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

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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. 1H-[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.

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...
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 oncocytic 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 antimitabolic 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 (RFC1), folypolyglutamate 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-Alrich (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-Alrich. 1H-[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% O₂, 5% CO₂ and 94% N₂ 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-
Sectional diameter (33). Mice were fed with a low-folate diet as
where tumor volume was calculated using the formula
and treated intraperitoneally (i.p.) on days 1, 2, 5 and 6 of each study
were included in each group. In group 1, saline was adminis-
tered i.p. on days 2 and 6, and 0.2 mg/kg GTN on days 1,
was defined as one course of
were treated with 2 courses and sacrificed 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

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-Beet
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 3.0x10^5 cells/dish and harvested. Cells were
re-suspended in PBS at 5.0x10^6 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 adminis-
tered 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 µg/kg PEM was administered
i.p. on days 2 and 6. In group 4, 50 µg/kg PEM was adminis-
tered i.p. on days 2 and 6, and 0.2 mg/kg GTN on days 1,
2, 5, and 6. In group 5, 50 µg/kg PEM was administered i.p.
only on days 2 and 6, and 0.2 mg/kg GTN and 10 µg/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 a^6/2,
where a 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 facto-
rial 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 evalu-
ated 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 signifi-
cant 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 expres-
sion 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
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...
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
Nitric oxide (NO) is an important signaling molecule, acting as a toxin, and an antioxidant in various biological processes. In cancer therapy, NO has been observed to enhance the cytotoxicity of PEM, a novel, multitargeted, antifolate, antineoplastic agent and a key drug in the treatment of non-squamous NSCLC. NO donors 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 the present study, we investigated the effect of NO on the expression of proteins which are associated with the metabolism of and resistance to PEM (32).

**Mechanism of Chemosensitivity and Resistance to PEM**

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 RFC 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 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.

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References


