

Carrier-Mediated Delivery Improves the Efficacy of 9-(2-Phosphonylmethoxyethyl)Adenine against Hepatitis B Virus

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Received December 20, 2000; accepted May 18, 2001

This paper is available online at <http://molpharm.aspetjournals.org>

ABSTRACT

We recently synthesized a lipophilic prodrug of 9-(2-phosphonylmethoxyethyl)adenine (PMEA), designated PMEAL-LO, and incorporated it into reconstituted lactosylated high-density lipoprotein (LacNeoHDL). In a rat model, LacNeoHDL-associated PMEAL-LO was internalized by the asialoglycoprotein receptor on parenchymal liver cells and converted into its active diphosphorylated metabolite. To further evaluate the therapeutic potential of the carrier-associated prodrug, we examined in this study the processing of ¹²⁵I-labeled PMEAL-LO-loaded LacNeoHDL by HepG2 cells. Upon incubation with HepG2 cells, PMEAL-LO-loaded LacNeoHDL became rapidly cell-associated. The association was saturable and of high-affinity ($K_d = 3.8 \pm 0.4$ nM). Asialofetuin, an established ligand for the asialoglycoprotein receptor, inhibited the association by >75%, which confirms the role of the asialoglycoprotein receptor. Association of the prodrug-loaded particles to HepG2 cells was coupled to degradation. Radiolabeled degradation

products appeared in the culture medium with a lag phase of 2 h. Asialofetuin and chloroquine inhibited secretion of degradation products by 75 to 80%, indicating that PMEAL-LO-loaded LacNeoHDL is internalized via the asialoglycoprotein receptor and lysosomally processed. The therapeutic potential of LacNeoHDL-associated PMEAL-LO was studied by measuring its effects on hepatitis B virus (HBV) replication in Hep AD38 cells (HBV-transfected HepG2 cells). LacNeoHDL-associated PMEAL-LO effectively inhibited HBV DNA synthesis. The EC_{50} value of carrier-associated PMEAL-LO was 35 times lower than that of free PMEAL (3.4 \pm 0.4 and 120 \pm 18 ng of PMEAL/ml, respectively). We conclude that the present results, combined with our earlier in vivo disposition data, underscore the therapeutic potential and utility of PMEAL-LO-loaded LacNeoHDL for treatment of chronic hepatitis B.

Chronic hepatitis B results from infection of parenchymal liver cells with hepatitis B virus (HBV) and is a wide-spread disease associated with a high degree of mortality and morbidity. Interferon- α and lamivudine are presently the only approved therapeutic agents for chronic hepatitis B. However, interferon- α has unsatisfactory response rates and can provoke serious side effects (Hoofnagle, 1998). Lamivudine is effective and well tolerated, but long-term treatment results in the emergence of virus drug resistance (Hagmeyer and Pan, 1999; Ono-Nita et al., 1999). Therefore, the development of effective and safe drugs for the treatment of chronic hepatitis B remains imperative. A promising candidate drug is the acyclic nucleoside phosphonate analog 9-(2-phosphonylmethoxyethyl)adenine (PMEA; adefovir). It inhibits the rep-

lication of human HBV in vitro (Yokota et al., 1991; Hejltink et al., 1994), and it has been shown that lamivudine-resistant HBV mutants remain sensitive to PMEAL (Xiong et al., 1998; Ono-Nita et al., 1999). Unfortunately, PMEAL displays unfavorable pharmacokinetics, in that the drug accumulates primarily in the kidneys; only a limited amount is taken up by liver (Naesens et al., 1992). The high kidney uptake of PMEAL is likely to result in nephrotoxicity, as has been shown for related nucleoside phosphonate analogs (Bischofberger et al., 1994; Smeijsters et al., 1996).

To enhance the therapeutic effects of PMEAL in the liver and concomitantly reduce nephrotoxicity, we developed a carrier-based strategy for the selective delivery of PMEAL to parenchymal liver cells. We designed the reconstituted lactosylated high-density lipoprotein (LacNeoHDL) carrier, a synthetic particle composed of lipids and lactosylated apoproteins (Bijsterbosch et al., 1994; 1996). LacNeoHDL is selec-

Financial support was granted by the Dutch Organization for Scientific Research (NWO) (to R.L.A.D.V.) and by the Fonds voor Wetenschappelijk Onderzoek-Vlaanderen (to J. N.).

ABBREVIATIONS: HBV, hepatitis B virus; PMEAL, 9-(2-phosphonylmethoxyethyl)adenine; LacNeoHDL, lactosylated reconstituted high density lipoprotein; BSA, bovine serum albumin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PMEAL-LO, conjugate of PMEAL and lithocholic acid-3 α -oleate; PBS, phosphate-buffered saline.

tively taken up by the asialoglycoprotein receptor. This receptor is uniquely localized on parenchymal liver cells (Ashwell and Harford, 1982), and remains expressed during acute and chronic hepatitis (Hyodo et al., 1993). PMEAL was incorporated into the lipid moiety of LacNeoHDL after conversion of the drug into a lipophilic prodrug (Fig. 1A), designated PMEAL-LO (de Vruhe et al., 1999). Because PMEAL-LO is present in the lipid moiety (Fig. 1B), it does not interfere with the recognition of the lactosylated apoproteins by the asialoglycoprotein receptor. In the prodrug, the linkage between PMEAL and the lithocholic acid-3 α -oleate lipid moiety is acid-labile. Uptake via the asialoglycoprotein receptor is linked to lysosomal processing, and the acid-labile linkage ensures the release of PMEAL in the acidic lysosomes. We showed recently in a rat model that LacNeoHDL-associated PMEAL-LO is rapidly and extensively taken up by the liver. The renal uptake was very low. Compared with PMEAL, the liver/kidney ratio was improved 500-fold (de Vruhe et al., 2000). In the liver, uptake occurred almost exclusively in parenchymal cells. We further showed that PMEAL is released from PMEAL-LO after internalization of the LacNeoHDL-associated prodrug. The drug is subsequently translocated from the lysosomal compartment to the cytosol, where it is con-

verted into its active diphosphorylated metabolite (de Vruhe et al., 2000).

To further evaluate the therapeutic potential of PMEAL-loaded LacNeoHDL, we examined in the present study the processing of the prodrug-loaded particles by HepG2 cells, human hepatoma cells that constitute a model system for human parenchymal liver cells. We investigated the receptor-mediated association and processing of PMEAL-LO-loaded LacNeoHDL by the cells and demonstrate the inhibitory effects of the prodrug-carrier complex on HBV replication in HBV-transfected HepG2 cells.

Materials and Methods

Reagents. PMEAL and PMEAL-LO were synthesized as described earlier (Holy and Rosenberg 1987; de Vruhe et al., 1999). Cholesteryl oleate (97%) was from Janssen Pharmaceuticals (Beerse, Belgium). Egg yolk phosphatidyl choline (98%) was from Fluka (Buchs, Switzerland). Lactosylated high-density lipoprotein apoproteins were prepared as described earlier (de Vruhe et al., 1999). Na¹²⁵I (carrier free) was from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). Asialofetuin was prepared as described earlier (Bijsterbosch and van Berkel, 1992). Bovine serum albumin (BSA, fraction V), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and tetracycline were from Sigma (St. Louis, MO). Penicillin, streptomycin, and gentamycin were from Roche Molecular Biochemicals (Mannheim, Germany). Eagle's modified Eagle's medium (EMEM) and Dulbecco's modified Eagle's medium were from Invitrogen (Carlsbad, CA). Fetal calf serum was from Hyclone Laboratories (Logan, UT). G418 was from Duchefa (Haarlem, The Netherlands). L-Glutamine was from Merck (Darmstadt, Germany). Ham's F12 medium and trypsin/EDTA were from BioWhittaker Europe (Verviers, Belgium). All other chemicals were of analytical grade.

Preparation of (PMEAL-LO-Loaded) LacNeoHDL. For the preparation of PMEAL-LO-loaded LacNeoHDL, a mixture of 0.5 mg of PMEAL-LO, 3.6 mg of phosphatidyl choline, and 1.8 mg of cholesteryl oleate was dispersed in sonication buffer (10 mM Tris-HCl buffer, pH 8.0, containing 0.1 M KCl, and 1 mM EDTA), and sonicated for 30 min at 49 to 52°C. Then, the temperature was lowered to 42 to 44°C. Sonication was continued, and 6 mg of lactosylated HDL apoproteins, dissolved in 4 M urea, were added in small portions over a period of 10 min. Sonication was stopped after a further 20 min, and large particles were removed by centrifugation. The PMEAL-LO-loaded LacNeoHDL particles were purified by gel permeation chromatography using a Superose-6 column (1.6 \times 50 cm) eluted with phosphate-buffered saline (PBS; 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl) supplemented with 1 mM EDTA. The composition of the purified preparations was similar to that of earlier preparations (de Vruhe et al., 1999, 2000). The prodrug-loaded particles were passed through a 0.45- μ m filter and stored at -80°C until use. Freezing and storage at -80°C does not affect the physical and biological properties of PMEAL-LO-loaded LacNeoHDL (de Vruhe et al., 2001). LacNeoHDL carrier without PMEAL-LO was prepared by omitting PMEAL-LO from the procedure described above.

Radioiodination of PMEAL-LO-Loaded LacNeoHDL. The lactosylated apoprotein of PMEAL-LO-loaded NeoHDL was labeled with ¹²⁵I using iodine monochloride as described previously (Bijsterbosch and van Berkel, 1992). Less than 2% of the labeled material was trichloroacetic acid-soluble.

Cell Culture. HepG2 cells were cultured at 37 °C in a humidified 5% CO₂/air atmosphere in EMEM supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, 10 μ g/ml insulin, 50 IU/ml penicillin, 50 μ g/ml streptomycin, and 50 μ g/ml gentamycin. Cells were subcultured once a week by detaching the cells with trypsin/EDTA (0.5 mg/ml and 0.2 mg/ml, respectively) followed by renewal of medium on the following day.

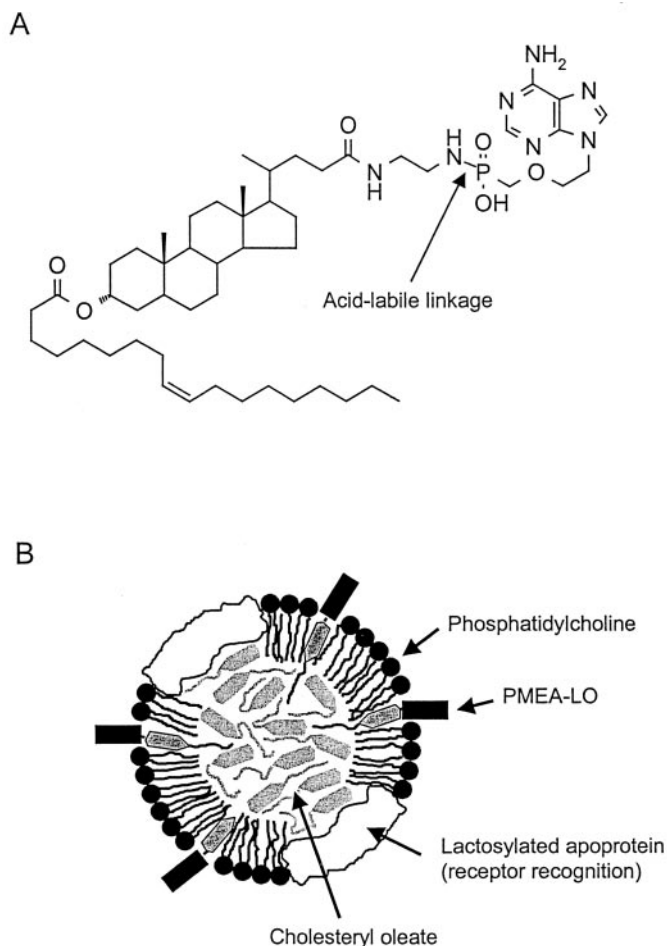


Fig. 1. Structures of PMEAL-LO and PMEAL-LO-loaded LacNeoHDL. A, the structure of PMEAL-LO: PMEAL is linked to lithocholic acid-3 α -oleate via an acid-labile phosphonoamidate bond. B, a hypothetical model of PMEAL-LO-loaded LacNeoHDL: A cholesteryl oleate core is surrounded by a monolayer of phosphatidyl choline, in which PMEAL-LO and lactosylated apoproteins (receptor recognition) are embedded.

Results

Hep AD38 cells were cultured at 37 °C in a humidified 5% CO₂/air atmosphere in EMEM/Ham's F12 (50/50) medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 5 mM L-glutamine, 50 IU/ml penicillin, 50 µg/ml streptomycin, 100 µg/ml kanamycin, 400 µg/ml G418, and 0.3 µg/ml tetracycline. Cells were subcultured once a week. Fresh medium was added every 3 days.

Determination of Association and Degradation of ¹²⁵I-labeled PMEAL-LO-loaded LacNeoHDL by HepG2 cells. HepG2 cells were seeded in 12-well culture plates and grown to subconfluence. The cells were washed three times with incubation medium [EMEM supplemented with 25 mM HEPES and 2% (w/v) BSA] and preincubated for 1 h at 37 °C in a humidified 5% CO₂/air atmosphere with incubation medium containing the indicated additions (asialofetuin, fetuin, or chloroquine). The experiments were started by adding ¹²⁵I-labeled PMEAL-LO-loaded LacNeoHDL to the cells. After incubation, the culture plates were placed on ice. Aliquots of 0.5 ml of the incubation medium were assayed for secreted radioiodinated degradation products, as described previously (van Berkel et al., 1981). Approximately two thirds of the degradation products consisted of iodine and the remainder consisted of iodinated tyrosine and/or tyrosine-containing oligopeptides. The cellular association of ¹²⁵I-labeled PMEAL-LO-loaded LacNeoHDL was determined by washing the cells three times with ice-cold wash buffer (0.15 M NaCl, 2.5 mM CaCl₂ and 25 mM Tris-HCl, pH 7.4) containing 0.2% (w/v) BSA, followed by three washes with the same buffer without BSA. The cells were then lysed with 1 ml of 0.1 N NaOH and the amounts of protein (method of Lowry et al., 1951, with BSA as standard) and radioactivity in the lysate were determined. One milligram of HepG2 protein represents 7.5 × 10⁶ cells.

Determination of the Replication of HBV in HepAD 38 cells. Hep AD38 cells were seeded in 12-well culture plates at a density of 1 × 10⁵ cells/cm². After 3 days of culture, the cells were washed five times with prewarmed phosphate-buffered saline. Subsequently, the cells were incubated with the EMEM/Ham's F12 culture medium described above, lacking tetracycline, but supplemented with the compounds under study. After 3 days, the medium was removed and replaced by fresh medium lacking tetracycline and supplemented with the compounds under study. After another 3 days of culture, viral DNA was extracted from the cells using a QIAGEN Blood and Cell Culture DNA Kit (QIAGEN, Hilden, Germany). The DNA was transferred to a nylon membrane and quantified by hybridization with a digoxigenin-labeled, HBV-specific probe as described in detail previously (Ying et al., 1999). The concentrations required to inhibit HBV DNA synthesis by 50% (EC₅₀) and 90% (EC₉₀) were calculated by interpolation.

Cytotoxicity Assay. HepG2 cells were seeded in 96-well culture plates at a density of 1 × 10⁵ cells/cm². After 3 days of culture, the culture medium was removed and replaced by medium supplemented with PMEAL-LO-loaded LacNeoHDL (0.04–0.2 µg PMEAL/ml) or an equal volume of PBS. After 6 days of culture (medium removed and replaced by fresh medium with PMEAL-LO-LacNeoHDL or PBS after 3 days), the viability of the cells was determined by measuring MTT reduction (Mosmann, 1983; Denizot and Lang, 1986) as follows. The medium was removed from the cells, and replaced by 125 µl of incubation medium [EMEM supplemented with 25 mM HEPES and 2% (w/v) BSA], containing 1 mg of MTT/ml. The cells were incubated for 3 h at 37°C in a humidified 5% CO₂/air atmosphere. Then, the incubation mixture was removed, and the blue formazan product of MTT reduction was dissolved in 125 µl of isopropanol containing 0.05 N HCl. The absorbance of the samples was measured using an automatic plate reader (Argus 300; Packard, Downers Grove, IL) set at 550 nm as test wavelength, and at 690 nm as reference wavelength. The absorbance values (A₅₅₀–A₆₉₀) of the samples were corrected for values measured in cells that had been cultured under the same conditions, and were incubated in parallel with incubation medium lacking MTT (absorbance values < 0.100).

Association of PMEAL-LO-Loaded LacNeoHDL with HepG2 Cells. To study the association of PMEAL-LO-loaded LacNeoHDL with HepG2 cells, the cells were incubated at 37°C with increasing amounts of ¹²⁵I-labeled PMEAL-LO-loaded LacNeoHDL. After 2 h of incubation, the association of radiolabel with the cells was determined. Figure 2 shows that the association of the prodrug-loaded particles to HepG2 cells is saturable and of high affinity (*K_d*, 0.63 ± 0.07 µg of apoprotein/ml). The involvement of the asialoglycoprotein receptor in the cellular association of PMEAL-LO-loaded LacNeoHDL was ascertained by preincubating the cells with asialofetuin, a galactose-terminated glycoprotein that specifically binds to the asialoglycoprotein receptor (Tolleshaug and Berg, 1980; Ashwell and Harford, 1982). Figure 3 shows that asialofetuin inhibits dose dependently the association of PMEAL-LO-loaded LacNeoHDL to HepG2 cells. At the maximal concentration tested (2 mg/ml), asialofetuin inhibited the association of the drug-carrier complex by > 75%. Native fetuin, which does not expose terminal galactose residues, displayed only minor inhibitory activity. At a concentration of 2 mg/ml, native fetuin was approximately as effective (28.5 ± 10.3% inhibition) as asialofetuin at 0.1 mg/ml (28.3 ± 1.0% inhibition).

Processing of PMEAL-LO-Loaded LacNeoHDL by HepG2 Cells. To become therapeutically active, the LacNeoHDL-associated PMEAL prodrug needs to be internalized and processed in the lysosomes. The internalization and processing of PMEAL-LO-loaded LacNeoHDL by HepG2 cells was examined by incubating the cells for different time periods with 0.5 µg/ml ¹²⁵I-labeled PMEAL-LO-loaded LacNeoHDL. Figure 4 (inset) shows that the radiolabeled particles rapidly associate with the cells. After 15 min of incubation, apoprotein was found to be associated at a concentration of 28.1 ± 3.0 ng/mg of cell protein. The association of the prodrug-labeled particles reached a maximal value of 80.4 ± 2.0 ng of apoprotein/mg of cell protein after 2 h of incubation. Association of PMEAL-LO-loaded LacNeoHDL to the HepG2 cells

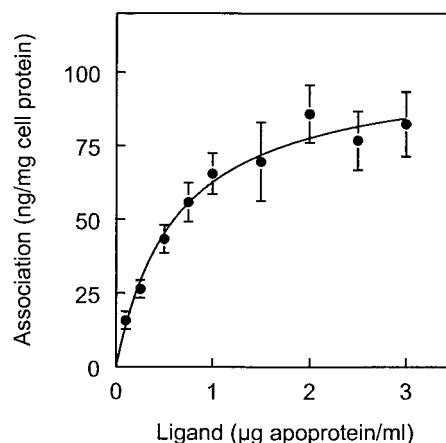


Fig. 2. Association of PMEAL-LO-loaded LacNeoHDL with HepG2 cells. HepG2 cells were incubated at 37°C with ¹²⁵I-labeled PMEAL-LO-loaded LacNeoHDL at concentrations up to 3 µg of apoprotein/ml. After 2 h of incubation, the amounts of cell-associated ¹²⁵I radioactivity were determined. Specific association is shown, which was obtained by subtracting the nonspecific association (determined in the presence of 2 mg/ml asialofetuin) from the total association. Values are means ± S.E.M. of four separate experiments.

was found to be coupled to degradation of the particles. Radiolabeled degradation products appeared in the culture medium with a lag phase of 2 h. After 24 h of incubation, degradation products derived from 431.4 ± 35.7 ng of apo-protein/mg of cell protein had been secreted.

The selectivity of the degradation was investigated in a competition experiment with asialofetuin. Figure 5 shows that the presence of asialofetuin (2 mg/ml) reduced the degradation by approximately 75%, whereas native fetuin had no effect at all. These findings indicate that the degradation results from asialoglycoprotein receptor-mediated uptake. To ascertain the involvement of the lysosomal apparatus, we examined the effects of chloroquine on the degradation. Chloroquine accumulates in lysosomes, and inhibits the lysosomal degradation of internalized ligands by raising the intra-

lysosomal pH (Seglen et al., 1979). Figure 5 shows that in the presence of 0.1 mM chloroquine, the degradation was reduced by >80%, which indicates that the degradation of PMEA-LO-loaded LacNeoHDL occurs in the lysosomal compartment.

Inhibition of the Replication of HBV in HBV-Transfected HepG2 cells by PMEA- LO-Loaded LacNeoHDL. The capacity of LacNeoHDL-associated PMEA-LO to inhibit the replication of HBV was examined using the HepAD38 cell line. HepAD38 cells are HepG2 cells that are stably transfected with a cDNA copy of pregenomic HBV RNA (Ladner et al., 1997). The replication of HBV in HepAD38 cells is controlled by tetracycline and is initiated by withdrawal of tetracycline from the culture medium. HepAD38 cells do express a functional asialoglycoprotein receptor, which was ascertained in a preliminary binding experiment with the established high-affinity ligand asialo-orosomucoid (not shown). Figure 6 compares the effects of LacNeoHDL-associated PMEA-LO on HBV DNA synthesis in HepAD38 cells with that of free PMEA. The carrier-associated PMEA pro-drug inhibited viral DNA synthesis at much lower concentrations than the free drug. The EC_{50} values were 3.4 ± 0.4 and 120 ± 18 ng of PMEA/ml, respectively (EC_{90} values were 250 ± 30 ng/ml and 4500 ± 500 ng/ml, respectively). The inhibitory effect of PMEA-LO-loaded LacNeoHDL on HBV synthesis was almost completely abolished by including 10 mM *N*-acetylgalactosamine in the incubation medium, whereas *N*-acetylglucosamine had no effect at all (Table 1). This finding indicates that the inhibition of viral DNA synthesis by PMEA-LO-loaded LacNeoHDL is mediated via a galactose-specific mechanism, i.e., the asialoglycoprotein receptor. *N*-Acetylgalactosamine and *N*-acetylglucosamine per se had no effect on viral DNA synthesis, nor did these compounds affect the inhibition of HBV DNA synthesis by free

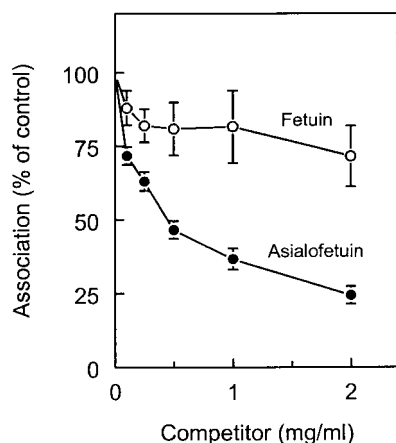


Fig. 3. Effects of asialofetuin and fetuin on the association of PMEA-LO-loaded LacNeoHDL to HepG2 cells. HepG2 cells were incubated at 37°C with 125 I-labeled PMEA-LO-loaded LacNeoHDL (0.5 μ g of apo-protein/ml) in the presence of the indicated amounts of asialofetuin (●) or fetuin (○). After 2 h of incubation, the amounts of cell-associated 125 I-radioactivity were determined. Values are means \pm S.E.M. of four separate experiments and are expressed as percentage of the association in control incubations (no competitor; 51.7 ± 6.8 ng/mg of cell protein).

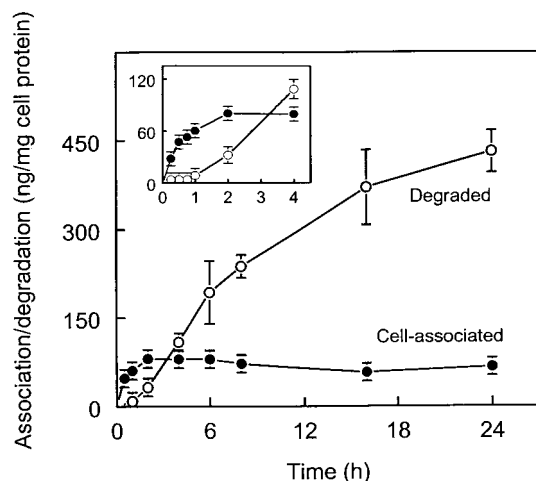


Fig. 4. Association and degradation of PMEA-LO-loaded LacNeoHDL by HepG2 cells. HepG2 cells were incubated at 37°C with 125 I-labeled PMEA-LO-loaded LacNeoHDL (0.5 μ g of apo-protein/ml). At the indicated time points, the cell-associated radioactivity (●) and the amounts of radiolabeled degradation products in the medium (○) were determined. The inserted graph shows details of cell association and release of degradation products up to 4 h of incubation. Values are means \pm S.E.M. of four separate experiments.

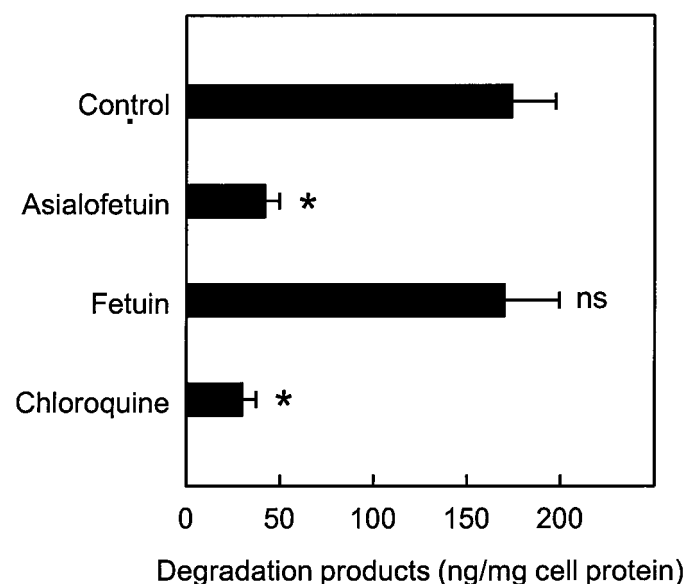


Fig. 5. Effects of asialofetuin, fetuin, and chloroquine on the degradation of PMEA-LO-loaded LacNeoHDL by HepG2 cells. HepG2 cells were incubated at 37°C with 125 I-labeled PMEA-LO-loaded LacNeoHDL (0.5 μ g of apo-protein/ml) in the presence of asialofetuin (2 mg/ml), fetuin (2 mg/ml), chloroquine (0.1 mM), or without further additions (control). After 6 h of incubation, the amounts of radioactively labeled degradation products in the medium were determined. Values are means \pm S.E.M. of four separate experiments. Differences with respect to the controls were tested for significance (paired *t* test): **P* < 0.05; ns, not significant.

PMEA (Table 1). The LacNeoHDL carrier alone had only limited effect on viral DNA synthesis. After addition of the carrier alone, at concentrations equivalent to carrier concentrations present when PMEAL-prodrug was added to 0.04 and 0.2 $\mu\text{g/ml}$, HBV DNA synthesis was $80 \pm 7\%$ and $76 \pm 1\%$ of the control values, respectively (means \pm S.E.M. of two independent experiments).

To ascertain that the observed reduction of HBV DNA in cells exposed to PMEAL and PMEAL-LO-loaded LacNeoHDL is virus-specific and not a nonspecific cytotoxic effect, the total amount of cellular DNA in the Hep AD38 cultures was also determined. In cultures incubated with PMEAL (5 $\mu\text{g/ml}$) and carrier-associated PMEAL-LO (0.2 μg of PMEAL/ml), the total cellular DNA contents were $90 \pm 3\%$ and $98 \pm 4\%$ of the control values, respectively. The lack of cytotoxicity of PMEAL-LO-loaded LacNeoHDL at effective anti-HBV concentrations was further demonstrated by incubating HepG2 cells with the prodrug-loaded carrier. After 6 days of incubation, the viability of the cells was evaluated by assessing their capacity to convert the tetrazolium salt MTT into a

blue-colored formazan (Mosmann, 1983; Denizot and Lang, 1986). Figure 7 shows that at concentrations of 0.04 to 0.20 μg of PMEAL/ml, which are highly effective against HBV replication, the prodrug-loaded particles do not affect the capacity of the cells to convert MTT. The formation of the blue formazan was similar in treated and control cells and required metabolically active cells, because at 0°C , no significant amounts of the formazan were produced. Furthermore, no morphological changes were observed in cells cultured with PMEAL-LO-loaded LacNeoHDL.

Discussion

We reported earlier on the *in vivo* disposition of PMEAL-LO-loaded LacNeoHDL in a rat model, and showed highly selective delivery of the prodrug-loaded particles to parenchymal liver cells via the asialoglycoprotein receptor. After internalization, PMEAL was released and converted into its active diphosphorylated metabolite (de Vruhe et al., 2000). In the present study, we demonstrate that PMEAL-LO-loaded LacNeoHDL is also efficiently internalized via the asialoglycoprotein receptor on HepG2 cells, human hepatoma cells that constitute a model system for human parenchymal liver cells. We further show that PMEAL-LO-loaded LacNeoHDL effectively inhibits HBV DNA synthesis in HBV-transfected HepG2 cells (the EC_{50} value is 35-fold lower than that of free drug), thus underscoring the therapeutic potential of the PMEAL prodrug-loaded particles.

PMEAL-LO-loaded LacNeoHDL associates with HepG2 cells with high affinity. The apparent dissociation constant was found to be 0.63 ± 0.07 μg of apoprotein/ml, which corresponds to 3.8 ± 0.4 nM (calculated from the physicochemical parameters listed in de Vruhe et al., 1999). The drug-carrier complex is designed to be taken up via the asialoglycoprotein receptor. To examine the involvement of this receptor, we performed competition experiments with asialofetuin. Fetuin is a glycoprotein with complex oligosac-

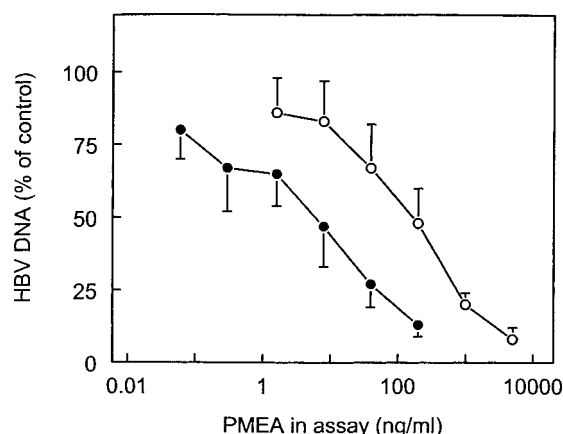


Fig. 6. Effects of PMEAL and PMEAL-LO-loaded LacNeoHDL on HBV synthesis in Hep AD38 cells. Hep AD38 were cultured in the presence of the indicated concentrations of PMEAL (○) or LacNeoHDL-associated PMEAL-LO (●; expressed as PMEAL equivalent). After 6 days, the intracellular HBV DNA levels were determined. Values are means \pm S.E.M. of five separate experiments and are expressed as percentage of the amounts of HBV DNA in control incubations (no additions).

TABLE 1

Effects of *N*-acetylgalactosamine and *N*-acetylglucosamine on the inhibition of HBV synthesis in HepAD38 cells by PMEAL-LO-LacNeoHDL and PMEAL.

Hep AD38 cells were cultured in the presence of PMEAL-LO-LacNeoHDL (0.04 μg of PMEAL/ml or 0.2 μg PMEAL/ml), PMEAL (5 $\mu\text{g/ml}$), or without PMEAL. The cultures were supplemented with 10 mM *N*-acetylgalactosamine (GalNac), 10 mM *N*-acetylglucosamine (GlcNac), or an equal volume of solvent (PBS). After 6 days, the amounts of HBV DNA in the cells were determined. Values are means \pm S.E.M. of three separate experiments and are expressed as percentage of the amounts of HBV DNA in control incubations (no PMEAL, PBS). Differences between GalNac/GlcNac incubations and PBS incubations were tested for significance (paired *t*-test).

	Viral DNA		
	PBS	GalNac	GlcNac
	% of control		
PMEAL-LO-LacNeoHDL: 0.04 μg of PMEAL/ml	15 \pm 4	72 \pm 12*	16 \pm 3 ^{ns}
PMEAL-LO-LacNeoHDL: 0.2 μg of PMEAL/ml	13 \pm 2	73 \pm 15*	12 \pm 13 ^{ns}
5 μg of PMEAL/ml PMEAL	9 \pm 3	10 \pm 3 ^{ns}	8 \pm 4 ^{ns}
No PMEAL	100 \pm 0	94 \pm 4 ^{ns}	98 \pm 10 ^{ns}

* $P < 0.05$; ns, not significant.

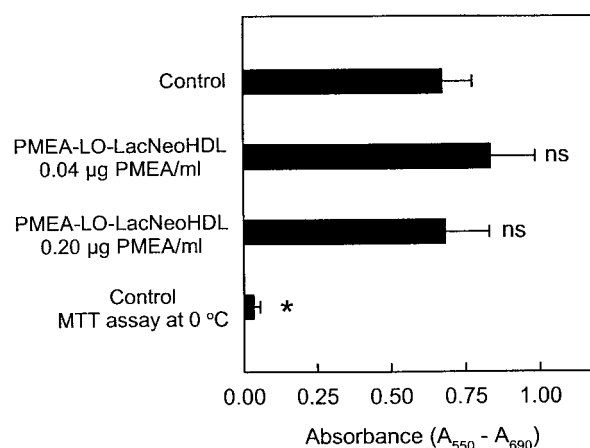


Fig. 7. Effects of PMEAL-LO-loaded LacNeoHDL on the viability of HepG2 cells. HepG2 cells were cultured in 96-well culture plates in medium supplemented with PMEAL-LO-loaded LacNeoHDL (0.04–0.2 μg PMEAL/ml) or an equal volume of PBS (control). After 6 days of culture, the viability of the cells was determined by incubating the cells for 3 h at 37°C with the tetrazolium salt MTT. PBS-treated cells were also incubated for 3 h with MTT at 0°C . The absorbance of the product of MTT reduction was measured in a plate reader at 550 nm (690 nm was used as reference wavelength). Values are means \pm S.E.M. of six cultures in two independent experiments. Differences with respect to the control were tested for significance (paired *t* test): * $P < 0.05$; ns, not significant.

charide chains terminated by sialic acid (Baenziger and Fiete, 1979; Nilsson et al., 1979). Removal of sialic acids exposes galactose residues, which are recognized by the asialoglycoprotein receptor (Tolleshaug and Berg, 1980; Ashwell and Harford, 1982). We found that asialofetuin effectively inhibits the association of PMEA-loaded LacNeoHDL to HepG2 cells, indicating that the asialoglycoprotein receptor is involved. Fetuin also inhibited the cell association to some extent, but was far less active (~20 times). The minor inhibitory activity of fetuin is probably caused by the presence, in the preparation, of small amounts of fetuin molecules with (partially) desialylated oligosaccharide chains and/or to low-affinity interaction of the penultimate galactose residue in fetuin (Baenziger and Fiete, 1979; Nilsson et al., 1979).

Association of radioiodinated PMEA-LO-loaded LacNeoHDL with HepG2 cells is followed by secretion of labeled degradation products into the medium. Asialofetuin and chloroquine effectively inhibited the secretion of degradation products, indicating that PMEA-LO-loaded LacNeoHDL is internalized via the asialoglycoprotein receptor and subsequently processed in the lysosomal compartment. Lysosomal processing of PMEA-LO-loaded LacNeoHDL is crucial, because PMEA-LO is designed to be hydrolyzed in the acidic lysosomes, thereby releasing PMEA. The prodrug-loaded particles associate rapidly with HepG2 cells, but the degradation products appear in the medium 2 h later. This lag phase probably reflects the time required for delivery to the lysosomal compartment, degradation, and excretion of degradation products. Summation of labeled degradation products in the medium and cell-associated PMEA-LO-loaded LacNeoHDL yields the total amount of internalized prodrug-loaded particles. From our earlier data on cellular protein and water contents (Bijsterbosch et al., 2000), we calculate that under the culture conditions used in our study (ligand concentration slightly below k_d), the intracellular PMEA (metabolite) concentration in the HepG2 cells raises to approximately 3 μM (i.e., 2 pmol/ 10^6 cells) during 24 h of culture. In the calculation, we did not account for loss of PMEA from the cells, but the reported intracellular half-lives are relatively long (5–18 h; Balzarini et al., 1991; Aduma et al., 1995). The inhibition constant for the active bisphosphorylated PMEA metabolite is approximately 0.1 μM (Xiong et al., 1998). The intracellular PMEA (metabolite) concentrations that can be attained using the LacNeoHDL carrier are therefore sufficiently high to exert a therapeutic effect, which was confirmed in the efficacy studies discussed below.

The therapeutic potential of the drug-carrier complex was evaluated by measuring the capacity of LacNeoHDL-associated PMEA-LO to inhibit the replication of HBV in the HepAD38 cell line. HepAD38 cells are HepG2 cells that are stably transfected with a cDNA copy of pregenomic HBV RNA, and replication of the virus is under the control of a tetracycline-responsive promoter (Ladner et al., 1997). The PMEA-LO-loaded LacNeoHDL inhibited viral DNA synthesis much more effectively than free PMEA. The EC_{50} value of carrier-associated PMEA-LO was 35 times lower than that of the free drug (3.4 ± 0.4 and 120 ± 18 ng of PMEA/ml, respectively) and is very similar to the EC_{50} value of lamivudine under our assay conditions (4.0 ± 0.5 ng/ml). The inhibition of viral DNA synthesis by PMEA-LO-loaded LacNeoHDL was virtually abolished by adding *N*-acetylgalactosamine to the medium, whereas *N*-acetylglucosamine had

no effect. This finding indicates that the inhibition is indeed mediated by a galactose-specific mechanism (i.e., the asialoglycoprotein receptor).

In summary, we demonstrate in this study that PMEA-LO-loaded LacNeoHDL is efficiently internalized via the asialoglycoprotein receptor on HepG2 cells and subsequently processed in the lysosomal compartment. PMEA-LO-loaded LacNeoHDL is designed to release PMEA once it is delivered to the lysosomes. By using HBV-transfected HepG2 cells, we demonstrate that the LacNeoHDL-mediated delivery of the prodrug indeed results in a highly effective inhibition of HBV synthesis. We conclude that the present results, in combination with the results provided by our earlier in vivo disposition study (de Vruhe et al., 2000), underscore the therapeutic potential and utility of PMEA-LO-loaded LacNeoHDL for the treatment of chronic hepatitis B.

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