Biochemical Pharmacology, Vol. 52, pp. 113–121, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00170-0

Specific Targeting of a Lipophilic Prodrug of Iododeoxyuridine to Parenchymal Liver Cells Using Lactosylated Reconstituted High Density Lipoprotein Particles

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ABSTRACT. We recently reported the conversion of the water-soluble antiviral drug iododeoxyuridine (IDU) into the lipophilic prodrug dioleoyl-iododeoxyuridine (IDU-Ol_2). The prodrug was incorporated into reconstituted high-density lipoprotein (NeoHDL) particles with physical and biological properties similar to those of native HDL. We also found, in initial experiments, that lactosylation of the prodrug-loaded NeoHDL increases its liver uptake. Because this offers the attractive perspective of using these particles for the delivery of drugs to the liver, we now analyze the characteristics and biological fate of lactosylated IDU-Ol_2-loaded NeoHDL. The particles (containing approximately 25 prodrug molecules) have the same size and charge as native HDL, indicating that lactosylation does not cause aggregation or oxidative modification. At 10 min after intravenous injection of lactosylated [^{3}H]IDU-Ol_2-loaded NeoHDL into rats, only 13.5 ± 2.8% of the dose was left in plasma and 75.9 ± 2.4% of the dose was recovered in the liver. The relative specific uptake by the liver was 1–2 orders of magnitude higher than that of any other tissue. The hepatic uptake of lactosylated [^{3}H]IDU-Ol_2-loaded NeoHDL was much higher than that of free [^{3}H]IDU (<20% of the dose). Both parenchymal liver cells and Kupffer cells express galactose-specific receptors. By isolating liver cells after injection of the prodrug-loaded particles, it was established that hepatic uptake occurred mainly (for 84.4 ± 3.8%) in parenchymal liver cells. Preinjection with asialofetuin substantially reduced the liver uptake of lactosylated [^{3}H]IDU-Ol_2-loaded NeoHDL, which points to uptake by the asialoglycoprotein receptor. Subcellular fractionation of the liver indicated that lactosylated [^{3}H]IDU-Ol_2-loaded NeoHDL does not merely associate to cells, but is internalized and delivered to the lysosomes. In conclusion, we show that IDU can be specifically targeted to the parenchymal liver cell. Conversion of the water-soluble parent drug into a lipophilic prodrug that is incorporated into a lactosylated reconstituted HDL particle, is an approach that may also be used to deliver other water-soluble drugs to the parenchymal liver cells. This may lead to more effective therapy for liver diseases such as hepatitis B. BIOCHEM PHARMACOL 52;1: 113–121, 1996.

KEY WORDS. asialoglycoprotein receptor; selective drug delivery; hepatitis B; antivirals; parenchymal liver cells; reconstituted lipoproteins

The selective delivery of a drug to its specific cellular target increases its therapeutic effectiveness and reduces undesired interactions with nontarget tissues [1, 2]. Drugs may be delivered to target cells by associating the drug to a carrier that is recognized by receptors present on the surface of these cells. If these receptors are only present on the target cells, highly specific delivery of the drug can be achieved.

In the liver, both Kupffer and parenchymal cells have receptors on their plasma membranes that specifically bind and internalize ligands with terminal D-galactose residues. The receptor on parenchymal cells is the classic asialoglycoprotein receptor [3, 4]. Kupffer cells express a receptor that binds galactose-exposing particles larger than 12 nm [5, 6]. This receptor (also referred to as the galactose-particle receptor) is different from the receptor on the parenchymal cells and is probably identical to a well-characterized receptor that also recognizes fucose [7, 8]. Because galactose-specific receptors show only a high expression on Kupffer and parenchymal liver cells, they are attractive targets for the delivery of drugs to these cells.

We showed previously that lipoproteins can be specifically targeted to the galactose-specific hepatic receptors [9, 10]. Lactosylation of the apoprotein moiety of HDL† induced rapid and highly specific uptake by the galactose receptor on parenchymal liver cells [10]. Lactosylation of LDL, on the other hand, resulted in uptake by the galactose

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† *Abbreviations:* IDU, 5-iodo-2′-deoxyuridine; IDU-OL_2, 3′,5′-dioleoyl-5-iodo-2′-deoxyuridine; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NeoHDL, HDL-like lipid particles.

Received 15 September 1995; accepted 20 February 1996.

receptors on Kupffer cells [9]. The different intrahepatic distributions of lactosylated LDL and lactosylated HDL can probably be ascribed to differences in the sizes of the particles [6]. Lipoproteins are attractive potential drug carriers [11–14]. They are spherical particles consisting of a core of apolar lipids surrounded by a phospholid monolayer, in which cholesterol and apoproteins are embedded. Highly lipophilic drugs can be incorporated into the apolar core and, thus, be transported, hidden inside the particles [11–14]. As drug carriers, lactosylated lipoproteins have advantages over galactose-exposing soluble molecules such as (neo)glycoproteins and lactosylated poly-L-lysine [15–17]. A (pro)drug incorporated into the lipid moiety of lactosylated lipoproteins is protected from the environment during transport in the circulation. Further, (pro)drugs incorporated in the lipid moiety are not likely to interfere with the receptor-mediated recognition of the lactose residues on the apoproteins.

A possible limitation for the use of lipoproteins as drug carriers may be their limited availability. We recently investigated the possibility of synthesizing lipoprotein-like lipid particles from commercially available lipids and isolated apoproteins. We succeeded in preparing particles with properties very similar to the naturally occurring human HDL [18]. More recently, we further investigated the potential use of reconstituted HDL particles, denoted NeoHDL, as drug carriers. We used the antiviral/antineoplastic drug IDU [19, 20] as model compound. IDU is not sufficiently lipophilic for incorporation into (neo)lipoproteins. We, therefore, synthesized the lipophilic prodrug IDU-Ol_2 (Fig. 1). The oleoyl residues were attached to IDU *via* an ester linkage [21]. Because esterases are ubiquitous, this type of linkage ensures release of the original, pharmacologically active drug at the site of delivery [22]. IDU-Ol_2 incorporated readily into NeoHDL, and the physical and biological properties of the prodrug-loaded particles were very similar to those of native HDL [21]. We also found, in initial studies, that lactosylation induced an increased liver association of the prodrug-loaded particles. This finding offers the attractive perspective of utilizing these particles as hepatotrophic carriers for lipophilic (pro)drugs. To further evaluate the feasibility of utilizing the particles as drug carriers, we analyzed in detail the physicochemical characteristics and biological fate of lactosylated IDU-Ol_2-loaded NeoHDL particles. We determined the tissue distribution of the prodrug-loaded particles to evaluate the specificity of liver uptake (i.e., to exclude major nonspecific uptake by extrahepatic tissues). In the liver, we identified the specific cell types and subcellular compartments involved in uptake, as well as the mechanism of hepatic uptake.

MATERIALS AND METHODS

Reagents

$Na^{125}I$ (carrier-free) was from Amersham International, Amersham, Bucks, U.K. [^{3}H]IDU-Ol_2 was synthesized as described earlier [21]. Cholesteryl oleate was obtained from

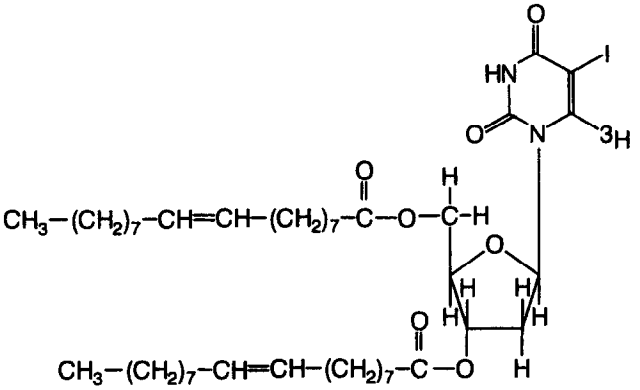

FIG. 1. 3′-5′-dioleoyl-5-iodo-2′-deoxyuridine (IDU-Ol_2).

Janssen (Beerse, Belgium). Egg yolk phosphatidyl choline was from Fluka (Buchs, Switzerland). Cholesterol and bovine serum albumin (fraction V) were obtained from Sigma (St. Louis, MO, U.S.A.). 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, U.S.A.). Asialofetuin was prepared as described in detail earlier [10]. All other reagents were of analytical grade.

Preparation of Lactosylated [^{3}H]IDU-Ol_2-Loaded NeoHDL

HDL-like lipid particles (NeoHDL) were prepared as described in detail earlier [21]. In brief, 3.6 mg of phosphatidyl choline, 0.9 mg of cholesterol, 1.8 mg of cholesteryl oleate, and 0.9 mg of [^{3}H]IDU-Ol_2 (specific radioactivity 5.5 mCi/nmol), dispersed in sonication buffer (10 mM Tris-HCl buffer, pH 8.0, containing 0.1 M KCl, 1 mM EDTA, and 0.025% NaN_3), were sonicated for 60 min at 49–52°C. Then, the temperature was lowered to 42–44°C. Sonication was continued, and 20 mg of HDL apoproteins, dissolved in 4 M urea, were added in small portions over a period of 10 min. After a further 20 min, the sonication was stopped and large particles were removed by centrifugation. The [^{3}H]IDU-Ol_2-loaded NeoHDL particles were purified by density gradient centrifugation and by FPLC using a Superose-6 column. The purified [^{3}H]IDU-Ol_2-loaded NeoHDL was subsequently lactosylated by incubating the particles (1.0 mg of protein/mL in 0.1 M sodium phosphate buffer, pH 7.0, containing 1 mM EDTA) under sterile conditions at 37°C with lactose and sodium cyanoborohydride to final concentrations of 100 mg/mL and 50 mg/mL, respectively. After 60 hr, the reaction was stopped by the addition of 0.2 volume of 0.6 M NH_4HCO_3. The lactosylated particles were exhaustively dialyzed against phosphate-buffered saline (10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl and 1 mM EDTA). Experiments with native HDL indicate that, under these conditions, apoprotein Al is the main apoprotein lactosylated.

Preparation of Radioiodinated Lactosylated [^{3}H]IDU-Ol$_2$-Loaded NeoHDL

[^{3}H]IDU-Ol$_2$-loaded NeoHDL, prepared as described in the previous section, was labeled with ^{125}I using iodine monochloride as described earlier [10]. The resulting double-labeled preparation, which contained approximately equal amounts of ^{125}I and ^{3}H, was subsequently lactosylated as described above.

Chemical Characterization of Lactosylated [^{3}H]IDU-Ol$_2$-loaded NeoHDL

The chemical composition of lactosylated [^{3}H]IDU-Ol$_2$-loaded NeoHDL was determined as follows. Protein was measured by the method of Lowry *et al.* [23], using bovine serum albumin as a standard. The amount of lactose was determined by the Anthrone assay [24]. Cholesterol and cholesteryl oleate were determined by an enzymatic method, as described earlier [25]. Phosphatidyl choline was assayed using a colorometric test kit provided by Boehringer Mannheim (Mannheim, Germany). The amount of [^{3}H]IDU-Ol$_2$ was determined by measuring radioactivity.

Determination of Plasma Clearance and Tissue Distribution

Male Wistar rats, weighing between 225 and 325 gram, were used. The animals were anesthesized by intraperitoneal injection of 15–20 mg of sodium pentobarbital, and the abdomen was opened. Radiolabeled prodrug-loaded lactosylated NeoHDL was 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 16,000 × *g*, and the plasma 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 [9]. At the indicated times, liver lobules were tied off and excised and, 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. Radioactivities in liver and other tissues were corrected for radioactivity in plasma present in the tissue at the time of sampling [26].

Determination of the Distribution Over Liver Cell Types

Rats were anesthesized and injected with radiolabeled prodrug-loaded lactosylated NeoHDL, as described above. The liver was perfused at 10 min after injection, and parenchymal, Kupffer, and endothelial cells were isolated from the liver as described in detail earlier [27]. Shortly before separation of the cells, a liver lobule was tied off and excised to determine the total liver uptake. The contributions of the various cell types to the total liver uptake was calculated as described previously [27]. As found with other ligands [9, 10, 27], no significant amounts of radioactivity were lost from the cells during the isolation procedure. This was checked in each experiment by comparing the calculated liver uptake (i.e., the summation of the contributions of the various cell types) with the value actually measured in the liver lobule.

Subcellular Fractionation

Rats were anesthesized and injected with radiolabeled prodrug-loaded lactosylated NeoHDL, as described above. Twenty minutes later, the liver was perfused with ice-cold 0.25 M sucrose containing 10 mM Tris-HCl buffer, pH 7.4. Subsequently, the liver was divided into subcellular fractions as described previously [28]. In brief, the liver was dispersed in 2 volumes of sucrose/Tris-HCl (see above) using a homogenizer of the Potter-Elvehjem type. Fractions enriched in nuclei, mitochondria, lysosomes, and microsomes were obtained by collecting pellets obtained after subjecting the homogenate to consecutive centrifugation steps of 5 min at 1200 × *g*, 5 min at 9,000 × *g*, 15 min at 22,000 × *g*, and 30 min at 210,000 × *g*, respectively (*g*-forces in middle of tubes), with the final supernatant being the cytosol fraction. The fractions were assayed for radioactivity, protein, and the activity of marker enzymes as described in detail earlier [28].

Determination of Proteins

Protein concentrations in cell suspensions and subcellular fractions were determined by the method of Lowry *et al.* [23], with a bovine serum albumin standard.

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. Gel slices were first digested with Soluene-350. Tissue samples were processed using a Packard 306 Sample Oxidizer. Some tissues (e.g., bone) were dissolved in 10 M NaOH at 95°C. In samples containing both ^{125}I and ^{3}H, the ^{125}I-radioactivity was counted in a Packard Auto-Gamma 5000 counter. The ^{3}H-radioactivity was subsequently measured as described above and corrected for the contribution of ^{125}I-radioactivity.

RESULT

Preparation and Characterization of Lactosylated [^{3}H]IDU-Ol$_2$-Loaded NeoHDL

Lactosylated NeoHDL particles, having incorporated the lipophilic prodrug [^{3}H]IDU-Ol$_2$ in the lipid moiety, were prepared as described earlier [21]. In short, phosphatidyl choline, cholesterol, cholesteryl oleate, [^{3}H]IDU-Ol$_2$ and HDL apoproteins were cosonicated. The resulting prodrug-loaded particles were purified by density gradient centrifu-

TABLE 1. Chemical composition of [^{3}H]IDU-OI_2-loaded lactosylated NeoHDL

	% of total weight
Protein	49.3 ± 4.4
Phosphatidyl choline	20.8 ± 2.4
Cholesterol	2.5 ± 0.8
Cholesteryl oleate	7.5 ± 0.4
Lactose	15.7 ± 1.5
[^{3}H]IDU-OI_2	4.2 ± 0.3

The chemical composition of [^{3}H]IDU-OI_2-loaded lactosylated NeoHDL was analyzed as described in Materials and Methods. Values given are means ± SEM of 3 different preparations.

gation and FPLC. [^{3}H]IDU-OI_2-loaded NeoHDL was subsequently provided with terminal D-galactosyl residues by incubation with lactose (D-galactosyl-D-glucose) and sodium cyanoborohydride. The latter reduces the Schiff's base formed between the glucose moiety of lactose and amino groups on NeoHDL, which results in covalent attachment of lactose to the apoproteins [29].

The chemical composition of lactosylated [^{3}H]IDU-OI_2-loaded NeoHDL is given in Table 1. 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-OI_2: 4.2 ± 0.3% of the total weight (12.2 ± 0.2% of the lipid moiety). From these data, it may be calculated that each particle contains approximately 25 IDU-OI_2 molecules.

We showed, earlier, that the physical properties of [^{3}H]IDU-OI_2-loaded NeoHDL are very similar to those of native HDL [21]. Because physical properties, such as size and electric charge, are crucial to the biological fate of a (modified) lipoprotein carrier [6, 14, 30], we investigated the effects of lactosylation on size and electric charge of [^{3}H]IDU-OI_2-loaded NeoHDL. For these studies, the apoproteins of the particles were also labeled with ^{125}I. This permitted monitoring of both the incorporated ^{3}H-labeled prodrug and the ^{125}I-labeled apoproteins. Figure 2A shows the elution profile of lactosylated [^{3}H]IDU-OI_2-loaded ^{125}I-NeoHDL on a calibrated Superose-6 column. Both ^{125}I and ^{3}H eluted at the same position as native HDL. This finding indicates that the size of the prodrug-loaded particles was similar to that of native HDL. Figure 2B shows the result of agarose gel electrophoresis of lactosylated [^{3}H]IDU-OI_2-loaded ^{125}I-NeoHDL. Lipoproteins subjected to this type of electrophoresis are separated primarily according to their electric charge. The main peak, which migrated 2–4 cm from the origin, contained approximately 85% of the ^{125}I-radioactivity and about 95% of the ^{3}H-radioactivity. The remaining 15% of the ^{125}I-radioactivity migrated more

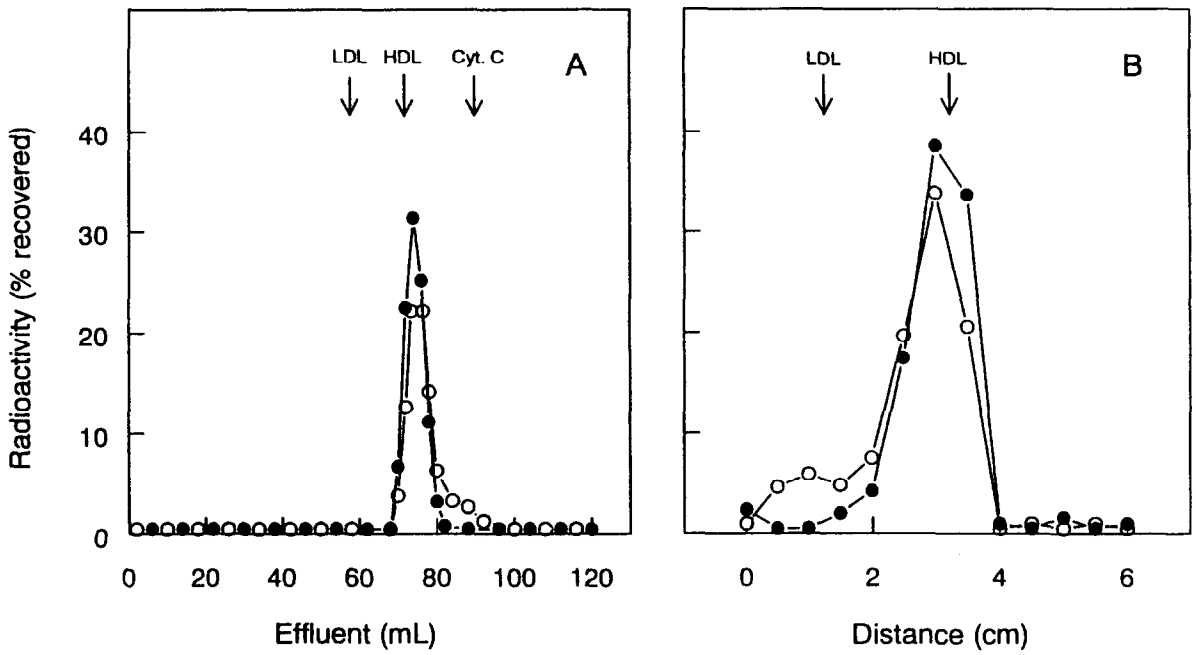

FIG. 2. Analysis of the physical properties of lactosylated [^{3}H]IDU-OI_2-loaded ^{125}I-NeoHDL by gel permeation chromatography (A) and gel electrophoresis (B). A: Lactosylated [^{3}H]IDU-OI_2-loaded ^{125}I-NeoHDL (0.10 mg of protein) was injected onto a Superose-6 FPLC column (60 × 1.8 cm). The 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/hr). 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 > 75%). The elution volumes of LDL, HDL, and cytochrome C, which were used to calibrate the column, are indicated by arrows. B: Lactosylated [^{3}H]IDU-OI_2-loaded ^{125}I-NeoHDL (5 μg 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 > 101%). Arrows indicate the positions of LDL and HDL markers.

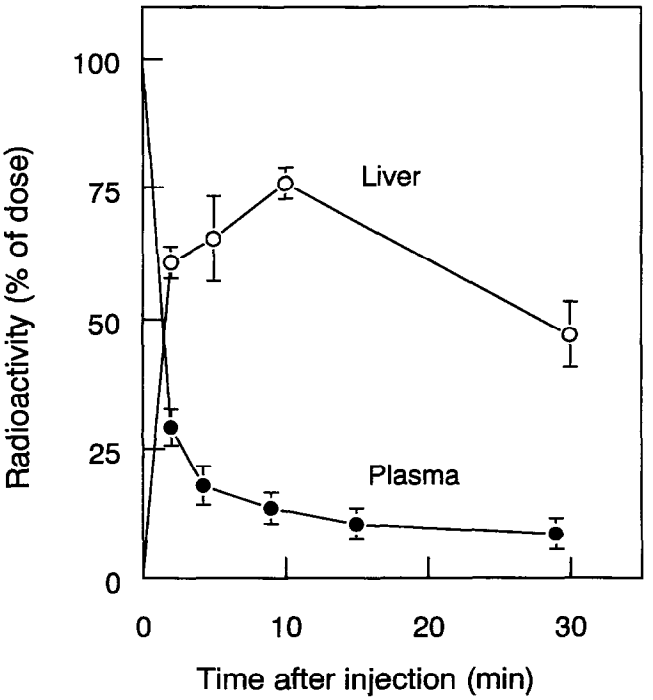

FIG. 3. Plasma clearance and liver association of lactosylated [^{3}H]IDU-Ol$_2$-loaded NeoHDL. Rats were intravenously injected with lactosylated [^{3}H]IDU-Ol$_2$-loaded NeoHDL (250 μg of protein/kg body weight). At the indicated times, the amounts of radioactivity in plasma (●) and liver (○) were determined. Values are means ± SEM of 3 rats.

slowly than the main peak, and probably represents iodinated apoproteins that had dissociated from the lactosylated NeoHDL particle. However, [^{3}H]IDU-Ol$_2$, which is the molecule of interest, is firmly associated with the main (NeoHDL) peak. Thus, the size and charge of lactosylated IDU-Ol$_2$-loaded NeoHDL are very similar to those of native HDL. Moreover, because ^{125}I and ^{3}H behaved similarly in the experiments, the results further indicate that, under the conditions employed, the particles are stable.

Plasma Clearance and Tissue Uptake of Lactosylated [^{3}H]IDU-Ol$_2$-loaded NeoHDL

To investigate the biological fate of lactosylated [^{3}H]IDU-Ol$_2$-loaded NeoHDL, rats were injected with the prodrug-loaded particles, and the plasma clearance and association of radioactivity to tissues were determined. Figure 3 shows that the injected radioactivity was very rapidly cleared from the bloodstream. At 10 min after injection, only 13.5 ± 2.8% of the dose was left in plasma. The decrease in plasma radioactivity coincided with an increase in hepatic radioactivity. At 10 min after injection, 75.9 ± 2.4% of the dose was recovered in the liver.

The results shown in Fig. 3 point to the liver playing a predominant role in the removal of lactosylated NeoHDL-associated IDU prodrug from the circulation. To investigate possible specific uptake by other organs and tissues, we determined the tissue distribution of lactosylated [^{3}H]IDU-Ol$_2$-loaded NeoHDL. The results are shown in Fig. 4, and are compared with the distribution of free [^{3}H]IDU. After injection of [^{3}H]IDU, the radioactivity distributed nonspecifically over the body. Most of the label was recovered in bulky tissues, such as muscles, skin, and bone, whereas the liver contained <20% of the dose. After injection of lactosylated [^{3}H]IDU-Ol$_2$-loaded NeoHDL, the relative specific radioactivity in the liver was found to be 1–2 orders of magnitude higher than that in any other tissue (excluding blood). Compared to the free drug, substantially lower amounts of IDU-Ol$_2$ were recovered in nonhepatic tissues. The ratio of liver uptake vs uptake by nonhepatic tissues (excluding blood) was 5.32 ± 0.57 after injection of the NeoHDL-associated prodrug, and 0.23 ± 0.01 after injection of the free drug.

Cellular and Subcellular Distribution of Lactosylated [^{3}H]IDU-Ol$_2$-Loaded NeoHDL in the Liver

In the liver, both Kupffer cells and parenchymal cells possess receptors that can bind and internalize galactose-containing ligands [3–6, 9, 10]. To identify the cell type(s) responsible for hepatic uptake, rats were injected with lactosylated [^{3}H]IDU-Ol$_2$-loaded NeoHDL, and parenchymal, Kupffer, and endothelial cells were isolated from the liver 10 min later. The cell isolation procedure was performed at a low temperature (8°C) to prevent processing of the internalized ligand. The results are shown in Table 2. The parenchymal cells were found to be the main site of uptake. These cells accounted for 84.4 ± 3.8% of total liver uptake, whereas Kupffer and endothelial cells contained much smaller amounts of radioactivity.

The mechanism of liver association of lactosylated [^{3}H]IDU-Ol$_2$-loaded NeoHDL was investigated by injecting rats with asialofetuin 1 min prior to injection of the prodrug-loaded particles. Asialofetuin specifically blocks uptake *via* the galactose-specific receptors on parenchymal liver cells [31]. Preinjection of the animals with asialofetuin (50 mg/kg body weight) inhibited the liver uptake of lactosylated [^{3}H]IDU-Ol$_2$-loaded NeoHDL considerably, and preinjection with the same dose of fetuin (which lacks terminal galactose residues) had no significant effect (Fig. 5). The reduction in hepatic uptake of radioactivity by asialofetuin was accompanied by a substantial increase in radioactivity in the blood plasma (not shown). These findings indicate that galactose-specific recognition sites in the liver are mainly responsible for uptake, and that [^{3}H]IDU-Ol$_2$ follows the fate of the lactosylated NeoHDL carrier. Because asialofetuin inhibits uptake by the asialoglycoprotein receptor, but not galactose-mediated uptake by Kupffer cells [31], this finding confirms the major role of parenchymal cells in the hepatic uptake of the prodrug-loaded lactosylated NeoHDL.

To investigate the intracellular processing of lactosylated [^{3}H]IDU-Ol$_2$-loaded NeoHDL, the liver was subjected to a

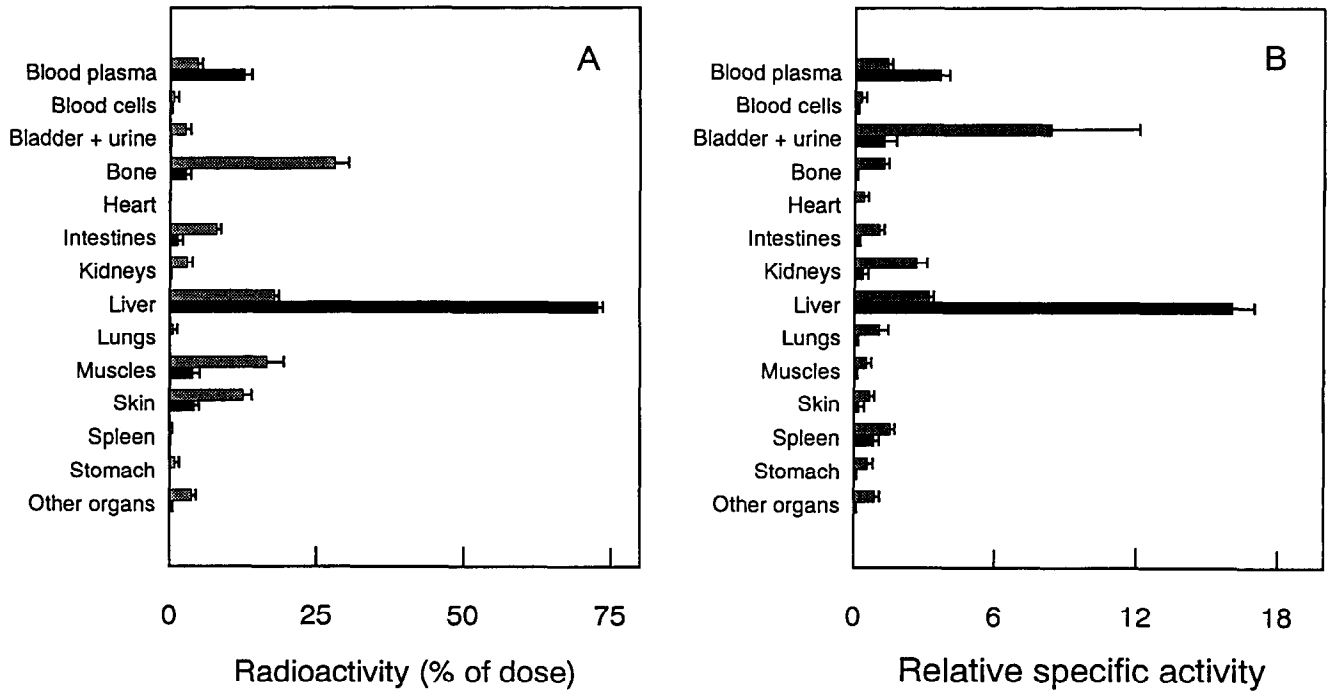

FIG. 4. Tissue distribution of lactosylated [^{3}H]IDU-Ol_2-loaded NeoHDL and [^{3}H]IDU. Rats were intravenously injected with lactosylated [^{3}H]IDU-Ol_2-loaded NeoHDL at a dose of 250 μg of protein/kg body weight (filled bar) or with an equivalent amount of underivatized [^{3}H]IDU (shaded bar). At 10 min after injection, the radioactivities in the indicated tissues and organs were determined. The results are expressed as % of the recovered amount of radioactivity (A) and as relative specific activity (B; % of total recovered radioactivity divided by % of total recovered weight). Recoveries of radioactivity and tissues in rats injected with lactosylated [^{3}H]IDU-Ol_2-loaded NeoHDL were 98.9 ± 0.9% and 97.2 ± 1.3%, respectively. Recoveries of radioactivity and tissues in rats injected with [^{3}H]IDU were 72.8 ± 3.4% and 100.1 ± 0.1%, respectively. Values are means ± SEM of 3 rats.

subcellular fractionation [28]. The distribution pattern of radioactivity showed the highest relative specific activity in the lysosomal fractions (Fig. 6). The lysosomal marker acid phosphatase also showed the highest relative specific activity in the lysosomal fraction, whereas the microsomal marker glucose-6-phosphatase had a clearly different distribution. This finding indicates that lactosylated [^{3}H]IDU-Ol_2-loaded NeoHDL does not merely associate with cells, but is internalized and transported to lysosomes.

DISCUSSION

We showed, recently, that the lipophilic prodrug IDU-Ol_2 can be efficiently incorporated into a reconstituted HDL particle (NeoHDL) with similar physicochemical properties to those of native HDL [21]. After intravenous injection into rats, the particles were relatively slowly cleared from the circulation, in a manner very similar to that of native HDL [21]. We also showed, in initial experiments, that lactosylation induced an increased liver association of the prodrug-loaded particles. In the present study, we further analyzed the characteristics and biological fate of IDU-Ol_2-loaded NeoHDL.

The lactosylated IDU-Ol_2-loaded NeoHDL particles contained a substantial amount of IDU-Ol_2: 4.2 ± 0.3% of the total weight, which corresponds to approximately 12% of the lipid moiety. Each particle may be calculated to contain approximately 25 IDU-Ol_2 molecules. Higher loads of the prodrug have not been tested, but may very well be possible. The physical properties of a lipoprotein carrier are crucial to its biological fate. Aggregation or introduction of negative charges (e.g., as a result of oxidative modification) will result in a rapid uptake by sinusoidal liver cells [14, 30]. Further, the size of a lactosylated lipoprotein particle largely determines its uptake by different liver cell types [6]. We, therefore, investigated the size and electric charge of the

TABLE 2. Uptake of intravenously injected lactosylated [^{3}H]IDU-Ol_2-loaded NeoHDL by liver cell types

Cell type	Uptake of lactosylated [^{3}H]IDU-Ol_2-loaded NeoHDL (% of total liver uptake)
Parenchymal cells	84.4 ± 3.4
Kupffer cells	10.6 ± 2.1
Endothelial cells	5.0 ± 2.3

Rats were injected with lactosylated [^{3}H]IDU-Ol_2-loaded NeoHDL at a dose of 250 μg of protein per kg body weight. Ten minutes later, parenchymal, endothelial, and Kupffer cells were isolated, and the association of radioactivity to each cell type was determined. Uptake by each cell type is expressed as the relative contribution to the total liver uptake. These values were calculated from the uptake per mg of cell protein and the contribution of each cell type to the total liver protein [27]. Values are means ± SEM of 3 rats.

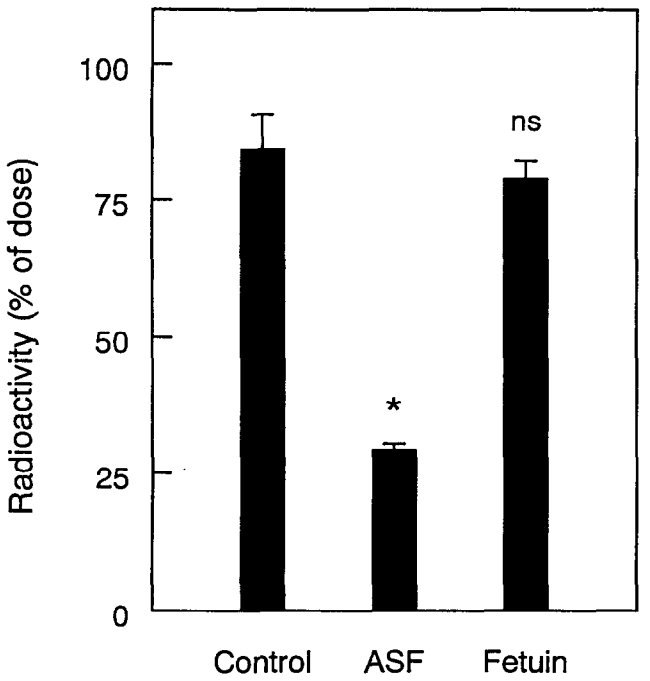

FIG. 5. Effect of asialofetuin and fetuin on the hepatic association of lactosylated [^{3}H]IDU-Ol_2-loaded NeoHDL. Rats were intravenously injected with lactosylated [^{3}H]IDU-Ol_2-loaded NeoHDL at a dose of 250 μg of protein/kg body weight. One min prior to injection, the animals received asialofetuin or fetuin, each at a dose of 50 mg/kg body weight. Controls were preinjected with solvent (phosphate-buffered saline). Ten min after injection, the radioactivity in the liver was determined. Differences with respect to the controls were tested for significance by Wilcoxon's two-sample test [37]. Values are means ± SEM of 3–4 rats. *$P < 0.05$; ns, not significant.

lactosylated prodrug-loaded NeoHDL particles. Analysis by gel permeation chromatography and agarose gel electrophoresis indicated that size and electric charge of the prodrug-loaded particles were very similar to those of native HDL. Thus, the particles were not aggregated or oxidatively modified, which reduces the risk of undesired uptake by sinusoidal liver cells. The [^{3}H]prodrug and [^{125}I]apoproteins of the particle behaved similarly in both assays, indicating that, under the conditions employed, the particles are stable. Further, the prodrug-loaded particles can be stored for at least 4 weeks at 4°C without noticeable effects on their physical properties or biological behavior.

Derivatization of IDU with oleic acid residues and subsequent incorporation of the prodrug into lactosylated NeoHDL drastically altered its biological fate. Underivatized IDU disappears very rapidly from the circulation after intravenous injection, and only a small proportion of the injected dose is recovered in the liver. The remainder was found to be evenly distributed over all tissues. IDU-Ol_2 incorporated into lactosylated NeoHDL also rapidly disappears from the circulation. In sharp contrast to the free drug, the cleared radioactivity was almost quantitatively recovered in the liver. The ratio of liver uptake vs uptake by nonhepatic tissues of the lactosylated NeoHDL-associated prodrug was 5.32 ± 0.57, (i.e., 23 times higher than that of the free drug; 0.23 ± 0.01). Binding of lactosylated IDU-Ol_2-loaded NeoHDL to the galactose receptors is followed by internalization and transport to the lysosomal compartment.

In the liver, parenchymal cells are mainly responsible for uptake of lactosylated IDU-Ol_2-loaded NeoHDL. In cell separation experiments, it was found that parenchymal cells contained approximately 85% of the total hepatic radioactivity. Preinjection of rats with asialofetuin, a specific competitor for uptake by the galactose receptor on parenchymal liver cells, substantially reduced liver uptake of lactosylated IDU-Ol_2-loaded NeoHDL. Native fetuin, which has no terminal galactose residues, had no significant effect. These findings indicate that the galactose residues of the prodrug-loaded particle mediate its hepatic uptake. Kupffer cells also express a galactose-specific receptor different from the receptor on parenchymal cells [5–8]. Asialofetuin does not inhibit galactose-mediated uptake by Kupffer cells [31]. Our finding that asialofetuin inhibits the hepatic uptake of lactosylated IDU-Ol_2-loaded NeoHDL, thus, provides corroborative evidence for uptake of the particles by parenchymal cells. Uptake of galactose-terminated lipoproteins by the two different hepatic galactose receptors depends on the spatial arrangement of the galactose residues on the particles, as well as on their size [6, 32–34]. The receptor on Kupffer cells can only bind and internalize galactose-terminated particles larger than 12 nm [6]. Lactosylated IDU-Ol_2-loaded NeoHDL eluted from the Superose-6 FPLC-column at the same position as native HDL (approx. 10 nm), which indicates that the prodrug-loaded particles are probably small enough to avoid substantial uptake by the galactose receptor on Kupffer cells.

Binding of lactosylated IDU-Ol_2-loaded NeoHDL to the galactose receptors is followed by internalization and transport to the lysosomal compartment, where the particles are processed. The oleoyl residues in IDU-Ol_2 are attached to IDU *via* an ester bond. This esterase-sensitive linkage was chosen to ensure release of the original, pharmacologically active drug at the site of delivery. The lysosomes contain a wide variety of hydrolytic enzymes, including esterases [35]. Upon *in vitro* incubation of lactosylated IDU-Ol_2-loaded NeoHDL with a lysosomal extract, IDU-Ol_2 was successively converted in the monoester and free IDU (M. K. Bijsterbosch, unpublished). It is anticipated that *in vivo* IDU-Ol_2-loaded lactosylated NeoHDL is similarly processed after it is internalized and delivered to the lysosomal compartment. Nucleosides such as IDU can easily pass through lysosomal membranes [36]. Thus, after processing of IDU-Ol_2 to IDU, the pharmacologically active IDU can become available to exert its action inside the cell.

Using lactosylated NeoHDL as carrier to target lipophilic prodrugs to the galactose receptor on parenchymal liver cells affords a number of advantages over previously published carrier systems, such as (neo)glycoproteins and lactosylated poly-L-lysine [15–17]. During transport in the cir-

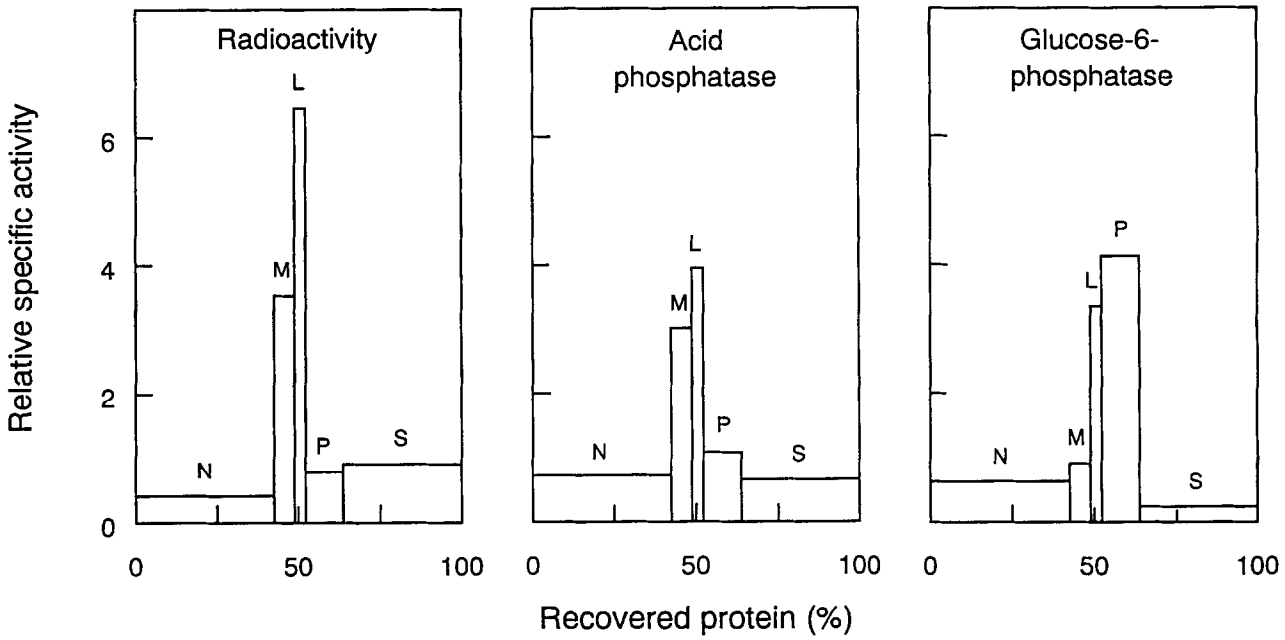

FIG. 6. Distribution patterns of radioactivity and marker enzymes over subcellular fractions of the liver after injection of lactosylated [^{3}H]IDU-Ol$_2$-loaded NeoHDL. Rats were injected with lactosylated [^{3}H]IDU-Ol$_2$-loaded NeoHDL (250 μg of protein per kg body weight). Twenty min after injection, the liver was perfused with ice-cold 0.25 M sucrose, containing 10 mM Tris-HCl buffer, pH 7.5, and divided into subcellular fractions by differential centrifugation, as described earlier [28]. The fractions were assayed for radioactivity, protein, and the activity of marker enzymes [28]; recoveries were >91%. Blocks from left to right represent: nuclear (N), mitochondrial (M), lysosomal (L), microsomal (P), and supernatant (cytosol: S) fractions. The relative protein concentration is given on the abscissa. The ordinate represents the relative specific activity (% of total recovered activity divided by % of total recovered protein).

culation, the lipophilic prodrug is hidden in the lipid moiety (probably the apolar core) and, thus, protected from the biological environment. Furthermore, as the lipophilic prodrugs are incorporated into the lipid moiety, high drug loads are possible without interfering with the receptor-mediated recognition of the lactose residues present on the apoproteins.

In conclusion, we show that IDU can be targeted highly specifically to parenchymal liver cells by incorporating its lipophilic prodrug into lactosylated NeoHDL, a particle that is recognized by galactose receptors on the target cell. These findings also have a wider significance, as the approach followed here may also be used to deliver other water-soluble drugs selectively to parenchymal liver cells. This may lead to more effective therapy for infectious diseases such as hepatitis.

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