Nitric oxide (NO) enhances pemetrexed cytotoxicity via NO-cGMP signaling in lung adenocarcinoma cells *in vitro* and *in vivo*

HIROKI NAGAI[1], HIROYASU YASUDA[2,3], YUKIMASA HATACHI[1], DENG XUE[2], TAKAHIKO SASAKI[4], MUTSUO YAMAYA[5], YUICHI SAKAMORI[1], YOUSUKE TOGASHI[1], KATSUHIRO MASAGO[1], ISAO ITO[1], YOUNG HAK KIM[1], TADASHI MIO[1] and MICHIAKI MISHIMA[1]

[1]Department of Respiratory Medicine, Graduate School of Medicine, Kyoto University, Kyoto, Kyoto 606-8507; [2]Clinical Practice, Innovation of New Biomedical Engineering Center, Tohoku University, Sendai, Miyagi 980-8574; [3]Sendai Advanced Medical Research Institute, Sendai, Miyagi 981-3135; Departments of [4]Respiratory Medicine, and [5]Advanced Preventive Medicine for Infectious Diseases, Tohoku University School of Medicine, Sendai, Miyagi 980-8574, Japan

Received January 15, 2012; Accepted March 14, 2012

DOI: 10.3892/ijo.2012.1461

Abstract. Pemetrexed (PEM) is a novel, multitargeted, antifolate, antineoplastic agent for the treatment of non-small cell lung cancer and malignant pleural mesothelioma. Additional effects of nitric oxide (NO) donors on the chemosensitivity of cancers have been reported. However, the effects of an NO donor on PEM-induced cytotoxicity remain unknown. In this study, we investigated the effects of the NO donors, NOC-18 on the cytotoxicity in A549 cells *in vitro* and of nitroglycerin (GTN), on the tumor growth of Lewis lung carcinoma cells in a murine syngraft model treated with PEM. The effects of NO donors on the expression of proteins associated with PEM metabolism, including thymidylate synthase (TS), reduced folate carrier 1 (RFC1), folylpolyglutamate synthase (FPGS), γ-glutamyl hydrolase (GGH) and multidrug resistance-related protein (MRP)5, and the effects of cyclic guanosine monophosphate (cGMP) signaling on these proteins were examined in A549 cells. Treatment with 100 nM NOC-18 for 3 days significantly enhanced PEM-induced cytotoxicity and increased the expression of RFC1 and FPGS in A549 cells. Treatment with 10 nM 8-bromo-cGMP (8-Br-cGMP) for 3 days also increased the expression of RFC1 and FPGS in A549 cells. 1*H*-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (10 μm) significantly reversed the increase in RFC1 and FPGS expression induced by 100 nM NOC-18 in A549 cells. Combination therapy with GTN and PEM significantly reduced tumor growth compared with PEM alone in the syngraft model. The enhanced antitumor effect of GTN plus PEM was significantly reversed by the concomitant addition of ODQ. These findings suggest that NO donors, such as NOC-18 and GTN, enhance the anticancer effects of PEM by increasing the RFC1 and FPGS expression and stimulating cGMP signaling pathways in cancer cells.

Correspondence to: Dr Hiroki Nagai, Department of Respiratory Medicine, Graduate School of Medicine, Kyoto University, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan
E-mail: hwithe@kuhp.kyoto-u.ac.jp

Abbreviations: ATP, adenosine triphosphate; 8-Br-cGMP, 8-bromo-cyclic guanosine monophosphate; cGMP, cyclic guanosine monophosphate; DETA, dipropylene triamine; FBS, fetal bovine serum; FPGS, folylpolyglutamate synthase; GGH, γ-glutamyl hydrolase; GTN, nitroglycerin; HIF-1α, hypoxia-inducible factor-1α; LLC, Lewis lung carcinoma; MRP, multidrug resistance-related protein; NO, nitric oxide; NSCLC, non-small cell lung cancer; ODQ, 1*H*-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one; PBS, phosphate-buffered saline; PEM, pemetrexed; RIPA, radioimmunoprecipitation assay; RFC1, reduced folate carrier 1; RT-PCR, reverse transcription-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SE, standard error; TS, thymidylate synthase; VEGF, vascular endothelial growth factor

Key words: nitric oxide, nitroglycerin, pemetrexed, hypoxia, cyclic guanosine monophosphate

Introduction

Lung cancer is the leading cause of cancer mortality worldwide, and approximately 85% of patients with lung cancer are classified as having non-small-cell lung cancer (NSCLC) (1). A variety of anticancer drugs has been developed for the treatment of lung cancer and has contributed to prolonging survival (1). However, even standard first-line platinum-based chemotherapy results in response rates of less than 40% and in a median overall survival of 8 to 14 months among patients with advanced NSCLC and good performance status (2,3). Thus, further advances in the treatment of NSCLC are of utmost importance.

In malignant solid tumors including NSCLC, tumor blood vessels are highly irregular and tortuous, with arteriovenous shunts, blind ends, lack of smooth muscle or innervation and an incomplete endothelial lining and basement membrane (4-7). Thus, low levels of oxygenation have been demonstrated in solid tumors but not in normal tissues (8-11).

Hypoxic conditions in solid tumors are associated with resistance to chemotherapy and radiotherapy (12,13). Hypoxic

conditions induce mutant p53 accumulation and hypoxia-inducible factor (HIF)-1α accumulation in cancer cells (14,15). The accumulation of mutant p53 in tumor tissues promotes chemoresistance to p53-dependent anticancer agents, including cisplatin and vinorelbine (16). Furthermore, HIF-1α activates the transcription of a number of genes that code for proteins involved in angiogenesis, cell growth, metastasis and chemoresistance (17,18). HIF-1α accumulation leads to cell cycle arrest via the crosstalk between HIF-1α and c-Myc, as well as the overexpression of vascular endothelial growth factor (VEGF) and the family of adenosine triphosphate (ATP)-binding cassette transporters, including P-glycoprotein and multidrug resistance-related proteins (MRPs). This subsequently results in the promotion of chemoresistance to taxanes (paclitaxel and docetaxel) and anthracyclines (adriamycin and daunorubicin) through the efflux of intracellular anticancer drugs to the outside of cancer cells (19-22).

Nitric oxide (NO) is a gaseous molecule that plays a unique role as a chemical messenger involved in vasodilator, neurotransmitter and anti-platelet activities (23,24). Under hypoxic conditions, NO has been reported to promote HIF-1α proteasomal degradation by the activation of HIF prolyl hydroxylase and HIF asparaginyl hydroxylase (25,26). HIF-1α degradation followed by a decrease in VEGF improves the delivery of anticancer drugs through vascular normalization and alteration of the oncotic pressure gradient (27).

Additionally, NO has been shown to reverse chemoresistance to doxorubicin due to the dysfunction of the MRP3 protein, via the nitration of tyrosine, in doxorubicin-resistant murine colon cancer cells *in vitro* (28). Treatment with nitroglycerin (GTN) has also been reported to significantly increase phosphorylated p53 at serine 15 and to enhance chemosensitivity to cisplatin in a syngraft mouse model bearing Lewis lung carcinoma (LLC) cells (29). Activated p53 in tumor tissues promotes the apoptosis of cancer cells by p53-dependent anticancer agents, including cisplatin and vinorelbine (16). Furthermore, NO-mimetics have been reported to attenuate hypoxia-induced drug resistance via cyclic guanosine monophosphate (cGMP)-dependent signaling (30). NO donors may be a promising novel therapy in combination with anticancer drugs.

The effects of a NO donor with anticancer drugs including docetaxel, amrubicin, cisplatin, and vinorelbine have been investigated, and clinical research is currently in progress (31). However, there have been no reports of the effect of NO donors on the chemosensitivity of antimetabolic cytotoxic drugs. Pemetrexed (PEM) is one of antimetabolic cytotoxic drugs and a novel, multitargeted antifolate and antineoplastic agent that is active in multiple tumor types, including NSCLC and malignant pleural mesothelioma. In clinical practice, PEM is a key cytotoxic drug for the treatment of NSCLC. In the present study, we investigated the effect of a NO donor on the cytotoxicity of PEM in lung adenocarcinoma cells *in vitro* and *in vivo*.

Materials and methods

Cell lines and experimental reagents. Human lung adenocarcinoma (A549 cells) and murine LLC cells were obtained from the Tohoku University Cell Resource Center for Biomedical Research (Sendai, Japan). A549 cells were tested and authenticated at the Tohoku University Cell Resource Center for Biomedical Research.

Primary antibodies against reduced folate carrier (RFC)1, folylpolyglutamate synthase (FPGS), γ-glutamyl hydrolase (GGH), thymidylate synthetase (TS) and MRP5 were purchased from Abcam (Cambridge, MA), and the monoclonal antibody against β-actin was from Sigma-Aldrich (St. Louis, MO). All secondary antibodies were purchased from Vector Laboratories (Burlingame, CA). A cell death detection kit (an enzyme-linked immunosorbent assay) was purchased from Roche Applied Sciences (Indianapolis, IN). The NO donor, NOC-18 [dipropylene triamine (DETA)/NONOate], was obtained from Calbiochem (La Jolla, CA). GTN was obtained from Nippon Kayaku (Tokyo, Japan). 8-Bromo-cGMP (8-Br-cGMP), which is a non-hydrolyzable analog of cGMP, was purchased from Sigma-Aldrich. 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), a selective and irreversible inhibitor of soluble guanylate cyclase, was purchased from Tocris Bioscience (Ellisville, MO). PEM was obtained from Toronto Research Chemicals (Toronto, ON, Canada).

Culture conditions. We used A549 cells, a cell line from human lung adenocarcinoma cells, for the *in vitro* experiments. For the *in vivo* study, we used LLC cells, a cell line from murine lung carcinoma cells, in a syngraft model. A549 cells were cultured under hypoxic conditions of 1% O_2, 5% CO_2 and 94% N_2 at 37˚C in minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin. LLC cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin.

Apoptosis assay. A549 cells were seeded at a density of 1,000 cells per well in 24-well plates on day 1, and treated with the NO donor, NOC-18, at a concentration of 10 or 100 nM from day 5 to day 7. The culture medium was changed every 24 h. A549 cells were exposed to PEM at a concentration of 200 μM for 3 h on day 8. We evaluated the apoptosis of A549 cells on day 10. All procedures were performed under hypoxic conditions. For evaluation of apoptosis, we used the Cell Death Detection ELISA Plus (Roche Applied Sciences), according to the manufacturer's instructions. This kit is based on a quantitative sandwich enzyme immunoassay principle, using mouse monoclonal antibodies directed against DNA and histones, which allows the apoptosis-specific detection and quantification of mononucleosomes and oligonucleosomes, respectively, that are released into the cytoplasm of apoptotic cells. Nucleosomes were photometrically detected at 405 nm by measuring peroxidase activity. The final absorbance was obtained by subtracting the observed absorbance of the negative control.

Western blot analysis. A549 cells were seeded in 100-mm culture dishes and incubated under hypoxic conditions until all cells reached 70-80% confluence. A549 cells were then treated with NOC-18 (10 or 100 nM), 8-Br-cGMP (10 nM), which is an analog of cGMP, or a combination of NOC-18 (100 nM) and ODQ (10 μM), which is a selective and irreversible inhibitor of soluble guanylate cyclase, for 3 days. A549 cells were washed with phosphate-buffered saline (PBS) and harvested on ice; protein extraction was then performed with radioimmunopre-

cipitation assay (RIPA) buffer. Total proteins were fractionated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), electrotransferred, and probed with specific antibodies against 5 proteins related to the metabolism of PEM: TS, RFC1, MRP5, FPGS and GGH (32). We examined the effect of the NO donor and NO-cGMP signaling on the expression of these proteins.

Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis. The A549 cells were seeded in 60-mm culture dishes and incubated under hypoxic conditions until all cells reached 70-80% confluence. The A549 cells were then treated with NOC-18 (10 or 100 nM), 8-Br-cGMP (10 nM), or a combination of NOC-18 (100 nM) and ODQ (10 μM) for 3 days. A549 cells were washed with PBS and harvested on ice, and then RNA extraction was performed with RNA-Bee buffer (Tel-Test Inc., Friendswood, TX). Total-RNA (1 μg from each sample) was then subjected to first-strand cDNA synthesis using the QuantiTect Reverse Transcription kit (Qiagen, Tokyo, Japan). We used the TaqMan® Gene Expression assay for the primers of human RFC1, TS, MRP5, FPGS and GGH (Applied Biosystems, Tokyo, Japan). The PCR reaction mixture consisted of cDNA obtained from each sample, TaqMan® Universal PCR Master Mix (Applied Biosystems), and the TaqMan Gene Expression Assay for each primer to a final volume of 20 μl. Cycling conditions were 50˚C for 2 min and 95˚C for 10 min, followed by 45 cycles at 95˚C for 15 sec and at 60˚C for 1 min. Gene expression values (relative mRNA levels) are expressed as the ratios of the genes of interest with respect to the internal reference gene, 18S ribosomal RNA (18S rRNA), to normalize the amount of RNA.

Animal studies. The animal studies were approved by the Animal Research Committee at Tohoku University and Kyoto University, and all procedures were carried out according to the European Commission for the use of experimental animals (EU Directive 2010/63/EU). LLC cells were cultured until numbering $3.0x10^7$ cells/dish and harvested. Cells were re-suspended in PBS at $5.0x10^5$ cells/0.1 ml and were injected subcutaneously into the right flank area of 6-week-old female NCr C57/BL6 mice (Japan SLC Inc., Shizuoka, Japan). After the average tumor volume reached 100 mm^3, the mice were randomly allocated to 5 treatment groups: saline (control, group 1); NO donor GTN (Millisrol®) alone (group 2); PEM alone (group 3); combination of PEM and GTN (group 4); or combination of PEM, GTN and ODQ (group 5). Seven mice were included in each group. In group 1, saline was administered intraperitoneally (i.p.) on days 1, 2, 5 and 6 of each study week. In group 2, 0.2 mg/kg GTN was administered i.p. on days 1, 2, 5 and 6. In group 3, 50 mg/kg PEM was administered i.p. on days 2 and 6. In group 4, 50 mg/kg PEM was administered i.p. on days 2 and 6, and 0.2 mg/kg GTN on days 1, 2, 5, and 6. In group 5, 50 mg/kg PEM was administered i.p. on days 2 and 6, and 0.2 mg/kg GTN and 10 mg/kg ODQ on days 1, 2, 5 and 6. One study week was defined as one course of treatment. Mice were treated with 2 courses and sacrificed on day 15. Tumors were measured every other day with a caliper, and tumor volume was calculated using the formula $ab^2/2$, where α is the maximum longitudinal length and b is the cross-sectional diameter (33). Mice were fed with a low-folate diet as a normal mouse diet contains 5-fold the amount of folate of a standard human diet, and excess folate uptake is known to lead to resistance to PEM (32).

Statistical analysis. Experimental results are expressed as the means ± standard error (SE). The comparison between the groups was evaluated using the Mann-Whitney U test. For multiple comparisons, results were analyzed by one-way factorial analysis of variance. A value of $p<0.05$ was considered to indicate a statistically significant difference.

Results

NOC-18 and PEM-induced apoptosis in A549 cells. NOC-18, which has a half-life of 21 h, was used as a NO donor. First, we evaluated the dose-dependency of NOC-18 on the apoptosis of A549 human lung adenocarcinoma cells. Culture medium with 100 nM NOC-18 was most effective at releasing apoptotic mononucleosomes and oligonucleosomes from A549 cells, compared with the control cells (no addition of NOC-18) and low-dose (10 nM) NOC-18 ($p<0.01$, Fig. 1A). Second, we evaluated the dose-dependency of NOC-18 on the apoptosis of A549 cells induced by treatment with 200 μM PEM for 3 h. When compared with PEM alone, treatment with the combination of PEM and 10 nM NOC-18 showed no statistically significant increase in apoptosis in A549 cells. However, treatment with the combination of PEM and 100 nM NOC-18 showed a statistically significant increase in apoptosis compared with PEM alone or the combination of PEM and the lower dose of NOC-18 ($p<0.01$, Fig. 1B).

NO donor and expression of RFC1 and FPGS. We examined the relative mRNA levels of *RFC1*, *TS*, *MRP5*, *FPGS* and *GGH* using real-time RT-PCR (Fig. 2A) and furthermore analyzed the relative expression of the 5 protein products using western blot analysis (Fig. 2B). Gene expression shown as the relative mRNA level is presented as the ratio of the gene of interest and the internal reference, 18S rRNA. Treatment with 100 nM NOC-18 significantly upregulated *RFC1* and *FPGS* mRNA as shown by real-time RT-PCR ($p<0.01$, Fig. 2A) and also increased the expression of these proteins as demonstrated by western blot analysis (Fig. 2B), compared with their respective controls; whereas, there were no differences in relative mRNA levels or protein expression for TS, GGH and MRP5.

The role of the NO-cGMP signaling pathway in the expression of RFC1 and FPGS. Treatment with 10 nM 8-Br-cGMP significantly upregulated the relative mRNA levels of *RFC1* and *FPGS* ($p<0.01$, Fig. 3A) and also enhanced protein expression (Fig. 3B) compared with the controls. By contrast, the addition of 10 μM ODQ to 100 nM NOC-18 significantly decreased the relative mRNA levels of *RFC1* and *FPGS* ($p<0.01$, Fig. 3A) and reduced the expression of RFC1 and FPGS (Fig. 3B) compared with NOC-18 alone.

GTN and the therapeutic efficacy of PEM in vivo. As shown in Fig. 4, treatment with GTN alone did not affect tumor growth, compared with the control. Treatment with PEM significantly attenuated tumor growth compared with the control at day 15 ($p<0.05$); thus, PEM was cytotoxic in NCr C57/BL6 mice fed

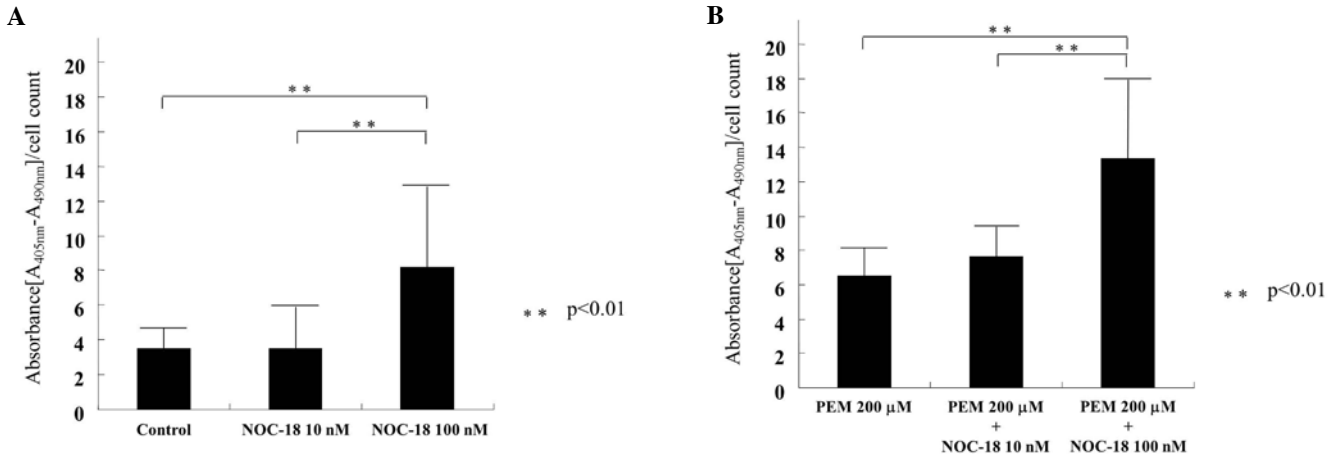

Figure 1. (A) Evaluation of apoptosis in A549 cells treated with NO donor alone and (B) NO donor-induced enhancement of apoptosis in A549 cells treated with PEM. (A) A549 cells were treated with NOC-18 (0, 10 or 100 nM) for 3 days and apotosis was examined. (B) A549 cells were treated with NOC-18 (0, 10 or 100 nM) for 3 days followed by PEM 200 μM and apotosis was examined. Apoptosis was expressed in terms of absorbance units. NOC-18 was used as the NO donor. A549 cells were cultured under hypoxic conditions (1% O_2, 5% CO_2 and 94% N_2 at 37˚C). Results are expressed as the means ± SE. **p<0.01 indicates statistically significant differences. NO, nitric oxide; PEM, pemetrexed.

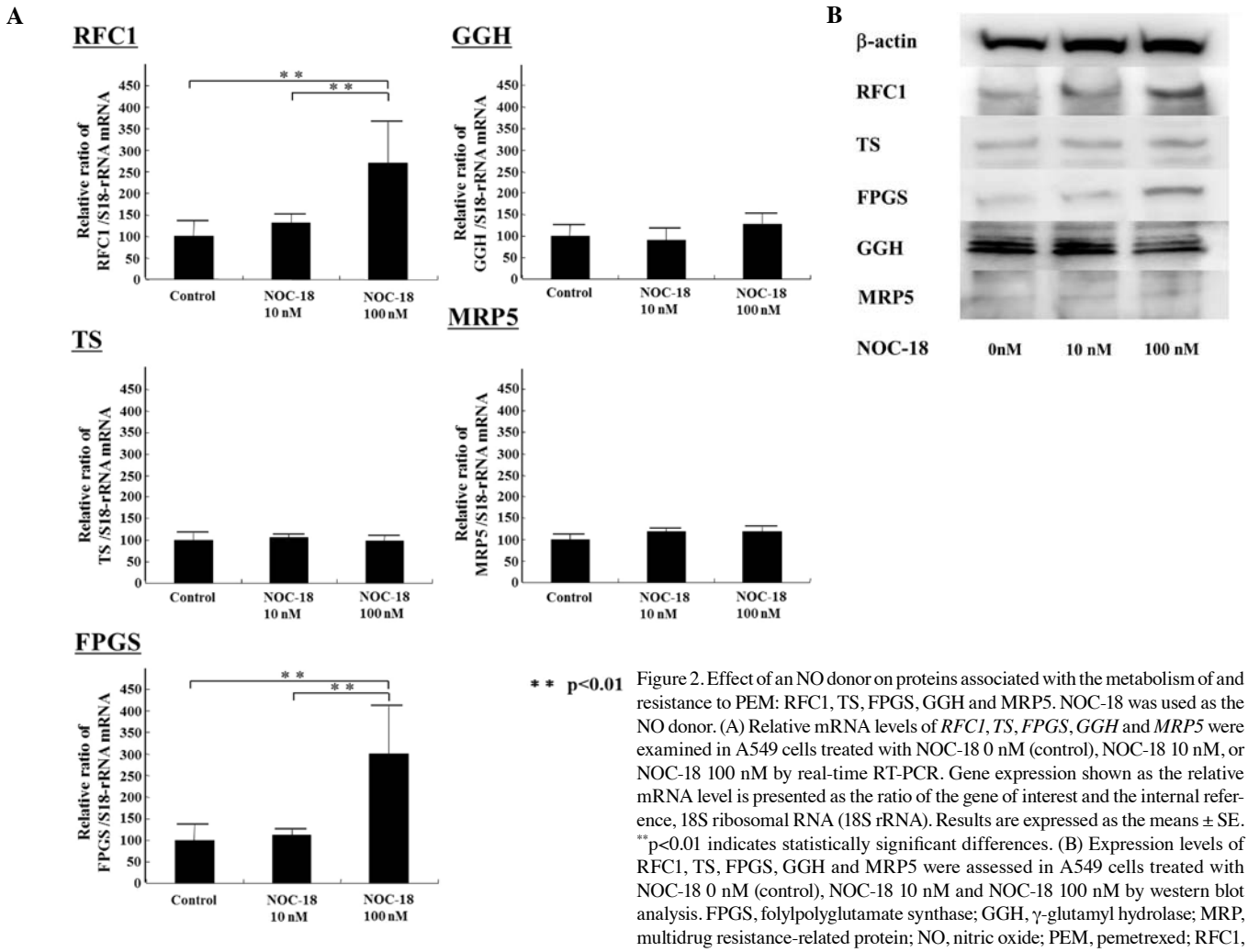

Figure 2. Effect of an NO donor on proteins associated with the metabolism of and resistance to PEM: RFC1, TS, FPGS, GGH and MRP5. NOC-18 was used as the NO donor. (A) Relative mRNA levels of *RFC1*, *TS*, *FPGS*, *GGH* and *MRP5* were examined in A549 cells treated with NOC-18 0 nM (control), NOC-18 10 nM, or NOC-18 100 nM by real-time RT-PCR. Gene expression shown as the relative mRNA level is presented as the ratio of the gene of interest and the internal reference, 18S ribosomal RNA (18S rRNA). Results are expressed as the means ± SE. **p<0.01 indicates statistically significant differences. (B) Expression levels of RFC1, TS, FPGS, GGH and MRP5 were assessed in A549 cells treated with NOC-18 0 nM (control), NOC-18 10 nM and NOC-18 100 nM by western blot analysis. FPGS, folylpolyglutamate synthase; GGH, γ-glutamyl hydrolase; MRP, multidrug resistance-related protein; NO, nitric oxide; PEM, pemetrexed; RFC1, reduced folate carrier 1; TS, thymidylate synthase.

a low-folate diet. Compared with treatment of PEM alone, the combination of PEM and GTN resulted in a significant decrease in tumor growth at day 15 (p<0.05). The combination treatment with PEM and GTN resulted in the lowest tumor growth rate of all the groups. By contrast, the combination of GTN, PEM and ODQ significantly increased tumor growth compared with

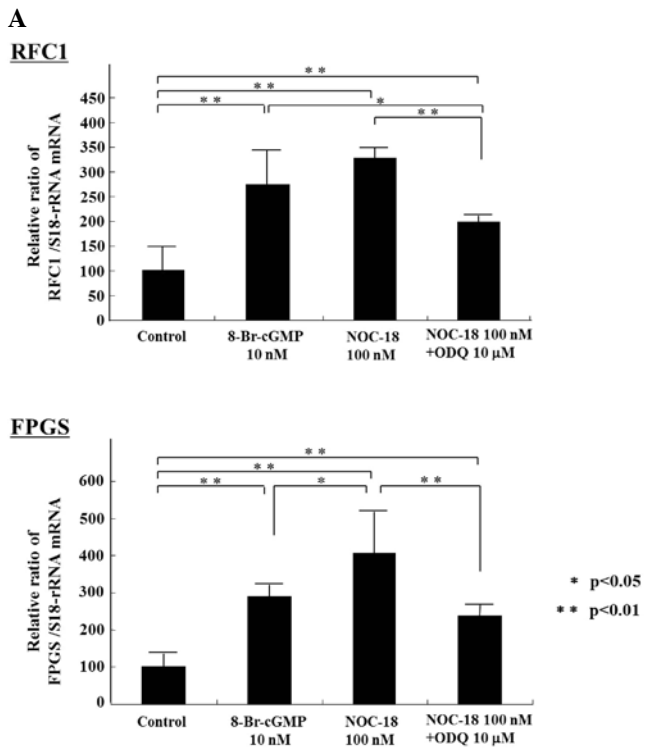

Figure 3. Effect of NO-cGMP signaling on NO-induced increase in RFC1 and FPGS. NOC-18 was used as the NO donor. 8-Br-cGMP was used as an analog of cGMP. ODQ was used as a selective and irreversible inhibitor of soluble guanylate cyclase. (A) Relative mRNA levels of *RFC1* and *FPGS* were examined in A549 cells treated with NOC-18 0 nM (control), NOC-18 100 nM, 8-Br-cGMP 10 nM, or a combination of NOC-18 100 nM and ODQ 10 μM by real-time RT-PCR. Gene expression shown as the relative mRNA level is presented as the ratio of the gene of interest and the internal reference, 18S ribosomal RNA (18S rRNA). Results are expressed as the means ± SE. *p<0.05 and **p<0.01 indicate statistically significant differences. (B) Expression levels of RFC1 and FPGS were assessed in A549 cells treated with NOC-18 0 nM (control), NOC-18 100 nM, 8-Br-cGMP 10 nM, or a combination of NOC-18 100 nM and ODQ 10 μM by western blot analysis. 8-Br-cGMP, 8-bromo-cyclic guanosine monophosphate; FPGS, folylpolyglutamate synthase; GGH, γ-glutamyl hydrolase; MRP, multidrug resistance-related protein; NO, nitric oxide; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PEM, pemetrexed; RFC1, reduced folate carrier 1; TS, thymidylate synthase.

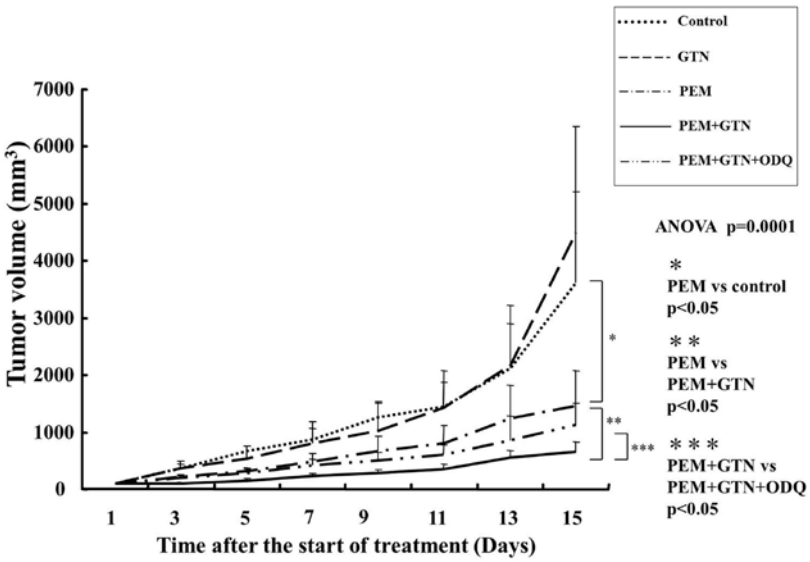

Figure 4. Tumor growth curve of LLC cell-derived syngraft tumors in C57/BL6 mice. GTN was used as an NO donor agent. ODQ was used as a selective and irreversible inhibitor of soluble guanylate cyclase. After the average tumor volume had reached 100 mm^3, the mice were treated with: saline; GTN alone; PEM alone; a combination of PEM and GTN; or a combination of PEM, GTN and ODQ. Tumors were measured every other day with a caliper, and tumor volume was calculated using the formula $ab^2/2$, where α is the maximum longitudinal length and *b* is the cross-sectional diameter. Error bars indicate the means ± SE. *p<0.05 indicates a statistically significant difference between the control and PEM group. **p<0.05 indicates a statistically significant difference between PEM and the combination of PEM and GTN. ***p<0.05 indicates a statistically significant difference between the combination of PEM and GTN and the combination of PEM, GTN and ODQ. GTN, nitroglycerin; LLC, Lewis lung carcinoma; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PEM, pemetrexed.

the combination of GTN and PEM. Therefore, the addition of ODQ to PEM and GTN reversed the GTN-induced increase in chemosensitivity to PEM. These *in vivo* results were consistent with our findings *in vitro*, suggesting that GTN improved the chemosensitivity to PEM through the NO-cGMP signaling pathway in our experimental animal model.

Discussion

In the present study, we demonstrated that efficacy of using NO donors, such as NOC-18 and GTN, with PEM for the treatment of lung adenocarcinoma. For this purpose, we used A549 cells *in vitro* and LLC cells *in vivo*. Our results showed

in vitro an additional effect of using 100 nM NOC-18 for 3 days on PEM-induced apoptosis in A549 cells. We also demonstrate *in vivo* that concomitant treatment with PEM and a clinical dose of GTN to treat ischemic heart disease causes a significant reduction in tumor growth compared with PEM alone. Treatment with 100 nM NOC-18 or 10 nM 8-Br-cGMP for 3 days significantly increased the expression of RFC1 and FPGS in A549 cells. The addition of 10 μM ODQ reversed the increase in RFC1 and FPGS expression at 3 days of treatment with 100 nM NOC-18 in A549 cells. Moreover, the addition of ODQ to the combination of PEM and GTN *in vivo* reversed GTN-induced improvement in chemosensitivity to PEM. NO donors improved the cytotoxicity of PEM via NO-cGMP signaling.

Recently there have been a number of reports on the efficacy of NO in anticancer therapy (31,34). NO donor drugs alone have been shown to induce apoptosis in several types of human cancer cells (35,36). The administration of GTN to prostate cancer patients has been shown to prolong the doubling time of prostate-specific antigen in a phase 2 trial (37). In addition, it has been reported that NO donor drugs are HIF-1α inhibitors and attenuate hypoxia-induced chemoresistance to taxanes and vinorelbine (34). In a randomized phase 2 trial, a combination of GTN plus vinorelbine and cisplatin was shown to improve the response rate and time to progression in comparison with vinorelbine and cisplatin alone in previously untreated patients with stage IIIB/IV NSCLC (38). Furthermore, the NO-cGMP signaling pathway has been reported to have a strong association with NO-induced attenuation of chemoresistance under hypoxic conditions (30). It is noteworthy that this attenuation was not found under normoxic conditions (31). Thus, NO donor drugs are promising candidates for anticancer therapy.

PEM is a novel, multitargeted, antifolate, antineoplastic agent and a key drug in the treatment of non-squamous NSCLC. Possible mechanisms for chemoresistance to antifolate agents in tumor cells include: impaired antifolate uptake due to the loss of RFC function; increased antifolate efflux due to the overexpression of ATP-driven multidrug resistance efflux transporters; overexpression of dihydrofolate reductase and mutations that decrease its affinity for antifolates; overexpression of TS and mutations that decrease its affinity for antifolates; defective antifolate polyglutamylation due to decreased FPGS expression and/or inactivating mutations; increased expression of GGH; and expansion of intracellular tetrahydrofolate cofactor pools (32). The overexpression of TS has been suggested as the most likely mechanism of chemoresistance to PEM (39). The loss of RFC1 expression has been reported to be associated with antifolate resistance in non-selected cell lines due to impaired antifolate uptake (40), and the loss of FPGS activity is the dominant mechanism of polyglutamylation-dependent antifolate in human leukemia cell lines due to impaired cellular retention (41). On the other hand, the loss of RFC gene expression in antifolate-resistant human leukemia cells has been shown to be reversed through cGMP-dependent activation of protein phosphatase 2A which is activated by NO (42,43).

In the present study, in order to determine the mechanism of NO-induced improvement in antitumor cytotoxity of PEM, we examined the effect of NO on the expression of proteins which are associated with the metabolism of and resistance to PEM: TS, RFC1, FPGS, GGH and MRP5 (32). TS is a primary target protein of PEM (32). RFC1 is a major PEM influx transporter (32). FPGS contributes to the polyglutamylation of PEM, which leads to its intracellular stability (32). GGH is an enzyme that counteracts the polyglutamylation of PEM and therefore reduces its intracellular stability (32). MRP5 is the main PEM efflux transporter (32). We found no difference in the expression of TS, GGH and MRP5 between the control and NO donor groups. On the other hand, the expression of RFC1 and FPGS was increased by treatment with a NO donor. In addition, 8-Br-cGMP, an analog of cGMP, also increased the expression of RFC1 and FPGS compared with the control, and the combination of a NO donor and ODQ, an inhibitor of soluble guanylate cyclase, reversed the NO-induced changes in RFC1 and FPGS expression. These findings show that the expression of RFC1 and FPGS was increased by the NO donor via NO-cGMP signaling. Increased RFC1 expression may lead to intracellular accumulation of PEM in A549 cells, and increased FPGS expression may promote the intracellular stability of PEM and retention of its active form. These findings suggest that NO from NOC-18 may enhance the cytotoxicity of PEM by increasing RFC1 and FPGS expression in A549 cells. In addition, in the *in vivo* experiment, the combination of GTN, PEM and ODQ significantly increased tumor growth compared with the combination of GTN and PEM, demonstrating the possibility that a NO donor may enhance the cytotoxicity of PEM by increasing RFC1 and FPGS expression via the NO-cGMP signaling pathway. However, we did not determine the actual intracellular concentration of PEM in A549 cells in this study, and therefore the effects of NOC-18 on the intracellular PEM concentration remain unclear. We intend to study the possible mechanism in future experiments.

NO is a signaling molecule, a toxicant, and an antioxidant under various conditions. Both pro- and anticancer activities have been described, depending on cell type and conditions, the source of NO, its concentration, rate of release, and other factors (44). In the present study, treatment with NOC-18 alone increased apoptosis in A549 cells *in vitro*, whereas treatment with GTN alone did not reduce tumor growth in LLC cells *in vivo*. A difference in the results between *in vitro* and *in vivo* studies regarding NO production and cancer cell proliferation has been reported previously (45). We believe that this difference is partly due to the variation in the efficacy of the NO donor among tumor cells and partly due to differences in the microenvironment around cancer cells in the 2 types of studies; for example, a lack of bystander cells, tumor-associated macrophages, vessels, interstitial pressure between cells and pH and oxygen gradients in the *in vitro* setting (34). Further studies are required to verify the efficacy of NO donors alone in the treatment of cancer.

In summary, this is the first study to demonstrate that NO donors enhance the cytotoxicity of PEM via NO-cGMP signaling and an increase in the expression of RFC1 and FPGS in lung adenocarcinoma cells. Furthermore, NO donors, such as GTN, may be promising agents to enhance chemotherapy. The efficacy of NO donor drugs combined with chemotherapy with PEM should be verified in a clinical setting in the future.

Acknowledgements

This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture (17790524, 19689018) of the Japanese Government.

References

1. National Comprehensive Cancer Network: NCCN Clinical Practice Guidelines in Oncology: Non-Small Cell Lung Cancer, 2011.
2. Schiller JH, Harrington D, Belani CP, *et al*: Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. N Engl J Med 346: 92-98, 2002.
3. Scagliotti GV, Parikh P, von Pawel J, *et al*: Phase III study comparing cisplatin plus gemcitabine with cisplatin plus pemetrexed in chemotherapy-naive patients with advanced-stage non-small-cell lung cancer. J Clin Oncol 26: 3543-3551, 2008.
4. Brown JM and Giaccia AJ: The unique physiology of solid tumors: opportunities (and problems) for cancer therapy. Cancer Res 58: 1408-1416, 1998.
5. Grunt TW, Lametschwandtner A and Staindl O: The vascular pattern of basal cell tumors: light microscopy and scanning electron microscopic study on vascular corrosion casts. Microvasc Res 29: 371-386, 1985.
6. Dewhirst MW, Tso CY, Oliver R, Gustafson CS, Secomb TW and Gross JF: Morphologic and hemodynamic comparison of tumor and healing normal tissue microvasculature. Int J Radiat Oncol Biol Phys 17: 91-99, 1989.
7. Shah-Yukich AA and Nelson AC: Characterization of solid tumor microvasculature: a three-dimensional analysis using the polymer casting technique. Lab Invest 58: 236-244, 1988.
8. Mueller-Klieser W, Vaupel P, Manz R and Schmidseder R: Intracapillary oxyhemoglobin saturation of malignant tumors in humans. Int J Radiat Oncol Biol Phys 7: 1397-1404, 1981.
9. Sutherland RM: Cell and environment interactions in tumor microregions: the multicell spheroid model. Science 240: 177-184, 1988.
10. Sutherland RM: Importance of critical metabolites and cellular interactions in the biology of microregions of tumors. Cancer 58: 1668-1680, 1986.
11. Hockel M and Vaupel P: Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. J Natl Cancer Inst 93: 266-276, 2001.
12. Vaupel P and Mayer A: Hypoxia in cancer: significance and impact on clinical outcome. Cancer Metastasis Rev 26: 225-239, 2007.
13. Moeller BJ and Dewhirst MW: HIF-1 and tumour radiosensitivity. Br J Cancer 95: 1-5, 2006.
14. Fels DR and Koumenis C: HIF-1alpha and p53: the ODD couple? Trends Biochem Sci 30: 426-429, 2005.
15. Hofer T, Wenger H and Gassmann M: Oxygen sensing, HIF-1alpha stabilization and potential therapeutic strategies. Pflugers Arch 443: 503-507, 2002.
16. Lowe SW, Ruley HE, Jacks T and Housman DE: p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 74: 957-967, 1993.
17. Liang BC: Effects of hypoxia on drug resistance phenotype and genotype in human glioma cell lines. J Neurooncol 29: 149-155, 1996.
18. Semenza GL, Roth PH, Fang HM and Wang GL: Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. J Biol Chem 269: 23757-23763, 1994.
19. Gordan JD, Thompson CB and Simon MC: HIF and c-Myc: sibling rivals for control of cancer cell metabolism and proliferation. Cancer Cell 12: 108-113, 2007.
20. Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD and Semenza GL: Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol 16: 4604-4613, 1996.
21. Comerford KM, Wallace TJ, Karhausen J, Louis NA, Montalto MC and Colgan SP: Hypoxia-inducible factor-1-dependent regulation of the multidrug resistance (MDR1) gene. Cancer Res 62: 3387-3394, 2002.
22. Comerford KM, Cummins EP and Taylor CT: c-Jun NH2-terminal kinase activation contributes to hypoxia-inducible factor 1alpha-dependent P-glycoprotein expression in hypoxia. Cancer Res 64: 9057-9061, 2004.
23. Rand MJ and Li CG: Nitric oxide as a neurotransmitter in peripheral nerves: nature of transmitter and mechanism of transmission. Annu Rev Physiol 57: 659-682, 1995.
24. Moncada S and Higgs EA: The discovery of nitric oxide and its role in vascular biology. Br J Pharmacol 147 (Suppl 1): S193-S201, 2006.
25. Callapina M, Zhou J, Schmid T, Kohl R and Brune B: NO restores HIF-1alpha hydroxylation during hypoxia: role of reactive oxygen species. Free Radic Biol Med 39: 925-936, 2005.
26. Hagen T, Taylor CT, Lam F and Moncada S: Redistribution of intracellular oxygen in hypoxia by nitric oxide: effect on HIF1alpha. Science 302: 1975-1978, 2003.
27. Dreher MR, Liu W, Michelich CR, Dewhirst MW, Yuan F and Chilkoti A: Tumor vascular permeability, accumulation, and penetration of macromolecular drug carriers. J Natl Cancer Inst 98: 335-344, 2006.
28. Riganti C, Miraglia E, Viarisio D, *et al*: Nitric oxide reverts the resistance to doxorubicin in human colon cancer cells by inhibiting the drug efflux. Cancer Res 65: 516-525, 2005.
29. Yasuda H, Nakayama K, Watanabe M, *et al*: Nitroglycerin may increase response to anticancer drugs in non-small cell lung cancer via reduction of HIF-1a pathway. Nitric Oxide 17: S15, A24, 2007.
30. Frederiksen LJ, Sullivan R, Maxwell LR, *et al*: Chemosensitization of cancer in vitro and in vivo by nitric oxide signaling. Clin Cancer Res 13: 2199-2206, 2007.
31. Yasuda H, Yanagihara K, Nakayama K, *et al*: Therapeutic applications of nitric oxide for malignant tumor in animal models and human studies. In: Nitric Oxide and Cancer: Prognosis, Prevention and Therapy. Bonavida B (ed). Springer Inc., New York, NY, pp419-441, 2010.
32. Assaraf YG: Molecular basis of antifolate resistance. Cancer Metastasis Rev 26: 153-181, 2007.
33. Carlsson G, Gullberg B and Hafstrom L: Estimation of liver tumor volume using different formulas - an experimental study in rats. J Cancer Res Clin Oncol 105: 20-23, 1983.
34. Yasuda H: Solid tumor physiology and hypoxia-induced chemo/radio-resistance: novel strategy for cancer therapy: nitric oxide donor as a therapeutic enhancer. Nitric Oxide 19: 205-216, 2008.
35. Bonavida B, Baritaki S, Huerta-Yepez S, Vega MI, Chatterjee D and Yeung K: Novel therapeutic applications of nitric oxide donors in cancer: roles in chemo- and immunosensitization to apoptosis and inhibition of metastases. Nitric Oxide 19: 152-157, 2008.
36. Huerta S, Chilka S and Bonavida B: Nitric oxide donors: novel cancer therapeutics (Review). Int J Oncol 33: 909-927, 2008.
37. Siemens DR, Heaton JP, Adams MA, Kawakami J and Graham CH: Phase II study of nitric oxide donor for men with increasing prostate-specific antigen level after surgery or radiotherapy for prostate cancer. Urology 74: 878-883, 2009.
38. Yasuda H, Yamaya M, Nakayama K, *et al*: Randomized phase II trial comparing nitroglycerin plus vinorelbine and cisplatin with vinorelbine and cisplatin alone in previously untreated stage IIIB/IV non-small-cell lung cancer. J Clin Oncol 24: 688-694, 2006.
39. Sigmond J, Backus HH, Wouters D, Temmink OH, Jansen G and Peters GJ: Induction of resistance to the multitargeted antifolate pemetrexed (ALIMTA) in WiDr human colon cancer cells is associated with thymidylate synthase overexpression. Biochem Pharmacol 66: 431-438, 2003.
40. Moscow JA, Connolly T, Myers TG, Cheng CC, Paull K and Cowan KH: Reduced folate carrier gene (RFC1) expression and anti-folate resistance in transfected and non-selected cell lines. Int J Cancer 72: 184-190, 1997.
41. Liani E, Rothem L, Bunni MA, Smith CA, Jansen G and Assaraf YG: Loss of folylpoly-gamma-glutamate synthetase activity is a dominant mechanism of resistance to polyglutamylation-dependent novel antifolates in multiple human leukemia sublines. Int J Cancer 103: 587-599, 2003.
42. Stark M and Assaraf YG: Loss of Sp1 function via inhibitory phosphorylation in antifolate-resistant human leukemia cells with down-regulation of the reduced folate carrier. Blood 107: 708-715, 2006.
43. Illi B, Dello Russo C, Colussi C, *et al*: Nitric oxide modulates chromatin folding in human endothelial cells via protein phosphatase 2A activation and class II histone deacetylases nuclear shuttling. Circ Res 102: 51-58, 2008.
44. Hofseth LJ, Hussain SP, Wogan GN and Harris CC: Nitric oxide in cancer and chemoprevention. Free Radic Biol Med 34: 955-968, 2003.
45. Edwards P, Cendan JC, Topping DB, Moldawer LL, MacKay S, Copeland E and Lind DS: Tumor cell nitric oxide inhibits cell growth in vitro, but stimulates tumorigenesis and experimental lung metastasis in vivo. J Surg Res 63: 49-52, 1996.