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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

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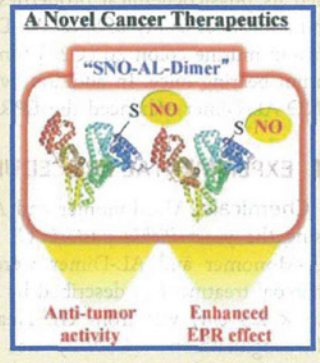
Supporting Information

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 $(GGGGS)_2$, 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

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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-Momomer).[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). Diethylenetriaminepentaacetic acid (DTPA) and EDTA were purchased from Nacalai Tesque (Kyoto, Japan). $^{111}InCl_3$ 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% CO_2 and 95% air) incubator, grown in 75 cm^2 flask (Falcon BD), and passaged when 75% confluency was reached. Male BALB/cAnNCrlCrlj 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 1×10^6 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 dichrosim (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 $O_2^{\bullet-}$, the xanthine/xanthine oxidase (X/XO) system was used to generate $O_2^{\bullet-}$ 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 ^{111}In by using DTPA anhydride as a

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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, ^{111}In-labeled protein was dissolved in saline, and the concentration of ^{111}In-labeled protein was adjusted by addition of nonradiolabeled protein to the solution. The ^{111}In-labeled protein solution was administrated 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 NOx. C26 tumor-bearing mice were produced with the method mentioned above. 100 μL SNO-AL-Dimer, SNO-AL-Monomer, or GSNO were administrated intravenously via the tail vein at dose of 1.3 μmol 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 μL of plasma was used for this study. The tumor and normal tissues were homogenized by use of BioMashaer. NO*x* (NO_2^- + NO_3^-) 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 μmol 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

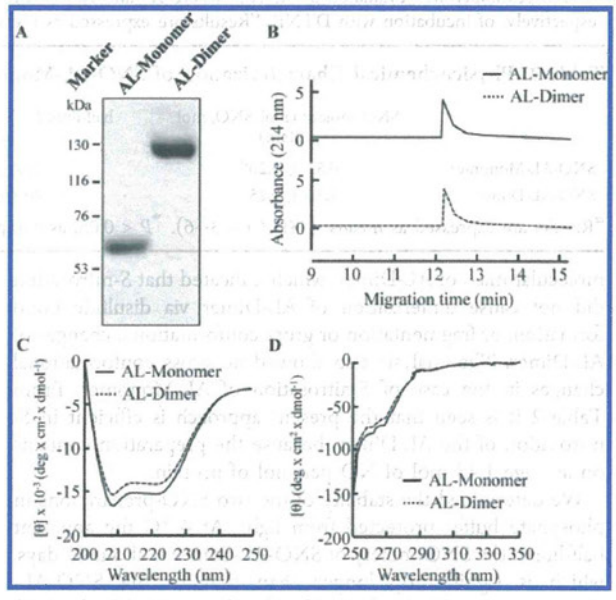

Figure 1. (A) Reduced SDS-PAGE (8%) of AL-Monomer and AL-Dimer visualized by CBB staining. (B) Capillary zone electrophoresis examination of AL-Monomer and AL-Dimer (both at a concentration of 10 mg/mL) in PBS (pH 7.4) solution. Far-UV (C) and near-UV (D) CD spectra of AL-Monomer and AL-Dimer in PBS (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 ^{34}Cys using DTNB, and as seen from Table 1, dimerization of HSA did not affect the accessibility of ^{34}Cys. Finally, we studied the scavenging activity of the two albumin forms against three different ROS, namely, DPPH, $O_2^{\bullet-}$, 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 ^{34}Cys (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

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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	$O_2^{\bullet-}$	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*

[a]The 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. [b]Results 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

[a]Results 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

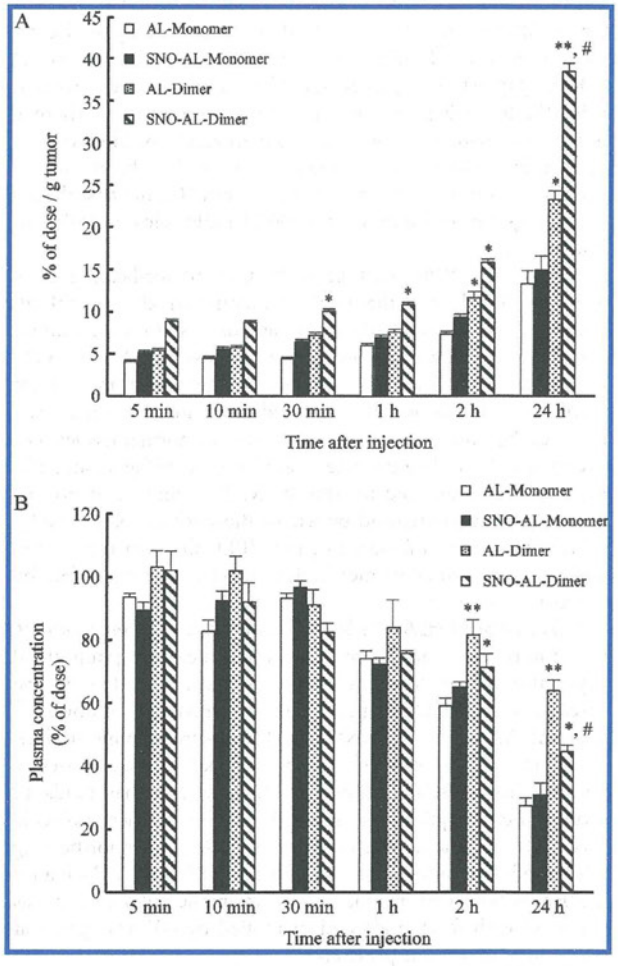

Figure 2. Tumor accumulation (A) and blood retention (B) of ^{111}In-labeled AL-Monomer and AL-Dimer with and without *S*-nitrosation after intravenous administration to C26 tumorbearing mice at a dose of 1 mg/kg. Results are means ± SD (n = 4). * $P < 0.05$, ** $P < 0.01$ compared with AL-Monomer or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

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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 extracelluar LDH release. As shown in Figure 3A, both AL-Dimer and AL-Monomer have a small cytotoxic effect, i.e., only 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.

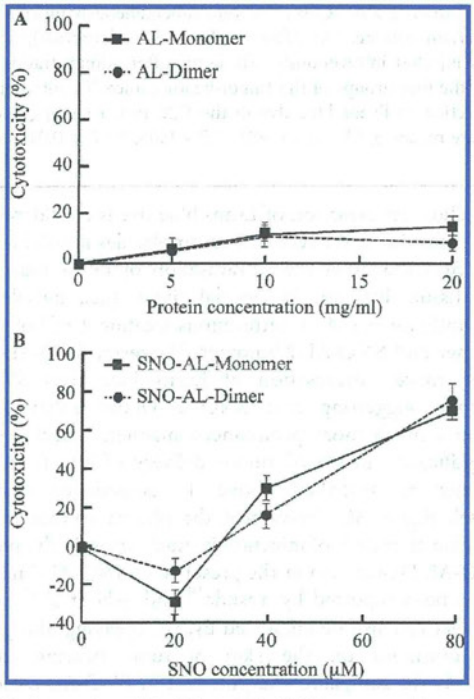

Figure 3. Cytotoxity of AL-Dimer and AL-Monomer without (A) and with (B) *S*-nitrosation. C26 cells were cultured at a density of 1×10^4 cells/well for 21 h in RPMI1640 supplemented with 10% FBS. After that period of time the medium was refreshed with AL-Dimer, AL-Monomer, SNO-AL-Dimer, or SNO-AL-Monomer 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 ± SD (n = 3–5).

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

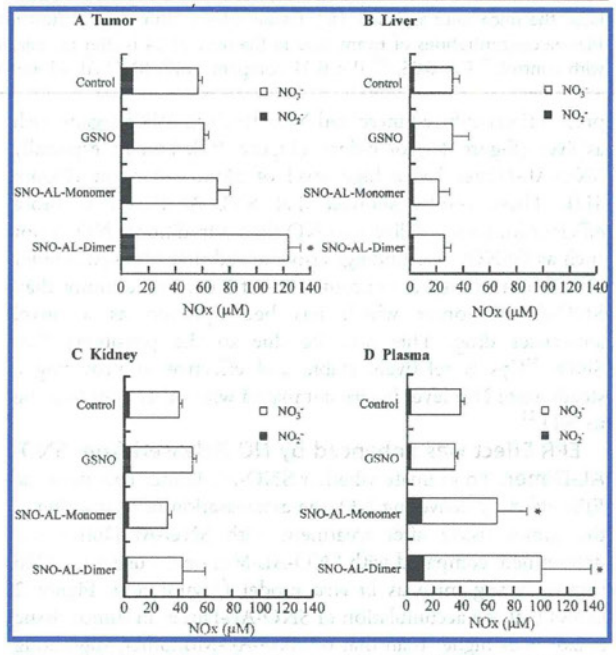

Figure 4. NOx levels in tumor tissue (A), liver (B), kidney (C), and plasma (D) at 6 h after administration of GSNO, SNO-AL-Monomer, or SNO-AL-Dimer (40 nmol NO/mouse) were determined by the HPLC flow-reactor system. Controls were mice which had not been treated with a NO-containing compound. Results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

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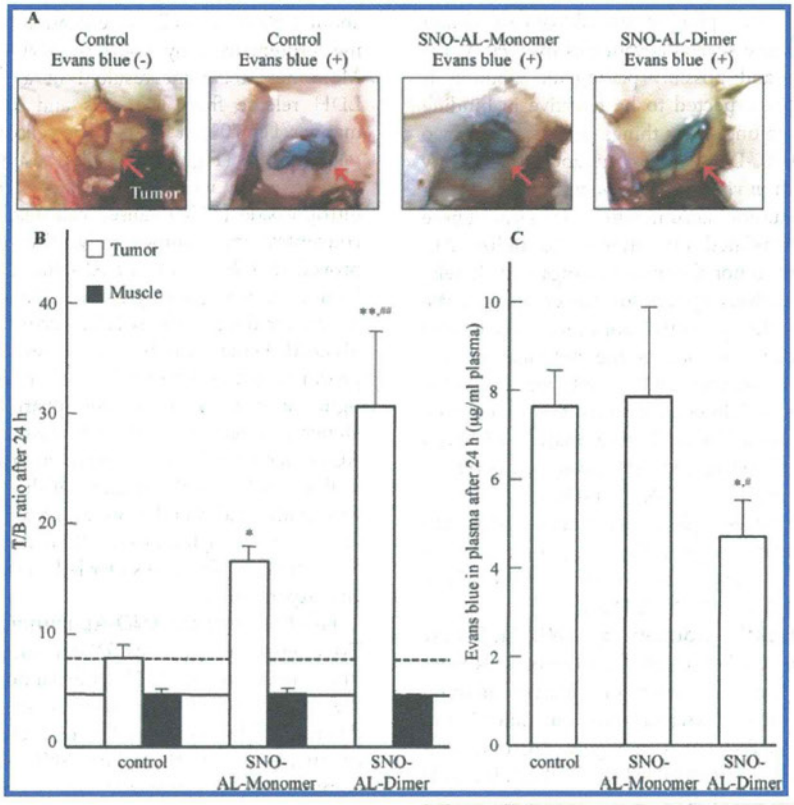

Figure 5. Extravasation of Evans blue induced by SNO-AL-Monomer and SNO-AL-Dimer. 1 $\times 10^6$ C26 cells were subcutaneously inoculated into the dorsal skin. The mice were kept for 10 days until the tumor had reached a certain volume. (A) After 10 days, saline (controls), SNO-AL-Monomer (40 nmol NO/mouse) 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 ± 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 Yasuda[10] 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

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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.

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Review

Macromolecular therapeutics in cancer treatment: The EPR effect and beyond

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ABSTRACT

In this review, I have discussed various issues of the cancer drug targeting primarily related to the EPR (enhanced permeability and retention) effect, which utilized nanomedicine or macromolecular drugs. The content goes back to the development of the first polymer–protein conjugate anticancer agent SMANCS and development of the arterial infusion in Lipiodol formulation into the tumor feeding artery (hepatic artery for hepatoma). The brief account on the EPR effect and its definition, factors involved, heterogeneity, and various methods of augmentation of the EPR effect, which showed remarkably improved clinical outcomes are also discussed. Various obstacles involved in drug developments and commercialization are also discussed through my personal experience and recollections.

Contents

1. Introduction: from the past to the present

From the time of the hypothetical concept of the *magic bullet* proposed by Paul Ehrlich at the end of the 19th century, almost 40–50 years elapsed before the appearance of practical clinical drugs such as sulfonamide and penicillin for the control of microbial infections. However, more than 100 years have passed since Ehrlich's concept to achieve advances in cancer treatment. The field of cancer chemotherapy began when, in 1943 during World War II in Bari, Italy, nitrogen mustard gas, a chemical warfare agent, was accidentally found to have antileukemic activity. However, research aimed at discovering acceptable, effective anticancer agents has not achieved its goal. Only in the last 10 years have we developed promising agents such as imatinib (Glivec), which is quite effective against chronic myelogenous leukemia. Although SMANCS (discussed below) was such a candidate, interest in it has been declining because of business

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reasons. Thus, antitumor drugs that would be effective against a wide range of solid tumors are not available, and ideal drugs that act against solid tumors do not yet exist. In fact, the overall cancer mortality rate has not changed very much in the past 50 years [1,2], whereas the mortality rate in patients with bacterial infections has dropped dramatically since antibiotics became available.

One of the fundamental difficulties in cancer therapy lies in the great genetic diversification seen in human cancers, even in individual patients in which the process of genetic alteration of patients' tumor would have progressed in the past 10–30 years by the time when the patient tumor was diagnosed. In the past 10–20 years, so-called molecular target drugs became popular as the least toxic but promising therapeutics. This trend, despite its being fashionable, is now rather problematic. In fact, these agents, such as the well-known example of molecular target drug, rofecoxib (Vioxx®, though it is not anticancer drug), with more than US$ 10 billion being paid as compensation for unexpected adverse effects, are not miracle drugs. The highly diverse genetic mutations in individual cancer patients, which were just mentioned, make the drug development strategy of utilizing a single molecular target, based on a tumor-specific molecular epitope or target enzyme, very difficult if not completely impossible [3–6].

Other concerns about molecular target anticancer agents include not only poor efficacy but also the cost of drugs to patients. Some drugs, for example, cost far more than interferon or other anticytokine agents. Many molecular target drugs for cancer cost almost US$ 10,000 per dose, or more than US$ 100,000 per year, yet the expected prolongation of life is a few weeks more than the expected survival time of 3 or 5 years for control subjects not receiving the drugs. One can find such information in various references [e.g., 7–12].

With regard to a new modality with potentially less cost, the drug-targeting method based on the enhanced permeability and retention (EPR) effect has a more universal application for solid tumors, so lower costs seem possible, with greater therapeutic effects on more types of tumors and fewer adverse effects. These reasons provide a good rationale for pursuing this method and encouraging such drug development. As this symposium issue will show, anticancer drugs that are more generally effective against solid tumors should be developed, and investigation of the EPR effect, which is based macromolecular therapeutics, will lead to ideal candidates. (Other examples, e.g., that of F. Kratz et al., will also appear in this special issue). Tables 1 and 2 summarize the definition of the EPR effect and the factors involved in solid tumors and inflammation.

One can, of course, argue that the heterogeneity of the EPR effect reduces its universal validity. The EPR effect is not perfect or effective for all solid tumors, because tumors of different patients vary greatly in actual clinical settings. For example, tumor diameters can be less than 1 cm to larger than 10 cm; and tumors can be highly hypoxic to normoxic, can have different pathological classes, are genetically diverse, can have partial or extensive necrosis, can have occluded or compressed vascular systems with or without blood coagulation in or around the tumor mass, and so on. This heterogeneity can be overcome in a number of ways. For instance, modulating the patient's hydrodynamic state by systemic (i.v.) infusion of angiotensin II leads to higher blood pressure on the laminar side and more effectively pushes a drug into the tumor interstitium. Section 3 in this article demonstrates the proof of this method in the clinical settings. We have also developed easier methods utilizing various vascular mediators, as given in Table 3.

Table 1
Profiles of the EPR effect.

Characteristics	Comments
Molecular size	Above 40 kDa; 800 kDa still shows an active EPR effect
Biocompatibility	No coagulation, no interaction with blood components and blood vessels, no cell lysis, no RES[a] clearance (e.g., macrophages). Protease bound protease-inhibitor is cleared in a few min even though biocompatible macromolecules.
Time required to achieve EPR effects	More than several hours in circulation in mice[b]. The trend can be seen even initial 30 min.
Drug retention time of macromolecular drugs in tumor	Mostly days to weeks[a], in great contrast to passive targeting of low MW drugs which is only few minutes[b].
pH (isoelectric point)/surface change	Weakly acidic to weakly cationic. Polycationic particles will disappear rapidly from circulation.

[a] Reticuloendothelial system, such as macrophages.
[b] Passive tumor targeting (visualization) can be observed by radiography with low MW contrast agents upon infusion into the artery. These images are visible for only a few minutes after the infusion, which is typical in passive targeting, but disappears in a few min. In contrast biocompatible nanomedicine exhibits prolonged tumor-retention period of days of weeks. Thus a great contrast to the passive targeting to the EPR effect.

Table 2
Factors and mediators involved in the EPR effect in cancer and inflammation, and their responsible enzymes or effectors.[a]

EPR effect-enhancing factors/ mediators	Enzymes responsible for factors	Comments: actions of enzymes and factors, or sources of factors
1. Bradykinin (kinin)	Kallikrein and other proteases, plasminogen activator produce bradykinin	Angiotensin I-converting enzyme (ACE) degrades kinin; ACE-inhibitor potentiates activity by blocking kinin degradation. Kinin induces NO synthase
2. Nitric oxide (NO)	Nitric oxide synthase (NOS), inducible isoform of NOS (iNOS)	Nitroglycerin, isosorbide dinitrate (ISDN, Nitrol®), and nitroprusside yield nitrate, and nitrite-reductase, which occurs in hypoxic tissue (tumor), generates NO in hypoxic tumors.
3. Prostaglandins (PGs)	Cyclooxygenase 2 (COX-2)	PGI_2 agonist/beraprost affect the EPR effect
4. Carbon monoxide (CO)	Heme oxygenase-1 (HO-1)	Hemin, NO, and ultraviolet light, and heat induce HO-1
5. Peroxynitrite ($ONOO^-$)	Generated by $NO + O_2^-$	Extremely rapid reaction
6. Matrix metalloproteinase (MMP), or collagenase (← proMMP)[b]	Procollagenase activation by $ONOO^-$	$ONOO^-$ activates pro-MMP[b] → MMP
7. Vascular endothelial growth factor (VEGF/VPF)	Nitric oxide synthase (NOS)	NO, endotoxin, and other cytokines can induce this VEGF
8. Tumor necrosis factor α (TNF-α) and TFG-β inhibitor	Cytokines, growth factor	Induces inflammation and normalization of tumor vasculature
9. Heat	Heat shock protein, HO-1 (HSP-32)	e.g. HO-1 and inflammation etc.

See text for detail.
[a] Above factors are most common mediators of inflammation and cancer that facilitate extravasation.
[b] proMMP: pro-matrix metalloproteinase (collagenase) is activated by $ONOO^-$ or by other proteases.

2. Our prototype polymer-conjugate drug, SMANCS, and a new strategy for intraarterial (i.a.) infusion: the ultimate tumor-targeted delivery

In 1979, we pioneered the development of the protein–polymer conjugate SMANCS, which is the antitumor protein drug neocarzinostatin (NCS) chemically conjugated with a synthetic copolymer of styrene–maleic acid copolymer (SMA) [13–15]. SMANCS exhibited unique properties compared with the parental NCS [14–18]. These properties included (i) prolongation of the plasma $t_{1/2}$ (by 20-fold); (ii) improved tumor-targeting capacity because of the EPR effect, i.e., a markedly higher (10- to 20-fold) intratumor concentration compared with the