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Short communication

Synthesis of a high functionality and quality lipid with gp130 binding hydrophobic peptide for the preparation of human glioma cell-targeted PEGylated liposomes

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Abstract

We developed high functionality and quality (HFQ) lipids for facile and rapid preparation of ligand-grafted PEGylated liposomes. Because HFQ lipids are designed to exhibit good water dispersibility, ligand-grafted PEGylated liposomes can be easily prepared using the post-insertion method. The aim of this study is to develop novel VTWTPQAWFQWV (VTW) peptide-HFQ lipid to target human glioma cells. In order to disperse in water, VTW-$(SG)_5$-lipid derivatives containing various amino acid residues with different charges were synthesized. Based on our previous work, $(SG)_5$, a serine-glycine repeated peptide with a discrete molecular weight, was used as a spacer between the VTW and lipid. Of the derivatives tested, VTW-K_3-$(SG)_5$-lipid with three lysine residues showed the highest dispersibility in water, and VTW-K_3-$(SG)_5$/PEGylated liposomes can be prepared using the post-insertion method. The sizes of PEGylated liposomes and VTW-K_3-$(SG)_5$/PEGylated liposomes were 69.5 ± 4.4 and 74.4 ± 5.0 nm, respectively. In addition, the zeta potentials of PEGylated liposomes and VTW-K_3-$(SG)_5$/PEGylated liposomes were -2.7 ± 1.6 and -1.3 ± 0.3 mV, respectively. We found that VTW-K_3-$(SG)_5$/PEGylated liposomes selectively associated with human glioma U251MG cells. We succeeded in developing water-dispersible VTW-K_3-$(SG)_5$-lipids for the preparation of VTW-grafted PEGylated liposomes for glioma cell targeting.

Keywords: liposomes; targeting; PEG; hydrophobic peptide; glioma

Introduction

Ligand-targeted liposomes have been widely investigated for the selective delivery of therapeutic agents such as anticancer drugs, plasmid DNA or siRNA to cancer cells [1-4]. It is known that polyethyleneglycol (PEG) ylation of the carrier surface is key to steric stabilization and reducing recognition by the reticuloendothelial system (RES) to prolong blood circulation times [5].

PEG is also commonly used as a spacer between lipids and ligand moieties such as antibodies, peptides, folate and sugars. Peptides in particular are useful ligands because numerous cell-binding peptides have been identified by phage display and combinatorial screening over the past decade [6]. Peptide-targeted carriers have therefore been developed as selective delivery vehicles for chemotherapeutic drugs and diagnostic agents [7]. However, the interaction between peptide ligands on the surface of carriers and their corresponding receptors can be affected by the steric hindrance of the PEG spacer, particularly in the case of hydrophobic peptides [8]. Moreover, conventional peptide-PEG-lipids show a broad molecular weight distribution. Because the dispersion of ligand peptide-PEG-lipids in water is dependent on their hydrophilicity/hydrophobicity balance, the post-insertion method may be infeasible if peptide ligands have hydrophobic properties.

Recently, we have reported high functionality and quality (HFQ) lipids for the preparation of active targeted PEGylated liposomes, and demonstrated the advantage of using serine-glycine repeats $(SG)_5$ spacer for peptide ligand presentation on the liposomal surface [9-11]. HFQ lipids have a discrete molecular weight and can be synthesized by solid phase peptide synthesis (SPPS). We synthesized KCCYSL (KCC) and GRGDS (RGD)-grafted lipids (KCC-$(SG)_5$-lipid and RGD-$(SG)_5$-lipid) by SPPS. This total synthetic method by SPPS for peptide-lipid preparation has advantages over post-conjugation of peptides to PEG-lipids, which can show molecular weight distribution, because SPPS is highly reproducible and side-reactions can be suppressed.

The preparation method used for peptide ligand-grafted liposome preparation is also important for clinical application. Microfluidic devices have recently been developed for the preparation of liposomal therapeutics under good manufacturing practice (GMP). Microfluidic devices enable simple, fast and highly reproducible preparation of liposomes by mixing lipids and drugs in a well-controlled manner. Ligand-

grafted liposomes can therefore be easily and reproducibly prepared by mixing functional lipid and pre-formed liposomes in a microfluidic channel [12], resulting in insertion of the functional lipid into liposomes if the lipid is highly dispersed in aqueous solution. A number of applications of microfluidic devices for the preparation of ligand-grafted liposomes have been reported, including transferrin and folic acid modification, which showed narrow size distribution and high reproducibility [12, 13]. There is therefore a distinct advantage to designing HFQ lipids that show good dispersibility in water. The KCC-$(SG)_5$-lipid and RGD-$(SG)_5$-lipid previously reported could be dispersed in water owing to the hydrophilic KCC and RGD ligand moiety; however, there are no reports of hydrophobic peptide ligands used in HFQ lipids, even conventional peptide-PEG lipids.

In the present study, we designed peptide-lipid derivatives with a hydrophobic peptide ligand and $(SG)_5$ spacer. We selected a hydrophobic peptide ligand, VTWTPQAWFQWV (VTW), which specifically binds to gp130 on human glioma U251MG cells (gp130 high expression), while showing low binding to human lung adenocarcinoma epithelial A549 cells (gp130 low expression) [14, 15]. We synthesized various VTW-$(SG)_5$-lipid derivatives with amino acid residues of different charge to tune their dispersibility in water. Furthermore, we prepared VTW-grafted PEGylated liposomes and evaluated their ability to target human glioma U251MG cells.

Material and methods

Materials

1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC), and rhodamine-DOPE were purchased from Avanti Polar Lipids (Alabaster, AL, USA). 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy (polyethylene glycol)-2000] ($mPEG_{2000}$-DSPE) was purchased from NOF (Tokyo, Japan). Rink Amide AM resin, the Fmoc-amino acids, N,N-dimethylformamide (DMF), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), trifluoroacetic acid (TFA) and triisopropylsilane (TIS) were purchased from Merck (Darmstadt, Germany). 1-Hydroxybenzotriazole (HOBt) was purchased from PEPTIDE INSTITUTE, Inc. (Osaka, Japan). N,N-Diisopropylethylamine (DIPEA) was purchased from

Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Dichloromethane (DCM), palmitic acid and cholesterol were purchased from NACALAI TESQUE Inc. (Kyoto, Japan). Piperidine, acetic anhydride and diethyl ether were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were reagent grade products obtained commercially.

Synthesis of VTW-$(SG)_5$-lipid derivatives

Dipalmitoyl-lysine-KSS-$(SG)_5$-K_3-VTWTPQAWFQWV (VTW-K_3-$(SG)_5$-lipid), dipalmitoyl-lysine-KSS-K_3-$(SG)_5$-VTWTPQAWFQWV (VTW-$(SG)_5$-K_3-lipid), dipalmitoyl-lysine-KSS-$(SG)_5$-K_2-VTWTPQAWFQWV (VTW-K_2-$(SG)_5$-lipid), dipalmitoyl-lysine-KSS-$(SG)_5$-VTWTPQAWFQWV (VTW-$(SG)_5$-lipid), and dipalmitoyl-lysine-ESS-$(SG)_5$-E_3-VTWTPQAWFQWV (VTW-E_3-$(SG)_5$-lipid) were synthesized using SPPS as previously reported, with slight modifications [9]. The Fmoc deprotection was performed with treatment with 20% piperidine for 20 min. The coupling of Fmoc-amino acids was performed in the presence of HBTU/HOBt/DIPEA for 30 min. Unreacted amino groups were acetylated in each step of the synthesis. After the coupling of Fmoc-Lys (Fmoc)-OH and deprotection of the Fmoc groups, palmitic acid (10 eq.) was coupled to the amino groups of the Lys residue in the presence of HBTU/HOBt/DIPEA. A Kaiser test was performed to confirm completion of the reaction. Lipids were cleaved and deprotected with TFA/TIS/H_2O (95/2.5/2.5) for 3 h. The crude products were precipitated in diethyl ether. The products were dispersed in water and dialyzed against water (MWCO: 6000-8000) for 4 days to remove acetylated peptides, and then lyophilized. The compounds were analyzed by MALDI-TOF-MS (Figure 1C-G).

Measurement of water dispersibility of VTW-$(SG)_5$-lipid derivatives

VTW-$(SG)_5$-lipid derivatives were dispersed in water at a concentration of 0.3 mM. After sonication and heating at 65 °C, the solution was centrifuged (7000×g, 25 °C, 1 min), and the supernatant was carefully removed. The residue was dried and desiccated overnight. The concentrations (mM) in Table 1 were calculated using the following formula:

Concentration (mM) = (total weight of lipid − (weight of tube with residue − weight of blank tube)/total weight of lipid) × 0.3

Preparation of liposomes

PEGylated liposomes were prepared by the lipid hydration method. DSPC, cholesterol and $mPEG_{2000}$-DSPE (55:35:10, molar ratio) were dissolved in methanol. 0.5 mol% rhodamine-DOPE was added to label the lipid membrane. A thin lipid film was formed by evaporation and vacuum desiccation overnight. The lipid film was hydrated at 65 °C in sterilized water, and sonicated for 3 min using a probe sonicator. VTW-K_3-$(SG)_5$/PEGylated liposomes were prepared by incubating PEGylated liposomes with 3 mol% of VTW-K_3-$(SG)_5$-lipid micelles at 60 °C for 1 h. The isotonic properties of the liposomes were adjusted by the addition of 10×phosphate buffered saline (10×PBS, pH 7.4). The liposome suspensions were filtered through 0.45-μm filters. The particle size and zeta potential were measured using a Zetasizer Nano ZS instrument (Malvern Instruments Ltd., Worcestershire, UK).

Cell culture

U251MG cells was provided from RIKEN Cell Bank (Tsukuba, Japan). A549 and MCF-7 cells were purchased from the European Collection of Cell Cultures (ECACC, Wiltshire, UK). Cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Bovogen, East Keilor, VIC, Australia), 100 U/mL penicillin and 100 ug/mL streptomycin (Wako, Osaka, Japan). The cells were maintained in an atmosphere of 5% CO_2 at 37 °C.

Cell association experiments

Cells were seeded in 24-well plates (5×10^4 cells/cm^2). After 24 h, cells were incubated in serum-free DMEM containing 25 μM of liposomes. After incubation, cells were washed two times with PBS, trypsinized, centrifuged (800×g, 25 °C, 3 min) and resuspended in PBS. Cells were analyzed using an LSR

Fortessa System (BD Biosciences, San Jose, CA, USA). After 24 h incubation with blank liposomes, cell viability in U251MG cells was assessed by WST-8 assay (Figure S1).

Confocal laser scanning microscopy

2.0×10^5 cells were seeded in a 35-mm glass based dish (Iwaki, Chiba, Japan). After 24 h, cells were incubated with serum-free DMEM containing 25 μM of liposomes. After 1 h, cells were washed with PBS and incubated with 200 nM Lysotracker® Green DND-26 (Life Technologies) for 1 h. After fixing with 4% PFA for 20 min, nuclei were stained with DAPI (5 μg/mL), and cells were mounted. Confocal images were acquired using a Carl Zeiss LSM710 (Germany).

Endocytosis pathway study and blocking experiment

U251MG cells were seeded in 24-well plates (5×10^4 cells/cm^2). After 24 h, cells were pre-incubated with 0.4 M sucrose, 200 μM genistein, 50 μM 5-(N-ethyl-N-isopropyl)-amiloride (EIPA), or at 4 °C for 30 min. Cells were then incubated with 25 μM of VTW-K_3-$(SG)_5$/PEGylated liposomes in the presence of inhibitors or at 4 °C for 90 min. In the blocking experiment, cells were pre-treated with peptides (200-fold of control peptide $(SG)_5$ or 100, 200 and 500-fold of blocking peptides, the final concentration of DMSO 1%) at 4 °C for 30 min. Cells were then incubated with 25 μM of liposomes at 4 °C for 60 min. After incubation, cells were washed two times with PBS, trypsinized, centrifuged (800×g, 25 °C, 3 min) and resuspended in PBS. Cells were analyzed using an LSR Fortessa System. After 24 h of pre-treatment with peptides, the toxicity of each peptide at a defined concentration was assessed by WST-8 assay (Figure S2).

Statistical analysis

ANOVA was used to analyze the statistical significance of differences between groups. For comparison between two groups, unpaired Student's t-test was used. Dunnett's test was used for multiple comparisons between control and treatment groups. Difference with $p<0.05$ was considered significant.

Results and discussion

Synthesis and evaluation of water dispersibility of VTW-$(SG)_5$-lipid derivatives

First, we synthesized VTW-$(SG)_5$-lipid based on the structure of previously developed HFQ lipids [9] (Figure 1A), however, VTW-$(SG)_5$-lipid could not be dispersed in water due to the hydrophobicity of the VTW peptide (Table 1). We therefore designed and synthesized VTW-$(SG)_5$-lipid derivatives with amino acid residues of different charge (lysine and glutamic acid), and compared their dispersibility in water (Figure 1B). As shown in Table 1, dispersibility in water increased with increasing number of K residues, with VTW-K_3-$(SG)_5$-lipid showing the greatest dispersibility in water (100%, 0.30 mM) of all derivatives. Interestingly, the introduction of K_3 behind the spacer (VTW-$(SG)_5$-K_3-lipid) reduced the dispersibility in water (68%, 0.20 mM). Introduction of E (VTW-E_3-$(SG)_5$-lipid) instead of K significantly reduced the dispersibility in water (25%, 0.07 mM). Islam et al reported the effects of short poly-amino-acid tags on the solubility of proteins, and poly-Lysine showed a greater effect on solubility than poly-Glutamic acid, owing to the higher isoelectric point of Lys [16]. This report supports our observations for VTW-$(SG)_5$-lipid derivatives dispersibility in water. In addition, the position of the Lys residue is an important factor, suggesting that addition of K_3 between the VTW and $(SG)_5$ portions of the lipid may enhance dispersibility.

Preparation and characterization of liposomes

Next, we prepared VTW-grafted PEGylated liposomes by post-insertion of 3 mol% of VTW-K_3-$(SG)_5$-lipid into pre-formed PEGylated liposomes. As shown in Table 2, this resulted in a slight increase in mean liposome diameter, from 69.5 ± 4.4 to 74.4 ± 5.0 nm. The zeta potentials were slightly negative for

PEGylated liposomes and VTW-K_3-$(SG)_5$/PEGylated liposomes. Therefore, post-insertion of 3 mol% of VTW-K_3-$(SG)_5$-lipid into PEGylated liposomes may have little effect on the size and zeta potential.

Selective binding of VTW-K_3-$(SG)_5$/PEGylated liposomes to U251MG cells

To evaluate the selectivity of VTW-K_3-$(SG)_5$/PEGylated liposomes, cellular association experiments with U251MG (gp130 high expression) and A549 and MCF-7 (gp130 low expression) cells [14, 17] were performed using flow cytometry. The mean fluorescence intensity of VTW-K_3-$(SG)_5$/PEGylated liposomes in U251MG cells at 3 h was 13.6-fold greater than that of PEGylated liposomes (Figure 2A). By comparison, the change in mean fluorescence intensity in A549 and MCF-7 cells at all time points was significantly lower than those observed for U251MG cells (Figure 2B and Figure S3). These results suggest that VTW-K_3-$(SG)_5$/PEGylated liposomes bind more selectively to human glioma U251MG cells than non-glioma cancer cells.

Intracellular distribution of VTW-K_3-$(SG)_5$/PEGylated liposomes

Intracellular distribution of VTW-K_3-$(SG)_5$/PEGylated liposomes in U251MG cells and A549 cells was evaluated by confocal microscopy. U251MG cells treated with VTW-K_3-$(SG)_5$/PEGylated liposomes showed greater fluorescence than those treated with PEGylated liposomes (Figure 3A). Negligible fluorescence was observed in A549 cells treated with PEGylated liposomes or VTW-K_3-$(SG)_5$/PEGylated liposomes (Figure 3B). These data support the findings of the cellular association experiment (Figure 2). VTW-K_3-$(SG)_5$/PEGylated liposomes (red fluorescence signals) were co-localized with the lysosome marker (green fluorescence signals) in U251MG cells, suggesting their rapid internalization and sorting to late endosomes/lysosomes.

Investigation of endocytosis pathway of VTW-K_3-$(SG)_5$/PEGylated liposomes

Ligand-targeted nanoparticles are predominantly taken up by endocytosis pathways, including clathrin- and caveolae-mediated endocytosis and micropinocytosis [18, 19]. To clarify the internalization mechanism of VTW-K_3-$(SG)_5$/PEGylated liposomes in U251MG cells, we conducted cellular uptake experiments using different inhibitors (Sucrose: clathrin-mediated endocytosis inhibitor; Genistein: caveolae-mediated endocytosis inhibitor; EIPA: macropinocytosis inhibitor). Sucrose, EIPA, and 4°C (inhibition of energy-dependent endocytosis) incubation significantly reduced the uptake of VTW-K_3-$(SG)_5$/PEGylated liposomes (Figure 4A). Wu et al reported that VTW peptide may bind to gp130, a highly expressed receptor in human glioma cells [14]. A competition experiment using gp130 binding peptides [14] (VTWTPQAWFQWVKKK (VTW) and TWSPEAWKKK (TWS)) was therefore performed. As shown in Figure 4B, binding of VTW-K_3-$(SG)_5$/PEGylated liposomes to U251MG cells was significantly inhibited by treatment with gp130 binding peptides depending on the amount of peptides. However, binding of VTW-K_3-$(SG)_5$/PEGylated liposomes to U251MG cells was not completely inhibited by pre-incubation of blocking peptides at a concentration of 500-fold molar excess of peptides, therefore, further experiments such as knockdown of gp130 gene would be needed to investigate their uptake mechanism in detail. These results suggest that VTW-K_3-$(SG)_5$/PEGylated liposomes bind to gp130 and are then taken up by U251MG cells via macropinocytosis or clathrin-mediated endocytosis. VTW peptide-mediated gene and protein delivery to human glioma cells has been reported [14, 15, 20], however, the endocytosis pathway was unclear. This is the first study reporting an internalization mechanism for VTW-grafted nanocarriers.

Conclusion

Total synthesis of HFQ lipids with a discrete molecular weight is possible using SPPS. In the present study, we designed and synthesized novel VTW-$(SG)_5$-lipid derivatives containing various amino acids to tune their dispersibility in water. As a consequence, it was established that VTW-K_3-$(SG)_5$-lipid

has high water dispersibility. VTW-K_3-$(SG)_5$/PEGylated liposomes can be prepared using the post-insertion method. A cell association study revealed that VTW-K_3-$(SG)_5$/PEGylated liposomes were taken up by human glioma U251MG cells via VTW recognition. We succeeded in developing VTW-HFQ lipid to prepare VTW-grafted PEGylated liposomes for targeting glioma cells. Although further studies about *in vivo* targeting ability of VTW-K_3-$(SG)_5$/PEGylated liposomes, these findings will be valuable for the targeting system to glioma using VTW-HFQ lipid.

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Disclosure

The author reports no conflicts of interest in this work.

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specific peptides and proteins, Int. J. Pharm. 422 (2012) 264–270.
doi:10.1016/j.ijpharm.2011.11.008.

337 **Table 1.** Dispersibility of VTW-$(SG)_5$-lipid derivatives in water

VTW-$(SG)_5$-lipid derivatives	Dispersed lipid% of total lipid	Concentration (mM)
Dipalmitoyl-lysine-KSS-$(SG)_5$-VTWTPQAWFQWV (VTW-$(SG)_5$-lipid)	0	0
Dipalmitoyl-lysine-KSS-$(SG)_5$-K_2-VTWTPQAWFQWV (VTW-K_2-$(SG)_5$-lipid)	45	0.13
Dipalmitoyl-lysine-KSS-$(SG)_5$-K_3-VTWTPQAWFQWV (VTW-K_3-$(SG)_5$-lipid)	100	0.30
Dipalmitoyl-lysine-KSS-K_3-$(SG)_5$-VTWTPQAWFQWV (VTW-$(SG)_5$-K_3-lipid)	68	0.20
Dipalmitoyl-lysine-ESS-$(SG)_5$-E_3-VTWTPQAWFQWV (VTW-E_3-$(SG)_5$-lipid)	25	0.07

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340 **Table 2.** Physicochemical properties of liposomes. Data represent mean ± standard deviation (n = 3).

	Particle size (nm)	ζ-potential (mV)	polydispersity index
PEGylated liposomes	69.5 ± 4.4	-2.7 ± 1.6	0.273 ± 0.096
VTW-K_3-$(SG)_5$/PEGylated liposomes	74.4 ± 5.0	-1.3 ± 0.3	0.261 ± 0.079

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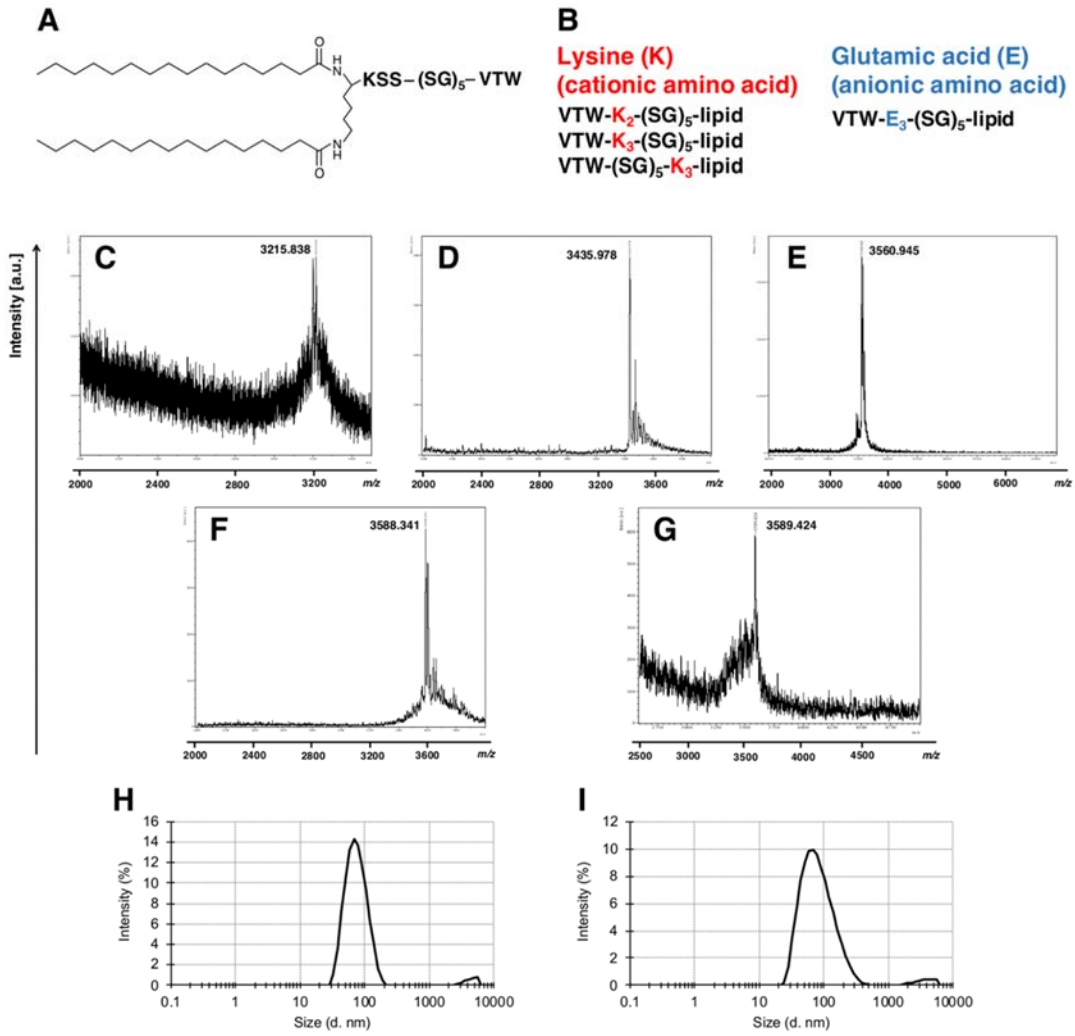

Figure 1. (A) Chemical structure of VTW-$(SG)_5$-lipid**.** (B) illustration of VTW-$(SG)_5$-lipid derivatives. (C) MALDI-TOF-MS spectrum of VTW-$(SG)_5$-lipid. Expected mass 3175.7. Found mass 3215.838 $[M + K]^+$. (D) MALDI-TOF-MS spectrum of VTW-K_2-$(SG)_5$-lipid. Expected mass 3432.1. Found mass 3435.978 $[M + 3H]^+$. (E) MALDI-TOF-MS spectrum of VTW-K_3-$(SG)_5$-lipid. Expected mass 3560.2. Found mass 3560.945 $[M + H]^+$. (F) MALDI-TOF-MS spectrum of VTW-$(SG)_5$-K_3-lipid. Expected mass 3560.2. Found mass 3588.341 $[M + Na]^+$. (G) MALDI-TOF-MS spectrum of VTW-E_3-$(SG)_5$-lipid. Expected mass 3564.0. Found mass 3589.424 $[M + Na]^+$. Size distribution of PEGylated liposomes (H) and VTW-K_3-$(SG)_5$/PEGylated liposomes (I) measured by dynamic light scattering.

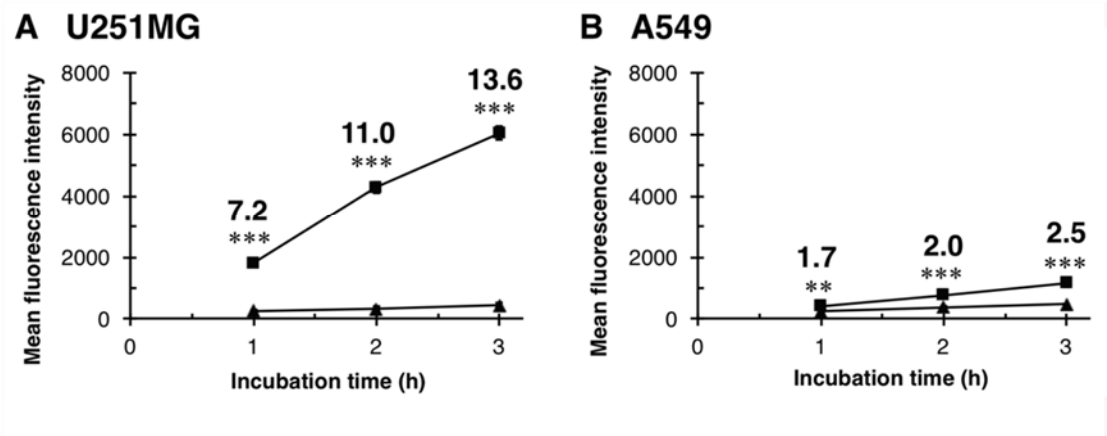

Figure 2. Selective binding of VTW-K_3-$(SG)_5$/PEGylated liposomes to U251MG cells. Cellular association time-course *in vitro* for 25 μM of PEGylated liposomes (*filled triangles*) and VTW-K_3-$(SG)_5$/PEGylated liposomes (*filled squares*) with U251MG cells (A) and A549 cells (B). Results are the mean ± standard deviation of triplicates. The values show the change in mean fluorescence intensity compared with PEGylated liposomes. An unpaired Student's t-test was performed to analyze the significance (**p<0.01, ***p<0.001).

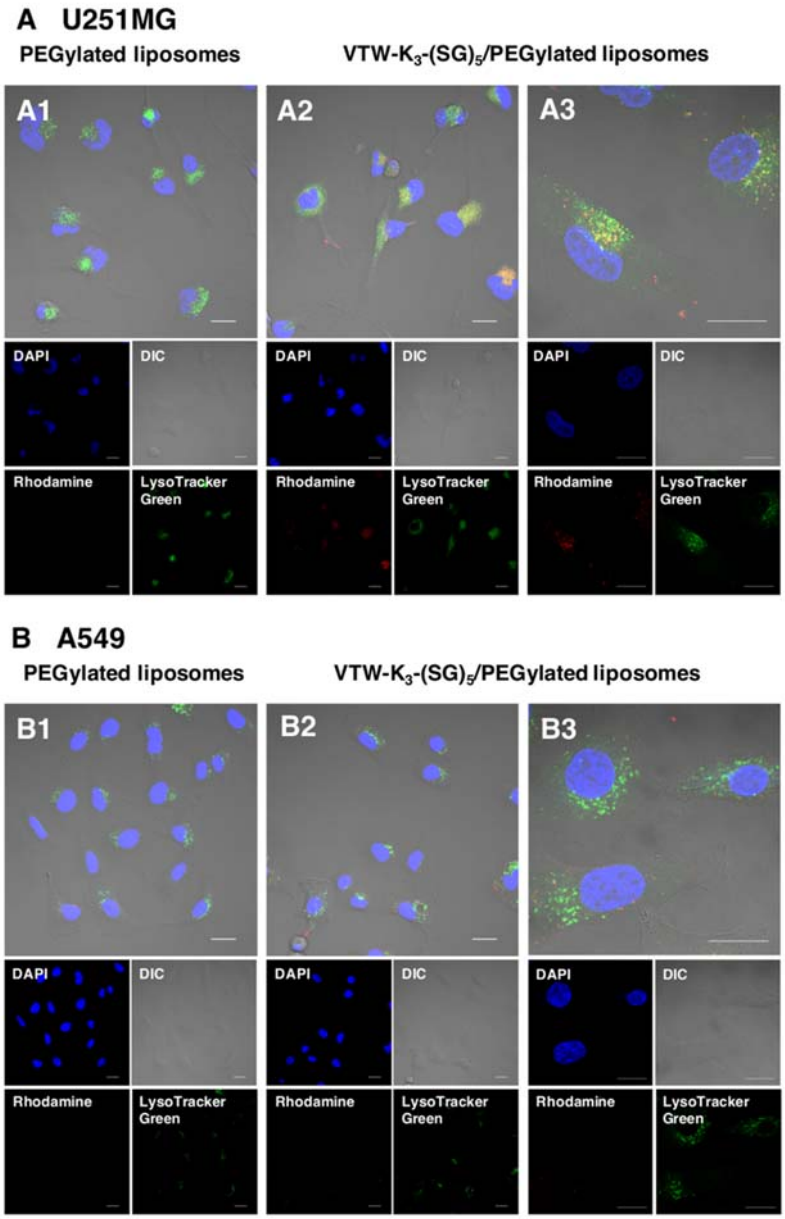

Figure 3. Confocal microscopy images of U251MG cells (A) and A549 cells (B). Cells were incubated with 25 μM of PEGylated liposomes or VTW-K_3-$(SG)_5$/PEGylated liposomes for 1 h. Nuclei and late endosomes/lysosomes were stained with DAPI (blue) and LysoTracker Green (green), respectively. Liposomes are indicated by red fluorescence (rhodamine). 40× (A1-2 and B1-2) and 100× (A3 and B3) oil-immersion objectives were used to visualize fluorescence (scale bar = 20 μm).

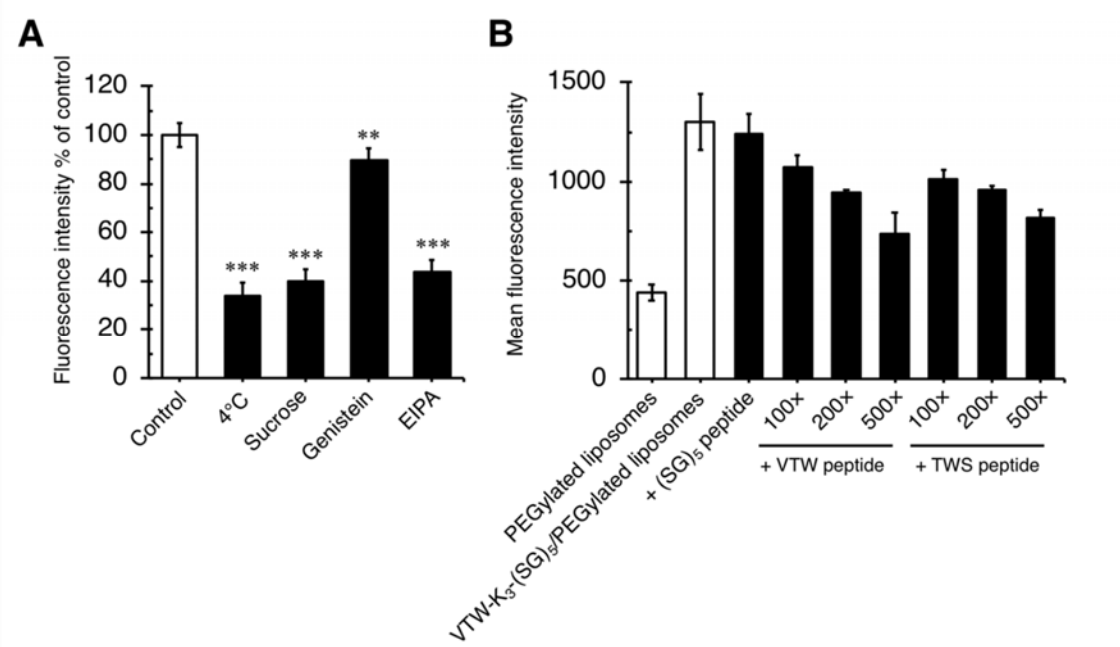

Figure 4. Endocytosis pathway study (A) and blocking experiment with VTW and TWS peptide (B) in U251MG cells. After pre-incubation with the inhibitors and peptides, the cells were incubated with 25 μM of VTW-K_3-$(SG)_5$/PEGylated liposomes. A group treated with VTW-K_3-$(SG)_5$/PEGylated liposomes only was used as a control. Results are expressed as the mean ± standard deviation of triplicates. One-way ANOVA and the Dunnett's test were performed to analyze the significance (**p<0.01, ***p<0.001).