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Modification of the Plasma Clearance and Liver Uptake of Steroid Ester-Conjugated Oligodeoxynucleotides by Association with (Lactosylated) Low-Density Lipoprotein

Erik T. Rump, Remco L. A. de Vrueh,* Muthiah Manoharan,† Ingrid H. E. Waarlo,* Richard van Veghel,* Erik A. L. Biessen,* Theo J. C. van Berkel* and Martin K. Bijsterbosch*‡*

*Division of Biopharmaceutics, Leiden/Amsterdam Center for Drug Research, P.O. Box 9503, 2300 RA Leiden, The Netherlands; and †ISIS Pharmaceuticals, Carlsbad, CA, 92008, U.S.A.

ABSTRACT. Low-density lipoprotein (LDL) has been proposed as carrier for the selective delivery of anticancer drugs to tumor cells. We reported earlier the association of several lipidic steroid-conjugated anticancer oligodeoxynucleotides (ODNs) with LDL. In the present study, we determined the stability of these complexes. When the complexes were incubated with a mixture of high-density lipoprotein and albumin, or with rat plasma, the oleoyl steroid-conjugated ODNs appeared to be more stably associated with LDL than the cholesteryl-conjugated ODN. Intravenously injected free lipid–ODNs were very rapidly cleared from the circulation of rats. The area under the curve (AUC) of the lipid–ODNs in plasma was <0.4 μg.min/mL. After complexation with LDL, plasma clearance of the lipid–ODNs was delayed. This was most evident for ODN-5, the ODN conjugated with the oleoyl ester of lithocholic acid (AUC = 6.82 ± 1.34 μg.min/mL). The AUC of ODN-4, a cholesteryl-conjugated ODN, was 1.49 ± 0.37 μg.min/mL. In addition, the liver uptake of the LDL-complexed lipid–ODNs was reduced. The lipid–ODNs were also administered as a complex with lactosylated LDL, a modified LDL particle that is selectively taken up by the liver. A high proportion of ODN-5 was transported to the liver along with lactosylated LDL (69.1 ± 8.1% of the dose at 15 min after injection), whereas much less ODN-4 was transported (36.6 ± 0.1% of the dose at 15 min after injection). We conclude that the oleoyl ester of lithocholic acid is a more potent lipid anchor than the other steroid lipid anchors. Because of the stable association, the oleoyl ester of lithocholic acid is an interesting candidate for tumor targeting of anticancer ODNs with lipoproteins. BIOCHEM PHARMACOL **59**;11:1407–1416, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. antisense oligodeoxynucleotides; low-density lipoprotein; lipidic oligodeoxynucleotides; liver; plasma proteins; selective delivery

Antisense ODNs§ directed against oncogenes that are implicated in the proliferation of tumor cells are an attractive novel therapeutic option for the treatment of cancer. Numerous studies with tumor cells in culture showed that such anticancer ODNs can very selectively inhibit the proliferation of these malignant cells [1]. Anticancer activity of antisense ODNs, due to inhibition of the expression of the target gene, has also been shown *in vivo* [2–6]. Despite these encouraging results, the therapeutic potential of anticancer ODNs has thus far not been fully exploited, because the delivery of ODNs to tumors is presently not optimal. The negatively charged ODNs do not readily penetrate the plasma membrane of tumor cells. Moreover, intravenously administered ODNs are *in vivo* mainly cleared from the circulation by the liver and kidneys [7–11]. A higher uptake of ODNs by tumors, and thus higher therapeutic efficacy, may be accomplished by association of ODNs with tumor cell-specific ligands. LDL is an attractive potential carrier for tumor-specific ODNs, as it has been shown that a variety of tumor cell types (e.g leukemic cells) internalize large amounts of LDL via the LDL receptor [12]. LDL is a spherical lipid/protein particle with a diameter of 23 nm, which functions in human plasma as the main cholesterol-transporting vehicle. LDL is recognized by the LDL receptor via apolipoprotein B-100, a glycoprotein present on the surface of LDL [13]. In addition to native human LDL, synthetic LDL-mimicking particles have recently been developed as a carrier system [14–16]. The LDL

‡ Corresponding author: Dr. Martin K. Bijsterbosch, Division of Biopharmaceutics, Leiden/Amsterdam Center for Drug Research, P.O. Box 9503, 2300 RA Leiden, The Netherlands. Tel. 31-71-5276038; FAX 31-71-5276032; E-mail: bijsterb@LACDR.Leidenuniv.nl

§ *Abbreviations:* AUC, area under the plasma curve; HDL, high-density lipoprotein; HSA, human serum albumin; LDL, low-density lipoprotein; ODN, oligodeoxynucleotide; and PS, phosphorothioate.

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receptor is a recycling receptor, which allows the delivery of large amounts of LDL-associated drugs. It has been calculated that intracellular drug concentrations in the μM range can easily be reached when a drug is delivered via the LDL receptor pathway [16].

As apolipoprotein B-100 is essential for the recognition of LDL by its receptor, the ODNs should preferably be associated with the lipid moiety of the particle. We therefore recently synthesized several lipidic steroid LDL anchors, which were conjugated to ODNs to enable the spontaneous association of the ODNs with LDL in solution [17]. In addition to cholesterol, the more lipidic oleoyl esters of lithocholic acid and cholenic acid were found to be very effective for LDL association. In the present study, we investigated the stability of these ODN/LDL complexes in order to identify the most potent LDL anchor. The exchange of the lipid–ODNs, complexed with LDL, to other plasma components was studied *in vitro*. We further examined *in vivo* in the rat the fate of the lipid–ODN/LDL complexes and the fate of complexes of lipid–ODNs with lactosylated LDL.

MATERIALS AND METHODS

Reagents

An 18-mer ODN complementary to the c-*myb* proto-oncogene [18] (5′-G*T*G* CCG GGG TCT TCG GGC-3′), provided with a C7 3′-amine linker and 3 PS linkages (*) at the 5′-end, was from Eurogentec. The ODN was ^{3}H-labeled and conjugated with lipidic steroid structures as described before [17]. ISIS-9388, a 3′-cholesteryl-conjugated PS–ODN specific for murine intercellular adhesion molecule-1 (5′-TGC ATC CCC CAG GCC ACC AU-3′) was synthesized and radiolabeled as described earlier [19]. Human and rat serum albumin were from Sigma. Ketamine (100 mg/mL; HCl salt) was from Eurovet. Hypnorm™ (0.315 mg/mL of fentanyl citrate and 10 mg/mL of fluanisone) and Thalomonal™ (0.05 mg/mL of fentanyl and 2.5 mg/mL of droperidol) were from Janssen-Cilag. Emulsifier Safe™ was from Packard. All other reagents were of analytical grade.

Isolation and Radioiodination of Lipoproteins

Human LDL (density 1.024–1.063 g/mL) and human HDL (density 1.063–1.210 g/mL) were isolated from the serum of fasted volunteers by density gradient ultracentrifugation [20] and were dialyzed against PBS (10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl) containing 1 mM EDTA. Radioiodination was performed at pH 10.0 with carrier-free ^{125}I as described by McFarlane [21]. Lactosylated LDL was prepared from LDL as described earlier [22]. Protein concentrations of the lipoproteins were determined by the method of Lowry *et al.* [23], with BSA as standard.

Determination of Exchange of Lipid–ODNs from LDL to HDL and Albumin

Equimolar amounts of LDL and lipid–[^{3}H]ODNs (200 pmol) were dissolved in 100 μL of PBS containing 1 mM EDTA and incubated for 2 hr at 37°. Subsequently, 50 μL of the mixture was injected onto a Superose 6 Precision Column (3.2 × 300 mm; Smart System, Pharmacia) and eluted at a flow rate of 50 μL/min with PBS. Fractions of 50 μL were collected, and the three main fractions containing the ODN/LDL complexes were pooled. An aliquot of 50 μL was subsequently incubated at 37° with 25 μL of a mixture of HDL and HSA, at a final ratio of LDL:HDL:HSA = 2:10:3 (w/w/w based on protein). At the indicated times, the incubation mixtures were injected onto the Superose 6 column, and the column was eluted as described above. The fractions were assayed for ^{3}H-radioactivity.

Determination of Exchange of Lipid–ODNs from LDL to Proteins in Rat Plasma

Lipid–[^{3}H]ODN/LDL complexes were prepared as described above, and aliqouts of 50 μL were subsequently incubated with 40 μL of citrated rat plasma. After incubation, the mixtures were injected onto the Superose 6 column, and the column was eluted as described above. The fractions were assayed for ^{3}H-radioactivity and cholesterol. The total cholesterol content of the fractions was determined colorimetrically.

Determination of Plasma Clearance and Liver Association

Male Wistar rats (180–230 g) were anaesthetized by subcutaneous injection of a cocktail of ketamine.HCl, fentanyl, droperidol, and fluanisone (75, 0.04, 1.1, and 0.75 mg/kg, respectively), and the abdomen was opened. Free lipid–[^{3}H]ODNs (4 μM in PBS) or complexes of free lipid–[^{3}H]ODNs with ^{125}I-LDL or lactosylated LDL (4 μM, complexes prepared as described above) were injected via the vena penis at a dose of 5 μg lipid–ODN/kg and 0.4 mg (lactosylated) LDL/kg. At the indicated times, blood samples of 400 μL were taken from the inferior vena cava and collected in heparinized tubes. The blood samples were centrifuged for 10 min at 500 *g*, and the serum was collected and assayed for ^{125}I-radioactivity. Next, 100 μL of 0.1 N HCl and 3 mL of Emulsifier Safe™ were added, and the ^{3}H-radioactivity was counted. The measured ^{3}H-radioactivity was corrected for the contribution of ^{125}I-radioactivity. 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 radioactivity in the liver at each time point was calculated from the radioactivities and weights of the liver samples and was corrected for radioactivity in plasma present in the tissue at the time of sampling (85 μL/g of fresh weight). All liver samples were counted for ^{125}I and for ^{3}H after combustion using a

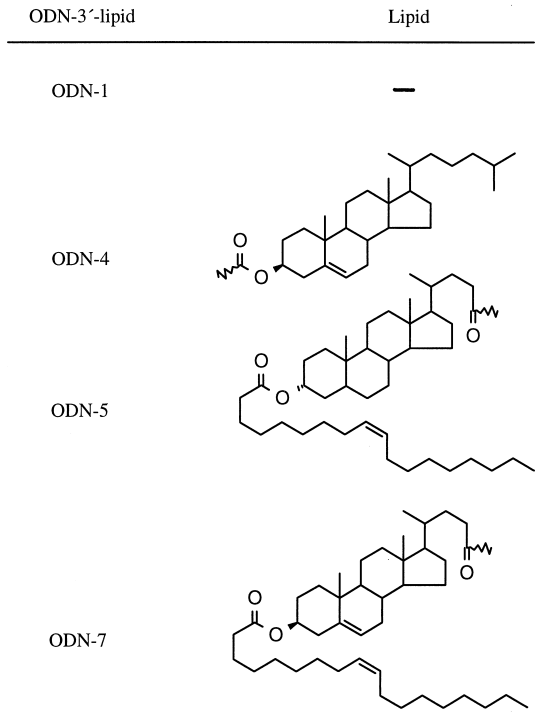

FIG. 1. Chemical structure of the lipid–ODNs used in this study. The ODNs were synthesized and purified as described previously [17].

Packard 306 Sample Oxidizer. The total amounts of radioactivity in plasma and liver were calculated using the equation: plasma volume (mL) = [0.0291 × body weight (g)] + 2.54 [24]. The plasma concentration–time AUC was calculated by computerized non-linear fitting (GraphPad Prism, Graphpad Software Inc.).

Analysis of Plasma Proteins by SDS–Polyacrylamide Gel Electrophoresis

Forty microliters of citrated rat plasma was diluted with 50 μL of PBS and injected onto the Superose 6 column. After fractionation, aliquots of 20 μL were separated in a 7.5% SDS–polyacrylamide gel under reducing conditions [25]. After electrophoresis, proteins were stained with Coomassie brilliant blue G-250.

RESULTS

Exchange of Lipid–ODNs from LDL to HDL and Albumin

We previously described the synthesis of three lipid–ODNs consisting of an ODN conjugated with a cholesteryl moiety or with oleoyl steroid ester moieties (Ref. 17; Fig. 1). In that earlier study, we demonstrated that these lipid–ODNs associate readily with isolated human LDL. The aim of the present study was to examine the stability of these lipid–ODN/LDL complexes. To be able to monitor exchange, ^{3}H-labeled lipid–ODNs were utilized. Lipid–[^{3}H]ODN/LDL complexes were prepared by incubating equimolar amounts of the two components for 2 hr at 37°. To separate small amounts of non-complexed lipid–ODNs from the ODN/LDL complex, the incubation was followed by size-exclusion chromatography on a calibrated Superose 6 Precision Column (see Table 1 for calibration details).

TABLE 1. Characteristics of plasma lipoproteins and albumin

Plasma component	Size [nm]†	Elution volume [mL]‡
Chylomicron*	>75	0.91
VLDL*	>30	0.91
LDL*	23	1.22
HDL*	10.9	1.57
HSA	4.2	1.68
RSA	4.2	1.68

*Human lipoproteins. VLDL, very low-densing lipoprotein.
†The indicated particle sizes were taken from Biessen *et al.* [44].
‡The elution volume was determined by size-exclusion chromatography of the isolated plasma components. RSA, rat serum albumin.

The elution profiles of the complexes of LDL with lipid–ODNs 4, 5, and 7 resembled that of LDL alone, indicating a similar size. As an example, the elution profile of the complex of ODN-5 and LDL is depicted in Fig. 2E. The complex has an elution volume (V_E) of 1.21 mL, which is very similar to that of LDL (Table 1 and Fig. 2F). To determine their stability, the ODN/LDL complexes were subsequently incubated at 37° with a mixture of HDL (to study exchange to another lipoprotein) and HSA (to study binding of ODNs to this major serum protein). After 25 min, redistribution of the lipid–[^{3}H]ODNs from LDL to HDL and/or HSA was investigated by subjecting the incubation mixtures to size-exclusion chromatography. Figures 2A–C depict the radioactivity and UV extinction chromatographic profiles. The UV signal between 1.50 and 1.80 mL represents HDL and HSA, which have V_Es of 1.57 and 1.68 mL, respectively (Table 1 and Fig. 2F). Only relatively small amounts of lipid–ODN were found at the position of HDL, and no significant binding to HSA was observed. Thus, most of the lipid–ODN remained associated with LDL, although the mixture contained HDL in relative excess (6-fold excess of lipoprotein surface area). The lipid–ODNs ODN-4, ODN-5, and ODN-7 have a partially PS-modified backbone (17% PS linkages). It was found in many studies that PS linkages in ODNs induce binding to serum proteins [26–30]. Therefore, we also examined the stability of complexes of LDL and ISIS-9388, a full PS ODN conjugated at the 3′-end with cholesterol (Fig. 1). Figure 2D shows that exchange of ISIS-9388 from LDL to HDL was slightly higher than observed for the other lipid–ODNs.

Exchange of Lipid–ODNs from LDL to (Lipo)proteins in Rat Plasma

To gain further insight into the stability of the ODN/LDL complexes under *in vivo* conditions, we studied the ex-

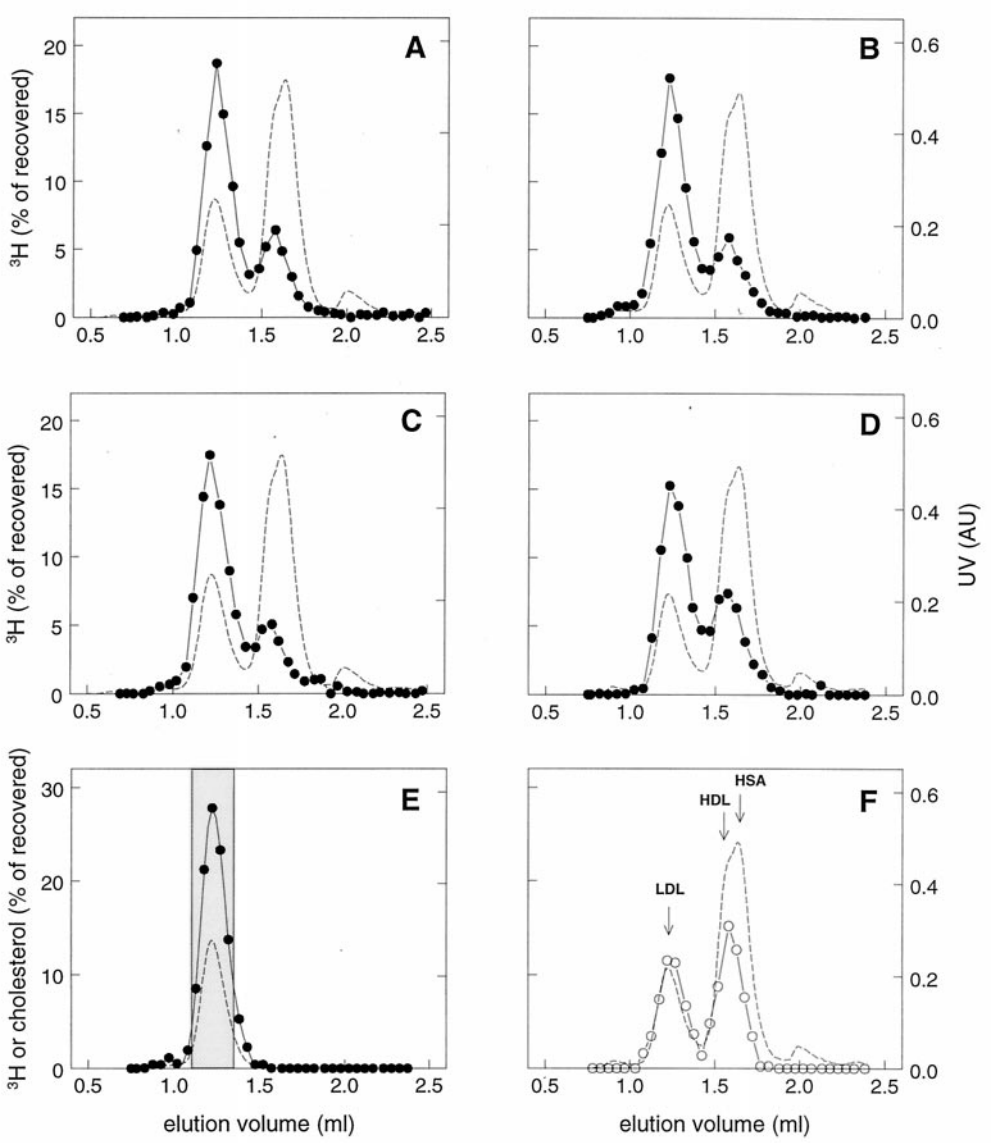

FIG. 2. Chromatographic profiles after incubation of lipid–ODN/LDL complexes with HDL aand HSA. Preformed lipid–ODN/LDL complexes were incubated with an HDL/HSA mixture for 25 min at 37°. The samples were subsequently injected onto a Superose 6 column (Pharmacia), and the fractions were monitored for UV absorption (254 nm, – – –) and assayed for ^{3}H-radioactivity (●) or cholesterol (○). The amounts of ^{3}H-radioactivity and cholesterol in the fractions are expressed as percentages of recovered radioactivity (recoveries were >95%). The gray zone in E represents the fractions containing 90% of the lipid–ODN/LDL complex, based on both UV absorption and ^{3}H-radioactivity. Panels A–D: HDL/HSA + complexes of LDL and ODN-4, ODN-5, ODN-7, and ISIS-9388 respectively; panel E: ODN-5/LDL complex; panel F: LDL/HDL/HSA mixture.

change rate of the lipid–ODNs from preformed lipid–ODN/LDL complexes to components in rat plasma. The chromatographic profile of rat plasma is shown in Fig. 3F. Triglyceride-rich lipoproteins (chylomicrons and very low-density lipoprotein), LDL, and HDL were detected by cholesterol and UV-absorption measurements, and eluted at 0.91, 1.21 and 1.57 mL, respectively. In addition to lipoproteins, peaks representing plasma proteins were detected at 1.25–1.40 mL and 1.70 mL. The peak at 1.25–1.40 mL primarily consisted of the high molecular weight protein α_1-macroglobulin (α_1M). This was illustrated by polyacrylamide gel electrophoresis of fractions eluting at 1.25–1.40 mL (Fig. 4). The 140 kDa α-chain and 40 kDa β-chain subunits of α_1M were visualized by Coomassie brilliant blue staining. The gel also shows a major protein band around 1.50 mL with a molecular weight of approximately 200 kDa, which probably represents a subunit of α^1-inhibitor III (α_1I_3). The peak at 1.70 mL consisted mainly of albumin and immunoglobulins (not shown). Lipid-[^{3}H]ODN/LDL complexes were incubated for 5 or 25 min with rat plasma and subsequently subjected to size-exclusion chromatography. The fractions were monitored for ^{3}H-radioactivity. Although HDL is the main lipoprotein present in rat plasma, no significant redistribution of the partially PS-modified lipid–ODNs (ODN-4, ODN-5, and ODN-7) from LDL to HDL (elution volume 1.57 mL) was seen. The

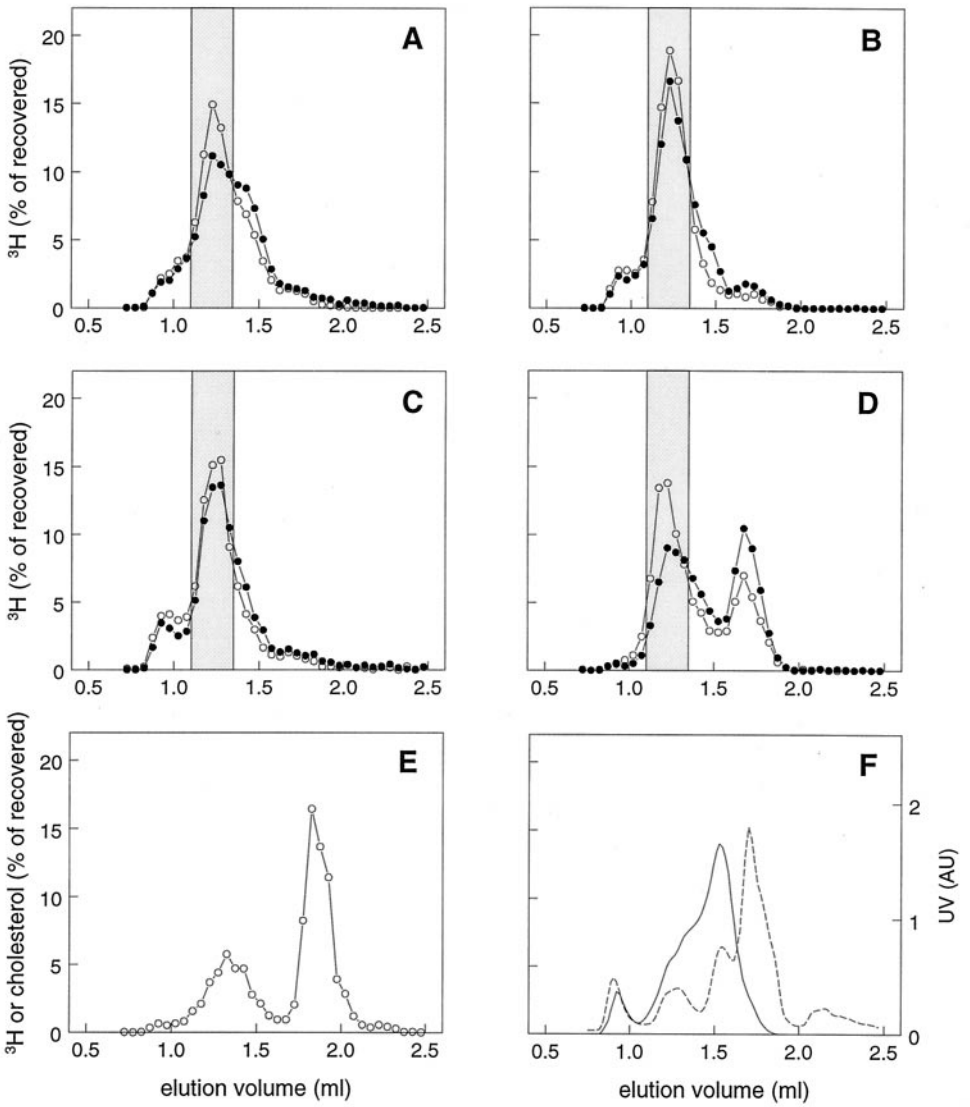

FIG. 3. Chromatographic profiles after incubation of lipid-ODN/LDL complexes within rat plasma. Preformed lipid–ODN/LDL complexess (A–D) or ODN-1 alone (E) were incubated with rat plasma for 5 min (○) or for 25 min (●) at 37°. The samples were subsequently injected onto a Superose 6 column (Pharmacia), and the fractions were assayed for ^{3}H-radioactivity. The ^{3}H-radioactivity in the fractions is expressed as a percentage of recovered radioactivity (recoveries were >95%). The gray zones in panels A–D indicate the fractions containing 90% of the lipid–ODN/LDL complex at t = 0 min, based upon both UV absorption and ^{3}H-radioactivity. Panel F depicts the chromatographic profile of rat plasma; the fractions were monitored for UV absorption (254 nm, – – –) and assayed for total cholesterol (——). Panel A: ODN-4; panel B: ODN-5; panel C: ODN-7; panel D: ISIS-9388; panel E: ODN-1.

lipid–ODNs did, however, redistribute to some extent to triglyceride-rich lipoproteins (elution volume 0.91 mL). Some redistribution to high molecular weight plasma proteins eluting at the position of α_1M (elution volume 1.25–1.40 mL) could also be observed (Fig. 3A–C). The oleoyl steroid ester-conjugated ODNs (ODN-5 and ODN-7) appeared to be most stably complexed to LDL, since after 25 min of incubation >70% of the radioactivity was still associated with the LDL fractions. At that time, only approximately 50% of the cholesteryl-conjugated ODN (ODN-4) was found to be associated with LDL. ODN-4 redistributed primarily to the high molecular weight proteins.

When the non-conjugated ODN (ODN-1) was incubated with rat plasma, a significant proportion also bound to high molecular weight proteins (Fig. 3E). Approximately 35% of the radioactivity eluted at 1.30–1.50 mL, whereas the remainder eluted as free ODN with a peak at 1.85 mL. This indicates that binding of the lipid–ODNs to high molecular weight proteins may not necessarily be ascribed to the lipid moiety of these ODNs. The three PS linkages in the ODN backbone may be responsible for protein binding as well.

Upon incubation with rat plasma, the full PS lipid–ODN ISIS-9388 dissociated more rapidly from a preformed complex with LDL than the partially PS-modified lipid–ODNs. After 25 min of incubation, less then 20% of the ISIS-9388 was found to be associated with LDL (Fig. 3D). Most of the fully PS-modified lipid–ODN was recovered in fractions

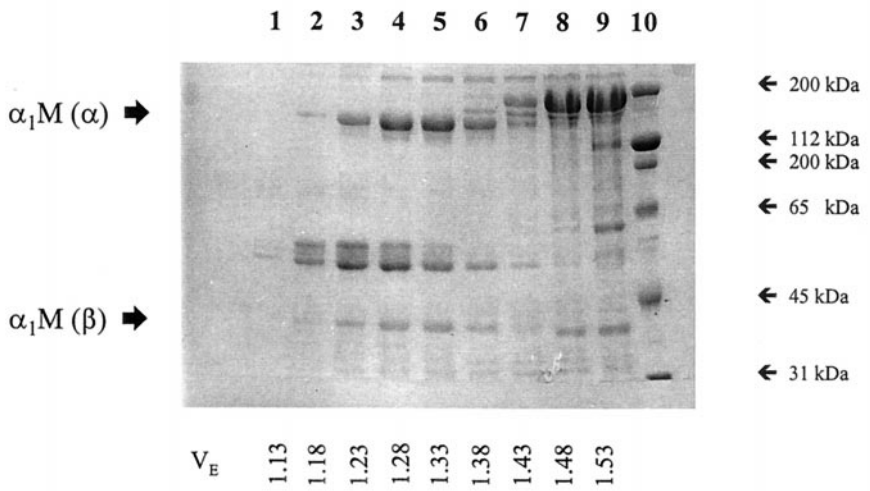

FIG. 4. SDS–polyacrylamide gel electrophoresis of fractions of size-exclusion chromatography. Fractions obtained after fractionation of rat plasma by size-exclusion chromatography were subjected to electrophoresis in a 7.5% SDS–polyacrylamide gel. Proteins were stained with Coomassie brilliant blue. The positions of the molecular weight markers (broad range Bio-rad) are indicated by arrows. Column fractions are designated according to their elution volume (V_E). Lanes 1–9: column fractions; lane 10: molecular weight markers.

containing high molecular weight proteins and in fractions rich in albumin and immunoglobulins (elution volume 1.70 mL). Although all the lipid–ODNs used in this study can be associated with the isolated human HDL (not shown), the ODNs, and especially the full PS ODN, have apparently much more affinity for non-lipoprotein plasma proteins. It should also be noted that no radioactivity eluted at the column volume after incubation of the partially PS-modified ODNs with plasma. This implies that no radiolabeled oligonucleotides were generated during the incubation, which indicates that the 3 PS linkages at the 5′-end and the amino linker/steroid lipid structures at the 3′-end effectively protect the ODNs against nuclease activity in the plasma.

Behavior of Lipid–ODN/LDL Complexes In Vivo

To establish which of the lipid steroid structures is the most effective LDL anchor *in vivo*, we first examined the plasma clearance of complexes of LDL and the lipid–ODNs in the rat. Since we found *in vitro* that the lipid–ODN with a full PS backbone (ISIS-9388) has a high affinity for plasma proteins, we only performed *in vivo* experiments with the partially PS-modified lipid–ODNs (ODN-4, ODN-5, and ODN-7). When injected without LDL, these lipid–ODNs were cleared very rapidly from the circulation (Fig. 5, A, C, and E). At 5 min after injection, more than 95% of the dose was cleared, and a significant proportion of the radioactivity (15–40% of the dose) was recovered in the liver (Fig. 5, B, D, and F). The plasma clearance of the lipid–ODNs complexed with LDL was studied utilizing double-labeled complexes ([^{3}H]ODN/^{125}I-LDL). This allows monitoring of both the lipid–ODN and the LDL carrier. Figures 5, A, C, and E shows that LDL was cleared very slowly from the circulation, with a concomitant low liver uptake (Fig. 5, B, D, and F). These findings are consistent with the previously reported half-life of 5–6 hr for LDL in the rat [31]. The lipid–ODNs in the [^{3}H]ODN/^{125}I-LDL complexes were cleared much slower than the non-complexed lipid–ODNs (Fig. 5, A, C, and E). At 5 min after injection, the percentages of complexed lipid–ODNs which had been cleared were 51% for ODN-4, and 39% and 24% for ODN-7 and ODN-5, respectively. In concordance, the plasma AUC of the complexed cholesteryl-conjugated ODN (ODN-4, AUC = 1.49 ± 0.37 μg.min/mL) was significantly lower than the plasma AUC of the LDL-associated oleoyl steroid ester-conjugated ODNs (ODN-7 and ODN-5; AUC = 4.61 ± 0.38 and 6.82 ± 1.34 μg.min/mL, respectively). The non-complexed lipid–ODNs had plasma AUC of <0.4 μg.min/mL. Compared to the free lipid–ODNs, the liver uptake of the lipid–ODNs complexed with LDL was reduced (Fig. 5, B, D, and F). However, the lipid–ODN/LDL complexes were not completely stable, since the clearance of the lipid–ODN did not completely resemble the clearance of LDL (Fig. 5, A, C, and E). The reduction of the clearance rate of the lipid–ODNs, achieved by complexation with LDL, was most evident for the oleoyl steroid ester-conjugated ODNs (ODN-5 and ODN-7). The cholesteryl-conjugated ODN (ODN-4) displayed the highest leakage from LDL.

Delivery of Lipid–ODNs to the Liver by Lactosylated LDL

We further explored the potential of lipoproteins to direct ODNs to specific liver cell populations by utilizing lactosylated LDL. In contrast to native unmodified LDL, lactosylated LDL is rapidly taken up by galactose-specific receptors on Kupffer cells [22]. We studied the behavior of complexes of this modified lipoprotein and two lipid–

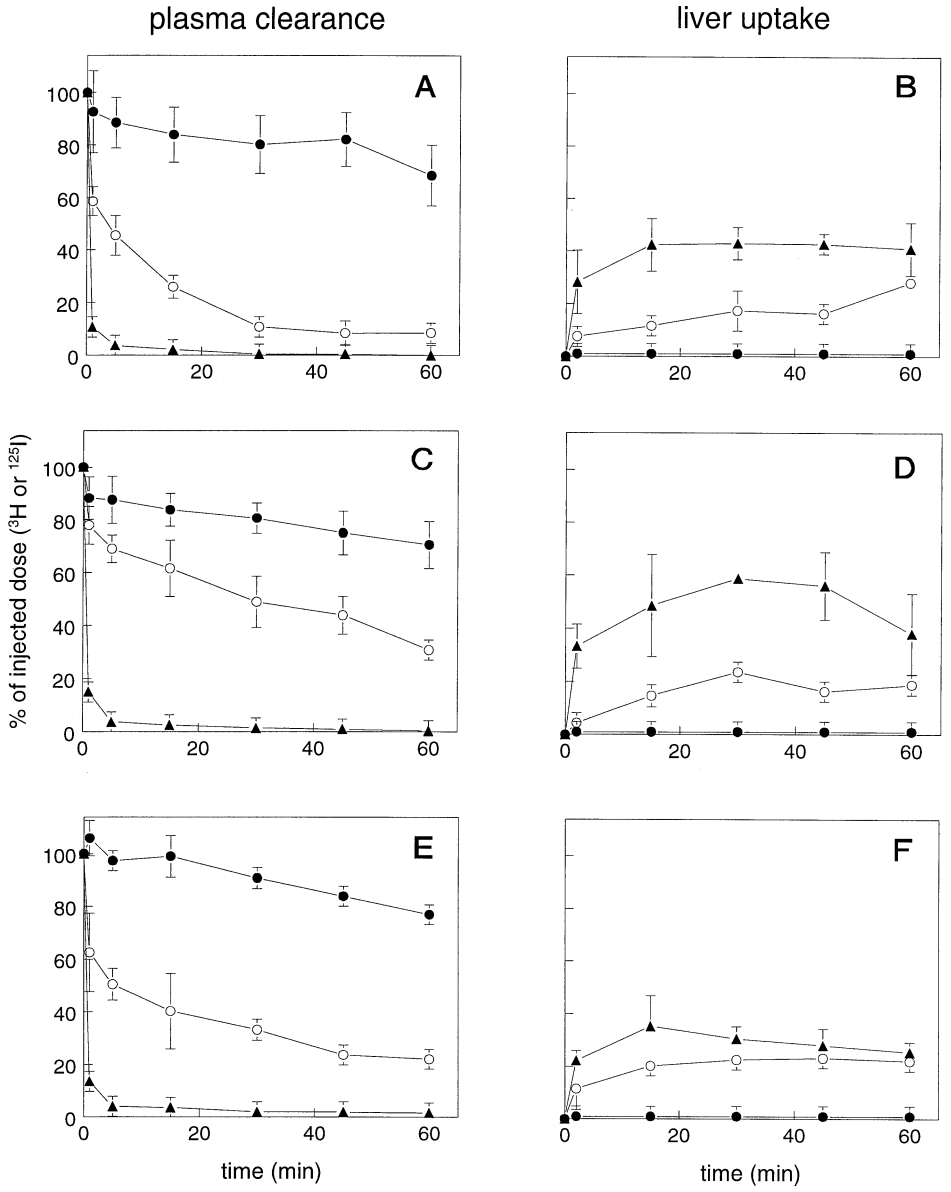

FIG. 5. Plasma clearance and liver association of lipid–ODNs and lipid–ODN/LDL complexese. Rats were injected with the free lilpid–[^{3}H]ODN (▲) or lipid-–[^{3}H]ODN^{125}I-LDL complexes (^{3}H, ○; ^{125}I, ●). At the indicated times, the amounts of radioactivity in plasma (A, C, and E) and liver (B, D, and F) were determined. Values are means ± SEM of either two (free lipid–ODN) or three (ODN/LDL complex) separate experiments. Panels A + B: ODN-4; panels C + D: ODN-5; panels E + F: ODN-7.

ODNs: ODN-4 and ODN-5. As depicted in Fig. 6, both lipid–ODNs were cleared rapidly from the circulation when administered as a complex with lactosylated LDL. The hepatic association of the lipid–ODNs was considerably higher when complexed with LacLDL than when complexed with native LDL (only 10–15% of the lipid–ODNs complexed with native LDL was associated with the liver at 15 min after injection; Fig. 5). This indicates that complexation of these lipid–ODNs with the hepatotropic carrier lactosylated LDL indeed enhances their liver uptake. Moreover, it appeared that the hepatic uptake of ODN-5 greatly exceeds the uptake of ODN-4 (69.1 ± 8.1% of the dose vs 36.6 ± 0.1% of the dose at 15 min after injection). This indicates that, compared to cholesterol, the oleoyl ester of lithocholic acid not only leads to a more avid association to LDL, but also allows a more specific targeting of ODNs to the liver by lactosylated LDL.

DISCUSSION

The approach of using LDL as a carrier for anticancer drugs was initially explored for conventional lipophilic drugs. In addition to these conventional drugs, the therapeutic efficacy of anticancer ODNs may be greatly enhanced using this approach. We and others have reported before that various lipid-conjugated ODNs can be associated with LDL in ratios of up to 50 ODN molecules per LDL particle [17, 32–36]. The association of conjugated ODNs with LDL is

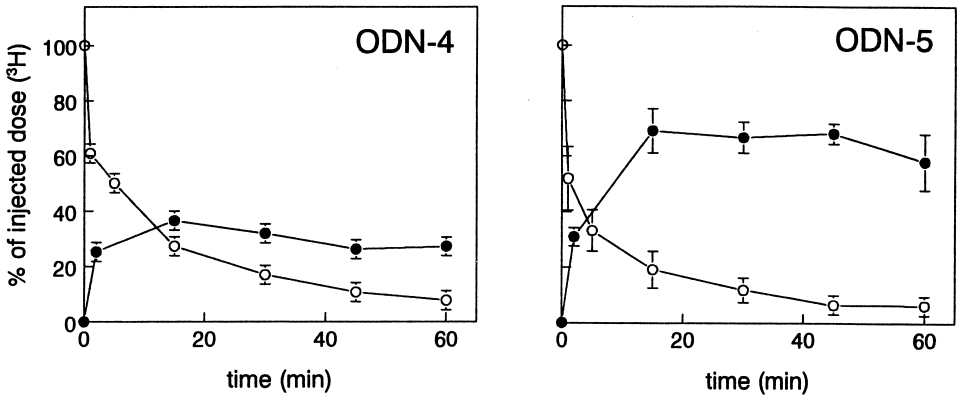

FIG. 6. Plasma clearance and liver association of lipid–ODN/lactosylated LDL complexes. Rats were injected with complexes of lactosylated LDL and lipid–[^{3}H]ODNs (left panel: ODN-4; right panel: ODN-5). At the indicated times, the amounts of radioactivity in plasma (○) and liver (●) were determined. Values are means ± variation of two separate experiments.

mediated by their lipid moieties, which intercalate in the phospholipid monolayer shell of the particle and which may even to some extent penetrate its apolar core. The lipid structures thus serve as LDL anchors. The oligonucleotide moieties of lipid–ODN conjugates remain outside the particle and are in principle accessible to nucleases. However, steric hindrance, due to the association of the lipid–ODN with the massive LDL particle, is likely to afford protection of the oligonucleotide against nuclease activity. For the effective delivery of these complexes to tumor cells via the LDL receptor *in vivo*, it is essential that lipid–ODNs in the circulation remain firmly associated with the LDL carrier via the LDL anchor. The stability of the complexes of LDL with lipid–ODNs has not yet been studied. To identify the most potent LDL anchor, we investigated herein the stability of complexes of LDL and an antineoplastic ODN conjugated with three different steroids. The ODNs used in our study were protected against 5′-exonuclease activity by three PS linkages at the 5′-end [28,37]. We therefore also assessed the effect of the degree of PS content upon the stability of lipid–ODN/LDL complexes by comparing a fully PS-modified cholesteryl-conjugated ODN with the partially PS-modified cholsteryl-conjugated ODNs.

We initially studied the stability of lipid–ODN/LDL complexes in a defined mixture containing both a lipoprotein (HDL) and the major plasma protein HSA. We found that the various ODN/LDL complexes are comparably stable in this mixture. The redistribution of the various LDL-complexed ODNs to HDL was very low and, especially for the partially PS-modified ODNs, binding to albumin was negligible. This finding differs from earlier observations from our laboratory with cholesteryl-conjugated phosphodiester ODNs [35]. This discrepancy is probably explained by the different analytical techniques used. In the earlier study, the incubation of the lipid–ODN/LDL complex with an HDL/albumin mixture was followed by density gradient centrifugation for 24 hr. This long analytical step probably allows a further redistribution. Furthermore, the high KBr concentrations, which are far from physiological but are necessary for gradient formation, may affect the association of the ODNs with LDL. The presently applied chromatographic procedure allows separation of the various lipoproteins, under physiological salt conditions, within 45 min. It thus appears that only 20–30% of the lipid–ODNs redistributed from LDL to HDL during the 25-min incubation time.

To study if other plasma components besides HDL and albumin might destabilize ODN/LDL complexes, incubations with plasma were performed. It was shown that the more lipidic oleoyl ester structures are better LDL anchors than the cholesteryl moiety. Compared to ODN-4, a higher percentage of ODN-5 and ODN-7 remained associated with LDL. The exchanged lipid–ODNs bound to triglyceride-rich lipoproteins and high molecular weight proteins. Exchange to HDL is very low, although HDL is the major lipoprotein present in the rat. PS-containing ODNs can avidly bind to serum proteins [26–30]. Indeed, we found binding to non-lipoprotein rat plasma proteins with all lipid-ODNs. A significant proportion of the partially PS-modified lipid–ODNs was recovered in the fractions containing the high molecular weight proteins. In addition, the fully PS-modified lipid-ODN, ISIS-9388, bound to proteins eluting at 1.70 mL and redistributed to a higher extent from LDL to plasma proteins than the partially PS-modified lipid–ODNs. This is likely to be the result of the high affinity of the full PS ODN for plasma proteins. However, the complex of ISIS-9388 and LDL appeared to be relatively stable in the HDL/HSA mixture, and no binding to albumin could be observed. Upon incubation with rat plasma, a large proportion of ISIS-9388 was recovered in the albumin-containing fractions. The concentration of albumin in plasma (40 mg/mL) was, however, much higher than in the HDL/HSA mixture (0.2 mg/mL), which might explain the lack of albumin binding in this latter mixture. In addition, ISIS-9388 may also bind to immunoglobulins like IgG that will elute at 1.70 mL [38].

The lipid–ODNs with only three PS linkages bound

primarily to high molecular weight proteins. The major high molecular weight protein present in rat plasma is α_1M, which has a molecular weight of 730 kDa [39]. The presence of α_1M in the fractions eluting at 1.25–1.40 mL was confirmed by polyacrylamide gel electrophoresis. Rat plasma also contains very small amounts of α_2-macroglobulin (α_2M, 705 kDa), which is also expected to elute at 1.30 mL. Cossum *et al.* suggested earlier that non-conjugated PS ODNs may bind to macroglobulins in rat plasma [27]. Our present data suggest that macroglobulin binding in rat plasma may also occur for the lipid-conjugated ODNs that we used.

Our *in vivo* studies indicate that partially PS-modified lipid–ODNs are, when administered without LDL, rapidly cleared from the circulation. A substantial amount of the lipid–ODN was recovered in the liver, which may be ascribed to recognition by scavenger receptors on liver cells [8, 40, 41]. When the lipid–ODNs were administered as ODN/LDL complexes, the plasma clearance was considerably delayed. Concomitantly, the liver uptake of the complexed lipid–ODNs was reduced. The clearance and liver uptake of the LDL particles in the complexes was not altered, which indicates that complexation with lipid–ODNs does not affect the integrity of the particle. Of all the steroid anchors tested, the lithocholic acid-3α-oleate structure most effectively reduced the clearance of the ODNs. The strong association of this latter lipid with the lipids of LDL is probably primarily responsible for the prolonged circulation. The experiments on the *in vivo* fate of complexes of lipid–ODNs and lactosylated LDL further underscore the strong association of the oleoyl ester of lithocholic acid with lipidic carriers. This lipid appears, therefore, to be a very suitable anchor for association of ODNs with lipidic carriers such as lactosylated LDL and lactosylated HDL [42]. The stable association of lipid–ODNs with these carriers, which are rapidly taken up by a receptor-mediated route, will allow the selective delivery of ODNs to specific liver cell types.

Taken together, we have shown in this study that the oleoyl ester of lithocholic acid is a better lipid anchor than the previously published cholesterol anchor. In this respect, it is also important to note that an ODN backbone modification which has a low affinity for plasma proteins (e.g. ODNs with a minimum of PS linkages or non PS ODNs such as peptide nucleic acids; Ref. 43) will also influence the stability of the association of ODNs with lipoproteins. Our data indicate that ODNs conjugated with oleoyl steroid esters remain firmly associated with LDL and may have an increased exposure to tumor cells due to the retarded plasma clearance. These lipid–ODNs are therefore interesting candidates for use in efficacy studies aimed at targeting ODNs *in vivo* to leukemia cells via the LDL receptor. In addition, the anchor structures are also very suitable for delivery of ODNs to specific liver cells using lactosylated lipidic carriers.

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