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A liposomal delivery system that targets liver endothelial cells based on a new peptide motif present in the Apo B-100 sequence

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Abstract
Liver dysfunction is associated with a variety of liver diseases, including viral or alcoholic hepatitis, fibrosis, cirrhosis, and portal hypertension. A targeted drug delivery system would be very useful in the treatment of these diseases. We herein describe the development of a system comprised of a new peptide-lipid conjugate for the efficient delivery of molecules to LEC. The RLTRKRGLK sequence (3359-3367), which mediates the association of LDL with arterial CSPG and an LDL receptor, was utilized as a ligand for achieving this goal. The peptide modified PEG-LPs (RLTR-PEG-LPs) were efficiently taken up by primary liver endothelial cells (Liver ECs) and other types of cells. In vivo biodistribution and confocal microscopy analysis showed that RLTR-PEG-LPs became widely accumulated in LECs within a short time. Distribution of RLTR-PEG-LPs was greatly reduced with a pretreatment of unlabeled RLTR-PEG-LPs, not cationic LPs, indicating that the sequence is important for LECs. The findings indicate that a reverse sequence of RLTR (KLGR) modified PEG-LPs (KLGR-PEG-LP) did the same pattern compared with RLTR-PEG-LPs, suggesting that the RKR or RXXR sequence might be essential for LECs targeting. Collectively RLTR-PEG-LPs and KLGR-PEG-LPs have the potential for delivering drugs to LECs.

Key words
ApoB-100 sequence, CSPG, LDL receptor, Liver endothelial cell, RLTR peptide, KLGR peptide
Introduction

The liver is the largest organ of the body and is probably the most important power and sewage treatment plant in the body. Two major types of cells populate the liver, namely, parenchymal and non-parenchymal cells. Approximately 80% of the liver volume is made up of parenchymal cells commonly referred to as hepatocytes (Ramadori et al., 2008). Sinusoidal endothelial cells, Kupffer cells and hepatic stellate cells are examples of non-parenchymal cells. Different types of liver diseases are associated with different types of liver cells. For example viral hepatitis and alcoholic hepatitis are associated with hepatocytes. Liver endothelial cell (LEC) dysfunction is associated with variety of liver diseases, including fibrosis, cirrhosis, and portal hypertension (Dominique et al., 2010). The defenestration of liver endothelial cells causes hyperlipidemia, because it becomes difficult for lipoproteins to reach hepatocytes (Rajkumar et al., 2010). Kupffer cells are associated with the progression of non-alcoholic steatosis and fibrosis. It has also been reported that hepatocellular stress caused by various diseases causes the release of different types of cytokines and chemokines by different types of cells which ultimately cause the transmigration of inflammatory cells towards their target, hepatocytes (Ramadori et al., 2008). Therefore, a selective drug delivery system would be an ideal approach for achieving a subsequent efficient therapy for treating different types of liver diseases.

A group of certain basic proteins or peptides have the ability to inhibit the binding of low density lipoprotein (LDL) to its receptor protein (Brown et al., 1978). This inhibition is caused by polycations interacting with the receptor. LDLs are associated with a negatively charged LDL receptor even though the net charge of this lipoprotein is also negative. This suggests that the net charge of the LDL is governed by the positive charge of the ApoB sequence. Two basic regions of similar size in ApoB-100 segments, namely 3147 through 3157 and 3359 through 3367 are part of the LDL receptor binding domain. This ApoB heterodimer binds to the LDL receptor and also binds with Glycopeaminoglycans (GAGs) with an affinity similar to that between LDL and GAGs (Urban et al., 1997). The ApoB-100 segment RLTRKRGLK (3359-3367) is a mediator of the association between LDL and arterial Chondroitin sulfate-rich proteoglycan (CSPG) (Urban et al., 1993). It has recently been reported that LEC express low density lipoprotein receptor protein-1 (LRP-1) which is a member of the LDL receptor gene family (Oie et al., 2011, Thomas et al., 1999). Another study has shown that LDL is taken up by both parenchymal and non-parenchymal cells (Marit et al., 1998).

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)). Based on these considerations, we selected the ApoB segment RLTRKRGLK (3359-3367) abbreviated here as RLTR for use as a novel ligand in designing a selective targeting system for hepatocytes.
Surprisingly, however, this carrier system was accumulated through the blood vessels in the liver. In order to examine the targeting ability of this RLTR modified liposome, our efforts were focused on two parameters, one being the cationic nature of this peptide and second the essential peptide sequence.

2. Materials and methods

2.1. Materials

Cholesterol (Chol), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), diethanolamine chloride (DC-6-14), Egg phosphatidylcholine (EPC), 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] (PEG₂₀₀₀-DSPE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). N-[(3-maleimide-1-oxopropyl)aminopropyl(polyethylene glycol-carbamyl)] distearoylphosphatidyl-ethanolamine (maleimide-PEG-DSPE) was purchased from Nippon Oil and Fat Co. (Tokyo, Japan). ³H-Cholesteryl hexadecyl ether (CHE) were purchased from New England Nuclear (USA). RLTRKRGLKGGC (RLTR in brief) and KLGRKRTLRCGC (KLGR in brief) peptides were purchased from Kurabo Industries, Osaka, Japan. Endothelial Cell Basal Medium (EBM-2) and other related growth factors were purchased from Lonza (Walkersville, MD, USA). Dulbecco's fetal bovine serum (FBS) was obtained from Hyclone Laboratories (Logan, UT, USA). All other chemicals used in this study were of analytical grade.

2.2. Animals

4-5 week old male ICR mice were purchased from Japan SLC (Shizuoka, Japan). The experimental protocols were reviewed and approved by the Hokkaido University Animal Care Committee in accordance with the guidelines for care and use of Laboratory animals. Animals were used without fasting in all experiments.

2.3. Conjugation of the RLTR peptide to PEG₂₀₀₀-DSPE

Peptides conjugated with Glycine-Glycine-Cysteine (GGC) sequence at the N-terminal were purchased from commercial sources. Actually the GGC linker was added to the N-terminal to facilitate the binding of the thiol group of cysteine residue to the pyrrole group of Maleimide-PEG₂₀₀₀-DSPE. The additional Gly-Gly (GG) amino acid was added to increase the flexibility of the peptide ligand attached on the top of Mal-PEG₂₀₀₀-DSPE. Conjugation was achieved by incubating a 1.2:1 molar ratio of RLTRKRGLKGGC peptide and maleimide-PEG-DSPE in deionized water at room temperature for 24 hrs. The conjugation of RLTR with PEG was confirmed 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 a 10
mg/ml solution of dihydroxybenzoic acid.

2.4. Preparation of liposomes

Liposomes (LPs) composed of EPC/Chol (molar ratio: 7/3) was prepared by the lipid hydration method. A control cationic LP was prepared using DC6-14, DOPE, and Cholesterol at a molar ratio of 4:3:3 (Ishiwata et al., 2000). RLTR peptide modified PEG-LPs (RLTR-PEG-LPs) were prepared by adding the required amount of RLTR-PEG to the lipid solution. 1 mol% Rhodamine-DOPE was incorporated, to serve as a label for the lipid component. A lipid film was produced 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 was incubated for 10 min to hydrate the lipid film. The glass tube was then sonicated for approximately 30 sec in a bath-type sonicator (AU-25C, Aiwa, Tokyo, Japan). The mean size and zeta potential of the prepared LPs were determined using a Zetasizer Nano ZS ZEN3600 instrument (Malvern Instruments Ltd., Worcestshire, UK).

2.5. Isolation of Primary liver endothelial cells (Liver ECs)

Liver endothelial cells (Liver ECs) were isolated as previously described (Hida et al., 2004, Akino et al., 2009, Ohga et al.). Briefly, the liver of a female KSN mouse was excised. The excised tissue was minced and digested with collagenase II (Worthington, Freehold, NJ). Blood cells were removed by a single sucrose step-gradient centrifugation with Histopaque 1077 (Sigma-Aldrich), and the resulting cell suspension was filtered. Endothelial cells were isolated using MACS according to the manufacturer's instructions using a FITC-anti-CD31 antibody. CD31-positive cells were sorted and plated on 1.5% gelatin-coated culture plates and grown in EGM-2MV (Clonetics, Walkersville, MD) and 10% fetal bovine serum. After subculturing for 2 weeks, the isolated ECs were purified by a second round of purification using FITC-BS1-B4 (Vector Laboratories, Burlingame, CA). All of the endothelial cells were split at a ratio of 1:3.

2.6. In vitro 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). After 24 hrs, the prepared rhodamine labeled PEG-LPs/RLTR-PEG-LPs were added and incubated for an additional 3 hrs. After the incubation, the cells were washed with PBS (pH 7.4) 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 supernatants were then collected. 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 590nm) using FP-750 Spectrofluorometer (JAS Co, Tokyo, Japan).

2.7. In vivo Biodistribution study
3H-Cholesterol hexadecyl ether (CHE) labeled LPs and RLTR-PEG-LPs were used to measure the biodistribution of liposomes in different organs in the mice. ICR mice were intravenously injected with 3H-labeled LPs or RLTR-PEG-LPs. After 25 min, the animals were sacrificed; the portal vein was cut and a needle was introduced into the vena cava and 10-15 ml of heparin containing PBS (40 units/ml) solution was used to remove the remaining blood and cell surface bound RLTR-PEG-LPs in the liver. Other organs, including the lungs and kidney were also collected and all of the collected organs were weighed. After weighing, the samples were solubilized in Soluene-350 (Perkin-Elmer Life Sciences, Japan) for overnight at 55 °C. Samples were decolorized by treatment with H₂O₂. The radioactivity of the samples was measured by using a liquid scintillation counting (LSC-6100, Aloka, Japan) after adding 10 ml of Hionic Flour (Perkin-Elmer Life Sciences, Japan) (Hatakeyama et al., 2004). Tissue accumulation of LPs was represented as the percentage of injected dose (%ID) per organ.

2.8. Confocal microscopy experiment

ICR mice were given intravenous injection of Rhodamine labeled RLTR-PEG-LPs and the mice were killed 25 min after the treatment. The liver was perfused as mentioned in the section 2.7 and then it was collected. The liver was then excised and washed with saline and sliced into 10-15 mm-sized blocks with scissors. The liver sections were then incubated with a 20 fold volume of a diluted solution of Hoechst 33342 (1mg/ml) and Isolectin B4 in HEPES buffer for 1 hr. The specimens were placed on a 35 mm glass base dish (IWAKI, Osaka, Japan) and observed by confocal laser scanning microscopy (A1 Confocal Laser Microscope System, Nikon Instruments Inc., Tokyo, Japan).

2.9. Inhibition assay

2.9.1 In vivo competitive inhibition study of RLTR-PEG-LPs

ICR mice were injected with unlabeled LPs and after 15 min, they were injected with cationic LP or RLTR modified PEG-LP or KLGR (reverse peptide sequence of RLTR) modified PEG-LP. After another 25 min of incubation the mice were sacrificed and the livers were perfused with 10 ml of a 40% heparin-PBS solution. The mice livers were then collected, sliced into 0.5mm x 0.5 mm pieces, stained with Hoechst 33342 and isolectin B4 and then observed by confocal microscopy (A1 Confocal Laser Microscope System, Nikon Instruments Inc., Tokyo, Japan).

2.9.2 In vitro competitive inhibition study of RLTR-PEG-LPs

For in vitro inhibition study unlabeled RLTR-PEG or KLGR-PEG modified LPs were used as inhibitors. We previously used an excess amount of free RLTR or KLGR peptide as an inhibitor but no significant inhibition was observed (data not shown). It was reported that the monomeric free peptide might not be sufficiently effective to inhibit the interactions of multiplex RLTR-PEG-LP or
KLGR-PEG-LP with the target receptor (Kibria et al., 2011). As a result we used unlabeled RLTR-PEG or KLGR-PEG modified LP as inhibitors in order to achieve multivalent attachment with the targeted receptors. Here 40,000 LECs were seeded in a 24-well plate and the plate was incubated overnight. After 24 hours, different concentrations of rhodamine labeled and unlabeled PEG-LPs (1:0, 1:5, 1:10, 1:20 and 1:50 respectively) were added and incubated for 3 hrs. After 3 hrs, the cells were washed 3 times with 1 ml of ice-cold phosphate buffer saline (PBS) which was supplemented with heparin (20 units/ml) to completely remove the surface-bound RLTR-PEG-LP and the intracellular fluorescence intensity of rhodamine was then determined (Kibria et al., 2011).

2.10. Statistical analysis
Comparisons between multiple treatments were made using one-way analysis of variance (ANOVA), followed by the ‘Dunnett test’. Pair-wise comparisons of subgroups were made using the student’s t-test. Differences among the means were considered to be statistically significant at a p-value of <0.05 and <0.01.

3. Results
3.1. Synthesis of RLTR-PEG-DsPE
The thiol group of the cystein residue in the RLTR peptide was conjugated by reaction with Mal-PEG2000-DsPE at 37°C for 24 hours (Reaction scheme is shown in the supplementary figure 1). MALDI-TOF MS analyses confirmed the synthesis of RLTR-PEG-DsPE.

3.2. The characteristic of RLTR-PEG-LPs and its cellular uptake
The selected RLTR peptide was attached to the top of PEG in PEGylated liposomes. PEG liposomes (PEG-LPs) and RLTR modified PEG liposomes (RLTR-PEG-LPs) were prepared by incorporating PEG-DsPE or RLTR-PEG-DsPE at levels of 1, 3, 5, or 10 mol% of the total lipid. The physical properties of the prepared LPS are shown in Table 1. To evaluate the effect of the RLTR peptide on cellular uptake, we next examined the cellular uptake of RLTR-PEG-LPs and PEG-LPs in primary liver endothelial cells (LECs) and in Hepa1-6 cell line. The RLTR peptide enhanced the cellular uptake of PEG-LPs and the maximum cellular uptake was observed within 3 mole% of RLTR peptide modification in both the cells (Fig. 1A-B).

3.3. In vivo selectivity of RLTR peptide
A biodistribution study of RLTR-PEG-LPs was carried out in order to confirm the targeting ability of RLTR-PEG modified LP. Compared to unmodified control LPS, RLTR-PEG-LPs were largely accumulated in the liver, with only negligible accumulation in the lung or spleen, within a very short
time (Fig. 2). The liver targeting ability of RLTR peptide was more than its ability to target lung or spleen. We then obtained an in vivo image of the liver to check the distribution pattern of this RLTR-PEG modified LP in liver.

We then performed an in vivo accumulation study to verify our hypothesis outlined in the introduction part. We investigated the intrahepatic distribution of RLTR-PEG-LPs by confocal microscopy. Rhodamine-labeled RLTR-PEG-LPs were widely distributed throughout the blood vessels (Fig. 3A), and these intensities were essentially merged with the signal for Isolectin B4, a marker of endothelial cells (Fig. 3B and C). These results demonstrate that RLTR-PEG-LPs efficiently target liver endothelial cells rather than hepatocytes. Furthermore, we compared the intrahepatic distribution pattern with RLTR-PEG-LPs and cationic LPs in order to evaluate the effect of the cationic charge of the liposomal surface. The size and zeta-potential of the Rhodamine-labeled RLTR-PEG-LPs and cationic LPs was 125 nm, 26mV and 132 nm, 22 mV respectively. The intrahepatic distribution pattern of the cationic LPs was quite different from that of the RLTR-PEG-LPs, in which the cationic LPs were gathered in particular spots (Fig. 3D). In addition, these dots did not overlap with liver endothelial cells (Fig.3E-F) indicating that they were taken up by non-parenchymal cells such as kupffer cells or were merely aggregated LPs.

3.4. In vivo inhibition study
In order to examine some possible mechanisms of unique targeting ability of RLTR peptide into liver endothelial cells, comparative studies of RLTR and its reversed peptide sequence named as KLGR were performed in the following part. Both RLTR-PEG-LPs and KLGR-PEG-LPs were accumulated along with the liver blood vessels (Fig. 4 A-B, G-H). Next, accumulation of both labeled RLTR-PEG-LPs and KLGR-PEG-LP along with the liver blood vessels were dramatically inhibited by a pre-treatment with unlabeled RLTR-PEG-LPs or KLGR-PEG-LPs (Fig. 4C-D, 4I-J), however, small portions of signal were remaining. In contrast, the accumulation of both labeled RLTR-PEG-LPs or KLGR-PEG-LP was not reduced by the pre-treatment with unlabeled cationic LPs (Fig. 4E-F, 4K-L). The results generated in this study suggest that cationic charge is not the reason for this uptake and may be both RLTR peptide and its reverse sequence, the KLGR peptide has some specificity for liver endothelial cells. Possible interpretations will be discussed in the discussion section.

3.5. In vitro comparative inhibition study of RLTR-PEG-LPs
This experiment was performed to support the in vivo inhibition data. We compared the cellular uptake of both RLTR-PEG-LPs and KLGR-PEG-LPs with that of cationic-LPs under the inhibition with unlabeled RLTR-PEG-LPs or cationic-LPs. A comparative inhibition study with labeled and
unlabeled LPs showed that the cellular uptake of labeled RLTR-PEG-LPs and KLGR-PEG-LPs were inhibited remarkably in the presence of a low amount of unlabeled RLTR-PEG-LPs and KLGR-PEG-LPs respectively (Fig. 5A-5B). On the other hand, the cellular uptake of labeled cationic LPs was not inhibited to a significant extent by unlabeled cationic LPs (Fig. 5C). The results generated in this study suggest that both the RLTR and KLGR peptide efficiently delivered the nanocarriers into cells via a specific route, while cationic LPs were randomly taken up by cells. This conclusion is further supported by the in vivo comparative inhibition study.

4. Discussion

It has been reported that hepatocytes also express LDL receptors (Marit et al., 1998) and hepatocytes comprise 80% of the total liver volume (Kniec et al., 2001). In a recent study it was also demonstrated that LDL is taken up by liver endothelial cells (Marit et al., 1998). In this present study we attempted to design a new ligand for hepatocytes based on the ApoB-100 sequence of LDL. The newly designed peptide ligand is referred to as RLTR. The size of the RLTR-PEG-LPs increased with the increase of the amount of RLTR-PEG. The surface charge of the PEG-LPs remained negative. However, the addition of the RLTR 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 RLTR-PEG of 1 mol% to 10 mol% (Table 1). This increment in surface charge of the RLTR-PEG-LPs reflects the cationic properties of the RLTR peptide. RLTR-PEG-LPs showed a remarkable enhancement in cellular uptake compared to PEG-LPs in different cell types including liver endothelial cell and Hepa 1-6 cell lines (Fig. 1). In the biodistribution study it was also observed that most of the RLTR modified PEG-LP had accumulated in the liver rather than other organs such as lung or spleen (Fig. 2). However, in confocal microscopy images it was observed that, RLTR-PEG-LPs accumulated at high levels in liver blood vessels (Fig. 3A-C) which indicate that these liposomes do not accumulate in hepatocytes. The cellular accumulation of RLTR-PEG-LPs was significantly higher than that of cationic LPs (Fig. 3D-F). Though the RLTR peptide is designed from LDL and there is previous report that LDL is rapidly taken up by LDL receptors, the peptide modified PEG-LP is highly accumulated in liver EC. It is possible that this carrier system has more specificity for liver EC, rather than hepatocytes. This surprising accumulation of this carrier system through blood vessels led us to conclude that this nanoparticle might be an ideal system for targeting the liver endothelial cells. The finding that cationic peptides and lipids accumulate at high levels in endothelial cells of liver is attractive and interesting. There are very few reports of the use of cationic or neutral nano carriers to target liver endothelial cells (Bartsch et al., 2004, Toriyabe et al., 2011). It should be noted that, these systems failed to achieve high accumulation in liver endothelial cells. Because of this, in this study we seized the opportunity to develop this nanocarrier to target liver EC. There are two possible reasons for the high accumulation of RLTR modified PEG-LP in liver EC.
Either the high cationic charge of the peptide is causing this high accumulation or the peptide sequence itself has specificity for liver endothelial cell. We then checked the specificity of both RLTR and its reverse sequence KLGR modified PEG-LP by both an *in vitro* and *in vivo* inhibition study. In the *in vivo* inhibition study we demonstrated that both the RLTR-PEG-LPs and KLGR-PEG-LPs uptake were inhibited by unlabeled RLTR-PEG-LPs and KLGR-PEG-LPs respectively (Fig. 4). However, the cationic LPs did not affect its uptake both in vivo or in vitro (Fig.4 and 5). Given these findings, the possibility of a higher accumulation in LEC is due to a higher cationic charge can be excluded. We then attempted to address the second possible reason of the higher accumulation in LEC, namely, the sequence of the peptide. We found that the sequence contain a motif RKR which remains the same both in the RLTR and KLGR peptide, although the sequence is completely reversed. It has been reported that stearylated polyarginine and its derivatives, i.e. stearyl-(RXR)$_4$ mediates the efficient plasmid transfection in several cell lines (Lehto et al., 2010). The RKR motif is similar as the RXR motif (Fig. 6). As our study shows that both the RLTR (RLTRKRGLK) and KLGR (KLGRKRRTLR) modified PEG-LP are equally efficient in targeting LEC, the possibility that the RKR sequence is responsible for this targeting cannot be completely excluded. There is another possibility, i.e. both peptides contain an RXXR motif. According to CendR theory this RXXR sequence is essential for a tissue penetrating property (Teesalu et al., 1997). As our peptide sequence RLTR (RLTRKRGLK) and KLGR (KLGRKRRTLR) both have the RXXR sequence and supporting CendR theory so there is another possibility that RLTR and KLGR are the true motifs for these two peptides for targeting LEC (Fig. 6). Both the peptide, RLTR and KTLR, modified LPs appear to have similar targeting abilities for liver EC. However, we were not able to identify the receptor or the key motif responsible for this targeting. Further study will be required to identify the motif or the receptor. LDL receptor, LRP-1, RXR or RXXR motif, which are mentioned in the introduction and discussion, are all possibilities.

5. Conclusion

Liposomes modified with the peptide sequence RLTRKRGLK or its reverse sequence KLGRKRRTLR, designed based on the ApoB-100 sequence, accumulated at high levels in liver endothelial cells via some as-yet-unidentified target receptors, and not via non-specific binding with the cell surface. The presence of RXR or RXXR motif in both peptides may explain their similar uptake by liver ECs. The RLTR or KLGR modified liposomes have the potential for use as a carrier system for the delivery of drugs to liver endothelial cells.

Acknowledgement
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Table 1
Physicochemical properties of the RLTR-PEG-LP

<table>
<thead>
<tr>
<th>% PEG</th>
<th>PEG-LP</th>
<th>Properties</th>
<th>RLTR-PEG-LP</th>
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<tr>
<td></td>
<td>size (nm)</td>
<td>z-potential (mV)</td>
<td>size (nm)</td>
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<tr>
<td>0</td>
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<td>-</td>
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<tr>
<td>10</td>
<td>110±11</td>
<td>-24±12</td>
<td>132±10</td>
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Data are presented as the mean ± SD (n=3)

Figure and Figure Legends
**Fig. 1.** Cellular uptake of RLTR-PEG-LPs. For the cellular uptake study, 40,000 cells/well were seeded in a 24-well plate. After 24 hr LPs modified with different mol% of PEG-DSPE or RLTR-PEG-DSPE were incubated with (A) Liver EC or (B) Hepa1-6 cells for 3 h and the cellular uptake efficiency of the prepared rhodamine labeled LPs were determined by measuring the fluorescence intensity of rhodamine. Cellular uptake is expressed as the mean ±SD (n=3) and Statistical analysis Vs LP was performed by One-way ANOVA followed by Dunnet-test. **P<0.01.

![Cellular uptake graph](image1)

**Fig. 2.** Biodistribution of $^3$H-CHE labeled RLTR-PEG-LPs and LPs in different organ. Male ICR mice were intravenously injected with labeled RLTR-PEG-LPs and LPs. After 25 min of incubation different organs of mouse were collected and radioactivity was measured. Tissue accumulation of LPs was represented as % of injected dose (ID). Here, % of ID is expressed as the mean ±SD (n=4). Statistical analyses were performed by the unpaired Student’s t-test, where *P<0.05.

![Biodistribution graph](image2)
Fig. 3. Representative intrahepatic distribution pattern of RLTR-PEG-LPs (A-C) and a cationic control LPs, in which the lipid composition was DC6-14/DOPE/Chol=4:3:3 (D-F). Green and red color represents blood vessels stained by Isolectin B4 and rhodamine labeled LPs respectively. Scale bars correspond to 50µm in all images.

Fig. 4. In vivo competitive inhibition studies of RLTR-PEG-LPs or KLGR-PEG-LPs using unlabeled RLTR-PEG-LPs or KLGR-PEG-LPs or cationic LPs. Green and red color represents blood vessels stained by Isolectin B4 and rhodamine labeled LPs respectively. Mice were pretreated with unlabeled RLTR-PEG-LPs or KLGR-PEG-LPs or cationic LPs 15 minutes before the second treatment with labeled RLTR-PEG-LPs or KLGR-PEG-LPs for another 25 minutes of incubation. Representative images of liver tissues with (A, B) labeled RLTR-PEG-LPs, (C, D) labeled RLTR-PEG-LPs pre-treated with unlabeled RLTR-PEG-LPs, (E, F) labeled
RLTR-PEG-LPs pre-treated with unlabeled cationic LPs, (G, H) labeled KLGR-PEG-LPs, (I, J) labeled KLGR-PEG-LPs pre-treated with unlabeled KLGR-PEG-LPs, (K, L) labeled KLGR-PEG-LPs pre-treated with unlabeled cationic LPs are shown. Scale bars correspond to 50µm in all images.

Fig. 5. In vitro cellular uptake inhibition studies using unlabeled (A) RLTR-PEG-LPs, (B) KLGR-PEG-LP and (C) cationic LPs. Different ratios of labeled and unlabeled LPs were co-incubated with 40,000 Liver ECs for 3 h. Data were expressed as the mean±SD (n=3). Statistical differences vs. RLTR-PEG-LP (1:0) or KLGR-PEG-LP (1:0) or control LP (1:0) were determined by one-way ANOVA followed by Dunnett test. **P < 0.01.

Fig. 6. Proposed theory for similar binding and cell penetration of RLTR and KLGR (reverse sequence of RLTR peptide) modified PEG-LPs. Where (A) shows binding of the RXR motif in both the peptide modified LPs with the receptor expressed by LEC and (B) shows binding of RXXR (RXXR represents either RLTR or RTLRT motif) of RLTR or KLGR modified LPs respectively to the receptor expressed by LEC.
Figure Legends (supplementary figure)

**Fig.1.** Conjugation of RLTR peptide with Maleimide-PEG$_{2000}$-DSPE. (A) Synthesis of RLTR-PEG-DSPE by coupling of thiol group of the cysteine residue of RLTR peptide (calculated MW: 1343.7) with Maleimide-PEG$_{2000}$-DSPE (calculated MW: 2936). Maleimide-PEG$_{2000}$-DSPE and the RLTR peptide (molar ratio 1:1:2) were dissolved in water at 37 °C and allowed to react for 24 h. MALDI-TOF MS spectra of (B) Maleimide-PEG$_{2000}$-DSPE and (C) RLTR-PEG$_{2000}$-DSPE, which confirmed that the conjugation was successful, as evidenced by the molecular shifts of free RLTR peptide from 1343.9 (observed MW) to 4371.74 (observed MW) which is the sum of the individually calculated MW of RLTR peptide and Maleimide-PEG$_{2000}$-DSPE.
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