Title	Size-controlled, dual-ligand modified liposomes that target the tumor vasculature show promise for use in drug-resistant cancer therapy
Author(s)	Takara, Kazuhiro; Hatakeyama, Hiroto; Kibria, Golam; Ohga, Noritaka; Hida, Kyoko; Harashima, Hideyoshi
Citation	Journal of Controlled Release, 162(1), 225-232 https://doi.org/10.1016/j.jconrel.2012.06.019
Issue Date	2012-08-20
Doc URL	http://hdl.handle.net/2115/50278
Type	article (author version)
Additional Information	There are other files related to this item in HUSCAP. Check the above URL.
File Information	JCR162-1_225-232.pdf

[Instructions for use](#)

Size-controlled, dual-ligand modified liposomes that target the tumor vasculature show promise for use in drug-resistant cancer therapy

Kazuhiro Takara^{1#}, Hiroto Hatakeyama^{1#}, Golam Kibria¹, Noritaka Ohga², Kyoko Hida², Hideyoshi Harashima^{1*}

¹Laboratory of Innovative Nanomedicine, Faculty of Pharmaceutical Sciences, and ²Division of Vascular Biology, Graduate School of Dental Medicine, Hokkaido University, Hokkaido, Japan

[#]K. Takara and H. Hatakeyama contributed equally to this work.

*Corresponding Author: Hideyoshi Harashima, Hokkaido University, Kita12 Nishi 6, Kita-ku, Sapporo City, Hokkaido, Japan 060-0812. Phone: +81-11-706-3919; Fax: +81-11-706-4879; E-mail: harasima@pharm.hokudai.ac.jp

Keywords: anti-angiogenesis, drug delivery, tumor endothelial cells, drug resistant tumor, dual ligand

Abstract

Anti-angiogenic therapy is a potential chemotherapeutic strategy for the treatment of drug resistant cancers. However, a method for delivering such drugs to tumor endothelial cells remains to be a major impediment to the success of anti-angiogenesis therapy. We designed liposomes (LPs) with controlled diameter of around 300 nm, and modified them with a specific ligand and a cell penetrating peptide (CPP) (a dual-ligand LP) for targeting CD13-expressing neovasculature in a renal cell carcinoma (RCC). We modified the LPs with an NGR motif peptide on the top of poly(ethylene glycol) and tetra-arginine (R4) on the surface of the liposome membrane as a specific and CPP ligand, respectively. The large size prevented extravasation of the dual-ligand LP, which allowed it to associate with target vasculature. While a single modification with either the specific or CPP ligand showed no increase in targetability, the dual-ligand enhanced the amount of delivered liposomes after systemic administration to OS-RC-2 xenograft mice. The anti-tumor activity of a dual-ligand LP encapsulating doxorubicin was evaluated and the results compared with Doxil[®], which is clinically used to target tumor cells. Even though Doxil showed no anti-tumor activity, the dual-ligand LP suppressed tumor growth because the disruption of tumor vessels was efficiently induced. The comparison showed that tumor endothelial cells (TECs) were more sensitive to doxorubicin by 2 orders than RCC tumor cells, and the disruption of tumor vessels was efficiently induced. Collectively, the dual-ligand LP is promising carrier for the treatment of drug resistant RCC via the disruption of TECs.

1. Introduction

Renal cancer patients, in which most frequent histology is renal cell carcinoma (RCC), typically respond poorly to chemotherapy [1,2]. This poor or complete lack of response to

chemotherapy in RCC can be mainly attributed to acquired drug resistance, including up-regulated P-glycoprotein (P-gp) which functions as an efflux pump for chemotherapeutic drugs [2]. Although interleukin (IL)-2 or interferon (IFN)- α based immunotherapy is approved for use, RCC is also resistant to this type of chemotherapy [3]. As a result, the resistance of cancer cells to chemotherapeutic treatment remains a major obstacle to the successful treatment of kidney cancer. Recently, new classes of drugs, sunitinib, sorafenib or bevacizumab, which target specific molecules that are related to the angiogenesis process, such as vascular endothelial growth factor (VEGF) and VEGF receptors (VEGFRs) have been approved for the treatment of RCC [3,4]. Although RCC patients suffer from side effects, the new class drugs appear to have improved clinical benefits [3,4]. This suggests that anti-angiogenic therapy has promise for the treatment of RCC. Further increases in therapeutic activity could be achieved by targeting the neovasculature with nanomedicines that contain ligands that are selective for a specific target.

Endothelial cells in angiogenic vessels express several proteins that are absent or barely detectable in established blood vessels, including α_v integrins, VEGFRs, and other types of membrane molecules, such as aminopeptidase N (CD13) [5]. It has been reported that ligand based liposomes that contain RGD or NGR motif peptides that are capable of targeting the neovasculatures can be used to deliver chemotherapeutic drugs [6–9]. The targeted liposomes showed efficient chemotherapeutic activity, particularly when the targeting was via internalizing ligands that facilitate the delivery of the therapeutic contents to an intracellular site of action via the endosome/lysosome pathway. However, because of the limited number of receptors and the recycling of endocytosis, receptor mediated endocytosis is a saturated pathway, which restricts the amount of liposomes that are available for cellular uptake and greatly decreases the magnitude of the pharmacological effect of such preparations.

To overcome this saturated pathway and to obtain further therapeutic efficacy, we developed a dual-ligand based poly(ethylene glycol) (PEG)-liposome (dual-ligand LP). The liposome was modified with a target ligand on the terminus of the PEG and a cell penetrating peptide (CPP) was attached to liposome surface [10,11]. Because it is masked by PEG, the CPP is not functional and opsonin-free in the systemic circulation. The recognition of target cells mediated by target ligands and subsequent cellular association permit the CPPs to allow the liposomes to be rapidly internalized by target cells, due to the close proximity of the liposomes to the surface of the target cells. As a result, the cellular uptake and the enhanced activity of the cargo of the dual-ligand LP is vastly superior compared to a liposome mono-modified with a specific ligand.

In the present study, we describe a novel therapy for RCC as a drug resistant tumor model that is achieved via targeting tumor blood vessels by a dual-ligand installed and

size-controlled liposomal system. The NGR motif peptide was employed as a specific ligand for targeting CD13, which is overexpressed in tumor blood vessels [6], and tetra-arginine (R4) was used as a CPP ligand. The advantage of targeting the neovasculature rather than RCC was verified by a direct comparison of the sensitivity to doxorubicin (DXR) in RCC and tumor endothelial cells (TECs) derived from RCC tissue. In an *in vivo* therapeutic study, to exclude the possibility of direct liposomal cytotoxicity to RCC, the size of liposomes was controlled, so that the liposomes were prevented from extravasation into tumor tissue via the enhanced permeability and retention (EPR) effect [12,13]. Finally, we compared the therapeutic efficacy of a dual-ligand LP and Doxil (Caelyx)[®], a clinically used liposomal system for delivering doxorubicin to tumor cells via the EPR effect [14].

2. Materials and Methods

2.1. Materials

Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (PEG-DSPE), cholesterol (Chol) and rhodamine-labeled DOPE (Rho-DOPE) were purchased from AVANTI Polar Lipids (Alabaster, AL, U.S.A.). PEG-DSPE with a functional maleimide moiety at the terminal end of PEG: *N*-[(3-maleimide-1-oxopropyl) aminopropyl polyethyleneglycol-carbamyl] distearoylphosphatidyl-ethanolamine (Mal-PEG-DSPE), egg phosphatidylcholine (EPC) and hydrogenated soybean phosphatidylcholine (HSPC) were purchased from Nippon Oil and Fat Co. (Tokyo, Japan). [³H]cholesteryl hexadecyl ether (CHE) was purchased from Perkin-Elmer Life Sciences Japan. Stearoylated tetraarginine (STR-R4) was purchased from PolyPeptide Laboratories (San Diego, CA, U.S.A.). NGR motif peptide, CYGGRGNG was obtained from Sigma Genosys Japan (Ishikari, Japan). The NGR motif peptide was conjugated with Mal-PEG-DSPE (NGR-PEG-DSPE) as described previously [10]. Alexa 647-conjugated griffonia simplicifolia isolectin B4 (GS-IB4-Alexa647) was purchased from Invitrogen. Hoechst33342 and Cell Counting Kit-8 was purchased from DOJINDO. RPMI 1640 were purchased from Sigma (St. Louis, MO, U.S.A.). Doxorubicin (DXR) was purchased from Wako (Osaka, Japan). All other chemicals used were commercially available reagent-grade products.

2.2. Preparation of LPs

A lipid film composed of EPC/Chol/Rho-DOPE (7:3:0.1 molar ratio) was prepared by evaporation, followed by hydration with PBS. The particle size was controlled by extrusion through polycarbonate membrane filter with a pore diameter of 0.4 μ m for large sized LPs, and subsequently through a 0.05 μ m pore diameter for small sized LPs. To modify the prepared liposomes with STR-R4, PEG-DSPE, or NGR-PEG-DSPE, they were incubated with the indicated amounts of STR-R4, PEG-DSPE, or NGR-PEG-DSPE for 60 min at 55°C, 950 rpm. The average diameter and the zeta-potential of the prepared liposomes were

determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS ZEN3600 (MALVERN Instruments, Worchestershire, U.K.).

2.3. Liposomal doxorubicin formulations

A lipid film composed of HSPC/Chol (7:3 molar ratio) was prepared by evaporation. The lipid film was hydrated with 155 mM ammonium sulfate (pH 5.5) at 65°C, and the particle size of liposomes was controlled by extrusion. The extruded liposomes were loaded on a Sepadex-G25 gel filtration column to exchange the outer buffer to PBS (pH 7.4). DXR was incubated with the extruded liposomes (1:10 wt/wt) at 60°C for 1 hr. After removing free DXR by gel filtration, the DXR loaded liposomes were modified with STR-R4, PEG-DSPE and NGR-PEG-DSPE, as described above. The loading efficiency of DXR in liposomes was determined by measuring the fluorescence of DXR (Ex=450 nm, Em=590 nm) of prepared liposomes following treatment with MeOH to disrupt the liposome structure. Doxil (doxorubicin encapsulated in small sized liposomes) were prepared as described previously [15].

2.4. Cell lines and culture

Renal cell carcinoma (RCC), OS-RC-2 cells (Riken Cell Bank, Tsukuba, Japan) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and penicillin (100 U/ml), streptomycin (100 µg/ml) at 37°C and 5% CO₂, respectively and used within 6 months of obtaining them from Riken Cell Bank.

2.5. Animal experiments and RCC xenograft model

Male 4-weeks-old BALB/cAJcl-*nu/nu* mice were purchased from CLEA Japan. OS-RC-2 cells (1×10^6 cells) in 70 µL PBS were s.c. inoculated into their back, and then grown until the tumor volume was 80-150 mm³. Tumor volume was calculated using the formula: $1/2 \times a \times b^2$, where *a* and *b* represent the largest and smallest tumor diameters, respectively. All experiments were approved by the Hokkaido University Animal Care Committee.

2.6. Fluorescence confocal microscopy and determination of vessel area

Liposomes labeled with rhodamine with the indicated lipid doses were intravenously injected into tumor-bearing mice. Tumor tissues were collected after 24 hr, and the endothelial cells and nucleus were then stained with GS-IB4-Alexa647 (20 µg/ml) and Hoechst 33342 (40 µM) in PBS for 1 hr. Tumor tissue images were collected by a confocal laser scanning microscope (Nikon A1) equipped with a $\times 20$ dry objective lens. The total pixels of blood vessels (green) in the tumor, liver and spleen were calculated using the ImagePro-plus software (Media Cybernetics Inc., Bethesda, MD, USA).

2.7. Biodistribution of liposomes

To evaluate the biodistribution of liposomes, the lipid membrane was labeled with [³H]CHE, as a lipid phase maker. Liposomes were administered to tumor-bearing mice via the tail vein at a dose of 0.5 µmol lipid. At 24 hr post-injection, the radioactivity in the blood

and tissues was measured, as described previously [16]. The blood concentration and tissue accumulation of liposomes are represented as the % of the injected dose (ID) per ml of blood and %ID per g tissue, respectively.

2.8. *In vivo therapeutic efficacy*

Liposomes encapsulating DXR were intravenously administered into tumor-bearing mice with indicated doses of mg DXR/kg body weight at indicated time points. Tumor volume and body weight were monitored at 3 days intervals after the doses.

2.9. *Isolation of mouse tumor endothelial cells (TECs)*

TECs were isolated, as described previously [17-22]. In a typical procedure, the TECs were isolated from OS-RC-2 tumors and dermal tissue in tumor-bearing mice using a magnetic cell sorting system (MACS; Milteny Biotec, Tokyo, Japan). The TECs were plated onto 1.5% gelatin-coated culture plates and grown in EGM-2MV (Clonetics, San Diego, CA) and 15% FBS. Diphtheria toxin (500 ng/ml; Calbiochem, San Diego, CA) was added to the TEC subcultures to kill any remaining human tumor cells.

2.10. *Cytotoxicity assay of RCC and TEC with free DXR*

OS-RC-2 and OS-RC-2 EC were incubated in 96-well plates (5×10^3 cells/well) with free DXR at the indicated doses for 8 hr. After removing DXR contained medium, cells were further cultured with fresh medium for 16 hr. The cells were then incubated with fresh medium containing 10% (v/v) Cell Counting Kit-8 reagent for an additional 2 hr. The absorbance (A) of each well was measured by a microplate reader (Thermo Scientific Varioskan Flash) at 450 nm. The percentage cytotoxicity = $[1 - (A \text{ of experimental wells} / A \text{ of control wells})] \times 100$.

2.11. *Statistical analysis*

Comparisons between multiple treatments were made using one-way analysis of variance (ANOVA), followed by the SNK test. Pair-wise comparisons between treatments were made using a student's t-test. A P-value of < 0.05 was considered significant.

3. Results

3.1. *Effect of PEG-liposome size on the distribution in tumor*

Generally, long-circulating liposomes with diameters of around 100 nm passively accumulate in tumor via the EPR effect [12,13]. This led us to assume that large sized liposomes might be suitable for vascular targeting. We first evaluated the effect of liposome size on distribution in the case of OS-RC-2 tumor tissue. PEG-LPs with an average diameter of either 100 nm or 300 nm were prepared as small PEG-LP or large PEG-LP, respectively (Fig. 1A and Table 1). After i.v. injection to tumor-bearing mice, the small PEG-LPs were mainly found to be exterior from the blood vessels (Fig. 1B). In contrast, large PEG-LPs were mainly found in close proximity to the blood vessels (Fig. 1C). These results suggest that the distribution of PEG-LPs in OS-RC-2 could be altered by controlling size of

PEG-LPs, and large LPs were used in further study for developing a system that targets blood vessels.

Fig. 1. Effect of liposome size on distribution in RCC tumor. (A) The size distribution of small (solid line) and large PEG-LP (dotted line) were determined by DLS measurements. (B) and (C) Upper images of unfixed tumor tissues that had been intravenously treated with either small PEG-LP or large PEG-LP labeled with rhodamine (red) (2.0 μmol lipid/mouse), respectively. Endothelial cells were labeled with lectin (green). The lower spectra were obtained from the arrow line in the upper images. Bars, 80 μm .

Table 1 Physical properties of the prepared liposomes

	Large size				Small size	
	PEG-LP	NGR-PEG-LP	R4/PEG-LP	R4/NGR-PEG-LP (Dual-ligand LP)	PEG-LP	Doxil
Diameter (nm)	298 $\pm$ 29	311 $\pm$ 18	296 $\pm$ 26	304 $\pm$ 17	100 $\pm$ 17	85 $\pm$ 3
PDI	0.209 $\pm$ 0.012	0.214 $\pm$ 0.028	0.225 $\pm$ 0.008	0.215 $\pm$ 0.025	0.089 $\pm$ 0.023	0.121 $\pm$ 0.021
Zeta-potential (mV)	-13 $\pm$ 3	-16 $\pm$ 2	-11 $\pm$ 4	-11 $\pm$ 3	-13 $\pm$ 3	-11 $\pm$ 2

Data are the means $\pm$ SD of at least three different preparations.

In case of large size, molar ratio of EPC/Cholesterol for biodistribution study or HSPC/Cholesterol for anti-cancer study was fixed at 7:3. Large size LPs were modified with 10 mol% PEG-DSPE or NGR-PEG-DSPE, and 2.5 mol% STR-R4. In case of small size, PEG-LP for biodistribution study was composed of EPC/Chol (7:3) with 5% PEG-DSPE, and Doxil for the anti-cancer study was composed of HSPC/Chol (3:2) with 5 mol% PEG-DSPE.

3.2. Accumulation and distribution of a dual-ligand LP in tumor

A dual-ligand formulation was developed using large size LPs. LPs were modified with either 10 mol% of PEG-DSPE (PEG-LP), 10 mol% NGR-PEG-DSPE (NGR-PEG-LP), or both of 10 mol% PEG-DSPE and 2.5 mol% STR-R4 (R4/PEG-LP; a dual-ligand LP),

respectively. A schematic illustration of these formulations is represented in Fig. 2A and the diameters of prepared formulations were comparable, as shown in Table 1. Although the NGR motif peptide contains one arginine residue, NGR-PEG-DSPE modification had no effect on the zeta-potential of the LPs, presumably because the presence of a mono arginine residue is not sufficient to alter the zeta-potential of the liposome, which is consistent with the previously reported results [10]. Since the PEG layer masked R4, the modification with R4 had no effect on zeta-potential. A biodistribution analysis in tumor bearing-mice was performed using LPs labeled with [³H]CHE. Even though neither NGR nor R4 modification showed an enhanced accumulation in tumor tissue, an increased amount of dual-ligand LP was observed in tumors compared to PEG-LP (Fig. 2B). In the case of blood and other organs, no significant difference was observed among the formulations, as shown in Supplementary Fig S1. We further investigated the distribution of a dual-ligand LP in RCC tumor by confocal microscopy. Tumor tissues were observed at 24 hr after the i.v. administration of rhodamine-labeled LPs. As shown in Fig. 2C, a few signals were detected in tumors that had been treated with PEG-LP. In the case of NGR-PEG-LP, the number of signals approached that of PEG-LP, which suggests that modification with specific NGR ligand had a minor effect on the targeting blood vessels in OS-RC-2 tumors. R4/PEG-LP showed no enhanced accumulation or distribution compared to PEG-LP, due to the fact that R4 was rendered non functional by masking by the PEG layer. However, dual modification with R4 and NGR resulted in a substantial increase in the LP signals in tumors, which suggests the dual modification synergistically functioned to target the tumor blood vessels. These results are consistent with the biodistribution study (Fig. 2B).

Fig. 2. Accumulation and localization of a dual-ligand LP in tumor. (A) Schematic illustration of prepared formulations. LPs of 300 nm in diameter were modified with either PEG, NGR modified PEG or R4. Dual-ligand LP was prepared by modified with both NGR modified PEG and R4. (B) Tumor accumulation at 24 hr after systemic administration of formulations labeled with [3 H] are represented by %ID/g tissue (the mean $\pm$ SD, n=4). **P<0.01. (C) Images of unfixed tumor tissues intravenously treated with each formulation labeled with rhodamine (0.5 μ mol lipid/mouse). Tumor endothelial cells were labeled with lectin (green). Arrow heads point red signals (liposomes) along with blood vessels.

3.3. Suppression of tumor growth by the dual-ligand LP

We next evaluated the anti-tumor effect of a dual-ligand LP. DXR was loaded to LPs by a pH gradient remote loading method. The encapsulation efficiency of DXR in all formulations exceeded 98%. As compared with PEG-LP, the single ligand modification showed no advantage for tumor suppression (Fig. 3). On the contrary, the dual ligand LP

significantly depressed tumor growth. This result is in good agreement with the distribution study (Fig. 2B and C). These findings indicated that the dual-ligand formulation can be used for targeting endothelial cells in OS-RC-2 tumors after systemic administration.

Fig. 3. Therapeutic effect of a dual-ligand LP in tumor. PBS or LPs containing 6 mg/kg of DXR were i.v.-injected on day 0 and 3. Tumor volume was monitored at indicated times. Dual-ligand LP showed better effect on the tumor growth than other formulations. ** $P < 0.01$, # $P < 0.01$ (PBS treated group versus LP-treated groups).

3.4. Comparison of dual-ligand with Doxil (Caelyx)[®]

We then compared the pharmacological efficacy of a dual-ligand LP with that of Doxil (Caelyx)[®] which has been approved for clinical use [14]. The diameters of the Doxil particles were controlled at around 100 nm (Table 1). A biodistribution analysis showed that uptake by liver and spleen was independent and dependent on the size of liposomes respectively, consistent with previous studies [23,24]. This accounts for the lower blood concentration for the dual-ligand LP compared to Doxil. As a result of its long blood circulation, Doxil accumulated at higher levels in tumors via the EPR effect than a dual-ligand LP did via the targeting of blood vessels (Fig. 4A). Since tumor suppression by a dual-ligand LP at a dose of 1.5 mg/kg DXR was comparable to DXR dose of 6.0 mg/kg, further studies were performed using liposomal DXR at a dose of 1.5 mg/kg considering side effects and clinical use (Fig. S2). Despite an enhanced accumulation in tumors, no anti-tumor effect was observed for the systemic treatment of Doxil (Fig. 4B). By contrast, tumor growth suppression was clearly observed for the case of treatment with a dual-ligand LP. As a result of the inhibition in tumor growth, the severe body weight loss shown for PBS and Doxil was improved (Fig. 4C).

The density of blood vessels in the tumor, liver and spleen was also observed as shown in Fig. 5A. Blood vessel density was clearly diminished as the result of treatment with the dual-ligand LP, while Doxil has no effect, similar to the controls. Even though liver and spleen are major clearance organs for the prepared formulations (Fig. S1), neither damage nor an abnormal morphology of blood vessels was observed in the liver and spleen (Fig. 5A). Moreover the quantification of the area of blood vessels also showed that blood vessels were disrupted exclusively in tumor tissue (Fig. 5B). No abnormal ALT value was observed in

Doxil and dual-ligand LP treated mice (Fig. S3). These results suggest that the dual-ligand LP specifically disrupted the neovasculture in OS-RC-2 tumors, but had no effect on normal endothelial cells in normal tissues such as the liver and spleen.

Fig. 4. Comparison of biodistribution, tumor growth and body weight change by dual-ligand LP with Doxil. (A) The biodistribution in tumor-bearing mice was determined using [^3H] labeled formulations. Blood concentration and tissue accumulation at 24 hr after systemic administration of formulations are represented by %ID/ml and %ID/g tissue (the mean $\pm$ SD, $n=4$), respectively. ** $P<0.01$, N.S.: not significant difference. (B) and (C) PBS or LPs containing 1.5 mg/kg of DXR were i.v.-injected on day 0, 1 and 2. Tumor volume and body weight (the mean $\pm$ SD, $n=4$) was monitored at indicated times. Body weight change was expressed as relative change versus Day 0. * $P<0.05$, ** $P<0.01$.

Fig. 5. Effect of cytotoxicity on blood vessels. PBS or LPs containing 1.5 mg/kg of DXR were intravenously dosed first 3 days. At 24 hr after final injection, tumor, liver and spleen were collected. Endothelial cells and nucleus in unfixed tissues were stained with lectin (green) and Hoechst33342 (blue), respectively. (A) Images were captured by a confocal microscopy. (B) The pixels for endothelial cells in tumor, liver and spleen were quantified (n=8-10). Blood vessels were disrupted only in tumor treated with the dual-ligand LP. **P<0.01. N.S.: Not significant difference.

3.5. Comparison of cytotoxicity in tumor cell and tumor endothelial cell

To elucidate the mechanisms responsible for the improved anti-tumor efficacy of the dual-ligand LP compared to Doxil, we investigated the sensitivity of OS-RC-2 tumor cells and TECs to free DXR. TECs from OS-RC-2 tumor tissue were successfully collected, as described previously [17-22]. As shown in Fig. 6, the TECs were more sensitive to DXR by 2 orders of magnitude than tumor cells. The EC₅₀ for DXR in TECs and OS-RC-2 tumor cells was calculated as 2.0 µg/ml and 95.1 µg/ml, respectively. The result strongly supports that the conclusion that the dual-ligand LP targeting TEC showed better tumor suppression than Doxil in OS-RC-2 tumor bearing mice.

Fig. 6. Comparison of sensitivity to DXR in RCC tumor cells and TECs. OS-RC-2 cells and TECs recovered from OS-RC-2 tumor tissue were incubated with free DXR at indicated concentrations for 8 hr. The cells were further incubated for 18 hr, followed by cell counting. EC₅₀ of TECs and tumor cells to DXR is 2.0 µg/ml and 95.1 µg/ml, respectively.

4. Discussion

It is now well recognized that liposomes (LPs) constitute drug delivery vehicles that can be used in cancer therapy [25]. Long-circulating liposomes, produced by modification with poly(ethylene glycol) (PEG) (PEG-LPs), the size of which is controlled at around 100 nm in diameter, are able to passively accumulate in tumors via the EPR effect [12,13]. Doxil (Caelyx)[®], PEGylated liposomal DXR, accumulates at high level in solid tumors and has less side effects compared with free DXR, and is clinically used in the treatment of AIDS-related Kaposi's sarcoma and ovarian carcinomas [14]. To achieve further chemotherapeutic efficacy, tumor targeting PEGylated liposomes were developed by attaching ligands that specifically target molecules that are specifically expressed on tumor cells [26].

Chemotherapeutic resistance in tumor cells is a serious obstacle in cancer therapy. Renal cell carcinomas (RCCs) are one of the most resistant tumors [1]. Tumor vasculature targeting by drug vehicles is a promising strategy for overcoming the resistance of tumor cells to drugs. In the present study, we used a 300 nm diameter liposome as a platform for developing a dual-ligand system for targeting endothelial cells, of which the size is larger than the one that is usually used, as shown in Fig. 7. We initially assumed that a large size could exclude the possibility of an anti-tumor effect by liposomes that accumulated in tumors via the EPR effect. As shown in Fig. 1, only a few liposomes were observed to be located outside of blood vessels in case of large sized particles, unlike small sized liposomes.

To develop an active targeting delivery system for tumor endothelial cells (TECs), the NGR motif peptide was employed as a ligand for CD13, which is overexpressed in TECs [6]. CD13 targeting systems with the NGR motif peptide showed enhanced therapeutic efficacy in lung, ovarian carcinoma and neuroblastoma [7,8,27]. Unexpectedly, in the OS-RC-2 tumor model, modification of the NGR motif resulted in a minor effect on targetability and tumor growth (Fig. 2 and 3). One possible reason for this is that the NGR motif peptide used in the present study is a linear form of which the binding affinity is inferior to that for the

cyclic form, leading to smaller amounts of liposomes being delivered. Another possible reason is that CD13 is not abundantly expressed in OS-RC-2 xenografts compared to other kinds of tumors. To induce the effect of a ligand on increasing the medicinal benefit of cargos, ligand tagged PEG-LPs should be internalized into target cells via endocytosis, followed by endosomal escape. However, specific receptor-mediated endocytosis proceeds in a saturable manner, due to limited number of receptors and the recycling of endocytosis, which restricts the amount of liposomes that are taken up by the target cells. To overcome this limitation, we proposed the use of a dual-ligand delivery system composed of a specific ligand and a cell penetrating peptide (CPP), as a cationic ligand [10,11]. Target ligands are conjugated at the top of the PEG chain and CPPs are grafted on the surface of the liposomes so as to be masked by PEG when circulating in the blood. After the TECs recognize the specific ligand, the liposomes must resist removal from the surface of TECs under the blood flow. In the dual-ligand design, the interaction of liposomes with target TECs mediated by a target ligand is supported by a CPP via strong cationic interactions with cell surfaces. Subsequently, the liposome is efficiently taken up by cells via the CPP, which is largely independent of the uptake mechanism associated with receptor-ligand interactions (Fig. 7)[11].

In addition, we employed a liposome with a diameter of 300 nm in the design of a dual-ligand formulation, not a 100 nm-diameter liposome, which is generally used for drug targeting to tumors [7-9]. We hypothesized that a large size would be more advantageous for targeting tumor endothelial cells than a small size by preventing the liposomes from extravasation to the tumor through permeable tumor blood vessels and would allow the liposomes to efficiently recognize the blood vessels. As we expected, PEGylated liposomes with diameters of 300 nm were detected mainly along the blood vessels, while PEGylated liposomes with a diameter of 100 nm were distributed both inside and outside of the blood vessels (Fig. 1). We also compared the distribution of large and small dual-ligand LPs. As shown in Fig. S4, The distribution for dual-ligand LPs in tumor tissues was well correlated with that for PEGylated liposomes (Fig. 1).

Additionally, it is possible that large liposomes represent an advantage for ligands to target vascular walls rather than small ones. It was reported that particles diameters > 200 nm appear to be more effective in adhering firmly to the margins of vascular walls under flow than particles with diameters of < 200 nm [28]. Therefore, the cellular binding affinity of a large dual-ligand LP was evaluated in comparison with the small one. As shown in Fig. S5, the relative K_d value of a large dual-ligand LP was around 10 times lower than that of the small one. This might account for this enhancement, since a large dual-ligand LP with a large surface would allow its ligands to interact more frequently with target molecules than a smaller size particle, which would result in multivalent and efficient binding. These results

suggest that a large size liposome would seem to be preferred for the dual-ligand formulation than small size one. To further clarify the advantage of a large size for dual-ligand mediated targeting, we also compared the tumor suppression of a dual-ligand LP with a diameter of 300 nm with a 100 nm diameter dual-ligand LP. As shown in Fig. S6, tumor growth suppression by the large dual-ligand LP was slightly superior compared to the small dual-ligand LP, even though higher amounts of a small dual-ligand LP was found in the tumor. Whereas the large size represented disadvantage regarding the increased accumulation of liposomes in the spleen (Fig.4A), no serious side effects were observed (Fig.5 and Fig. S2). Taking these results into consideration, a large liposome appears to be preferred for a dual-ligand formulation to target tumor endothelial cells than a small sized particle, therefore further evaluations were performed using a large-diameter dual-ligand LP.

Fig. 7. Schematic diagram of the strategy used to develop the dual-ligand LP. Doxil accumulates in tumors via the EPR effect. The size of a dual-ligand LP is controlled around 300 nm and specific ligands and CPPs are modified on the top of PEG chain and on the surface of liposomes, respectively. CPPs should not be functional and free from opsonins due to steric hinderance of the PEG layer in the blood circulation. While after arriving at the target tumor endothelial cells, cellular association via the specific ligands (1) allows CPPs to exert their powerful ability to internalize the liposomes into cells due to proximity of the liposomes to the surface of target cells (2). 1 g of tumor tissue contains 10^8 cells, and tumor endothelial cells constitute approximately 2 % of tumor tissue. Therefore, the relative required dose of DXR by targeting OS-RC-2 is estimated approximately 2380 fold higher than that by targeting tumor endothelial cells to kill the objective cells. Despite the differences, the

delivery of Doxil is only 3-fold larger than the dual-ligand LP, which clearly accounts for the absence of an anti-tumor effect of Doxil in RCC tumor.

A dual-ligand LP of 300 nm encapsulating DXR represented an enhanced anti-tumor effect compared to Doxil (Fig. 4B). If 1 g tumor tissue contains 10^8 cells [29], we estimate that the availability of DXR in tumor cells would be 5 %ID/ 10^8 cells, since the amount of liposome in a tumor via the EPR effect was approximately 5% ID/g tumor (Fig.4A). On the other hand, tumor endothelial cells constitute only approximately 2 % of the total tumor tissue (2×10^6 cells/g tumor) [18]. Because 1.5 %ID/g tumor of liposomes was found in the case of the dual-ligand LP, the availability of DXR in TEC is calculated as 0.75%ID/ 10^6 cells, which means the concentration of DXR in TEC would be expected to be at least 10-fold higher than that in OS-RC-2 cells. Furthermore, cytotoxicity analyses indicated that TECs derived from OS-RC-2 tissue are approximately 2 orders more sensitive to DXR than OS-RC-2 cells (Fig. 6). Taking these facts into consideration, targeting TECs should be around 3 orders of magnitude more efficient in terms of exerting cytotoxicity by DXR than targeting OS-RC-2 kidney cancer tissue (Fig. 7). Even though a dual-ligand LP efficiently disrupted blood vessels, tumor growth was partially inhibited, presumably because surviving OS-RC-2 cells could generate new blood vessels. For further therapeutic efficacy, the inhibition of factors such as VEGF from OS-RC-2 cells should be used in combination with the above described therapy.

In summary, here we report on a novel anti-neovasculature therapy for drug-resistant renal cell carcinomas based on a unique delivery system comprised of large-sized liposomes that had been modified with a dual-ligand. We also directly compared the cytotoxicity between tumor cells and tumor endothelial cells. The findings clearly show that targeting the neovasculature is 3-orders more efficient than tumor cells in a drug resistance tumor. The results provide a promising basis for further anti-angiogenic chemotherapy, which may be valuable for future clinical applications for drug-resistant cancer.

Acknowledgments

This study was supported by grants from the Special Education and Research Expenses of the Ministry of Education, Culture, Sports, Science and Technology of Japan. The authors wish to thank Dr. Milton S. Feather for his helpful advice in writing the English manuscript.

References

- [1] N.J. Vogelzang, W.N. Stadler. Kidney cancer, *Lancet*. 352 (1998) 1691–1696.
- [2] E. Soto-Vega, C. Arroyo, Y. Richaud-Patin, M. García-Carrasco, L.G. Vázquez-Lavista, L. Llorente. P-glycoprotein activity in renal clear cell carcinoma, *Urol. Oncol.* 27 (2009) 363–366.

- [3] T.E. Hutson, Targeted therapies for the treatment of metastatic renal cell carcinoma: clinical evidence, *The Oncologist* 16 (2011) 14–22.
- [4] G.C. Lorenzo, C. Buonerba, M. Biglietto, F. Scognamiglio, B. Chiurazzi, F. Riccardi, G. Carteni, The therapy of kidney cancer with biomolecular drugs, *Cancer Treat Rev.* 36 (2010) S16–S20.
- [5] J. Stevens, B. St. Croix, Tumor endothelial markers, in: W.D. Figg, J. Folkman, (Eds.), *Angiogenesis; An integrative approach from science to medicine*, Springer, 2008, pp333–342.
- [6] W. Arap, R. Pasqualini, E. Ruoslahti, Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model, *Science* 279 (1998) 377–380.
- [7] F. Pastorino, C. Brignole, D. Di Paolo, B. Nico, A. Pezzolo, D. Marimpietri, G. Pagnan, F. Piccardi, M. Cilli, R. Longhi, D. Ribatti, A. Corti, T.M. Allen, M. Ponzoni, Targeting liposomal chemotherapy via both tumor cell-specific and tumor vasculature-specific ligands potentiates therapeutic efficacy, *Cancer Res.* 66 (2006) 10073–10082.
- [8] E.A. Murphy, B.K. Majeti, L.A. Barnes, M. Makale, S.M. Weis, K. Lutu-Fuga, W. Wrasidlo, D.A. Cheresch, Nanoparticle-mediated drug delivery to tumor vasculature suppresses metastasis, *Proc. Natl. Acad. Sci. U. S. A.* 105 (2008) 9343–9348.
- [9] Y. Murase, T. Asai, Y. Katanasaka, T. Sugiyama, K. Shimizu, N. Maeda, N. Oku, A novel DDS strategy, "dual-targeting", and its application for antineovascular therapy, *Cancer Lett.* 287 (2010) 165–171.
- [10] K. Takara, H. Hatakeyama, N. Ohga, K. Hida, H. Harashima, Design of a dual-ligand system using a specific ligand and cell penetrating peptide, resulting in a synergistic effect on selectivity and cellular uptake, *Int. J. Pharm.* 396 (2010) 143–148.
- [11] G. Kibria, H. Hatakeyama, N. Ohga, K. Hida, H. Harashima, Dual-ligand modification of PEGylated liposomes shows better cell selectivity and efficient gene delivery, *J. Control. Release* 153 (2011) 141–148.
- [12] Y. Matsumura, H. Maeda. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumorotropic accumulation of proteins and the antitumor agent smancs, *Cancer Res.* 46 (1986) 6387–6392.
- [13] H. Hatakeyama, H. Akita, H. Harashima, A multifunctional envelope type nano device (MEND) for drug and gene delivery to tumors based on the EPR effect: a strategy for overcoming the PEG dilemma, *Adv. Drug Deliv. Rev.* 63 (2011) 152–160.
- [14] M.E. Davis, Z. Chen, D.M. Shin, Nanoparticle therapeutics: an emerging treatment modality for cancer, *Nat. Rev. Drug Discov.* 7 (2008) 771–782.
- [15] T.A. ElBayoumi, V.P. Torchilin, Tumor-specific anti-nucleosome antibody improves therapeutics efficacy of doxorubicin-loaded long-circulating liposomes against primary and metastatic tumor in mice, *Mol. Pharm.* 6 (2009) 246–254.

- [16]H. Hatakeyama, H. Akita, K. Maruyama, T. Suhara, H. Harashima, Factor governing the in vivo tissue uptake of transferring coupled polyethylene glycol liposomes in vivo, *Int. J. Pharm.* 281 (2004) 25–33.
- [17]K. Hida, Y. Hida, D.N. Amin, A.F. Flint, D. Panigrahy, C.C. Morton, M. Klagsbrun, Tumor-associated endothelial cells with cytogenetic abnormalities, *Cancer Res.* 15 (2004) 8249–8255.
- [18]N. Ohga, K. Hida, Y. Hida, C. Muraki, K. Tsuchiya, K. Matsuda, Y. Ohiro, Y. Totsuka, M. Shindoh, Inhibitory effects of epigallocatechin-3 gallate, a polyphenol in green tea, on tumor-associated endothelial cells and endothelial progenitor cells, *Cancer Sci.* 100 (2009) 1963–1970.
- [19]K. Matsuda, N. Ohga, Y. Hida, C. Muraki, K. Tsuchiya, T. Kurosu, T. Akino, S.C. Shih, Y. Totsuka, M. Klagsbrun, M. Shindoh, K. Hida, Isolated tumor endothelial cells maintain specific character during long-term culture, *Biochem. Biophys. Res. Commun.* 394 (2010) 947–954.
- [20]T. Akino, K. Hida, Y. Hida, K. Tsuchiya, D. Freedman, C. Muraki, N. Ohga, K. Matsuda, K. Akiyama, K. Harabayashi, M. Klagsbrun, M. Shindoh, Cytogenetic abnormalities of tumor-associated endothelial cells in human malignant tumors, *Am. J. Pathol.* 175 (2009) 2657–2667.
- [21]C. Muraki, N. Ohga, Y. Hida, H. Nishihara, Y. Kato, K. Tsuchiya, K. Matsuda, Y. Totsuka, M. Shindoh, K. Hida K, Cyclooxygenase-2 inhibition causes antiangiogenic effects on tumor endothelial and vascular progenitor cells, *Int. J. Cancer* 130 (2012) 59–70.
- [22]T. Kurosu, N. Ohga, Y. Hida, N. Maishi, K. Akiyama, W. Kakuguchi, T. Kuroshima, M. Kondo, T. Akino, Y. Totsuka, M. Shindoh, F. Higashino, K. Hida, HuR keeps an angiogenic switch on by stabilising mRNA of VEGF and COX-2 in tumour endothelium, *Br. J. Cancer* 104 (2011) 819–829.
- [23]K. Maruyama, T. Yuda, A. Okamoto, S. Kojima, A. Suganaka, M. Iwatsuru, Prolonged circulation time in vivo of large unilamellar liposomes composed of distearoyl phosphatidylcholine and cholesterol containing amphipathic poly(ethylene glycol), *Biochim. Biophys. Acta.* 1128 (1992) 44–49.
- [24]D. Liu, A. Mori, L. Huang, Role of liposome size and RES blockade in controlling biodistribution and tumor uptake of GM₁-containing liposomes, *Biochim. Biophys. Acta.* 1104 (1992) 95–101.
- [25]V.P. Torchilin, Recent advances with liposomes as pharmaceutical carriers, *Nat. Rev. Drug Discov.* 4 (2005) 145–160.
- [26]T.M. Allen, Ligand-targeted therapeutics in anticancer therapy, *Nat. Rev. Cancer* 2 (2002) 750–763.

- [27] Y. van Hensbergen, H.J. Broxterman, Y.W. Elderkamp, J. Lankelma, J.C. Beers, M. Heijn, B. Epie, K. Hoekman, H.M. Pinedo, A doxorubicin-CNGRC-peptide conjugate with prodrug properties, *Biochem. Pharmacol.* 63 (2002) 897–908.
- [28] F. Gentile, A. Curcio, C. Indolfi, M. Ferrari, P. Decuzzi, The margination propensity of spherical particles for vascular targeting in the microcirculation, *J. Nanobiotechnology* 6 (2008) 1–9.
- [29] J.Y. Zheng, D. Chen, J. Chan, D. Ui, E. Ko, S. Pang, Regression of prostate cancer xenografts by a lentiviral vector specifically expressing diphtheria toxin A, *Cancer Gene Ther.* 10 (2003) 764–770.

Figure legends

Fig. 1. Effect of liposome size on distribution in RCC tumor. (A) The size distribution of small (solid line) and large PEG-LP (dotted line) were determined by DLS measurements. (B) and (C) Upper images of unfixed tumor tissues that had been intravenously treated with either small PEG-LP or large PEG-LP (2.0 μmol lipid/mouse), respectively. Endothelial cells were labeled with lectin (green). The lower spectra were obtained from the arrow line in upper images. *Bar*, 80 μm .

Fig. 2. Accumulation and localization of a dual-ligand LP in tumor. (A) Schematic illustration of prepared formulations. LPs of 300 nm in diameter were modified with either PEG, NGR modified PEG or R4. Dual-ligand LP were prepared by modified with both NGR modified PEG and R4. (B) Tumor accumulation at 24 hr after systemic administration of formulations labeled with [^3H] are represented by %ID/g tissue (the mean $\pm$ SD, $n=4$). $^{**}P<0.01$. (C) Images of unfixed tumor tissues intravenously treated with each formulation labeled with rhodamine (0.5 μmol lipid/mouse). Tumor endothelial cells were labeled with lectin (green). Arrow heads point red signals (liposomes) along with blood vessels.

Fig. 3. Therapeutic effect of a dual-ligand LP in tumor. PBS or LPs containing 6 mg/kg of DXR were i.v.-injected on day 0 and 3. Tumor volume was monitored at indicated times. Dual-ligand LP showed better effect on the tumor growth than other formulations. $^{**}P<0.01$, $^{\#}P<0.01$ (PBS treated group versus LP-treated groups). A Dual-ligand LP showed significant tumor growth suppression compared to the other formulations.

Fig. 4. Comparison of biodistribution, tumor growth and body weight change by dual-ligand LP with Doxil. (A) The biodistribution in tumor-bearing mice was determined using [^3H] labeled formulations. Blood concentration and tissue accumulation at 24 hr after systemic administration of formulations are represented by %ID/ml and %ID/g tissue (the mean $\pm$ SD, $n=4$), respectively. $^{**}P<0.01$, N.S.: not significant difference. (B) and (C) PBS or LPs containing 1.5 mg/kg of DXR were i.v.-injected on day 0, 1 and 2. Tumor volume and body weight (the mean $\pm$ SD, $n=4$) was monitored at indicated times. Body weight change

was expressed as relative change versus Day 0. *P<0.05, **P<0.01.

Fig. 5. Effect of cytotoxicity on blood vessels. PBS or LPs containing 1.5 mg/kg of DXR were intravenously dosed first 3 days. At 24 hr after final injection, tumor, liver and spleen were collected. Endothelial cells and nucleus in unfixed tissues were stained with lectin (green) and Hoechst33342 (blue), respectively. (A) Images were captured by a confocal microscopy. (B) The pixels for endothelial cells in tumor, liver and spleen were quantified (n=8-10). **P<0.01. N.S.: Not significant difference.

Fig. 6. Comparison of sensitivity to DXR in RCC tumor cells and TECs. OS-RC-2 cells and TECs recovered from OS-RC-2 tumor tissue were incubated with free DXR at indicated concentrations for 8 hr. The cells were further incubated for 18 hr, followed by cell counting. EC50 of TECs and tumor cells to DXR is 2.0 µg/ml and 95.1 µg/ml, respectively.

Fig. 7. Schematic diagram of the strategy used to develop the dual-ligand LP. Doxil accumulates in tumors via the EPR effect. The size of a dual-ligand LP is controlled around 300 nm and specific ligands and CPPs are modified on the top of PEG chain and on the surface of liposomes, respectively. CPPs should not be functional and free from opsonins due to steric hinderance of the PEG layer in the blood circulation. While after arriving at the target tumor endothelial cells, cellular association via the specific ligands (1) allows CPPs to exert their powerful ability to internalize the liposomes into cells due to proximity of the liposomes to the surface of target cells (2). 1 g of tumor tissue contains 10^8 cells, and tumor endothelial cells constitute approximately 2 % of tumor tissue. Therefore, the relative required dose of DXR by targeting OS-RC-2 is estimated approximately 2380 fold higher than that by targeting tumor endothelial cells to kill the objective cells. Despite the differences, the delivery of Doxil is only 3-fold larger than the dual-ligand LP, which clearly accounts for the absence of an anti-tumor effect of Doxil in RCC tumor.

Table

Table 1 Physical properties of the prepared liposomes

	Large size				Small size	
	PEG-LP	NGR-PEG-LP	R4/PEG-LP	R4/NGR-PEG-LP (Dual-ligand LP)	PEG-LP	Doxil
Diameter (nm)	298 ± 29	311 ± 18	296 ± 26	304 ± 17	100 ± 17	85 ± 3
PDI	0.209 ± 0.012	0.214 ± 0.028	0.225 ± 0.008	0.215 ± 0.025	0.089 ± 0.023	0.121 ± 0.021
Zeta-potential (mV)	-13 ± 3	-16 ± 2	-11 ± 4	-11 ± 3	-13 ± 3	-11 ± 2

Data are the means ± SD of at least three different preparations.

In case of large size, molar ratio of EPC/Cholesterol for biodistribution study or HSPC/Cholesterol for anti-cancer study was fixed at 7:3. Large size LPs were modified with 10 mol% PEG-DSPE or NGR-PEG-DSPE, and 2.5 mol% STR-R4. In case of small size,

PEG-LP for biodistribution study was composed of EPC/Chol (7:3) with 5% PEG-DSPE, and Doxil for the anti-cancer study was composed of HSPC/Chol (3:2) with 5 mol% PEG-DSPE.