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Effective delivery of an angiogenesis inhibitor by neovessel-targeted liposomes

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ABSTRACT

Angiogenesis is critical for tumor growth and metastasis, and several angiogenesis inhibitors have been developed for the treatment of cancer. Previously, we identified angiogenic vessel-homing peptide, Ala-Pro-Arg-Pro-Gly (APRPG), by use of a phage-displayed peptide library. APRPG peptide-modified liposomes have been revealed to be useful for the delivery of encapsulated drugs to angiogenic vasculature in tumor-bearing animals. In the present study, to assess the usefulness of APRPG-PEG-modified liposomes as a carrier of angiogenesis inhibitors in vitro and in vivo, we designed and validated APRPG-PEG-modified liposomal angiogenesis inhibitor. SU1498, an inhibitor of vascular endothelial growth factor (VEGF) receptor tyrosine kinase, was successfully encapsulated into the liposomes. APRPG-PEG-modified liposomal SU1498 inhibited VEGF-stimulated endothelial cell proliferation in vitro. Moreover, APRPG-PEG-modified liposomal SU1498 significantly decreased tumor microvessel density in Colon26 NL-17 cell-bearing mice and prolonged the survival time of the mice. These findings suggest that APRPG-PEG-modified liposomes effectively deliver SU1498 to angiogenic endothelial cells in tumors and thus inhibit tumor-induced angiogenesis.

1. Introduction

Solid tumors require blood supply for the maintenance of nutrients and oxygen. Therefore, angiogenesis, the development of new blood vessels, is critical for the tumor progression (Folkman, 1972). Angiogenesis could contribute to not only primary tumor growth but also blood-borne metastasis. Therefore, inhibition of angiogenesis is expected to suppress primary tumor growth and hematogenous metastasis (Holmgren et al., 1995). A number of studies have led to the identification of several regulators of angiogenesis; some of which represent therapeutic targets (Bergers and Benjamin, 2003). Based on these findings, various angiogenesis inhibitors have been developed and running in clinical trials (Shimizu and Oku, 2004). Vascular endothelial growth factor (VEGF) and its receptors are well-characterized pro-angiogenic molecules and would be the target for antiangiogenic therapy (Ferrara et al., 2003). Bevacizumab (Avastin), an anti-human VEGF-A monoclonal antibody, shows the significant antitumor effect and has been approved as an anticancer drug by the US Food and Drug Administration (Hurwitz et al., 2004). Besides bevacizumab, several small-molecule inhibitors of receptor tyrosine kinases (RTK), such as VEGF receptors (VEGFR) or basic fibroblast growth factor receptors, have been developed as an anticancer agent (Mendel et al., 2003).

By the way, pharmacokinetics and pharmacodynamics are critical issues for the development of novel drugs. Drug delivery systems (DDS) are known to improve the pharmacological properties of certain drugs such as anticancer and antifungal drugs (Allen and Cullis, 2004). In cancer treatment, liposomes are widely used as drug carriers (Torchilin, 2007), since they have several favorable characteristics as a carrier of anticancer agents: they can entrap both hydrophobic and hydrophilic compounds; they can reduce the severe side effects; and they tend to accumulate in tumor tissues through the angiogenic endothelium by the enhanced permeability and retention (EPR) effect (Maeda et al., 2000). In fact, several anticancer drugs such as doxorubicin were entrapped into the liposomes, and the liposomal doxorubicin has been known to reduce the side effects and to deliver the drug to tumor tissues (Hofheinz et al., 2005). Moreover, many investigations have shown that liposomes can be modified with various

Abbreviations: APRPG-PEG-Lip-SU1498, APRPG-PEG-modified liposomal SU1498; DDS, drug delivery systems; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; DSPE-PEG-APRPG, distearoylphosphatidylethanolamine-polyethyleneglycol-APRPG peptide conjugate; EPR, enhanced permeability and retention; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; HUVECs, human umbilical vein endothelial cells; PEG-Lip-SU1498, PEG-modifiedliposomal SU1498; POPC, palmitoyloleoylphosphatidylcholine; RTK, receptor tyrosine kinase; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

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targeting tools such as antibodies, peptides, or carbohydrates in order to effectively deliver drugs to the target organs (Kondo et al., 2004; Managit et al., 2003; Park et al., 2002). For example, it has been shown that anti-HER2 immunoliposomes selectively bind to and internalize in HER2-overexpressing cancer cells in vitro, and doxorubicin-loaded anti-HER2 immunoliposomes show the marked therapeutic effects in HER2-overexpressing xenograft models (Park et al., 2002).

For the purpose to obtain a targeting tool to tumor neovessels, we previously isolated a peptide, Ala-Pro-Arg-Pro-Ala (APRPG), homing to tumor angiogenic vasculature by in vivo biopanning with a phage-displayed peptide library (Oku et al., 2002). Then, we utilized APRPG peptide for delivering liposomes to the angiogenic site in tumor-bearing animals. In fact, APRPG peptide-modified liposomes highly accumulated in tumor tissues, and doxorubicin-encapsulated APRPG peptide-modified liposomes significantly suppressed tumor growth through damaging the angiogenic endothelial cells (Maeda et al., 2004; Oku et al., 2002). In the present study, we aimed to develop a liposomal antiangiogenic agent targeted effectively to tumor neovasculature and investigated the effect of APRPG-modified liposomal antiangiogenic agent, namely SU1498, a RTK inhibitor of VEGFR2 (Boguslawski et al., 2004), in tumor-bearing mice.

2. Materials and methods

2.1. Materials

VEGF receptor tyrosine kinase inhibitor SU1498 was purchased from LC laboratories (San Diego, CA, USA). APRPG peptide-conjugated polyethyleneglycol (M.W. 2000)-distearoylphosphatidylethanolamine (DSPE-PEG-APRPG) was synthesized as described previously (Maeda et al., 2004). Dipalmitoylphosphatidylcholine (DPPC), palmitoyloleoylphosphatidylcholine (POPC), and dipalmitoylphosphatidylglycerol (DPPG) were the products of Nippon Fine Chemical Co. Ltd. (Takasago, Hyogo, Japan).

2.2. Preparation of liposomal SU1498

Liposomes were similarly prepared as described previously (Maeda et al., 2004) except that SU1498 was used as an entrapping drug instead of doxorubicin in the present experiment. In brief, lipids and SU1498 (DPPC:POPC:DPPG:cholesterol:SU1498:DSPE-PEG or DSPE-PEG-APRPG = 10:10:2:2:1:2 as a molar ratio) in chloroform/methanol solution were poured into round-bottom flask, and the organic solvent was removed by the evaporation. The resulting thin lipid film was further dried under reduced pressure. Liposomes were prepared by the hydration of the lipid film with 0.3 M sucrose solution by vortexing, brief sonication and freeze-thawing for three cycles with liquid nitrogen. Then, the size of the liposomes was adjusted by extrusions through a 100-nm pore size polycarbonate membrane filter. The particle size and ζ-potential of the liposomes were measured with ZETASIZER (Malvern Instruments, Worcs, UK).

2.3. Entrapment of SU1498 into liposomes

The liposomes containing SU1498 were prepared as described above. The liposome solutions were fractionated by a gel filtration chromatography with PD10 column (GE healthcare UK Ltd., Buckinghamshire, UK). The eluted samples were collected as 2 mL in each fraction, and the amount of SU1498 was determined by measuring the absorbance at 350 nm in the each fraction in the presence of 1% reduced Triton X-100. The entrapment efficiency was calculated as follow: Amount of SU5416 in liposome fraction (fractions 1 and 2)/total amount of SU5416 detected after gel filtration chromatography.

2.4. Cell proliferation assay

Human umbilical vein endothelial cells (HUVECs, Clonetics, Walkersville, MD, USA) were cultured in endothelial growth medium-2 (EGM-2, Clonetics) at 37 °C in a humidified atmosphere of 5% CO_2 in the air. Colon26 NL-17 mouse colon carcinoma cells were cultured in DMEM/Ham F12 medium supplemented with 10% FBS (Japan BioSerum, Tokyo, Japan) at 37 °C in a CO_2 incubator.

HUVECs were seed on gelatin-coated 35 mm dishes at 1.0×10^5 cells/dish and incubated overnight. After replacing of culture medium to endothelial basal medium-2 (EBM-2, Clonetics) supplemented with 0.5% fetal bovine serum (FBS, Clonetics), the cells were treated with free SU1498 dissolved in DMSO, PEG-modified liposomal SU1498 (PEG-Lip-SU1498), and APRPG-PEG-modified liposomal SU1498 (APRPG-PEG-Lip-SU1498) at 1 μM of the final concentration of SU1498 for 3 h. Then, recombinant human $VEGF_{165}$ (20 ng/ml, BD biosciences, San Diego, CA, USA) was added to the cells, and the cells were incubated for another 48 h. Colon26 NL-17 cells were seeded (3.0×10^4 cells/35 mm dish), and the cells were incubated overnight in DMEM/Ham F12 medium supplemented with 10% FBS at 37 °C. Then, the cells were treated with the samples and further incubated for 48 h. Finally, the viable cells were stained with crystal violet, and the dye was extracted with 33% acetic acid and measured at absorbance of 570 nm as described previously (Goto et al., 2005).

2.5. Analysis of microvessel density in tumor tissues

Colon26 NL-17 cells (1.0×10^6 cells) were implanted subcutaneously into the posterior flank of 5-week-old BALB/c male mice (Charles River Japan, Tokyo, Japan). From days 3 to 11 after tumor implantation, each sample, namely, PEG-Lip-SU1498 (5 mg/kg as SU1498), APRPG-PEG-Lip-SU1498 (5 mg/kg as SU1498), and 0.3 M sucrose solution (control), was injected intravenously every other day. On day 13, the mice were sacrificed under anesthesia with diethyl ether, and the tumors were excised. The tumor tissues were mounted on OCT compound (Sakura Finetechnochemical Co. Ltd., Tokyo, Japan) and frozen at −80 °C. The tumor tissue sections (10 μm) were prepared with microtome (HM 505E, Microm, Walldorf, Germany) and mounted onto Matsunami adhesive silane (MAS)-coated slide glass (MATSUNAMI, Osaka, Japan). Immunohistochemical staining against CD31 was performed described previously (Yonezawa et al., 2007) with some modifications. The sections were fixed with ice-cold acetone, washed with phosphate buffered saline (PBS), and blocked endogenous peroxidase activity with 3% H_2O_2 in PBS. Non-specific protein bindings were blocked with 1% bovine serum albumin (BSA) dissolved in PBS. Then, a biotinylated murine anti-CD31 monoclonal antibody (BD Pharmingen, San Diego, CA, USA) was added to the sections and secondary staining was performed with VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. These sections were rinsed and counterstained with Mayer's hematoxylin (Wako Chemical, Osaka, Japan).

For quantification of tumor blood vessels, three of high vessel density areas (hot spots) per section were selected and captured using Olympus IX71 (×10 magnification, 0.54 mm^2, Olympus Co. Ltd., Tokyo, Japan). CD31 positive area was quantified with ImageJ software (http://rsb.info.nih.gov/ij/index.html).

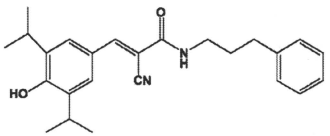

Fig. 1. Structure of SU1498 [(*E*)-3-(3,5-diisopropyl-4-hydroxyphenyl)-2-[(3-phenyl-*n*-propyl)aminocarbonyl]acrylonitrile].

2.6. Therapeutic experiment

Colon26 NL-17-bearing mice were prepared as described above. Each liposomal SU1498 or 0.3 M sucrose solution (control) was administered by the following two different schedules; (A) intravenously injected from days 3 to 11 every other day (5 mg/kg/day, total 5 times 25 mg/kg as SU1498) after tumor implantation; (B) intraperitoneally injected from days 1 to 12 every day (2.5 mg/kg/day, total 12 times 30 mg/kg as SU1498) after tumor implantation. Since SU1498 is almost insoluble in water, we could not examine the effect of the free drug on tumor in vivo. The animals were cared for according to the guidelines for the care and use of laboratory animals of the University of Shizuoka.

2.7. Statistical analysis

Data was statistically analyzed by Student's *t*-test followed by *F*-test (StatView, version 4.5; Abacus Concepts, Inc., Berkeley, CA), and $p < 0.05$ was considered as significant.

3. Results

3.1. Entrapment of SU1498 into liposome and liposomal characterization

To investigate whether angiogenic vessel-targeted liposomes is useful for delivery of angiogenesis inhibitors, we first prepared liposomal SU1498, an inhibitor of VEGFR2 tyrosine kinase. The chemical structure of SU1498 [(*E*)-3-(3,5-diisopropyl-4-hydroxyphenyl)-2-[(3-phenyl-*n*-propyl)aminocarbonyl]acrylonitrile] is shown in Fig. 1. We examined liposomal composition for effective entrapment of SU1498 into liposomes and determined the basic lipid component as follows; DPPC:POPC:DPPG:cholesterol: SU1498 = 10:10:2:2:1. Then, the entrapment efficiency of SU1498 into PEG- or APRPG-PEG-modified liposomes was measured. Approximately 75% of SU1498 was detected in liposome fractions (fractions 1 and 2) but not detected in other fractions (Fig. 2, Table 1). In addition, each liposome size and ζ-potential after extrusion was approximately 160 nm and −3 mV, respectively (Table 1).

3.2. Cell proliferation assay

Next, to examine the antiangiogenic activity of liposomal SU1498, cell proliferation assay of VEGF-stimulated HUVECs was performed. APRPG-PEG-Lip-SU1498 strongly suppressed endothelial cell proliferation induced by the treatment with VEGF, while PEG-Lip-SU1498 suppressed partially as well as free SU1498

Table 1
Characteristics of liposomal SU1498

	Entrapment (%)	Particle size (nm)	ζ-potential (mV)
PEG-Lip-SU1498	74.6	178	−2.32
APRPG-Lip-SU1498	78.7	160	−4.50

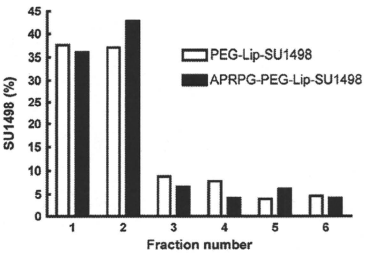

Fig. 2. Entrapment efficiency of SU1498 into the liposomes. Liposomal SU1498 was prepared as described in Section 2, and the prepared liposomes were fractionated by gel filtration chromatography. The fractionated samples were measured with the turbidity. Liposomes were eluted at fractions 1 and 2. The liposomes were solubilized with reduced Triton X-100, and the amount of SU1498 in the liposome fractions was determined with absorbance at 350 nm.

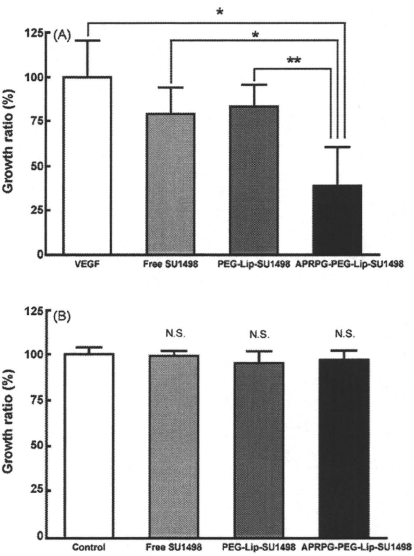

Fig. 3. Suppression of VEGF-induced endothelial cell proliferation by the treatment with APRPG-PEG-Lip-SU1498. (A) HUVECs were seeded (1.0×10^5 cells) on gelatin-coated dishes and incubated overnight at 37 °C. After the change of medium to EBM-2 containing 0.5% FBS, the cells were treated with free SU1498, PEG-Lip-SU1498, and APRPG-PEG-Lip-SU1498 (1 μM as SU1498) for 3 h. VEGF (20 ng/ml) was added to the cells, and the cells were further incubated for 48 h. (B) Colon26 NL-17 cells were seeded (3.0×10^4 cells) and incubated overnight. Then, the cells were treated with free SU1498, PEG-Lip-SU1498, and APRPG-PEG-Lip-SU1498 (1 μM as SU1498) and incubated for 48 h. Finally, the survival cell density was determined by crystal violet staining (absorbance at 570 nm). The cell growth ratio induced by the treatment with VEGF was calculated. The data show the mean ± S.D. ($n = 4$). The asterisks show significantly differences: $^*p < 0.05$; $^{**}p < 0.01$.

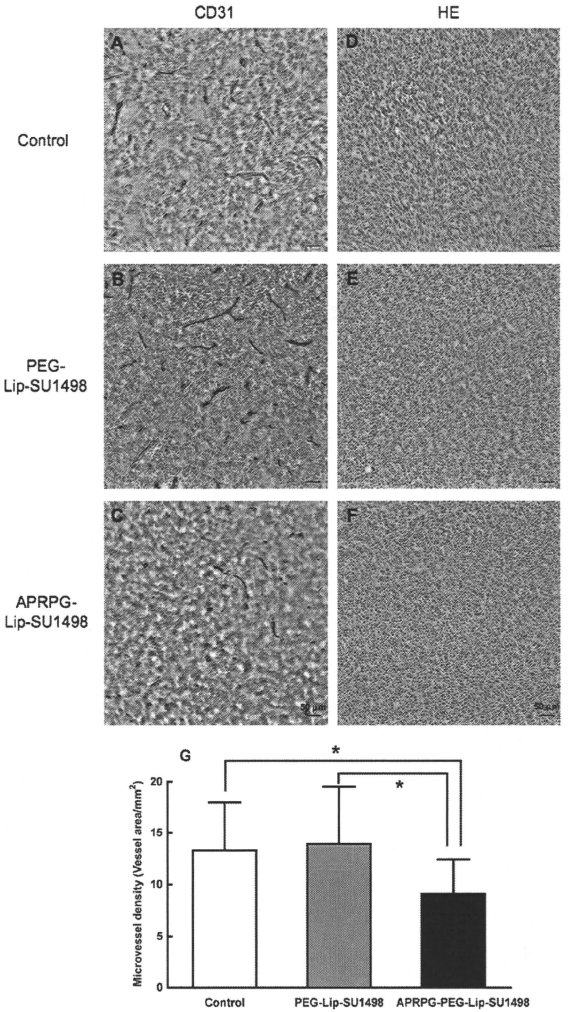

Fig. 4. Suppression of tumor angiogenesis by the treatment with APRPG-modified liposomal SU1498. Colon26 NL-17-bearing mice were injected intravenously with each sample (5 mg/kg as SU1498) from days 3 to 11 every other day after tumor implantation. The tumors were excised at day 13, and the tumor tissue frozen sections were prepared. Immunohistochemical staining of endothelial cells was performed with an antibody specific for CD31 (A–C), and histological analysis was performed with hematoxylin and eosin (D–F). MVD (vessel area/mm^2) was calculated with ImageJ software (G). Scale bars represent 20 μm. The data show the mean ± S.D. The asterisks show significantly difference: $^*p < 0.05$.

Table 2
Survival time of tumor-bearing mice treated with liposomal SU1498

	Mean survival days ± S.D.	
	Schedule A	Schedule B
Control	32 ± 10	47 ± 8
PEG-Lip-SU1498	45 ± 4]#	53 ± 14
APRPG-Lip-SU1498	52 ± 2 *]	59 ± 6 *

The asterisks show significantly difference: * $p<0.05$ versus Control; # $p<0.05$.

(Fig. 3A). On the contrary, free SU1498, PEG-Lip-SU1498, and APRPG-PEG-Lip-SU1498 did not suppress the proliferation of Colon26 NL-17 carcinoma cells (Fig. 3B). These results suggest that liposomalization of SU1498 does not alter the inhibitory activity of it against VEGF-signaling, and APRPG peptide-modification of liposomes enhances the effect of SU1498 maybe through the increase in availability of the drug to HUVECs.

3.3. Antiangiogenic effect of neovasculature-targeted liposomal SU1498 in vivo

Since liposomal SU1498 showed antiangiogenic activity in vitro, we further examined the effect of angiogenic vessel-targeted liposomal SU1498 in vivo. Antiangiogenic activity of APRPG-PEG-Lip-SU1498 was examined in solid tumor-bearing mice. We performed immunohistochemical staining for CD31, which is an endothelial cell marker, and analyzed microvessel density in tumors of Colon26 NL-17-bearing mice after the treatment of APRPG-PEG-Lip-SU1498. The treatment with APRPG-PEG-Lip-SU1498 decreased microvessel density in the tumors compared to control and to that with PEG-Lip-SU1498 (Fig. 4). The data indicate that targeted delivery of angiogenesis inhibitors to tumor endothelial cells enables to enhance the antiangiogenic activity in tumor-bearing mice.

Since inhibition of angiogenesis can suppress tumor growth and metastasis, the effect of liposomal SU1498 on the survival time of Colon26 NL-17-bearing mice was examined. The tumor-bearing mice were administered with each sample by two different schedules as described above: schedule A (i.v. administration) is commonly used in liposomal studies (Sadzuka et al., 2000); schedule B (i.p. administration) has been used as schedule of the treatment with VEGF RTK inhibitors (Fong et al., 1999; Laird et al., 2000; Koyanagi et al., 2003). Both the treatments did not significantly suppress the tumor volume of the Colon26 NL-17-bearing mice and did not cause the marked body weight loss of the mice (data not shown). In contrast, in terms of survival time, there were significant differences between the groups: The treatment with APRPG-PEG-Lip-SU1498 elongated the survival time of the mice compared with other treated groups in schedule A (Fig. 5A, Table 2). However, in schedule B, although APRPG-PEG-Lip-SU1498 tended to prolong the mean survival days, there were not significant differences between PEG- and APRPG-PEG-Lip-SU1498 (Fig. 5B, Table 2).

4. Discussion

In this study, we evaluated the usefulness of tumor vasculature-targeted liposomes as drug carriers of angiogenesis inhibitors. SU1498, known as a potent inhibitor of VEGF receptor tyrosine kinase, has been shown to inhibit VEGF-induced migration and invasion of endothelial cells (Strawn et al., 1996). In addition to the anti-receptor activity, it has been also shown that SU1498 stimulates accumulation of phosphorylated extracellular signal-regulated kinase (ERK) and inhibits their activity in endothelial cells (Boguslawski et al., 2004). We attempted to develop liposomal SU1498, because RTK inhibitors of VEGF are representative antiangiogenic agents, SU1498 has been shown not to affect other RTKs (Strawn et al., 1996), and SU1498 is a hydrophobic compound which can be encapsulated into lipid barrier of liposomes such as amphotericin B or taxol (Bekersky et al., 2000; Yang et al., 2007). In fact, SU1498 did not show suppression of proliferation of Colon26 NL-17 carcinoma cells and was efficiently incorporated into the liposomes, and liposomal SU1498 had the adequate particle size and ζ-potential.

Modification of liposomes with APRPG peptide has been shown to enable to target tumor vasculature (Maeda et al., 2004; Oku et al., 2002). APRPG-PEG-Lip-SU1498 was significantly suppressed the VEGF-induced proliferation of HUVECs in vitro and the tumor microvessel density in an in vivo experiment compared with PEG-Lip-SU1498. Furthermore, by the intravenously treatment with APRPG-PEG-Lip-SU1498, the survival time of the tumor-bearing mice was prolonged, although the significant prolongation was not observed in the case of the intraperitoneally administration. In Fig. 5, the survival time of control mice in two separate experiments was a bit different. However, the survival time in each experiment would be comparable. SU1498 has been shown the antitumor effect by starting the treatment from 1 day post cell inoculation. Therefore, we started the treatment 1 day post tumor implantation when the angiogenesis would not start yet in schedule B. It is

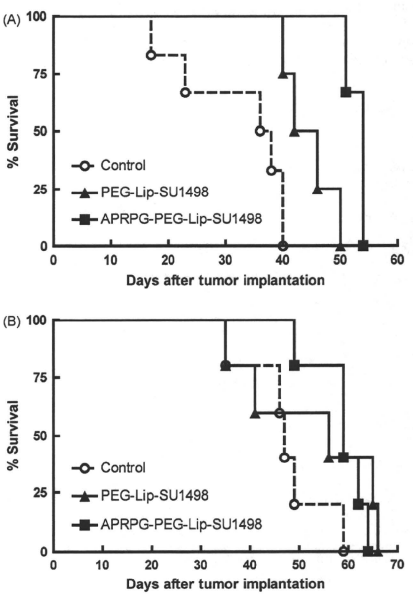

Fig. 5. Kaplan–Meier survival curve of Colon26 NL-17-bearing mice by treatment with APRPG-PEG-modified liposomal SU1498. 0.3 M sucrose (control), PEG-Lip-SU1498, and APRPG-PEG-Lip-SU1498 were injected into the Colon26 NL-17-bearing mice on two different schedules as follows: (A) intravenously injected (5 mg/kg as SU1498) from days 3 to 11 every other day; or (B) intraperitoneally injected (2.5 mg/kg as SU1498) from days 1 to 12 every day ($n=5$). The asterisks show significantly difference: *$p<0.05$ versus control; #$p<0.05$.

thought that the differences may affect the antiangiogenic activity, because it has been reported that biodistribution and pharmacokinetics of PEG-liposomes is different between when the liposomes are administered intravenously and intraperitoneally (Sadzuka et al., 1997). Since we previously showed that APRPG-modified liposomes highly accumulated in tumor tissues and bind to angiogenic endothelial cells in vivo (Maeda et al., 2006; Oku et al., 2002), these results can be explained that APRPG-modified liposomes effectively delivered SU1498 to angiogenic endothelial cells and suppressed the tumor angiogenesis. Our data for the first time indicate the usefulness of APRPG-modified liposomes for targeted delivery of angiogenesis inhibitors. Besides APRPG-modified liposomes, tumor vasculature-targeted liposomes have been shown to be effective carrier of cytotoxic anticancer drugs (Kondo et al., 2004; Pastorino et al., 2003). Such liposomes could be applied to drug delivery of various types of antiangiogenic agents.

PEG-Lip-SU1498 did not show significant antiangiogenic effect in the tumor-bearing mice. Since PEG-modified liposomes are known to be stable in blood circulation, it appears to deliver SU1498 to tumor tissues through endothelial cell layer by EPR effect (Maeda et al., 2000). Therefore, not just passive targeting, actively targeting to angiogenic endothelial cells may be an important factor in drug delivery of angiogenesis inhibitors.

In conclusion, we showed that APRPG-PEG-Lip-SU1498 suppressed tumor angiogenesis and prolonged the survival times of tumor-bearing mice, indicating that APRPG-modified liposomes effectively deliver SU1498 to angiogenic endothelial cells. The present study suggest that angiogenic vessel-targeted liposomes are useful carriers of angiogenesis inhibitors for antiangiogenic cancer therapy.

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Antiangiogenic cancer therapy using tumor vasculature-targeted liposomes encapsulating 3-(3,5-dimethyl-1H-pyrrol-2-ylmethylene)-1,3-dihydro-indol-2-one, SU5416

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Abstract

Previously, we identified angiogenic vessel-homing peptide Ala-Pro-Arg-Pro-Gly (APRPG), and showed that APRPG-modified liposomes could selectively target to tumor neovasculature. Here, we designed an APRPG-modified liposome encapsulating SU5416, an angiogenesis inhibitor, to overcome the solubility problem, and to enhance the antiangiogenic activity of SU5416. Liposomal SU5416 appeared to have the appropriate characteristics, such as particle size and stability in serum. It showed a significantly lower hemoglobin release than SU5416 dissolved in a Cremophor EL-containing solvent. Compared with peptide-unmodified liposomal SU5416, the APRPG-modified liposomal SU5416 significantly suppressed tumor growth and with no remarkable side effects. Thus, targeted delivery of antiangiogenic drugs with tumor vasculature-targeted liposomes may be useful for antiangiogenic cancer therapy.

Keywords: Angiogenesis; Drug delivery systems; SU5416; Antiangiogenic therapy; APRPG-modified liposomes

1. Introduction

Angiogenesis is the development of new blood vessels from pre-existing vessels, and is an attractive target for cancer therapy because it is essential for tumor growth and hematogenous metastasis [1]. Vascular targeting therapy is divided into two main types: (i) antiangiogenic approach, which prevents the processes of angiogenesis in tumors, through inhibitors of angiogenic signaling; and (ii) antivascular approach, which impairs the established neovasculature using a vascular disrupt agent [2].

Vascular endothelial growth factor (VEGF) and its receptors are the best-characterized signal pathway in angiogenesis and are regarded as a target molecule for the antiangiogenic approach [3]. In

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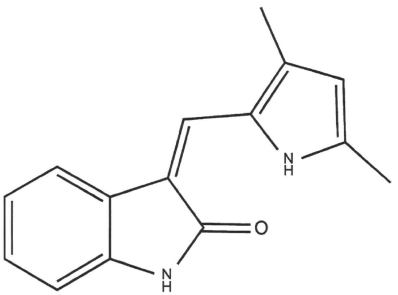
fact, several drugs that inhibit VEGF signal transduction have been developed. For example, bevacizumab, a humanized anti-VEGF-A monoclonal antibody, and SU11248, a small molecule inhibitor against receptor tyrosine kinases (RTKs) of VEGF receptor (VEGFR) and platelet-derived growth factor receptor (PDGFR), have both been approved for cancer treatment [4].

Z-3-[(2,4-dimethylpyrrol-5-yl)methylidenyl]-2-indolinone (SU5416) is a potent inhibitor of VEGFR-2 tyrosine kinase [5]. The structure of SU5416 is shown in Fig. 1. This inhibitor has been shown to suppress VEGF-mediated angiogenesis in vitro and in vivo through the inhibition of autophosphorylation of VEGFR-2 by blocking the AMP-binding site within the kinase domain of the receptor [6]. It has been reported that SU5416 has no direct cytotoxic properties to cancer cells but inhibits tumor growth in numerous tumor xenograft models [7]. In Phase I and II trials, the therapeutic efficacy of SU5416 has been shown in combination with certain anticancer drugs. In a Phase III clinical trial, however, SU5416 showed no significant clinical benefit, and some patients showed striking responses induced by the toxicity of the solvent with Cremophor EL (CrEL) that was used to dissolve SU5416 for clinical administration [7–9]. Since CrEL has been known to induce various undesirable effects such as anaphylactic shock or hemolysis [10,11], coadministration with dexamethasone or other steroids is required to prevent hypersensitivity reactions [12]. Therefore, much effort has been devoted to improving the aqueous solubility of some agents to forgo using CrEL. For further enhancement of antiangiogenic effects and reduction of the side effects of SU5416, drug delivery systems (DDS) can be an important factor. However, studying antiangiogenic drugs in the field of DDS is not sufficient.



Molecular Formula: $C_{15}H_{14}N_2O$

Molecular Weight: 238.3

Fig. 1. Structure of 3-(3,5-dimethyl-1H-pyrrol-2-ylmethylene)-1, 3-dihydro-indol-2-one, SU5416.

Liposomes are small lipid vesicles and one of the most advanced drug nanocarriers in DDS studies [13]. As drug carriers, liposomes have various favorable characteristics for cancer therapy, such as low toxicity, long-term blood circulation, and accumulation in inflamed tissues and tumors by enhanced permeability and retention (EPR) effect [14,15]. Liposomal formulation of hydrophobic drugs has been shown to overcome the solubility problem and the solvent-induced side effect [16]. In addition, liposomes can be modified with various molecules, such as antibodies, carbohydrates, or peptides, to selectively target several kinds of cells [17]. In our previous studies, we identified angiogenic vessel-homing peptide Ala-Pro-Arg-Pro-Gly (APRPG), and utilized it in liposomal drug delivery. APRPG peptide-modified liposomes directly targeted angiogenic endothelial cells, and doxorubicin-incorporated APRPG-modified liposomes significantly suppressed tumor growth through the disruption of tumor neovasculature [18–20]. These studies raise the possibility that APRPG-modified liposomes are also useful drug carriers for targeted delivery of antiangiogenic drugs.

In this study, to overcome the solubility problem and to enhance the antiangiogenic effect of SU5416, we designed the SU5416-incorporated APRPG-modified liposome. We evaluated the characteristics of liposomal SU5416 as a liposomal drug, such as its encapsulation efficiency, stability in serum, VEGF inhibitory activity, and hemolytic activity in vitro. Subsequently, the therapeutic effect of APRPG-modified liposomal SU5416 in tumor-bearing mice was examined.

2. Materials and methods

2.1. Cell culture and materials

Colon26 NL-17 carcinoma cells were cultured in DMEM/Ham's F12 medium (WAKO, Osaka, Japan) supplemented with streptomycin (100 μg/ml), penicillin (100 U/ml), and 10% heat-inactivated fetal bovine serum (FBS, Japan Bio Serum Co., Ltd., Tokyo, Japan) at 37 °C in a 5% CO_2 atmosphere. Human umbilical vein

endothelial cells (HUVECs, Takara Bio Inc., Otsu, Shiga, Japan) were maintained in endothelial growth medium-2 (EGM-2, Cambrex Corporation, Walkersville, MD, USA) at 37 °C under 5% CO_2 in a humidified chamber. HUVECs used in this study were between passages 4 and 7. The lipids for preparing liposomes were the products of Nippon Fine Chemical, Co., Ltd., (Takasago, Hyogo, Japan).

2.2. Preparation of liposomal SU5416

Liposomes were prepared as described previously [19]. In brief, dipalmitoylphosphatidylcholine (DPPC), palmitoyl-oleoylphosphatidylcholine (POPC), cholesterol, and SU5416 solutions in chloroform were mixed (10:10:5:1 as a molar ratio) and dried under reduced pressure to make a thin lipid film. A distearoylphosphatidylethanolamine polyethyleneglycol (DSPE-PEG) or APRPG peptide-conjugated DSPE-PEG (DSPE-PEG-APRPG) solution was respectively, added to the initial lipid solutions in the proportion of 10-mol % to PC for the modification of the liposomes with PEG or PEG-APRPG. The thin lipid films were hydrated with 20 mM HEPES-buffered saline (pH 7.4), and the liposome solutions were frozen and thawed for three cycles with liquid nitrogen. The liposome size was then adjusted by extrusion through 100 nm-pore sized polycarbonate filters. The particle size and ζ-potential of liposomal SU5416 was measured using ZETASIZER (Malvern Instruments, Worcs, UK).

2.3. Determination of entrapment efficiency of SU5416 into liposomes

Liposomal SU5416 were prepared as described above. The prepared liposomes were fractionated by gel filtration chromatography using PD-10 column (GE Healthcare, UK. Ltd., Buckinghamshire, UK) according to the manufacturer's instruction. The turbidity of each fraction was determined by measuring the absorbance at 750 nm to define the liposome fractions. The amount of SU5416 in each fraction was quantified by absorption at 440 nm using high performance liquid chromatography (HPLC, HITACHI, Tokyo, Japan) equipped with ODS-80Ts column (Tosoh Corporation, Tokyo, Japan). The mobile phase for the HPLC analysis was composed of methanol and 35 mM KH_2PO_4 (3:1).

2.4. Stability of liposomal SU5416 in presence of serum

The prepared liposome solutions were incubated in the presence or absence of 50% FBS for 1 h at 37 °C. After that, the liposomes were separated by gel filtration chromatography using Sepharose™ 4 Fast Flow (Amersham Biosciences, Uppsala, Sweden) as described previously [21], and the amount of SU5416 in the liposome fractions was determined using HPLC as described above.

2.5. Cell proliferation assay

HUVECs were seeded (7500 cells/well) on a gelatin-coated 96-well plate and incubated overnight. After the change of medium to 0.5% FBS-containing endothelial basal medium-2 (EBM-2, Cambrex Corporation), the cells were treated with SU5416 (dissolved in DMSO), PEG-liposomal SU5416 (PEG-Lip-SU5416), or APRPG-PEG-liposomal SU5416 (APRPG-Lip-SU5416) and incubated for 3 h at 37 °C. Then, recombinant human $VEGF_{165}$ (20 ng/ml as final concentration, BD biosciences, San Diego, CA, USA) was added to the each well, and the cells were further incubated for 48 h. Colon26 NL-17 cells were seeded (3000 cells/well) on a 96-well plate in DMEM/Ham's F12 supplemented with 10% FBS and incubated overnight. Then, the cells were treated with the samples and further incubated for 48 h at 37 °C. The cell viability was measured with TetraColorOne™ (Seikagaku, Tokyo, Japan) according to the manufacturer's instruction.

2.6. Hemolytic assay

Free SU5416 was dissolved in the following components: polyethylene glycol 400; CrEL (Nakalai Tesque, Kyoto, Japan); benzyl alcohol; and dehydrated ethanol (45:31.5:2:21.5 w/w %) as described previously [7], and the SU5416 solution was diluted with 0.45% sodium chloride before treatment. Hemolytic assay was performed as described previously [22] with some modification. In brief, blood was obtained from 6-week-old BALB/c male mice (Japan SLC, Shizuoka, Japan). Red blood cells were collected by centrifugation (2000*g*, 5 min, 4 °C, five times) of the blood. The pellet was resuspended in 20 mM HEPES-buffered saline (pH 7.4) to give a 5% (v/v) solution. The suspension was added to HEPES-buffered saline, free SU5416, PEG-Lip-SU5416, or APRPG-Lip-SU5416 and incubated for 30, or 60 min at 37 °C. After centrifugation, the supernatants were transferred to a 96-well plate. Hemolytic activity was determined by measuring the absorption at 570 nm. Control samples of 0% lysis (in HEPES buffer) and 100% lysis (in 1% Triton X-100) were employed in the experiment.

2.7. Therapeutic experiment

Colon26 NL-17 carcinoma cells were subcutaneously implanted (1.0×10^6 cells) into the posterior flank of 4-week-old BALB/c male mice. HEPES-buffered saline (Control), free SU5416, PEG-Lip-SU5416, or APRPG-Lip-SU5416 was intravenously injected every other day (3 mg/kg/day as SU5416) from day 5 to day 13 after tumor implantation. The tumor size and body weight were monitored daily as described previously [19]. The animals were cared for according to the Guidelines for the Care and Use of Laboratory Animals of the University of Shizuoka.

2.8. Statistical analysis

Statistical analysis of the experiments was performed by unpaired Student's *t*-test using KaleidaGraph software (HULINKS, Tokyo, Japan).

3. Results

3.1. Characterization of liposomal SU5416

To investigate whether liposomal SU5416 has appropriate characteristics as a liposome agent, we examined its entrapment efficiency into the liposomes, particle size, ζ-potential, and its stability in the presence of serum. In gel filtration chromatography analysis, liposome fractions were defined by turbidity (absorption at 750 nm). More than 85% of SU5416 was detected in the liposome fractions of Control (non-modification), PEG- and APRPG-modified liposomes (Fig. 2). SU5416-encapsulated liposomes had approximately 130 nm of particle size and −3.0 mV of ζ-potential, respectively (Table 1). The notable change of particle size and the leakage of SU5416 from the liposomes were not observed until 14 days after preparation of the liposomes (Fig. 3). We also examined the stability of liposomal SU5416 in the presence of serum. PEG-Lip-SU5416 and APRPG-Lip-SU5416 were incubated with or without serum, and liposomal SU5416 was fractionated by gel filtration chromatography. After the incubation with serum, more than 85% of SU5416 in comparison with PBS alone were detected in the liposome fractions, fraction 5–10 (Fig. 4). These analyses revealed that SU5416 was effectively and stably encapsulated in the liposomes, and PEG-Lip- and APRPG-Lip-SU5416 stably existed in the presence of serum.

3.2. Growth inhibitory activity of liposomal SU5416

SU5416 has been shown to suppress endothelial cell proliferation through the inhibition of VEGF signal transduction [5]. To confirm that liposomal SU5416 has similar growth inhibitory activity against VEGF-stimulated endothelial cells, we performed a cell proliferation assay. PEG- and APRPG-Lip-SU5416 significantly inhibited endothelial cell proliferation induced by treatment with VEGF in a concentration dependent manner as well as free SU5416 (Fig. 5A). On the contrary, free SU5416 and liposomal SU5416 did not suppress the proliferation of Colon26 NL-17 carcinoma cells (Fig. 5B). These data suggest that encapsulated SU5416 maintains an inhibitory activity against VEGF signal transduction.

3.3. Suppression of hemolysis by liposomalization of SU5416

Since SU5416 is a hydrophobic compound, it is dissolved in the solvent containing CrEL for use in the

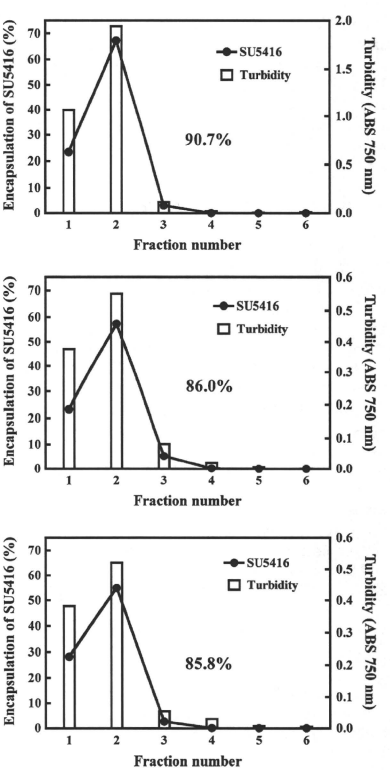


Fig. 2. Entrapment of SU5416 into Control, PEG- or APRPG-modified liposomes. Control liposomal SU5416 (a), PEG-modified liposomal SU5416 (b), and PEG-APRPG-modified liposomal SU5416 (c) were fractionated by gel filtration chromatography with PD-10 column. The turbidity (bar, left *Y* axis) was determined by measurement of the absorption at 750 nm, and the amount of SU5416 (dot, right *Y* axis) was measured using HPLC (absorption at 440 nm). The calculated entrapment efficiency is indicated in each graph.

Table 1
Particle size and ζ-potential of liposomal SU5416

	Particle size (nm)	ZP (mV)
PEG-Lip-SU5416	131.8 ± 14	−3.0 ± 2.2
APRPG-Lip-SU5416	142.6 ± 28	−3.0 ± 1.0

The data indicate the means ± SD.
ZP, ζ-potential.

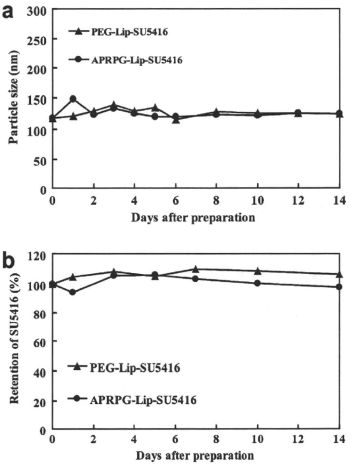


Fig. 3. Stability of liposomal SU5416 in particle size and entrapment efficiency. PEG- and APRPG-modified liposomal SU5416 were incubated until day 14 at 4 °C. The particle size of PEG-Lip-SU5416 (closed triangle) and APRPG-Lip-SU5416 (closed circle) was measured at indicated times (a). The amount of SU5416 into the liposomes was determined after gel filtration chromatography, and the relative entrapment efficiency was calculated as compared to that of the day 0 (b).

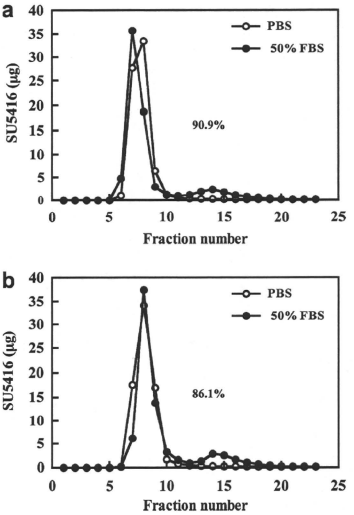


Fig. 4. Retention of SU5416 into liposomes in the presence of serum. PEG- (a) and APRPG-modified liposomal SU5416 (b) were incubated with (open circle) or without 50% fetal bovine serum (closed circle) for 1 h at 37 °C. Liposomal SU5416 was fractionated by gel filtration chromatography. The amount of SU5416 was measured using HPLC. The retention efficiency of SU5416 is indicated in each graph.

clinical studies. CrEL has been shown to induce some undesirable effects such as hemolysis [16]. To determine whether liposomalization of SU5416 precludes these side effects, we examined its hemolytic activity. Free SU5416 dissolved in the solvent induced remarkable hemolysis. In contrast, PEG- and APRPG-Lip-SU5416 showed a significantly low hemolytic activity (Fig. 6).

3.4. Tumor growth suppression by treatment with APRPG-modified liposomal SU5416 in tumor-bearing mice

Finally, the effect of APRPG-Lip-SU5416 in Colon26 NL-17 carcinoma cell-bearing mice was examined. APRPG-Lip-SU5416 significantly suppressed tumor growth compared with control ($p < 0.05$), free SU5416 ($p < 0.05$), and PEG-Lip-SU5416-treatment ($p < 0.01$, Fig. 7a). However, free SU5416 and PEG-Lip-SU5416 showed no tumor growth suppression under the present experimental conditions. SU5416- and liposomal SU5416-treatment did not affect the body weight changes of the mice, an indicator of a side effect (Fig. 7b). Although most of the mice showed shock-like behavior by injection intravenously with SU5416 dissolved in the CrEL-containing solvent, the behavior was not induced by liposomal SU5416 (data not shown).

4. Discussion

In this study, we attempted to develop neovasculature-targeted liposomal SU5416 to overcome the problem of solubility and to enhance the antiangiogenic activity of SU5416 through an active targeting strategy. Liposomal SU5416 has an appropriate particle size and an almost neutral electronic charge. These characteristics have been known to affect liposome distribution. In fact, it has been reported that liposomes having a particle size of approximately 100 nm and a neutral charge accumulate in inflammation region such as tumors through enhanced permeability and retention (EPR) effect [15]. It is also known that hydrophobic agents incorporated into the liposomal membrane transfer to plasma lipoproteins in the bloodstream. Therefore, we examined the stability of liposomal SU5416 in

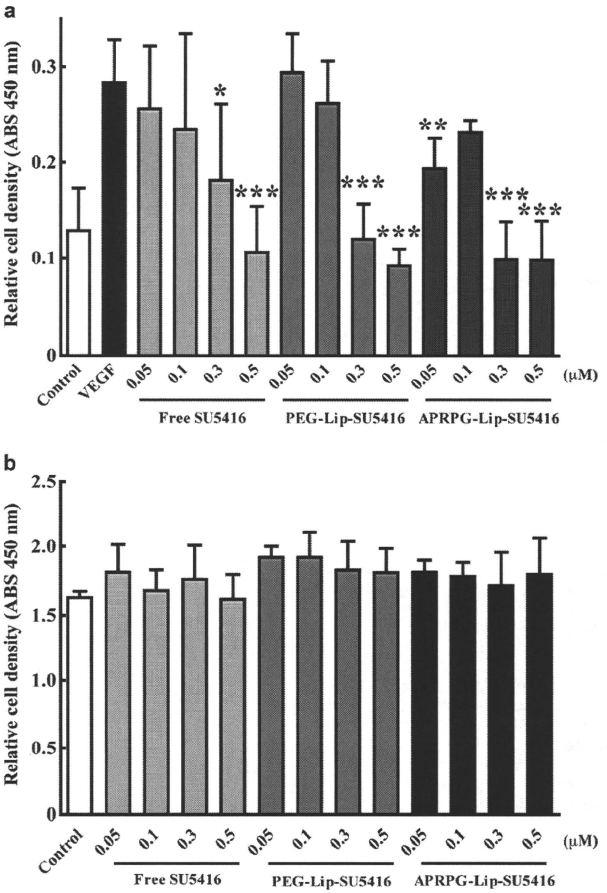


Fig. 5. Inhibited effect of liposomal SU5416 on VEGF-induced endothelial cell growth. (a) HUVECs (7500 cells/well) were seeded on a 96-well plate. The culture medium was changed to EBM-2 containing 0.5% FBS, and the cells were treated with free SU5416, PEG-Lip-SU5416, or APRPG-Lip-SU5416 at indicated concentration and incubated for 3 h at 37 °C. Then, the cells were added to $rhVEGF_{165}$ (20 ng/mL as final concentration) and further incubated for 48 h. (b) Colon26 NL-17 cells (3000 cells/well) were also seeded on a 96-well plate and incubated overnight. The cells were treated with these samples and further incubated for 48 h. Finally, cell viability was determined with TetraColor ONE™. The bars indicate the means ± SD. ($n = 4$), and the significant differences are indicated as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus VEGF-treated group.

the presence of serum, and observed it to be quite stable there. In addition, liposomalization of SU5416 maintained the antiangiogenic activity of SU5416. These findings suggest that SU5416-incorporated liposomes can adequately function as a liposomal drug.

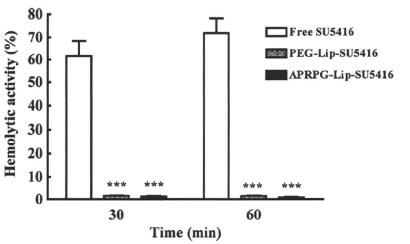


Fig. 6. Reduction of solvent-induced hemolysis by liposomalization of SU5416. Red blood cells were collected by centrifugation of the blood and resuspended in HEPES-buffered saline. The cell suspension was added to HEPES-buffered saline, free SU5416, PEG-Lip-SU5416, or APRPG-Lip-SU5416 and incubated for 30 or 60 min at 37 °C. After centrifugation, hemolytic activity was determined by measuring the absorbance (570 nm) of the supernatant. Control samples of 0% lysis (in HEPES buffer) and 100% lysis (in 1% Triton X-100) were employed in the experiment. The bars indicate the means ± SD. ($n = 4$). Significant difference is shown as follow: $^{***}p < 0.001$ versus free SU5416.

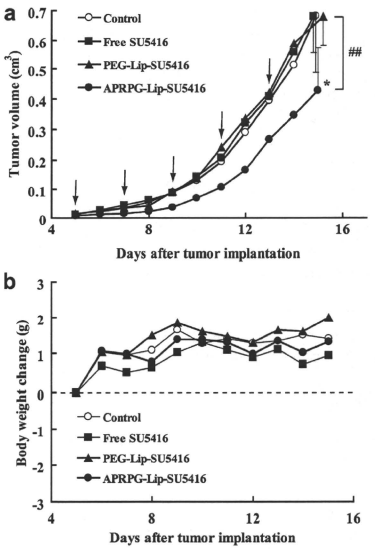


Fig. 7. Suppression of tumor growth by treatment with APRPG-modified liposomal SU5416 in tumor-bearing mice. Colon26 NL-17 carcinoma cells were implanted s.c. into the left posterior flank of 4-week-old BALB/c male mice ($n = 5$–6 per group). The mice were injected i.v. with HEPES buffer (Control, open circle), free SU5416 (3 mg/kg, closed square), PEG- (closed triangle) or APRPG-modified liposomal SU5416 (as SU5416 dosage, 3 mg/kg, closed circle) on days 5, 7, 9, 11, and 13 after tumor implantation. Tumor volume (a) and body weight change (b) were determined as described in the Section 2. Arrows show the days of injection. The data indicate the means ± SD, and the significant differences are indicated as follows: $^{*}p < 0.05$ versus control and free SU5416; $^{\#\#}p < 0.01$ versus PEG-Lip-SU5416.

We found that the liposomal SU5416 did not induce hemolysis in vitro and shock-like behaviors when it was intravenously injected. SU5416 is dissolved in the solvent containing CrEL that has been shown to induce various side effects [11]. Liposomes have also been used to formulate a variety of poorly water soluble drugs [23,24]. For example, by formulation into liposomes, paclitaxel, an anticancer drug used by dissolving in a mixture of 50% ethanol and 50% CrEL, has improved solubility, pharmacokinetics, and antitumor activity yet avoided any solvent-induced side effects [25,26]. Our findings suggest that liposomalization of SU5416 can overcome the solubility problem and decrease the risk of side effects caused by a solvent.

In an in vivo experiment, although APRPG-Lip-SU5416 did not exhibit any dramatic antitumor effect, it showed a statistically significant antitumor activity and without any prominent side effect. These results suggest that APRPG-modified liposomes may enhance antiangiogenic activity through targeted delivery of SU5416 to angiogenic endothelial cells in vivo. The previous study has shown that free SU5416 can suppress tumor growth by frequent injection at a high dose (10–25 mg/kg) [6], and therefore it is not thought to suppress tumor growth under the present treatment conditions (3 mg/kg/day, 5×). In addition, PEG-Lip-SU5416 also did not show the antitumor activity. One of the possible difference between APRPG-Lip-SU5416 and PEG-Lip-SU5416 is whether or not the liposomes directly target tumor endothelial cells [18,19]. PEG or other polymer modification is useful for a drug delivery system by the prolongation of drug circulation in the blood [27,28]. Since PEG liposomes accumulate in tumor tissues through the endothelial cell layer by the EPR effect, PEG-Lip-SU5416 seems to be weakly associated with angiogenic endothelial cells in the tumors. Our data suggest that active targeting to angiogenic endothelial cells may be an useful strategy to enhance the therapeutic effect of angiogenesis inhibitors. To improve the effect, it may be necessary to optimize liposome formulation (ligand

density, lipid composition, etc.) or to modify other ligands (antibodies, peptides, etc.).

In conclusion, we have shown that (i) SU5416 can be formulated in liposomes; (ii) Liposomal SU5416 can be administered without remarkable side effects; and (iii) APRPG-Lip-SU5416 exhibits higher antitumor activity than PEG-Lip-SU5416. Thus, tumor vasculature-targeted liposomes may be useful for drug delivery of antiangiogenic drugs, and the development of such DDS may advance antiangiogenic cancer therapy.

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