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Synthesis of a Lipophilic Daunorubicin Derivative and Its Incorporation into Lipidic Carriers Developed for LDL Receptor-Mediated Tumor Therapy

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Purpose. Many tumors express elevated levels of LDL receptors (apoB, E receptors) on their membranes. Selective delivery of anti-neoplastic drugs to tumors by incorporation of these drugs into LDL or LDL-resembling particles should improve the efficacy of tumor therapy and minimize the severe side-effects. Since the apolipoproteins on the particles are essential for the LDL receptor recognition, drugs should preferably be incorporated into the lipid moiety. Most anti-tumor agents are too hydrophilic for incorporation into these carriers.

Methods. We synthesized LAD, a lipophilic prodrug of daunorubicin, by coupling the drug via a lysosomally degradable peptide spacer to a cholesteryl oleate analog.

Results. The overall yield of the synthesis was 50% with a purity of >90%. Radioactively ([^{3}H]) labeled LAD was obtained via a slightly modified procedure (yield 40%). The octanol/water partition coeffient of LAD is 30-fold higher than that of daunorubicin. LAD could be incorporated into triglyceride-rich lipid emulsions and small liposomes, which, if provided with apoE, have been demonstrated earlier to be cleared *in vivo* via the LDL receptor. The liposomes contained approximately 10 molecules of LAD per liposomal particle. Analysis of differently sized LAD-containing emulsions suggests that LAD associates with the surface of lipidic particles. In the presence of human serum, LAD did not dissociate from the emulsion particles, indicating a firm association of LAD with the carrier.

Conclusions. The coupling of a cholesterol ester analog to daunorubicin results in a lipophilic prodrug that can be firmly anchored into lipidic carriers. LAD-loaded emulsions and liposomes provided with recombinant apoE will be tested in the near future for their ability to deliver LAD to tumor tissue *in vivo* via the LDL receptor.

KEY WORDS: low-density lipoprotein receptor; daunorubicin derivative; drug carrier; tumor therapy.

INTRODUCTION

Experimental pharmacotherapeutic cancer treatments aim to improve the efficacy of anti-tumor drugs and to minimize the drug-related side-effects. A very promising approach is to selectively deliver drugs to tumor cells via specific markers. The receptor for low density lipoprotein (LDL) is such a marker, since many tumors show relatively elevated levels of LDL receptors on their membranes. Especially myeloid leukemic cells in the acute phase, but also colon-, kidney-, and brain tumors, as well as tumors of gynecological origin have been shown to express high levels of the LDL receptor (1). Because of the high expression of LDL receptors on tumor cells, LDL has been proposed as a carrier for the selective delivery of drugs to tumor cells.

LDL consists of a core of cholesterol esters surrounded by a monolayer of mainly phospholipids and some cholesterol. A large part of the surface is covered by apolipoprotein B (apoB). Binding of LDL to its receptor is followed by internalization and degradation of the particle in the lysosomes (2). Since apoB is essential for the LDL receptor recognition, drugs should preferably be incorporated into the lipid moiety. In many studies, however, the incorporation procedures used caused subtle changes in the complex structure of apoB, which induced *in vivo* rapid uptake of the drug-LDL complexes by other uptake mechanisms than the LDL receptor pathway (3,4). To avoid uptake by these additional mechanisms, and to increase the pharmaceutical feasibility of LDL receptor-mediated tumor therapy, artificial particles aimed at resembling LDL in biological behavior were synthesized (5-11). In our laboratory, we developed triglyceride-rich lipid emulsions and small liposomes. These particles were provided with recombinant-produced apolipoprotein E (apoE), and showed LDL receptor-mediated uptake in rats and mice (10,11). ApoE that is associated with small lipidic particles is a high-affinity ligand for the LDL receptor (12–15).

Because of the lipidic nature of LDL and LDL-resembling particles, only lipophilic drugs can be incorporated into these particles. As most anti-tumor drugs are too hydrophilic for incorporation into lipidic particles, LDL receptor-mediated tumor therapy depends on the development of new lipophilic anti-tumor drugs, or on the modification of well-characterized and registered drugs with a sufficiently lipophilic moiety. In the present study, we derivatized the anthracycline anti-tumor drug daunorubicin (DNR) with a cholesteryl ester analog, with the aim to obtain a lipophilic prodrug that can be firmly incorporated into our apoE-containing LDL-resembling particles. Daunorubicin is resistant to lysosomal enzymes (16,17), which is a prerequisite for drug targeting via the LDL receptor pathway.

The free amino group of the sugar moiety of the daunorubicin was used for derivatization. However, as modification of this group can reduce the cytotoxicity of the drug (18), it is essential that the coupling is reversible. We therefore intercalated a lysosomally degradable tetrapeptide spacer, consisting of two repetitive units of leucine and alanine (ALAL), between the drug and the lipophilic anchor. In studies in which various spacers were tested, this tetrapeptide spacer was the most effective in the controlled release of anti-neoplastic drugs from protein carriers (19–21). For instance, Trouet et al. demonstrated

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ABBREVIATIONS: ALAL, Alanine-leucine-alanine-leucine; ApoB, Apolipoprotein B; ApoE, Apolipoprotein E; C, Cholesterol; CETP, Cholesterol ester transfer protein; CO, Cholesterol oleate; EYPC, egg yolk phosphatidylcholine; DIPEA, N,N-diisopropylethylamine; DNR, Daunorubicin; HDL, high density lipoprotein; LAD, conjugate of 3α-O-(oleoyl)-5β-cholanic acid and ALAL-daunorubicin; LCO, 3α-O-(oleoyl)-5β-cholanic acid; LDL, low density lipoprotein; LPC, lysophosphatidylcholine; TG, triolein.

that upon incubation with lysosomal enzymes 75% of the daunorubicin in an albumin-ALAL-daunorubicin complex was released from the complex within 10 h (19).

The resulting lipophilic prodrug of daunorubicin, a conjugate of 3α-O-(oleoyl)-5β-cholanic acid and ALAL-daunorubicin (LAD), was incorporated into triglyceride-rich lipid emulsions and small liposomes. These particles mimic LDL when provided with apoE. The extent and stability of the incorporation of LAD into these particles was studied, using ^{3}H-labeled LAD

MATERIALS AND METHODS

Materials

Daunorubicin (Cerubidine®) was purchased from Rhône-Poulenc Rorer S.A., Anthony-Cedex, France. [1α,2α(N)−^{3}H]-cholesteryl oleate ([^{3}H]CO) and [1−^{14}C]cholesteryl oleate ([^{14}C]CO) were from Amersham International, Amersham, Buckinghamshire, UK. [^{3}H(G)]Daunorubicin was from New England Nuclear Research Products, Boston, MA, USA. 9-Fluorenylmethyloxycarbonyl-Ala-Leu-Ala-Leu-N-Hydroxysuccinimide ester (Fmoc-ALAL-OSu) was from Bachem, Bubendorf, Switserland. Egg yolk phosphatidylcholine (EYPC, 98%) and triolein (TG, 99%) were obtained from Fluka, Buchs, Switserland. Cholesteryl oleate (CO, 97%) was from Janssen Chimica, Beerse, Belgium. Cholesterol (C, >99%) and L-α-lysophosphatidylcholine (LPC, 99%) were from Sigma, St. Louis, MO, USA. Cholesterol oxidase, cholesterol esterase, peroxidase type II (200 units/mg), and Precipath®L were from Boehringer Mannheim, Mannheim, Germany. Human recombinant apolipoprotein E_3 was a generous gift from Dr. T. Vogel, Biotechnology General Ltd., Rehovot, Israel. Toluene and 1,2-dichloroethane (DCE) were distilled from P_2O_5. N,N-Dimethylformamide (DMF) was stirred overnight with CaH_2, and then distilled under reduced pressure. Both solvents were stored over molecular sieves (4 Å). All other chemicals and solvents were of analytical grade.

Pentafluorophenyl (PFP)-activated 3α-O-(oleoyl)-5β-cholanic acid (PFP-LCO) was synthesized from litocholic acid as follows. The carboxylic acid of litocholic acid was first protected with 4-bromophenacyl bromide. Subsequently, the oleic acid chain was introduced at the 3-OH, using oleoylchloride in the presence of dimethylaminopyridine. After hydrolysis of the carboxylic acid-protecting group, the steroid was activated with PFP in the presence of dicyclohexylcarbodiimide.

Methods

Reaction mixtures were analyzed by thin layer chromatography (TLC) on Silica gel 60-F_{254} (0.2 mm-thick preformed layers on aluminium sheets; Merck, Darmstadt, Germany) using the solvent: NH_4OH:CH_3OH:dichloromethane (DCM) (1:10:89; v/v/v). Daunorubicin (derivatives) were directly visible by their orange/red colour. Synthesized compounds were analyzed by ^{13}C-and ^{1}H-nuclear magnetic resonance (NMR) spectroscopy, and by Fast Atom Bombardment Mass Spectrometry (FAB-MS). ^{1}H NMR and ^{13}C NMR spectra were recorded using a Jeol JNM-FX 200, a Bruker WM-300, or a Bruker DMX-600 spectrometer. ^{13}C NMR spectra were monitored using the attached proton test technique, utilizing CD_3OD:$CDCl_3$ (10:90) as solvent. ^{1}H NMR: Chemical shifts (δ) are given in ppm relative to tetramethylsilane as an internal standard. Positive and negative ion FAB-MS was carried out using a JEOL JMS SX/SX102A four-sector mass spectrometer, coupled to a JEOL MS-MP7000 data system. The samples were dissolved in triethanolamine and loaded onto a stainless steel probe and bombarded with xenon atoms with an energy of 3 KeV. The concentration of dissolved DNR was determined by measuring the absorbance at 475 nm in PBS (10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl; $\epsilon = 8704$) (19). ^{3}H- and ^{14}C radioactivities were counted in Emulsifier Safe® (aqueous samples), or Ultima Gold® or HIONIC Fluor® (organic samples) scintillation cocktails in a Packard 1500 Tri-Carb liquid scintillation analyzer. The apparatus and all scintillation cocktails were from Packard Instrument Company Inc., Downers Grove, IL, USA.

Synthesis of ([^{3}H])LAD: 1] Synthesis of H_2N-Ala-Leu-Ala-Leu-([^{3}H])DNR

The method used to couple DNR to the tetrapeptide spacer was adapted from Law *et al.* (22). Step 1A: To a solution of DNR (4.5 mg, 8.5 μmol) in 10 ml dry DCE and 600 ml dry DMF, 60 mg (85 μmol) of Fmoc-ALAL-OSu was added portionwise over a time period of 2 hours. The reaction mixture was kept in the dark and was stirred overnight at room temperature. The progress of the reaction was monitored by TLC (DNR: Rf = 0.13, Fmoc-ALAL-DNR: Rf = 0.82). Step 1B: To deprotect the NH_2 moiety, piperidine was added dropwise to the reaction mixture (final volume 2.5 % (v/v)), and the stirred mixture was left overnight at rT in the dark. The progress of the reaction was monitored by TLC (H_2N-ALAL-DNR: Rf = 0.29). H_2N-ALAL-DNR was purified by column chromatography (Silica gel 60, h × ø; 8 × 5 cm) with a gradient of 10-40% CH_3OH in DCM (v/v). Yield: 5 mg (65%). The compound was dried in vacuo and stored at −20°C until use. ^{13}C {^{1}H} NMR, 50.1 MHz, δ (ppm): 135.17 (C-2), 120.05 (C-1), 118.97 (C-3), 101.04 (C-1'), 76.80 (C-9), 69.94 (C-7), 68.77 (C-4'), 67.81 (C-5'), 56.80 (O-$\underline{C}H_3$), 52.97, 53.62, 50.55 (3× C_α(A,L)), 40.74, 40.59 (2× C_β(L)), 35.68 (C-8), 33.32 (C-10), 24.88 (C-14), 17.08 (C-6'). MS(FAB): 918 $(M + Na)^+$, exact mass calculated for $C_{45}H_{61}N_5O_{14}Na$ 918.4113, found 9-18.4123.

H_2N-ALAL-[^{3}H]DNR was synthesized from [^{3}H]DNR as described above with minor modifications. To 275 μg of [^{3}H]DNR (specific radioactivity: 17 GBq/mmol), dissolved in 1 ml 2.5% DMF in DCE (stirred), was added 1.8 mg of Fmoc-ALAL-OSu portionwise over a time period of 2 hours. The progress of the reaction was monitored by TLC followed by determination of the radioactivity. After 18 h at rT, piperidine was added dropwise (2.5 % v/v) and left to react overnight. The product was purified by Sephadex LH-20 column chromatography (h × ø; 90 × 0.7 cm) with CH_3OH:DCM (1:2; v/v) as eluent. The radiochemical purity of the product was > 90% as judged by TLC.

Synthesis of ([^{3}H])LAD: 2] Coupling of 3α-O-(oleoyl)-5β-cholanic acid (LCO) to H_2N-Ala-Leu-Ala-Leu-([^{3}H])DNR

To a solution of H_2N-ALAL-DNR (20 mg, 22 μmol) in 10 ml CH_3OH:DCM (1:9; v/v) were added 40 μl (223 μmol) of N,N-diisopropylethylamine (DIPEA) and 72 mg (112 μmol) of PFP-activated 3α-O-(oleoyl)-5β-cholanic acid. The reaction mixture was kept in the dark at room temperature and stirred overnight. The progress of the reaction was monitored by TLC (LCO-ALAL-DNR: Rf = 0.67). LCO-ALAL-DNR (LAD) was purified by column chromatography (Silica gel 60, h × ø; 8 × 5 cm) with a gradient of 2–4% CH_3OH in DCM yielding 26.7 mg of product (80%). The product was dried in vacuo and stored at −20°C until use. The molair extinction coefficient of LAD was determined in CH_3OH:DCE (1: 9, v/v) at 475 nm: $\epsilon = 5335$. ^1H NMR, 400 MHz, δ (ppm): 7.97 (1H, d, J=7.6 Hz, H-1), 7.72 (1H, t, J=8.1 Hz, H-2), 7.34 (1H, d, J=8.5 Hz, H-3), 5.41 (1H, d, J=3.7 Hz, H-1′), 5.27 (2H, br m, vinyl H), 5.18 (1H, br s, H-7), 4.64 (1H, br m, H-3″), 4.00 (3 H, s, $OC\underline{H}_3$), 3.59 (1H, br s, H-4′), 2.35 (3H, s, C-14), 0.86 (3H, t, $C\underline{H}_3$-oleoyl), 0.57 (3H, s, H-18″).

^{13}C {^1H} NMR, 150.3 MHz, δ (ppm): 212,99 (C-13), 187.04 (C-5), 186.53 (C-12), 175.56, 174.20, 173.93, 173.78 (4× $\underline{C}$=O), 173.30 ($\underline{C}$=O oleoyl), 160.80 (C-4), 156.00 (C-6), 155.34 (C-11), 135.60 (C-2), 135.36, 134.32, 134.24, 120.71, 111.23, 110.97 (6× Cq), 129.52, 129.77 (2× $\underline{C}H$ oleoyl), 119.62 (C-1), 118.31 (C-3), 100.68 (C-1′), 76.32 (C-9), 74.19 (C-3″), 69.37 (C-7), 68.21 (C-4′), 67.43 (C-5′), 56.33 (O-$\underline{C}H_3$), 56.24, 55.93 (C-14″, C-17″), 53.35, 52.31 (2× C_α(L)), 50.33, 49.88 (2× C_a(A)), 45.75 (C-3′), 40.01, 39.87 (2×, C_β(L)), 35.11 (C-8), 32.90 (C-10), 30.14 (C-2′), 24.62 (C-14), 16.53, 16.48, 16.43 (C-6′, 2× C_β(A)), 13.77 ($\underline{C}H_3$ oleoyl), 11.80 (C-18″). MS(FAB): 1518.5 $(M-H)^-$, exact mass calculated for $C_{87}H_{130}N_5O_{17}$ 1518.9526, found 1518.9535.

LCO-Ala-Leu-Ala-Leu-[^3H]DNR was synthesized from H_2N-ALAL-[^3H]DNR as described above. An aliquot of approximately 0.5 μmol of H_2N-ALAL-[^3H]DNR was dissolved in 280 μl CH_3OH:DCE (1:9 v/v), together with 5 μmol DIPEA, and 5 μmol PFP activated LCO. After the reaction was completed (monitored by TLC and counting of the radioactivity), the product was isolated by Sephadex LH-20 column chromatography (h × ø; 90 × 0.7 cm) using CH_3OH:DCM (1: 2) as eluens. The crude product was then further purified by TLC (3.8% CH_3OH in DCM). Spots containing LCO-ALAL-DNR were scraped off and extracted with CH_3OH:DCM (1:9, v/v). MS(FAB): 1519.1 $(M-H)^-$. The final yield was 500 μg (63%) with a radiochemical purity of >95% as determined by TLC. The specific activity was calculated to be 1.822 GBq/mmol, using $\epsilon = 5335$ (see above). LCO-ALAL-[^3H]DNR was stored in EtOH:DCE (1:9 v/v) at −20°C.

Determination of the Partition Coefficient (Log P)

Aliquots of [^3H]DNR, H_2N-ALAL-[^3H]DNR, and [^3H]LAD (approximately 1 μCi) were each dried under N_2 in a glass vial. The compounds had been purified by TLC immediately before the experiment. Then, 1 ml of 50 mM of sodium phosphate buffer, pH 7.4, and 1 μl of 1-octanol were added, and the mixtures were gently shaken for 18–22 h at 37°C. Samples of 100 and 200 μl of the octanol and aqueous phase were counted for radioactivity in 3.5 ml of scintillation cocktail. More than 95% of each compound was recovered in both phases. The partition coefficient P is defined as the concentration of the compound in the octanol phase divided by the concentration of the compound in the aqueous phase.

Preparation and Characterization of LAD-containing Liposomes

LAD-containing liposomes were prepared by sonication using a procedure described by Rensen *et al.* (10). EYPC (50 mg) and 1.9 mg CO were mixed in a 20 ml glass vial with 0-200 μg of LAD, containing 0.2 μCi [^3H]LAD. The solvent was evaporated under N_2 (1h, rT), followed by vacuum desiccation overnight at 4°C. The lipids and prodrug were hydrated in 11.4 ml of 10 mM Tris-HCl buffer, pH 8.0, containing 0.1 M KCl. The mixture was subsequently sonicated at an output of 18μm (amplitude of sonic probe) for 1 h under argon, using a Soniprep 150 (MSE Scientific Instruments, Crawley, W. Sussex, UK), equipped with a water bath to maintain the temperature at 54°C. After sonication, the liposomes were purified and concentrated by density gradient ultracentrifugation at 285,000 × g, for 18 h at 4°C, according to Redgrave *et al.* (23). Following ultracentrifugation, the liposomes, visible as a narrow opalescent layer at a density of 1.016–1.040 g/ml, were isolated by aspiration with glass capillary pipettes and were stored under argon at room temperature. The amount of LAD in the preparation was determined by measuring the radioactivity of [^3H]LAD. To calculate the number of molecules of LAD per liposomal particle, it was assumed that the phospholipid bilayer length is 39×10^{-10} m (24), and that the polar head of a EYPC occupies a surface of 4.2×10^{-19} m^2 (24). The amounts of EYPC and CO were determined using enzymatic kits for phospholipids, and esterified cholesterol, respectively (Boehringer Mannheim, Mannheim, Germany). Precipath®L was used as standard in both assays. The size of the particles and the homogeneity of the population was determined by photon correlation spectroscopy at 27°C, using a Malvern 4700c submicron particle analyzer, at an angle of 90° (Malvern Instruments, Malvern, Worcs, UK).

Preparation and Characterization of LAD-containing Emulsions

Emulsions were prepared as described by Rensen *et al.* (11) from TG, EYPC, LPC, CO, and C at a weight ratio of 70: 22.7: 2.3: 3.0: 2.0 (100 mg of total lipid, containing a trace amount of [^3H]LAD (approximately 1 μg)). The solvent was evaporated under N_2 (1h, room temperature), followed by vacuum desiccation overnight at 4°C. Subsequently, the lipids and prodrug were hydrated in 10 ml of sonication buffer: 10 mM HEPES, 2.4 M NaCl, 1 mM EDTA, pH 7.4 (density 1.10 g/ml). The mixture was sonicated at an output of 18μm (amplitude of sonic probe) for 30 min at 54°C under argon (see preparation liposomes). The emulsion was fractionated into three differently sized populations by consecutive density ultracentrifugation steps. After centrifugation for 22 min at 71,000 × g at 20°C, a fraction containing large emulsion particles (fraction 1) was removed from the top of the tube by aspiration. The aspirated volume was replaced by NaCl buffer of similar density (i.e. 1.006 g/ml), and emulsion fractions 2 and 3 were similarly isolated after subsequent centrifugation steps at 285,000 × g for 22 min and 4–5 h, respectively. All emulsion fractions were characterized for ^3H radioactivity, size, homogeneity and lipid composition (as described in the section above). For the calculations on the behavior of LAD as surface- or core component, it was assumed that all particles were spherical, that the phospholipid monolayer was 3.9 nm, and that the density of all emulsions was similar (1.006 g/ml).

Determination of Redistribution of LAD from Emulsion to Serum Components

Aliquots of 250 μl of emulsion fraction 2 (see above), containing 2.5 mg of triglyceride, and 6 nCi [^{3}H]LAD or 0.2 μCi of [^{3}H]CO, were incubated with 2.5 ml of freshly isolated human serum for 5, 30, 120 or 300 min at 37°C. The incubations were stopped by adding 1.3 ml of ice-cold PBS and solid KBr was added to a density of 1.21 g/ml. The mixtures were then subjected to density gradient ultracentrifugation for 18 h (4°C, 285,000 × g) according to Redgrave *et al.* (23). The emulsion, which was concentrated at the top of the tube, was removed by aspiration (fraction 20). The remainder of the gradient was fractionated into 0.6 ml aliquots, starting at the bottom of the centrifugation tube. ^{3}H Radioactivity in all fractions was counted. The top fractions 16–19, and in particular fraction 20, contained the emulsion particles, while the fractions 5–10 and 11–15 contained high-density-lipoproteins (HDL) and LDL, respectively.

RESULTS

Synthesis and Properties of LAD

The synthesis of LAD was accomplished in a two-step procedure, which is depicted in Figure 1. First, the Leu-Ala-Leu-Ala peptide was coupled via its carboxyl group to the amino group of DNR (1A). The carboxyl group of the peptide was activated by hydroxysuccinimide, and its amino group was protected by Fmoc. After coupling of the peptide to DNR, the aminogroup of the peptide was deprotected by the addition of piperidine (1B). In the second step, H_2N-Ala-Leu-Ala-Leu-DNR was coupled to the lipophilic anchor 3α-O-(oleoyl)-5β-cholanic acid (LCO). The coupling was accomplished by adding the pentafluorophenyl-activated ester of LCO. The resulting prodrug LCO-Ala-Leu-Ala-Leu-DNR (LAD) was obtained with an overall yield of 56% (purity >90%). The structure of LAD, presented in Figure 2, was confirmed by NMR and mass spectroscopic analysis. Tritium-labeled LAD was synthesized from ^{3}H-labeled DNR by a procedure similar to the one described above. Two independent batches of [^{3}H]LAD were prepared with a final yield after purification of 39.3 ± 0.8 % and a purity of 95%. The specific radioactivity was 2.1 ± 0.3 GBq per mmol of LAD. After five months of storage of [^{3}H]LAD at −20 °C in DCE containing 10% (v/v) EtOH, more than 90% of the radioactivity was still associated with the drug.

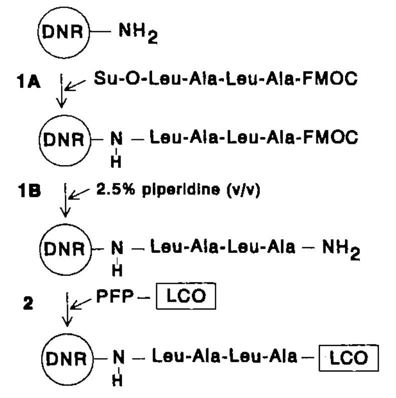

Fig. 1. Synthesis of LAD: a conjugate of 3α-O-(oleoyl)-5β-cholanic acid and alanyl-leucyl-alanyl-leucyl-daunorubicin.

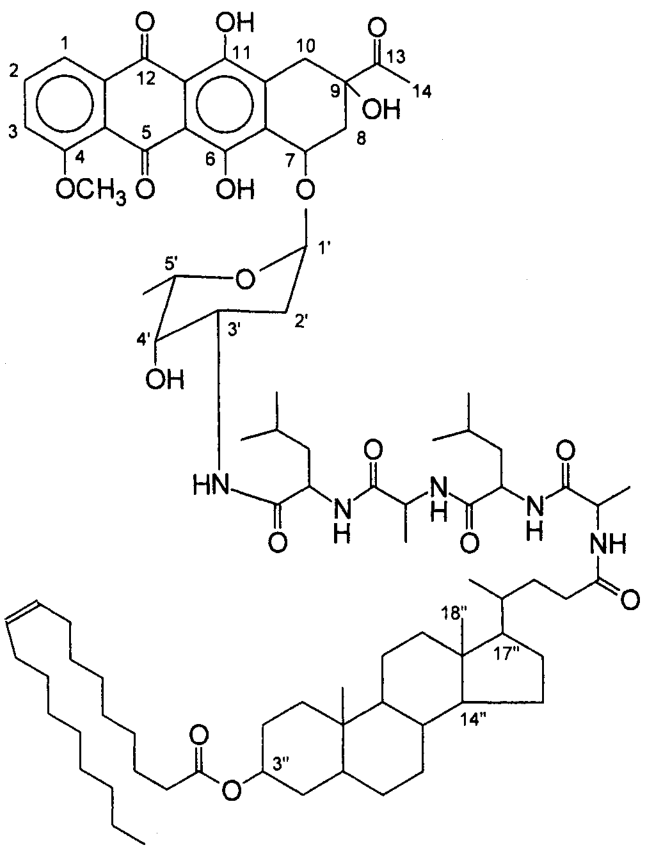

Fig. 2. Structure of LAD.

To monitor the effect of the derivatization of DNR on its lipophilicity, we measured the octanol/water partition coefficients (log P values) of the original drug DNR, the intermediate product H_2N-ALAL-DNR, and LAD. The partition coefficient of LAD was found to be 30-fold higher than that of DNR. The partition coefficient of the H_2N-ALAL-DNR intermediate is increased 5-fold as compared to DNR (Table I).

Incorporation of LAD into Artificial LDL-like Lipid Particles

To evaluate the ability of LAD to be transported by lipidic carriers, we examined the incorporation of LAD into small

Table I. Octanol/water Distribution of Daunorubicin and Its Lipophilic Derivatives

	Log P[a,b]
DNR	1.00 ± 0.01
H_2N-ALAL-DNR	1.71 ± 0.09
LAD	2.52 ± 0.04

[a] The partition coefficient P of a compound is calculated by dividing its concentration in octanol by its concentration in the aqueous phase.
[b] Values are means ± variance of two independent determinations.

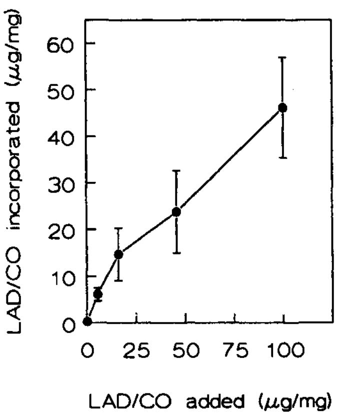

Fig. 3. Incorporation of LAD in liposomes. The indicated amounts of LAD, including 0.2 μCi [^{3}H]LAD, were sonicated with 50 mg egg-yolk phosphatidylcholine (EYPC) and 1.9 mg cholesteryl oleate (CO). The resulting liposomes were purified by density-gradient ultracentrifugation. The incorporation of LAD is expressed as μg LAD incorporated per mg liposomal CO, and is given as a function of the amount of LAD added per mg CO added. The presented values are means of 3 determinations ± S.E.M.

liposomes and triglyceride-rich lipid emulsions. Both types of particles can mimic LDL when provided with apoE (10,11). Figure 3 shows that various amounts of LAD could be efficiently incorporated into the liposomes. The LAD-loaded liposomes prepared with 100 mg LAD/mg CO formed a homogeneous population of particles (low polydispersity: 0.180 ± 0.006) with a size of 29.3 ± 0.3 nm (mean ± S.E.M.; n = 13). The molar ratio of phospholipid to neutral lipid (EYPC: CO) in these particles was 18.9 ± 0.7 (mean ± S.E.M., n = 10). Liposomes prepared with 100 mg LAD/mg CO contained 2.1 mg LAD per mg PL, which is, assuming that 1 mg of phospholipid represents 7.6×10^{13} particles, equivalent to 10 ± 1.1 (mean ± S.E.M., n=8) molecules of LAD per liposomal particle. As the population of liposomes was very homogeneous (polydispersity <0.2), there are probably no large differences in drug load between particles within the population.

LAD was also incorporated in triglyceride-rich emulsions by co-sonication with the lipids. The resulting particles were separated into three populations with sizes of 50 ± 1.7 nm, 76.2 ± 1.3 nm, and 145.5 ± 4.9 nm (characteristics summarized in Table II). Of the added amount of LAD, 9.8 ± 1.0%, 33.6 ± 2.3%, and 39.7 ± 2.8% were recovered in the emulsion fractions 1–3, respectively. By relating the amount of LAD incorporated in each emulsion to the surface and volume of the particles in each population (represented by the amounts of phospholipid and triglyceride, respectively), conclusions can be drawn about the location of LAD inside the particle. Figure 4 shows clearly that the distribution of LAD over the surface and volume of the particle is very similar to that of the phopholipid surface marker, whereas the triglyceride core marker has a strikingly different distribution. This indicates that in these particles LAD is likely to be located at the surface and not in the core of the emulsion particles.

Table II. Characteristics of LAD-containing Triglyceride-rich Emulsion Fractions

	Emulsion 1[a]	Emulsion 2[a]	Emulsion 3[a]
Mean diameter (nm)	145.5 ± 4.9	76.2 ± 1.3	50.0 ± 1.7
Polydispersity	0.15 ± 0.01	0.12 ± 0.01	0.22 ± 0.01
Composition (% w/w)			
- Triolein	82.6 ± 0.3	76.8 ± 0.4	65.2 ± 0.6
- Phosphatidylcholine	13.7 ± 0.4	18.2 ± 0.4	30.1 ± 0.6
- Cholesterol (ester)	3.7 ± 0.1	5.0 ± 0.1	4.7 ± 0.1

[a] All values are means ± SEM of four experiments.

Redistribution of LAD from Emulsion to Serum Components

The feasibility to use LAD-containing lipidic particles as drug-carrier complexes depends strongly on the ability of LAD to remain associated with these particles in the circulation. Both spontaneous redistribution of LAD to serum (lipo)proteins and transfer protein-mediated redistribution of LAD may occur. The latter because the coupling of a cholesteryl ester analog to daunorubicin might convert the drug into a substrate for cholesterol-ester-transfer proteins (CETP) present in serum. To study the redistribution of LAD from a triglyceride-rich emulsion to serum components, emulsion fraction 2 labeled with [^{3}H]LAD or [^{3}H]cholesteryl oleate was incubated with freshly isolated human serum. After incubation, the mixtures were subjected to density ultracentrifugation to separate the emulsion particles from lipoproteins. Figure 5 shows the amounts of LAD and cholesteryl oleate recovered in the serum lipoprotein fractions (LDL + HDL). LAD was clearly not transferred from the emulsion- to the lipoprotein fractions in a time-dependent manner. After 5 min of incubation, 88% of the amount of LAD originally present in the emulsion was recovered in the emulsion fractions. The remainder was equally divided over all fractions, including the lipoprotein fractions. Longer incubation times (up to 5 h) did not significantly change the amount of LAD recov-

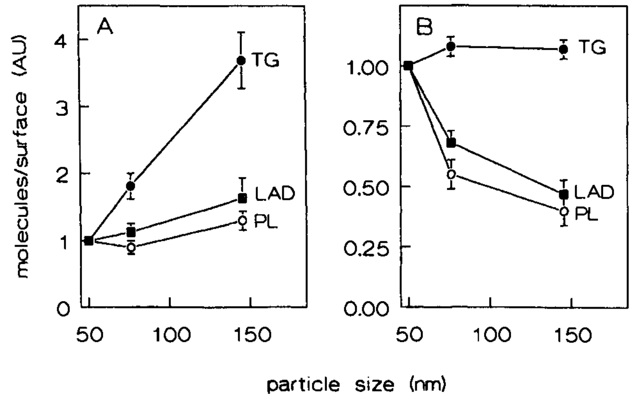

Fig. 4. Incorporation of LAD into homogeneous emulsions of different size; behavior as surface- or core component. [^{3}H]LAD was incorporated into three homogeneous emulsion populations with different particle sizes, which consisted of a shell of mainly phospholipids (PL) and a core of triglycerides (TG). The amount of LAD incorporated into each emulsion, and the amounts of phospholipids and triglycerides in each emulsion were determined. Then, the ratios of PL (○), TG (●), and LAD (■) per surface unit (A) and volume unit (B) of the emulsions were calculated. The data are presented in arbitrary units (AU) with the ratios of the smallest-sized emulsion set at 1.0. Values are means ± S.E.M of 4 experiments.

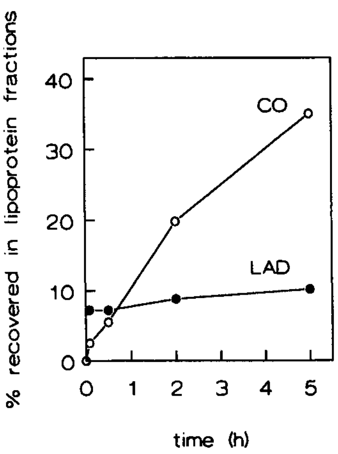

Fig. 5. Redistribution of LAD and cholesteryl oleate from emulsion particles to serum lipoproteins LAD-containing emulsion fractions 2, radiolabeled with either [^{3}H]LAD or [^{3}H]cholesteryl oleate were incubated with freshly isolated human serum for 5, 30, 120 or 300 min at 37°C. Subsequently, the incubation mixtures were subjected to density ultracentrifugation. The gradients were fractionated and the radioactivity in each fraction was determined. HDL and LDL were positioned in fraction 5 to 15, while the emulsion was mainly present in fraction 20. The percentage of the recovered amount of [^{3}H]LAD (●) or [^{3}H]CO (○) radioactivity in fraction 5–15 was plotted against the incubation time (total recoveries in fraction 1–20 were >95%).

ered in the lipoprotein- or emulsion fractions. Cholesteryl oleate, which was added to control for CETP activity, was transferred from the emulsion to lipoprotein fractions in a time-dependent manner. After 5 min of incubation, 97% of the amount of CO originally present in the emulsion was still recovered in the emulsion fractions, while after 5 h, 35% of the CO was transferred from the emulsion to the serum lipoproteins. Incubation with rat serum, which lacks CETP (30), did not result in transfer of CO from the emulsion to the lipoproteins (not shown). It can be concluded that LAD is not a substrate for CETP.

DISCUSSION

The successful development of LDL receptor-mediated tumor targeting requires lipophilic anti-tumor drugs that can be incorporated into LDL, or into the recently developed artificial LDL-mimicking particles. Unfortunately, the availability of lipophilic anti-tumor drugs is limited. We conjugated daunorubicin via a lysosomally degradable tetrapeptide to a cholesterol ester analog, 3α-O-(oleoyl)-5β-cholanic acid. Earlier attempts to increase the lipophilicity of daunorubicin analogs with only fatty acyl chains did not result in a firm anchoring of the (pro)drug into the LDL (4,25). In contrast to earlier studies (26), we coupled the anthracycline to a lipophilic anchor via a linkage that is biodegradable (19). The synthesis of the prodrug (LAD) was accomplished in a two-step reaction procedure with an overall yield of >50%. Tritium-labeled LAD was synthesized from [^{3}H]DNR with an overall yield of approximately 40% (radiochemical purity 95%). The derivatisation of DNR with the tetrapeptide-lipid moiety increased the lipophilic character of the drug substantially, resulting in a 30-fold enhanced octanol/water distribution.

LAD was incorporated into liposomes by cosonication of LAD with the liposomal lipid components. On the addition of 100 μg LAD per mg CO, approximately 10 molecules of LAD could be incorporated per liposomal particle. Considering the non-saturated incorporation curve, it is likely that even higher amounts of LAD can be incorporated per liposomal particle. It can be calculated that the phospholipid bilayer of the small liposomes (28 nm) used in this study represents 60% of the total volume of the particles. This provides a relatively large incorporation volume for lipophilic drugs like LAD. The incorporation of LAD did not significantly affect size nor homogeneity of the liposome population. The sizes of LAD-loaded liposomes and liposomes containing no drug (10) were 29.3 ± 0.3 nm and 28.9 ± 0.9 nm, respectively (polydispersities: 0.180 ± 0.006 and 0.198 ± 0.001, respectively). The incorporation of LAD into the liposomes had also no effect on the ability of the liposomes to acquire apoE. When LAD-loaded liposomes were incubated with apoE, approximately 6 molecules became firmly associated per liposome. These findings are very similar to those in our earlier study, in which we examined the interaction of apoE with liposomes without the prodrug (10).

LAD could also be incorporated into emulsions with different sizes. Upon enrichment with apoE, the clearance of the smallest population of particles (50 nm) is almost completely dependent on the LDL receptor (11). This suggests that this emulsion population might be successfully used as an LDL-resembling carrier of anti-tumor drugs. Comparison of the LAD-loaded emulsions with emulsions containing no drug (11) indicates that the incorporation of LAD had no significant effect on the sizes and the composition of the emulsions. By comparing the amounts of LAD in the differently sized emulsion populations, it was found that the extent of incorporation of LAD into the particles correlates with the available surface of the particles and not with the available volume. It can therefore be concluded that LAD is present in the surface region of the emulsions. This finding implicates that the small liposomes, which have a relatively large surface/mass ratio, are probably more suitable carriers for LAD than the triglyceride-rich emulsions.

Inadequate anchoring of a drug into a lipidic carrier often results in the redistribution of the drug to lipidic serum components, such as lipoproteins, which leads to a less efficient targeting (3,27,28). Furthermore, the use of a cholesterol oleate analog as anchor, as is the case for LAD, might induce a protein-mediated redistribution of the prodrug by the cholesteryl ester transfer protein (CETP). In humans, this transfer protein is responsible for transport of cholesterol esters between lipoproteins in the circulation (29,30). For the study of the redistribution of LAD we chose a LAD-loaded emulsion rather than LAD-liposomes, because the emulsion (density 1.006 g/ml) is easier to separate from lipoproteins (densities: 1.027–1.172 g/ml) by density gradient ultracentrifugation than the LAD-liposomes (density 1.016–1.058 g/ml). In the presence of human or rat serum, 10–15% was lost from the emulsion instantaneously after mixing with the serum. However, more than 85% of the incorporated LAD remained associated to the emulsion, even after 5 hours of incubation. Preliminary *in vivo* experiments in mice indicated that after 4 h of circulation, 70% of the initially incorporated drug was still associated with the carrier. Cholesteryl oleate was transferred from the emulsion to lipoproteins upon incubation with human serum, which indicates the presence of active CETP. As LAD was not exchanged, we conclude that LAD is not a substrate for CETP.

In conclusion, in the present study we synthesized LAD, a lipophilic prodrug of daunorubicin that can be efficiently and stably incorporated into triglyceride-rich lipid emulsions and small liposomes. It had been demonstrated earlier that these particles, if provided with apoE, are cleared *in vivo* via the LDL receptor pathway. LAD-loaded emulsions and liposomes provided with recombinant apoE will be tested in the near future on their ability to deliver LAD *in vivo* to tumor tissue via the LDL receptor pathway. The present study may also be extended by derivatizing cytotoxic analogs of daunorubicin with the 3α-O-(oleoyl)-5β-cholanic acid anchor. This is expected to yield a variety of lipophilic antitumor agents that can be used for the further development of the LDL receptor-mediated tumor therapy.

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