Synthesis of a Lipophilic Prodrug of 9-(2-Phosphonylmethoxyethyl)adenine (PMEA) and Its Incorporation into a Hepatocyte-Specific Lipidic Carrier

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Purpose. 9-(2-Phosphonylmethoxyethyl)adenine (PMEA), a potent inhibitor of Hepatitis B virus replication, is in vivo hardly taken up by parenchymal liver cells (the site of infection). Our aim is to examine whether lactosylated reconstituted HDL (LacNeoHDL), a lipidic particle that is specifically internalized by parenchymal liver cells, is a suitable carrier for the selective delivery of PMEA to this cell type.
Methods. To incorporate PMEA into LacNeoHDL, we synthesized a lipophilic prodrug (PMEA-LO) by coupling PMEA via an acid-labile phosphonamidate bond to lithocholic acid-3α-oleate.
Results. The yield of the synthesis was 52% ($[^3H]$PMEA-LO: 24%). $[^3H]$PMEA-LO readily incorporated into LacNeoHDL (13 molecules/particle) without affecting the size and net negative charge of the carrier. Further, incubation studies at lysosomal pH showed $[^3H]$PMEA was completely released from the carrier whereas, at neutral pH or in plasma, appreciable release was not observed.
Conclusions. The conjugation of PMEA with lithocholic acid-3α-oleate results in a lipophilic prodrug that readily associates with LacNeoHDL. The association of the prodrug does not affect the physicochemical properties of the particle, and PMEA is released from the carrier at lysosomal pH. These findings indicate that by using the prodrug approach, LacNeoHDL is a suitable carrier to deliver PMEA to parenchymal liver cells.

KEY WORDS: chronic hepatitis B; PMEA; acid-labile prodrug; drug carrier; asialoglycoprotein receptor.

INTRODUCTION

Chronic hepatitis B is a liver disease caused by an infection of the parenchymal liver cell with the hepatitis B virus (HBV). If left untreated, the disease can eventually lead to cirrhosis and hepatocellular carcinoma (1). Presently, interferon-alpha is the only clinically approved therapeutic agent. Unfortunately, interferon-alpha is far from optimal, due to a low response rate and a number of dose-dependent side-effects (2). Therefore, several alternative anti-HBV drugs have been developed, of which the nucleoside analogs appear to be the most promising. One of these analogs, showing good efficacy in HBV infected cell lines, is the acyclic nucleoside phosphonate analog 9-(2-phosphonylmethoxyethyl)adenine (PMEA) (3). However, *in vivo*, most of the administered PMEA accumulates in the kidneys, and $<5\%$ of the drug is taken up by the liver, the primary site of HBV infection (4). This is likely to result in serious nephrotoxicity, as was demonstrated recently for the structurally related compounds (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine ((*S*)-HPMPA) and (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine ((*S*)-HPMPC) (5,6). Selective delivery of PMEA to the parenchymal liver cell may represent a promising approach to improve its therapeutic efficacy against chronic hepatitis B.

Because of its unique localization and abundant expression on parenchymal liver cells, the asialoglycoprotein receptor represents an attractive target for selective delivery of drugs to this cell type (7). The asialoglycoprotein receptor specifically recognizes proteins, polymers, and particles with exposed galactose and N-acetyl-galactosamine residues (8). After binding to the receptor, ligands are rapidly internalized and transported to the lysosomes.

We demonstrated recently that NeoHDL, artificial high density lipoprotein (HDL)-like particles constructed of lipids and isolated apoproteins, can be specifically targeted to the asialoglycoprotein receptor by lactosylation of the apoproteins (9). Lactosylated NeoHDL (LacNeoHDL) therefore constitutes an attractive carrier system for the delivery of lipophilic (pro)-drugs to parenchymal liver cells, as lipophilic (pro)drugs can be incorporated into the lipid moiety of the particles without interfering with the receptor-mediated recognition of the lactosylated apoproteins. This constitutes a major advantage over other galactose-exposing soluble carrier systems, such as lactosaminated serum albumin and poly-L-lysine, in which both drug and galactose are located in close vicinity of each other (10,11). Galactose-exposing liposomes, a particulate carrier system, have also been described. However, in the liver these particles are primarily taken up by Kupffer cells (via the galactose-particle receptor), rather than parenchymal liver cells (12).

Although some highly lipophilic drugs can spontaneously incorporate into lipoproteins, most antiviral drugs, including PMEA, are too hydrophilic. These compounds need to be chemically modified with a lipophilic residue to induce association. We recently investigated the feasibility of this approach, using the antiviral drug iododeoxyuridine as model compound. We found that incorporation of the dioleoyl prodrug of iododeoxyuridine (IDU-OI_2) into LacNeoHDL leads to a high and selective uptake of the drug by parenchymal liver cells (13).

In the present study, we investigated whether the aforementioned strategy can also be used to incorporate PMEA into LacNeoHDL. Lithocholic acid-3α-oleate (3α-(oleoyloxy)-5β-cholanic acid) was chosen as lipophilic anchor because it was recently demonstrated that this steroid ester induces spontaneous association of hydrophilic compounds, such as antisense

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ABBREVIATIONS: DCM, dichloromethane; DiPEA, *N,N*-diisopropylethylamine; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.HCl; HBV, hepatitis B virus; (*S*)-HPMPA, (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine; (*S*)-HPMPC, (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine; IDU-OI_2, dioleoyl-iododeoxyuridine; Lac(Neo)HDL, lactosylated (reconstituted) high density lipoprotein; LDL, low density lipoprotein; MeOH, methanol; PBS, phosphate-buffered saline, pH 7.4; PMEA, 9-(2-phosphonylmethoxyethyl)adenine; PMEA-NH_2, ethylenediamino-PMEA; PMEA-LO, conjugate of PMEA-NH_2 and lithocholic acid-3α-oleate; (RP)-TLC, (reversed-phase)-thin layer chromatography; TEA, triethylamine.

oligodeoxynucleotides and daunorubicin, with lipoprotein(-like) particles (14,15). PMEA was covalently coupled to lithocholic acid-3α-oleate via an acid-labile phosphonamidate bond (16), and the resulting lipophilic prodrug (PMEA-LO) was subsequently incorporated into LacNeoHDL. The acid-labile linkage of PMEA to the lipophilic anchor was chosen to trigger the release of the drug once the complex is delivered to the acidic lysosomes. The physico-chemical properties of the prodrug-loaded carrier were investigated, as well as the release of PMEA from the complex at lysosomal pH.

MATERIALS AND METHODS

Reagents

9-(2-Phosphonylmethoxyethyl)adenine (PMEA) was kindly provided by Dr. J. Balzarini (Rega institute, Leuven, Belgium). [Adenine-2,8-^{3}H]-PMEA (0.33 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide. HCl (EDC) was obtained from Pierce (Rockford, IL). *N,N*-diisopropylethylamine (DiPEA, 99.5%, redistilled) was from Aldrich (Brussels, Belgium). The pentafluorophenyl ester of 3α-(oleoyloxy)-5β-cholanic acid (lithocholic acid-3α-oleate) was synthesized as described earlier (14). Dichloromethane (DCM) was dried by refluxing with P_2O_5, distilled, and stored over molecular sieves (4Å). Methanol (MeOH) was dried by refluxing with magnesium, distilled, and stored over molecular sieves (3Å). *N,N*-Dimethylacetamide and dioxane were stored over molecular sieves (4Å). All other reagents were of analytical grade.

General Procedures

Thin layer chromatography analyses were performed on Silica gel 60 F_{254} (TLC) or RP18 F_{254s} (RP-TLC) aluminium sheets (Merck, Darmstadt, Germany). Compounds were visualized by UV (254 nm), or by spraying with ninhydrin for detection of primary/secondary amines or $MnCl_2$ for steroids (14). ^{1}H-NMR spectra (200 MHz), ^{31}P-NMR spectra (80.7 MHz), and ^{13}C-NMR spectra (50.1 MHz) were measured using a Jeol JNM-FX 200. ^{1}H-NMR spectra (300 MHz) were recorded on a Bruker WM-300 spectrometer. ^{13}C-NMR spectra were monitored using the attached proton test technique. ^{1}H and ^{13}C chemical shifts (δ) are given in ppm relative to tetramethylsilane as internal standard and ^{31}P chemical shifts are given in ppm relative to 85% H_3PO_4 as external standard.

Synthesis of ([^{3}H])PMEA-LO: 1] Synthesis of Ethylenediamino-([^{3}H])PMEA (PMEA-NH_2)

PMEA (56 mg, 205 μmol) was dissolved in 2 ml of an aqueous solution of 0.5 M ethylenediamine (pH 6.1; adjusted with concentrated HCl). The subsequent addition of a 5-fold molar excess of EDC (196 mg, 1.02 mmol) started the reaction. After stirring overnight at room temperature, the mixture was lyophilized. The residue was taken up in 2.5 ml of H_2O, and divided into 5 equal portions. PMEA-NH_2 was subsequently purified from each portion using a Macherey-Nagel Nucleosil C18-column (5 μm, 250*4.6 mm). After injection of the sample, the column was washed with H_2O (20 min), followed by a linear gradient (10 min) from 0–50% acetonitrile (flow: 0.75 ml/min; Detection by A_{260}). Finally, PMEA-NH_2 (elution at approximately 20%) was lyophilized and stored at −20°C. The purity was >90% as judged by TLC using MeOH/NH_4OH (100/1.5, v/v) as solvent. Yield: 55 mg (85%); ^{1}H NMR (D_2O, 200 MHz): 8.19, 8.18 (2H, s, H-8 and H-2), 4.41 (2H, t, H-1′), 3.92 (2H, t, H-2′), 3.54 (2H, d, H-4′), 2.50–2.33 (4H, m, H-1″ and H-2″). ^{31}P NMR (D_2O): 19.176 (1P, s, HN*P*CH).

[^{3}H]PMEA-NH_2 was synthesized as described above, except that 126 μg of [^{3}H]PMEA (0.79 mCi/mg) and 366 μl of 0.25 M of ethylenediamine were used, and that a 100-fold molar excess of EDC was added. The HPLC fractions containing [^{3}H]PMEA-NH_2 were pooled and the absorption at 260 nm was measured. Finally, the pooled fractions were divided into aliquots of 50 nmol of [^{3}H]PMEA-NH_2, lyophilized and stored at −20°C. The (radiochemical) purity of the product was >90% as judged by TLC (MeOH/NH_4OH; 100/1.5). Yield of radioactivity: 70–80%.

Synthesis of ([^{3}H])PMEA-LO: 2] Conjugation of 3α-(oleoyloxy)-5β-Cholanic Acid and ([^{3}H])PMEA-NH_2

To a solution of PMEA-NH_2 (31 mg, 98 μmol) in 1.8 ml of H_2O were added 6 ml of *N,N*-dimethylacetamide and a solution of 66.5 mg (82 μmol) of the pentafluorophenyl ester of 3α-(oleoyloxy)-5β-cholanic acid, dissolved in 4.8 ml of dioxane. The reaction was initiated by adding 60 μl of DiPEA. After stirring overnight at room temperature, the reaction mixture was diluted with 4 ml of H_2O, and the conjugate was extracted twice with 14 ml of MeOH/DCM (2/5, v/v). The organic layers were pooled and the solvents were removed *in vacuo*. Finally, the residue was dissolved in dimethylsulfoxide (10 mg/ml) and stored at −20°C. The purity was >90% as judged by RP-TLC, using MeOH/DCM/TEA (45/53/2, v/v/v) as solvent. Yield: 57 mg (61%); ^{1}H NMR ($CDCl_3$/MeOD; 9/1, 300 MHz): 8.24, 8.22 (2H, s, H-8 and H-2), 5.32 (2H, br m, vinyl H), 4.72 (1H, br m, H-3, lithochol), 4.38 (2H, t, H-1′), 3.85 (2H, t, H-2′), 3.64 (2H, br s, H-4′), 3.44–3.01 (4H, m, H-1″ /H-2″), 0.88 (3H, t, H_{18}-oleoyl), 0.62 (3H, s, H_{18}-lithochol). ^{13}C {^{1}H} NMR ($CDCl_3$): 175.3 (C=O,amide), 173.3 (C=O,ester), 155.4 (C-6), 152.1 (C-2), 149.5 (C-4), 118.5 (C-5), 129.9/129.7 (C=H,oleoyl), 74.1 (C_3-lithochol) 70.9 (C-2′), 42.7 (C-1′), 39.6/37.5 (C-1″/C-2″), 14.0 (C_{18}-oleoyl), 12.0 (C_{18}-lithochol).

For the synthesis of radiolabeled PMEA-LO, [^{3}H]PMEA-NH_2 (50 nmol, 10 μCi) was dissolved in 2.3 μl of 20 mM NaOH. The reaction was subsequently started by adding 7.7 μl of *N,N*-dimethylacetamide, containing 0.038 μl DiPEA, and 7 μl of dioxane, containing 35 μg (43 nmol) of the pentafluorophenyl ester of 3α-(oleoyloxy)-5β-cholanic acid. After standing overnight at room temperature, [^{3}H]PMEA-LO was purified by RP-TLC using MeOH/DCM/TEA (45/53/2; v/v/v) as solvent. Material at the same position as the PMEA-LO marker was scraped off and extracted thrice with 500 μl DCM/MeOH (1/1; v/v). The organic layers were pooled and used immediately for incorporation experiments. The (radiochemical) purity of the product was 90%, as judged by RP-TLC (MeOH/DCM/TEA; 45/53/2). Yield of radioactivity: 30%

Preparation of Lactosylated HDL Apoproteins

HDL was isolated from 250 ml of freshly isolated human serum (Blood bank, Leiden, The Netherlands) by precipitation with sodium phosphotungstate and $MgCl_2$ as described in detail earlier (17). The isolated HDL (10 mg of protein/ml) was subsequently lactosylated as described previously (13). Protein was measured by the Bicinchoninic acid (BCA) protein assay of Pierce, using bovine serum albumin (fraction V; Sigma, St.Louis, MO) as a standard. Lactose was assayed as described previously (13). Finally, the lactosylated HDL apoproteins were isolated by delipidation with diethylether/ethanol (3/2, v/v), dialyzed against 5 mM NH_4HCO_3, lyophilized, and stored under argon at −20°C.

Preparation of [^{3}H]PMEA-LO-Loaded LacNeoHDL

[^{3}H]PMEA-LO-loaded LacNeoHDL was prepared essentially as was described earlier for NeoHDL (18). [^{3}H]PMEA-LO (0.5 mg, specific radioactivity: 10–35 dpm/ng) was cosonicated at 49–52°C with 3.6 mg of egg yolk phosphatidylcholine (98%; Fluka, Buchs, Switzerland). and 1.8 mg of cholesteryl oleate (97%; Janssen, Beerse, Belgium). The sonication was stopped after 30 min, and the temperature was lowered to 42°C. Sonication was continued for 30 min, and 6 mg of lactosylated HDL apoproteins, dissolved in 1 ml 4 M ureum, were added in 10 equal portions over the first 10 min. The resulting particles were purified by size-exclusion chromatography using a Superose® 6 column (1.6 × 50 cm) eluted with phosphate-buffered saline, pH 7.4 (PBS), containing 1 mM EDTA. Finally, [^{3}H]PMEA-LO-loaded LacNeoHDL was passed through a Millipore filter (0.45 μm), and stored at 4°C.

Characterization of [^{3}H]PMEA-LO-Loaded LacNeoHDL

Cholesteryl oleate and egg yolk phosphatidylcholine were assayed as described previously (13). Protein and lactose were determined as described above. The amount of [^{3}H]PMEA-LO was determined by measuring radioactivity. The size of [^{3}H]PMEA-LO-loaded LacNeoHDL was determined by gel permeation chromatography using a SMART system equipped with a Superose® 6 PC 3.2/30 column (Pharmacia Biotech, Uppsala, Sweden). The column was eluted with PBS, containing 0.01% sodium azide, at a flow rate of 50 μl/min. The injection volume was 50 μl, and fractions of 100 μl were collected for analysis. The mean diameter of [^{3}H]PMEA-LO-loaded LacNeoHDL was calculated from a quadratic equation, which was derived from polynomial regression between the Stoke's diameter and the partition coefficient (K_{AV}) of 6 standards: LDL (24.2 nm), thyroglobulin (17.0 nm), ferritin (12.2 nm), HDL (9.5 nm), lactate dehydrogenase (8.16 nm) and bovine serum albumin (7.1 nm) (9,19,20). The correlation coefficient of the regression curve was 0.999. The net negative charge of [^{3}H]PMEA-LO-loaded LacNeoHDL was determined by gel electrophoresis using a 0.75% (w/v) agarose gel at pH 8.8 (75 mM Tris-HCl, 80 mM Hippuric acid and 0.65 mM EDTA).

Determination of the Release of [^{3}H]PMEA from [^{3}H]PMEA-LO-Loaded LacNeoHDL

Aliquots of 82.5 μl of [^{3}H]PMEA-LO-loaded LacNeoHDL, dissolved in PBS, containing 1 mM EDTA (30 μg/ml of [^{3}H]PMEA-LO), were mixed with either 17.5 μl of 0.1 M sodium citrate, pH 4.4 (final pH: 4.7), or 17.5 μl of 0.1 M sodium phosphate, pH 7.4. To study the release in plasma, 25 μl of [^{3}H]PMEA-LO-loaded LacNeoHDL, dissolved in PBS, containing 1 mM EDTA (26 μg/ml of [^{3}H]PMEA-LO), was mixed with 75 μl of freshly isolated rat plasma. At the indicated times, 50 μl samples were analyzed by gel permeation chromatography using the SMART system described above. Fractions eluting between 2.0–2.4 ml, which contain the low-molecular weight products, were pooled. To identify [^{3}H]PMEA as the primary hydrolysis product, a 100 μl sample was taken and analyzed on a Jasco-HPLC system equipped with a Macherey-Nagel Nucleosil C18 column (5 μm, 250*4.6 mm). The column was eluted with 5% acetonitrile in 50 mM KH_2PO_4 and 5 mM tetrabutylammonium hydrogen sulfate (pH 6.0; adjusted with 10 M NaOH), at a flow rate of 1 ml/min. UV detection (260 nm) was performed using a Jasco UV-975 detector (Tokyo, Japan). Data were processed using Borwin™ chromatography software (JMBS Developpements, Le Fontanil, France).

Determination of the Specific Radioactivity of [^{3}H]PMEA-LO

As direct determination of the specific radioactivity of [^{3}H]PMEA-LO proved to be difficult, it was determined indirectly by measuring the specific radioactivity of the [^{3}H]PMEA moiety after acid hydrolysis of the prodrug. To this end, carrier-associated [^{3}H]PMEA-LO (30 μg/ml) was subjected to acid hydrolysis (6 h), gel permeation chromatography, and HPLC analysis as described in the previous section. The amount of PMEA in the peak was calculated by relating its UV-signal to the UV-peak signals obtained after injecting a series of PMEA standards into the HPLC system. The PMEA standards gave a linear increase signal between 20 and 400 ng (correlation coefficient: >0.997). From these results, the specific radioactivity of the released [^{3}H]PMEA (dpm/ng) could be calculated, and the specific radioactivity of [^{3}H]PMEA-LO was calculated from the difference in molecular weights (PMEA: 273 and PMEA-LO: 938 g/mol).

Determination of Radioactivity

SMART and HPLC samples and TLC scrapings were counted in 2, 15 and 10 ml of Emulsifier Safe™, respectively. Gel slices were first overnight digested with 0.4 ml Soluene-350, and then counted in 4 ml of Hionic Fluor™. All samples were counted in a Packard Tri-Carb 1500 liquid scintillation counter. The apparatus, scintillation cocktails, and Soluene-350 were from Packard (Downers Grove, IL).

RESULTS

Synthesis of ([^{3}H])PMEA-LO

PMEA-LO was synthesized via a two-step reaction (Fig. 1). First, ethylenediamino-PMEA (PMEA-NH_2) was synthesized using a procedure earlier described for the synthesis of ethylenediamino-nucleotides (21). The water-soluble carbodiimide EDC was used to couple PMEA via a phosphonamidate bond to ethylenediamine. PMEA-NH_2 was subsequently purified by reversed-phase chromatography to a purity of >90%. In the second step, PMEA-NH_2 was treated with a molar short measure of the pentafluorophenyl ester of lithocholic acid-3α-oleate in the presence of the organic base DiPEA to furnish the desired product, PMEA-LO. Since the use of a molar short measure resulted in a quantitative coupling of the lipid to

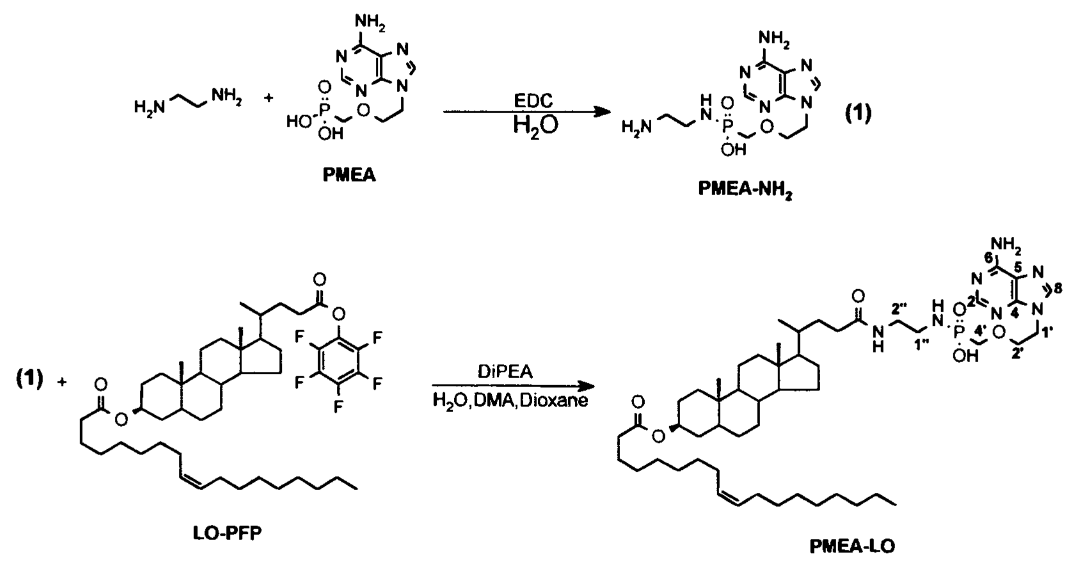

Fig. 1. Two-step synthesis of PMEA-LO using ethylenediamine as spacer.

PMEA-NH_2, PMEA-LO could subsequently easily be purified by extraction with MeOH/DCM (2/5; v/v). The overall yield of PMEA-LO was 52% and the purity was >90%. The structure of PMEA-LO was confirmed by ^{1}H-and ^{13}C-NMR. The method described above was also used to synthesize tritium-labeled PMEA-LO, starting from [^{3}H]PMEA, except that [^{3}H]PMEA-LO was purified by means of preparative reversed phase thin layer chromatography instead of extraction. The final yield of [^{3}H]PMEA-LO was 24% and the radiochemical purity was 90%.

Preparation and Characterization of [^{3}H]PMEA-LO-Loaded LacNeoHDL

[^{3}H]PMEA-LO was incorporated in lactosylated NeoHDL by means of cosonication with lipids and lactosylated HDL apoproteins. This procedure is similar to the ones described earlier for the preparation of NeoHDL and IDU-Ol_2-loaded (Lac)NeoHDL (9,13,18). In our previous studies, IDU-Ol_2-loaded LacNeoHDL was obtained by preparation of prodrug-loaded NeoHDL, followed by lactosylation of the particles. In the present study, the preparation procedure was significantly shortened by using HDL apoproteins that had been lactosylated beforehand. The lactosylated HDL apoproteins could be obtained at large scale by reductive lactosamination of HDL and subsequent delipidation of the lactosylated apoproteins. SDS-polyacrylamide gel electrophoresis of the apoproteins revealed lactosylated apoprotein A-I was the main constituent (>85%). Based on the lactose and protein content, it can be calculated that approximately 28 lactose residues are coupled to one apoprotein A-I molecule. After sonication, the [^{3}H]PMEA-LO-loaded LacNeoHDL particles were purified by gel permeation chromatography.

The chemical composition of [^{3}H]PMEA-LO-loaded LacNeoHDL is given in Table I. The particles contained a substantial amount of PMEA-LO: 2.6 ± 0.3% of the total weight (approximately 5% of the lipid moiety). Based on its size and composition, and assuming that its density is similar to that of native HDL, it can be calculated that each particle comprises approximately 13 PMEA-LO molecules. The final purified preparation of the particles contained 46.6 ± 8.1, 51.2 ± 2.8%, 49.4 ± 5.2%, and 27.2 ± 2.8% of the added lactosylated apoproteins, egg yolk phosphatidylcholine, cholesteryl oleate, and PMEA-LO, respectively (mean ± SEM, n = 4). The lower recovery of the prodrug, compared to the other constituents, is probably due to the harsh conditions during sonication (e.g., locally high temperatures), which results in hydrolysis and/or exchange of tritium from the prodrug to water. Analysis of the sonication mixture by high-resolution gel permeation chromatography indicated that approximately 20% of the radiolabel in the prodrug had become water-soluble and was subsequently lost from the particles (data not shown).

Physical properties, such as size and net negative charge, are crucial for the biological fate of a drug carrier. The size of [^{3}H]PMEA-LO-loaded LacNeoHDL was determined by high resolution gel permeation chromatography. Figure 2 shows the elution profiles of the apoproteins, the lipid constituent cholesteryl oleate, and the radiolabeled prodrug. These different particle components exhibit a similar elution pattern, which clearly demonstrates that they form one unity. The mean elution volume was used to calculate the size of the particles, and it was found that [^{3}H]PMEA-LO-loaded LacNeoHDL particles have a diameter of 11.0 ± 0.3 nm (mean ± SEM, n = 4). The net negative charge of [^{3}H]PMEA-LO-loaded LacNeoHDL was studied by agarose gel electrophoresis of the prodrug-loaded

Table I. Chemical Composition of [^{3}H]PMEA-LO-Loaded LacNeoHDL

Constituent	% of total weight[a]
Protein	35.5 ± 4.1
Egg yolk phosphatidylcholine	33.7 ± 2.7
Cholesteryl oleate	16.0 ± 0.9
Lactose	12.1 ± 1.1
[^{3}H]PMEA-LO	2.6 ± 0.3

[a] The chemical composition of [^{3}H]PMEA-LO-loaded LacNeoHDL was determined as described in Materials and Methods. Values are expressed as the mean ± SEM of four different preparations.

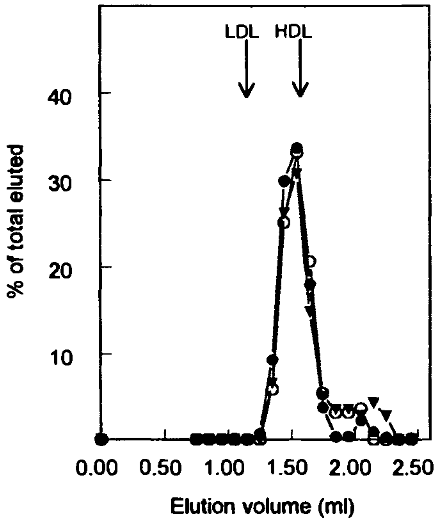

Fig. 2. Analysis of [^{3}H]PMEA-LO-loaded LacNeoHDL by gel permeation chromatography. [^{3}H]PMEA-LO-loaded LacNeoHDL was analyzed by gel permeation chromatography using the SMART system described in Materials and Methods. Fractions of 100 μl were collected and assayed for protein (○), cholesteryl oleate (▼) and [^{3}H]PMEA-LO (●). Egg yolk phosphatidylcholine exhibited a similar elution pattern as the other three constituents, but was left out for clarity. The results are expressed as % of total eluted (recoveries: >76%). The elution volumes of LDL and HDL are indicated with arrows.

particles. The prodrug, lipid and protein moiety displayed an identical behavior (Fig. 3), which confirms that these three constituents form one unity. The electrophoretic mobility (relative to bromophenol blue) of [^{3}H]PMEA-LO-loaded LacNeoHDL was 0.36 ± 0.01 (mean ± variation, n = 2), which is lower than that of native HDL (R_f: 0.57).

Release of [^{3}H]PMEA from [^{3}H]PMEA-LO-loaded LacNeoHDL

In order to become pharmacologically active, it is crucial PMEA is released from the prodrug-loaded carrier. We therefore chose to include a phosphonamidate bond in the PMEA prodrug, which is acid-labile and should trigger the release of PMEA once the complex is exposed to the acidic environment of the lysosomes. The release of [^{3}H]PMEA from the prodrug-loaded carrier was studied by incubating [^{3}H]PMEA-LO-loaded LacNeoHDL at 37°C at pH 4.7, the ambient pH in the lysosomes (22). To examine the pH-sensitivity of the release, the prodrug-loaded carrier was also incubated at pH 7.4, the physiological pH. To separate released [^{3}H]PMEA from the prodrug-loaded carrier, samples were subjected to gel permeation chromatography. Figure 4 shows the elution profiles of the (pro)drug, and the core lipid cholesteryl oleate. After 6 hours of incubation at pH 4.7, the release of [^{3}H]PMEA from the prodrug-loaded carrier was almost complete (approximately 90%), whereas at pH 7.4 only a small fraction of total prodrug was released. In contrast, the elution profile of cholesteryl oleate was not affected by 6 hours of incubation at pH 4.7. This finding indicates the particle remains intact under the acidic conditions, and the observed release of PMEA from the carrier was solely the result of hydrolysis. To ascertain the released radioactivity represents PMEA, a sample was subjected to reversed HPLC. Figure 5 shows the applied radioactivity eluted as PMEA, indicating the released radioactivity indeed represents PMEA. Finally, to examine the stability of the carrier-associated prodrug in the circulation, the release of [^{3}H]PMEA from the prodrug-loaded

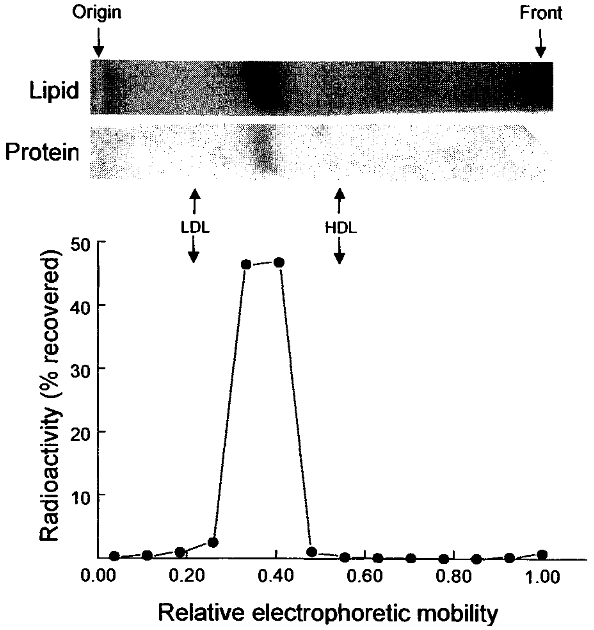

Fig. 3. Analysis of [^{3}H]PMEA-LO-loaded LacNeoHDL by gel electrophoresis. [^{3}H]PMEA-LO-loaded LacNeoHDL was subjected to electrophoresis in a 0.75% (w/v) agarose gel at pH 8.8 (75 mM Tris-Hippuric buffer). One lane was cut in 0.5 cm slices that were assayed for ^{3}H-radioactivity. The radioactivity in each slice is given as % of total recovered radioactivity (recovery: >80%). Two other lanes were stained for protein and lipid with Coomassie Brilliant Blue and Sudan Black, respectively. The electrophoretic mobilities (relative to the front marker Bromophenol blue) of LDL and HDL are indicated with arrows.

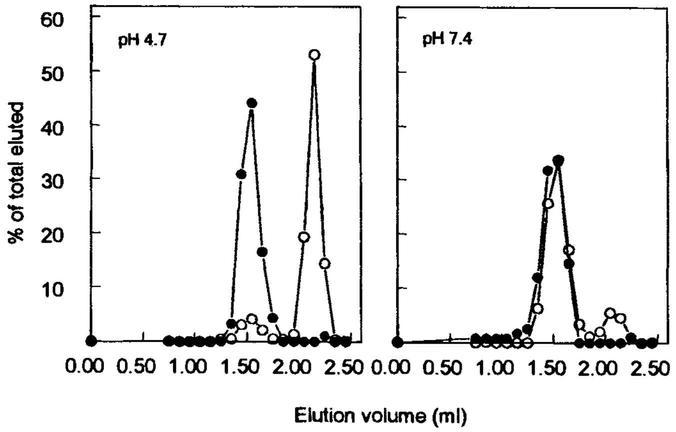

Fig. 4. Release of [^{3}H]PMEA from [^{3}H]PMEA-LO-loaded LacNeoHDL at lysosomal and neutral pH. [^{3}H]PMEA-LO-loaded LacNeoHDL was incubated at pH 4.7 (left panel) and pH 7.4 (right panel) at 37°C. After 6 hours, samples were analyzed by gel permeation chromatography as described in Materials and Methods. Fractions of 100 μl were collected and assayed for ^{3}H-radioactivity (○) and cholesteryl oleate (●) as described in materials and methods. The results are expressed as % of total eluted (recoveries: >68%).

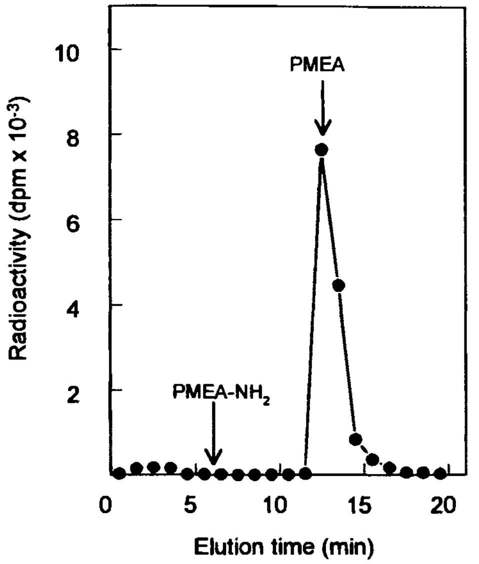

Fig. 5. HPLC analysis of radioactivity released upon acidic incubation of [^{3}H]PMEA-loaded LacNeoHDL. [^{3}H]PMEA-LO-loaded LacNeoHDL was incubated at 37°C for 6 hours at pH 4.7. The reaction mixture was analyzed by gel permeation chromatography as in Fig.4. Fractions containing released radioactivity (2.0–2.4 ml) were pooled and analyzed by HPLC as described in Materials and Methods. Fractions eluting from the HPLC column (1 ml) were collected and assayed for ^{3}H-radioactivity. The elution times of PMEA and ethylenediamino-PMEA standards in this system are indicated with arrows.

carrier was studied by incubating [^{3}H]PMEA-LO-loaded LacNeoHDL at 37°C with freshly isolated rat plasma. When the prodrug-loaded carrier was incubated for 3 hours, only 5.0 ± 0.6% of the [^{3}H]PMEA was released (mean ± SEM, n = 3), which predicts the prodrug will not be hydrolyzed in the circulation.

DISCUSSION

Carrier-mediated delivery of PMEA to parenchymal liver cells should result in a significant increase in its efficacy against chronic hepatitis B, and concomitantly reduce nephrotoxic side-effects. In the present study, we synthesized a lipophilic prodrug of PMEA and investigated its incorporation into LacNeoHDL.

The lactosylated NeoHDL carrier was designed for the selective delivery of lipophilic (pro)drugs to the parenchymal liver cells (9). PMEA is water-soluble. To induce association with the carrier, PMEA was coupled to lithocholic acid-3α-oleate via an ethylenediamine spacer, yielding a lipophilic prodrug (PMEA-LO). Lithocholic acid-3α-oleate was chosen as a lipophilic anchor, because attachment of a number of hydrophilic compounds to this molecule was found to induce a stable incorporation of the compounds into LDL or LDL-mimicking particles (14,15). Moreover, lithocholic acid-3α-oleate is, like LacNeoHDL, composed of endogenous components and thus completely biodegradable and non-toxic. PMEA-LO contains an acid-labile phosphonamidate bond (16), which was meant to trigger the release of PMEA once the prodrug-loaded carrier enters the acidic lysosomes. The overall yield of the synthesis was 52% and the purity was <90%. Tritium-labeled PMEA-LO was synthesized at the same purity, but at a lower yield (24%).

PMEA-LO was incorporated into LacNeoHDL by cosonicating the prodrug with its lipid components and lactosylated HDL apoproteins. Based on weight, PMEA-LO-loaded LacNeoHDL contained 2.6 ± 0.3% of prodrug, which corresponds to approximately 13 molecules per particle. The size and net negative charge of PMEA-LO-loaded LacNeoHDL were determined, because both properties play a crucial role in the biological fate of particulate carriers. In addition to the already mentioned asialoglycoprotein receptor on parenchymal liver cells, Kupffer cells also express a galactose-recognizing receptor (23). Only galactose-exposing particles larger than 15 nm exhibit a high affinity for this receptor, whereas smaller galactose-exposing carriers are not taken up (9,24). Based on gel permeation chromatography, we calculated a size of 11.0 ± 0.3 nm for [^{3}H]PMEA-LO-loaded LacNeoHDL. The PMEA prodrug-loaded particles should therefore be sufficiently small to avoid uptake by the galactose receptor on Kupffer cells. Negative charges on a carrier can induce rapid uptake by scavenger receptors present on liver endothelial and Kupffer cells (25). The relative electrophoretic mobility of PMEA-LO-loaded LacNeoHDL was lower than that of native HDL, which strongly suggests the prodrug-loaded carrier will not be recognized by scavenger receptors. In the present study, PMEA-LO-loaded LacNeoHDL was prepared on a laboratory scale. However, large-scale preparation for therapeutic purposes is probably feasible. A method for large-scale preparation of NeoHDL, suitable for therapeutic use, has been reported (26). The product could easily be stabilized by lyophilization in the presence of sucrose. Moreover, the carrier was well tolerated in humans after intravenous infusion, and during a 3-month follow-up period no antibodies against the carrier were detected (27).

Finally, we studied the release of PMEA from PMEA-LO-loaded LacNeoHDL. Ligands are internalized via the asialoglycoprotein receptor rapidly traverse through the endosomal compartment, and reach within 10 min their final destination, the lysosomes (28). Therefore, we chose to study the release of PMEA at pH 4.7, the ambient pH in the lysosomes (22). Within 6 hours of incubation, PMEA was almost completely liberated from LacNeoHDL. The release of PMEA occurred only under acidic conditions; at neutral pH and in freshly isolated rat plasma no appreciable release was observed. Thus, PMEA will remain associated with the carrier during transport in the circulation, and be released when the prodrug-loaded carrier is delivered to the lysosomes. The carrier itself remained intact during the incubation at low pH. From this latter, in combination with the observed [^{3}H]PMEA release, we conclude that [^{3}H]PMEA-LO is present on the surface, i.e. in the phospholipid shell of the LacNeoHDL particles. Since no degradation products were found in cellular extracts after receptor-mediated endocytosis of PMEA (29), the released PMEA is probably stable in the lysosomes.

For the following reasons, it is anticipated that the released PMEA can subsequently escape to the cytosol and exert its pharmacological action. Inside the acidic lysosomes, a small proportion of PMEA will be fully protonated, and thus uncharged. Uncharged PMEA is likely to leave the lysosomes by passive diffusion. Once in the cytosol, the translocated PMEA is rapidly deprotonated and the existing concentration gradient of unprotonated PMEA between lysosomes and cytosol is maintained. A similar explanation was recently proposed by

Connelly *et al.* for the lysosomal release of (*S*)-HPMPC, a related nucleoside phosphonate analog, in Vero cells (30).

In conclusion, we have synthesized PMEA-LO, an acid-labile lipophilic prodrug of PMEA. The prodrug readily incorporates into LacNeoHDL, without affecting the physico-chemical properties of the carrier. The observed *in vitro* hydrolysis indicates PMEA will be released in its pharmacologically active form once the prodrug-loaded carrier enters the lysosomes of the parenchymal liver cell. Thus, the present data suggests effective carrier-mediated delivery of PMEA to parenchymal liver cells by the LacNeoHDL carrier is feasible *in vivo*.

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