

Figure 2 Increase in intracellular hydrogen peroxide (H_2O_2) by means of treatment with polyethylene glycol-conjugated D-amino acid oxidase (PEG-DAO) plus D-Ala and restoration of bactericidal activity of chronic granulomatous disease-like neutrophils. Mouse peritoneal neutrophils were pretreated with $10 \mu\text{mol/L}$ dichlorofluorescein diacetate, a fluorescent molecular probe for H_2O_2 , and were then incubated with (a) increasing concentrations of H_2O_2 or (b) increasing concentrations of PEG-DAO (1–100 mU/mL) plus a fixed amount of 10 mmol/L D-Ala, followed by incubation for 30 min at room temperature. The fluorescence intensity of neutrophils was measured by means of a flow cytometer. (c) Normalized fluorescence intensity of cells in (b) representing intracellular reactive oxygen species. (d) Mouse peritoneal neutrophils were pretreated with $10 \mu\text{mol/L}$ diphenylene iodonium (DPI) and were then incubated with opsonized *Staphylococcus aureus* to allow phagocytosis for 30 min. After removal of non-phagocytosed bacteria in the supernatant, neutrophils were treated with PEG-DAO plus 10 mmol/L D-Ala for 30 min. Viable bacteria inside the neutrophils were counted as described in Materials and methods. Values are means \pm SE ($n = 4$). **Indicates statistically significant differences ($P < 0.01$) by Student's *t*-test

Restoration of bactericidal activity of human CGD neutrophils by PEG-DAO treatment

We examined the bactericidal activity of PEG-DAO with neutrophils from a patient with CGD, as they are known to have little antibacterial effect. We first checked the

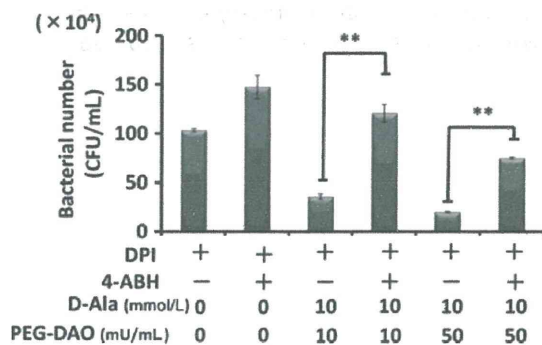


Figure 3 Polyethylene glycol-conjugated D-amino acid oxidase (PEG-DAO) restored the bactericidal activity of diphenylene iodonium (DPI)-treated neutrophils, which depended on myeloperoxidase (MPO) activity. Mouse peritoneal neutrophils were treated with both DPI, an inhibitor of nicotinamide adenine dinucleotide phosphate oxidase, and 4-aminobenzoic acid hydrazide (4-ABH), an inhibitor of MPO. Cells were then incubated with opsonized *Staphylococcus aureus* to allow phagocytosis. After removal of non-phagocytosed bacteria, neutrophils were treated with PEG-DAO for 30 min. The number of viable bacteria was derived from the count of colonies on agar plates, as described in Materials and methods. Values are means \pm SE ($n = 4$). **Indicates statistically significant differences ($P < 0.01$) by Student's *t*-test

intracellular oxidant status before and after PEG-DAO treatment. In agreement with the results shown in Figure 2, treatment with PEG-DAO plus D-Ala increased the intracellular oxidative state (Figure 4a). CGD neutrophils showed less bactericidal activity as compared with healthy neutrophils. However, treatment with 10 or 30 mU/mL PEG-DAO restored the bactericidal activity of these CGD neutrophils to a considerable extent in the presence of 10 mmol/L D-Ala (Figure 4b).

Discussion

Patients with CGD have a genetic defect in NADPH oxidase, which results in deficient H_2O_2 generation and hence poor antimicrobial defense. Consequently, neutrophils from patients with CGD fail to kill bacteria, and chronic inflammation may result.^{2,3} CGD neutrophils continue to evidence normal migration, phagocytosis and MPO enzyme activity; only their H_2O_2 -generating capacity is severely impaired.¹⁰ We thus hypothesized that supplementation with H_2O_2 would restore this function of CGD neutrophils.

H_2O_2 itself shows bactericidal activity *in vitro*, but intravenous injection of H_2O_2 does not result in antibacterial activity against bacteria *in vivo* because of the presence of excess catalase in the systemic circulation.¹¹ In other words, circulating H_2O_2 *in vivo* is rapidly cleared by catalase, so no H_2O_2 is available for targeting to infected or

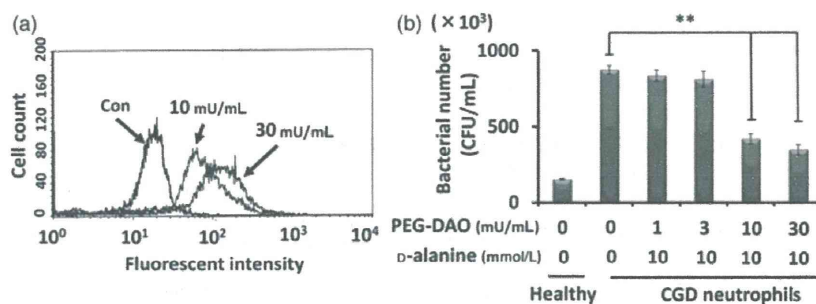


Figure 4 Restoration of bactericidal function of human chronic granulomatous disease (CGD) neutrophils by polyethylene glycol-conjugated D-amino acid oxidase (PEG-DAO) treatment. (a) Human CGD neutrophils were pretreated with 10 μ mol/L dichlorofluorescein diacetate, an intracellular marker of oxystress, and were then incubated with PEG-DAO (10 or 30 mU/mL) and 10 mmol/L D-Ala for 30 min at room temperature. The fluorescence intensity of the neutrophils was measured by using a fluorescence flow cytometer. (b) CGD neutrophils were incubated with *Staphylococcus aureus* to allow phagocytosis. After removal of the non-phagocytosed *S. aureus* by centrifugation, neutrophils were treated with PEG-DAO and D-Ala for 30 min followed by incubation with 0.2% Tween-20 to liberate bacteria from the neutrophils. Serial dilutions of bacteria were then mixed with Trypticase soy agar in Petri dishes and incubated overnight at 37°C, and bacterial colonies was counted. Values are means \pm SE ($n = 3$). **Indicates statistically significant differences ($P < 0.01$) by Student's *t*-test

inflamed sites. Furthermore, H₂O₂ may harm mammalian cells that have no catalase or other antioxidants.¹¹ Thus, new strategies to deliver the H₂O₂ to the target site are required, which may provide a new strategy of H₂O₂-dependent treatment of infection and cancer.

To achieve the delivery of H₂O₂ to an infected or inflamed site, we prepared PEG-DAO which retained a degree of H₂O₂-generating activity that was comparable with that of native DAO.^{6,7} Also, DAO derived from a porcine source can be used in humans, because PEGylation reduces the antigenicity of this enzyme. PEG-DAO accumulates preferentially in tumor tissue because of the EPR effect, the mechanism of which is based on the highly enhanced extravasation of macromolecules in the tumor and inflamed tissues. In addition, impaired lymphatic clearance of such macromolecules from interstitial space makes this effect more distinct. This increased vascular permeability is induced partly by overproduction of inflammatory vascular mediators such as bradykinin, nitric oxide and many others.^{2,8}

The concentrations of D-amino acids, which are substrates of PEG-DAO, are extremely low in mammalian blood plasma, so H₂O₂ generation by PEG-DAO alone in systemic circulation is quite limited. However, we can induce H₂O₂ generation with an intravenous injection of D-amino acids. PEG-DAO at first accumulates predominantly at the inflamed site because of the EPR effect. After several hours of PEG-DAO infusion via an intravenous route allowing PEG-DAO to accumulate more selectively at the disease site, at that time, the PEG-DAO concentration in blood is very low, and D-amino acid is infused subsequently via the intravenous route. Thus, PEG-DAO is preferable for delivery of H₂O₂ to the inflamed sites or cancer tissue, and avoids systemic generation of H₂O₂.⁶

PEG-DAO plus D-Ala, as mediated by H₂O₂, showed bacteriostatic activity against *S. aureus* in a dose-dependent manner via production of H₂O₂ (Figure 1). These results indicate that PEG-DAO can serve as an antibacterial agent if it is selectively delivered to an infected site. This delivery became a possibility as a result of the prolonged plasma half-life of PEG-DAO and the EPR effect.

When PEG-DAO plus D-Ala was supplied to NADPH oxidase-deficient neutrophils, it increased the amount of

H₂O₂ inside the cells (Figures 2 and 4). The fluorescent oxystress probe DCFH-DA, which effectively enters neutrophils, contains a diacetate group that is quickly hydrolyzed, so DCFH-DA becomes reactive to H₂O₂ and then fluorescent.¹² The higher fluorescence intensity of DCFH-DA thus indicates a higher oxidative state inside cells, but does not reflect the oxidative state outside cells. As expected, PEG-DAO treatment or H₂O₂ treatment increased the intracellular level of H₂O₂ in a dose-dependent manner (Figure 2a). Furthermore, in the presence of D-Ala, addition of PEG-DAO also increased the amount of intracellular H₂O₂ in a dose-dependent manner (Figures 2a and b). These results clearly indicate that PEG-DAO treatment can supply exogenous H₂O₂ efficiently to neutrophils and that this restored level of intracellular H₂O₂, as H₂O₂ is converted to the hypochlorite ion by MPO, would facilitate potent bactericidal activity of neutrophils from a CGD patient.

We also examined whether PEG-DAO treatment would restore the bactericidal activity of CGD-like neutrophils. We prepared neutrophil mimics, which were similar to neutrophils in patients with CGD, by pretreatment with 10 μ mol/L DPI, which achieves its effects by inhibiting NADPH oxidase and thus suppressing H₂O₂ generation.¹³ In this experiment, we used PEG-DAO plus D-Ala and examined the effect of this treatment on phagocytosed bacteria. We observed a significant increase in the number of viable bacteria inside these neutrophils after DPI treatment (Figure 2d). In this setting, the bactericidal activity of DPI-treated neutrophils was similar to that of neutrophils from a CGD patient. However, PEG-DAO treatment of DPI-treated neutrophils greatly suppressed the number of viable bacteria inside neutrophils, almost to the number in healthy neutrophils (Figure 2d).

Most MPO exists in vacuoles in neutrophils,¹⁴ and MPO oxidizes the chloride ion, with H₂O₂, to produce hypochlorous acid, one of the most potent bactericidal molecules in biological systems. We therefore hypothesized that restoration of bactericidal activity of DPI-treated neutrophils by treatment of PEG-DAO and D-Ala was mediated by the function of MPO. Consistent with our hypothesis, 4-ABH, an MPO inhibitor, suppressed the bactericidal activity of

the neutrophils, even in the treatment of PEG-DAO plus D-Ala (Figure 3).

As a more important result, we found that PEG-DAO plus D-Ala treatment restored the bactericidal activity of neutrophils from a CGD patient. Although the CGD neutrophils showed decreased bactericidal activity, in great contrast to healthy neutrophils (Figure 4), treatment with PEG-DAO plus D-Ala restored the bactericidal activity of these CGD neutrophils (Figure 4).

Our results thus demonstrated that H₂O₂ supplementation via PEG-DAO plus D-Ala would protect against bacterial infection. In our experiments, we used porcine DAO to prepare PEG-DAO. For porcine DAO, the K_{cat} and K_m values for D-proline are 43.3 s⁻¹ and 2 mmol/L, respectively, whereas the corresponding values for D-Ala are 6.4 s⁻¹ and 3.1 mmol/L.¹⁵ Thus, using D-proline may be preferable to using D-Ala to treat CGD neutrophils, although this issue requires additional investigation. Furthermore, H₂O₂ can enter the cytosol of neutrophils and be converted to hypochlorous acid by MPO. These observations suggest that H₂O₂ supplementation via enzymatic action may become a plausible approach for treatment of patients with CGD. Less useful therapeutic strategies for CGD exist as yet, despite the great advances in the development of antimicrobial agents. The previously reported pharmacokinetics of PEG-DAO indicated an effective targeting ability to solid tumors as a result of the EPR effect. Bacterial components such as bacterial proteases and endotoxin facilitate the vascular permeability, and thus leakage of blood components such as albumin from circulating blood. Consequently, these macromolecules will accumulate at the infected or inflamed site.^{16,17} Although the accumulation property of macromolecular proteins at the tumor and inflamed tissue is similar, accumulation of PEG-DAO at the inflamed tissue in the CGD mouse model of human patients is yet to be determined. PEG-DAO pharmacokinetics may be similarly beneficial for targeting to the inflamed granuloma tissue in patients with CGD, although more studies are needed to confirm this possibility.

Author contributions: All authors participated in the design, interpretation of the studies, analysis of the data and review of the manuscript. HNa conducted the experiments and wrote the manuscript with HM; TM and HNu supplied the critical sample; and JF and HM discussed the content constructively for the experiments and revised the manuscript.

ACKNOWLEDGEMENTS

The use of blood samples from a patient with CGD was approved by the Institutional Review Board of University of Miyazaki, Japan. This work was supported by Grant-in-Aid for Scientific Research 21791016 from the

Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

- Hohn DC, Lehrer RI. NADPH oxidase deficiency in X-linked chronic granulomatous disease. *J Clin Invest* 1975;55:707-13
- Johnston RB Jr, Keele BB Jr, Misra HP, Lehmeyer JE, Webb LS, Baehner RL, Rajagopalan KV. The role of superoxide anion generation in phagocytic bactericidal activity. Studies with normal and chronic granulomatous disease leukocytes. *J Clin Invest* 1975;55:1357-72
- Repine JE, Clawson CC. Quantitative measurement of the bactericidal capability of neutrophils from patients and carriers of chronic granulomatous disease. *J Lab Clin Med* 1977;90:522-8
- Burton K. The stabilization of D-amino acid oxidase by flavin-adenine dinucleotide, substrates and competitive inhibitors. *Biochem J* 1951;48:458-67
- Mosebach KO. [Mechanism of action of some factors affecting D-amino acid oxidase. III. Reactions and significance of nascent hydrogen peroxides.] *Hoppe Seylers Z Physiol Chem* 1957;309:206-18
- Fang J, Sawa T, Akaike T, Maeda H. Tumor-targeted delivery of polyethylene glycol-conjugated D-amino acid oxidase for antitumor therapy via enzymatic generation of hydrogen peroxide. *Cancer Res* 2002;62:3138-43
- Fang J, Deng D, Nakamura H, Akuta T, Qin H, Iyer AK, Greish K, Maeda H. Oxystress inducing antitumor therapeutics via tumor-targeted delivery of PEG-conjugated D-amino acid oxidase. *Int J Cancer J Int Cancer* 2008;122:1135-44
- Matsumura Y, Maeda H. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumorotropic accumulation of proteins and the antitumor agent smancs. *Cancer Res* 1986;46:6387-92
- Fang J, Nakamura H, Maeda H. The EPR effect: unique features of tumor blood vessels for drug delivery, factors involved, and limitations and augmentation of the effect. *Adv Drug Deliv Rev* 2011;63:136-51
- Gougerot-Pocidalo MA, Elbim C, Dang PM, El Benna J. [Primary immune deficiencies in neutrophil functioning]. *Presse Med* 2006;35:871-8
- Shenep JL, Stokes DC, Hughes WT. Lack of antibacterial activity after intravenous hydrogen peroxide infusion in experimental *Escherichia coli* sepsis. *Infect Immun* 1985;48:607-10
- Lebel CP, Bondy SC. Sensitive and rapid quantitation of oxygen reactive species formation in rat synaptosomes. *Neurochem Int* 1990;17:435-40
- Ellis JA, Mayer SJ, Jones OT. The effect of the NADPH oxidase inhibitor diphenyleneiodonium on aerobic and anaerobic microbicidal activities of human neutrophils. *Biochem J* 1988;251:887-91
- Rosen H, Crowley JR, Heinecke JW. Human neutrophils use the myeloperoxidase-hydrogen peroxide-chloride system to chlorinate but not nitrate bacterial proteins during phagocytosis. *J Biol Chem* 2002;277:30463-8
- Molla G, Sacchi S, Bernasconi M, Pilone MS, Fukui K, Polegioni L. Characterization of human D-amino acid oxidase. *FEBS Lett* 2006;580:2358-64
- Xing J, Moldobaeva N, Birukova AA. Atrial natriuretic peptide protects against *Staphylococcus aureus*-induced lung injury and endothelial barrier dysfunction. *J Appl Physiol* 2011;110:213-24
- Kamata R, Yamamoto T, Matsumoto K, Maeda H. A serratal protease causes vascular permeability reaction by activation of the Hageman factor-dependent pathway in guinea pigs. *Infect Immun* 1985;48:747-53

(Received October 28, 2011, Accepted February 16, 2012)

S-Nitrosated Human Serum Albumin Dimer is not only a Novel Anti-Tumor Drug but also a Potentiator for Anti-Tumor Drugs with Augmented EPR Effects

Yu Ishima,^{†,‡} Di Chen,[†] Jun Fang,^{§,¶} Hiroshi Maeda,[#] Ai Minomo,[†] Ulrich Kragh-Hansen,[¶] Toshiya Kai,^{†,||} Toru Maruyama,^{†,‡} and Masaki Otagiri^{*,†,§,¶}

[†]Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, and [‡]Center for Clinical Pharmaceutical Science, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan

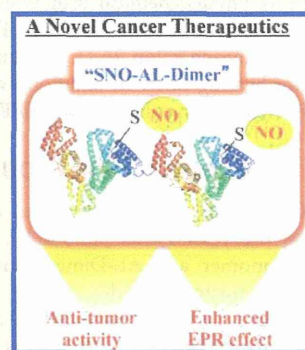
[§]Faculty of Pharmaceutical Sciences and [#]Drug Delivery System Research Institute, Sojo University, 4-22-1 Ikeda, Kumamoto 860-0082, Japan

[¶]Department of Medical Biochemistry, University of Aarhus, DK-8000 Aarhus C, Denmark

^{||}Tohoku Nipro Pharmaceutical Corporation, 428 Okanouchi, Kagamiishimachi, Iwasegun, Fukushima 969-0401, Japan

Supporting Information

ABSTRACT: Macromolecules have been developed as carriers of low-molecular-weight drugs in drug delivery systems (DDS) to improve their pharmacokinetic profile or to promote their uptake in tumor tissue via enhanced permeability and retention (EPR) effects. In the present study, recombinant human serum albumin dimer (AL-Dimer), which was designed by linking two human serum albumin (HSA) molecules with the amino acid linker (GGGS)₂, significantly accumulated in tumor tissue even more than HSA Monomer (AL-Monomer) and appearing to have good retention in circulating blood in murine colon 26 (C26) tumor-bearing mice. Moreover, we developed S-nitrosated AL-Dimer (SNO-AL-Dimer) as a novel DDS compound containing AL-Dimer as a carrier, and nitric oxide (NO) as (i) an anticancer therapeutic drug/cell death inducer and (ii) an enhancer of the EPR effect. We observed that SNO-AL-Dimer treatment induced apoptosis of C26 tumor cells *in vitro*, depending on the concentration of NO. In *in vivo* experiments, SNO-AL-Dimer was found to specifically deliver large amounts of cytotoxic NO into tumor tissue but not into normal organs in C26 tumor-bearing mice as compared with control (untreated tumor-bearing mice) and SNO-AL-Monomer-treated mice. Intriguingly, S-nitrosation improved the uptake of AL-Dimer in tumor tissue through augmenting the EPR effect. These data suggest that SNO-AL-Dimer behaves not only as an anticancer therapeutic drug, but also as a potentiator of the EPR effect. Therefore, SNO-AL-Dimer would be a very appealing carrier for utilization of the EPR effect in future development of cancer therapeutics.



INTRODUCTION

Lack of efficiency in delivering therapeutic agents to a target tumor region leading to severe toxic side effects is most often a barrier to the clinical application of novel therapeutic anticancer drugs in cancer therapy. To limit the side effects of cancer chemotherapy, drug delivery systems (DDS) are being developed with specific targeting for cancers and increased retention in tumor tissues,^{1–3} and/or to enhance the stability and half-life of low-molecular-weight drugs such as nitric oxide (NO) in the circulation.⁴ Previous reports have shown that macromolecules such as human serum albumin (HSA) and synthetic polymers seem to be useful as carriers of low-molecular-weight drugs in DDS systems to improve the pharmacokinetic profile of the drugs or to promote the uptake of drugs in tumor tissue via enhanced permeability and retention (EPR) effects.^{5–7} These findings have inspired research toward the development of DDS with specific targeting potential for tumor tissues in cancer therapy.

NO has been reported to cause DNA damage and direct cell death at high concentrations.^{8,9} Therefore, it has been realized that NO has potential for cancer therapy, and it is emerging as an antioncogenic agent which, in contrast to conventional therapeutic agents,^{10,11} is able to overcome the often-seen tumor cell resistance to treatment. Although there are several NO donors, such as S-nitrosoglutathione (GSNO) and NO-donating nonsteroidal anti-inflammatory drugs (NO-NSAIDs), which were shown to induce apoptosis of tumor cells *in vitro*,^{12,13} their clinical use is limited by some problems, such as toxicity at high concentrations due to lack of specificity for tumor cells or side effects occurring during long-term application.^{14–16} As a consequence, binding of NO to a

Received: September 29, 2011

Revised: November 23, 2011

Published: January 7, 2012

potential carrier to form a stable NO-releasing DDS is proposed as an approach that would overcome these problems.

Recombinant human serum albumin dimer (AL-Dimer), in which the C-terminus of one HSA molecule was linked to the N-terminus of another HSA molecule by the amino acid linker (GGGGS)₂, was designed and successfully produced by the yeast *Pichia pastoris*. It has been reported that AL-Dimer has a longer circulation time than the monomeric form of HSA (AL-Monomer).¹⁷ Because of that finding, we presumed that AL-Dimer would be a good candidate to improve the pharmacokinetic profile of a low-molecular-weight drug such as NO which otherwise has a very short plasma half-life *in vivo*. Moreover, AL-Dimer is expected to have an enhanced accumulation in solid tumor via the EPR mechanism^{18,19} due to its large molecular weight (130 kDa). Therefore, it is possible that AL-Dimer could be of great clinical use as a new DDS material with a superior plasma retention property (e.g., prolonged plasma half-life) as well as with increased tumor specific accumulation.

In this study, we developed a novel DDS system of NO, a potential anticancer therapeutic, using AL-Dimer as a carrier, namely, S-nitrosated AL-Dimer (SNO-AL-Dimer), and examined its physicochemical properties. Then, the biodistribution and anticancer ability of SNO-AL-Dimer was evaluated both *in vitro* in murine colon 26 (C26) tumor cells and *in vivo* in C26 tumor-bearing mice. In addition, we also investigated whether SNO-AL-Dimer enhanced the EPR effect by delivering NO.

EXPERIMENTAL PROCEDURES

Chemicals. AL-Monomer and AL-Dimer were synthesized using the yeast *Pichia pastoris* (*P. pastoris*) (strain GS115).¹⁷ AL-Monomer and AL-Dimer were defatted by means of a charcoal treatment as described by Chen.²⁰ Sephadex G-25 (φ 1.6 × 2.5 cm) was from GE Healthcare (Kyoto, Japan). S-Nitrosoglutathione (GSNO) was purchased from Dojindo Laboratories (Kumamoto, Japan). 1,4-Dithiothreitol (DTT) was from Sigma-Aldrich (St. Louis, MO). Isoamyl nitrite (IAN) was bought from Wako Chemicals (Osaka, Japan). Diethylene-triaminepentaacetic acid (DTPA) and EDTA were purchased from Nacalai Tesque (Kyoto, Japan). ¹¹¹InCl₃ was a gift from Nihon Medi-Physics Co., Ltd. (Hyogo, Japan).

Cells and Animals. C26 cells were cultured in RPMI-1640 containing 10% fetal bovine serum (San kou junyaku, Co., Ltd., Japan), 100 units/mL penicillin, and 100 μg/mL streptomycin, incubated in a humidified (37 °C, 5% CO₂ and 95% air) incubator, grown in 75 cm² flask (Falcon BD), and passaged when 75% confluency was reached. Male BALB/cAnNCrCrlj mice (5 weeks old, 17–22 g) were purchased from Charles River Laboratories, Japan. A C26 solid tumor model was established by subcutaneously implanting of 1 × 10⁶ C26 cells into the back of the mice.

Synthesis of SNO-HSAs. SNO-HSAs were prepared according to a previous report.²¹ In brief, the HSA forms (20 mg/mL) were incubated with 3 mM DTT for 5 min at 37 °C. Afterward, the DTT was removed quickly by Sephadex G-25 gel filtration and eluted with 0.1 M potassium phosphate buffer (pH 7.4) containing 0.5 mM DTPA. DTT-treated HSAs (20 mg/mL) were incubated with 3 mM isoamyl nitrite (IAN) for 60 min protected from light at 37 °C. SNO-HSAs were purified by Sephadex G-25 gel filtration, eluted with phosphate buffered saline (PBS) (pH 7.4). These samples were stored at –80 °C until used in the experiments.

Physicochemical Characterization of HSAs. *CD Spectra.* Circular dichroism (CD) spectra of AL-Monomer and AL-Dimer were measured using J-720-type spectropolarimeter (JASCO, Tokyo, Japan). For the calculation of mean residue ellipticity [θ], the molecular weight was considered as 66.5 kDa for AL-Monomer and 130 kDa for AL-Dimer.¹⁷

Capillary Zone Electrophoresis. The P/ACE MDQ system (Beckman Instruments, Fullerton, CA, USA) in conjunction with a photodiode array detector was employed to determine the net charge of AL-Dimer compared with AL-Monomer. Capillaries with total capillary length of 60 cm (50 cm to the detector) were used. New capillaries were initialized by flushing with water (5 min), 0.1 M NaOH (5 min), water (5 min), and run buffer (5 min) before use. Between analyses the capillary was rinsed with 0.1 M NaOH (3 min), water (3 min), and run buffer (3 min). Injection was performed at a pressure of 0.5 psi for 5 s, and detection was performed at a wavelength of 214 nm.

SDS-PAGE. The purity of AL-Monomer and AL-Dimer was examined by 8% reduced sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Gross conformational changes induced by S-nitrosation of AL-Monomer and AL-Dimer were assessed by means of nonreducing SDS-PAGE (8%) at 4 °C in the dark. The bands were stained by using Coomassie Brilliant Blue (CBB).

Anti-Oxidant Activity of HSAs. For 1,1'-diphenyl-2-picrylhydrazyl (DPPH) radicals, the radical scavenging activity of the HSAs (0.67 mg/mL; AL-Monomer 10 μM; AL-Dimer 5 μM) was determined from the decrease in the absorbance of DPPH radicals at 517 nm due to their scavenging of an unpaired electron of the stable DPPH radical in a mixture of 10 mL ethanol, 10 mL of 50 mM 2-(N-morpholino)ethanesulfonic acid (Mes) buffer (pH 5.5), and 5 mL of 0.5 mM DPPH in ethanol.²² For O₂^{•-}, the xanthine/xanthine oxidase (X/XO) system was used to generate O₂^{•-} to measure the superoxide-scavenging activity of the HSAs. Briefly, 1 mL of solution contained 100 μg/mL X, 0.01 U/mL XO, 250 μM DTPA, and 500 μM luminol in the absence or presence of 0.67 ng/mL of HSAs (AL-Monomer 10 nM; AL-Dimer 5 nM). The chemiluminescence (CL) response was continuously recorded for 4 min at room temperature using Mini Lumat LB 9506 luminometer (EG&G Berthold, Germany). CL was obtained from the slope of the counts versus the time graph. To evaluate the NO scavenging activity of the HSAs, we measured the amount of the S-nitroso moiety of HSAs after incubation with NO by means of HPLC coupled with a flow reactor assay, as previously reported.²³ Briefly, 10 mg/mL HSAs (AL-Monomer 150 μM; AL-Dimer 75 μM) was incubated with 5 mM NOC7 for 10 min in PBS at 25 °C in the dark. To stop the reaction, 1.5 mM N-ethylmaleimide was added to these reaction solutions; the resulting solutions were kept at –80 °C until analysis.

Determination of SNO-Moiety and Stability of SNO-HSAs. The amounts of the SNO-moiety of the SNO-HSAs were quantified by HPLC coupled with a flow reactor system, as previously reported.²³ The stability of the SNO-moiety on storage was examined as follows. SNO-HSAs were dissolved in PBS with 0.5 mM DTPA (pH 7.4) and protected from light at 4 or 25 °C. At appropriate times after the start of incubation, aliquots of the SNO-HSA solutions were taken, and the SNO-moieties were determined by HPLC as mentioned above.

Pharmacokinetic Experiments. The HSAs and SNO-HSAs were labeled with ¹¹¹In by using DTPA anhydride as a

bifunctional chelating agent.^{24,25} The pharmacokinetics and body distribution of HSAs and SNO-HSAs were carried out in a C26 solid tumor model. In brief, ¹¹¹In-labeled protein was dissolved in saline, and the concentration of ¹¹¹In-labeled protein was adjusted by addition of nonradiolabeled protein to the solution. The ¹¹¹In-labeled protein solution was administered intravenously via the tail vein at a dose of 1 mg/kg. At scheduled times after the intravenous injection, the mice were sacrificed, and blood was collected from the inferior vena cava. Tumor tissue was collected for tumor distribution. The radioactivity of each sample was counted using a well-type NaI scintillation counter ARC-2000 (Aloka, Tokyo, Japan).

Cell Death Induced by the HSAs. For detection of C26 cell death induced by the HSAs with and without S-nitrosation, the cells were seeded in flat-bottomed 96-well plates (1×10^4 cells per well). After culturing for 21 h, the media were refreshed and HSAs with different NO concentrations were added into the plates. After further 24 h, cell death was evaluated by the lactate dehydrogenase (LDH) assay from Wako Pure Chemical (Osaka, Japan). The percentage of dead cells was determined using the following formula: $(T - L)/(H - L) \times 100$, where T is the absorbance at 490 nm of supernatants of HSA-treated cells, using 630 nm as a reference, L is the absorbance of supernatants of control untreated cells, and H is the absorbance corresponding to the maximal (100%) LDH release of Triton-lysed cells.

In Vivo Measurement of NO_x. C26 tumor-bearing mice were produced with the method mentioned above. 100 μ L SNO-AL-Dimer, SNO-AL-Monomer, or GSNO were administered intravenously via the tail vein at dose of 1.3 μ mol NO/kg. At 6 h after the intravenous injection, the mice were sacrificed, and blood was collected from inferior vena cava, followed by collection of tumors as well as normal tissues, i.e., liver and kidney. The collected blood was centrifuged, and 100 μ L of plasma was used for this study. The tumor and normal tissues were homogenized by use of BioMasher. NO_x (NO₂⁻ + NO₃⁻) level was measured by the HPLC flow-reactor system, and NO_x, the oxidative metabolites of NO, were quantified by the Griess reaction.

Effect of SNO-HSAs on Solid Tumor Vascular Permeability. C26 tumors obtained from at least six mice per group at 10 days after tumor cell inoculation were used for the *in vivo* vascular permeability experiments, as previously reported.²⁶ SNO-AL-Monomer and SNO-AL-Dimer were administered i.v. via the tail vein at doses of 1.3 μ mol NO/kg. To assess vascular permeability in solid tumors, Evans blue dye that binds to serum albumin quickly *in vivo* to behave as a macromolecule was injected i.v. at a dose of 10 mg/kg into the tumor-bearing mice at 6 h after administration of the SNO-HSAs. At 24 h after the Evans blue dye injection, the dye in the solid tumor was extracted with formamide and quantified by UV absorption at 620 nm, as described previously.⁵

Statistical Analysis. The statistical significance of collected data was evaluated using the ANOVA analysis followed by Newman-Keuls method for more than 2 means. Differences between the groups were evaluated by Student's *t* test. $P < 0.05$ was regarded as statistically significant.

RESULTS AND DISCUSSION

Physicochemical and Structural Properties of AL-Dimer Compared with AL-Monomer. Physicochemical properties of macromolecular carriers including molecular weight and electric charge influence their biological properties,

such as plasma half-life, tumor accumulation, and tumor cell uptake which are important factors for tumor targeted carriers.²⁷ Therefore, we examined the physicochemical and structural properties of AL-Dimer. First, SDS-PAGE was used to evaluate the molecular weight and purity of AL-Dimer generated by the *P. pastoris* system. From Figure 1A, it is seen

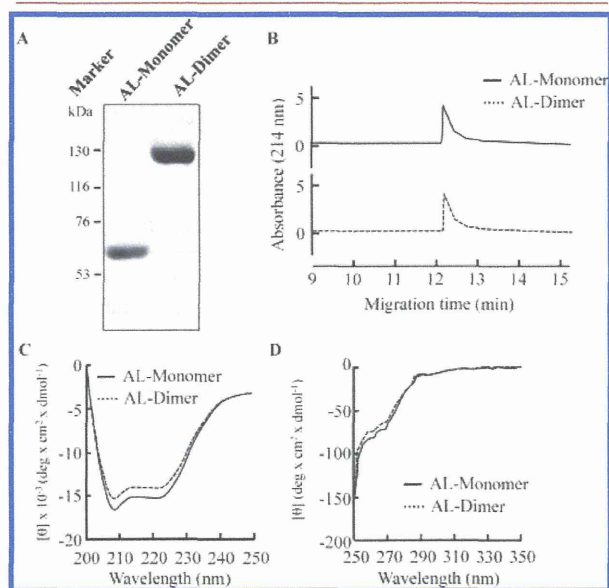


Figure 1. (A) Reduced SDS-PAGE (8%) of AL-Monomer and AL-Dimer visualized by CBB staining. (B) Capillary zone electrophoresis examination of AL-Monomer and AL-Dimer (both at a concentration of 10 mg/mL) in PBS (pH 7.4) solution. Far-UV (C) and near-UV (D) CD spectra of AL-Monomer and AL-Dimer in PBS (pH 7.4) solution at 25 °C.

that under reducing conditions AL-Dimer shows as a single band with a molecular mass of 130 kDa, which is approximately twice the molecular weight of AL-Monomer. This finding indicates a successful generation of pure AL-Dimer. The net charge of HSA was not modified by dimerization, because capillary zone electrophoresis shows that the net charge related to the amount of protein is the same for AL-Dimer and AL-Monomer (Figure 1B). The conformational structure of the two albumin forms were evaluated by CD spectra. As shown in Figure 1C and D, AL-Dimer has CD spectra which are only slightly different from those of AL-Monomer. These results suggest that AL-Dimer has a conformational structure similar to that of AL-Monomer. Furthermore, we checked the effect of AL-Dimerization on the accessibility of ³⁴Cys using DTNB, and as seen from Table 1, dimerization of HSA did not affect the accessibility of ³⁴Cys. Finally, we studied the scavenging activity of the two albumin forms against three different ROS, namely, DPPH, O₂^{•-}, and NO (Table 1). In all three examples, AL-Dimer has a significantly higher scavenging activity than AL-Monomer, suggesting small but facilitating changes in the microenvironment of other antioxidative amino acid residues in AL-Dimer than ³⁴Cys (Figure 1C and D).

Physicochemical Properties of SNO-AL-Dimer Compared with SNO-AL-Monomer. Nonreducing SDS-PAGE was used to confirm the purity of SNO-AL-Dimer after S-nitrosation reaction (Supporting Information Figure 1). It revealed only a single band for SNO-AL-Dimer with the same

Table 1. Physicochemical Characterization of AL-Monomer and AL-Dimer

	Number of free Cysteine (mol SH/mol protein)	SH accessibility to DTNB ^a (A_5/A_{60} /mol SH)	radical scavenging activities (%)		
			DPPH	O ₂ [*]	NO
AL-Monomer	1	0.73 ± 0.08 ^b	36 ± 6.4	30 ± 9.4	26 ± 8.4
AL-Dimer	2	0.68 ± 0.13	47 ± 4.4*	45 ± 8.6*	37 ± 6.6*

^aThe accessibility was evaluated as A_5/A_{60} , where A_5 and A_{60} is the sample absorbance at 405 nm after 5 and 60 min (maximal absorbency), respectively, of incubation with DTNB. ^bResults are expressed as means ± SD ($n = 3-9$). * $P < 0.05$, as compared with AL-Monomer.

Table 2. Physicochemical Characterization of SNO-AL-Monomer and SNO-AL-Dimer

	SNO moiety (mol SNO/mol protein)	half-lives ($T_{1/2}$) in PBS at 4 °C (day)	half-lives ($T_{1/2}$) in PBS at 25 °C (day)	decomposition products
SNO-AL-Monomer	0.52 ± 0.20 ^a	25.4 ± 12.2	21.3 ± 7.4	AL-Monomer
SNO-AL-Dimer	1.48 ± 0.25*	46.8 ± 15.1*	39.0 ± 11.9*	AL-Dimer

^aResults are expressed as means ± SD ($n = 3-6$). * $P < 0.05$, as compared with AL-Monomer.

molecular mass of AL-Dimer, which indicated that S-nitrosation did not cause dimerization of AL-Dimer via disulfide bond formation, or fragmentation or gross conformational changes of AL-Dimer. The analysis also showed no gross conformational changes in the case of S-nitrosation of AL-Monomer. From Table 2 it is seen that the present approach is efficient in S-nitrosation of the AL-Dimer, because the preparation contains on average 1.48 mol of NO per mol of protein.

We determined the stability of the two SNO-preparations in phosphate buffer protected from light. At 4 °C the apparent half-life of the SNO-moiety of SNO-AL-Dimer is about 47 days, which is significantly longer than that of the SNO-AL-Monomer (i.e., 25 days) (Table 2). Also at 25 °C, the half-lives were long, i.e., 39 and 21 days for SNO-AL-Dimer and SNO-AL-Monomer, respectively. This result provided evidence that the SNO-HSAs are fairly stable compounds in neutral buffer even at room temperature.

Accumulation of AL-Dimer in Tumor Tissue Compared with AL-Monomer. The adverse side effects of anticancer drugs, resulting from lack of specificity in conventional therapies, usually limit the increase of dose intensity which is required to eradicate the cancerous growth.²⁸⁻³⁰ One of the developing strategies for the enhancement of tumor specific delivery of the therapeutics is albumin-based drug delivery technology.^{1,31} Albumin has emerged as a drug carrier in the past 10 years for drug targeting and for improving the pharmacokinetic profile of various therapeutic drugs.³¹⁻³³ Examples are the antitumor therapeutic drug doxorubicin-albumin conjugate,³⁴ and the use of cationized albumin as drug carrier for blood-brain barrier transport.^{35,36} In order to clarify whether intravenously injected AL-Dimer accumulate in solid tumor tissue, we performed a biodistribution analysis of AL-Dimer given to C26 tumor-bearing mice. As shown in Figure 2A, it was observed that at 24 h after injection the accumulation of AL-Dimer in the tumor tissue was 1.8 times higher than that of AL-Monomer. Moreover, AL-Dimer showed a much higher level of blood retention compared with that of AL-Monomer (Figure 2B). These findings are consistent with a previous study showing that proteins with molecular weights ranging from 12 to 150 kDa correlated with the rate of tumor uptake, and that long circulation times lead to enhanced tumor uptakes of the protein.³⁴ Thus, AL-Dimer seems to be a good candidate as a drug carrier, because in addition to a prolonged blood retention it has a good accumulation in tumor tissue.

Thus, the physicochemical characterization showed that AL-Dimer has a conformational structure and net charge which in

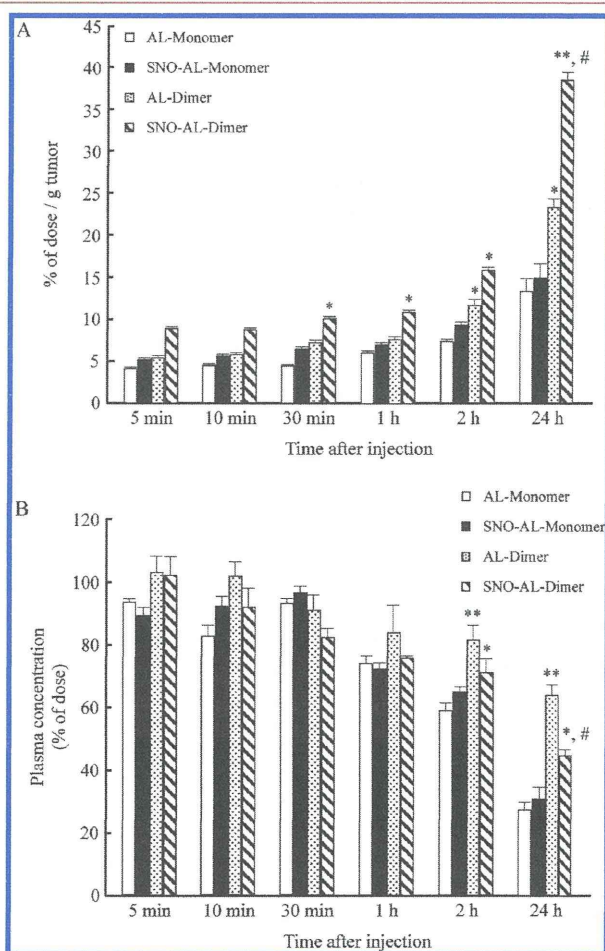


Figure 2. Tumor accumulation (A) and blood retention (B) of ¹¹¹In-labeled AL-Monomer and AL-Dimer with and without S-nitrosation after intravenous administration to C26 tumor-bearing mice at a dose of 1 mg/kg. Results are means ± SD ($n = 4$). * $P < 0.05$, ** $P < 0.01$ compared with AL-Monomer or SNO-AL-Monomer, # $P < 0.05$ compared with AL-Dimer.

principle are similar to those of AL-Monomer. Furthermore, the pharmacokinetics and biodistribution studies very importantly showed that AL-Dimer has superior blood retention properties and tumor targeting potential. Therefore, based on

these findings, we found that exploiting recombinant AL-Dimer as a drug carrier would have several advantages over HSA: (a) the use of commercial and possibly pathogenic albumin is avoided; (b) AL-Dimer is expected to be effective in binding albumin-binding drugs, among other things because it has two molecules of ^{34}Cys . (c) AL-Dimer could enhance the stability of therapeutic drugs both *in vitro* and *in vivo* and improve their blood retention and tumor accumulation *in vivo*. These potential advantages stimulated our attempt to utilize AL-Dimer as a carrier of antitumor therapeutic drugs and thereby to develop a new drug delivery system for cancer therapy. We chose to use NO as the potential anticancer agent and developed SNO-AL-Dimer to confirm the potential of AL-Dimer as a carrier in anticancer DDS. First, for evaluating whether S-nitrosation of AL-Dimer changes its pharmacokinetic parameters, we performed a biodistribution analysis of SNO-AL-Dimer in C26 tumor-bearing mice. Promisingly, the results showed that the accumulation of SNO-AL-Dimer in tumor tissue was 2.6 and 1.6 times higher than that of SNO-AL-Monomer and AL-Dimer, respectively, suggesting that S-nitrosation of AL-Dimer further enhanced its EPR effect, probably via NO release from SNO-AL-Dimer.

C26 Tumor Cell Death Induced by SNO-AL-Dimer Compared with SNO-AL-Monomer. C26 tumor cells were used for investigating the cytotoxicity of different albumin preparations, and cell death was assayed by the quantification of extracellular LDH release. As shown in Figure 3A, both AL-Dimer and AL-Monomer have a small cytotoxic effect, i.e., only

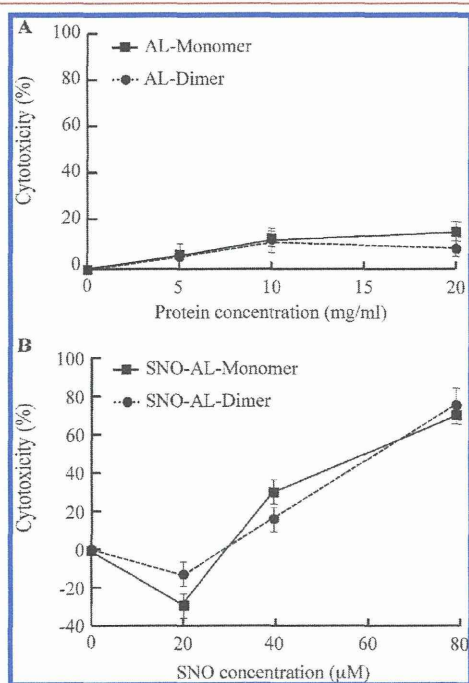


Figure 3. Cytotoxicity of AL-Dimer and AL-Monomer without (A) and with (B) S-nitrosation. C26 cells were cultured at a density of 1×10^4 cells/well for 21 h in RPMI1640 supplemented with 10% FBS. After that period of time the medium was refreshed with AL-Dimer, AL-Monomer, SNO-AL-Dimer, or SNO-AL-Monomer for 24 h, and cell death was assessed by measurement of extracellular LDH activity. In (B), the protein concentrations were approximately 2–9 mg/mL. Results are means \pm SD ($n = 3-5$).

about 10% of the cells were dead after 24 h of exposure to 20 mg protein/mL. By contrast, SNO-AL-Dimer or SNO-AL-Monomer treatment resulted in a much more pronounced LDH release from the cells and in a NO-dose dependent manner, i.e., 70% or more of the cells died in the presence of 80 μM of SNO (Figure 3B). This result is in agreement with a previous study which reports that NO donors such as sodium nitroprusside (SNP) caused cell death depending on the NO concentration.¹² Importantly, the present findings clearly proved that NO, but not AL-Dimer, is the tumor cell death inducer, thus suggesting that AL-Dimer is a suitable carrier for anticancer drugs such as NO. Accordingly, we considered that SNO-AL-Dimer may become a new NO donor with superior pharmacological (cytotoxic) effect. Further, because of the significantly improved *in vivo* pharmacokinetics of SNO-AL-Dimer as described above, it is reasonable to expect that SNO-AL-Dimer would show a much improved antitumor effect, as well as a substantial reduction of the toxicity profile compared with traditional NO donors which are known to cause adverse side effects at high concentrations during long-term treatment.^{14,15} The data obtained so far led us to perform the following *in vivo* experiments.

NO Delivery by SNO-AL-Dimer for Tumor Targeting.

To evaluate whether AL-Dimer successfully delivers NO to tumor tissue *in vivo*, a C26 solid tumor model was utilized, and the delivery of NO into tumor tissue by SNO-AL-Dimer was determined by quantification of NOx level in the tumor and compared with those of SNO-AL-Monomer and the commercially available NO donor GSNO. From Figure 4A it can be seen that intravenous treatment with SNO-AL-Dimer, but not with SNO-AL-Monomer or GSNO, caused a significant increase in the tumor level of NOx. By contrast, none of the

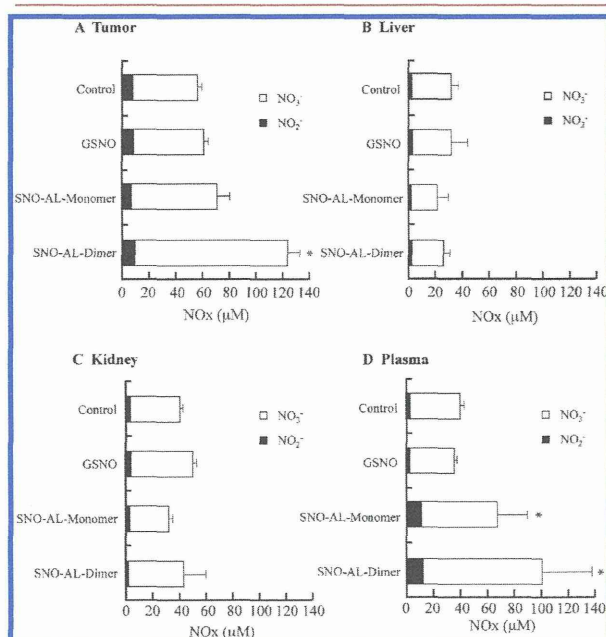


Figure 4. NOx levels in tumor tissue (A), liver (B), kidney (C), and plasma (D) at 6 h after administration of GSNO, SNO-AL-Monomer, or SNO-AL-Dimer (40 nmol NO/mouse) were determined by the HPLC flow-reactor system. Controls were mice which had not been treated with a NO-containing compound. Results are means \pm SD ($n = 3-11$). * $P < 0.05$ compared with control.

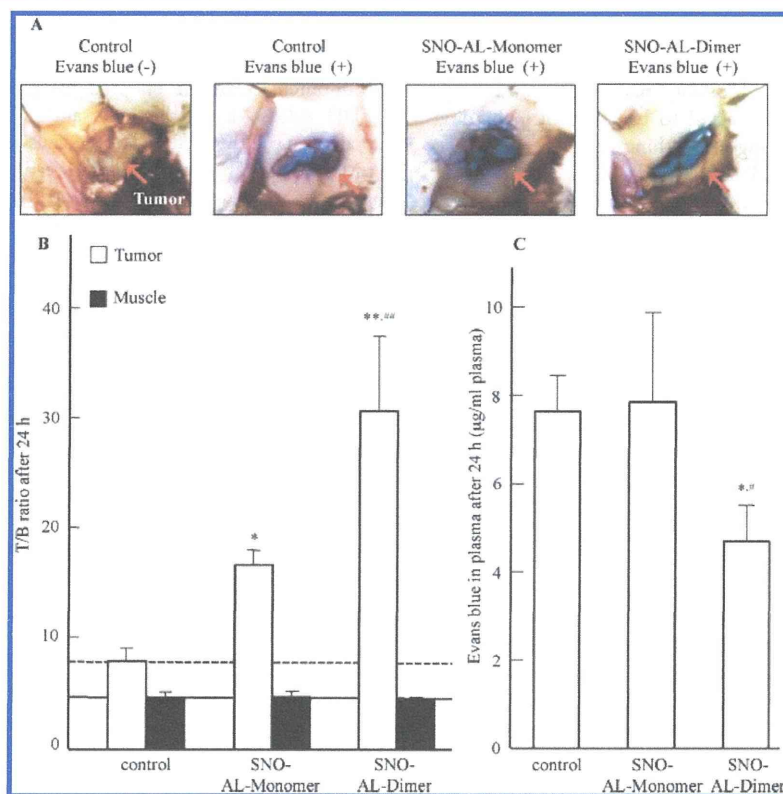


Figure 5. Extravasation of Evans blue induced by SNO-AL-Monomer and SNO-AL-Dimer. 1×10^6 C26 cells were subcutaneously inoculated into the dorsal skin. The mice were kept for 10 days until the tumor had reached a certain volume. (A) After 10 days, saline (controls), SNO-AL-Monomer (40 nmol NO/mouse) or SNO-AL-Dimer (40 nmol NO/mouse) was injected intravenously. Six hours after administration of the different solutes, Evans blue dye was injected i.v. at a dose of 10 mg/kg into three of the four groups of the tumor-bearing mice. Twenty-four hours later, the mice were assessed. (B) Tissue–blood ratio at 24 h after intravenous injection of Evans blue dye in the C26 tumor-bearing mice. (C) Plasma concentrations of Evans blue in the mice at 24 h after i.v. injection. Results are means \pm SD ($n = 6$ –8). * $P < 0.05$, ** $P < 0.01$ compared with control, # $P < 0.05$, ## $P < 0.01$ compared with SNO-AL-Monomer.

preparations induced increased NO x levels in other organs such as liver (Figure 4B) or kidney (Figure 4C). Finally, especially SNO-AL-Dimer has a high level of blood retention (Figure 4D). These results showed that SNO-AL-Dimer is more efficient for tumor delivery of NO than a traditional NO donor such as GSNO. The findings also showed that SNO-AL-Dimer caused a much higher concentration of NO x in the tumor than SNO-AL-Monomer which has been proven as a novel anticancer drug. This may be due to the possibility that SNO- 34 Cys is relatively stable and effective in providing a steady-state NO level *in vivo* compared with other sites binding to NO.²¹

EPR Effect was Enhanced by NO Released from SNO-AL-Dimer. To evaluate whether SNO-AL-Dimer enhances the EPR effect by delivering NO, the extravasation of Evans blue in the tumor tissue after treatment with SNO-AL-Dimer was determined, compared with SNO-AL-Monomer, using the C26 tumor-bearing mice as *in vivo* model (Figure 5A). Figure 2 shows that the accumulation of SNO-AL-Dimer in tumor tissue is 2.6 times higher than that of SNO-AL-Monomer, suggesting that S-nitrosation of AL-Dimer further enhanced its EPR effect. This enhanced effect on EPR is a most important finding in our study, and we used the Evans blue method to confirm it. Evans blue dye binds to albumin noncovalently *in vivo* after i.v. injection, and the albumin–dye complex (molecular mass of ca. 70 kDa) is extravasated in the tumor tissue due to the EPR

effect. Thus, extravasation of Evans blue dye is considered to be a marker for the EPR effect of macromolecules in solid tumor.²⁶ Figure 5B shows that the extravasation of Evans blue dye in tumor tissue, but not in normal tissue (i.e., muscle), was significantly increased by intravenous treatment of both SNO-AL-Dimer and SNO-AL-Monomer. However, SNO-AL-Dimer induced more extravasation of Evans blue than SNO-AL-Monomer, suggesting that SNO-AL-Dimer enhances EPR effect in a much more pronounced manner, probably due to the significantly increased tumor delivery of NO from SNO-AL-Dimer as described above. In accordance with that proposal, Figure 5C shows that the plasma concentration of Evans blue after 24 h of injection is much lower in the presence of SNO-AL-Dimer than in the presence of SNO-AL-Monomer.

It has been reported by Yasuda¹⁰ and Seki et al.³⁷ that the EPR effect can also be enhanced by, i.e., applying nitroglycerin (NG) ointment on the skin of tumor-bearing animals. Therefore, we compared the potential of SNO-AL-Dimer and NG on augmentation of the EPR effect. As the result, both the NO x level in tumor tissue (Supporting Information Figure 2) and the extravasation of Evans blue (Supporting Information Figure 3) are 2-fold higher in the animals given SNO-AL-Dimer than in the animals treated with NG. In light of these results, we propose that SNO-AL-Dimer possesses a powerful EPR effect. To elaborate this idea further, it is necessary to investigate the effect of SNO-AL-Dimer on the tumor

accumulation and antitumor activity of other anticancer drugs such as Doxil or Abraxane. Our drug delivery system is also open for linking SNO-AL-Dimer to human antitumor protein toxins such as human RNase, tBID (truncated BH3-interacting domain death agonist), granzyme B, and AIF (apoptosis-inducing factor). The facile production and broad applicability make our drug delivery system a viable vehicle for anticancer drugs.

Conclusion. Our study had three major findings. First, SNO-AL-Dimer can accumulate in tumor tissue (Figure 2). Second, SNO-AL-Dimer delivers NO selectively to tumor tissue, resulting in cytotoxicity to the tumor cells (Figure 3 and Figure 4). Third, SNO-AL-Dimer behaves as a powerful enhancer of the EPR effect via NO release in the tumor (Figure 5). These findings lead to the idea that SNO-AL-Dimer possesses the clinical possibility for being not only an anticancer drug, but also a valuable tumor targeting delivery system due to enhancement of the EPR effect. In addition, we thus believe that using SNO-AL-Dimer as a carrier of anticancer drugs might be expected to improve the therapeutic potential due to the superior blood retention properties and tumor specific accumulation.

■ ASSOCIATED CONTENT

Supporting Information

Additional experimental methods and figures as described in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

*Tel.: +81-96-371-4150; Fax: +81-96-362-7690; E-mail: otagirim@ph.sojo-u.ac.jp.

■ ACKNOWLEDGMENTS

This research was supported by Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science (JSPS) (KAKENHI 18390051 and 22790162). This work was in part supported by grants from the Uehara Memorial Fund. Thanks are also due to members of the Gene Technology Center in Kumamoto University for their important contributions to the experiments.

■ REFERENCES

- (1) Kerr, D. J., and Los, G. (1993) Pharmacokinetic principles of locoregional chemotherapy. *Cancer Surv.* 17, 105–22.
- (2) Reddy, L. H. (2005) Drug delivery to tumours: recent strategies. *J. Pharm. Pharmacol.* 57, 1231–42.
- (3) Moses, M. A., Brem, H., and Langer, R. (2003) Advancing the field of drug delivery: taking aim at cancer. *Cancer Cell* 4, 337–41.
- (4) Katayama, N., Nakajou, K., Komori, H., Uchida, K., Yokoe, J., Yasui, N., Yamamoto, H., Kai, T., Sato, M., Nakagawa, T., Takeya, M., Maruyama, T., and Otagiri, M. (2008) Design and evaluation of S-nitrosylated human serum albumin as novel anticancer drug. *J. Pharmacol. Exp. Ther.* 325, 69–76.
- (5) Matsumura, Y., and Maeda, H. (1986) A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumorotropic accumulation of proteins and the antitumor agent smancs. *Cancer Res.* 46, 6387–92.
- (6) Maeda, H., and Matsumura, Y. (1989) Tumorotropic and lymphotropic principles of macromolecular drugs. *Crit. Rev. Ther. Drug Carrier Syst.* 6, 193–210.

- (7) Maeda, H., Seymour, L. W., and Miyamoto, Y. (1992) Conjugates of anticancer agents and polymers: advantages of macromolecular therapeutics in vivo. *Bioconjugate Chem.* 3, 351–62.

- (8) Kiziltepe, T., Hideshima, T., Ishitsuka, K., Ocio, E. M., Raje, N., and Catley, L. (2007) JS-K, a GST-activated nitric oxide generator, induces DNA double-strand breaks, activates DNA damage response pathways, and induces apoptosis in vitro and in vivo in human multiple myeloma cells. *Blood* 110, 709–18.

- (9) Ishima, Y., Yoshida, F., Kragh-Hansen, U., Watanabe, K., Katayama, N., Nakajou, K., Akaike, T., Kai, T., Maruyama, T., and Otagiri, M. (2011) Cellular uptake mechanisms and responses to NO transferred from mono- and poly-S-nitrosated human serum albumin. *Free Radic. Res.* 45, 1196–206.

- (10) Yasuda, H. (2008) Solid tumor physiology and hypoxia-induced chemo/radio-resistance: Novel strategy for cancer therapy: Nitric oxide donor as a therapeutic enhancer. *Nitric Oxide* 19, 205–16.

- (11) Yasuda, H., Yamaya, M., Kubo, H., and Sasaki, H. (2006) 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, 688–94.

- (12) Messmer, U. K., Reimer, D. M., and Brune, B. (1996) Nitric oxide-induced apoptosis: p53-dependent and p53-independent signaling pathways. *Biochem. J.* 319, 299–305.

- (13) Ulrich, C. M., Bigler, J., and Potter, J. D. (2006) Non-steroidal anti-inflammatory drugs for cancer prevention: promise, perils and pharmacogenetics. *Nat. Rev. Cancer* 6, 130–40.

- (14) Hryniuk, W. A., Figueredo, A., and Goodyear, M. (1987) Applications of dose intensity to problems in chemotherapy of breast and colorectal cancer. *Semin. Oncol.* 11, 3–11.

- (15) Hogg, N. (2000) Biological chemistry and clinical potential of S-nitrosothiols. *Free Radic. Biol. Med.* 28, 1478–86.

- (16) Wallace, J. L., Del, and Soldato, P. (2003) The therapeutic potential of NO-NSAIDs. *Fundam. Clin. Pharmacol.* 17, 11–20.

- (17) Matsushita, S., Chuang, V. T., Kanazawa, M., Tanase, S., Kawai, K., Maruyama, T., Suenaga, A., and Otagiri, M. (2006) Recombinant human serum albumin dimer has high blood circulation activity and low vascular permeability in comparison with native human serum albumin. *Pharm. Res.* 23, 882–91.

- (18) Maeda, H., Wu, J., Sawa, T., Matsumura, Y., and Hori, K. (2000) Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. *J. Controlled Release* 65, 271–84.

- (19) Tanaka, T., Shiramoto, S., Miyashita, M., Fujishima, Y., and Kaneo, Y. (2004) Tumor targeting based on the effect of enhanced permeability and retention and the mechanism of receptor-mediated endocytosis. *Int. J. Pharm.* 227, 39–61.

- (20) Chen, R. F. (1967) Removal of fatty acids from serum albumin by charcoal treatment. *J. Biol. Chem.* 242, 173–81.

- (21) Ishima, Y., Sawa, T., Kragh-Hansen, U., Miyamoto, Y., Matsushita, S., Akaike, T., and Otagiri, M. (2007) S-nitrosylation of human variant albumin Liprizzi (R410C) confers potent antibacterial and cytoprotective properties. *J. Pharmacol. Exp. Ther.* 320, 969–77.

- (22) Sassa, H., Takaishi, Y., and Terada, H. (1990) The triterpene celastrol as a very potent inhibitor of lipid peroxidation in mitochondria. *Biochem. Biophys. Res. Commun.* 172, 890–7.

- (23) Akaike, T. (2000) Mechanisms of biological S-nitrosation and its measurement. *Free Radic. Res.* 33, 461–9.

- (24) Yamasaki, Y., Sumimoto, K., Nishikawa, M., Yamashita, F., Yamaoka, K., Hashida, M., and Takakura, Y. (2002) Pharmacokinetic analysis of in vivo disposition of succinylated proteins targeted to liver nonparenchymal cells via scavenger receptors: importance of molecular size and negative charge density for in vivo recognition by receptors. *J. Pharmacol. Exp. Ther.* 301, 467–77.

- (25) Peters, T., Jr. (1996) *All about Albumin: Biochemistry, Genetics, and Medical Applications*, Academic Press, San Diego.

- (26) Wu, J., Akaike, T., and Maeda, H. (1998) Modulation of enhanced vascular permeability in tumors by a bradykinin antagonist, a cyclooxygenase inhibitor, and a nitric oxide scavenger. *Cancer Res.* 58, 159–65.