Synthesis of the Dioleoyl Derivative of Iododeoxyuridine and Its Incorporation into Reconstituted High Density Lipoprotein Particles

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ABSTRACT: We investigated the potential use of reconstituted HDL particles (NeoHDL) as a carrier for lipophilic (pro)drugs. The antiviral drug iododeoxyuridine (IDU) was used as model compound. [^{3}H]-IDU was derivatized with two oleoyl residues to dioleoyl[^{3}H]iododeoxyuridine ([^{3}H]IDU-Ol$_2$), and the lipophilic prodrug was incorporated into NeoHDL by cosonication of [^{3}H]IDU-Ol$_2$ with lipids and HDL apoproteins. NeoHDL particles with the same density, size, and electrophoretic mobility as native HDL were obtained, which contained 7.3 ± 0.8% (w/w) [^{3}H]IDU-Ol$_2$ (about 30 molecules of prodrug per particle). NeoHDL-associated [^{3}H]IDU-Ol$_2$ was stable during 2 h of incubation with human plasma; the prodrug was not appreciably hydrolyzed, nor exchanged with LDL. After intravenous injection of [^{3}H]-IDU-Ol$_2$-loaded ^{125}I-NeoHDL into rats, [^{3}H]IDU-Ol$_2$ disappeared more rapidly from the circulation than the ^{125}I-apoproteins (78.0 ± 8.0% vs 30.1 ± 4.5% of the dose cleared from plasma in 60 min, respectively). The hepatic association of the prodrug was higher than that of the apoproteins (21.6 ± 0.5 vs 5.2 ± 1.0% of the dose at 10 min after injection, respectively). As selective clearance and uptake of lipid esters is also observed with native HDL, this suggests that, *in vivo*, prodrug-loaded NeoHDL may be subject to physiological HDL-specific processing. Lactosylated [^{3}H]IDU-Ol$_2$-loaded ^{125}I-NeoHDL, which contains galactose residues that can be recognized by galactose receptors on parenchymal liver cells, was rapidly cleared from plasma. At 10 min after injection, only about 10% of the injected ^{125}I-apoprotein and ^{3}H-prodrug was left in plasma, and approximately 75% of the injected amount of both labels was recovered in the liver. We conclude that it is possible to convert a hydrophilic drug, like IDU, into a lipophilic prodrug that can be efficiently incorporated into a reconstituted HDL particle with similar properties as native HDL. The same approach may be applied for other water-soluble drugs. In particular, with lactosylated NeoHDL, an efficient delivery system to the liver can be achieved, which allows a more effective treatment of diseases like hepatitis B.

The selective delivery of drugs to their specific cellular targets increases their therapeutic effectivity and reduces undesired interactions with nontarget tissues. A number of soluble molecules, like antibodies and lectins, and particulate systems, such as liposomes and nanoparticles, have been proposed as carriers for drugs (Poznansky & Juliano, 1984; Tomlinson, 1987). Furthermore, in the past decade it has become clear that lipoproteins are also attractive potential carriers (Counsell & Pohland, 1982; Shaw et al., 1987; Bijsterbosch & van Berkel, 1990; Firestone, 1994).

Lipoproteins are spherical lipid/protein complexes responsible for the transport of lipids in the circulation. As endogenous carriers, they are not immunogenic and escape recognition by the reticuloendothelial system ("stealth behavior"). Structurally, they consist of an apolar core, composed of cholesteryl oleate and/or triglycerides, surrounded by a monolayer of phospholipids in which cholesterol and specific apoproteins are embedded. A variety of lipophilic compounds can be incorporated in the lipid moiety of lipoproteins and thus be transported, hidden inside these particles (Counsell & Pohland, 1982; Shaw et al., 1987; Bijsterbosch & van Berkel, 1990; Firestone, 1994). Lipoproteins may therefore be utilized as carriers for lipophilic (pro)drugs. The distribution of a drug associated with a lipoprotein will depend on the metabolic fate of its lipoprotein carrier. Lipoproteins are removed from the circulation by specific receptors that recognize their apoproteins (Bijsterbosch & van Berkel, 1990). In addition, lipoproteins can be directed to nonlipoprotein receptors by chemical modification of the apoproteins. We have shown, for example, that by reductive lactosamination low density lipoprotein (LDL)[1] and high denstity lipoprotein (HDL) can be directed to galactose receptors present on Kupffer cells and liver parenchymal cells, respectively (Bijsterbosch et al.,1989; Bijsterbosch & van Berkel, 1992).

A possible limitation for the use of lipoproteins as drug carriers may be their limited availability. In a recent study we therefore investigated the possibility to synthesize, from commercially available lipids and isolated apoproteins, lipoprotein-like lipid particles. We succeeded in preparing

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[1] Abbreviations: DMAC, dimethylacetamide; EDTA, ethylenediaminetetraacetic acid; HDL, high density lipoprotein; HPLC, high performance liquid chromatography; IDU, 5-iodo-2′-deoxyuridine. IDU-Ol$_1$, 5′-oleoyl-5-iodo-2′-deoxyuridine; IDU-Ol$_2$, 3′,5′-dioleoyl-5-iodo-2′-deoxyuridine; LDL, low density lipoprotein; PBS, phosphate-buffered saline; TLC, thin layer chromatography.

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particles with properties very similar to the naturally occuring human HDL (Schouten et al., 1993).

In the present study, we extended this earlier work and investigated whether such a reconstituted HDL particle, denoted NeoHDL, can actually be used to transport lipophilic drugs. A number of drugs, for instance, cyclosporin A and some porphyrin derivatives, incorporate spontaneously into lipoproteins (Mraz et al., 1986; De Smidt et al., 1993). Most drugs, however, are not sufficiently lipophilic for incorporation into (neo)lipoproteins. To be incorporated, these drugs have to be converted to a lipophilic prodrug. It has been shown earlier that antineoplastic drugs can be incorporated in LDL after derivatization with lipophilic residues (Firestone et al., 1984; Vitols et al., 1985; De Smidt & van Berkel, 1990). In the present study, we used 5-iodo-2′-deoxyuridine (IDU) as a model compound. IDU is a drug with antiviral activity, and it may also be used as radiosentisizer in the radiotherapy of tumors (Prusoff & Goz, 1974; Santos et al., 1992). IDU is not very lipophilic, and we therefore synthesized a lipophilic prodrug by derivatizing IDU with two oleoyl residues. The resulting lipophilic compound, 3′,5′-dioleoyl-5-iodo-2′-deoxyuridine (IDU-Ol_2), was incorporated into NeoHDL, and the physicochemical properties and biological fate of the prodrug-loaded particles were investigated. By using ^{3}H-labeled IDU-Ol_2 and radioiodination of apoproteins, the incorporated prodrug and apoprotein moiety could be monitored simultaneously.

EXPERIMENTAL PROCEDURES

Reagents and Materials. 2′-Deoxy[6-^{3}H]uridine (25.5 Ci/mmol) was obtained from New England Nuclear Research Products, Boston, Ma. Na^{125}I (carrier free) was supplied by Amersham International, Amersham, Bucks, U.K. Oleoyl chloride, 2′-deoxyuridine, 5-iodo-2′-deoxyuridine, cholesteryl oleate, and Dowex 1 X8 (200−400 mesh) were from Janssen, Beerse, Belgium. Egg yolk phosphatidylcholine was from Fluka, Buchs, Switzerland. Cholesterol and bovine serum albumin (fraction V) were obtained from Sigma, St. Louis, Mo. Lactose was supplied by Merck, Darmstadt, Germany. Sodium cyanoborohydride was from Aldrich, Brussels, Belgium. Emulsifier Safe and Hionic Fluor scintillation cocktails and Soluene-350 were from Packard, Downers Grove, IL. All other reagents were of analytical grade. Thin layer chromatography (TLC) plates (silica 60-F_{254} preformed 0.2-mm-thick layers on aluminium sheets) were obtained from Merck, Darmstadt, Germany.

Synthesis of 5-Iodo-2′-deoxy[6-^{3}H]uridine ([^{3}H]IDU). ^{3}H-Labeled and unlabeled 2′-deoxyuridines were dissolved in 1 N HNO_3 and mixed to a final concentration of 25 mM (5.7 mg/mL; specific radioacticity 50 mCi/mmol). An aliquot of 0.6 mL of this solution was refluxed for 3 h at 70 °C under continuous stirring with 30 mg of I_2 and 0.3 mL of $CHCl_3$. Then, $CHCl_3$ and unreacted iodide were removed by extraction with diethyl ether. The aqueous phase was mixed with 10 mL of 90 mM NaOH and applied to a column of 2 mL of Dowex-1 (X8; 200−400 mesh) in the formate form. The column was subsequently washed with 10 mL of 10 mM NaOH. Fractions were collected and assayed for the presence of (modified) pyrimidine base by measuring radioactivity and absorbance at 288 nm. Less than 5% of the applied radioactivity and UV-absorbing material eluted from the column during application of the diluted aqueous phase and subsequent washing of the column. Virtually all of the applied radioactivity and UV-absorbing material were subsequently eluted from the column by 0.1 M formic acid (total recoveries >90%). The eluted material showed a number of spots upon TLC (solvent: ethyl acetate saturated with 50 mM sodium phosphate buffer, pH 6.0). The major spot, which contained approximately 65% of the applied radioactivity, was at the same position (R_f 0.25) as the 5-iodo-2′deoxyuridine marker (2′-deoxyuridine: R_f 0.06). The column fractions were lyophilized and further purified by preparative TLC on silica gel 60 using the solvent system described above. Material at the same postion as 5-iodo-2′-deoxyuridine marker was scraped off and extracted with methanol. The (radiochemical) purity was >95% as judged by TLC (solvent: methanol/water/ammonia, 80:40:8) and HPLC (column: Nucleosil 120 7C_{18}; eluent: 10% acetonitrile in 0.1 M sodium acetate buffer, pH = 5.45).

Synthesis of 3′,5′-Dioleoyl-5-iodo-2′-deoxyuridine (IDU-Ol_2). To 71 mg of 5-iodo-2′-deoxyuridine (0.2 mmol), dissolved in 8 mL of dry dimethylacetamide (DMAC), were added 1.67 mL of dry pyridine and 0.33 mL of oleoyl chloride (1.0 mmol). After standing for 24 h at 65 °C, the reaction mixture was transferred to a separatory funnel containing 80 mL of H_2O and 40 mL of $CHCl_3$. The organic phase was washed once with 80 mL of 10% (w/v) $NaHCO_3$ and twice with 80 mL of H_2O, and subsequently the solvent was evaporated. The resulting yellowish oil showed two spots upon analysis by TLC (solvent: methanol/dichloromethane, 5:95): a major spot with $R_f = 0.75$ and a minor spot with $R_f = 0.20$ (iododeoxyuridine marker: $R_f < 0.05$). The material was further purified by column chromatography over silica gel 60 (230−400 mesh; column dimensions 0.8 × 12.0 cm) by applying a gradient of 0−5% (v/v) methanol in dichloromethane. Fractions containing the major and the minor product were pooled and analyzed by NMR and mass spectroscopy. The major product was identified as 3′,5′-dioleoyl-5-iodo-2′-deoxyuridine (IDU-Ol_2), and the minor product as 5′-oleoyl-5-iodo-2′-deoxyuridine (IDU-Ol_1).

IDU-Ol_2: yield 79 mg (45%); ^{1}H-NMR (200 MHz, $CDCl_3$): δ 7.96 (s, 1H: C*H*-6), 6.26 (t, 1H: C*H*-1′), 5.32 (t, 4H: C*H* C_9/C_{10}-oleoyl), 5.20 (m, 1H: C*H*-3′), 4.50−4.26 (m, 3H: C*H*-4′, CH_2-5′), 2.52−2.24 (m, 6H: CH_2-2′, CH_2 C_2-oleoyl), 2.00 (d, 8H: CH_2 C_8/C_{11}-oleoyl), 1.61 (m, 4H: CH_2 C_{17}-oleoyl), 1.28 (m, 40H: CH_2 C_3-C_7/C_{12}-C_{16}-oleoyl), 0.86 (t, 6H: CH_3-oleoyl). Mass: 882.5 (calculated: 883.0).

IDU-Ol_1: yield 19 mg (15%); ^{1}H-NMR (200 MHz, $CDCl_3$): δ 7.96 (s, 1H: C*H*-6), 6.23 (t, 1H: C*H*-1′), 5.32 (t, 2H: C*H* C_9/C_{10}-oleoyl), 4.46−4.14 (m, 4H: C*H*-3′, C*H*-4′, CH_2-5′), 2.52−2.28 (m, 4H: CH_2-2′, CH_2 C_2-oleoyl), 2.00 (d, 4H: CH_2 C_8/C_{11}-oleoyl), 1.64 (m, 2H: CH_2 C_{17}-oleoyl), 1.26 (m, 20H: CH_2 C_3-C_7/C_{12}-C_{16}-oleoyl), 0.86 (t, 3H: CH_3-oleoyl). Mass: 618.2 (calculated: 618.6).

NMR spectra were measured at 200 MHz using a JEOL JNM-FX 200 spectrometer. ^{1}H chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard. Mass spectra were measured by plasma desorption mass spectrometry in positive ionization mode.

Synthesis of 3′,5′-Dioleoyl-5-iodo-2′-deoxy[6-^{3}H]uridine ([^{3}H]IDU-Ol_2). To 1 mg of [^{3}H]IDU (2.8 μmol; specific radioactivity 13.5 mCi/mmol), dissolved in 0.11 mL of dry DMAC, were added 9 μl of oleoyl chloride (28 μmol) and 23 μl of dry pyridine. After standing for 20 h at 65 °C, the reaction mixture was transferred to a glass vial containing

0.6 mL of $CHCl_3$. The organic phase was washed once with 1.2 mL of 10% (w/v) $NaHCO_3$ and thrice with 1.2 mL of H_2O, and subsequently the solvent was evaporated. The resulting yellowish oil was further purified by TLC on silica gel 60 (dichloromethane/methanol, 95:5). Material at the same position as the IDU-Ol_2 marker (approximately 80% of the applied radioactivity) was scraped off and extracted with dichloromethane/methanol (95:5). The radiochemical purity of the product was >96% as judged by TLC using the solvent systems dichloromethane/methanol (95:5) and ethyl acetate saturated with 50 mM sodium phosphate buffer, pH 6.0.

Determination of Partition Coefficients. Aliquots of 100 nmol of [^{3}H]IDU or [^{3}H]IDU-Ol_2 were dried in 4 mL stoppered glass vials. Then, 1.0 mL of 1-octanol and 1 mL of 50 mM sodium phosphate buffer (pH 7.4) were added, and the mixtures were shaken for 16 h at 37 °C. Samples of the octanol and the aqueous phase were then assayed for radioactivity, and the partition coefficients P (amount in octanol/amount in aqueous phase) were calculated.

Preparation and HDL and HDL Apoproteins. Human HDL (density 1.063−1.210 g/mL) was isolated by two repetitive centrifugations as described earlier (Redgrave et al., 1975). HDL was subsequently depleted of apoE-containing material using a Sepharose−heparin column (Weisgraber & Mahley, 1980). To isolate apoproteins, HDL was dialyzed against water and freeze-dried. The lyophilized material was extracted with ethanol/diethyl ether (3:1) for 16 h at 4 °C and, subsequently, centrifuged for 5 min at 2000g. The supernatant was aspirated, and the pellet was subjected to two similar extractions for 4 h each. The final pellet was washed with ether, dried, and stored under nitrogen at −20 °C.

Preparation of [^{3}H]IDU-Ol_2-Loaded NeoHDL. The following lipids, dissolved in $CHCl_3$, were mixed in a 20 mL glass scintillation counting vial: 3.6 mg of phosphatidylcholine, 1.8 mg of cholesteryl oleate, 0.9 mg of cholesterol, and 0.9 mg of [^{3}H]IDU-Ol_2. The solvent was evaporated under a stream of nitrogen. Subsequently, 10 mL of sonication buffer (10 mM Tris-HCl buffer, pH 8.0, containing 0.1 M KCl, 1 mM EDTA, and 0.025% NaN_3), degassed and saturated with nitrogen, was added, and the contents of the vial was sonicated with a macrotip (14-μm output) under a stream of nitrogen. The temperature was maintained at 49−52 °C. The sonication was stopped after 60 min, and the temperature was lowered to 42−44 °C. Sonication was continued, and 20 mg of HDL apoproteins, dissolved in 1 mL of 4 M urea, were added in 10 equal portions over a period of 10 min. After all protein was added, the mixture was sonicated for a further 20 min. The sonication mixture was then centrifuged at 12000g for 5 min to remove large particles. Subsequently, the preparation was subjected to density gradient ultracentrifugation as described earlier (Redgrave et al., 1975). Particles with a density ranging from 1.08 to 1.18 g/mL (50−75% of the applied radioactivity) were pooled and concentrated. The NeoHDL was further purified by FPLC using a Superose-6 column (Pharmacia, Uppsala, Sweden). The column (60 × 1.8 cm) was eluted with 0.1 M sodium phosphate buffer, pH 7.0, containing 0.5 M NaCl and 10 mM EDTA (flow rate 24 mL/h). Fractions eluting at the the same position as native HDL (60−90% of the applied radioactivity) were collected, pooled, and concentrated.

Physicochemical Characterization of [^{3}H]IDU-Ol_2-Loaded NeoHDL. The chemical composition of [^{3}H]IDU-Ol_2-loaded NeoHDL was determined as follows. Protein was measured by the method of Lowry et al. (1951), using bovine serum albumin as a standard. Cholesterol and cholesteryl oleate were determined by an enzymatic method as described earlier (Nagelkerke et al., 1986). Phosphatidylcholine was determined using a colorometric test kit provided by Boehringer Mannheim, Mannheim, Germany. [^{3}H]IDU-Ol_2 was assayed by measuring its radioactivity.

The size of [^{3}H]IDU-Ol_2-loaded NeoHDL was determined by FPLC (Superose-6 column) and by photon correlation spectroscopy, using a Malvern 4700c submicron particle analyzer, at an angle of 90°.

Radioiodination of [^{3}H]IDU-Ol_2-Loaded NeoHDL. [^{3}H]-IDU-Ol_2-loaded NeoHDL was labeled with ^{125}I using iodine monochloride as described earlier (Bijsterbosch & van Berkel, 1992). The resulting double-labeled preparations contained approximately equal amounts of ^{125}I and ^{3}H. The distribution of ^{125}I over apoproteins and lipids in [^{3}H]IDU-Ol_2-loaded ^{125}I-NeoHDL was determined by extraction as described by Folch et al. (1957). The apoprotein moiety contained 98.9 ± 0.2% of the ^{125}I, and 0.5 ± 0.1% was present in the lipids. The remaining 0.6 ± 0.1% was free ^{125}I (means ± SEM of 3 determinations).

Lactosylation of [^{3}H]IDU-Ol_2-Loaded NeoHDL. [^{3}H]IDU-Ol_2-loaded NeoHDL (1 mg/mL in 0.1 M sodium phosphate buffer, pH 7.0, containing 1 mM EDTA) was incubated under sterile conditions at 37 °C with lactose and sodium cyanoborohydride to final concentrations of 100 and 50 mg/mL, respectively. After 60 h, the reaction was stopped by the addition of 0.2 volume of 0.6 M NH_4HCO_3. Subsequently, sodium cyanoborohydride and unbound lactose were removed by exhaustive dialysis against PBS (10 mM sodium phoshate buffer, containing 0.15 M NaCl and 1 mM EDTA).

In Vivo Plasma Clearance and Liver Association. Male Wistar rats (225−325 g) were used. The animals were anesthesized by intraperitoneal injection of 15−20 mg of sodium pentobarbital, and the abdomen was opened. Radiolabeled ligands were injected via the vena penis. At the indicated times, blood samples of 0.2−0.3 mL were taken from the inferior vena cava and collected in heparinized tubes. The samples were centrifuged for 2 min at 16000g and assayed for radioactivity. The total amount of radioactivity in plasma was calculated using the equation: plasma volume (mL) = [0.0219 × body weight (g)] + 2.66 (Bijsterbosch et al., 1989). At the indicated times, liver lobules were tied off and excised. At the end of the experiment the remainder of the liver was removed. The amount of liver tissue tied off successively did not exceed 15% of the total liver mass. Radioactivity in the liver at each time point was calculated from the radioactivities and weights of the liver samples and corrected for radioactivity in plasma present in the tissue at the time of sampling (85 μL/g fresh weight; Caster et al., 1955).

Determination of Radioactivity. Samples containing ^{3}H were counted in a Packard Tri-Carb 1500 liquid scintillation counter, using Emulsifier Safe or Hionic Fluor scintillation cocktails. Liver samples, TLC scrapings, and gel slices were first digested with Soluene-350. In samples containing both ^{125}I and ^{3}H, the ^{125}I-radioactivity was counted in a Packard Auto-Gamma 5000 counter, and the ^{3}H-activity was mea-

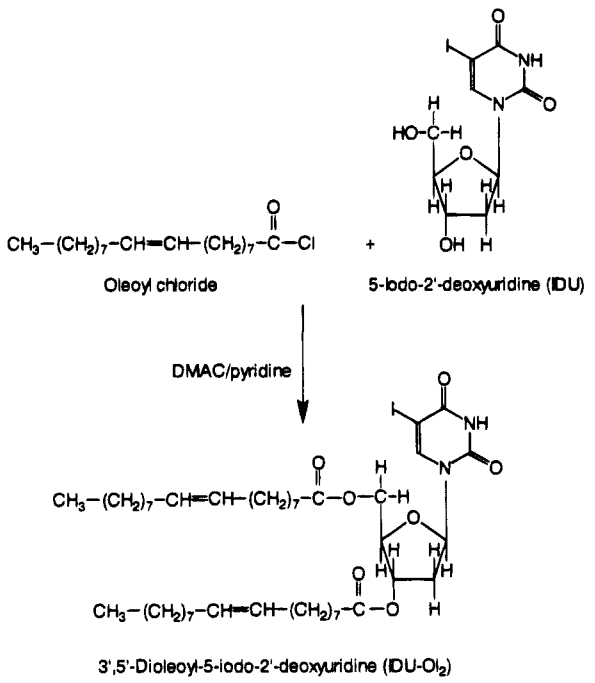

FIGURE 1: Synthesis of IDU-Ol_2.

sured as described above and corrected for the contribution of ^{125}I.

Assay of the Stability of NeoHDL-Associated IDU-Ol_2. [^{3}H]IDU-Ol_2-loaded NeoHDL (10 μg of protein/mL) was incubated with serum or phosphate-buffered saline at 37 °C in aliquots of 0.16 mL. After 2 h, the incubation was terminated by the addition of 0.6 mL of chloroform/methanol (1:2). Phases were separated by adding 0.2 mL of chloroform and 0.2 mL of 0.28 N HCl. The upper (aqueous) phase was taken off, and the lower (organic) phase was washed thrice with 0.5 mL of 0.1 N HCl. The aqueous phase was combined with the wash fluids and counted for radioactivity. The organic phase was subjected to TLC (solvent: methanol/dichloromethane, 5:95). Approximately 10 μg of IDU-Ol_2 and IDU-Ol_1 were added as carrier and to enable detection. The IDU-Ol_2 and IDU-Ol_1 spots were scraped off and counted for radioactivity.

RESULTS

Synthesis of and Characterization of (^{3}H-Labeled) IDU-Ol_2. The synthesis of IDU-Ol_2 was based on a previously described procedure for the preparation of dioleoylfluorodeoxyuridine (Nishizawa et al., 1965). In short, oleoyl chloride was allowed to react with IDU in a mixture of dimethylacetamide and pyridine (Figure 1). IDU-Ol_2 was subsequently purified from the reaction mixture by extraction and silica column chromatography. The identity of IDU-Ol_2 was confirmed by ^{1}H-NMR and mass spectroscopy. To be able to monitor the biological fate of the (pro)drug, a ^{3}H-labeled derivative was synthesized. First, [^{3}H]IDU was synthesized from 2′-deoxy[6-^{3}H]uridine by electrophilic substitution, essentially as described by Prusoff (1959). The UV spectrum of the product was identical to that of reference IDU (a maximum at 279 nm and a minimum at 254 nm), but clearly different from that of 2′-deoxyuridine (Figure 2). The radiochemical purity was >95%, as estimated by thin layer chromatography and HPLC. [^{3}H]IDU was subsequently derivatized with oleoyl chloride as described above.

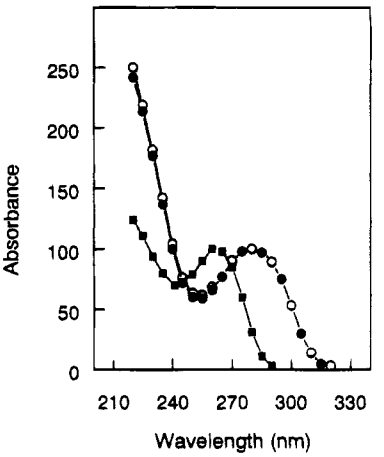

FIGURE 2: Spectral properties of [^{3}H]iododeoxyuridine and deoxyuridine. Iododeoxyuridine (●), [^{3}H]iododeoxyurine (○), and deoxyuridine (■) were dissolved in 0.1 M NH_4OH, and their absorption in the ultraviolet region was determined spectrophotometrically. The results are expressed as % of the maxima at 262 nm (■) and 279 nm (○, ●).

Table 1: Chemical Composition of [^{3}H]IDU-Ol_2-Loaded NeoHDL; Comparison with Native ApoE-Depeleted HDL[a]

	% of total wt	
	[^{3}H]IDU-Ol_2-loaded NeoHDL	native apoE-free HDL
protein	52.3 ± 0.2	48.9 ± 3.9
phosphatidylcholine	26.5 ± 0.9	28.9 ± 1.4
cholesterol	3.6 ± 0.4	2.9 ± 0.5
cholesteryl oleate	10.3 ± 0.6	19.4 ± 0.6
[^{3}H]IDU-Ol_2	7.3 ± 0.8	

[a] The chemical compositions of [^{3}H]IDU-Ol_2-loaded NeoHDL and native apoE-depleted HDL were analyzed as described in the Experimental Procedures. Values given are means ± SEM of 3 different preparations.

[^{3}H]IDU-Ol_2 was identical to unlabeled IDU-Ol_2 as judged by its chromatographic behavior in two TLC systems. Measurement of the partition coefficients of [^{3}H]IDU-Ol_2 and [^{3}H]IDU confirmed that the prodrug was much more lipophilic than the parent compound: log P values were found to be 4.07 ± 0.01 and −0.65 ± 0.01, respectively (means ± SEM of 3 determinations).

Preparation of [^{3}H]IDU-Ol_2-Loaded NeoHDL. The procedure used to prepare [^{3}H]IDU-Ol_2-loaded NeoHDL was based on previously described methods for the preparation HDL-like particles (Atkinson & Small, 1986; Pittman et al., 1987; Jonas et al., 1989; Schouten et al., 1993). In brief, a lipid emulsion was obtained by cosonication of phophatidylcholine, cholesterol, cholesteryl oleate, and [^{3}H]IDU-Ol_2. Subsequently, HDL apoproteins were added to the emulsion, and sonication was continued for another 30 min. The resulting [^{3}H]IDU-Ol_2-loaded NeoHDL was purified by density gradient centrifugation and FPLC.

Physicochemical Characterization of [^{3}H]IDU-Ol_2-Loaded NeoHDL. The chemical composition of [^{3}H]IDU-Ol_2-loaded NeoHDL is given in Table 1, and is compared with the composition of native apoE-depleted HDL. The formation of the prodrug-loaded particles was very reproducible; only small variations were found in the compositions of different preparations. The particles contained a substantial amount of IDU-Ol_2: 7.3 ± 0.8% of the total weight (15.4 ± 1.6% of the lipid moiety). From the size, density, and composition

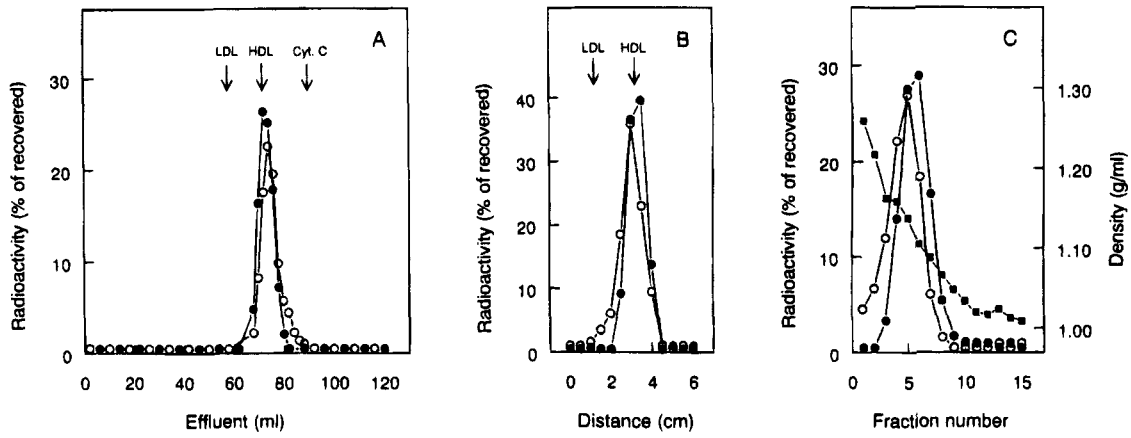

FIGURE 3: Analysis of the physical properties of [^{3}H]IDU-Ol$_2$-loaded ^{125}I-NeoHDL by gel chromatography (A), gel electrophoresis (B), and density gradient centrifugation (C). (A) [^{3}H]IDU-Ol$_2$-loaded ^{125}I-NeoHDL (0.25 mg of protein) was injected onto a Superose-6 column (60 × 1.8 cm), and the column was eluted with 0.1 M sodium phosphate buffer, pH 7.0, containing 0.5 M NaCl and 10 mM EDTA (flow rate 6 mL/h). Fractions of 2.0 mL were collected and assayed for ^{3}H (●) and ^{125}I (○). The results are expressed as % of the recovered radioactivity (recoveries >96%). The elution volumes of LDL, HDL, and cytochrome *c*, which were used to calibrate the column, are indicated by arrows. (B) [^{3}H]IDU-Ol$_2$-loaded ^{125}I-NeoHDL (0.025 mg of protein) was subjected to electrophoresis in a 0.75% (w/v) agarose gel at pH 8.8 (75 mM Tris−hippuric acid buffer). The gel was cut in slices that were assayed for ^{3}H (●) and ^{125}I (○). The radioactivity in each slice is given as % of the recovered radioactivity (recoveries >97%). Arrows indicate the positions of LDL and HDL markers. (C) [^{3}H]IDU-Ol$_2$-loaded ^{125}I-NeoHDL (0.25 mg of protein) was subjected to density gradient centrifugation (Redgrave et al., 1975). The gradient (12.0 mL) was fractionated in fractions of 0.8 mL. The fractions were assayed for ^{3}H (●) and ^{125}I (○), and their densities (■) were measured. The results are expressed as % of the recovered radioactivity (recoveries >97%).

of the prodrug-loaded NeoHDL, it can be calculated that each particle contains about 30 IDU-Ol$_2$ molecules. The results further show a remarkable similarity with the composition of native HDL.

The size of the prodrug-loaded particles was studied using photon correlation spectroscopy, and compared to that of native HDL. The mean sizes of [^{3}H]IDU-Ol$_2$-loaded NeoHDL and native apoE-depleted HDL were found to be 9.7 ± 0.6 and 9.4 ± 0.7 nm, respectively (means ± SEM of 3 preparations). The physical properties of [^{3}H]IDU-Ol$_2$-loaded NeoHDL were further studied by size exclusion chromatography, agarose gel electrophoresis, and density gradient centrifugation. For these studies, the apoproteins of the particles were also labeled with ^{125}I. This allowed the monitoring of both the incorporated ^{3}H-labeled prodrug and the ^{125}I-labeled apoproteins. Figure 3A shows the elution profile of [^{3}H]IDU-Ol$_2$-loaded ^{125}I-NeoHDL on a calibrated Superose-6 FPLC column. Both ^{125}I and ^{3}H eluted at the same position as native HDL. This finding indicates that the size of [^{3}H]IDU-Ol$_2$-loaded NeoHDL is similar to that of native HDL, which corroborates the results of the analysis by photon correlation spectroscopy. Figure 3B shows the result of agarose electrophoresis of [^{3}H]IDU-Ol$_2$-loaded ^{125}I-NeoHDL. Lipoproteins subjected to this type of electrophoresis are separated mainly according to their electric charge. Both ^{125}I and ^{3}H were recovered at the same position as the native HDL marker. The results of density gradient centrifugation of [^{3}H]IDU-Ol$_2$-loaded ^{125}I-NeoHDL are shown in Figure 3C. More than 90% of both ^{125}I and ^{3}H radioactivity was recovered at a density between 1.088 and 1.218 g/mL, which corresponds to the density of native HDL. Thus, our findings indicate that size, density, and charge of IDU-Ol$_2$-loaded NeoHDL are very similar to those of native HDL. Moreover, because in all experiments ^{125}I and ^{3}H behaved similarly, the results further indicate that under the conditions employed the particles are stable.

Stability of IDU-Ol$_2$-Loaded NeoHDL in Serum in Vitro. The stability of NeoHDL-associated IDU-Ol$_2$ in serum was tested by incubating prodrug-loaded NeoHDL in rat serum at 37 °C. After 2 h of incubation, the amounts of [^{3}H]IDU-Ol$_2$, [^{3}H]IDU-Ol$_1$, and water-soluble [^{3}H]metabolites were determined. Table 2 shows that during incubation the amount of IDU-Ol$_2$ decreased only slightly, with a concomittant small increase in the amount of water-soluble metabolites.

Exchange of NeoHDL-associated IDU-Ol$_2$ with other serum proteins was studied by incubating the prodrug-loaded particle with human serum for 1 h at 37 °C. After incubation, the distribution of IDU-Ol$_2$ over serum proteins was determined by density gradient centrifugation. Human serum was used in this experiment to be able to detect possible exchange with LDL (rat serum contains mainly HDL and very little LDL). Figure 4 shows that, although a high amount of LDL was present in the incubation mixture, hardly any exchange of the prodrug from NeoHDL to LDL had occurred.

In Vivo Fate of [^{3}H]IDU-Ol$_2$-Loaded NeoHDL; Effect of Lactosylation. To investigate the biological fate of the prodrug-loaded particles, rats were injected with [^{3}H]IDU-Ol$_2$-loaded ^{125}I-NeoHDL. The plasma clearance of both labels was monitored. Because of the major role of the liver in the metabolism of native HDL (Glass et al., 1985), we also measured the association of radioactivity with the liver. The results are shown in Figure 5A. As judged by the behavior of the ^{125}I-labeled apoproteins, the particles were slowly cleared from the circulation, just as native ^{125}I-labeled HDL (Schouten et al., 1988). Only a small proportion of the ^{125}I became associated with the liver (5.2 ± 1.0% of the dose at 10 min after injection). The ^{3}H-radioactivity disappeared more rapidly from plasma, and it associated to a higher extent with the liver.

For comparison, Figure 5B shows the fate of underivatized [^{3}H]IDU after intravenous injection into rats. The labeled drug is very rapidly cleared from the circulation, and a relatively small amount was recovered in the liver. The remainder of the dose, as has been shown previously, was

Table 2: Stability of NeoHDL-Associated IDU-Ol_2 in Phosphate-Buffered Saline and Rat Serum *in Vitro*[a]

	% of total radioactivity		
incubation	IDU-Ol_2	IDU-Ol_1	water-soluble metabolites
control	97.9 ± 0.2	2.1 ± 0.2	0.0 ± 0.0
PBS, 2 h	97.3 ± 0.2	1.8 ± 0.2	0.9 ± 0.3
serum, 2 h	95.7 ± 0.4	0.8 ± 0.3	3.5 ± 0.2

[a] [^{3}H]IDU-Ol_2-loaded NeoHDL (10 μg of protein/mL) was incubated with rat serum or phosphate-buffered saline (PBS) at 37 °C. After 2 h, the amounts of [^{3}H]IDU-Ol_2, [^{3}H]IDU-Ol_1, and water-soluble [^{3}H]metabolites were determined by extraction and thin layer chromatography. Controls were extracted immediately after mixing prodrug-loaded NeoHDL with serum or PBS. The results are expressed as % of the total radioactivity and are means ± SEM of 3 incubations.

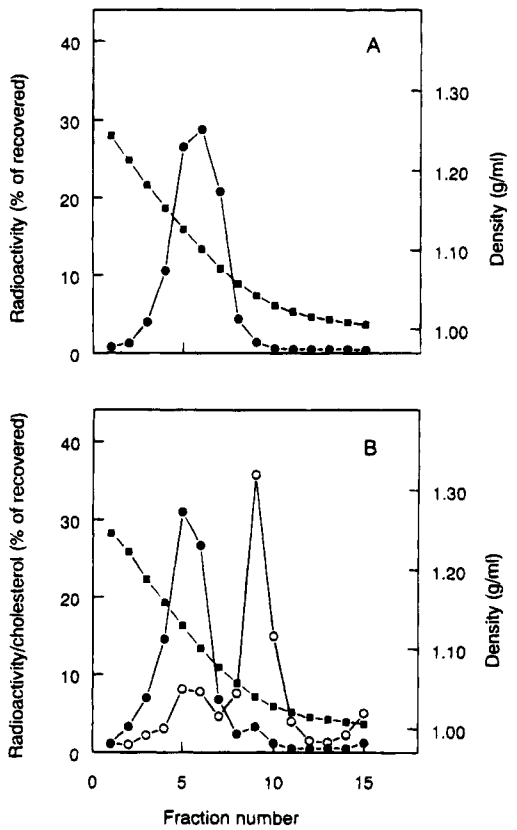

FIGURE 4: Density gradient centrifugation of [^{3}H]IDU-Ol_2-loaded NeoHDL incubated with saline (A) or serum (B). [^{3}H]IDU-Ol_2-loaded NeoHDL (0.05 mg of protein/mL) was incubated for 1 h at 37 °C with phosphate-buffered saline (A) or human serum (B). Aliquots of 2 mL of the incubation mixtures were subjected to density gradient centrifugation (Redgrave et al., 1975). The gradients (12.0 mL) were fractionated in fractions of 0.8 mL. The fractions were assayed for ^{3}H (●) and cholesterol (○; only B), and their densities (■) were measured. The results are expressed as % of the recovered amounts of radioactivity and cholesterol (recoveries >98%).

nonspecifically distributed over the total body (Biessen et al., 1994).

We showed in previous studies that lactosylation of native HDL and NeoHDL, a procedure by which the particles are provided with terminal galactose residues, leads to very rapid uptake of the particles by galactose-specific receptors on parenchymal liver cells (Bijsterbosch & van Berkel, 1992; Schouten et al., 1994). To investigate whether a high liver uptake of the prodrug can be induced via this pathway, [^{3}H]-IDU-Ol_2-loaded ^{125}I-NeoHDL was lactosylated and injected into rats. Figure 6 shows that lactosylation of the prodrug-loaded particle dramatically alters its biological fate. After injection of lactosylated [^{3}H]IDU-Ol_2-loaded ^{125}I-NeoHDL, both labels were equally rapidly cleared from the circulation. At 10 min after injection, only 11.5 ± 1.0% and 7.5 ± 2.1% of the injected ^{3}H- and ^{125}I-activity were left in plasma, respectively. At that time, the liver contained 74.9 ± 9.2% and 75.3 ± 6.6% of the injected amounts of ^{3}H and ^{125}I, respectively.

DISCUSSION

To enable incorporation of IDU into NeoHDL, we synthesized a lipophilic prodrug of IDU. The approach to prepare a lipophilic prodrug from a water-soluble parent compound by the coupling of lipophilic residues was applied in a number of earlier studies (De Smidt & van Berkel, 1990; Firestone et al., 1984; Vitols et al., 1985). In these studies, lipophilic prodrugs of antineoplastic compounds like nitrogen mustard, doxorubicin, methotrexate, and floxuridine were incorporated in LDL, and it was found that the resulting prodrug–LDL complexes can be recognized by LDL receptors on cultured cells. In the present study, IDU was derivatized with two oleoyl residues. Oleyl residues were chosen as lipophilic "anchor" as they are natural components of lipoproteins. The residues were attached via an ester linkage. As esterases are ubiquitous, this type of linkage ensures release of the original, pharmacologically active drug at the site of delivery (Sinkula & Yalowski, 1975). In previous studies, a series of 5′-mono esters of IDU were synthesized, and it was found that the lipophilicity of the prodrug is determined by the choice of side chain (Narurkar & Mitra, 1988; Ghosh & Mitra, 1991). In these earlier studies, relatively short aliphatic chains were used, which resulted in a moderate degree of lipophilicity (log P < 1.4). The oleoyl 3′,5′-diester prepared in the present study was much more lipophilic (log P > 4.0).

The lipophilic prodrug was incorporated into neoHDL by including it in the lipid mixture used to prepare the particles. This procedure resulted in the reproducible formation of particles containing a substantial amount of IDU-Ol_2. The prodrug accounted for 7.3 ± 0.8% of the total weight, which corresponds to approximately 15% of the lipid moiety. It was calculated that each particle contains about 30 prodrug molecules. Higher loads have not been tested, but may very well be possible. The composition further shows a remarkable similarity with that of native HDL. In the present study, the particles were prepared from commercially available components and isolated apoproteins. In the future, recombinant apoproteins may be used to prepare fully artificial particles. Various physical properties of [^{3}H]IDU-Ol_2-loaded NeoHDL were studied and compared with those of native HDL. To allow the simultaneous monitoring of both the incorporated prodrug and the apoproteins of the particles, the apoproteins of the [^{3}H]prodrug-loaded NeoHDL were labeled with ^{125}I. We demonstrate by two different methods that the size of [^{3}H]IDU-Ol_2-loaded NeoHDL is very similar to that of native HDL. Photon correlation spectroscopy indicated sizes for the prodrug-loaded particle and native HDL of 9.7 ± 0.6 and 9.4 ± 0.7 nm, respectively. Furthermore, [^{3}H]IDU-Ol_2-loaded ^{125}I-NeoHDL eluted at the same position as native HDL on a Superose-6 FPLC column. The density and electrical charge of [^{3}H]IDU-Ol_2-loaded ^{125}I-NeoHDL were determined by density gradient centrifugation

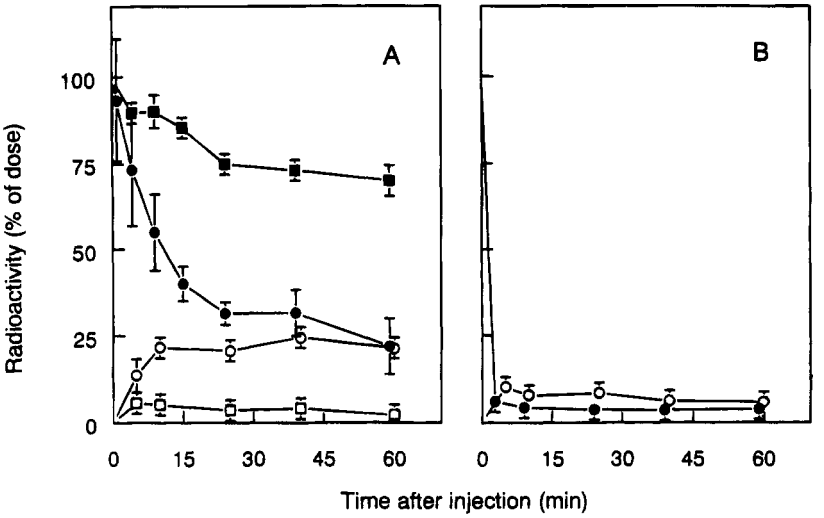

FIGURE 5: Plasma clearance and liver association of [^{3}H]IDU-Ol$_2$-loaded ^{125}I-NeoHDL (A) and [^{3}H]IDU (B). Rats were intravenously injected with [^{3}H]IDU-Ol$_2$-loaded ^{125}I-NeoHDL (A) or [^{3}H]IDU (B). The animals received 5 μg of IDU or an equivalent amount of IDU-Ol$_2$ per kg body weight. At the indicated times, the amounts of radioactivity in plasma and liver were determined. Values are means ± SEM of 3 rats. ●, ^{3}H in plasma; ○, ^{3}H in liver; ■, ^{125}I in plasma; □, ^{125}I in liver.

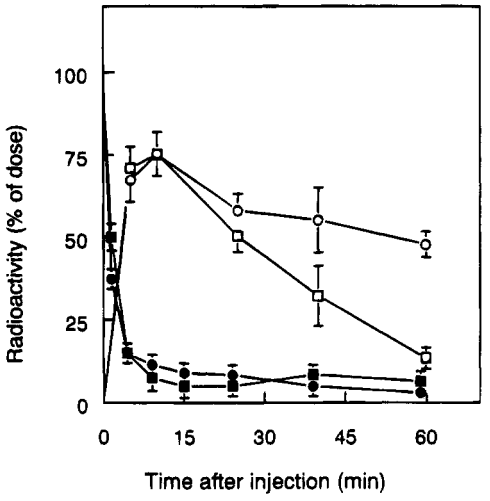

FIGURE 6: Plasma clearance and liver association of lactosylated [^{3}H]IDU-Ol$_2$-loaded ^{125}I-NeoHDL. Rats were intravenously injected with lactosylated [^{3}H]IDU-Ol$_2$-loaded ^{125}I-NeoHDL at a dose equivalent to 5 μg of IDU per kg body weight. At the indicated times, the amounts of radioactivity in plasma and liver were determined. Values are means ± SEM of 3 rats. ●, ^{3}H in plasma; ○, ^{3}H in liver; ■, ^{125}I in plasma; □, ^{125}I in liver.

and agarose gel electrophoresis, respectively. It was found that the prodrug-loaded particles had the same characteristics as native HDL. As in all assays the prodrug and apoproteins of the particle behaved similarly, it can be concluded that under the conditions employed the particles are stable.

The stability of the the prodrug-loaded particle in serum was investigated by *in vitro* incubation studies. Upon incubation with rat serum, NeoHDL-associated IDU-Ol$_2$ was not rapidly metabolized. Furthermore, it was found that the prodrug does not rapidly exchange with other lipoproteins, such as LDL, during *in vitro* incubation with human serum.

Derivatization of IDU and the subsequent incorporation of the prodrug into neoHDL drastically altered the biological fate of the drug. Underivatized IDU is rapidly cleared from the circulation after injection, and 10−15% of the injected amount was found in the liver. We showed earlier that the remainder is distributed nonspecifically over the total body. Major recovery sites were bulky tissues like muscles and skin (Biessen et al., 1994). As judged by the behavior of the apoproteins, IDU-Ol$_2$-loaded NeoHDL particles are only slowly cleared from the circulation, just like native HDL. Only a very small proportion became associated with the liver. As even a limited oxidative modification of lipoproteins results in a substantial hepatic uptake of lipoprotein particles (Van Berkel et al., 1991), this result indicates that no significant oxidative damage was provoked to the particles during preparation. The labeled prodrug, however, disappeared more rapidly from the circulation, and it associated to a higher extent with the liver. As mentioned above, NeoHDL-associated IDU-Ol$_2$, during *in vitro* incubations in serum, is not rapidly hydrolyzed nor does it readily exchange with LDL. Apparently, NeoHDL-associated IDU-Ol$_2$ *in vivo* is subject to selective release from HDL. It has been reported for a number of tissues, including liver, that cholesterol esters from native HDL are selectively taken up without a parallel uptake of the apoproteins (Glass et al., 1985). The rate of selective cellular uptake of various cholesterol esters can be up to 40-fold higher than the uptake of apoproteins (Sattler & Stocker, 1993). The observed higher rate of plasma clearance and liver uptake of IDU-Ol$_2$ from the circulation may thus, at least in part, be explained by HDL-specific natural processing of the prodrug-loaded particles.

We showed, earlier, that if (neo)HDL is provided with terminal galactose residues by reductive lactosamination, the resulting lactosylated (Neo)HDL is recognized by galactose receptors on parenchymal liver cells (Bijsterbosch & van Berkel, 1992; Schouten et al., 1994). In the present study, we show that lactosylated IDU-Ol$_2$-loaded NeoHDL is very rapidly cleared from the circulation. Within 10 min after injection, approximately 90% of the apoproteins as well as the prodrug is cleared from the circulation. The cleared radioactivity is largely (>80%) recovered in the liver. The hepatic association of prodrug and apoproteins was very similar. This finding indicates that, in the case of lactosylated IDU-Ol$_2$-loaded NeoHDL, the particles are taken up as an entity. In preliminary experiments it was found that preinjection of asialofetuin substantially reduced the rate of plasma clearance and liver uptake of both the prodrug and the apoproteins of lactosylated IDU-Ol$_2$-loaded NeoHDL (data

not shown). As asialofetuin specifically inhibits uptake by the asialoglycoprotein receptor on parenchymal liver cells (Van Berkel et al., 1987), this finding indicates that this receptor is mainly responsible for the hepatic uptake of lactosylated IDU-Ol_2-loaded NeoHDL. The approach to use lactosylated NeoHDL as a carrier to target lipophilic prodrugs to the galactose receptor on parenchymal liver cells affords a number of advantages over previously published carrier systems like (neo)glycoproteins and lactosylated poly-L-lysine (Jansen et al., 1993; Biessen et al., 1994). During transport in the circulation, the lipophilic prodrug is hidden in the lipid moiety (probably the apolar core), protected from the biological environment. Furthermore, as the lipophilic prodrugs are incorporated in the lipid moiety, high drug loads are possible without interfering with the receptor-mediated recognition of the lactose residues that are present on the surface of the apoproteins.

In conclusion, our findings indicate that it is possible to convert a hydrophilic drug like IDU into a lipophilic prodrug that can be efficiently incorporated into a reconstituted HDL particle with similar physicochemical properties as native HDL. The prodrug-loaded particles are *in vitro* stable in serum, their *in vivo* behavior resembles that of native HDL, and lactosylation induces selective uptake by parenchymal liver cells. The latter result is particularly interesting, as this approach may also be used to target other lipophilic derivatives of water-soluble drugs highly specifically to liver parenchymal cells. This may lead to a more effective therapy of infectious diseases like hepatitis B.

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REFERENCES

Atkinson, D., & Small, D. M. (1986) *Annu. Rev. Biophys. Biophys. Chem. 15*, 403−456.

Biessen, E. A. L., Beuting, D. M., Vietsch, H., Bijsterbosch, M. K., & van Berkel, Th. J. C. (1994) *J. Hepatol.* (in press).

Bijsterbosch, M. K., & van Berkel, Th. J. C. (1990) *Adv. Drug Delivery Rev. 5*, 231−251.

Bijsterbosch, M. K., & van Berkel, Th. J. C. (1992) *Mol. Pharmacol. 41*, 404−411.

Bijsterbosch, M. K., Ziere, G. J., & van Berkel, Th. J. C. (1989) *Mol. Pharmacol. 36*, 484−489.

Caster, W. O., Simon, A. B., & Armstrong, W. D. (1955) *Am. J. Physiol. 183*, 317−321.

Counsell, R. E., & Pohland, R. C. (1982) *J. Med. Chem. 25*, 1115−1120.

De Smidt, P. C., & van Berkel, Th. J. C. (1990) *Cancer Res. 50*, 7476−7482.

De Smidt, P. C., Versluis, A. J., & van Berkel, Th. J. C. (1993) *Biochemistry 32*, 2916−2922.

Firestone, R. A. (1994) *Bioconjugate Chem. 5*, 105−113.

Firestone, R. A., Pisano, J. M., Falck, J. R., McPhaul, M. M., & Krieger, M. (1984) *J. Med. Chem. 27*, 1037−1043.

Folch, J., Lees, M., & Stanley, G. H. S. (1957) *J. Biol. Chem. 226*, 497−509.

Ghosh, M. K., & Mitra, A. K. (1991) *Pharm. Res. 8*, 771−775.

Glass, C., Pittman, R. C., Civen, M., & Steinberg, D. (1985) *J. Biol. Chem. 260*, 744−750.

Jansen, R. W., Kruijt, J. K., van Berkel, Th. J. C., & Meijer, D. K. F. (1993) *Hepatology 18*, 146−152.

Jonas, A., Kezdy, K. E., & Wald, J. H. (1989) *J. Biol. Chem. 264*, 4818−4824.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265−275.

Mraz, W., Reble, B., Kemkes, B. M., & Knedel, M. (1986) *Transplant. Proc. 18*, 1281−1284.

Nagelkerke, J. F., Bakkeren, H. F., Kuipers, F., Vonk, R. J., & van Berkel, Th. J. C. (1986) *J. Biol. Chem. 261*, 8908−8913.

Narurkar, M. M., & Mitra, A. K. (1988) *Pharm. Res. 5*, 734−737.

Nishizawa, Y., Casida, J. E., Anderson, S. W., & Heidelberger, C. (1965) *Biochem. Pharmacol. 14*, 1605−1619.

Pittman, R. C., Glass, D. K., Atkinson, D., & Small, D. M. (1987) *J. Biol. Chem. 262*, 2435−2442.

Poznanski, M. J., & Juliano, R. L. (1984) *Pharmacol. Rev. 36*, 277−336.

Prusoff, W. H. (1959) *Biochim. Biophys. Acta 32*, 295−296.

Prusoff, W. H., & Goz, B. (1974) *Fed. Proc. 32*, 1679−1687.

Redgrave, T. G., Roberts, D. C. K., & West, C. E. (1975) *Anal. Biochem. 65*, 42−49.

Santos, O., Pant, K. D., Blank, E. W., & Ceriani, R. L. (1992) *J. Nucl. Med. 33*, 1530−1534.

Sattler, W., & Stocker, R. (1993) *Biochem. J. 294*, 771−778.

Schouten, D., Kleinherenbrink-Stins, M. F., Brouwer, A., Knook, D. L., & van Berkel, Th. J. C. (1988) *Biochem. J. 256*, 615−621.

Schouten, D., van der Kooij, M., Muller, J., Pieters, M. N., Bijsterbosch, M. K., & van Berkel, Th. J. C. (1993) *Mol. Pharmacol. 44*, 486−492.

Shaw, J. M., Shaw, K. V., Yanovich, S., Iwanik, M., Futch, W. S., Rosowsky, A., & Schook, L. B. (1987) *Ann. N.Y. Acad. Sci. 507*, 252−271.

Sinkula, A. A., & Yalowski, S. H. (1975) *J. Pharm. Sci. 64*, 181−210.

Tomlinson, E. (1987) *Adv. Drug Delivery Rev. 1*, 87−198.

Van Berkel, Th. J. C., Dekker, C. J., Kruijt, J. K., & Van Eijk, H. G. (1987) *Biochem. J. 243*, 715−722.

Van Berkel, Th. J. C., de Rijke, Y. B., & Kruijt, J. K. (1991) *J. Biol. Chem. 266*, 2282−2289.

Vitols, S. G., Masquelier, M., & Peterson, C. O. (1985) *J. Med. Chem. 28*, 451−454.

Weisgraber, K. H., & Mahley R. W. (1980) *J. Lipid Res. 21*, 316−325.