Augmentation of the Enhanced Permeability and Retention Effect with Nitric Oxide–Generating Agents Improves the Therapeutic Effects of Nanomedicines

Waliul Islam¹, Jun Fang², Takahisa Imamura³, Tomas Etrych⁴, Vladimir Subr⁴, Karel Ulbrich⁴, and Hiroshi Maeda¹,⁵,⁶

Abstract

Enhanced permeability and retention (EPR) effect–based nanomedicine is a promising strategy for successful anticancer therapy. The EPR effect is based on tumor blood flow. Because advanced large tumors, as frequently seen in clinical settings, are heterogeneous, with regions of defective vasculature and blood flow, achieving the desired tumor drug delivery is difficult. Here, we utilized the EPR effect to increase drug delivery. To augment the EPR effect for improved therapeutic effects of nanomedicine, we exploited vascular mediators—the nitric oxide (NO) generators nitroglycerin (NG), hydroxyurea, and l-arginine. These compounds generate NO in tumor tissues with relatively high selectivity. Using different nanosized drugs in our protocol significantly increased (1.5–2 times) delivery of nanomedicines to different solid tumor models, along with markedly improving (2–3-fold) the antitumor effects of these drugs. Also, in 7,12-dimethylbenz[a]anthracene–induced advanced end-stage breast cancer, often seen in clinical settings, 2 mg/kg polymer-conjugated pirarubicin (P-THP) with NG (0.2 mg/mouse) showed better effects than did 5 mg/kg P-THP, and 5 mg/kg P-THP used with NG resulted in cures or stable tumors (no tumor growth) for up to 120 days. Moreover, in a murine autochthonous azoxymethane/dextran sulfate sodium-induced colon cancer model, NO donors markedly improved the therapeutic effects of P-THP even after just one injection, results that were comparable with those achieved with three weekly P-THP treatments. These findings strongly suggest the potential usefulness of NO donors as EPR effect enhancers to improve the therapeutic efficacy of nanomedicines. Mol Cancer Ther; 17(12): 2643–53. ©2018 AACR.

Introduction

Current clinical results of conventional chemotherapy are far from successful, even though the use of conventional chemotherapeutics goes back more than 70 years (1). The lack of tumor selectivity of such anticancer drugs is the major problem. Therefore, development of truly tumor-targeting drugs is an urgent need in cancer chemotherapy.

Along this line, molecular drugs have attracted much attention in the past two decades, because they target particular molecules or focus on specific oncopgenes or growth factors that are highly expressed by tumor cells. However, recent clinical results have indicated disappointing outcomes of molecular target drugs, especially for solid tumors (2, 3). The intrinsic heterogeneity of tumors and numerous mutations in individual patients may account for the failure of these treatments (4, 5).

In view of this background information, we have been investigating a more universal tumor-targeting strategy. We utilize the unique anatomical and pathophysiologic features of solid tumors, which are highly permeable to macromolecular drugs because of large gaps between vascular endothelial cells, so molecules larger than 40 kDa selectively enter and accumulate in tumor tissues. This phenomenon does not occur in normal tissues (6). Matsumura and Maeda first reported this phenomenon in 1986 and named it the enhanced permeability and retention (EPR) effect (7), which has become a pivotal principle in the development of macromolecular anticancer drugs, or nanomedicines. The EPR effect may be applied in cancer chemotherapy by using nanosized drugs that make use of liposomes, polymeric micelles, polymer conjugates, and nanoparticles (8, 9).

Today, the EPR effect is a well-recognized phenomenon and has been validated in various solid tumor models and well as in cancer patients (6, 8–11). However, the EPR effect depends on tumor blood vessels and blood flow. Tumors that are rich in blood vessels usually demonstrate good EPR effects and responses to treatment, whereas tumors with poor blood flow have inferior delivery of drugs, even macromolecular drugs. For example, tumors such as prostate cancer, pancreatic cancer, and metastatic liver cancer in humans are hypovascular. Pancreatic cancers are
also rich in stroma and coagulation factors and have poor blood circulation (6, 10). All these facts result in poor blood flow and consequently insufficient delivery of macromolecular drugs to tumors and thus unsatisfactory therapeutic effects. Additional enhancement of the EPR effect and tumor drug delivery is essential to achieve better therapeutic outcomes.

To augment the EPR effect, therefore, we have exploited vascular mediators involved in the EPR effect, the clinical factors nitric oxide (NO), bradykinin (BK), and carbon monoxide (CO; refs. 6, 12). Tumor blood vessels are not static but rather dynamic. Blood flow in tumors can therefore be enhanced by using vascular mediators such as NO. We previously demonstrated that by using angiographic techniques in tumor patients, we visualized blood vessels of massive metastatic liver cancers more clearly after administration of Nitrol, an NO-releasing agent (13). A separate study of transplanted solid tumors in mice also showed significantly increased tumor blood flow as well as macromolecular drug delivery to tumors after use of another NO donor, nitroglycerin (NG; ref. 14). We also described results similar to those achieved with NO by using other vascular mediators such as inhibitors of angiotensin I–converting enzyme (i.e., enalapril), CO, and other agents (6, 10, 15–20). However, very few studies have used such enhancers of the EPR effect to determine whether they will improve therapeutic effects in vivo.

In this study, we selected three NO donors—NG, L-arginine (L-Arg), and hydroxyurea (HU)—all of which are now used in clinical settings and generate NO in a tumor-selective manner. We investigated their effects on the EPR effect in various solid tumor models including carcinogen-induced autochthonous tumors, which are biologically and pathologically more similar to clinical human tumors.

Here, we tested two types of polymeric anticancer drugs developed by our group. One is N-(2-hydroxypropyl)methacrylamide copolymer (pHPMA)–conjugated pirarubicin (P-THP), which contains a tumor pH-responsive hydrazone bond and shows excellent antitumor effects against various solid models (21). Others are polymeric nanoprobes used in photodynamic therapy (PDT), i.e., pH-PMMA-conjugated pyropheophorbide-a (P-PyF) and HPMA-conjugated zinc protoporphyrin (ZnPp, PZP), both demonstrated high tumor accumulation and favorable tumor imaging (22, 23). In this study, we carefully analyzed the enhancement of the drug distribution in tumors and the therapeutic effect of these drugs resulting from the use of NO donors, and we discuss the mechanisms involved in these effects.

Materials and Methods

Materials

L-Arg and NG were purchased from AY Pharmaceuticals Co., Ltd. and Sanwa Kagaku Kenkyusho Co., Ltd., respectively. HU, azoxymethane (AOM), isoflurane, and Evans blue were purchased from Wako Pure Chemical Industry. Dextran sodium sulfate (DSS) was purchased from MP Biomedicals, LLC. The polymer conjugate of HPMA polymer (Mw = 28,000, PDI = 1.78, hydrazide content = 5.8 mol%) with pirarubicin (THP) via hydrazone bonding was prepared as described previously; this conjugate is abbreviated as P-THP (21). We synthesized PZP and P-PyF as previously described (22, 23). We prepared BSA-conjugated rhodamine B isothiocyanate; BSA was from Nacalai Tesque, Inc. Rhodamine B isothiocyanate and 7,12-dimethylbenz[a]anthracene (DMBA) were purchased from Sigma. Other reagents and solvents of reagent grade were from commercial sources and used without further purification.

Animals, cells, and tumor models

Female Sprague–Dawley (SD) rats (5 weeks old), male ddY mice, male BALB/c mice, male C57/Bl mice, and male ICR mice (all 6 weeks old) were purchased from SLC. All animals were maintained at 22 ± 1°C and 55 ± 5% relative humidity with a 12-hour light/dark cycle. All experiments were approved by the animal ethics committees and carried out according to the Laboratory Protocol for Animal Handling of Sojo University. For the S180 solid tumor model, murine sarcoma S180 cells (2 × 105), maintained by weekly passage in ddY mouse ascites, were implanted subcutaneously in the dorsal skin of ddY mice. Mouse colon cancer C26 cells and melanoma B16-F10 cells were maintained in in vitro cultures by using RPMI-1640 and DMEM, respectively (Wako Pure Chemical Industry), supplemented with 10% FBS (Nichiirei Biosciences Inc.) under 5% CO2/air at 37°C. The cultured cells were collected and suspended in physiologic saline to a concentration of 2 × 106 cells/mL, and 0.1 mL of cell suspension (2 × 106 cells) was implanted into the dorsal skin of BALB/c mice and C57/Bl mice, to obtain the solid tumor models of C26 tumors and B16 tumors, respectively.

Generation of NO in tumors and normal tissues after administration of NO donors

We used the C26 tumor model to investigate NO generation. We performed the experiments at 12 to 14 days after injection of tumor cells, when tumors were 10 to 15 mm in diameter. NO donors were NG, 0.1 mg/mouse, which was applied to normal skin as ointment, and L-Arg, 50 mg/mouse, and HU, 50 mg/kg, which were injected i.p. into mice. After 4 and 24 hours, mice were killed and tumors and normal tissues, e.g., blood, kidney, and liver, were collected. We obtained serum by centrifuging blood (4,000 × g, 20 minutes). We added 0.4 mL of 0.01 mol/L phosphate-buffered 0.15 mol/L saline (PBS) to 100 mg of each tissue and homogenized the samples. Supernatants of tissue homogenates were used to measure NO via the Griess method (24) with a nitrite/nitrate assay kit (Dojindo Laboratories).

Augmentation of accumulation of nanomedicines in tumors by using NO donors

We used both S180 and C26 tumors in mice to investigate enhancement of nanomedicine accumulation. We performed the experiments when tumor diameters reached to approximately 10 mm. We injected P-THP i.v. after NO donors were applied as described in the previous section. At 24 hours after i.v.-administered P-THP, mice were killed, blood samples were withdrawn, and tissues were dissected after perfusion with 20 mL of PBS. P-THP distributed in each tissue was then extracted and analyzed by high performance liquid chromatography (HPLC) as reported previously (21, 25).

We similarly investigated the body distributions of the nanoprobes PZP and P-PyF. Briefly, when tumor diameters measured approximately 10 mm, we injected 5 mg/kg (PyF equivalent) P-PyF or 15 mg/kg (ZnPp equivalent) PZP dissolved in physiologic saline i.v. via tail veins. At 24 hours after these injections, mice were killed, and tumors and normal tissues were then
In vivo therapeutic effects of nanomedicines plus NO donors. Evaluation of antitumor effects of P-THP with EPR enhancers in implanted tumor models. In both S180 tumor and C26 tumor, when tumors had diameters of about 10 mm, and 5 mg/kg or 15 mg/kg THP-equivalent of P-THP was injected i.v. at 0.1 mL/mouse. NO donors were applied as described above, immediately after administration of 5 mg/kg-P-THP. Tumor volumes and body weights were recorded throughout the experimental period. Tumor volume (mm$^3$) was calculated as $(W^2 \times L)/2$ by measuring the length $(L)$ and width $(W)$ of the tumor. A humane endpoint was established as growth of tumor to 2 cm in diameter or body weight loss of the mice of $>10\%$.

In the B16 tumor model, similarly when tumor diameters measured about 10 mm, 2 mg/kg or 5 mg/kg (THP-equivalent) of P-THP was injected i.v. at 0.1 mL/mouse. Various NO donors were investigated at 2 mg/kg, in combination with P-THP, as described above.

Augmentation of the PDT effect of the nanoprobes with EPR enhancers in the C26 and other tumor models. When tumor diameters reached to approximately 10 mm, P-PyF dissolved in physiologic saline at indicated concentrations was administered i.v. Control mice were injected with physiologic saline at indicated concentrations was administered i.v. NO donors were applied immediately after P-PyF administration as described above. Control mice were injected with physiologic saline. At 24 and 48 hours after administration of P-PyF, tumors were irradiated via an endoscopic fiber optics system with xenon light at 400 to 700 nm (MAX-303; Asahi Spectra) for 5 minutes (36 J/cm$^2$).

We also investigated lung metastasis in the C26 tumor model, which occurs at 30 to 40 days after tumor inoculation into the dorsal skin (26, 27). Briefly, with the same treatment protocol as above, 40 days after tumor inoculation, mice were killed and lungs were excised. Metastatic lung tumors were detected by using ex vivo imaging with the IVIS system (IVIS XR; Caliper Life Sciences); tumor nodules were identified macroscopically, and numbers of metastatic nodules and each diameter were counted.

For the PDT group using AOM/DSS-induced colon cancer model which is described in the following section, 15 mg/kg PZP (ZnP complex) was injected i.v. similarly at 0, 14, and 25 days, a total of 3 times. In the combination group with the EPR effect enhancers, NG ointment was applied to normal skin at 0.1 mg/mouse during administration of PZP. Tumor-selective generation of NO by NG, L-Arg, and HU was confirmed by using fluorescent or non-fluorescent nanomedicines. The humane endpoint in this model was when mice lost $>10\%$ of their body weight with severe diarrhea and hematochezia.

Improvement in therapeutic efficacy of nanomedicines by using various NO donors in the AOM/DSS-induced autochthonous colon tumor model. AOM at 10 mg/kg (0.3 mL/mouse) was injected i.p. into ICR mice, and 1 week later 2% DSS was fed for 7 days in drinking water. Ten weeks after AOM administration, 3 mice were randomly selected to determine formation of tumors in the colon by macroscopic and microscopic histology or by injecting BSA conjugated with rhodamine or Evans blue, after which tumor nodules were visualized by fluorescence or normal light, respectively.

After confirmation of tumor nodule formation in model mice, they were grouped randomly. For treatment groups, 15 mg/kg P-THP was injected i.v. via the tail vein, in the absence or presence of NO donors as described above. In a separate experiment, 15 mg/kg P-THP was injected once at 0, 14, and 25 days, a total of 3 times.

Effect of NO donors in the DMBA-induced advanced breast cancer model. To establish the DMBA-induced breast cancer model, 10 mg of DMBA was dissolved in 1 mL of corn oil and was administered to SD rats only once via the oral route. Breast tumors appeared after 12 to 14 weeks of DMBA administration. Drug treatments were started when tumors were relatively large (2–3 cm in diameter), a model of advanced cancer as frequently seen in clinical settings. P-THP was injected at 2 or 5 mg/kg (THP equivalent) i.v., 0.5 mL/rat, once each on days 0, 14, and 21, a total of three injections, and in some experiments, an extra injection was given on day 70 after the first treatment. In the group receiving 2 mg/kg P-THP, NG ointment was administered at 0.2 mg/rat. The humane endpoint for this rat model study was a tumor diameter of about 4 cm or a body weight loss of $>10\%$.

Measurement of tumor blood flow
A laser Doppler flow meter (ALF21; Advance Co. Ltd.) was used to measure tumor blood flow in tumor. NG, L-Arg, and HU were applied as described above at 0.1 mg/mouse, 50 mg/mouse, and 50 mg/kg, respectively. With animals under isoflurane anesthesia, tumor blood flow was measured every 15 to 30 minutes. Many areas of solid tumors, as many as 10 areas, were chosen for this measurement. The average tumor blood flow was then calculated.

Statistical analyses
All data are expressed as mean ± SD. Data were analyzed by using ANOVA followed by the Bonferroni correction. A difference was considered statistically significant when $P < 0.05$.

Results
Tumor-selective generation of NO by NG, L-Arg, and HU
As Fig. 1 shows, NG at 0.1 mg/mouse significantly increased the amount of NO in tumor tissue at 4 hours after administration, which continued for at least 24 hours (Fig. 1A). However, in normal tissues, i.e., liver, kidney, and plasma, although we found a slightly increased NO production, it was not significant (Fig. 1A). Similarly, administration of L-Arg (50 mg/mouse) and HU (50 mg/kg) resulted in tumor-selective generation of NO (Fig. 1B and C).

Increased accumulation of macromolecular drugs in tumors by NO donors
After confirming tumor-specific production of NO induced by NO donors, we investigated the body distribution of the polymeric drug (P-THP) that we developed in our laboratory and that showed high tumor accumulation because of the EPR effect (21) in a murine model of implanted colon cancer (C26). The results clearly revealed that P-THP, at 24 hours after being injected i.v., had markedly enhanced concentrations in tumors by the EPR.
effect and in plasma (prolonged plasma half-life) compared with most normal tissues (Fig. 1D), results that are in concordant to those shown in Fig. 1A–C. More important, administration of NO donors immediately after P-THP injected i.v. resulted in a significant increase (1.5–2 times) in drug accumulation in tumors, whereas no significant changes in drug distribution in normal tissues were found (Fig. 1D–F). The augmentation of the EPR effect caused by these NO donors seems to be dose-dependent. However, no significant difference was obtained for the different concentrations used (i.e., NG at 0.1 and 1 mg/mouse; L-Arg at 30 and 90 mg/mouse; and HU at 50 and 100 mg/kg; Fig. 1D–F). We thus performed subsequent studies by using NG at 0.1 mg/mouse, L-Arg at 50 mg/mouse, and HU at 50 mg/kg.

Furthermore, we also confirmed the EPR effect by using the fluorescence nanophotosensitizer probe, P-PyF (23). NO donors significantly increased accumulation of P-PyF in tumor tissues but not in normal tissues (Fig. 2A). This increased tumor accumulation was also clearly illustrated by in vivo imaging: a much more intense fluorescence signal was detected in mice treated with NO donors compared with control mice (no NO donors; Fig. 2B).

Similar results were found with mice bearing sarcoma (S180) tumors by using another polymeric nanophotosensitizer probe, PZP, with the same dose and treatment protocols (Supplementary Fig. S1).

Improved therapeutic effects of polymeric nanodrugs as affected by NO donors

Therapeutic effects in s.c.-implanted tumor models. We then investigated whether administration of NO donors enhanced the therapeutic effects of polymeric nanodrugs by using the polymeric anticancer drug P-THP and nanophotosensitizer probes, P-PyF and P-ZnP (PZP).

In the colon cancer C26 model, as shown in Fig. 3A–C, P-THP alone produced dose-dependent inhibition of tumor growth. One
injection of 5 mg/kg P-THP did not significantly suppress tumor growth, whereas at 15 mg/kg P-THP markedly suppressed tumor growth compared with untreated controls. When 5 mg/kg P-THP was combined with NO donors, a significantly enhanced therapeutic effect was achieved, which was similar to or better than that of 15 mg/kg P-THP (Fig. 3A–C). This finding indicates 3-fold intensified therapeutic effects by NO donors. Moreover, NO donors alone at the doses applied did not produce significant antitumor effects.

We next tested the effect of combination therapy in the murine melanoma B16 model, which is a highly aggressive tumor that all mice died within 20 days after tumor inoculation. We confirmed improved therapeutic effects for the combination therapy of NO donors with the polymeric drugs. The results showed significant potentiation of antitumor effect with each NO donor (Fig. 3D–F), and the survival of mice was also significantly increased by this combination therapy (~30% prolongation of survival span compared with controls; Supplementary Fig. S2).

Then, we utilized PDT with the nanophotosensitizer probe P-PyF. A therapeutic benefit was similarly observed in each NO donor. That is, PDT with 2 mg/kg P-PyF in the C26 tumor model did not have an apparent antitumor effect, whereas 5 mg/kg P-PyF induced marked suppression of tumor growth. The combination therapy of 2 mg/kg P-PyF with NO donors with light resulted in almost the same therapeutic effect as that achieved with 5 mg/kg P-PyF + light (Fig. 4A–C).

In this experiment, we also observed metastatic tumor nodules in the lung of the C26 tumor and visualized by fluorescence imaging. As Fig. 4D shows, we observed strong fluorescence in metastatic lung tumors in mice receiving P-PyF injection (no PDT), which suggested formation of tumor nodules in the lung, in which the fluorescent probe P-PyF accumulated by virtue of the EPR effect. We confirmed this finding by counting the tumor
nODULES IN THE LUNG (FIG. 4D AND E). PDT TREATMENT REDUCED LUNG METASTASIS BY P-PyF IN A DOSE-DEPENDENT MANNER, AND THE COMBINATION OF THE LOW CONCENTRATION OF P-PyF (2 mg/kg) WITH NO DONORS SIGNIFICANTLY LOWERED THE NUMBERS OF METASTATIC NODULES, AN EFFECT THAT WAS COMPARABLE WITH THE EFFECT OF THE HIGH CONCENTRATION OF P-PyF (5 mg/kg).

WE OBTAINED SIMILAR RESULTS WITH THE IMPLANTED SARCOMA S180 MODEL, IN WHICH THE COMBINATION OF NO DONORS WITH P-THP MARKEDLY INCREASED THE THERAPEUTIC EFFECT OF P-THP (SUPPLEMENTARY FIG. S3).

IN ADDITION, DURING ALL THESE EXPERIMENTS, ANIMALS REVEALED NO APPARENT LOSS OF BODY WEIGHT.

Therapeutic effects in the AOM/DSS-induced autochthonous murine colon cancer model. We then investigated the effect of the combination of P-THP plus NO donors in a murine colon cancer model induced by a chemical carcinogen (AOM/DSS); this model is more like naturally formed colon carcinoma than artificially inoculated models. Ten weeks after AOM/DSS was administered, multiple tumor nodules were found in the colons of mice and were visualized by using the macromolecular fluorescent dye rhodamine-BSA as well as by an Evans blue-albumin complex (Supplementary Fig. S4); treatment was then carried out. At 30 days after treatment with P-THP in combination with/or without NO donors, we found many large tumor nodules in untreated control mice (Fig. 5A), whereas P-THP treatment markedly reduced the numbers of tumor nodules as well as tumor sizes (Fig. 5A–C; Supplementary Fig. S4). More important, P-THP combined with NO donors markedly reduced colon tumor nodules in both number and size (Fig. 5B and C), and in some mice (3/8), no tumor nodules were seen (a cure; Fig. 5A; Supplementary Fig. S4).

With the same model, we also examined the effect of NG on PDT by using the polymeric PDT nanophotosensitizer probe P2ZP. As Fig. 5D–F shows, PDT via endoscopic irradiation significantly reduced the number and size of tumor nodules in the colon, and
combination with NG further enhanced the therapeutic effect, although no significant difference was observed.

In these experiments, we found no apparent loss of body weight of the animals.

**Therapeutic effects in the DMBA-induced rat breast cancer model.**

In this study, we began treatment when the tumor was relatively large (20–25 mm in diameter), which mimics large advanced tumors in clinical settings. Rats without treatment showed rapid tumor growth that resulted in huge tumors in 3 weeks (Fig. 6). Injections of P-THP every 5 days (one injection each time) markedly suppressed tumor growth in a dose-dependent manner; the lower concentration of P-THP (2 mg/kg) stabilized tumor sizes for up to 60 days (Fig. 6); the higher concentration of P-THP (5 mg/kg), however, resulted in apparent regression in tumor size, and this effect continued for up to 2 months, then tumor regrowth was observed. However, an additional P-THP treatment (on day 70) with NG effectively suppressed and controlled the tumor growth till 120 days (Fig. 6). Thus, combination of P-THP with NG significantly accelerated the reduction in tumor size. One rat had no apparent tumors at 60 days after treatment, and other rats had very small tumors (<1 cm diameter) that did not grow; this effect continued for at least 4 months, which indicates that the treatment resulted in complete regression.

In these experiments, we found no apparent loss of body weight of the animals.

**Increase in tumor blood flow as affected by NO donors.**

To analyze the possible mechanisms involved in augmentation of the EPR effect as caused by NO, we explored the tumor blood flow. As Supplementary Fig. S5 illustrates, use of NO donors increased tumor blood flow, up to 1.3 to 2 times more, by 4 hours after their administration.

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Figure 4.

Augmentation of the effect of PDT on C26 tumors by using P-PyF and NO donors. Different concentrations (PDT-L, 2 mg/kg; PDT-H, 5 mg/kg) of P-PyF were injected i.v. when tumor diameters measured about 10 mm. Immediately after the injection of 2 mg/kg P-PyF, an NO donor (NG, 0.1 mg/mouse; L-Arg, 50 mg/mouse; or HU, 50 mg/kg) was administered. After 24 and 48 hours of P-PyF injection, light irradiation (120 mW/cm², 5 minutes, 36 J/cm²) was performed. Tumor growth was measured every 3 or 5 days. A-C, The effects of NG, L-Arg, and HU, respectively. D, Fluorescence imaging demonstrating lung metastases (3 mice in each group) of C26 tumors implanted in the dorsal skin, with imaging performed at 40 days after tumor injection (28 days after administration of P-PyF). No fluorescence was detected without P-PyF, whereas strong fluorescence was seen after P-PyF injection; after PDT, fluorescence decreased, with this decrease being correlated with the reduced tumor growth shown in A-C and the suppressed number of metastatic tumor nodules in the lung (E). Data are mean ± SD; n = 4–8. *, P < 0.05 and **, P < 0.01. See text for details.
Discussion

Nanomedicine is becoming a promising therapeutic modality for cancer because of its tumor-targeting capability that drives the EPR effect. According to the EPR effect, macromolecular drugs, or nanomedicines, accumulate in tumor tissues at relatively high concentrations, i.e., 5% to 10% of the total injected drug (7, 28–30). Conventional low-molecular-weight drugs, however, commonly accumulate in tumor tissues at less than 0.1%, or 1%, of injected drugs (7, 28, 31). In this context, targeting of drugs to tumors has shown great advances. Namely, we can obtain 10 to 50 times higher concentrations of macromolecular drugs in tumors than those of conventional anticancer drugs by virtue of the EPR effect.

However, in view of the above-described findings, e.g., >80% of total nanomedicines are not distributed in tumor. We thus anticipated that further augmentation of tumor-targeted drug delivery, more than the normal EPR effect, can be possible. One challenge is so-called active targeting that utilizes active motifs to recognize tumor cells or tumor-related molecules. However, massive studies of molecular genetics of human tumors have revealed formidable mutations occur and nullify the target (4, 5). Also, molecules utilized for active targeting, such as folate receptors and RGD motifs, are usually found not solely in tumors but also are expressed in many normal tissues, which increases off-target accumulation. For example, folate-conjugated nanoparticles did not have better accumulation than control nanoparticles in...
Figure 6. Enhanced therapeutic effects of P-THP as caused by NO donors in the DMBA-induced advanced breast cancer model. Oral administration of 10 mg of DMBA induced rat breast tumors. After 12 to 14 weeks of DMBA administration, when tumors were large (2–3 cm in diameter), P-THP was injected i.v. at 2 or 5 mg/kg (THP equivalent) once each on days 0, 14, and 21, a total of three injections. In some experiments, an extra injection was added on day 70 after the first treatment. The group given 2 mg/kg P-THP was treated with NG ointment at 0.2 mg/rat. Arrows indicate P-THP injections. Data are mean ± SD, n = 6–8. See text for details.

Improved Nanomedicine Effects via the EPR Effect and NO

tumors (32), and folate conjugates were mostly captured by the liver, which is the organ that stores excess folate (33). In addition, targeting ligands also always have large impacts on the circulation kinetics and pharmacokinetics of nanocarriers, the results being shorter plasma half-lives and poor EPR-effect-based accumulation of drugs in tumors (34–37). Consequently, despite often promising in vitro findings, a very small number of studies indicated that active targeting really improves accumulation of nanomedicines in tumors (28, 30, 35–39).

In these regards, we have been studying vascular mediators involved in the EPR effect, i.e., BK, NO, CO, and prostaglandins, to determine their potentials as EPR enhancers (6, 10, 12). In our investigations here, we specifically focused on NO, which we previously showed increased vascular permeability and blood flow (14, 40, 41). Here, we selected three NO donors, i.e., NG, L-Arg, and HU. These agents are used clinically and present no obstacles for clinical drug development. Moreover, they generate NO selectively in tumor tissues. For example, NG becomes nitrite immediately after absorption, and nitrite circulates. Under hypoxic conditions at a weakly low pH (e.g., pH 6–7), as in cardiac infarct tissue or tumor tissues, nitrite will be converted to NO (14, 41, 42). This process does not occur in normoxic normal tissues. Thus, tumor-selective permeability will be enhanced, as we demonstrated in a previous study (14) as well as in the present study (Fig. 1A).

L-Arg is the substrate of nitric oxide synthase (NOS) during NO generation (40, 41). Tumor and inflammatory tissues express high levels of NOS, especially the inducible form of NOS that is mostly derived from infiltrated macrophages (40, 43). Thus, producing NO in tumors with relatively high specificity by using L-Arg is a reasonable expectation. Present results support this expectation (Fig. 1B).

HU is a medicament used for sickle-cell anemia, chronic myelogenous leukemia, cervical cancer, and polychytemia vera. With regard to its therapeutic mechanisms, it is an alkylating agent in cancer treatment. Maeda and colleagues, however, found NO generation from HU, which is also believed to be one of its therapeutic mechanisms (44, 45). Namely, HU generates NO via NOS, as HU is the intermediate in NO production from L-Arg (44, 45), and HU may thus demonstrate tumor-selective NO production. The present study also confirmed this hypothesis about NO production from HU in tumors (Fig. 1C).

By using these NO donors, we observed enhanced delivery of nanomedicines to tumors about 2 times greater than that without NO donors (Figs. 1D–F and 2). More important, we found no apparent increase in drug accumulation in normal tissues (Figs. 1D–F and 2). We thus confirmed the superior therapeutic modality of the combination therapy of nanomedicines with these NO donors.

As we expected, we found an increased therapeutic effect in three implanted murine tumor models, i.e., S180, C26, and B16 (Figs. 3 and 4; Supplementary Figs. S2 and S3). The combination treatment with NO donors resulted in about 2- to 3-fold improvement of therapeutic effect (Figs. 3 and 4). These results are paralleled with increased drug accumulations found in tumors (Figs. 1D–F and 2). The improved therapeutic effect was seen not only in primary tumors but also in metastatic tumors (Fig. 4D and E). Further, in a chemically induced murine colon cancer model that is similar to cancers seen in clinical situations, a marked therapeutic effect was seen after treatment with NO donors combined with P-THP at a dose that did not produce an apparent therapeutic effect (Fig. 5B and C).

In most of the preclinical studies, treatments were started when tumors were relatively small (2–5 mm). However, many clinical cancers being encountered are large and widely disseminated. Thus, small early-stage tumor model may not reflect the usual clinical settings of human cancers. Here, we used advanced tumor models that can mimic advanced, late-stage clinical tumors. For this purpose, in this study, we performed experiments with the DMBA-induced rat breast cancer model, and treatments began when tumors were large (e.g., 2–2.5 cm in diameter). Although treatment with P-THP alone significantly suppressed tumor growth, its therapeutic effect was clearly demonstrated and tumors are mostly stable with no or little increase in tumor size (Fig. 6). However, when the combination therapy with NG was used, a significant decrease in tumor size was observed, complete regression occurred in one of five rats, and tumors remained small without additional
growth, an effect that continued for at least 4 months (Fig. 6). Therefore, we believe that this combination therapy utilizing NO donors as EPR enhancers has great potential for successful tumor-targeted chemotherapy using nanomedicines.

With regard to the mechanisms involved in the augmented therapeutic effect of nanomedicines as induced by NO donors, restoration of tumor blood flow was believed to be a major factor, which was proved in our previous study (14) as well as in the present study (Supplementary Fig. S5). Yasuda and colleagues also reported that NG increased the vulnerability of hypoxic tumors to chemotherapy by increasing tumor blood flow as well as by suppressing hypoxia-inducible factor-1α, vascular endothelial growth factor, and P-glycoprotein (46) via NO production. Also, this benefit of the combination therapy with NG was confirmed in clinical settings (46). Multiple mechanisms thus may operate in the improved antitumor effect of nanomedicines as related to NO donors.

**Conclusion**

In this study, we evaluated the potential of NO donors as potent enhancers of the EPR effect to improve the therapeutic effect of polymeric anticancer drugs or nanomedicines. By using different solid tumor models, including chemically induced tumor models, we clearly demonstrated increased polymeric drugs tumor accumulation into tumor when combined with NO donors, i.e., NG, L-Arg, and HU (Figs. 1D–F and 2), which paralleled the improved therapeutic effect (Figs. 3–6). These findings strongly suggested the importance and applicability of NO donors for successful anticancer treatment with nanomedicines based on EPR effect, and we anticipate clinical application of these tumor-selective NO donors in the future.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: H. Maeda

Development of methodology: W. Islam, J. Fang, K. Ulbrich, H. Maeda

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): W. Islam, J. Fang, T. Inamura, H. Maeda

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): W. Islam, J. Fang, T. Itoh, H. Maeda

Writing, review, and/or revision of the manuscript: W. Islam, J. Fang, H. Maeda

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): W. Islam, J. Fang, H. Maeda

Study supervision: W. Islam, J. Fang, H. Maeda

Other (synthesis of polymer conjugates): V. Subr

Other (in development and methodology part only partial contribution): K. Ulbrich

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**References**


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