HCT116 cells showed rounded morphology and formed small clusters (Fig. 4A, left), CT26CL25 cells made net-like structures and displayed elongated morphology (Fig. 4A, right). Independently of the morphologies of the colonies, Saq-NO treatment during 72 h strongly inhibited Matrigel growth in both types of colon cancer cells, whereas the inhibitory potential of the novel modified drug was greater than that of the unmodified Saq (Fig. 4A). In a similar manner Saq-NO was found to be superior in decreasing migration, invasion, and adhesive properties of cells compared with its parent compound (Fig. 4B). To confirm the permanent reduction of metastatic properties, the cells were pretreated with drugs, and formation of lung metastases was examined. At day 18, one animal in the control group died, and 2 animals displayed massive lung metastases (>120 nodules per mouse, diameter~3 mm each). Pretreatment of the cells with Saq resulted in a lower presence of metastases (n1 = 120 nodules, diameter ~3 mm; n2 = 90 nodules; and n3 = 30 nodules, diameter ~2 mm), but its efficacy was significantly less than that of Saq-NO. In the group of animals receiving Saq-NO-pretreated cells only, one animal exhibited mild metastases (n = 59, diameter ~1 mm). Taken together, these results show that Saq-NO exerted its tumoricidal activity against colon cancer cells through inhibition of proliferation and a decrease in metastatic potential.

Role of NO in the Antitumor Effects of NO-Modified Compounds. We next compared the release of NO into the culture medium of CT26CL25 cells in the presence of the NO-modified drugs or NO donors such as SNAP, SIN-1, SNP, and DETA NONOate. Although both GIT-27NO and Saq-NO released lower amounts of NO than the NO donors, they exhibited more powerful reduction of cell viability than the latter (Fig. 5, A and B). Even if GIT-27NO formed higher amounts of nitrites than Saq-NO, the diminished number of viable cells in cultures did not correlate with accumulated nitrites. Only GIT-27NO efficiently induced NO release inside the cells (Fig. 5C). On the other hand, both NO-modified compounds induced production of ROS (Fig. 5D). More sensitive measurement of NO release performed by flow cytometric analysis confirmed the previous results (Fig. 5, E and F). Concomitant treatment with hemoglobin, an extracellular scavenger of NO, resulted in restored viability of cells exposed to GIT-27NO, confirming the contribution of released NO to the tumoricidal action of the drug (Fig. 5G). NO rapidly reacts with ROS, forming peroxynitrites; therefore, we next scavenged them by adding MEG sulfate. MEG sulfate restored cell viability in GIT-27NO-treated but not in Saq-NO-treated (Fig. 5H) cultures, confirming the crucial role of NO-induced peroxynitrites in GIT-27NO-mediated toxicity.
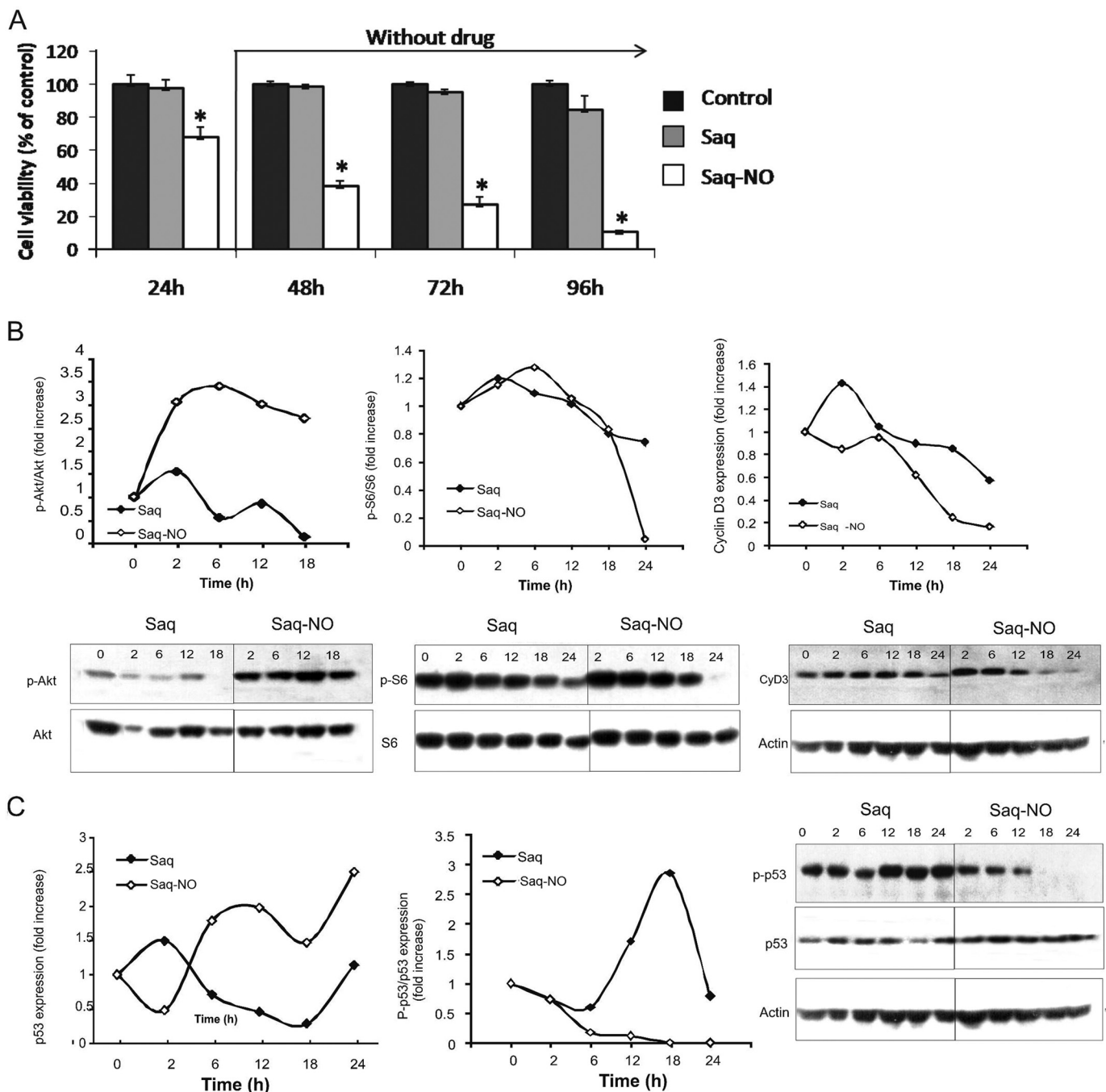
Fig. 3. Saq-NO suppressed cell proliferation. A, CT26CL25 cells were treated for 24 h with Saq or Saq-NO or left untreated (control), and cell viability in the absence of the drugs was measured by CV after an additional 24, 48, and 72 h. The data are presented as the mean ± S.D. from a representative of three independent experiments. ∗ $p < 0.05$ refers to untreated cultures. Cells were treated with an IC_{50} dose of Saq-NO and an equal dose of Saq for the indicated time and Akt, S6 protein, cyclin D3 (B), p53, and p-p53 Ser20 (C) expression at the indicated time points was analyzed by Western blot. Densitometric analysis of data from a representative of three experiments was presented as fold increase relative to that of the control.

Discussion

In our study, we demonstrated in two different colon cancer cell lines strong sensitivity to treatment with two compounds designed by covalent attachment of a NO moiety to the original anti-inflammatory or antiviral core drug. The mode of action of NO-modified drugs was profoundly altered compared with that of the original drugs. In agreement with previous data (Maksimovic-Ivanic et al., 2008; Mijatovic et al., 2008, 2010; Donia et al., 2009), the anti-inflammatory agent VGX-1027 did not affect colon cancer cell viability.

Analyses of cell cycle distribution as well as AnnV-PI double staining revealed that GIT-27NO triggered apoptosis in both colon cell lines tested. However, in contrast to its action in Ta3H mammary cells, this process involves caspase activation as well as enhanced expression of caspases 8 and 3 at the gene level accompanied by mitochondrial membrane depolarization in mouse but not in Bax-deficient human colon cell lines (Mijatovic et al., 2010). Different intracellular profiles of the apoptotic process pulsed by the treatment as a reflection of intrinsic specificity of tested lines did not obviously
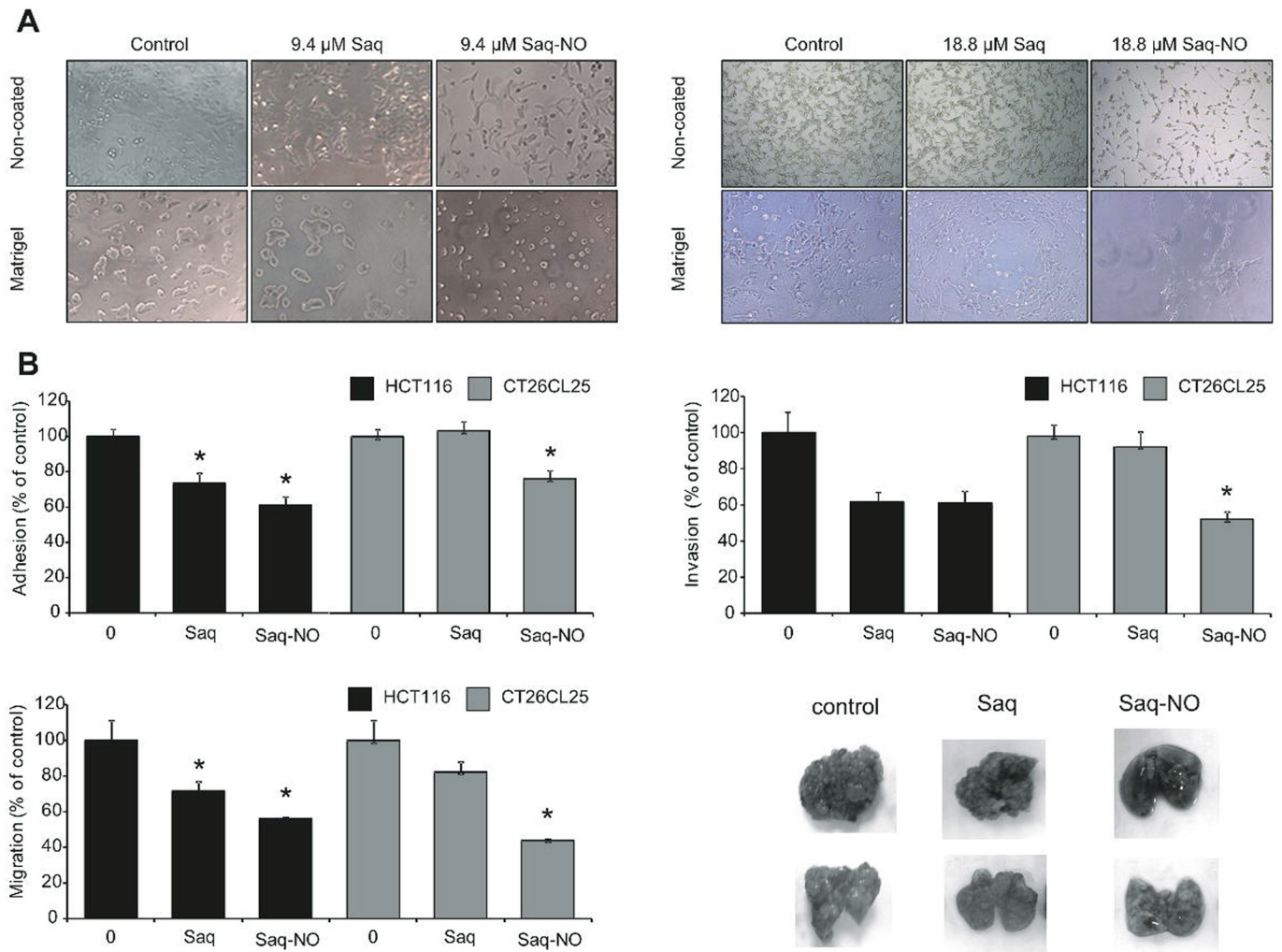
Fig. 4. Saq-NO abrogates metastatic potential of the cells. A, light microscopy and Matrigel growth of cells after 72 h of treatment with Saq or Saq-NO for HCT116 (right) and CT26CL25 (left) cells. B, cells were pretreated for 24 h with Saq or Saq-NO or left untreated (0), and adhesion was measured after 1 h, and migration and invasion were examined after 36 or 24 h, respectively. The data are presented as the mean ± S.D. from a representative of two independent experiments. ∗, $p < 0.05$ refers to untreated cultures. Representative photographs of lungs from animals that received control or Saq- or Saq-NO-pretreated tumor cells are presented.

disturb the prevalence of the proapoptotic signal and completion of the dying process (Mijatovic et al., 2010). This cytotoxic mode of action coincided with a slight effect on the rate of cell proliferation that failed to influence the metastatic properties of either cell line when GIT-27NO was administered at subtoxic doses (not shown). As demonstrated previously, the cytocidal effects of GIT-27NO are related to the intracellular introduction of high levels of NO (Maksimovic-Ivanic et al., 2008; Mijatovic et al., 2010). Even if GIT-27 NO released lower amounts of NO compared with corresponding doses of exogenous NO donors, the uptake of NO in colon cancer cells exposed to GIT-27NO was impressively high. Subsequently, cell damage promoted by the toxic amount of NO released from GIT-27NO and other free radicals generated in this cascade were reflected in cell viability. Furthermore, the cytocidal effects of GIT-27NO on colon cancer cells were successfully blocked by the peroxynitrite scavenger MEG sulfate, confirming the crucial role of released NO and peroxynitrite in drug-mediated toxicity. Of interest, the intrinsic properties of HCT116 cells in redox status make them more tolerant to the oxidative stress generated by applied

doses of GIT-27NO (Dang et al., 2005), thus requiring a prolonged period of exposure to the drug for induction of massive apoptotic cell death.

On the other hand, Saq-NO decreased the viability of colon cancer cells primarily through inhibition of cell proliferation but not through induction of cell death (Maksimovic-Ivanic et al., 2009). Moreover, a short pulse exposure of the colon cancer cells to Saq-NO was sufficient to induce irreversible loss of their dividing potential, indicating permanent changes in cell physiology. A long-lasting blockade in cell division was observed upon prolonged Saq-NO treatment, which significantly affected the metastatic potential of both cell lines independently of their previously documented differences in metastasis (Sahai and Marshall, 2003). We previously showed that permanent inhibition of cellular proliferation correlated with induction of differentiation and up-regulated p53 expression in cancer cells of neuroectodermal origin (Maksimovic-Ivanic et al., 2009). The phenomenon described was connected to p53 involvement in the process of differentiation of transformed cells. Strong up-regulation of p53 through de novo synthesis rather than disturbed proteo-
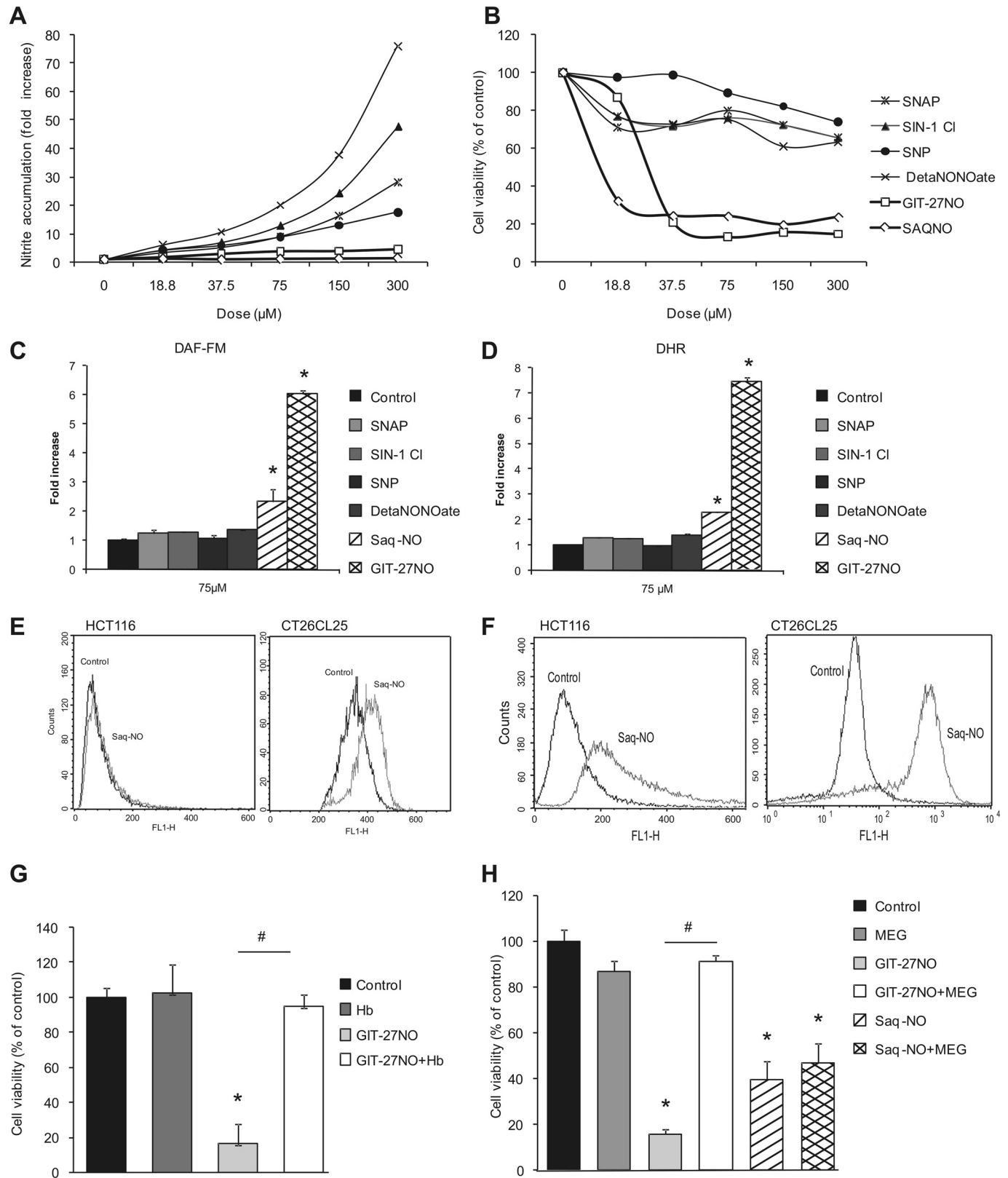
Fig. 5. The contribution of NO, ROS, and RNS to the tumoricidal effects of the drugs. Cells (10^4 cells/well) were treated with a range of doses of the indicated NO donors, Saq-NO, or GIT-27NO for 24 h, after which accumulation of nitrites was determined by the Griess reaction (A), and cell viability was examined by CV test (B). The data are presented as mean values and SD was less than 10%. Intracellular NO production was determined by DAF-FM indicator and ROS/RNI production by DHR staining. Intensity of fluorescence was analyzed by a Chameleon multiplate reader (C and D) or FACSCalibur flow cytometer (E and F). Cells were cultivated with IC_{50} concentrations of the drugs in the presence of hemoglobin (Hb) (25 μM) (G) or MEG (100 μM) (H) for 24 h, and viability was measured by CV. The data are presented as the mean $\pm$ S.D. from a representative of three independent experiments *, $p < 0.05$, refers to untreated cultures.

litical degradation can be involved in decreased cellular proliferation and also lost of malignant properties. In concordance with our previous data, transient up-regulation of Akt activity was detected (Maksimovic-Ivanic et al., 2009; Mijatovic et al., 2011). However, inhibition of the downstream part of the PI3K/Akt pathway manifested through down-regulation of S6 protein observed in both lines with Saq-NO treatment could be essential for both loss of proliferative potential and decreased metastatic properties. The same effect was described in prostate cancer LNCap cells upon treatment with this drug (Mojic et al., 2012). The upstream regulator of S6 protein, S6K, regulates these processes (Fenton and Gout, 2011), offering an explanation for the decreased capacity of the cell lines tested to adhere, migrate, and invade. In addition, it was recently reported that Saq was able to inhibit metastatic properties through inhibition of angiogenesis and matrix metalloproteinase-2 and -9 expression (Sgadari et al., 2002; Toschi et al., 2011). Moreover, the results obtained in vitro were confirmed in a syngeneic metastatic model of colon cancer, in which pretreatment of CT26CL26 cells with Saq-NO for 24 h was sufficient to permanently affect their metastatic potential.

The absence of highly destructive RNS concurs with the cytostatic mode of action of the compound (Calcerrada et al., 2011). It is evident that oxidative stress triggered by Saq-NO may not be triggered by the discrete quantity of NO released that probably falls within concentration ranges required by this gas to regulate cell physiology (Hickok and Thomas, 2010; Singh and Gupta, 2011). The efficacy of certain newly synthesized drugs against colon cancer cells in tumor transplant studies in animal cells is improving (Maksimovic-Ivanic et al., 2008, 2009; Mijatovic et al., 2008, 2010, 2011; Donia et al., 2009, 2011; Rothweiler et al., 2010; Canducci et al., 2011). Because NO is a short-lived and highly reactive molecule, the advantage of GIT-27NO over other NO donors is related to its ability to directly deliver NO into the cell and therefore prevent its loss before reaching a target. Moreover, rapid amplification of the toxic signal through formation of peroxynitrite makes it highly effective in inducing cell death.

In contrast to GIT-27NO, Saq-NO was created from a drug that already possessed anticancer properties. Addition of a NO moiety to Saq markedly enhanced the anticancer action of Saq in a manner apparently independent of NO release. We demonstrate for the first time in these colon cancer cell lines that their high sensitivity to Saq-NO was associated with suppression by the drug of migratory and invasive capacities. These findings reveal an additional and important antineoplastic property of Saq-NO, which along with its direct cytostatic effects and chemo- and immunosensitizing properties qualify it as an important chemotherapeutic agent worthy of further research and potentially of clinical evaluations.

Authorship Contributions

Participated in research design: Mijatovic, Maksimovic-Ivanic, Zocca, McCubrey, and Nicoletti.

Conducted experiments: Mojic, Mijatovic, Maksimovic-Ivanic, Miljkovic, Stankovic, Mangano, Travali, Donia, and Fagone.

Contributed new reagents or analytic tools: Al-Abed.

Performed data analysis: Mojic, Mijatovic, and Maksimovic-Ivanic.

Wrote or contributed to the writing of the manuscript: Mojic, Mijatovic, Maksimovic-Ivanic, Stosic-Grujicic, Zocca, and Nicoletti.

References

Bernstein WB and Dennis PA (2008) Repositioning HIV protease inhibitors as cancer therapeutics. *Curr Opin HIV AIDS* **3**:666–675.

Bower M, Fox P, Fife K, Gill J, Nelson M, and Gazzard B (1999) Highly active anti-retroviral therapy (HAART) prolongs time to treatment failure in Kaposi's sarcoma. *AIDS* **13**:2105–2111.

Calcerrada P, Peluffo G, and Radi R (2011) Nitric oxide-derived oxidants with a focus on peroxynitrite: molecular targets, cellular responses and therapeutic implications. *Curr Pharm Des* **17**:3905–3932.

Canducci F, Ceresola ER, Saita D, Al-Abed Y, Garotta G, Clementi M, and Nicoletti F (2011) The new and less toxic protease inhibitor saquinavir-NO maintains anti-HIV-1 properties in vitro indistinguishable from those of the parental compound saquinavir. *Antiviral Res* **91**:292–295.

Chow WA, Jiang C, and Guan M (2009) Anti-HIV drugs for cancer therapeutics: back to the future? *Lancet Oncol* **10**:61–71.

Coimbra M, Kuijpers SA, van Seters SP, Storm G, and Schiffelers RM (2009) Targeted delivery of anti-inflammatory agents to tumors. *Curr Pharm Des* **15**:1825–1843.

Dang DT, Chen F, Kohli M, Rago C, Cummins JM, and Dang LH (2005) Glutathione S-transferase pi1 promotes tumorigenicity in HCT116 human colon cancer cells. *Cancer Res* **65**:9485–9494.

Donia M, Maksimovic-Ivanic D, Mijatovic S, Mojic M, Miljkovic D, Timotijevic G, Fagone P, Caponnetto S, Al-Abed Y, McCubrey J, et al. (2011) In vitro and in vivo anticancer action of saquinavir-NO, a novel nitric oxide-derivative of the protease inhibitor saquinavir, on hormone resistant prostate cancer cells. *Cell Cycle* **10**:492–499.

Donia M, Mijatovic S, Maksimovic-Ivanic D, Miljkovic D, Mangano K, Tumino S, Biondi A, Basile F, Al-Abed Y, Stosic-Grujicic S, et al. (2009) The novel NO-donating compound GIT-27NO inhibits in vivo growth of human prostate cancer cells and prevents murine immunoinflammatory hepatitis. *Eur J Pharmacol* **615**:228–233.

Fagone P, Mangano K, Coco M, Perciavalle V, Garotta G, Romao CC, and Nicoletti F (2012) Therapeutic potential of carbon monoxide in multiple sclerosis. *Clin Exp Immunol* **167**:179–187.

Fenton TR and Gout IT (2011) Functions and regulation of the 70kDa ribosomal S6 kinases. *Int J Biochem Cell Biol* **43**:47–59.

Flick DA and Gifford GE (1984) Comparison of in vitro cell cytotoxic assays for tumor necrosis factor. *J Immunol Methods* **68**:167–175.

Gallagher DJ and Kemeny N (2010) Metastatic colorectal cancer: from improved survival to potential cure. *Oncology* **78**:237–248.

Gao J, Liu X, and Rigas B (2005) Nitric oxide-donating aspirin induces apoptosis in human colon cancer cells through induction of oxidative stress. *Proc Natl Acad Sci USA* **102**:17207–17212.

Giuliani F, De Vita F, Colucci G, and Pisconti S (2010) Maintenance therapy in colon cancer. *Cancer Treat Rev* **36**:S42–S45.

Gupta AK, Cerniglia GJ, Mick R, McKenna WG, and Muschel RJ (2005) HIV protease inhibitors block Akt signaling and radiosensitize tumor cells both in vitro and in vivo. *Cancer Res* **65**:8256–8265.

Hickok JR and Thomas DD (2010) Nitric oxide and cancer therapy: the emperor has NO clothes. *Curr Pharm Des* **16**:381–391.

Kashfi K and Rigas B (2007) The mechanism of action of nitric oxide-donating aspirin. *Biochem Biophys Res Commun* **358**:1096–1101.

Kleinman HK, McGarvey ML, Liotta LA, Robey PG, Tryggvason K, and Martin GR (1982) Isolation and characterization of type IV procollagen, laminin, and heparan sulfate proteoglycan from the EHS sarcoma. *Biochemistry* **21**:6188–6193.

Labianca R and Merelli B (2010) Screening and diagnosis for colorectal cancer: present and future. *Tumori* **96**:889–901.

Lebbé C, Blum L, Pellet C, Blanchard G, Vérola O, Morel P, Danne O, and Calvo F (1998) Clinical and biological impact of antiretroviral therapy with protease inhibitors on HIV-related Kaposi's sarcoma. *AIDS* **12**:F45–F49.

Maksimovic-Ivanic D, Mijatovic S, Harhaji L, Miljkovic D, Dabideen D, Fan Cheng K, Mangano K, Malaponte G, Al-Abed Y, Libra M, et al. (2008) Anticancer properties of the novel nitric oxide-donating compound (S,R)-3-phenyl-4,5-dihydro-5-isoxazole acetic acid-nitric oxide in vitro and in vivo. *Mol Cancer Ther* **7**:510–520.

Maksimovic-Ivanic D, Mijatovic S, Miljkovic D, Harhaji-Trajkovic L, Timotijevic G, Mojic M, Dabideen D, Cheng KF, McCubrey JA, Mangano K, et al. (2009) The antitumor properties of a nontoxic, nitric oxide-modified version of saquinavir are independent of Akt. *Mol Cancer Ther* **8**:1169–1178.

Mijatovic S, Maksimovic-Ivanic D, Mojic M, Malaponte G, Libra M, Cardile V, Miljkovic D, Harhaji L, Dabideen D, Cheng KF, et al. (2008) Novel nitric oxide-donating compound (S,R)-3-phenyl-4,5-dihydro-5-isoxazole acetic acid-nitric oxide (GIT-27NO) induces p53 mediated apoptosis in human A375 melanoma cells. *Nitric Oxide* **19**:177–183.

Mijatovic S, Maksimovic-Ivanic D, Mojic M, Timotijevic G, Miljkovic D, Mangano K, Donia M, Di Cataldo A, Al-Abed Y, Cheng KF, et al. (2011) Cytotoxic and immune-sensitizing properties of nitric oxide-modified saquinavir in iNOS-positive human melanoma cells. *J Cell Physiol* **226**:1803–1812.

Mijatovic S, Maksimovic-Ivanic D, Mojic M, Timotijevic G, Miljkovic D, Donia M, Libra M, Coco M, McCubrey J, Al-Abed Y, Korac A, et al. (2010) Induction of caspase-independent apoptotic-like cell death of mouse mammary tumor TA3Ha cells in vitro and reduction of their lethality in vivo by the novel chemotherapeutic agent GIT-27NO. *Free Radic Biol Med* **48**:1090–1099.

Mojic M, Mijatovic S, Maksimovic-Ivanic D, Dinic S, Grdovic N, Miljkovic D, Stosic-Grujicic S, Tumino S, Fagone P, Mangano K, et al. (2012) Saquinavir-NO-targeted

S6 protein mediates sensitivity of androgen-dependent prostate cancer cells to TRAIL. *Cell Cycle* **11:**1174–1182.

Niehues T, Horneff G, Megahed M, Schroten H, and Wahn V (1999) Complete regression of AIDS-related Kaposi's sarcoma in a child treated with highly active antiretroviral therapy. *AIDS* **13:**1148–1149.

Rigas B and Williams JL (2008) NO-donating NSAIDs and cancer: an overview with a note on whether NO is required for their action. *Nitric Oxide* **19:**199–204.

Rothweiler F, Michaelis M, Brauer P, Otte J, Weber K, Fehse B, Doerr HW, Wiese M, Kreuter J, Al-Abed Y, et al. (2010) Anticancer effects of the nitric oxide-modified saquinavir derivative saquinavir-NO against multidrug-resistant cancer cells. *Neoplasia* **12:**1023–1030.

Rothwell PM, Fowkes FG, Belch JF, Ogawa H, Warlow CP, and Meade TW (2011) Effect of daily aspirin on long-term risk of death due to cancer: analysis of individual patient data from randomised trials. *Lancet* **377:**31–41.

Rothwell PM, Wilson M, Elwin CE, Norrving B, Algra A, Warlow CP, and Meade TW (2010) Long-term effect of aspirin on colorectal cancer incidence and mortality: 20-year follow-up of five randomised trials. *Lancet* **376:**1741–1750.

Sahai E and Marshall CJ (2003) Differing modes of tumour cell invasion have distinct requirements for Rho/ROCK signalling and extracellular proteolysis. *Nat Cell Biol* **5:**711–719.

Schütt M, Zhou J, Meier M, and Klein HH (2004) Long-term effects of HIV-1 protease inhibitors on insulin secretion and insulin signaling in INS-1 β cells. *J Endocrinol* **183:**445–454.

Sgadari C, Barillari G, Toschi E, Carlei D, Bacigalupo I, Baccarini S, Palladino C, Leone P, Bugarini R, Malavasi L, et al. (2002) HIV protease inhibitors are potent anti-angiogenic molecules and promote regression of Kaposi sarcoma. *Nat Med* **8:**225–232.

Sgadari C, Monini P, Barillari G, and Ensoli B (2003) Use of HIV protease inhibitors to block Kaposi's sarcoma and tumour growth. *Lancet Oncol* **4:**537–547.

Singh S and Gupta AK (2011) Nitric oxide: role in tumour biology and iNOS/NO-based anticancer therapies. *Cancer Chemother Pharmacol* **67:**1211–1224.

Stojanovic I, Cuzzocrea S, Mangano K, Mazzon E, Miljkovic D, Wang M, Donia M, Al Abed Y, Kim J, Nicoletti F, et al. (2007) In vitro, ex vivo and in vivo immunop- harmacological activities of the isoxazoline compound VGX-1027: modulation of cytokine synthesis and prevention of both organ-specific and systemic autoimmune diseases in murine models. *Clin Immunol* **123:**311–323.

Stosic-Grujicic S, Cvetkovic I, Mangano K, Fresta M, Maksimovic-Ivanic D, Harhaji L, Popadic D, Momcilovic M, Miljkovic D, Kim J, et al. (2007) A potent immunomodulatory compound, (*S,R*)-3-phenyl-4,5-dihydro-5-isoxazole acetic acid, prevents spontaneous and accelerated forms of autoimmune diabetes in NOD mice and inhibits the immunoinflammatory diabetes induced by multiple low doses of streptozotocin in CBA/H mice. *J Pharmacol Exp Ther* **320:**1038–1049.

Toschi E, Sgadari C, Malavasi L, Bacigalupo I, Chiozzini C, Carlei D, Compagnoni D, Bellino S, Bugarini R, Falchi M, et al. (2011) Human immunodeficiency virus protease inhibitors reduce the growth of human tumors via a proteasome-independent block of angiogenesis and matrix metalloproteinases. *Int J Cancer* **128:**82–93.

Van Geelen CM, de Vries EG, and de Jong S (2004) Lessons from TRAIL-resistance mechanisms in colorectal cancer cells: paving the road to patient-tailored therapy. *Drug Resist Updat* **7:**345–358.

Wolpin BM and Mayer RJ (2008) Systemic treatment of colorectal cancer. *Gastroenterology* **134:**1296–1310.

Yeh RK, Chen J, Williams JL, Baluch M, Hundley TR, Rosenbaum RE, Kalala S, Traganos F, Benardini F, del Soldato P, et al. (2004) NO-donating nonsteroidal antiinflammatory drugs (NSAIDs) inhibit colon cancer cell growth more potently than traditional NSAIDs: a general pharmacological property? *Biochem Pharmacol* **67:**2197–2205.

Zhang Y, Yuan J, Zhang HY, Simayi D, Li PD, Wang YH, Li F, and Zhang WJ (2012) Natural resistance to apoptosis correlates with resistance to chemotherapy in colorectal cancer cells. *Clin Exp Med* **12:**97–103.

Address correspondence to: Dr. Ferdinando Nicoletti, Department of Bio-Medical Sciences, University of Catania, Via Androne 83, 95124 Catania, Italy. E-mail: ferdinic@unict.it