Recombinant high-density lipoprotein complex as a targeting system of nosiheptide to liver cells

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Abstract
Nosiheptide is a lipophilic peptide of significant anti-hepatitis B virus (anti-HBV) activity in cell culture, but has poor distribution to liver in vivo. In this study, recombinant high-density lipoprotein (rHDL) complexes of nosiheptide were constructed to target this anti-HBV agent to hepatocytes. The optimized rHDL–nosiheptide complex had a high drug-loading efficiency (>80%) and a diameter smaller than 30 nm. The concentration of nosiheptide in an optimized rHDL–nosiheptide complex to achieve 50% virus inhibition (IC50) in HepG2 2.2.15 cells was 0.63 mg/ml, which was 40 times lower than the IC50 of nosiheptide in control liposome (2.5 mg/ml) and 200 times lower than the IC50 of the free nosiheptide (12.5 µg/ml). The complex targeted most of the administered nosiheptide to the liver within 30 min after i.v. injection to male Wistar rats. Together, this report provides early evidence that it is feasible to develop efficient, HDL-based drug delivery systems against HBV, utilizing apolipoprotein A-I as the targeting moiety.

Keywords: Apolipoprotein, HDL, liver targeting, nosiheptide, HBV

Introduction
Hepatitis B (HB) is a viral infection that affects about 400 million people around the globe and causes approximately one million deaths per year (Leemans et al. 2007). The prognosis of HB relies on agents that interfere with the life cycle of the pathogen, hepatitis B virus (HBV). Most of the commercially available anti-HBV agents are nucleoside or nucleotide analogues that serve as inhibitors of the HBV reverse transcriptase. However, the efficacy of such drugs is limited to the long-term palliation of HBV replication and is further challenged by drug resistance due to virus mutations. Consequently, alternative chemical identities continue to be heavily investigated for anti-HBV activity, including the recently approved interferon alpha and its pegylated derivatives.

One attractive strategy to improve the activity of anti-HBV agents is to target them into HBV-infected cells by drug delivery systems that recognize receptors on the surface of hepatocytes (Rensen et al. 2001). For example, Bijsterbosch and coworkers (Bijsterbosch et al. 1989, 1994, 1996; Bijsterbosch and Van Berkel 1992) have demonstrated that lactosylated lipoproteins can serve as a liver-targeting system via galactose-specific hepatic receptors (Ashwell and Harford 1982). More recently, Hattori and Kawakami (2000) reported a lactosylated liposome formulation that
was capable of improving the hepatic distribution of probucol. The suppression of the targeting by galactosylated bovine serum albumin suggested that the targeting was mediated by the asialoglycoprotein receptor (ASGPr; Spies 1990).

High-density lipoprotein (HDL) represents another promising system for hepatic targeting. The endogenous HDL takes up cholesterol and phospholipids from peripheral tissues and delivers them to hepatocytes via the apolipoprotein A-I (apoAI), which is the major lipoprotein component in HDL. ApoAI binds to the scavenger receptor, class B, type I (SR-BI) expressed on the surface of hepatocytes (Acton et al. 1996; Wei et al. 1996; Martinez et al. 2003), followed by endocytosis and selective translocation of cholesteryl esters and phospholipids into the hepatocyte cytosol. However, HDL has not yet been widely applied to deliver anti-HBV agents due to difficulties in preparing large quantities of the human HDL apolipoproteins. To overcome this technical problem, we have overexpressed the human apoAI in *Pichia pastoris* (Feng et al. 2004, 2006).

Nosiheptide, produced by *Streptomyces actuosus*, is a sulfur-containing polypeptide antibiotic that inhibits the growth of Gram positive bacteria (Benazet et al. 1980). Recently, we have discovered the significant anti-HBV activity of nosiheptide in cell culture (Wu et al. 1997; Cai et al. 2005). Compared with anti-HBV drugs in clinical use, nosiheptide is exceptionally lipophilic (Pascard et al. 1977), making it a promising cargo molecule for HDL. Herein, we report the construction of the recombinant HDL–nosiheptide complex (rHDL–nosiheptide complex) comprising apoAI, phosphatidylcholine, and the lipophilic antibiotic nosiheptide in place of cholesterol. We demonstrate that the rHDL–nosiheptide complex significantly improved the anti-HBV activity of nosiheptide in cell culture and enhanced the hepatic distribution of nosiheptide in rats.

Material and method

*Materials*

ApoAI and nosiheptide were prepared as previously described (Zhou et al. 1991; Feng et al. 2006). Egg yolk phosphatidylcholine (PC) and sodium deoxycholate (SDC) were purchased from Sigma (Shanghai, P.R. China). All other chemical reagents were obtained commercially and were of analytical grade. One liter of stock PBS buffer (0.15 M, pH adjusted to 7.4 by concentrated HCl) contained 8 g NaCl, 0.2 g KCl, 1.56 g Na₂HPO₄·H₂O, and 0.2 g KH₂PO₄. PBS buffers used in the following experimental procedures were prepared by diluting the stock PBS buffer with water. The HBV-infected HepG₂ 2.2.15 cell line, which secreted HB surface antigen (HBsAg) and HB “e” antigen (HBeAg), was from the Institute of Medicinal Biotechnology, Chinese Academy of Medical Science (Beijing, P.R. China). Male Wistar rats were from the Animal Test Center of Fudan University (Shanghai, P.R. China).

*Preparation of nosiheptide formulations by sonication*

A mixture of 100 mg PC and appropriate amount of nosiheptide were dissolved in 5 ml organic solvent (chloroform/methanol = 2/1, v/v) in a 100 ml round-bottom flask. The solvent was evaporated using a rotary evaporator at 30°C, followed by removal of the solvent residue using N₂ flow to form a thin film at the bottom of the flask. For liposome formulations of nosiheptide without apoAI, the film was hydrated with 5 ml PBS buffer (0.05 M, pH 7.4); for rHDL–nosiheptide complexes, the film was hydrated with a mixture of 5 ml PBS buffer (0.05 M, pH 7.4) and 3 ml apoAI solution (5 mg/ml) in PBS (0.05 M, pH 7.4). The suspension was sonicated for 5 min with intermittent cooling in an ice bath. The nosiheptide formulation was separated from the unencapsulated nosiheptide by a Sephacryl S-200 column (1.6 x 60 cm) eluted with 0.05 M PBS (Cai et al. 2005). The nosiheptide formulation was harvested from the eluant and its volume was measured.

*Preparation of nosiheptide formulations by sodium deoxycholate dialysis*

A thin film of PC and nosiheptide was prepared as described above. For liposome formulations of nosiheptide without apoAI, the film was hydrated with 5 ml SDC solution (100 mg/ml) in 0.05 M PBS (pH 7.4); for rHDL–nosiheptide complexes, the film was hydrated with a mixture of 5 ml SDC solution in 0.05 M PBS (pH 7.4) and 3 ml apoAI solution (5 mg/ml) in 0.05 M PBS (pH 7.4). The mixture was agitated in a 24°C water bath for 30 min. SDC and the unencapsulated nosiheptide were removed by dialysis (dialysis bags purchased from Shanghai Shisheng Cellular Biotechnology Inc., P.R. China; MWCO = 6000) in 0.05 M PBS buffer (pH 7.4, x 100 volume) for 22 h (dialysis buffer refreshed at 1, 2, 4, 6, 10, 14, and 18 h). The nosiheptide formulation was harvested and its volume was measured. For animal studies, the optimized rHDL–nosiheptide complex was concentrated by dialysis in PEG of high osmotic pressure to increase the concentration of nosiheptide above 0.5 mg/ml.

*HPLC analysis of nosiheptide*

Nosiheptide was analyzed by HPLC (Waters 1525) using a reverse-phase column (YWG, C18, 4.6 mm x 200 mm) coupled with a UV detector (λ = 313 nm; Horii and Oka 2000). The mobile
Quantification of the drug-loading efficiency of nosiheptide

An aliquot of a nosiheptide formulation was dissolved in 10% TritonX-100 and analyzed with HPLC as mentioned above. The drug-loading efficiency (%) was calculated using the formula: $C \times V/A_0 \in 100$, where $C$ is the nosiheptide concentration in its formulation as determined by HPLC, $V$ is the volume of the harvested formulation after sonication or dialysis, and $A_0$ is the amount of nosiheptide consumed for the preparation of the formulation (Feng et al. 2008).

Characterization of the morphology and particle size of nosiheptide formulations

The morphology of nosiheptide formulations was characterized by transmission electron microscopy (TEM) on a Philips CM 200 electron microscope (200 kV, 2 Å resolution; FEI Company, Hillsboro, Oregon, USA) following the manufacturer’s recommendations. The hydrodynamic diameter of nosiheptide formulations was determined by photon correlation spectrometry on a NICOMP 380 ZLS submicron particle sizer (Particle Sizing Systems, Santa Barbara, CA, USA) using the following parameters: He–Ne laser, 90° angle, and 23°C.

Anti-HBV activity assay in HepG2 2.2.15 cells

The HBV-infected HepG2 2.2.15 cells commonly used for anti-HBV assays (Zhai et al. 1990; Kumar et al. 2006) were cultured in DMEM medium (Gibco, USA) containing 10% FCS and maintained in a humidified atmosphere containing 5% CO₂ at 37°C. The cells were seeded in 24-well plates (~1 × 10⁵ cells in 1 ml medium per well) and cultured for 72 h. The cells were then cultured in the medium containing appropriate concentrations of freshly prepared nosiheptide formulations for another 9 days. The inhibition of the secretion of HBsAg and HBeAg was evaluated by the decrease of absorbance ($\lambda = 450$ nm) using the ELISA diagnostic kits (Shanghai Kehua Biotech Co. Ltd, Shanghai, P.R. China) according to the manufacturer’s recommendations. Rate of inhibition (inhibition %) was calculated using the formula: (absorbance of the control group – absorbance of the experimental group)/(absorbance of the control group) × 100. As a control for cytotoxicity, cell survival was determined by the MTT (3-(4,5)-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide) assay.

Results and discussion

Preparation of rHDL–nosiheptide complexes

In order to enhance drug targeting to hepatocytes, we desire to prepare rHDL–nosiheptide complexes of small size for the following reasons. First, a small size decreases the clearance of lipidic particulates by the reticular endothelial system comprising fixed macrophages in liver (Kupffer cells), spleen, and lymph nodes. Secondly, a small size would facilitate the exposure of apoAI on the complex surface and hence enhances its recognition by SR-BI. Thus, sonication and detergent dialysis, each being a common technique to prepare small lipidic colloids, were used to construct prototype rHDL–nosiheptide complexes, starting with egg PC, nosiheptide, and apoAI in the ratio of 1/1/0.15 (w/w/w). As shown by the TEM studies (Figure 1(A),(B)), the prototype rHDL–nosiheptide complexes prepared by dialysis using the detergent SDC had a mean diameter around 100 nm, which was significantly smaller than that (~200 nm) of the complexes prepared by sonication. In addition, a control liposomal formulation of
nosiheptide without apoAI had a larger size of 300 nm in diameter (Figure 1(C)), suggesting that apoAI facilitates the formation of smaller lipidic colloids containing nosiheptide.

In an effort to improve the drug-loading efficiency of the rHDL–nosiheptide complexes, the effects of the PC/nosiheptide ratio and of the formulation procedure (sonication vs. SDC dialysis) on the drug-loading efficiency were investigated. As shown in Table I, the drug-loading efficiency was improved by the increase of the PC/nosiheptide ratio, whereas there is no significant difference in drug-loading efficiency between sonication and SDS dialysis. The high drug-loading efficiency (>80%) at PC/nosiheptide ratio of 33 or higher strongly suggests that hydrophobic interactions between PC and the lipophilic drug nosiheptide could be the major driving force of the drug-loading process.

rHDL–nosiheptide complexes, which started with a PC/nosiheptide ratio of 33 and which were prepared by SDC dialysis, were selected for further studies due to their smaller size, high drug-loading efficiency, and sufficient nosiheptide concentration for biological assays. When such complexes were prepared at a higher SDC/PC ratio (Table II and Figure 2), the particle size further decreased. Photon correlation spectrometry studies (Table II) showed that the mean hydrodynamic diameter of the dialyzed rHDL complexes was reduced below 30 nm when the SDC/PC ratio was increased to 4:1. Furthermore, TEM showed that a large portion of the optimized complex (starting with 3 mg nosiheptide, 100 mg PC, 15 mg apoAI, and 400 mg SDC) ranged from 10 to 20 nm in diameter, similar to that of the endogenous HDL. Segrest et al. (1999) reported that at a diameter of 10–20 nm, the lipidic contents of HDL complexes would be fully encapsulated by apoAI. Most of the optimized rHDL–nosiheptide complexes appeared as solid particulates under TEM, suggesting that they assumed the structure of mixed micelles similar to that of the natural HDL. TEM studies (Figure 2(B)) also showed that a small portion of the particulates were smaller than 10 nm in diameter. The optimized complex maintained a high drug-loading efficiency (>80%).

Table I. Effects of PC/nosiheptide (w/w) ratio and formulation procedure (sonication or SDC dialysis) on the drug-loading efficiency (mean ± SD, n = 3) of rHDL–nosiheptide complexes.

<table>
<thead>
<tr>
<th>PC/nosiheptide</th>
<th>Sonication</th>
<th>SDC dialysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>100:1</td>
<td>95.7 ± 1.0</td>
<td>93.8 ± 1.1</td>
</tr>
<tr>
<td>50:1</td>
<td>92.8 ± 1.6</td>
<td>90.8 ± 1.3</td>
</tr>
<tr>
<td>33:1</td>
<td>86.8 ± 2.1</td>
<td>81.5 ± 2.4</td>
</tr>
<tr>
<td>25:1</td>
<td>72.8 ± 2.0</td>
<td>72.5 ± 2.1</td>
</tr>
<tr>
<td>20:1</td>
<td>64.1 ± 2.3</td>
<td>60.4 ± 2.3</td>
</tr>
</tbody>
</table>

Table II. Effect of SDC/PC ratio on the particle size (mean ± SD, n = 20) of the dialyzed rHDL–nosiheptide complexes.

<table>
<thead>
<tr>
<th>SDC/PC</th>
<th>Hydrodynamic diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>88.1 ± 10.6</td>
</tr>
<tr>
<td>2:1</td>
<td>55.6 ± 8.9</td>
</tr>
<tr>
<td>4:1</td>
<td>29.3 ± 3.6</td>
</tr>
</tbody>
</table>

Figure 1. TEM (× 52,000) images of prototype nosiheptide formulations (starting PC/nosiheptide = 1/1, w/w). (A) Prototype rHDL–nosiheptide complex prepared by sonication. (B) Prototype rHDL–nosiheptide complex prepared by SDC dialysis (SDS/PC = 1/1, w/w). (C) A sonicated control nosiheptide liposome without apoAI.

Figure 2. TEM (× 52,000) images of rHDL–nosiheptide complexes (starting PC/nosiheptide = 33/1, w/w) prepared by SDC dialysis. (A) SDC/PC = 1/1, w/w. (B) SDC/PC = 4/1, w/w.
Stability of the optimized rHDL–drug complex

Different conditions were tested for the storage of the optimized rHDL–nosiheptide complex (starting with 3 mg nosiheptide, 100 mg PC, 15 mg apoAI, and 400 mg SDC). Our preliminary observations (Table III and Figure 3) showed no noticeable changes of the complex morphology for up to 18 months when stored at −20°C in a sealed container filled with nitrogen together with the cryoprotective agent DMSO (1/10, v/v).

In vitro anti-HBV activity analysis

The anti-HBV activities of the free nosiheptide, a dialyzed control nosiheptide liposome without apoAI, and the optimized rHDL–nosiheptide complex were evaluated in HepG2 2.2.15 cells (Zhai et al. 1990; Kumar et al. 2006) using the HBeAg and HBsAg ELISA assays. As shown in Table IV, the concentration of nosiheptide in optimized rHDL–nosiheptide complex to achieve 50% virus inhibition (IC50) was 0.63 μg/ml, which was 40 times lower than the IC50 of nosiheptide in control liposome (2.5 μg/ml) and 200 times lower than the IC50 of the free nosiheptide (12.5 μg/ml). Control rHDL or liposome preparations without nosiheptide showed no anti-HBV activities (data not shown). These results demonstrated the drastically higher anti-HBV activity of the optimized rHDL–nosiheptide complex, and suggested its ability to target nosiheptide into hepatocytes.

Tissue distribution of rHDL–nosiheptide complex in male Wistar rats

The optimized rHDL–nosiheptide complex was administered (0.5 mg nosiheptide per rat in 1 ml) intravenously to male Wistar rats and the distribution of nosiheptide in the liver and plasma was determined 30 min after injection (Figure 4). The hepatic distribution of the optimized rHDL–nosiheptide complex accounted for most of the administered nosiheptide (73.0 ± 3.58%), and was seven times as much as that in plasma (11.5 ± 0.592%). When the free nosiheptide was administered for comparison, the distribution was 16.7 ± 0.84% in liver and 52.5 ± 3.15% in plasma. These results demonstrated that the rHDL–nosiheptide complex targeted nosiheptide to the liver. Since the reported hepatic uptake of nontargeted, small PC/cholesterol liposome (PC/cholesterol = 2/1, 30–50 nm in diameter; Allen and Everest 1983) is much slower (~12% at 30 min postinjection) than that of the optimized rHDL–nosiheptide complex, our results strongly suggest that

Table III. Stability of the optimized rHDL–nosiheptide complex under different storage conditions.

<table>
<thead>
<tr>
<th>Storage conditions</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Room temperature (RT), open system</td>
<td>Coagulation after one day, dark yellow color after seven days</td>
</tr>
<tr>
<td>RT, sealed container filled with N2</td>
<td>Coagulation after one day</td>
</tr>
<tr>
<td>4°C, sealed container filled with N2</td>
<td>Coagulation after seven days</td>
</tr>
<tr>
<td>−20°C, sealed container filled with N2</td>
<td>No coagulation after 18 months, cracks and chaps on complex surface</td>
</tr>
<tr>
<td>−20°C, sealed container filled with N2, DMSO (1/10, v/v) added</td>
<td>No coagulation after 18 months, no cracks or chaps on complex surface</td>
</tr>
</tbody>
</table>

Figure 3. TEM (×52,000) images of dialyzed rHDL–nosiheptide complexes (starting with 3 mg nosiheptide, 100 mg PC, 15 mg apoAI, and 400 mg SDC) after storage for 18 months at −20°C in the sealed, nitrogen-filled containers (A) with or (B) without the cryoprotective agent DMSO (1/10, v/v).

Figure 4. Distribution of the optimized rHDL–nosiheptide complex and free nosiheptide in male Wistar rats 30 min after i.v. injection. Each group consisted of six or more animals. The error bars represent the SD of the mean. ‘The rest’ denotes the percentage of administered nosiheptide that was unaccounted for by nosiheptide recovered from liver and plasma.
Table IV. Anti-HBV activity of nosiheptide formulations in HepG2 2.2.15 cells.

<table>
<thead>
<tr>
<th>Nosiheptide concentration (µg/ml)</th>
<th>Free nosiheptide</th>
<th>Nosiheptide liposome</th>
<th>rHDL–nosiheptide complex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HBsAg HbeAg</td>
<td>HBsAg HbeAg</td>
<td>HBsAg HBeAg</td>
</tr>
<tr>
<td>0.32</td>
<td>NT NT</td>
<td>NT NT</td>
<td>32.0 ± 1.6 0</td>
</tr>
<tr>
<td>0.63</td>
<td>NT NT</td>
<td>NT NT</td>
<td>51.8 ± 1.2 45.5 ± 2.6</td>
</tr>
<tr>
<td>1.25</td>
<td>NT NT</td>
<td>46.9 ± 2.6 15.1 ± 2.3</td>
<td>68.3 ± 2.0 53.5 ± 1.5</td>
</tr>
<tr>
<td>2.5</td>
<td>NT NT</td>
<td>55.4 ± 1.2 36.2 ± 1.7</td>
<td>76.9 ± 1.4 68.4 ± 2.4</td>
</tr>
<tr>
<td>5.0</td>
<td>NT NT</td>
<td>65.4 ± 3.0 36.8 ± 2.5</td>
<td>NT NT</td>
</tr>
<tr>
<td>12.5</td>
<td>50.0 ± 1.7</td>
<td>18.0 ± 1.1</td>
<td>NT NT</td>
</tr>
<tr>
<td>25.0</td>
<td>57.0 ± 1.9</td>
<td>16.0 ± 1.3</td>
<td>NT NT NT</td>
</tr>
<tr>
<td>50.0</td>
<td>60.0 ± 2.1</td>
<td>21.0 ± 1.5</td>
<td>NT NT NT</td>
</tr>
</tbody>
</table>

NT, not tested.

a significant portion of the optimized rHDL–nosiheptide complex was targeted to the parenchymal hepatocytes via apoAI.

Conclusion

Recombinant HDL complexes were constructed to target the lipophilic anti-HBV agent nosiheptide to hepatocytes. The optimized rHDL–nosiheptide complex had a high drug-loading efficiency and a diameter smaller than 30 nm. The complex drastically enhanced the anti-HBV activity of nosiheptide in HepG2 2.2.15 cells, and targeted most of the administered nosiheptide to liver within 30 min after i.v. injection to rats. Together, this report provides early evidence that it is feasible to develop efficient, HDL-based drug delivery systems against HBV, utilizing apoAI as the targeting moiety. Further in vivo studies on the targeting and anti-HBV activities of this delivery system are on the way and will be reported in due course.

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References

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