DOI:10.1158/1535-7163.MCT-07-2037

Molecular Cancer Therapeutics

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Mol Cancer Ther 2008;7:510-520. Published online March 17, 2008.

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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*

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Abstract

Preclinical studies have shown that nitric oxide (NO)–donating nonsteroidal anti-inflammatory drugs possess anticancer activities. Here, we report *in vitro* and *in vivo* studies showing the antitumor effect of the NO-donating isoxazole derivative (*S,R*)-3-phenyl-4,5-dihydro-5-isoxazole acetic acid (GIT-27NO). GIT-27NO, but not the NO-deprived parental compound VGX-1027, significantly affected viability of both rodent (L929, B16, and C6) and human (U251, BT20, HeLa, and LS174) tumor cell lines. GIT-27NO triggered either apoptotic cell death (e.g., L929 cells) or autophagic cell death (C6 and B16 cells). Moreover, GIT-27NO hampered the viability of cisplatin-resistant B16 cells. NO scavenger hemoglobin completely prevented GIT-27NO-induced death, indicating that NO release mediated the tumoricidal effect of the compound. Increase in intracellular NO upon on the treatment was associated with intensified production of reactive oxygen species, whereas their neutralization by antioxidant *N*-acetylcysteine resulted in partial recovery of cell viability. The antitumor activity of the drug was mediated by the selective activation of mitogen-activated protein kinases in a cell-specific manner and was neutralized by their specific inhibitors. *In vivo* treatment with GIT-27NO significantly reduced the B16 melanoma growth in syngeneic C57BL/6 mice. The therapeutic effect occurred at dose (0.5 mg/mouse) up to 160 times lower than those needed to induce acute lethality (80 mg/mouse). In addition, a dose of GIT-27NO five times higher than that found effective in the melanoma model was well tolerated by the mice when administered for 4 consecutive weeks. These data warrant additional studies to evaluate the possible translation of these findings to the clinical setting. [Mol Cancer Ther 2008;7(3):510–20]

Introduction

Nitric oxide (NO) is a highly reactive molecule implicated in numerous physiologic and pathologic processes, which plays an important role in nonspecific antitumor immune response (1, 2). However, in some circumstances, NO may also lead to tumor expansion and metastases (1, 3, 4). The outcome of tumor-NO interactions depends on the source and quantity of NO, duration of the exposure, and characteristics of the cells (1). By acting as electron donor or electron acceptor, NO may react with inorganic molecules, structures in DNA, prosthetic groups, or proteins, consequently modifying enzymatic and transcriptional factor activities. By generating qualitatively new radicals, NO indirectly mediates further destruction of cellular components (3–5).

A major challenge for designing novel antitumor agents is generation of compounds with improved efficacy, lower side effects, and potential synergism with currently available antineoplastic drugs. The pleiotropic effects of NO on tumor cell growth and survival has suggested that NO-releasing donors could represent a new class of anticancer agents. Along this line of research, particular interest has been focused on the generation of NO-donating nonsteroidal anti-inflammatory drugs (NSAID) that consist of NSAID to which an NO-donating group is covalently attached via an aromatic or aliphatic spacer (6). Although these drugs share some of pharmacologic properties with parental compounds, current data indicate that their structural modification is responsible for enhanced potency and diminished toxicity (7). Preclinical studies have shown that NO-NSAIDs are effective in Alzheimer's disease, cardiovascular, rheumatologic, and lung diseases (7). Combination of cyclooxygenase inhibition property of NSAIDs with tumoricidal potential of NO makes these drugs suitable candidates for treatment of malignant diseases (6). Different NO-NSAIDs have been shown to exert antitumor effects both *in vitro* and *in vivo* (8–14).

Received 9/24/07; revised 12/26/07; accepted 1/10/08.

Grant support: Serbian Ministry of Science grant 143029.

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Note: D. Maksimovic-Ivanic and S. Mijatovic equally contributed to this work.

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doi:10.1158/1535-7163.MCT-07-2037

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We have recently shown that the isoxazoline compound (*S*,*R*)-3-phenyl-4,5-dihydro-5-isoxazole acetic acid (GIT-27NO), VGX-1027, possesses strong immunomodulatory properties both *in vitro* and *in vivo* (15, 16). Considering that NO-donating drugs often have enhanced pharmacologic activity compared with their parent compounds, we synthesized a novel isoxazoline compound VGX-1027 to which a NO releasing moiety was covalently attached (Fig. 1).

In the present study, we have investigated the potential antitumor effects of both parental and NO-conjugated compounds *in vitro* on different murine and human tumor cell lines as well as in *in vivo* model of melanoma in C57BL/6 mice.

Materials and Methods

Reagents, Cells, and Animals

Acridine orange was obtained from Labo-Moderna. All other chemicals used in experiments were purchased from Sigma. GIT-27NO (mol/L = 328 g/mol) and VGX-1027 (mol/L = 205 g/mol) were stored at 4°C as a 5 mg/mL stock solution in 2.5% DMSO-H_2O and were diluted in culture medium immediately before use. Control cell cultures contained the amount of DMSO corresponding to its content in the solution with the highest concentration of GIT-27NO used in the particular experiment.

Rat glioma C6 and human glioblastoma U251 cell lines were kind gifts from Dr. Pedro Tranque (Universidad de Castilla-La Mancha). Murine melanoma B16, human adenocarcinoma HeLa, human breast cancer BT20, and human colon cancer LS174 cell lines were kind gifts from Dr. Sinisa Radulovic (Institute for Oncology and Radiology of Serbia), whereas mouse fibrosarcoma L929 was obtained from the European Collection of Animal Cell Cultures. Cells were grown in HEPES-buffered RPMI 1640 supplemented with 5% FCS, 2 mmol/L glutamine, 0.01% sodium pyruvate, 5×10^{-5} mol/L 2-mercaptoethanol, and antibiotics (culture medium) at 37°C in a humidified atmosphere with 5% CO_2. After the conventional trypsinization procedure, the cells were seeded at 1×10^4 per well in 96-well plate, 2×10^5 per well in 6-well plate, or 3×10^4 per well in 4-well chamber slide, cultivated overnight, and then

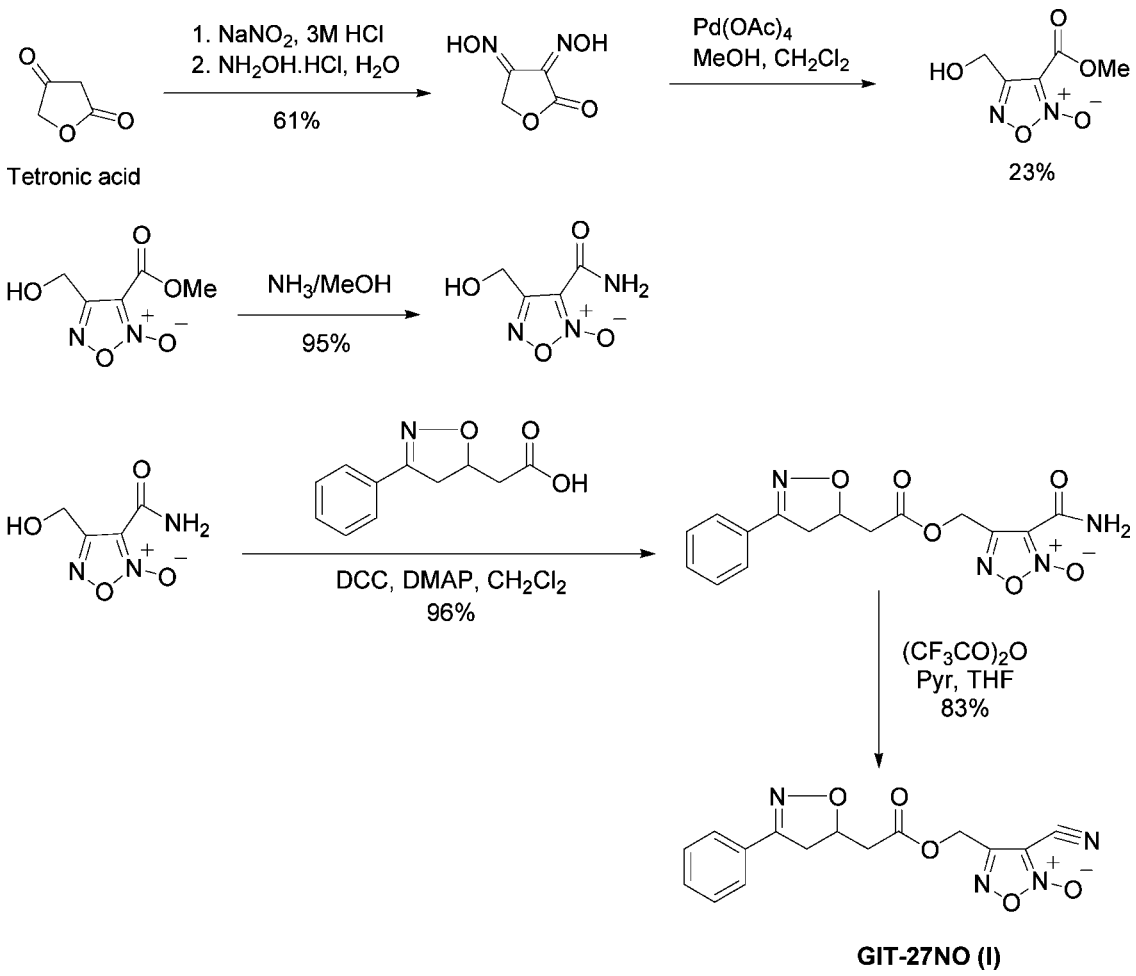


Figure 1. Synthesis of GIT-27NO.

DOI:10.1158/1535-7163.MCT-07-2037

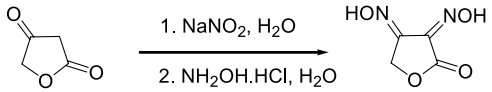
exposed to drug. Resistance to cisplatin was established by continuous cultivation of B16 melanoma in the presence of increasing low concentration of cisplatin (10 nmol/L - 10 μmol/L) as described elsewhere (17).

Inbred C57BL/6 mice were obtained from our own facility at the Institute for Biological Research "Sinisa Stankovic." Outbred, 5-week-old male CD1 mice purchased from Harlan (San Pietro al Natisone) were kept at the animal house of the Department of Biomedical Sciences, University of Catania. Both C57BL/6 and CD1 mice were kept under standard laboratory conditions (nonspecific pathogen free) with free access to food and water. The handling of animals and the study protocol were in accordance with international guidelines and approved by the local Institutional Animal Care and Use Committee.

Synthesis of GIT-27NO

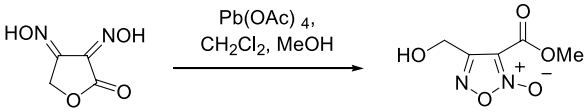
GIT-27NO was synthesized in five steps as described below:

1. Synthesis of 3,4-bis[hydroxylimino]-2-oxotetrahydrofuran



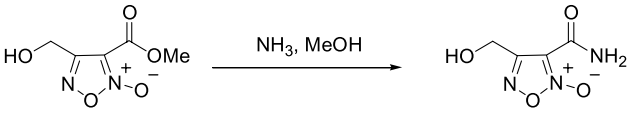
Sodium nitrite (6.9 g, 100 mmol/L) was added in portions to a stirred solution of tetronic acid (10 g, 100 mmol/L) in 3 mol/L HCl (60 mL). The purple solution was stirred for 15 min at room temperature and then added dropwise to a vigorously stirred solution of hydroxylamine HCl (13.9 g, 200 mmol/L) in water (150 mL). The purple coloration gradually fades at room temperature and 3,4-bis[hydroxylimino]-2-oxotetrahydrofuran (9.11 g, 63%) precipitates as white needles (18).

2. Synthesis of 4-hydroxymethyl-2-oxyfurazan-3-carboxymethylester



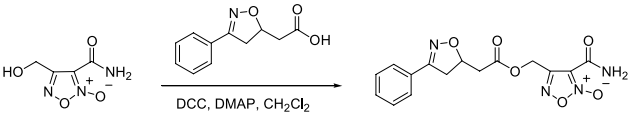
To a mixture of 3,4-bis[hydroxylimino]-2-oxotetrahydrofuran (5.5 g, 38.2 mmol/L), CH_2Cl_2 (60 mL), and methanol (21 mL) was added $Pb(OAc)_4$ (16.9 g, 38.2 mmol/L) at 5°C to 10°C. After stirring at room temperature for 2 h, triethylamine (5 mL, 38.2 mmol/L) was added and stirred for an additional 30 min. CH_2Cl_2 (150 mL) was added and washed with water. The CH_2Cl_2 extract was dried with Na_2SO_4 and concentrated *in vacuo*. The residue was stirred with isopropyl acetate and filtered. The filtrate was recrystallized from isopropyl acetate to afford 4-hydroxymethyl-2-oxyfurazan-3-carboxymethylester (1 g, 15%). ^{1}H NMR (80 MHz, C_3D_6O) δ 3.94 (3H, s), 4.85 (2H, s). ^{13}C NMR (20 MHz, C_3D_6O) δ 52.6, 56.2, 107.9, 156.9, 157.8.

3. Synthesis of 4-hydroxy-2-oxyfurazan-3-carboxylic acid amide



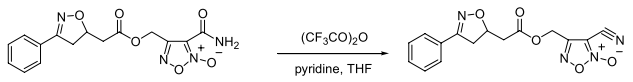
To 4-hydroxymethyl-2-oxyfurazan-3-carboxymethylester (611 mg, 3.51 mmol/L) was added 2 mol/L ammonia in MeOH (25 mL). The solution was stirred for 2 h at room temperature. The reaction mixture was concentrated *in vacuo* to afford 4-hydroxy-2-oxyfurazan-3-carboxylic acid amide (551 mg, 98%). ^{1}H NMR (80 MHz, C_3D_6O) δ 4.80 (2H, s). ^{13}C NMR (20 MHz, C_3D_6O) δ 56.1, 111.3, 157.1, 158.8.

4. Coupling VGX-27 with the NO-donating agent



To a stirred solution of 4-hydroxy-2-oxyfurazan-3-carboxylic acid amide (170 mg, 1.07 mmol/L), (3-phenyl-4,5-dihydro-isoxazol-5-yl) acetic acid (262 mg, 1.28 mmol/L), and DMAP (65 mg, 0.53 mmol/L) in CH_2Cl_2 (10 mL) was added DCC (264 mg, 1.28 mmol/L). After stirring at room temperature for 24 h, diethyl ether (50 mL) was added and the mixture was filtered through Celite. Concentration of the filtrate followed by flash column chromatography (4:1 ethyl acetate/hexane) of the residue afforded the ester (356 mg, 96%). ^{1}H NMR (80 MHz, C_3D_6O) δ 2.78 (d, J = 5 Hz, 1H), 2.91 (d, J = 5 Hz, 1H) 3.30 (d, J = 8 Hz, 1H), 3.62 (d, J = 8 Hz, 1H), 5.14 (m, 1H), 5.48 (s, 2H), 7.42 (m, 3H), 7.68 (d, J = 8Hz, 2H). ^{13}C NMR (20 MHz, C_3D_6O) δ 39.2, 39.4, 57.1, 77.5, 110.7, 126.6, 128.7, 130.0, 154.9, 155.8, 156.5, 169.3.

5. Synthesis of GIT-27NO



Trifluoroacetic anhydride (0.3 mL, 2.02 mmol/L) was added dropwise to a stirred and ice-salt cooled solution of (3-phenyl-4,5-dihydro-isoxazol-5-yl) acetic acid-4-carbamoyl-5-oxyfurazan-3-ylmethyl ester (348 mg, 1.01 mmol/L), dry pyridine (0.15 mL, 2.02 mmol/L) in THF (10 mL). The cooling bath was removed, and stirring was continued for 1 h at room temperature. The reaction mixture was poured into water, acidified with 10% HCl (aqueous), and extracted with ethyl acetate. The combined organic layer was dried with Na_2SO_4 and evaporated to afford a residue that was purified by flash chromatography

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to afford the nitrile (275 mg, 83%). ^{1}H NMR (80 MHz, $CDCl_3$) δ 2.79 (d, J = 5 Hz, 1H), 2.93 (d, J = 5 Hz, 1H), 3.12 (d, J = 8 Hz, 1H), 3.55 (d, J = 8 Hz, 1H), 5.11 (m, 1H), 5.29 (s, 2H), 7.38 (m, 3H), 7.59 (d, J = 8 Hz, 2H). ^{13}C NMR (20 MHz, $CDCl_3$) δ 39.2, 40.2, 56.2, 76.2, 96.5, 105.2, 126.8, 128.9, 130.6, 152.5, 156.8, 169.2.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide and Crystal Violet Assay

The viability of the cells, seeded in flat-bottomed 96-well plates (1×10^4 cells per well) and after 24 h of incubation in the presence of different concentration of either VGX-1027 or GIT-27NO, was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test or crystal violet staining for adherent cells as described previously (19). The results are presented as percentage of control values obtained in untreated cultures.

Determination of Cell Death

For detection of necrotic cell death, the cells were seeded in flat-bottomed 96-well plates (1×10^4 cells per well) and the lactate dehydrogenase (LDH) assay was done after 6, 18, and 24 h of incubation as described elsewhere (19). The percentage of dead cells was determined using the following formula: $[(E - C) / (T - C)] \times 100$, where E is the experimental absorbance of treated cultures measured at 492 nm, C is the absorbance of supernatants of control untreated cells, and T is the absorbance corresponding to the maximal (100%) LDH release of Triton-lysed cells.

Cell cycle analysis and apoptosis detection were done as described elsewhere (19). For detection of autophagy, the cells were cultured in six-well plates (2×10^5 per well), detached, and stained with 1 μg/mL acridine orange for 15 min at room temperature (20). At the end of incubation period, the cells were washed and resuspended in PBS. Green (510-530 nm) and red (>650 nm) fluorescence emission from 10^4 cells illuminated with blue (488 nm) excitation light was measured with a FACSCalibur and analyzed using CellQuest software.

Measurement of Reactive Oxygen Species Release, Intracellular NO, and Nitrite Accumulation

The cells were stained with 1 μmol/L dihydrorhodamine-123 20 min before the treatment with the compound. After the cultivation period, cells were washed and resuspended in PBS, and analysis of reactive oxygen species (ROS) release was done with a FACSCalibur and analyzed using CellQuest Pro software (21).

For intracellular NO detection, detached cells were washed and incubated 1 h at 37°C with 2 μmol/L NO indicator DAF-FM diacetate (Molecular Probes) in phenol red– and serum-free RPMI 1640. The cells were then washed and incubated for additional 15 min at 37°C in fresh RPMI 1640 for completion of deesterification of the intracellular diacetates. Finally, the cells were resuspended in PBS and analyzed by FACSCalibur. Nitrite accumulation as indicator of NO release was measured by Griess reaction as described previously (22).

Immunocytochemical Analysis of Nitrotyrosine

The detection of nitrotyrosine expression was done by the immunocytochemical procedure as described previously (19). The cells were cultivated in glass chamber slides (3×10^4 per well) and stained with anti-nitrotyrosine antibody (1:1,000 in PBS; Sigma). Recognition of nitrotyrosine was done with rabbit extravidin-peroxidase staining kit according to the manufacturer's instructions (Sigma) 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).

Cell-Based ELISA and Western Blot Analysis of Mitogen-Activated Protein Kinase Activation

Activation of mitogen-activated protein kinases [MAPK; p38 MAPK, extracellular signal-regulated kinase (ERK), and Jun NH_2-terminal kinase (JNK)] was determined by a slightly modified method for cell-based ELISA by Versteeg et al. (23) and carried out as described previously (19). The phosphorylation status of ERK1/2, p38, and JNK was analyzed by Western blotting followed by chemiluminescent detection using the PhosphoPlus p44/42 MAPK (Thr^{202}/Tyr^{204}) Antibody Kit and antibodies specific for phospho-SAPK/JNK (Thr^{183}/Tyr^{185}), SAPK/JNK (56G8), phospho-p38 (Thr^{180}/Tyr^{182}), and p38 (all from Cell Signaling Technology) according to the manufacturer's instruction. Resulting bands densities were measured by Scion Image Beta 4.0.3 software (Scion). The results are calculated as ratio of band density of activated/total protein form and presented as relative expression in comparison with the control value, which was arbitrarily set to 1.

Induction of Melanoma in C57BL/6 Mice and Treatment with GIT-27NO

Primary tumors were induced by s.c. injection of 2×10^5 B16 melanoma cells in the dorsal right lumbosacral region of syngeneic C57BL/6 mice. The mice were randomized into the GIT-27NO or the vehicle-treated group soon after the challenge with the tumor cells. Tumor growth was observed daily, and the treatment with GIT-27NO started from day 10 after implantation. GIT-27NO was prepared immediately before treatment and was injected i.p. at a dose of 0.5 mg/mouse for 14 consecutive days. The animals were observed until day 30. Mice were sacrificed and tumor growth was determined by three-dimensional measurements of individual tumors from each mouse. Tumor volume was calculated using the following formula: $[0.52 \times a \times b^2]$, where a is the longest and b is the shortest diameter (24).

Acute Toxicity

To define the acute toxicity of GIT-27NO, the test compound was administered i.p. to CD1 mice at the single doses of 5, 10, 20, 40, and 80 mg/mouse. Control groups were treated i.p. with vehicle (DMSO 80% for 80 mg dose and DMSO 50% for 40, 20, 10, and 5 mg doses). Mortality was evaluated every hour for the first 4 h and then every 10 h until 72 h after dosing.

Subacute Toxicity

To define the subacute toxicity of GIT-27NO, three experimental groups of CD1 mice were treated i.p. with 2.5 mg/mouse GIT-27NO or vehicle or left untreated. There were seven mice for each group. Treatment was done daily for 4 consecutive weeks. During the study period, the mice

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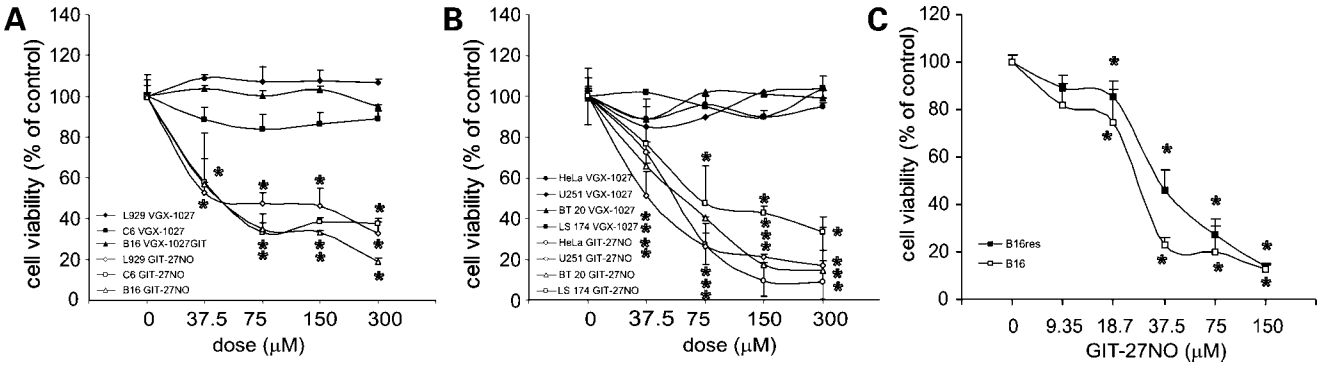

Figure 2. GIT-27NO down-regulates tumor cell viability. Cells (1×10^4 per well) were treated with different concentrations of either VGX-1027 or GIT-27NO (**A** and **B**). B16 and cisplatin-resistant B16 cells were treated with different concentrations of GIT-27NO (**C**). After 24 h of cultivation, cell viability was determined by crystal violet test. Mean ± SD of three independent experiments. *, $P < 0.05$, untreated cultures.

were weighed twice a week. Clinical appearance of the mice was evaluated daily.

After 4 weeks of continuous treatment, the mice were bled from the tail vein for biochemical analyses and individual blood samples were obtained for hematologic biochemical analyses (glucose, cholesterol, urea, GOT, and triglycerides) carried out as described elsewhere (15).

Statistical Analysis

The results are presented as mean ± SD of triplicate observations from one representative of at least three experiments with similar results, except if indicated otherwise. The significance of the differences between various treatments was assessed by ANOVA followed by Student Newman-Keuls test or Mann-Whitney *U* test for evaluating of GIT-27NO efficacy *in vivo*. $P < 0.05$ was considered to be significant.

Results

Synthesis of GIT-27NO

GIT-27NO was synthesized in two stages. First 4-hydroxymethyl-2-oxyfurazan-3-carboxylic acid amide, the NO-donating agent, was prepared in three steps and then coupled to VGX-1027 (25). The new product was reduced to yield the desired compound GIT-27NO (Fig. 1).

Evaluation of Antitumor Activity of GIT-27NO versus VGX-1027

The three malignant rodent cell lines (mouse fibrosarcoma L929, rat astrocytoma C6, and mouse melanoma B16) and the four human cell lines (adenocarcinoma HeLa, breast carcinoma BT20, colon carcinoma LS174, and glioblastoma U251) were incubated for 24 h with different concentrations of either GIT-27NO or VGX-1027 and cell viability was then determined by crystal violet test. As shown in Fig. 2A and B, whereas the parental compound VGX-1027 did not affect the viability of tumor cells, GIT-27NO led to dose-dependent reduction in number of viable cells. B16 cell line that acquired resistance to cisplatin by continuous cultivation in the presence of low concentration of this drug was as sensitive to GIT-27NO treatment as maternal B16 cell line (Fig. 2C). Diminished tumor cell viability by GIT-27NO was associated with reduction of mitochondrial respiration as estimated by MTT (data not shown). Thus, it was evident that chemical modification of inert substance resulted in generation of new compound with strong tumoricidal potential against large spectrum of different cell lines as well as against melanoma cells resistant to one of most frequently used cytostatic drugs (cisplatin).

GIT-27NO Induces Distinct Types of Tumor Cell Death

As shown in Fig. 3A, GIT-27NO treatment induced LDH release in all three cell lines tested in a dose-dependent manner. However, significant amount of LDH was detectable not before 18 h of cultivation with GIT-27NO (data not shown), suggesting that observed effect could be ascribed to the end stage of apoptotic or autophagic cell death rather than to initial necrotic cell death. The analysis of cellular DNA content done after 24 h of cultivation with the GIT-27NO revealed significantly increased proportion of hypodiploid cells in L929 cultures (Fig. 3B), whereas the percentage of C6 and B16 cells in sub-G compartment was significantly lower (<10%), indicating the importance of other type of cell death triggered by the drug in those cultures (Fig. 3B). Concordantly, cells with condensed chromatin and shrinked nucleus were visible in cultures exposed to GIT-27NO, confirming the prevalence of apoptotic cell death in L929 cultures in contrast to B16 and C6 cells (Supplementary Fig. S1).[5] An alternative pathway of cell death known as autophagy was therefore analyzed in GIT-27NO-treated cells. The development of acidic autophagosomes as a hallmark of autophagy was identified by flow cytometric analysis of acridine orange–stained cells. The intensity of red fluorescence was significantly increased in C6 and B16 cell lines but not in L929 cells on GIT-27NO treatment (Fig. 3C). The relevance of autophagic cell death in diminishing viability of the cells

[5] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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exposed to GIT-27NO was further confirmed by using specific autophagic inhibitors such as bafilomycin A and 3-methyladenine. 3-Methyladenine inhibits autophagic sequestration through the inhibition of phosphatidylinositol-3-kinase, whereas bafilomycin A, a vacuolar H^+-ATPase inhibitor, blocks the fusion of autophagosomes with lysosomes (20). As judged by MTT assay, the viability of GIT-27NO-treated cells was recovered in the presence of both autophagic inhibitors tested, thus confirming the contribution of autophagic cell death in GIT-27NO cytotoxicity towards C6 and B16 cells (Fig. 3D). Restored viability of C6 cells in the presence of the 3-methyladenine was confirmed by light microscopy (Supplementary Fig. S2).[5] Collectively, these results suggest that GIT-27NO exerts specific antitumor effects by activating either apoptotic or autophagic cell death.

Cytotoxicity of GIT-27NO Depends on ROS and Reactive Nitrogen Species Generation

To assess the role of NO in antitumor activity of the drug, we analyzed its ability to release NO. GIT-27NO failed to release NO and form nitrites in the absence of cells, whereas significant amount of nitrites was detected after the 24 h cells treatment (Fig. 4A, *left*). These data suggest that GIT-27NO, unlike conventional NO donors such as SNP, SNAP, GSNO, and SIN-1 (26), does not release NO spontaneously. Concordantly, significant amount of intracellular NO was detected by DAF-FM diacetate indicator even after 2-h treatment of cells with GIT-27NO (Fig. 4A, *right*). To further test the contribution of NO to the antitumor effect of GIT-27NO, tumor cells were cultured concomitantly in the presence of the drug and NO scavenger, hemoglobin. The viability of cells treated with

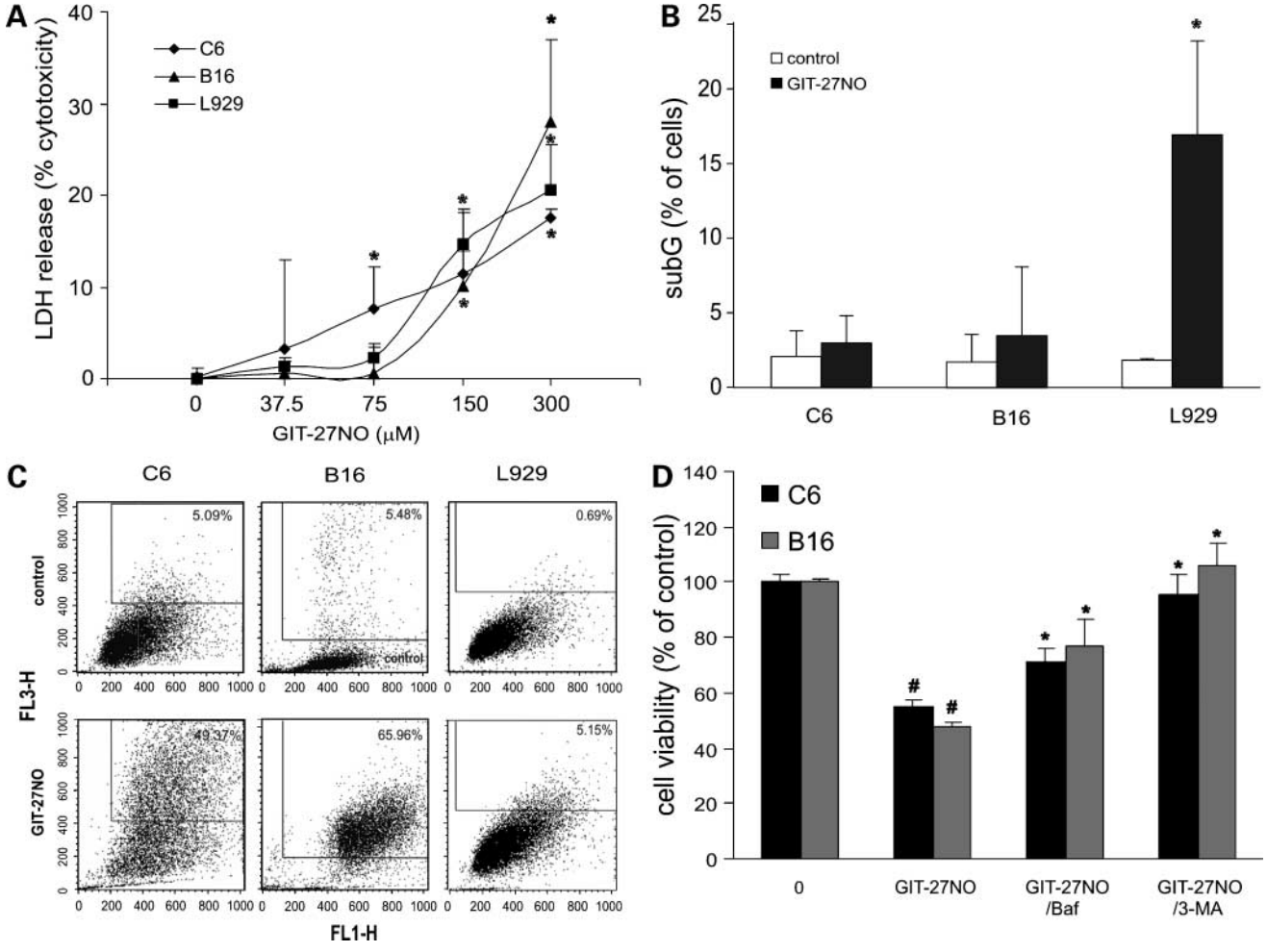

Figure 3. GIT-27NO induces different type of cell death in C6, B16, and L929 cells. **A,** cells were incubated with different doses of GIT-27NO for 24 h, and LDH release assay was done. *, $P < 0.05$. After 24 h of incubation with 75 μmol/L GIT-27NO, cells were stained with propidium iodide and cell cycle was analyzed by flow cytometry (**B**). After 24 h of cultivation without or with GIT-27NO (75 μmol/L), cells were stained with acridine orange and analyzed by flow cytometry for the presence of orange-red acidic autophagic vesicles (**C**). Cells were incubated without or with GIT-27NO (75 μmol/L), 3-methyladenine (1 mmol/L), and/or bafilomycin A (0.5 μmol/L) for 24 h, as indicated, and MTT assay was done (**D**). #, $P < 0.05$, untreated cells; *, $P < 0.05$, GIT-27NO-treated cells.

DOI:10.1158/1535-7163.MCT-07-2037

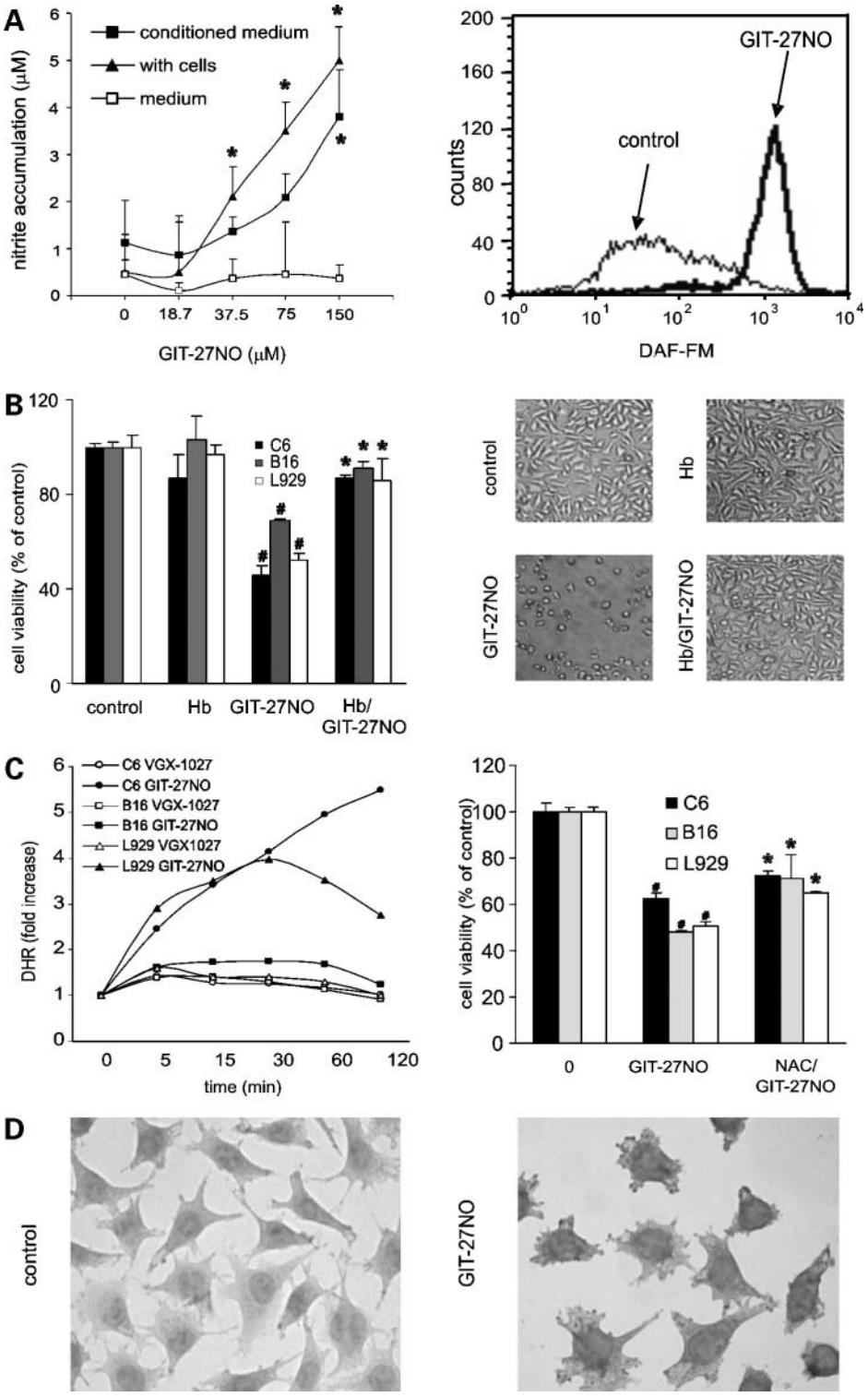

Figure 4. Cytotoxic activity of GIT-27NO is NO mediated. **A,** accumulation of nitrites was detected after 24 h of incubation of C6 cells, cell-conditioned medium, or medium alone with indicated concentrations of GIT-27NO. Intracellular NO was detected by flow cytometry of DAF-FM diacetate fluorescence after 2 h of incubation of C6 cells without (control) or with GIT-27NO (75 μmol/L; *right*). **B,** C6 cells were incubated without (control) or with hemoglobin (12.5 μmol/L) and/or GIT-27NO (75 μmol/L), and after the 24 h of incubation, MTT assay (*left*) or light microscopy analyses (magnification, ×400; *right*) were done. **C,** production of ROS was detected by flow cytometry of dihydrorhodamine-123 fluorescence in indicated time points and expressed as fold increase of geometric means of untreated cells (*left*). The viability of cells incubated without or with GIT-27NO (75 μmol/L) in the presence or absence of antioxidant *N*-acetylcysteine (2.5 μmol/L) was determined after 24 h by MTT assay (*right*). #, $P < 0.05$, untreated cells; *, $P < 0.05$, GIT-27NO-treated cells. **D,** presence of nitrotyrosine residues in C6 cells incubated for 24 h without (control) or with GIT-27NO (75 μmol/L) detected by immunocytochemistry and analyzed by light microscopy (magnification, ×630).

GIT-27NO in this condition was almost completely restored, indicating that NO is crucial for drug-mediated cytotoxicity (Fig. 4B, *left*). This phenomenon was further visualized by light microscopy (Fig. 4B, *right*). Because hemoglobin is an extracellular scavenger, these results suggest that either cell membrane and/or some soluble cell products were responsible for NO release from the drug. To prove this, after 24 h of incubation of equal number of cells, culture medium (conditioned medium) was mixed with GIT-27NO and accumulation of nitrites was measured

DOI:10.1158/1535-7163.MCT-07-2037

after additional 24 h. As seen on Fig. 4A, significant amounts of nitrites were detected in the presence of conditioned medium, indicating the involvement of soluble cellular products in the process of NO liberation from the drug.

Because NO can trigger the oxidative stress (27), we therefore investigated the possible generation of ROS in GIT-27NO-treated cells. Relative to control cells, as judged by intracellular dihydrorhodamine-123 fluorescence (Fig. 4C, *left*), the intracellular production of ROS was markedly elevated even after the shortest treatment of cells with GIT-27NO but not with VGX-1027. Neutralization of ROS by the scavenger *N*-acetylcysteine partly recovered the viability of GIT-27NO-treated cells, indicating limited role of ROS in tumoricidal effect of the compound (Fig. 4C, *right*). On the other hand, significant formation of nitrotyrosine was detected by immmunocytochemistry on treatment with GIT-27NO compared with untreated cells (Fig. 4D). This indicates the occurrence of nitration process as a consequence of binding of NO_2 group to the phenol ring in tyrosine. Together, these data suggest that interplay between reactive nitrogen species and ROS could be responsible for drug-induced cytotoxicity.

Antitumor Activity of GIT-27NO Is Mediated by Differential Activation of MAPK Pathways

MAPKs are family of serine-threonine kinases responsible for conversion of extracellular signals to important cellular programs of survival, proliferation, and death (28, 29). Therefore, we analyzed the influence of GIT-27NO on the activation of these signaling pathways. As determined by cell-based ELISA, various signaling molecules were targeted by GIT-27NO in the different cell lines.

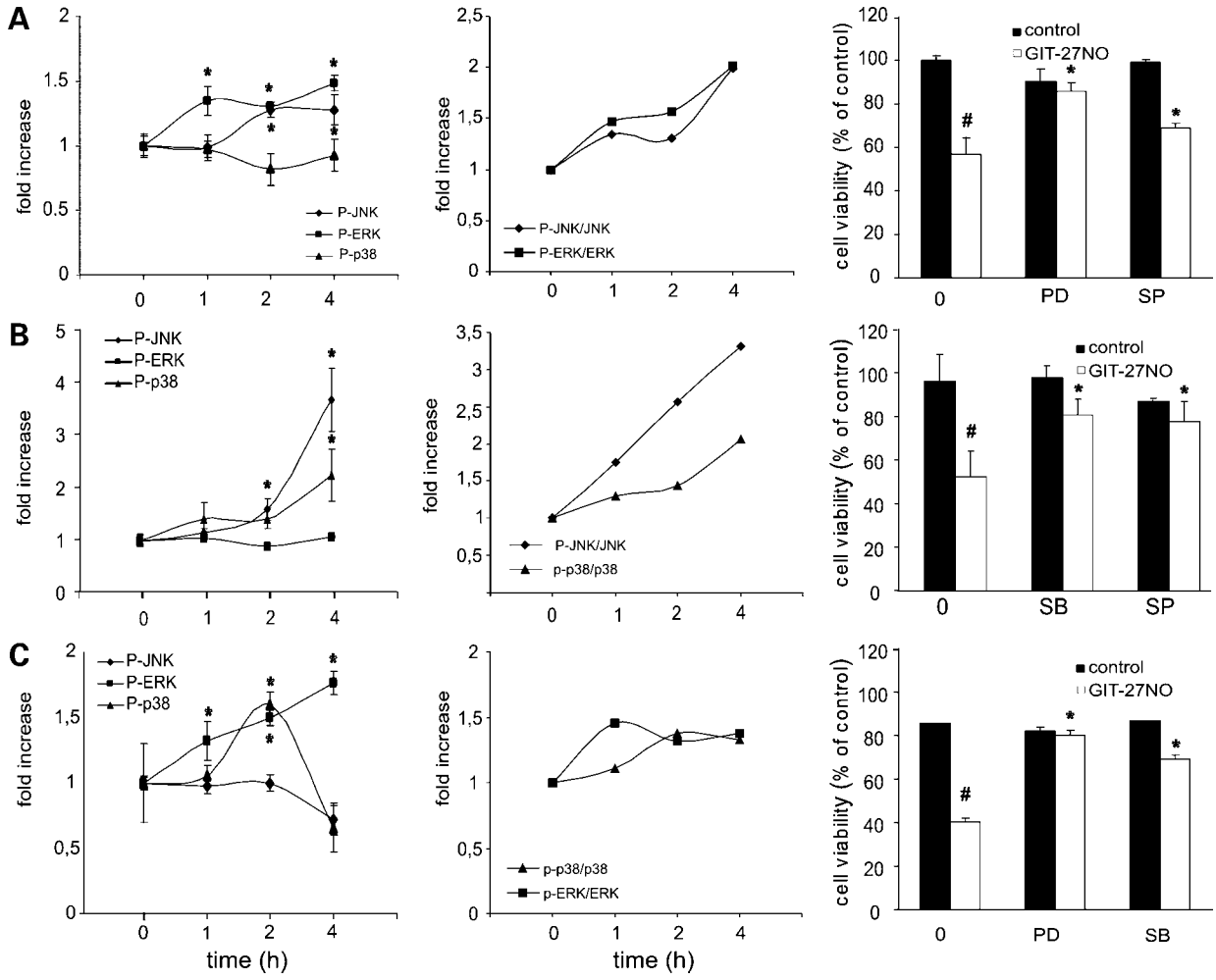

Figure 5. GIT-27NO differentially activates MAPKs. Cells were incubated with 75 μmol/L GIT-27NO and activation of JNK, ERK, and p38 MAPK in (**A**) C6, (**B**) B16, or (**C**) L929 cells was assessed by cell-based ELISA at indicated time points (*left*). Data are fold increase relative to those obtained in untreated control cultures. *, $P < 0.05$. Activation of kinases was evaluated by Western blot and densitometric analysis of data from a representative experiment was presented as fold increase relative to control (*middle*). Cells were incubated without (control) or with GIT-27NO (75 μmol/L) in the presence or absence of specific inhibitors PD98059 (*PD*; 50 μmol/L), SP600125 (*SP*; 0.75 μmol/L), and SB202190 (*SB*; 20 μmol/L) and MTT assay was done after 24 h of incubation in C6 (**A**, *right*), B16 (**B**, *right*), or L929 (**C**, *right*) cells. #, $P < 0.05$, untreated cells; *, $P < 0.05$, GIT-27NO-treated cells.

DOI:10.1158/1535-7163.MCT-07-2037

Table 1. Effects of *in vivo* GIT-27NO treatment on B16 melanoma tumor growth

Group	No. animals	Mean tumor volume*	Mortality†
Control	27	2.97 ± 6.4	2
GIT-27NO	25	0.82 ± 1.2‡	0

*Tumor volumes were determined at the end of follow-up period (30 days after B16 melanoma cell implantation) and volume was calculated as indicated in Materials and Methods.
†Mortality was presented as number of animals died from disease during the experiment.
‡$P < 0.05$, vehicle-treated control animals.

Whereas GIT-27NO induced continuous activation of ERK1/2 and JNK in C6 cells (Fig. 5A, *left*), it did not affect the activation of ERK1/2 in B16 cells but exhibited a significant time-dependent activation of JNK and p38 (Fig. 5B, *left*). Finally, treatment of L929 cells with GIT-27NO led to sustained activation of ERK1/2 and significant activation of p38, without affecting the expression of JNK (Fig. 5C, *left*). The activation of specific kinases was further confirmed by Western blot analysis (Fig. 5A-C, *middle*). In an effort to assess the biological significance of the activation of MAPKs in the reduction of cell viability, we used known pharmacologic inhibitors of these molecules: PD98059 for ERK, SP600125 for JNK, and SB202190 for p38 activation. Treatment of C6 cells with specific ERK and JNK inhibitors (Fig. 5A, *right*), B16 cells with JNK and p38 inhibitors (Fig. 5B, *right*), and L929 cells with ERK and p38 inhibitors (Fig. 5C, *right*) significantly improved the survival of tumor cell lines after the 24 h of incubation with the drug, indicating the role of MAPKs in drug action. The data thus suggest that ability of GIT-27NO to affect different signaling pathways was determined by cell specificity.

GIT-27NO Inhibits the Tumor Growth in C57BL/6 Mice Bearing B16 Melanoma

Having shown the cytotoxic properties of GIT-27NO *in vitro*, we evaluated the *in vivo* antitumor properties of the drug in B16 melanoma-bearing mice. As illustrated in Table 1, a short-course treatment with GIT-27NO (0.5 mg/mouse/d for 10 days), which started from the day 10 after tumor implantation, at the time when the first tumors were palpable in the group, was associated with a significant reduction of tumor growth compared with the control tumor-bearing animals. The effect was stable even after drug withdrawal. There were no significant differences in the incidence and the mean volume of the tumors between the GIT-27NO and the vehicle-treated group before commencing the treatment on day 10th.

In vitro and *In vivo* Toxicity of GIT-27NO

Although GIT-27NO was toxic for primary astrocytes and fibroblasts *in vitro* (Supplementary Fig. S3A),[5] this toxicity occurred at lower extent than that observed in malignant cells. That this *in vitro* toxicity does not preclude the *in vivo* use of the compound is proven by the acute and subacute toxicity studies carried out in CD1 mice. The acute toxicity test revealed that first dose of GIT-27NO capable to induce death from 24 to 72 h (mean ± SD, 54 ± 21.3) after administration was 80 mg/mouse, which was 160 times higher than effective daily dose used *in vivo*. As regard the subacute toxicity study, it was shown that when administered daily to CD1 mice for 4 consecutive weeks at a dose five times higher (2.5 mg/mouse) than that found effective in the melanoma model, GIT-27NO was well tolerated as judged from their clinical appearance and gain in body weight that were indistinguishable from that of vehicle-treated or untreated controls (Supplementary Figs. S3B and S3C).[5] In a similar manner, no significant differences were found among these three groups in the blood values of glucose, cholesterol, urea, GOT, and triglycerides (Supplementary Table S1).[5]

Discussion

Numerous evidences revealed strong antineoplastic properties of NSAIDs modified by covalent attachment of NO (6, 8, 9, 12, 30). In this study, we showed for the first time that chemical modification of parental isoxazole acetic acid derivative VGX-1027 created a qualitatively new NO-donating compound possessed strong antitumor potential. Treatment of different murine and human tumor cell lines with GIT-27NO led to significant reduction of their viability. Moreover, the powerful anticancer activity of GIT-27NO is highlighted by the preserved sensitivity of melanoma cell line that had developed resistance to one frequently used anticancer agent such as cisplatin. However, the parental compound VGX-1027 did not affect the survival of tumor cells when tested at the same (Fig. 2) or even at higher concentrations than GIT-27NO (data not shown).

Induction of apoptosis (31) is a frequent mechanism of action of anticancer drugs. Thus, it was shown that NO-aspirin was capable to induce apoptosis in colon cancer cells, whereas the other drug, NO-sulindac, was responsible for apoptotic death of bladder and prostate carcinoma (9, 10). Several evidences indicate that the capacity of the tumor cells to develop resistance to pharmacologically induced apoptosis plays a key role in determining their resistance to chemotherapy (32). This phenomenon could be explained by the overexpression of signaling molecules with antiapoptotic properties (33). Induction of the other type of cell death, autophagy, was recently reported as an anticancer mechanism of action of radiation or different drugs, such as tamoxifen, aloe-emodin, arsenic trioxide, and temozolomide (19, 20, 34–36). Extremely interesting feature of GIT-27NO is its potential to adapt the tumoricidal mechanism to cell type. L929 cells underwent to process of apoptotic cell death. In contrast, slight apoptosis coincided with induction of autophagy in cultures of C6 and B16 cell lines. Treatment of cells with specific inhibitors of autophagy, 3-methyladenine and bafilomycin A, restored cell viability, thus suggesting that in our conditions autophagy serves as destroying rather than salvaging mechanism. Interactions between apoptotic and autophagic cell death are well documented. These two types of cell

DOI:10.1158/1535-7163.MCT-07-2037

death could coexist in the cell, and depending on circumstances, they could trigger or inhibit each other (32). For example, although aloe-emodin is considered as apoptosis-inducing agent, we and others have shown that this drug preferentially acts through induction of autophagic cell death in glioma cell line (19, 37). The epilogue of apoptotic and autophagic cell death could be the accidental death known as necrosis. Moreover, in apoptosis-deficient cells, autophagy promotes necrosis (38). Therefore, the observed late LDH release after GIT-27NO treatment is the consequence of both types of programmed cell death rather then primary necrosis.

Unlike other exogenous donors of NO, GIT-27NO was only capable of releasing NO in the presence of cells or cell-conditioned medium. Furthermore, the NO scavenger, hemoglobin, almost completely neutralized the effect of the compound, indicating the crucial role of cell-dependent liberation of extracellular NO in suppression of tumor cell growth. It is known that NO may shift the electron transport chain in more reduced state, promoting the O_2^- formation and subsequent generation of other ROS and reactive nitrogen species responsible for further destruction of cells (4, 28). It was shown previously that ROS and reactive nitrogen species mediated apoptotic and autophagic cell death (5, 20, 39). Treatment of cells with parental compound VGX-1027 did not release ROS, whereas significant amounts of these molecules were determined in the presence of GIT-27NO even after 5 min of incubation. Dihydrorhodamine-123 also detected peroxynitrite, which is the product of reaction of O_2^- and NO and is actually one of the most reactive and toxic molecules (3, 5) responsible for nitration of tyrosine, a target for protein activation and consequent signaling propagation (4). NO release triggered the production of reactive radicals and, as a consequence, led to reduction of cell viability that was further verified by the antioxidant-*N*-acetylcysteine treatment. This is consistent with the ability of NO-donating aspirin to induce apoptosis in human colon cancer cells through induction of oxidative stress (10). However, it was recently shown that antitumor property of NO-NSAIDs was not the consequence of attached NO but could be ascribed to the NO carrier (40). Structure of our compound did not include spacer, and as confirmed, the observed toxicity was clearly related to drug NO release. Therefore, comparative analyses of our results with GIT-27NO with those obtained with NO-NSAIDs should be carefully evaluated.

NO and $ONOO^-$ can both alter cell signaling pathways involved in regulation of proliferation, differentiation, and cell death processes (28). GIT-27NO differentially modified the activity of members of MAPK family depending on the cell type. Whereas in L929 cells GIT-27NO induced time-dependent up-regulation of ERK1/2 and p38, treatment of B16 cells resulted in strong phosphorylation of JNK and p38. In C6 glioma cells, GIT-27NO simultaneously up-regulated JNK and ERK, whereas the activity of p38 was not affected. It was shown that strong activation of ERK1/2 in L929 cells induced by cisplatin led cells to apoptosis, and its suppression neutralized the antitumor potential of the drug (41). Therefore, up-regulated ERK1/2 could be the possible reason for preferential apoptosis of L929 cells on treatment with GIT-27NO.

In agreement with our *in vitro* data, the proof of concept study carried out in a preclinical model of melanoma shows that GIT-27NO is capable of favorably influencing the course of the disease even when administered under a ''therapeutic'' regime to mice that have already developed tumor. The observation that this therapeutic effect of GIT-27NO occurs at doses up to 160 times lower to those provoking 100% lethality in mice and that prolonged treatment with a five times higher dose of GIT-27NO than that capable of inhibiting melanoma growth *in vivo* was well tolerated from the mice anticipates a favorable safety profile of the compound and warrant additional preclinical studies to evaluate the anticancer profile of GIT-27NO and the potential translation of these findings to the clinical setting.

Acknowledgments

We thank Drs. T. Simic, A. Savic-Radojevic, M. Pljesa-Ercegovac, and M. Matic (Institute for Biochemistry, School of Medicine, Belgrade University) for valuable help in Western blot analysis.

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