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A new peptide motif present in the protective antigen of anthrax toxin exerts its efficiency on the cellular uptake of liposomes and applications for a dual-ligand system

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ABSTRACT

Protective antigen (PA) is a nontoxic protein present in anthrax toxin. Domain 4 of PA (PA-D4) acts as a receptor binding site for tumor endothelial marker 8 (TEM8). In this study, KYND motif from PA-D4 was utilized as a ligand against TEM8. The efficiency of KYND motif on cellular association was assessed by evaluating the cellular uptake of PEGylated liposomes (PEG-LPs) in TEM8 positive and negative cells. The peptide was attached on the top of the PEG of PEG-LP. Compared to PEG-LP, KYND modified PEG-LP (KYND-PEG-LP) enhanced the cellular uptake to a greater extent in all cell lines. Based on the inhibition assay, no receptor involvement was observed in the cellular association of KYND-PEG-LP, suggesting that KYND motif functions as a cell penetrating peptide (CPP) which facilitated the internalization of PEG-LP via clathrin mediated endocytosis pathway. Further enhancement of cellular uptake was observed when KYND-PEG-LP was combined with octaarginine (R8) on the surface of lipid membrane as dual-CPP ligand formulation, however, when PEG-LP combined with only R8, only negligible enhancement was observed. These findings suggest that two CPP ligands act in a synergistic fashion; therefore the dual-CPP ligand based liposomal formulation can be assumed to be an effective delivery system.

1. Introduction

Anthrax is a lethal infectious disease caused by *Bacillus anthracis*, a Gram-positive, spore-forming, rod shaped bacterium that primarily infects herbivores such as cattle and deer (Keim and Smith, 2002; Turnbull, 2002). The anthrax toxin, a three-part toxin, consists of protective antigen (PA; 83 kDa, 735 amino acid residues), lethal factor (LF; 90 kDa), and edema factor (EF; 89 kDa), in which PA acts as a cellular receptor-binding component for tumor endothelial marker 8 (TEM8) and capillary morphogenesis protein 2 (CMG2) (Bradley et al., 2001; Scobie and Young, 2005; Werner et al., 2006), where the segment of the amino acid sequence responsible for binding with either TEM8 or CMG2, is still unclear, but would be in two different positions of PA. Due to its receptor binding capability, PA has the potential to serve a target for structure based drug therapies. PA has four domains in its structure, as follows: domain 1 (residues 1–258), domain 2 (residues 249–487), domain 3 (residues 488–595) and domain 4 (residues 596–735) (Petosa et al., 1997; Young and Collier, 2007). Further studies on PA revealed that domain 4 (residues 596–735) directly binds to cellular receptors like TEM8 (Bradley et al., 2001; Petosa et al., 1997; Singh et al., 1991). It has also been reported that domain 4 of PA (PA-D4) contains two loops in its structure in the form of a small loop (residues 679–693) and a large loop (residues 704–723) and it was proposed that the large loop does not interact directly with receptors (Brossier et al., 1999; Varughese et al., 1999). This suggests that the small loop (residues 679–693) of PA-D4 would be more important for receptor binding and that residue 682 might play a major role in receptor recognition (Varughese et al., 1999). The small loop of PA-D4 was found to be the domain that is responsible for binding with TEM8. As a result, the small loop of PA-D4 appears to be an ideal candidate for utilization as a specific ligand for drug delivery systems.

Based on these considerations, we selected the amino acid residues 679–688, KKYNDKLPLY (abbreviated hereafter as KYND) from the small loop of PA-D4 for use as a novel ligand in the design of a selective targeting system. It is well known that liposomes are suitable nano-carriers that have the capacity to deliver drug particles to various target cells in vitro or diseased tissues in vivo (Puri et al., 2009; Du et al., 2007). Hence, in our design, we attached the selected KYND peptide to the top of poly ethylene glycol (PEG) of PEGylated liposomes and the cellular interaction efficiency of this ligand was evaluated based on the cellular uptake of the ligand-incorporated PEGylated liposomes. Cell-penetrating

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peptides (CPPs), short peptide sequences, are currently in use for drug and gene delivery (El-Sayed et al., 2009). CPPs, also known as protein transduction domains (PTDs) have the capability to enter into the cells either alone or in conjugation with small molecules or bulky cargos such as peptides, proteins, oligonucleotides, plasmid DNA (pDNA) or liposomes. In previous studies, we reported on the development of stearylated octaarginine (STR-R8) modified on the surface of liposomes encapsulating plasmid DNA (pDNA), small interfering RNA (siRNA), or proteins, which can be efficiently internalized by cells (Kogure et al., 2004; Nakamura et al., 2007; Suzuki et al., 2007). We recently proposed a dual-ligand liposomal delivery system modified with a specific ligand on the top of the PEG chain and STR-R8, as a CPP ligand, on the surface of liposomes (Takara et al., 2010). Even though the cellular uptake by target cells of PEGylated liposomes modified with either a specific ligand or STR-R8 was enhanced to only a minor extent, the dual-ligand formulation showed an increased specificity and its cellular uptake by target cells expressing target molecules was efficient, due to the synergistic effect of the dual-ligand formulation.

In the present study, we also incorporated STR-R8 on the surface of the PEGylated liposomes along with the selected KYND peptide for use in a dual-CPP ligand formulation. The cellular uptake of the dual-CPP ligand modified PEGylated liposomes was evaluated the data compared with the uptake for a single-CPP ligand version.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (EPC), cholesterol (Chol), N-(lissamine rhodamine B sulfonyl)-1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (rhodamine-DOPE), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (PEG-DSPE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). N-[(3-maleimide-1-oxopropyl) aminopropyl polyethyleneglycol-carbamyl] distearoylphosphatidyl-ethanolamine (maleimide-PEG-DSPE) was purchased from Nippon Oil and Fat Co. (Tokyo, Japan). Stearylated octaarginine (STR-R8) and Octaarginine (R8) peptide were purchased from Polypeptide Laboratories (San Diego, CA, USA) and Genixtalk Co. (Osaka, Japan), respectively. Endothelial Cell Basal Medium (EBM-2) and other related growth factors were purchased from Lonza (Walkersville, MD, USA). KKYNDKLPLYGC peptide (KYND in brief) was purchased from Sigma Genosys Japan (Ishikari, Japan). Dulbecco's Modified Eagle's Medium (DMEM), amiloride, filipin III from *Streptomyces filipinensis* were obtained from Sigma–Aldrich Co. Ltd. (St. Louis, MO, USA). Heparin and sucrose were purchased from Wako Pure Chemical Industries (Osaka, Japan). Fetal bovine serum (FBS) was purchased from Hyclone Laboratories (Logan, UT, USA). Rabbit anti-mouse and human TEM8 antibody (cat. no. ab21270) was purchased from Abcam Inc. (San Francisco, CA, USA) and Alexa Fluor 488 labeled goat anti-rabbit IgG (cat. no. A11008) was purchased from Invitrogen (Carlsbad, CA, USA). All other chemicals used in this study were of analytical grade.

2.2. Conjugation of KYND peptide with PEG-lipid

The KYND peptide was conjugated with maleimide-PEG-DSPE (1:1 molar ratio) in deionized water at room temperature for 24 h. The conjugation of KYND with PEG was confirmed by determining the molecular weight of KYND-PEG-DSPE by matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) MS (Bruker Daltonics, Germany) using acetonitrile:water = 7:3 with 0.1% of trifluoroacetate as the matrix solution, supplied with 10 mg/ml of dihydroxybenzoic acid.

2.3. Preparation of liposomes

Liposomes (LPs) composed of EPC/Chol (molar ratio: 7/3) were prepared by the lipid hydration method. A lipid film was formed by evaporation of the solvents (chloroform and ethanol) from a lipid solution in a glass tube. HEPES buffer (10 mM, pH 7.4) was added and the solution incubated for 10 min to hydrate the lipid film. The glass tube was then sonicated for approximately 30 s in a bath-type sonicator (AU-25 C, Aiwa, Tokyo, Japan). During the formation of the film, 1 mol% rhodamine-DOPE was incorporated, to serve as a lable for the lipid component. To modify the prepared LPs with STR-R8, PEG-DSPE and KYND-PEG-DSPE, the required amount of STR-R8, PEG-DSPE, or KYND-PEG-DSPE was also added to the lipid solution.

2.4. Characterization of liposomes

The mean size and zeta potential of the prepared LPs were determined using a Zetasizer Nano ZS ZEN3600 instrument (Malvern Instruments Ltd., Worchestershire, UK).

2.5. Cell culture

Human cervical carcinoma cells (HeLa) and mouse pancreas endothelial cells (MS-1) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, penicillin (100 units/ml), streptomycin (100 μg/ml). The cells were cultured under an atmosphere of 5% CO_2 and 95% humidity at 37 °C. Human umbilical vein endothelial cells (HUVEC) were cultured in EBM-2 medium supplemented with 2% FBS (v/v), penicillin (100 units/ml), streptomycin (100 μg/ml) and growth factors under an atmosphere of 5% CO_2 and 95% humidity at 37 °C.

2.6. Evaluation of expression of TEM8

Cells were washed with PBS (pH 7.4) and detached by treatment with trypsin–EDTA. The detached cells were incubated with the anti TEM8 antibody for 20 min at 4 °C, followed by incubation with the Alexa Flour 488 labeled secondary antibody for 20 min at 4 °C. Ten thousand cells per sample were analyzed using a FACSVantageSE flow cytometer (BD, San Jose, CA, USA).

2.7. Cellular uptake study

For the cellular uptake study, 40,000 cells were seeded in a 24-well plate (Corning incorporated, Corning, NY, USA) (40,000 cells/well) and the plate incubated overnight at 37 °C in an atmosphere of 5% CO_2 and 95% humidity. After 24 h, the prepared rhodamine labeled LPs were added and incubated for an additional 3 h at 37 °C in an atmosphere of 5% CO_2 and 95% humidity. After the incubation, the cells were washed 3 times with 1 ml of ice-cold phosphate buffer saline (PBS) supplemented with heparin (20 units/ml) to completely remove the surface-bound KYND-PEG-LP, as reported previously (Khalil et al., 2004) and then treated with Reporter Lysis Buffer (Promega Corp., Madison, WI, USA) followed by centrifugation at 12,000 rpm for 5 min at 4 °C to remove debris. The cellular uptake efficiency of the prepared rhodamine labeled LPs were determined by measuring the fluorescence intensity of rhodamine (excitation at 550 nm and emission at 590 nm) using FP-750 Spectrofluorometer (JAS Co, Tokyo, Japan).

2.8. The uptake mechanism study of KYND modified PEG-LPs

2.8.1. Inhibition assay in presence of free peptide

To examine the effect of excess amount of free peptides on the cellular uptake of LPs, a cellular uptake experiment was performed

in the presence of the free KYND peptide. Excess KYND peptide (25 fold vs. peptide used in conjunction with modified LPs) in Kreb's buffer was added to the well plate prepared as described above and incubated for 1 h at 37 °C in an atmosphere of 5% CO_2 and 95% humidity. After 1 h, LPs were added and incubated for 3 additional hours. The cells were washed 3 times with 1 ml of ice-cold phosphate buffer saline (PBS) supplemented with heparin (20 units/ml) to completely remove the surface-bound KYND-PEG-LP and the fluorescence intensity of rhodamine was then determined.

2.8.2. Inhibition assay in presence of unlabeled PEG-LPs modified with KYND

To investigate the involvement of the receptor in the cellular uptake pathway of KYND-PEG-LP, 40,000 HUVECs were seeded in a 24-well plate and the plate was incubated overnight at 37 °C in an atmosphere of 5% CO_2 and 95% humidity. Next day, 10 mol% KYND modified rhodamine labeled and unlabeled PEG-LPs were added using different concentrations (1:0, 1:5, 1:10, 1:20 and 1:50, respectively) and incubated for next 3 h. After 3 h, the cells were washed 3 times with 1 ml of ice-cold phosphate buffer saline (PBS) supplemented with heparin (20 units/ml) to completely remove the surface-bound KYND-PEG-LP and the fluorescence intensity of rhodamine was then determined. Same experiment was conducted using R8 peptide grafted on the top of PEG of PEG-LPs (R8-PEG-LPs).

2.8.3. Inhibition assay in presence of inhibitors

To investigate the mechanism of internalization of KYND motif, 40,000 Hela cells were seeded in a 24-well plate (Corning incorporated, Corning, NY, USA) and the plate was incubated overnight at 37 °C in an atmosphere of 5% CO_2 and 95% humidity. Before transfection, the cells were washed with 1 ml of PBS and were pre-incubated with Kreb's buffer in the absence or presence of the following inhibitors for various times: Amiloride (5 mM) for 30 min; sucrose (1 M) for 30 min or Filipin III (400 μg/ml) for 1 h at 37 °C (Mudhakir et al., 2008; Khalil et al., 2004; Hansen et al., 1993). KYND modified PEG-LP was added and the cells were incubated for 1 h at 37 °C in the presence or absence of inhibitors. The cells were washed 3 times with 1 ml of ice-cold phosphate buffer saline (PBS) supplemented with heparin (20 units/ml) to completely remove the surface-bound KYND-PEG-LP and the fluorescence intensity of rhodamine was then determined, as described above.

2.9. Statistical analysis

Pair-wise comparisons of subgroups were made using a Student's *t*-test. Comparisons between multiple treatments were made using one-way analysis of variance (ANOVA), followed by the 'Dunnett test. Factorial analysis was done by two-way ANOVA. Differences among the means were considered to be statistically significant at a p-value of <0.05.

3. Results

3.1. Synthesis of KYND-PEG-DSPE

KYND-PEG_{2000}-DSPE was synthesized after conjugation of the KYND peptide (calculated MW 1441.68 and observed MW 1444.04) with maleimide-PEG-DSPE (calculated Mn 2936) in water (Fig. 1A). MALDI-TOF MS analyses were performed for the KYND peptide and maleimide-PEG-DSPE (Fig. 1B), which confirmed that the conjugation was successful, as evidenced by the molecular shifts in the MALDI-TOF MS analysis (Fig. 1C).

3.2. Effect of the KYND peptide as single ligand on the cellular uptake of PEGylated LPs

To evaluate the KYND mediated cellular uptake of PEG-LPs, we confirmed the expression of TEM8 in HUVEC, MS-1, and Hela cells. As shown in Fig. 2, HUVEC showed TEM8 expression, while MS-1 and Hela were determined to be TEM8 negative. The modified LPs were prepared by incorporating PEG-DSPE or KYND-PEG-DSPE at levels of 1, 5, 10, or 15 mol% of the total lipid. The physical properties of the prepared LPs are shown in Table 1. The findings indicate that, the size of the prepared LPs gradually decreased with an increase in the amount of PEG or KYND-PEG used, indicating that the KYND peptide had no remarkable effect on the size of the LPs. The surface charge of the PEG-LPs remains negative, similar to that for a bare liposome and almost equal, even when a higher amount of PEGylation was employed. However, addition of the KYND peptide resulted in a gradual increase in the surface charge of the PEG-LPs and a large difference in surface charge was observed between increments of KYND-PEG of 1 mol% to 5 mol% (Table 1). This increment in surface charge of the KYND-PEG-LPs is indicative of the cationic properties of the KYND peptide. To evaluate the effect of the KYND peptide on cellular uptake, we examine the cellular uptake efficiency of PEG-LPs that had been modified with the KYND peptide. In HUVEC, TEM8 positive cells, the findings indicated that PEGylation had a slight inhibitory effect on the cellular uptake of LPs, however, the KYND peptide gradually enhanced the cellular uptake of PEG modified LPs and the maximum cellular uptake was observed for PEG-LPs modified with 10% KYND (Fig. 3A). On the other hand, in MS-1 and Hela cells, KYND modification also significantly enhanced the cellular uptake of PEG-LPs despite a lack of TEM8 expression, as shown in Fig. 3B and C.

3.3. Evaluation of uptake route of KYND modified PEG-LPs

3.3.1. Effect of free peptide on cellular uptake of KYND-PEG-LPs

KYND modification resulted in a remarkable enhancement in the cellular uptake of PEG-LPs in all cell lines tested. To investigate the enhancement of cellular uptake through receptor interactions, an inhibition assay was performed by adding the free peptide prior to the addition of KYND modified PEG-LPs. For this study, we prepared PEG-LPs modified with 10 mol% of the KYND peptide due to its maximum enhancement in cellular uptake, as shown in Fig. 3. The inhibition assay showed no inhibition in the cellular uptake of the KYND modified PEG-LPs in the presence of free peptide at 3 h post incubation (Fig. 4). These results indicate that the KYND peptide might not function via interactions with a specific receptor.

3.3.2. Effect of peptide modified unlabeled PEG-LPs on the cellular uptake of labeled PEG-LPs

The cellular uptake of KYND modified PEG-LPs was evaluated by increasing the concentration of unlabeled KYND-PEG-LPs, as shown in Fig. 5. The results showed that the cellular uptake of rhodamine labeled KYND-PEG-LPs was inhibited remarkably upon addition of unlabeled KYND-PEG-LPs (Fig. 5A). It was interestingly observed that the unlabeled R8-PEG-LPs have significant inhibitory effect on the cellular uptake of labeled R8-PEG-LPs (Fig. 5B). After getting these results, the cellular uptake of labeled KYND-PEG-LPs was also evaluated in presence of unlabeled R8-PEG-LPs and it was found that the cellular uptake of labeled KYND-PEG-LPs was surprisingly inhibited by R8-PEG-LPs (Fig. 5C). The results generated in this study demonstrated that both of R8 peptide and KYND motif peptide are interacting with the same molecules present on the cell surface. Therefore, it can be conferred that KYND motif peptide functions in a non-specific manner and no specific receptor is involved in the internalization process of KYND modified PEG-LPs.

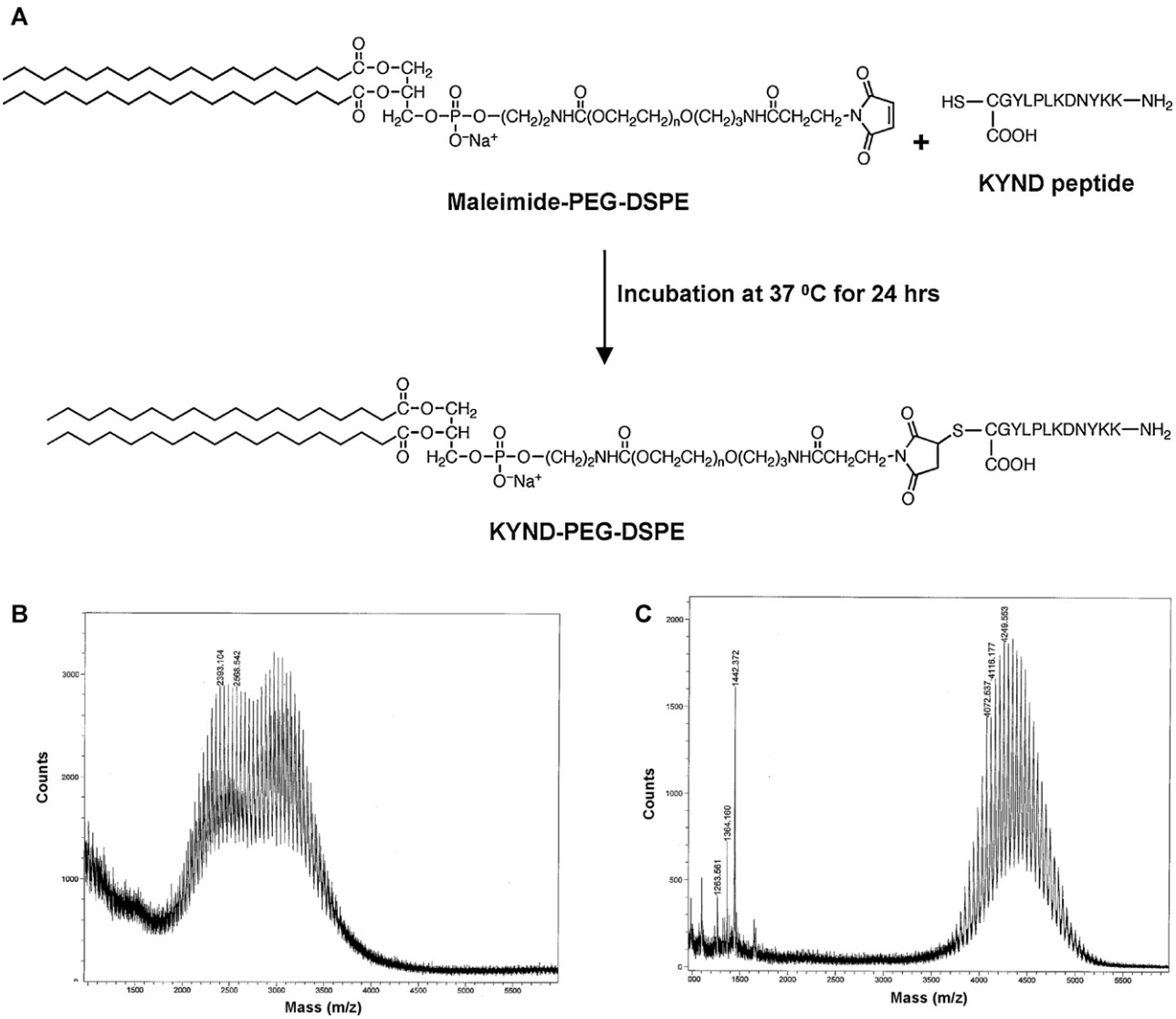

Fig. 1. Conjugation of KYND with maleimide-PEG-DSPE. (A) Route for the synthesis of KYND-PEG-DSPE. maleimide-PEG-DSPE and the KYND peptide (molar ratio 1:1) were dissolved in water at 37 °C and allowed to react for 24 h. MALDI-TOF MS spectra of (B) maleimide-PEG-DSPE and (C) KYND-PEG-DSPE.

3.3.3. Investigation of cellular uptake mechanism using inhibitors

The cellular uptake route of PEG-LP modified with KYND was investigated by evaluating the contribution of different endocytic pathways. Inhibitors that specifically block macropinocytosis, clathrin-mediated endocytosis and caveolar endocytosis were used to determine the mechanism of KYND-PEG-LP uptake. Specifically, a hypertonic medium (sucrose) was used to inhibit clathrin-mediated endocytosis via dissociation of the clathrin lattice (Heuser and Anderson, 1989). Amiloride inhibits macropinocytosis by inhibiting the Na^+/H^+ exchange required for macropinocytosis

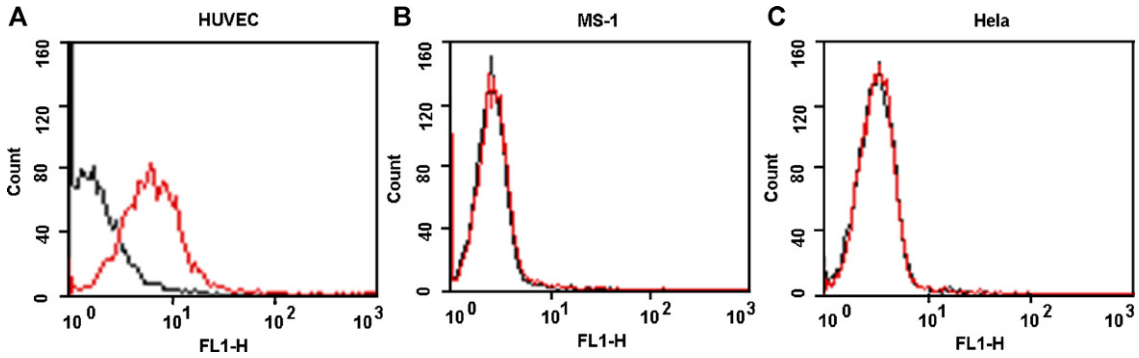

Fig. 2. Expression level of TEM8. The expression of TEM8 on (A) HUVEC, (B) MS-1, and (C) Hela cells was confirmed by flow cytometry analysis as described in Section 2. Black lines indicate non-treatment, and red lines indicate the results obtained with the anti TEM8 antibody treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 1
Physical properties of prepared LPs.

Formulation	PEG-LP		KYND-PEG-LP	
Amount of PEG-lipid	Diameter (nm)	ζ-Potential (mV)	Diameter (nm)	ζ-Potential (mV)
0 mol%	103 ± 11	−28 ± 7		
1 mol%	89 ± 4	−31 ± 6	86 ± 6	−23 ± 6
5 mol%	90 ± 13	−35 ± 0	84 ± 6	16 ± 6
10 mol%	91 ± 12	−35 ± 7	85 ± 7	25 ± 5
15 mol%	87 ± 4	−33 ± 2	75 ± 3	24 ± 4

The data are expressed as the mean ± SD value for at least three different preparations. The amount of PEG-lipid 0 mol% means PEG-unmodified LP.

(Hewlett et al., 1994). Filipin inhibits caveolar uptake through cholesterol depletion (Lamaze and Schmid, 1995). For this study, we prepared PEG-LPs modified with 10 mol% of the KYND peptide. The cellular uptake of KYND-PEG-LP was inhibited by sucrose (Fig. 6) but in contrast, the presence of amiloride or filipin did not inhibit the uptake of KYND-PEG-LP. This study reveals that KYND modified PEG-LPs were internalized into cells via clathrin-mediated endocytosis pathway.

3.4. Cellular uptake of PEGylated LPs modified with dual-ligand

We recently reported on the development of a dual-ligand PEGylated liposomal formulation modified with a specific ligand on the top of the PEG and oligoarginine, as a CPP ligand, on the surface of lipid membrane (Takara et al., 2010). Since the oligoarginine was masked by the PEG layer, the oligoarginine resulted in no enhancement in the cellular uptake of PEGylated liposomes. However, in a dual-ligand formulation, the interactions between a specific ligand and the target molecule resulted oligoarginine being in close proximity to the cell surface, which permits oligoarginine to internalize the liposomes efficiently into the target cells. In this study, we assessed whether or not the dual-ligand concept is applicable for the combination of KYND, as a CPP ligand modified on the top of PEG and oligoarginine on the surface of liposomes. To observe the effect of the dual-ligand formulation on cellular uptake, we additionally incorporated 1 mol% stearylated octaarginine (STR-R8) on the surface of the LPs modified with 5 mol% PEG or KYND-PEG. The average diameter and ζ-potential of R8 modified PEG-LP and R8 modified KYND-PEG-LP were 86 ± 1 nm and −20 ± 9 mV, and 92 ± 1 nm and 32 mV, respectively. The addition of STR-R8 on the surface of lipo-

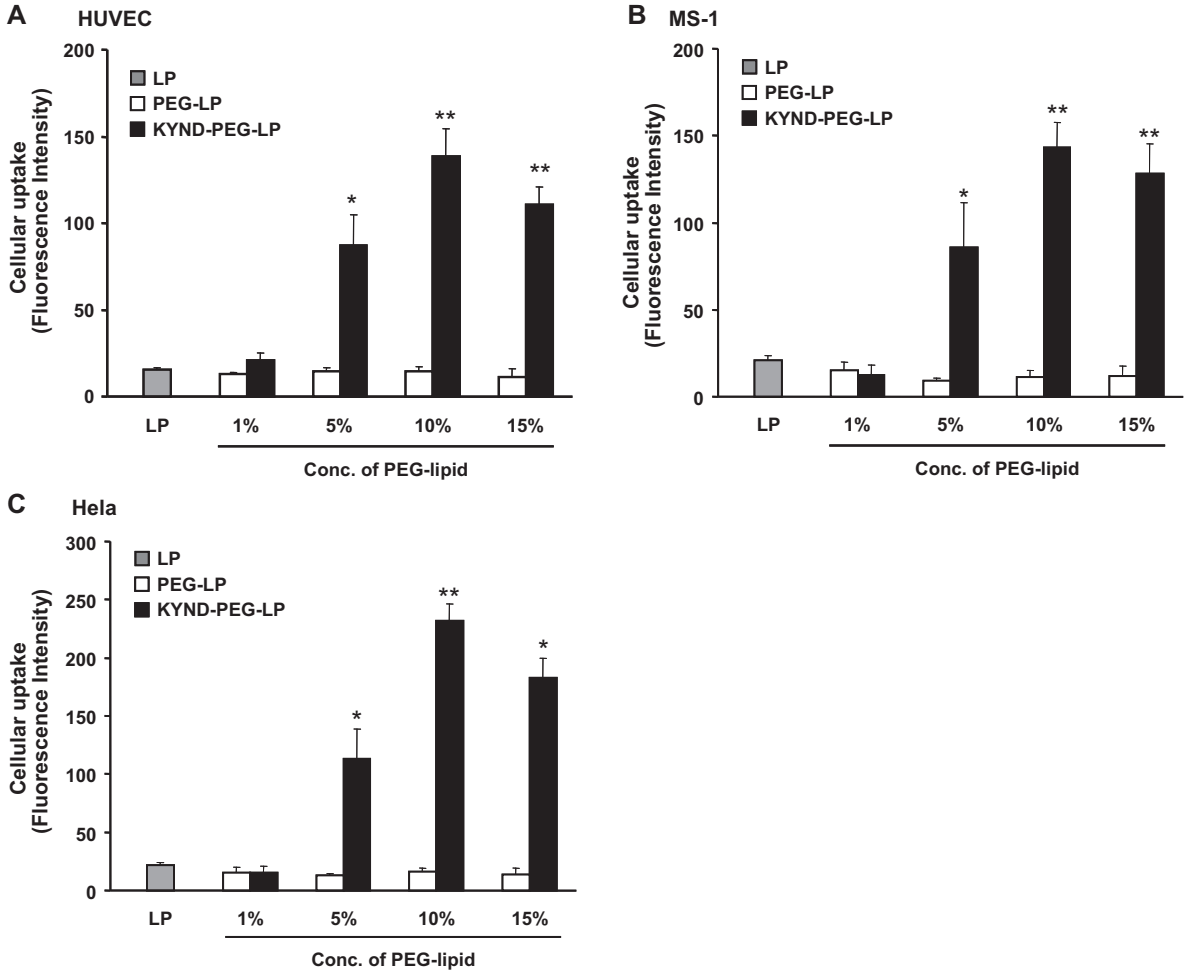

Fig. 3. Cellular uptake of KYND modified PEGylated liposomes. Different formulations of LPs were incubated with (A) HUVEC, (B) MS-1, or (C) Hela cells for 3 h and the amount of cellular uptake was determined as described in Section 2. Cellular uptake is expressed as the mean ± SD ($n = 3$). The statistical differences vs. LP were determined by one-way ANOVA followed by 'Dunnett test. $^{*}P < 0.05$; $^{**}P < 0.01$.

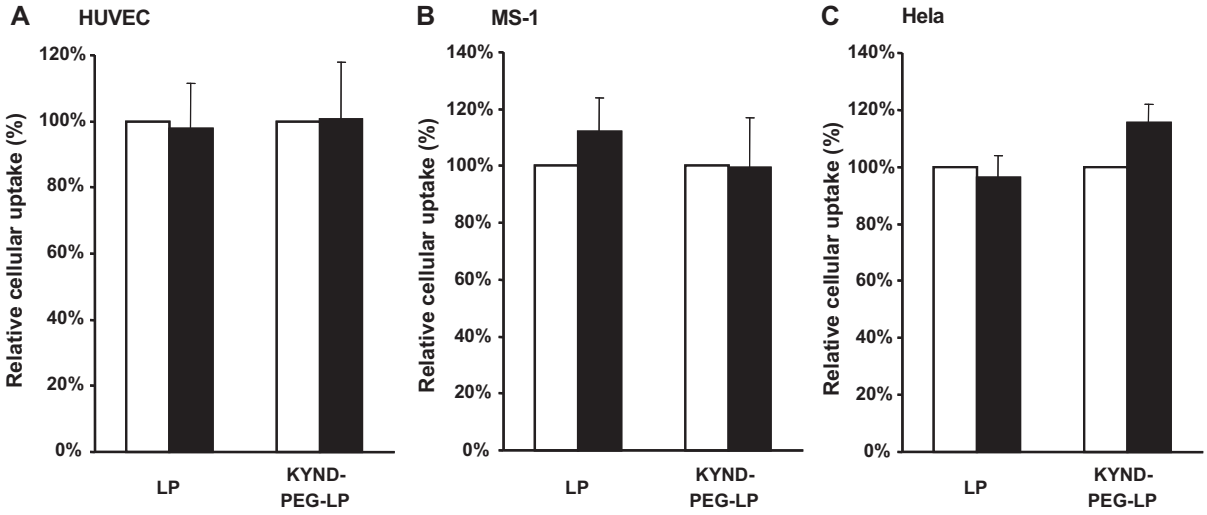

Fig. 4. Effect of free peptide on the cellular uptake of KYND modified PEG-LP. An excess amount of KYND peptide (25-fold vs. peptide used in LP formulation) was incubated for 1 h, and KYND modified PEG-LP was subsequently incubated with (A) HUVEC, (B) MS-1, or (C) Hela cells for 3 h. The amount of cellular uptake was determined as described in Section 2. Open bars and closed bars indicate the cellular uptake of LPs without free peptide and with free peptide, respectively. The relative cellular uptake was expressed as percentage of fluorescence measured in the absence of the free peptide.

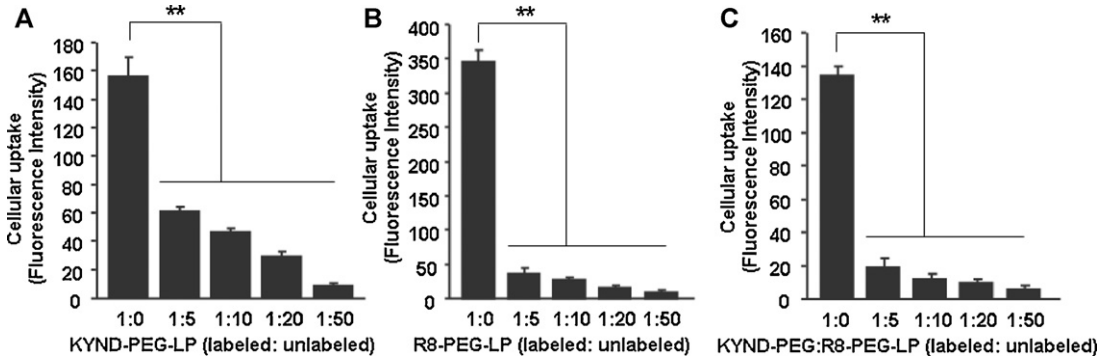

Fig. 5. Effect of peptide modified unlabeled PEG-LPs on the cellular uptake of peptide modified labeled PEG-LPs. Different concentrations of peptide modified unlabeled PEG-LPs are added to the specific amount of peptide modified rhodamine labeled PEG-LPs and incubated with 40,000 HUVEC for 3 h. Inhibition of cellular uptake of (A) rhodamine labeled KYND-PEG-LPs after incubation with unlabeled KYND-PEG-LPs, (B) rhodamine labeled R8-PEG-LPs after incubation with unlabeled R8-PEG-LPs and (C) rhodamine labeled KYND-PEG-LPs after incubation with unlabeled R8-PEG-LPs. The amount of cellular uptake was determined as described in Section 2. The statistical differences vs. KYND-PEG-LP (1:0) or R8-PEG-LP (1:0) were determined by one-way ANOVA followed by Dunnett test. $^{**}P < 0.01$.

somes had no influence on particle diameter, and slightly increased the ζ-potential compared to liposomes without STR-R8 (Table 1). As shown in Fig. 7, R8 modification resulted in only a minor effect on the cellular uptake of PEGyalated LPs, presumably because the R8 moiety is not able to interact with the cell surface due to steric hindrance by the PEG layer, and the KYND mediated cellular uptake of PEG-LP was observed. In the dual-ligand formulations modified with both KYND and R8, cellular uptake in all cell lines was greatly enhanced compared to KYND-PEG-LP, even though R8 was hindered by the PEG layer. These results suggest that a dual-ligand formulation comprised with two kinds of CPP ligands also exerted a synergistic effect on cellular association and uptake.

4. Discussion

It is well known that liposomes are very effective drug carriers for therapeutics. In a liposomal drug delivery system, polyethylene glycol (PEG)-modified liposomes are well known to be useful drug carriers for cancer therapy for drugs such as Doxil, PEGylated liposomes encapsulating doxorubicin (Perez et al., 2002; Sekiya and Imamura, 2008; Minisini et al., 2008). PEG modified liposomes have long-circulation time through avoidance of trapping by a reticuloendothelial system (RES) such as the liver and spleen (Sakakibara et al., 1996; Lasic, 1996). Through the EPR effect based on the structural features of the neovasculature, long-circulating liposomes can passively accumulate in tumor tissue. However, PEGylation inhibits cellular uptake and following the endosomal escape of liposomes a significant loss of pharmacological effect of the drugs is usually observed. To improve selectivity and cellular uptake, the modification of targeting ligands on the top of PEG in PEGylated liposomes (active targeting) is a potentially valuable approach to solving this problem. For tumor targeting, transferrin, antibodies, and peptides have been widely investigated as selective ligands for the targeting of over-expressed molecules in tumor cells and tumor endothelial cells such as the transferrin receptor (Ishida et al., 2001; Suzuki et al., 2008), the folic acid receptor (Stephenson et al., 2004; Yamada et al., 2008), membrane-type1 matrix metalloproteinase (Hatakeyama et al., 2007), aminopeptidase N (Pastorino et al., 2003; Garde et al., 2007), and Integrin $\alpha v\beta 3$ (Danhier et al., 2009; Dijkgraaf et al., 2007).

Angiogenesis has an important role in tumor growth and metastasis. Therefore, anti-angiogenic therapy by targeting tumor endothelial cells represents an attractive and important strategy in cancer therapy (Wu and Chang, 2010; Chang et al., 2009; Ogawara et al., 2009; Corti and Ponzoni, 2004). In the present study, we attempted to create a novel targeting ligand for TEM8 on tumor

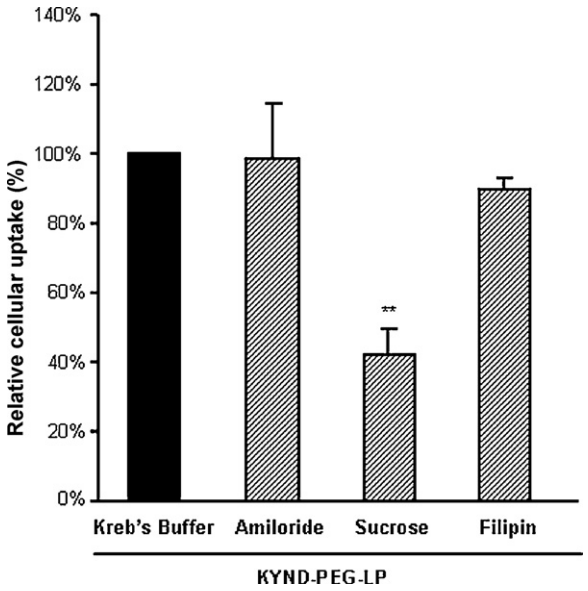

Fig. 6. Cellular uptake of KYND modified PEG-LP in the presence of different specific inhibitors. Hela cells were incubated with rhodamine labeled KYND-PEG-LP for 1 h in the absence (control) or presence of amiloride, hypertonic medium and filipin, respectively. Closed bar indicated the cellular uptake of KYND-PEG-LP. The relative cellular uptake was expressed as percentage of fluorescence measured from control.

endothelial cells. It was reported that the protective antigen (PA) of the anthrax toxin from *Bacillus anthracis* has the ability to recognize TEM8 (Bradley et al., 2001; Scobie and Young, 2005). Furthermore, domain 4 of PA (PA-D4) might be the optimal region for recognizing TEM8 (Singh et al., 1991; Brossier et al., 1999). Based on the previous reports, we designed a new peptide sequence, KKYNDKLPLY, derived from the PA-D4 which was predicted to act as a ligand for TEM8. The selected KYND peptide was attached to the top of PEG in PEGylated liposomes. The efficiency of the selected KYND peptide was estimated by evaluating the cellular uptake of the prepared PEGylated liposomes by TEM8 positive HUVEC cells, and TEM8 negative MS-1 and Hela cells. KYND modification altered the surface charge of PEGylated liposomes generated by lysine residues, resulting in a particle with a strong positive charge (Table 1). As shown in Fig. 3, PEGylation on the surface of the LPs (PEG-LPs) had a slight inhibitory effect on cellular uptake, indicating that PEGylation generates an aqueous layer on the surface of the LPs which inhibited the interaction of LPs with the cell surface. However, it was found that KYND modified PEG-LPs (KYND-PEG-LPs) showed a remarkable enhancement in cellular uptake compared to PEG-LPs in, not only TEM8 positive HUVEC cells, but also TEM8 negative MS-1 and Hela cells. The cellular uptake of PEG-LPs gradually increased with increasing mol% of KYND and the maximum enhancement was determined to be 10 mol% KYND modified PEG-LP but 15 mol% KYND modified PEG-LP showed an attenuation in cellular uptake in all cell lines. This can be explained either by the inhibition of the cellular association of LPs as the result of higher amounts of PEGylation or that the KYND peptide possesses any cytotoxicity on the cell lines used. The protein concentration was measured from the same samples as were used in the cellular uptake experiments and no remarkable difference in protein concentration was observed in the different LP preparations compared with non-treatment (data not shown), which indicates that the KYND peptide at all tested concentrations showed no toxic effects on cellular viability. From these results, it appears that higher amounts of PEGylation are responsible for the reduction in the cellular uptake of 15 mol% KYND modified PEG-LP. We have also evaluated the cellular uptake of PEG-LPs modified with reverse sequence of KYND motif peptide (briefly called NDK peptide) and the results showed that NDK motif peptide is not working as efficiently as KYND motif peptide (Supplementary Fig. 1), indicating that in KYND motif peptide, terminal cationic group might be responsible for electrostatic interactions with cell surface molecules which eventually exhibited the higher amount of cellular uptake. Furthermore, the excess amount of free KYND peptide had no influence on the cellular uptake of KYND modified PEG-LPs (Fig. 4) which indicated that either there is no specific receptor involved in the uptake pathway of KYND modified PEG-LPs or free monomeric KYND is not effective enough to inhibit the interactions of multiplex KYND-PEG-LPs with the target receptor. To confirm the receptor interactions, we have evaluated the cellular uptake of KYND modified rhodamine labeled PEG-LPs in presence of different concentrations of KYND modified unlabeled PEG-LPs (Fig. 5A). In this study, it was observed that the cellular uptake of KYND modified labeled PEG-LPs was inhibited by the addition of higher amount of KYND modified unlabeled PEG-LPs which indicated that there might have any specific receptor responsible for interaction with KYND motif peptide. These results provided the contradictory idea about the findings of the inhibition study in presence of free KYND motif peptide (Fig. 4). Therefore, the same experiment has been performed using octaarginine (R8) peptide which is a well known CPP, as shown in Fig. 5B. In this experiment, it was found that the cellular uptake of rhodamine labeled R8 modified PEG-LPs (R8-PEG-LPs) was remarkably inhibited after addition of unlabeled R8-PEG-LPs which might be due to the inhibition of electrostatic interactions of R8 peptide (present in labeled PEG-LPs) with cell surface molecules by higher concentration of R8 peptide present in unlabeled PEG-LPs. After getting these results, the same experiments has also been conducted using rhodamine labeled KYND-PEG-LPs and unlabeled R8-PEG-LPs (Fig. 5C). It was observed that the cellular uptake of KYND-PEG-LPs was significantly inhibited by the presence of higher concentration of R8 modified PEG-LPs. These results indicated that both of KYND motif peptide and R8 peptide enhance the cellular uptake of PEG-LPs by sharing the same cell surface molecules. Upon considering the results obtained from these experiments, it can be concluded that the KYND peptide motif selected for use is not a specific ligand for TEM8, but, rather, a CPP. According to definition of CPP, CPP consists of arginine or lysine residues (Mitchell et al., 2000; Wender et al., 2000) and amino acids with bulky hydrophobic side chains (Rothbard et al., 2002). KYND peptide we selected is composed of lysine residues and hydrophobic amino acids such as leucine and proline. Taking these facts into consideration, contrary to expectation, it was assumed that the KYND motif peptide acts not as a specific ligand for TEM8 but as a novel CPP peptide. The internalization of KYND peptide was significantly inhibited when cells were incubated in presence of sucrose (Fig. 6), which indicated that the internalization of new CPP is governed mainly by clathrin mediated endocytosis pathway. The selected sequence within PA-D4 of the anthrax toxin might be responsible for the attachment with target molecules and invasion inside of cells rather than the selectivity of the anthrax toxin. It is expected that other regions of PA-D4 would generate the selectivity for TEM8. As a result of our study, we conclude that the KYND motif peptide from anthrax toxin is, in fact, a novel CPP.

We recently reported on the development of a dual-ligand liposomal formulation modified with a selective ligand and a CPP peptide as a cationic ligand (Takara et al., 2010). Although targetability is achieved by introducing a specific ligand into the PEGylated liposomes, the amount of cellular uptake of the liposomes is restricted since receptor mediated endocytosis is a saturated pathway. To overcome this, we designed a dual-ligand formulation, in which a specific ligand is modified on the top of PEG, and an oligoarginine, as a CPP ligand, is modified on the surface of the liposome. In the dual-ligand formulation, oligoarginine on the surface is partially or fully masked by the PEG layer, which

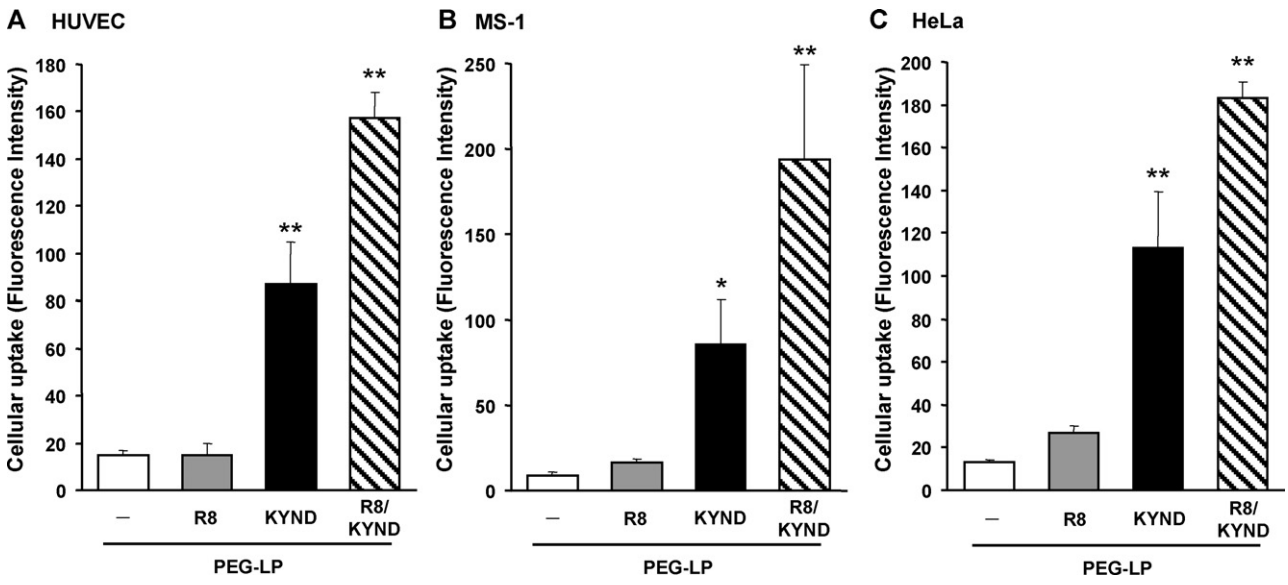

Fig. 7. Cellular uptake of dual-ligand PEGylated liposomes. Different formulations of PEG-LPs were incubated with (A) HUVEC, (B) MS-1, or (C) Hela cells for 3 h and the amount of cellular uptake was then determined as described in Section 2. Cellular uptake is expressed as the mean ± SD ($n = 3$). The statistical differences vs. unmodified PEG-LP were determined by one-way ANOVA followed by Dunnett test. $^{*}P < 0.05$, $^{**}P < 0.01$.

resulted in inactivity for cellular association. However, after the specific binding of the ligand with target molecules, the oligoarginine comes into close proximity to the cell surface, which permits oligoarginine to associate efficiently with the target cells. It should be noted that a liposomal carrier should be rationally designed so as to control the topology of the functional devices to permit their synergistic function. In the present study, we investigated whether a carrier designed with two types of CPP ligands would be applicable for a dual-ligand formulation by using a KYND motif peptide on the top of PEG and R8 as an oligoarginine on the surface of liposomes.

The relative values for cellular uptake derived form Fig. 7 were calculated and the results are shown in Table 2. STR-R8 modification on the surface membrane of PEG-LP had negligible or minor effects on cellular uptake due to the inhibition of interactions of the R8 moiety with the cellular surface by the PEG layer. On the other hand, KYND modification increased the cellular uptake of PEG-LP by 6–10-fold. Furthermore, in the case of a dual-ligand formulation (R8/KYND-PEG-LP), the efficiency of cellular uptake was 10–20-fold compared to PEG-LP (Fig. 7). Additionally, factor analysis by two-way ANOVA finds the both KYND dependent ($P < 0.001$) and R8 dependent ($P < 0.05$) uptake, and also indicates the interaction ($P < 0.05$) in three cells, which suggests the effect of R8 on cellular uptake is exerted mainly in the case of KYND-modified PEG-LP. It supposes that the dual-ligand formulation enhanced cellular uptake in a synergistic manner. The synergistic effect may result from that the R8 moiety, which is masked by PEG layer, is able to associate with the cellular surface after the binding of KYND on top of PEG to cells. We have also evaluated the cellular uptake of R8 and KYND modified PEG-LP (R8-PEG/KYND-PEG-LP) using HUVEC in which both of R8 and KYND motif peptide were grafted on the top of PEG, as shown in Supplementary Fig. 2. Presenting on the top of PEG, either R8 (R8-PEG-LP) or KYND motif peptide (KYND-PEG-LP) showed their maximum efficiency on the cellular uptake of PEG-LP (Supplementary Fig. 2A). In combination, presenting on the top of PEG, both of R8 and KYND motif peptide (R8-PEG/KYND-PEG-LP) also exhibited their maximum efficiency on cellular uptake of PEG-LP (Supplementary Fig. 2B); however, this effect seemed to be the additional effect of R8-PEG-LP and KYND-PEG-LP, not the synergistic effect. On the other hand, it was found that presenting on the surface of the lipid membrane PEG-LP, R8 (R8/PEG-LP) could not work efficiently (Supplementary Fig. 2B). However, compared to KYND-PEG-LP, R8/KYND-PEG-LP showed further enhancement of cellular uptake, though R8 was masked by PEG layer and this enhancement was also comparable to the uptake amount of R8-PEG/KYND-PEG-LP. These results indicated that after electrostatic interactions of KYND, R8 comes to close proximity of cell surface and exerted its synergistic effect on the cellular uptake of PEG-LP, which is important from the view point of designing a successful dual-ligand based delivery system.

Recently, we reported that the dual-ligand formulation composed of a specific ligand on the top of PEG and a CPP on the surface of liposome exerted synergistic effect on the cellular uptake of PEG-liposome (Takara et al., 2010). It seems reasonable to conclude that the dual-ligand formulation containing a combination of, not only a specific ligand and a CPP ligand, but also two kinds of CPP ligands functions in a synergistic manner and topology control of the attached ligands facilitated the enhancement of cellular uptake.

Table 2
Relative comparisons of cellular uptake obtained by the indicated formulations.

Cell line	PEG-LP	R8/PEG-LP	KYND-PEG-LP	R8/KYND-PEG-LP
HUVEC	1.0	1.0	5.9	10.7
MS-1	1.0	1.8	9.5	21.4
Hela	1.0	2.0	8.6	14.0

The uptake amount obtained by PEG-LP is set as the control.

5. Conclusion

A small fragment containing a series of amino acid residues (KKYNDKLPLY) that are found in one of the protein components of the anthrax toxin called protective antigen was selected for testing in this study. The selected peptide motif possesses strong capability for enhancing the cellular uptake of liposomes in a non-specific manner. Liposomes modified with the selected peptide were internalized via clathrin mediated endocytosis pathway. As a result of this study, a new nature origin cell-penetrating peptide (CPP) was identified from domain 4 of the protective antigen in the anthrax toxin. Furthermore, using the new CPP ligand, a dual-CPP ligand based PEGylated liposomal delivery system was developed which showed a synergistic effect on the cellular association of liposomes compared

to the single KYND peptide or STR-R8 modified PEGylated liposomes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2011.03.010.

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