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Research paper

# Liver targeting and anti-HBV activity of reconstituted HDL–acyclovir palmitate complex

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**Abstract**

High-density lipoprotein (HDL) particles deliver cholesterol from periphery tissues to liver in human body through specifically binding to a receptor on the surface of hepatocytes. Therefore, HDL particles can be potentially used to target drugs to liver. We synthesized the acyclovir palmitate as the pro-drug of acyclovir and incorporated it into recombinant HDL to form reconstituted HDL (rHDL)–acyclovir palmitate complex. The efficiency of the encapsulation of acyclovir palmitate was high, reached 97% when the acyclovir palmitate and phosphatidylcholine (PC) ratio was 1:20. In an in vitro HBV inhibition assay, 0.0022 μmol/ml rHDL–acyclovir palmitate showed 20% inhibition of HBV. To reach the same level of inhibition, 20 times, 40 times, and 200 times more acyclovir palmitate liposome, acyclovir liposome, and free acyclovir were needed, respectively. Rat body distribution study showed that 71.2% of the dose was recovered in the liver, 10.2% of the dose was in the plasma, and 18.6% was in the rest of the body at 30 min after injection. These results indicated that rHDL–acyclovir palmitate complex had strong liver targeting property, suggesting that reconstituted HDL could be used to deliver drugs to treat liver diseases.


*Keywords:* Apolipoprotein A-I; HDL; Liver targeting; Acyclovir; Acyclovir palmitate

## 1. Introduction

Drugs with a targeting moiety can accumulate in the targeted organ or tissue selectively. Drug targeting has many advantages, such as increase of drug concentration in the targeted sites and decrease of negative effects on non-target compartments, reduction of drug dose required to achieve a therapeutic effect as well as the cost of therapy [1]. Studies of suitable targeting carriers have become an important field of pharmaceutical research. Recently, use of lipoproteins as potential drug carriers has attracted a great deal of attention. Lipoproteins are spherical particles consisting of a core of apolar lipids surrounded by a phospholid monolayer, in which cholesterol and apoproteins are embedded. Highly hydrophobic drugs can be incorporated into the apolar core and, thus, be transported, hidden inside the particles [2]. Because lipoproteins are endogenous molecules, as drug carriers, these particles are completely biodegradable, do not trigger immunological responses, and can escape recognition and elimination by the reticuloendothelial system.

Low density lipoproteins (LDL) were initially being considered as drug carriers, probably due to their high lipid contents. But LDL is generally considered ''bad cholesterol'' lipoproteins limit their use [3,4]. However, high density lipoproteins (HDL) have more advantages than LDL. HDL is generally considered ''good cholesterol'' lipoproteins. It is an important class of lipoproteins and plays a major role in the transport of cholesterol from various tissues to the liver (so-called 'reverse cholesterol transport'). So different methods have been developed for the preparation of reconstituted HDL (rHDL) subspecies, which were used as models to better understand the metabolic role and fates of the different HDL particles [5–7].

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Even though other studies have shown that liposomes, polymers, and proteins like albumin can target liver as drug carriers [2], HDL–drug complex has the advantage of specific interaction with liver cells via cell surface receptors. This type of specific interaction may provide more specific targeting. It was reported in 1992 that there is a specific recognition receptor SR-BI of apolipoprotein A-I (apoA-I) on hepatocyte surface. HDL takes up cholesterol from peripheral tissues and delivers it back to liver via apoA-I, which binds to its receptor on the liver cell membrane [8,9]. Therefore, HDL has the potential to be used as a liver targeting drug carrier. To investigate the possibility of rHDL being used as a carrier for delivering antitumor drugs to hepatocyte cells, Lou prepared the recombinant complex of HDL and aclacinomycin (rHDL-ACM) by sonication of apoproteins from human serum and ACM as well as PC. Their results showed that rHDL-ACM could bind on SMMC-7721 cell [10].

The main limitation to develop rHDL as therapeutic agents or targeting carriers is the difficulty to isolate large quantities of HDL apolipoproteins from human serum [11]. Apolipoprotein A-I (ApoA-I) is the major protein of HDL particles. But up to now human blood is the only source of ApoA-I, however, isolating ApoA-I from serum has many problems. To overcome these problems and to obtain large quantities of apoA-I for research and therapeutic use, we successfully expressed human ApoA-I in *Pichia pastoris* [12,13]. Then we prepared the recombinant HDL–drug complex (rHDL–drug complex) by using PC, recombinant apoA-I and hydrophobic drugs instead of the cholesterol. In this report we used the antiviral drug acyclovir as a model compound. Acyclovir is not sufficiently hydrophobic to be incorporated into the complex. We therefore synthesized the hydrophobic pro-drug – acyclovir palmitate, and used it in the study. We demonstrated that the rHDL–drug complex improved the biological distribution of acyclovir and had good liver cell targeting property.

## 2. Materials and methods

### 2.1. Materials

Acyclovir was provided by Friendy Co. of Shanghai. Phosphatidylcholine (PC, from fresh egg yolk) and sodium deoxycholate (SDC) were purchased from Sigma. Recombinant ApoA-I was prepared through fermentation in our laboratory. All other chemical reagents were purchased from commercial source and were of analytical grade. PBS buffer (1 L) contained NaCl 8 g, KCl 0.2 g, $Na_2HPO_4{\cdot}H_2O$ 1.56 g, $KH_2PO_4$ 0.2 g and was adjusted to pH 7.4 with dilute KOH.

Liver cancer cell line $HepG_2$ 2.2.15 was maintained in our laboratory. The HepG2 2.2.15 cell line, which secreted HBsAg and HBeAg, was from Institute of Medicinal Biotechnology, Chinese Academy of Medical Science (Beijing, China). The ELISA diagnostic kits for HBsAg and HBeAg were purchased from Shanghai Kehua Biotech Co. Ltd.

Male Wistar rats (170–220 g) were provided by the Test Animal Center of Fudan University.

### 2.2. Preparation of the pro-drug acyclovir palmitate

Aacyclovir (10 mmol) was added into a 250 ml round-bottom flask and dissolved with 80 ml pyridine. Palmitoyl chloride (10 mmol) was then added under stirring and then reacted at 60 °C for 48 h. Water (4 °C, 400 ml) was added when the reaction temperature was down to about 25 °C. The precipitate was collected by filtration and washed with 4 °C water. Then the solid was dissolved in methanol and crystallized [14].

### 2.3. Preparation of rHDL–prodrug acyclovir palmitate complex

The rHDL–acyclovir palmitate complex was prepared according to similar reported procedures [15]. A mixture of 100 mg PC and different amounts of the acyclovir palmitate was dissolved in 5 ml solvent (chloroform:methanol = 2:1 v/v) in a 100 ml round-bottom flask. Te solvent was evaporated with a rotary evaporator at 30 °C and a thin film was formed. The trace solvent residue was finally removed with a stream of nitrogen gas. Five milliliters of SDC solution (100 mg/ml in PBS) alone or with 3 ml recombinant ApoA-I (5 mg/ml in PBS) was added and mixed thoroughly. The mixture was shaken at 24 °C in a water-bath for 30 min. The free SDC and unencapsulated acyclovir palmitate were removed by dialysis in PBS buffer for 22 h. The prepared rHDL–acyclovir palmitate complex was collected and the volume was measured.

### 2.4. Preparation of acyclovir liposome

Acyclovir liposome was prepared with reverse phase evaporation vesicles (REV) [16]. PC (100 mg) was dissolved in 6 ml chloroform and the solvent was evaporated with a rotary evaporator to form a thin film. Six milliliters of ethyl ether was added to dissolve the thin film. Two milliliters of acyclovir solution (20 mg/ml, in 0.05 M PBS) was added and the resulting mixture was sonicated for 5 min on an ice-bath. The solvent was then evaporated with a rotary evaporator until the liposome was formed. The unencapsulated drug was removed by Sephadex-25 (1.0/30 cm) column.

### 2.5. The particle size and morphology

The particle size of rHDL–drug complex was determined by Nicomp_ 380 ZLS laser diffraction sizer (PSS Nicomp, Santa Barbara, CA, USA). The measurement conditions were: He–Ne laser; angle, 90°; temperature, 23 °C; The morphology of the complex was examined with an electron microscope (Philips CM 200).

### 2.6. HPLC analysis

The concentrations of acyclovir and acyclovir palmitate were determined by HPLC with a reverse phase column (YWG C18 4.6 × 200 mm). For the measurement of acyclovir, the mobile phase was 10% methanol in water with a rate of 1 ml/min and the detection wavelength was 254 nm. For the measurement of acyclovir palmitate, the mobile phase was 75% acetonitrile in water with a rate of 1 ml/min and the detection wavelength was 259 nm [17,18].

### 2.7. Determination the encapsulation efficiency

The encapsulation efficiency of acyclovir and acyclovir palmitate in the complex was determined by dissolving the complex with 10% Triton X-100. The acyclovir and acyclovir palmitate contents were determined by HPLC as described. Encapsulation efficiency (%) = elemental drug in complex/elemental drug added.

### 2.8. Anti-HBV activity in vitro

Anti-HBV activity in vitro was performed using the HepG2 2.2.15 cell line [19]. The cells were cultured in DMEM (Gibco) containing 100 ml/L calf serum and maintained in a humidified atmosphere containing 5% $CO_2$ at 37 °C. Cells were seeded in 24-well plates. After incubation for 72 h, the drugs were added and cultured continually for 9 d. The survived cells were determined by MTT (3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenyltetrazoliumbromide). The OD was determined with HBsAg and HbeAg Kit and calculated as:

The inhibition rate = OD(control cell) − OD(test cell)/OD(control cell).

### 2.9. Tissue distribution of acyclovir and rHDL–acyclovir palmitate complex

Male Wistar rats weighing between 170 and 220 g were used. One milliliter (300 μg/ml) of drug or rHDL–acyclovir palmitate complex was injected via the vena penis. After 30 min the animals were anesthetized by ether. Blood samples of 0.5 ml were taken from vena penis and the tissues of liver were collected.

The blood sample was centrifuged at 12,000 rpm for 10 min. Serum (0.3 ml) was mixed with 0.3 ml of 10% Triton X-100. After 30 min, 3 ml of methanol was added followed by centrifugation at 12,000 rpm for 20 min. The supernatant was collected and analyzed by HPLC to determine the concentrations of acyclovir or acyclovir palmitate.

Tissues of 0.5 g liver were homogenized, mixed with 1.0 ml of 10% Triton X-100, and stirred for 30 min. After centrifugation, the supernatant was collected and analyzed by HPLC to determine the concentrations of acyclovir or acyclovir palmitate.

The percentages of acyclovir palmitate in the rest of the body were calculated by subtracting the percentages in liver and blood from 100%.

## 3. Results and discussion

### 3.1. Preparation of acyclovir palmitate

We used acyclovir as a model drug to investigate the liver targeting properties of rHDL. Because acyclovir is not hydrophobic enough to be encapsulated into the rHDL complex efficiently, the more hydrophobic acyclovir palmitate pro-drug was prepared and used in this study. Acyclovir palmitate was synthesized in 72.2% yield and its structure (Fig. 1) was confirmed by mass spectrometry, NMR and other methods.

### 3.2. Preparation of rHDL–acyclovir palmitate

The rHDL–acyclovir palmitate complex was prepared and the encapsulation efficiency was studied. In general, hydrophobic drugs are bound to PC with high affinity. The encapsulation efficiency of acyclovir palmitate was very high because of its high hydrophobic nature. It reached about 98% when the acyclovir palmitate/PC ratios were low. In Table 1 are listed the encapsulation efficiencies at different acyclovir palmitate/PC ratios. It shows that the encapsulation efficiency decreased with the increase of acyclovir palmitate/PC ratio. However, when the acyclovir palmitate/PC ratio is low, less drug is encapsulated. Therefore, the optimal ratio of acyclovir palmitate/PC needs to be determined for a specific application. The ratio of 1:20 for acyclovira palmitate and PC was used in this case based on high encapsulation efficiency and relatively high drug content. In contrast, the encapsulation efficiency of acyclo-

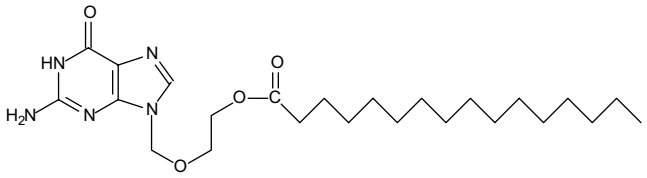


Fig. 1. Chemical structure of acyclovir palmitate, synthesized from acyclovir and palmitoyl chloride.

Table 1
Effects of the ratio of prodrug/PC (w/w) on encapsulation efficiency (E.E)

| Acyclovir palmitate/PC | Encapsulation efficiency (%) |
|---|---|
| 1:100 | 98.3 |
| 1:50 | 97.9 |
| 1:20 | 96.6 |
| 1:10 | 92.2 |
| 1:5 | 85.6 |
| Acyclovir/PC (1:2.5) | 20 |

vira, which is much less hydrophobic, was about 20%, because of low affinity with PC.

### 3.3. Particle size and morphology of rHDL–drug complex and liposome

The particle size of rHDL–acyclovir palmitate complex and its morphology were also examined (Fig. 2a–c). The mean particle size of rHDL–acyclovir palmitate complex was about 33.5 nm. The acyclovir liposome was about 350 nm. Native HDL is formed from apoA-I, phospholipids and cholesterol. The crystal structure of lipid-free apoA-I shows that apoA-I is comprised of an N-terminal four-helix bundle and two C-terminal helices [20]. When the size of the complex of phospholipids and apoA-I reached 10–20 nm, the particle was wrapped by apoA-I [21]. Therefore, the rHDL–acyclovir palmitate particles in which the PC was surrounded by apoA-I exhibited smaller particle size than the particles with no apoA-I. In liver different particles size liposome will be taken up by different type cells of liver. Later we will investigate the effect of different particle size on anti-HBV activity in vivo.

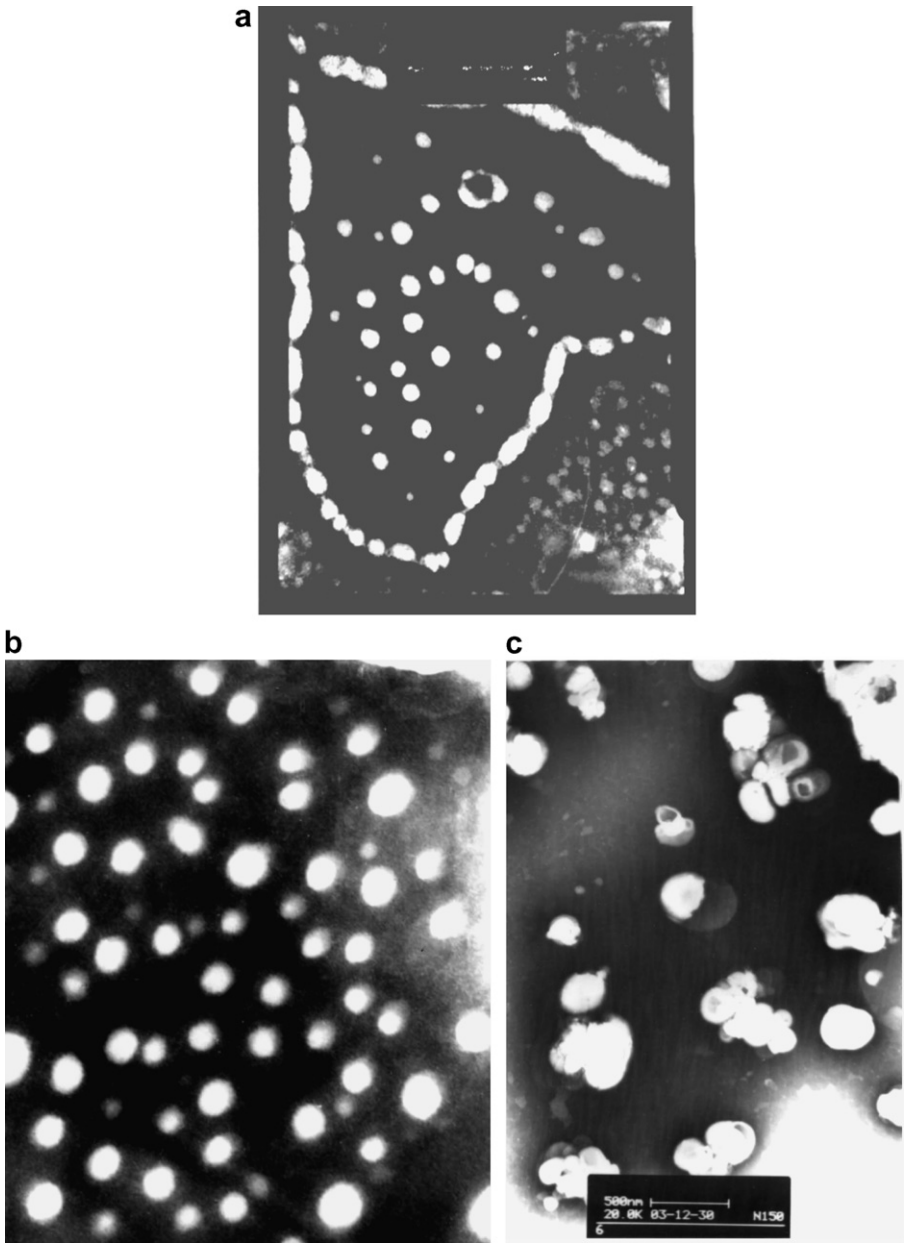


Fig. 2. (a) The morphology of the rHDL–acyclovir palmitate liposome with apoA-I (74,000×), examined with an Philips CM200 electron microscope. The mean particle size was 33.5 nm. (b) The morphology of the acyclovir liposome without apoA-I (20,000×). The mean particle size was 61 nm. (c) The morphology of the acyclovir liposome and the mean particle size was 350 nm.

### 3.4. The stability of rHDL–acyclovir palmitate

Reconstruct HDL–acyclovir palmitate was stable when it was protected by DMSO, sealed in N2 and stored at −20 °C. The shape of complex was well preserved and no agglutination occurred after 6 months.

### 3.5. Anti-HBV activity in vitro

The anti-HBV activities of different lipid–drug complexes were investigated. Liver cancer cells ($HepG_2$ 2.2.15) were seeded in 24-well plates. After incubation for 72 h, preparations of liposome without drug, acyclovir liposome, acyclovir palmitate liposome and rHDL–acyclovir palmitate were added to the cells, respectively. The cells were cultured continually for 9 days. Their HBV inhibition rates as determined by HBeAg and HBsAg assays are shown in Table 2. As shown, markedly different concentrations of the drug were required to achieve the same level (20%) of virus inhibition (Fig. 3). The HBV inhibition reached 20% when the concentration of acyclovir palmitate was 0.0022 μmol/ml, in the form of rHDL–acyclovir palmitate complex. Comparing with rHDL–acyclovir palmitate complex, to reach the same level of virus inhibition, 20 times the concentration of acyclovir palmitate in the form of acyclovir palmitate liposome without apoA-I, 40 times of acyclovir in the form of acyclovir liposome, 200 times of free acyclovir were required. Table 2 also shows the anti-HBV activities of free acyclovir and free acyclovir palmitate.

These results showed that drug complexes containing ApoA-I have better anti-HBV activities than other drug-containing liposomes.

### 3.6. Tissue distribution of acyclovir and rHDL–acyclovir palmitate complex

To investigate the liver targeting property of rHDL–drug complex, rHDL–acyclovir palmitate complex was administered to rats and the concentrations of acyclovir palmitate in liver and serum were determined 30 min after dosing. The total serum volume of a rat was calculated by the equation: serum volume (mL) = 0.0219 body weight (g) + 2.66. And the amount of acyclovir palmitate in blood was calculated by the serum volume and serum concentration, which was determined by HPLC. The percentages of acyclovir palmitate in liver, serum and rest of the rat body were 71.2%, 10.2% and 18.6% (which was obtained by 100–71.2–10.2%), respectively. The amount of acyclovir palmitate in liver was seven times that in serum and four times that in the rest of the body. In addition, the tissue distribu-

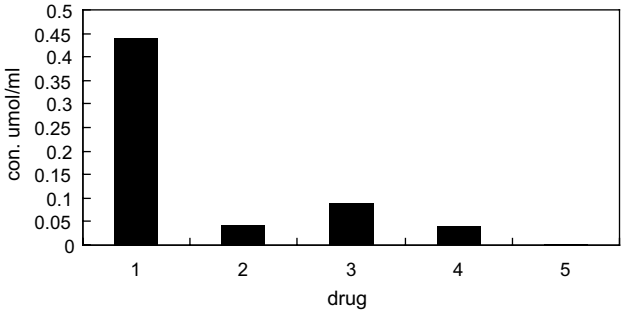


Fig. 3. To reach 20% inhibition of HBV, the drug concentrations needed. 1, free acyclovir; 2, free acyclovir palmitate; 3, acyclovir liposome; 4, acyclovir palmitate liposome without apoA-I; 5, rHDL–acyclovir palmitate.

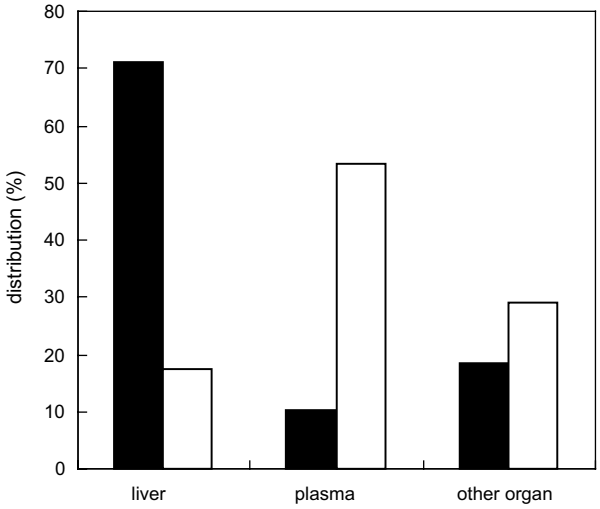


Fig. 4. Distribution of free acyclovir palmitate and rHDL–acyclovir palmitate in rats. Black bar, rHDL–acyclovir palmitate; white bar, free acyclovir palmitate.

Table 2
In vitro anti-HBV activity

| Con. | Liposome no drug | | Acy | | Acy-pal | | Acy lip no apoA I | | Acy palmitate lip no ApoA-I | | rHDL–acy palmitate | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | HbsAg | HBeAg | HbsAg | HBeAg | HbsAg | HBeAg | HbsAg | HBeAg | HbsAg | HbeAg | HBsAg | HBeAg |
| 0.0022 μM | | | | | | | | | | | 19.1% | 19.5% |
| 0.0033 μM | | | | | | | | | | | 23.7% | 27.6% |
| 0.0066 μM | | | | | | | | | | | 68.3% | 53.5% |
| 0.0444 μM | | | | | 19.1 | 20.0 | 7.5 | 8.3 | 20.3 | 29.3 | | |
| 0.0888 μM | | | | | | | 18.2 | 40.5 | 52.6 | 42.4 | | |
| 0.444 μM | | | 18.0 | 20.7 | | | | | | | | |
| 1.25% | 0 | 0 | | | | | | | | | | |
| 2.5% | 0 | 0 | | | | | | | | | | |
| 5.0% | 0 | 0 | | | | | | | | | | |

tion of free acyclovir palmitate was also measured. The distribution of free acyclovir palmitate was 17.5% in liver, 53.3% in blood and 29.2% in the rest of the body. In this case, the serum had the highest concentration and the liver had the lowest (Fig. 4). These results demonstrated that the rHDL–acyclovir palmitate complex, which contained apoA-I, had the property to target liver cells.

## 4. Conclusion

Specific disease tissue targeting can increase the effectiveness of the drug and decrease the toxicity. We used acyclovir as a model drug to investigate the liver targeting properties of rHDL and prepared its more hydrophobic form, acyclovir palmitate, to be used in this study. The anti-HBV assay of different forms of lipid–drug complexes showed that the HBV inhibition reached 20% when the concentration of acyclovir palmitate was 0.0022 μM and when acyclovir palmitate was complexed with rHDL. To reach the same level of virus inhibition, 20 times acyclovir palmitate liposome, 40 times acyclovir liposome, and 200 times free acyclovir were required. Body distribution study of the rHDL–acyclovir palmitate complex showed that the majority of acyclovir palmitate (71.2%) was targeted to the liver. The amount of acyclovir palmitate in liver was seven times that in serum and four times that in the rest of the body. These results from in vitro and in vivo assays demonstrated that rHDL containing ApoA-I had strong liver targeting property. Our initial study provided the proof of the concept for further development of ApoA-I as a targeting moiety for liver-acting drugs.

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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.ejpb.2007.07.005.

## References

[1] D.K.F. Meijer, R.W. Jansen, G. Molema, Drug targeting systems for antiviral agents: options and limitations, Antiviral Res. 18 (1992) 215–258.
[2] P.C.N. Rensen, L.A. Remco, K. Johan, Recombinant lipoproteins: lipoprotein-like lipid particles for drug targeting, Adv. Drug Deliv. Rev. 47 (2001) 251–276.
[3] B.M. Samadi, G. Favre, P. Canal, Low density lipin for cytotoxic drug targeting: improved activity of elliptinium derivative against B16 melanoma in mice, Br. J. Cancer 68 (1993) 319–326.
[4] R. A Firestone, Low density lipoprotein as a vehicle for targeting antitumor compounds to cancer cells, Bioconjug. Chem. 5 (1994) 105–113.
[5] D. Atkinson, D.M. Small, Recombinant lipoproteins: implications for structure and assembly of native lipoproteins, Annu. Rev. Biophys. Biophys. Chem. 15 (1986) 403–456.
[6] C.G. Brouillette, G.M. Anantharamaia, Structural models of human apolipoprotein A-I, Biochim. Biophys. Acta 1256 (1995) 103–129.
[7] C.E. Matz, A. Jonas, Micellar complexes of human apolipoprotein A-I with phosphatidyl cholines and cholesterol prepared from cholate-lipid dispersions, J. Biol. Chem. 257 (1982) 4535–4540.
[8] Q. Wei, M.P. Wu, P.F. Chen, Cooperation of HDL receptor and hepatic lipase in the selectives uplake of HDL2-CE by rat hepatic simusoidal cells, Acta Biochem. Biophys. Sin. 28 (6) (1996) 661–664.
[9] L.O. Martines, S. Jacquet, J.P. Esteve, Ectopic β-chain of ATP synthase is apolipoprotein A-I receptor in hepatic HDL endocytosis, Nature 421 (6918) (2003) 75–79.
[10] B. Lou, X.L. Liao, M.P. Wu, High-density lipoprotein as a potential carrier for delivery of a lipophilic antitumoral drug into hepatoma cells, World J. Gastroenterol. 11 (7) (2005) 954–959.
[11] E.R. Sherman, D.C. Steven, Rapid chromatographic purification of apolipoprotein AI and A-II, Anal. Biochem. 149 (1985) 166–168.
[12] M.Q. Feng, Q.S. Cai, D.X. Song, High yield and secretion of recombinant human apolipoprotein AI in *Pichia pastoris*, Prot. Expr. Purif. 46 (2006) 337–342.
[13] M.Q. Feng, Q.S. Cai, D.X. Song, Expression of human apolipoprotein AI in *Pichia pastoris*, Chin. J. Biotech. 20 (6) (2004) 906–911.
[14] Q.S. Cai, M.Q. Feng, H. Huang, High yield synthesis, structure confirm of acyclovir palmitate and its role in inhibiting hepatitics in vivo. Fudan University, J. Med. Sci. 34 (4) (2007) 486–490.
[15] M.S. Nagarsenker, V.Y. Londhe, G.D. Nadkarni, Preparation and evaluation of liposomal formulations of tropicamide for ocular delivery, Int. J. Pharm. 190 (1990) 63–67.
[16] Y. hattori, S. Kawakami, Controlled biodistribution of galactosylated liposomes and incorporated probucol in hepatocyte-selective drug targeting, J. Control. Release 69 (2000) 367–377.
[17] S.L. Law, K.J. Huang, C.H. Chiang, Acyclovir-containing liposomes for potential ocular delivery corneal penetration and absorption, J. Control. Release 63 (8) (2000) 135–140.
[18] K.Y. Hostetler, S. Parker, C.N. Sridhar, Acyclovir diphosphate dimyristoylglycerol, Proc. Natl. Acad. Sci. USA 90 (1993) 11835–11839.
[19] M.A. Sells, Z. Zelent, G. Acs, Production of Hepatitis B virus particles in HepG2 cells transfected with cloned Hepatitis B virus DNA, Proc. Natl. Acad. Sci. USA 84 (1987) 1005–1009.
[20] A.A. Ajees, G.M. Anantharamaiah, V.K. Mishra, Crystal structure of human apolipoprotein A-I: insights into its protective effect against cardiovascular diseases, Proc. Natl. Acad. Sci. USA 103 (7) (2006) 2126–2131.
[21] P. Jere, M.K. Segrest, A.E. Jones, A detail molecular belt model for apolipoprotein A-I in discoidal high density lipoprotein, J. Biol. Chem. 274 (45) (1999) 31755–31758.