phobic interactions with the surface of a hydrophobic binding region since π-term slopes of about 0.5 are typical for such an event. If the substituents become very hydrophobic, however, the linear relationship with lipophilicity changes to a parabolic one,[8] indicating that at least for such substituents hydrophobic influences are more likely connected with transport processes.

It is evident from the results of this study that 4-hydroxyquinoline-3-carboxylic acids, containing relatively small substituents, interact in a very similar manner with the three dehydrogenase enzymes and that significant differences among the enzymes are only observed if one explores binding sites at greater distances from the quinoline nucleus. Since the analysis clearly does not reflect the observed activities of larger, nonpolar substituents, it appears that such substituents afford a shift in binding during the course of interacting at more unique sites on mitochondrial malate dehydrogenase in particular. More importantly, the principal component analysis has demonstrated that the inhibition of ascites cell respiration by the quinolines incorporating nonaromatic substituents does not result from inhibition of lactate dehydrogenase or the malate dehydrogenases as the primary targets, thus indicating the involvement of additional or alternative sites of action. Irrespective of the implications for the specific data evaluated in this investigation, the potential utility of multivariate techniques such as principal component analysis as tools for assessing the interrelatedness of multiple biological test systems has been further substantiated.

Selective Uptake of a Toxic Lipophilic Anthracycline Derivative by the Low-Density Lipoprotein Receptor Pathway in Cultured Fibroblasts

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N-(*N*-Retinoyl)-L-leucyldoxorubicin 14-linoleate (r11-DOX), a new lipophilic derivative of doxorubicin, was synthesized and incorporated into low-density lipoprotein (LDL). The drug–LDL complex contained 100–200 drug molecules/LDL particle. When cultured normal human fibroblasts were incubated with ^{125}I-LDL-incorporated drug, there was a perfect correlation between the cellular uptake plus degradation of ^{125}I-LDL and the cellular drug accumulation. The presence of excess native LDL inhibited the cellular uptake and degradation of ^{125}I-LDL and the drug accumulation to the same extent. In contrast, methylated LDL, which does not bind to the LDL receptor, did not alter the cellular uptake and degradation of ^{125}I-LDL nor did it alter the drug accumulation. When LDL receptor-negative fibroblasts from a patient with the homozygous form of familial hypercholesterolemia were incubated with the drug–^{125}I-LDL complex, cellular drug accumulation was very low. The drug–LDL complex inhibited the growth of cultured normal human fibroblasts. The drug incorporated into methylated LDL was much less toxic. These findings suggest that r11-DOX incorporated into LDL is delivered to cells selectively by the LDL receptor pathway. This might be of value in the treatment of leukemia, since we have previously found that leukemic cells exhibit higher LDL receptor activity than white blood cells and bone marrow cells from healthy subjects.

The main problem in cancer chemotherapy is not lack of activity but of selectivity. Thereby, toxic effects on normal cells limit the possibilities to treat patients. A possible way of reducing the undesired effects of antineoplastic drugs and concentrating the toxic effects to the malignant cells could be to link a potent anticancer agent to a carrier with a high affinity for the malignant cells. We have focused our attention on the possibility of using low-density lipoprotein (LDL) as a drug carrier in the treatment of leukemia.

LDL is the major cholesterol-carrying lipoprotein in human plasma. The spherical LDL particles with a diameter of 220 Å contain a lipid core of about 1500 cholesteryl ester molecules surrounded by a polar shell of free cholesterol, phospholipids, and protein.[1] Human cells express cell-surface receptors for LDL.[1] Once bound to its receptor, LDL is internalized and degraded in lysosomes. The lipid core of LDL yields unesterified cholesterol, which is used for membrane synthesis, whereas the protein part of LDL is degraded to amino acids.[1]

The reason why LDL is of special interest as a carrier for cytotoxic drugs is that leukemic cells isolated from patients with acute myelogenous leukemia have much higher LDL receptor activities (measured as the high-affinity degradation of ^{125}I-LDL) than normal white blood cells and nucleated bone marrow cells.[2] Gynecologic cancer cells also possess high LDL receptor activity both when assayed in monolayer culture and in membrane preparations from tumor-bearing nude mice.[3] Recently, an enhanced receptor-mediated uptake of LDL by tumor tissue in vivo was demonstrated in an animal model.[4]

The free and esterified cholesterol of LDL can be extracted with heptane and the particles can be reconstituted with exogenous hydrophobic compounds.[5] Such reconstituted LDL binds to the LDL receptor with the same affinity as native LDL and is internalized and degraded intracellularly. We took advantage of this fact and incorporated a very lipophilic cytotoxic agent, *N*-(trifluoroacetyl)adriamycin 14-valerate (AD 32) into LDL.[6] *N*-(Trifluoroacetyl)adriamycin 14-valerate is poorly soluble in water and has been withdrawn from further clinical trials because of lung toxicity due to the vehicle used to administer it—a mixture of ethanol and castor oil.[7] When white blood cells were incubated with the *N*-(trifluoroacetyl)adriamycin 14-valerate–LDL complex, cells with high LDL-receptor activity accumulated more drug than

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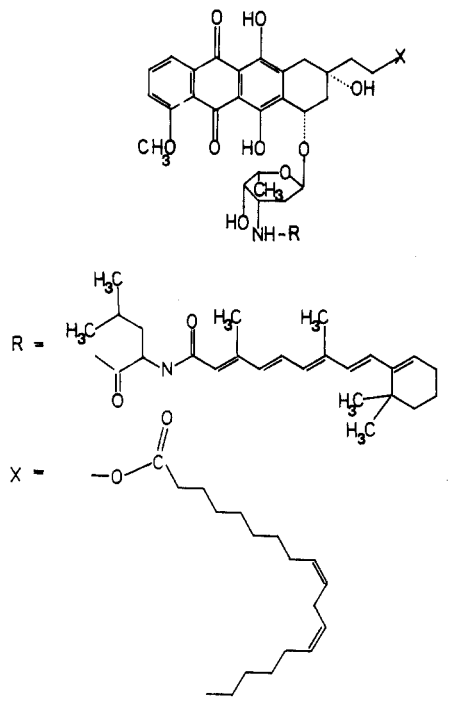

Figure 1. Structural formula of *N*-(*N*-retinoyl)-L-leucyldoxorubicin 14-linoleate; R = *N*-retinoyl-L-leucyl residue, X = linoleyl residue.

did cells with low receptor activity.[6] This was also observed when aclacinomycin A was incorporated into LDL by a mixing technique and the drug uptake studied in cultured human glioma cells.[8] However, in both cases a large part of the cellular drug uptake probably occurred by leakage of drug from the drug–LDL complex to the cells since the cellular drug accumulation markedly exceeded that which could be explained by uptake and degradation of the ^{125}I-LDL complex.

In the present study, we report the synthesis of a lipophilic anthracycline derivative which can be incorporated into LDL in such a way that it is selectively transported into cells by the LDL receptor pathway.

Chemistry. We have studied the possibility of converting doxorubicin into a derivative which could be incorporated into LDL. This drug was chosen because it has the broadest antitumoral spectrum of all antineoplastic drugs presently used.[9] Furthermore, doxorubicin is resistant to lysosomal enzymes,[10] which is a prerequisite for retaining its cytotoxic effects after being taken up into the lysosomes by the LDL receptor pathway. Finally, its chemical structure is assessible to suitable modifications. Doxorubicin can be esterified at the C-14 position and the primary amino group on its sugar moiety is suitable for an amide-type linkage (Figure 1). Krieger et al. have demonstrated that it is possible to reconstitute LDL with exogenous hydrophobic compounds containing cis unsaturated long-chain fatty acids or polyisoprenoids.[5] Consequently, we linked an unsaturated fatty acyl chain (linoleic acid) to the C-14 position of doxorubicin. The doxorubicin 14-linoleate derivative was synthesized from 14-bromodoxorubicin and sodium linoleate. We increased the lipophilicity of the compound by blocking the amino group with a retinoyl residue. However, the presence of

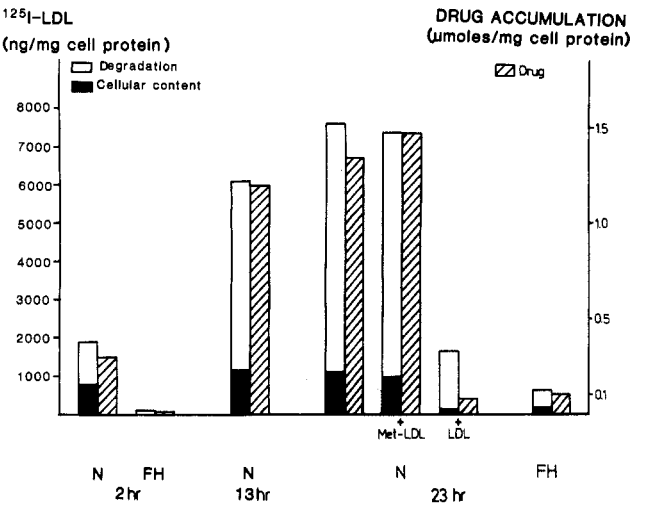

Figure 2. Cellular drug accumulation and cellular content and degradation of ^{125}I-LDL on incubation at 37 °C of normal fibroblasts (N) and receptor-negative fibroblasts from a subject with familial hypercholesterolemia (FH) with rll-DOX–^{125}I-LDL (9 μg/mL of LDL, 1.75 μM of drug) and the effects of adding excess (200 μg/mL) native and methylated LDL (Met-LDL). Each value shows the mean result from duplicate incubations; the range was less than ±10%.

a free amino group is crucial for the cytotoxic effects of anthracyclines.[11] Therefore, the amino acid leucine was intercalated as a spacer arm between the amine and the retinoyl residue, since this leads to a slow release of free anthracycline due to enzymatic cleavage of the peptide bonds in the lysosomes.[12,13]

Biological Data

Uptake and Degradation of rll-DOX–^{125}I-LDL. Figure 2 shows the cellular drug accumulation and the cellular uptake and degradation of ^{125}I-LDL when normal and receptor-negative human fibroblasts were incubated with rll-DOX–^{125}I-LDL (9 μg/mL of LDL, 1.75 μM of the drug). During the incubation, normal fibroblasts took up and degraded ^{125}I-LDL and accumulated the drug. After 2 h of incubation, the amount of parent drug corresponded to about 70% of the total cellular drug uptake. The rest consisted of two as yet unidentified metabolites. With time, the fraction present as metabolites increased. After 23 h of incubation, doxorubicin could also be detected. The ^{125}I-LDL part of the complex was taken up and degraded at the same rate as native ^{125}I-LDL (not shown). The presence of excess native LDL inhibited the uptake and degradation of the ^{125}I-LDL part of the complex. As estimated from the data in Figure 2, the high-affinity receptor-mediated uptake and degradation of ^{125}I-LDL were about 80% of the total. The remainder was probably due to unspecific bulk fluid endocytosis.[14] The presence of excess native LDL also strongly inhibited the cellular drug accumulation. In contrast, methylated LDL, which does not bind to the LDL receptor,[15] did not inhibit cellular uptake and degradation of ^{125}I-LDL nor did it reduce cellular drug accumulation. The perfect correlation be-

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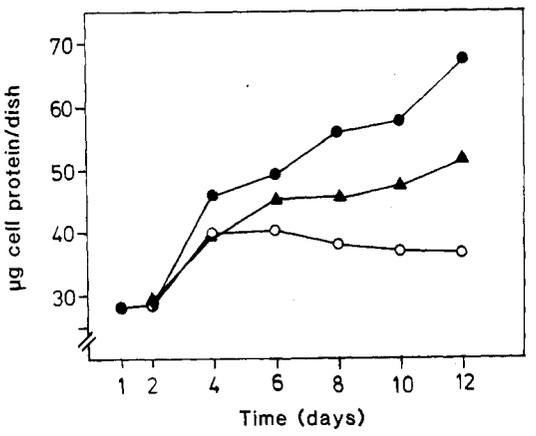

Figure 3. Time course for the growth-inhibitory effect of rll-DOX–LDL (○) (5 μg/mL of LDL, 0.67 μM of drug) and rll-DOX-methyl-LDL (▲) (7.9 μg/mL of Met-LDL, 0.67 μM of drug) upon incubation of normal fibroblasts at 37 °C. (●) = control. Each value represents the average of duplicate incubations; the range was less than ±10%.

tween cellular uptake and degradation of ^{125}I-LDL and cellular drug accumulation as well as the effects of native and methylated LDL in excess strongly support the hypothesis of a receptor-mediated uptake of rll-DOX–^{125}I-LDL. Furthermore, receptor-negative fibroblasts took up and degraded very little ^{125}I-LDL and accumulated far less drug than normal fibroblasts probably as a result of bulk fluid endocytosis.[14]

Cellular Growth Inhibition by rll-DOX–LDL. In the presence of rll-DOX–LDL (5 μg/mL of LDL, 0.66 μM of the drug) no growth of normal fibroblasts occurred after 4 days of incubation as shown in Figure 3. When the cells were incubated with rll-DOX incorporated into methylated LDL (7.9 μg/mL of methyl-LDL, 0.66 μM of the drug), the cells continued to grow over the time period studied, although at a slower rate than untreated cells. Thus, the toxicity data are also consistent with a receptor-mediated uptake of the complex. The difference in toxicity between the native and methylated LDL complexes was not as pronounced as could be expected from the accumulation data in Figure 2. However, it has to be noted that the experiments shown in Figures 2 and 3 were performed under quite different conditions. The experiment shown in Figure 2 was designed to obtain subconfluent cells possessing high LDL receptor activity during the short incubation period according to the schedule of Brown and Golstein.[16] However, in order to study the toxicity of the drug–LDL complexes, the incubation period had to be extended to several days. As seen in Figure 3, the cell growth rate was lower both at the beginning and at the end of the incubation than on days 2–4. The LDL receptor activity in cultured cells is very much dependent on the cell growth rate.[8,17] Thus, rapidly dividing cells express more LDL receptors than slowly dividing cells. In the experiment shown in Figure 3, the LDL receptor activity was of the same magnitude as that in the experiment shown in Figure 2 only on days 2–4 (not shown). Both before and after days 2–4 it was lower. This may explain the less pronounced difference between native and methylated rll-DOX–LDL complexes in the toxicity experiment as compared to the drug-uptake experiment. Besides receptor-mediated uptake of native LDL, both native and methylated LDL can enter cells by bulk fluid endocytosis. This is supported by the data in Figure 2, showing that receptor-negative fibroblasts with time accumulated small but significant amounts of LDL-incorporated drug. The relative significance of this nonspecific process diminishes with increased receptor activity.

The data do not provide any evidence as to whether the toxicity was exerted by rll-DOX as such or by its metabolites. It has not been possible to study the cellular uptake and toxicity of free rll-DOX because of its low water solubility. Chemical blockade of the amino group of anthracyclines markedly reduces the cytotoxic effects of the drugs.[11] However, previous work has shown that when an amino acid is intercalated between the amino group of an anthracycline and an acyl residue, there will be a slow release of the parent drug intracellularly, probably as a result of lysosomal hydrolysis.[12,13] It is therefore possible that the cytotoxic effect of rll-DOX was exerted by metabolites and free doxorubicin was, in fact, detected in the cells.

Krieger et al. have shown that 25-hydroxycholesteryloleate, a toxic cholesterol ester derivative, incorporated into LDL exerts LDL receptor-mediated toxicity.[18] A benzo[*a*]pyrene analogue incorporated into LDL that requires exposure to UV light to be toxic is also LDL-receptor selective.[19] However, the present study demonstrates that also cancer chemotherapeutics can be modified, incorporated into LDL and selectively delivered to cells by LDL receptors in vitro.

Studies are now in progress to investigate the in vivo fate of the rll-DOX–LDL complex and to see if the drug can be targeted to malignant cells with high LDL receptor activity in vivo.

Experimental Section

Materials. Retinoic acid (99% pure, no. R-2625), linoleic acid (sodium salt, 99% pure, no. L-8134), and *N*,*N*′-dicyclohexylcarbodiimide (no. D-3128) were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium ^{125}[I]iodide (carrier free, pH 7–11) was purchased from the Radiochemical Centre (Amersham, U.K.). Falcon tissue culture dishes, 35 × 10 mm, were from Becton Dickinson and Co. (Oxnard, CA), and 90 × 20 mm tissue culture dishes were obtained from A/S Nunc (Roskilde, Denmark). Fetal calf serum, L-glutamine, tissue culture medium RPMI 1640, and pencillin–streptomycin were obtained from Gibco-Biocult Ltd. (Paisley, Scotland). 14-Bromodoxorubicin hydrochloride was kindly supplied by Professor F. Arcamone, Milan, Italy.

Solutions in organic solvents were dried over Na_2SO_4 and filtered. Thin-layer chromatography (TLC) on silica gel 60 F_{254} (no. 5735, Merck, Darmstadt, BRD) was used for identification purposes and to test homogeneity. The TLC solvent systems used were chloroform–methanol (95:5 by volume) for hydrophobic compounds and chloroform–methanol–water (120:20:1 by volume) and dichloromethane–methanol–water–formic acid (85:12:1:2 by volume) for more polar compounds.

Chemical Synthesis. Doxorubin 14-Linoleate (1). This derivative was synthesized according to the method described by Arcamone et al.[20] for preparing doxorubicin 14-octanoate. In brief, 14-bromodoxorubicin hydrochloride (15-fold molar excess) was added to dried sodium linoleate in anhydrous acetone. The mixture was stirred at refluxing temperature for 2 h and filtered and the filtrate was evaporated. The crude solid was dissolved in methanol and 0.1 M HCl and the product was extracted with chloroform. The chloroform phase was evaporated to a small volume and the product was precipitated with excess diethyl ether and centrifuged. The supernatant contained large amounts of

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linoleic acid and the product was separated from the fatty acid by repeated precipitation with diethyl ether as described above. Finally, the pure product (yield 90%) was carefully dried under vacuum. The infrared (KBr) spectrum of the compound was consistent with the assigned structure.

***N*-L-Leucyldoxorubicin 14-Linoleate (2).** The synthesis was performed as described previously for the coupling of L-leucine to doxorubicin.[12,13] In brief, 1 was reacted with the *N*-hydroxysuccinimide ester of trityl-L-leucine (10% molar excess of the ester). The product was deprotected in concentrated acetic acid, neutralized with ammonia, extracted with chloroform, and finally purified by silica gel chromatography (Kieselgel 60, no. 7734, Merck, Darmstadt, BRD) with increasing concentrations of methanol in chloroform as the mobile phase. The product was eluted with 4% methanol in chloroform with a yield of approximately 50%.

***N*-(*N*-Retinoyl)-L-leucyldoxorubicin 14-Linoleate (3).** Retinoic acid was linked to the terminal amino group of **2** with use of carbodiimide as condensing agent. Retinoic acid (29 mg, 96 μmol) in 3 mL of dichloromethane was activated by dicyclocarbodiimide (10 mg, 48.5 μmol) at 4 °C to the corresponding symmetrical anhydride of the fatty acid. After 4 h, 30 mg (32.6 μmol) of **2** was added and the coupling reaction was carried out a t room temperature. After 60 h, the solvent was evaporated and the crude solid was dissolved in 2 mL of hexane–chloroform (80:20 by volume) and applied to a Sep-Pak silica cartridge, and the unreacted activated retinoic acid was eluted with hexane–chloroform (80:20). The percentage of hexane in the mixture was reduced stepwise and retinoic acid was eluted with hexane–chloroform (50:50). Hexane–chloroform (20:80 by volume) eluted the product. After evaporation of the solvent, small amounts of contaminating retinoic acid were separated from the product by dissolving the crude solid in benzene and precipitating the acid by the addition of 10–30 μL of 0.1 M NaOH. The benzene was evaporated, and the product was dissolved in chloroform and washed with H_2O. The chloroform phase was suspended with Na_2SO_4, filtered, evaporated, and dried under vacuum. The product (Figure 1), yield 50–80%, was readily soluble in benzene and carbon tetrachloride and slightly less soluble in diethyl ether. It was stored dissolved in benzene at −20 °C.

Lipoproteins. Human LDL (density 1.019–1.063 g/mL) and lipoprotein-deficient serum (density > 1.215 g/mL) were prepared by ultracentrifugation of serum from healthy subjects.[6] LDL was iodinated [6] and methylated[15] as described previously. All concentrations of LDL given refer to protein.

Incorporation of rll-DOX. LDL was extracted with heptane and the drug was incorporated as described previously for other hydrophobic compounds.[5] In brief, 1.9 mg of LDL, ^{125}I-LDL, or methylated LDL was lyophilized in the presence of insoluble starch, and the neutral lipids were removed by extracting three times with heptane. The heptane-extracted LDL was then reconstituted by the following sequential steps: addition of 200 μL of carbon tetrachloride containing 2–3 mg of the drug at 4 °C, evaporation of the solvent under nitrogen at 4 °C, solubilization of the reconstituted LDL in 10 mM Tricine (pH 8.4), and removal of the starch and unincorporated material by centrifugation. The rll-DOX–LDL complex (protein recovery 50–75%, drug recovery 5–10%) contained 100–200 drug molecules/LDL particle, corresponding to a drug/LDL protein weight ratio ranging from 0.13 to 0.26. When the complex was dialyzed extensively against 0.15 M NaCl containing 0.3 mM EDTA for 48 h, the drug/LDL protein weight ratio was not altered. During incubation of cells with the complex, rll-DOX constituted 90% of the total fluorescence in the medium after 23 h at 37 °C as determined by HPLC analysis.

Cell Incubations. Fibroblasts from a healthy 13-year-old girl (abdominal wall biopsy) were grown in monolayer culture. Fibroblasts from a patient with the LDL receptor-negative form of homozygous familial hypercholesterolemia (GM 1915) were obtained from The Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, NJ and grown in monolayer culture. The cells were maintained in a humidified incubator (5% CO_2/95% air) at 37 °C in a growth medium consisting of RPMI-1640 medium supplemented with antibiotics (100 IU of penicillin + 100 μg of streptomycin/mL), L-glutamine (1%), and 10% fetal calf serum. For experiments, confluent cell monolayers in 175-cm^2 stock flasks were detached with a solution containing 0.05% trypsin and 0.02% EDTA and seeded (day 0) at a concentration of 270 000 cells/dish into 90-mm petri dishes containing 8 mL of growth medium. On day 3, the medium was replaced by 8 mL of fresh medium. On day 5, each monolayer was washed with 8 mL of RPMI-1640, and 5 mL of RPMI-1640 medium (antibiotics + glutamine as above) containing 10% human lipoprotein deficient serum (medium A) was added. The experiments were started on day 7 after the cells had been incubated for 48 h in medium A. The incubations were performed in 4 mL of fresh medium A and terminated by collecting the medium and washing the dishes at 4 °C, three times with 10 mL of phosphate-buffered saline (PBS), pH 7.4, containing 0.2% (w/v) bovine serum albumin, and three times with 10 mL of PBS alone. The cells were harvested with a rubber policeman in 2 mL of H_2O and sonicated with a 100-W ultrasonic disintegrator (cat. no. 7100 MSE, London, England). Aliquots, were taken for determination of radioactivity, protein, and drug concentration. Blank incubations were performed as above but in the absence of cells.

To study the effect of drug–LDL complexes on cell growth, normal human fibroblasts were seeded (day 0) at a concentration of 50 000 cells/dish in 35-mm petri dishes in 2 mL of medium A. On day 1 the medium was changed to 1 mL of fresh medium A and the indicated additions. The medium was changed daily thereafter. After the indicated incubation time, the cells were washed three times with 3 mL of PBS, detached with 1.2 mL of 0.5 M NaOH, and then stored at −20 °C until assayed for cell protein as a measure of cell growth.

Cellular Uptake and Degradation of ^{125}I-LDL. The cellular content of ^{125}I-LDL was determined from the radioactivity in aliquots of the washed detached cells. The cellular degradation of ^{125}I-LDL was quantitated from an aliquot of the incubation medium as described earlier.[14] In brief, undegraded, high molecular weight ^{125}I-LDL was precipitated with trichloroacetic acid (TCA) and the degradation of ^{125}I-LDL was determined from the TCA-soluble radioactivity after removal of free iodine by oxidation with H_2O_2 and extraction with chloroform. The high-affinity degradation of ^{125}I-LDL was calculated as the difference in cellular degradation of ^{125}I-LDL in the presence and absence of excess unlabeled LDL as described previously.[14] Radioactivity was measured in a Packard autogamma scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, IL).

Assays. The drug content of LDL complexes and cellular drug accumulation were determined by high-performance liquid chromatography using fluorometric detection as described earlier.[21] The samples, frozen at −20 °C until assayed, were extracted with a chloroform–methanol mixture (4:1 by volume). The Lichrosorb Si-60 column (Hibar, 25 cm × 4 mm, Merck) was eluted with a mixture of chloroform, acetic acid, methanol, and 0.3 mM $MgCl_2$ (973.4:15:13:0.6 by volume) at a flow rate of 1.5 mL/min. With this flow rate, the sample front eluted at 1.5 min and rll-DOX at a retention time of 2.2 min.

Standard curves were prepared by extraction of rll-DOX in the presence of an appropriate amount of cell protein or LDL. Unidentified metabolites were quantitated as drug equivalents, assuming identical fluorescence properties. Protein content was measured by the method of Lowry et al.,[22] using bovine serum albumin as standard.

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Registry No. 1, 94731-64-3; 2, 94731-65-4; 3, 94731-66-5; 14-bromodoxorubicin hydrochloride, 29742-67-4; sodium linoleate, 822-17-3; trityl-L-leucine *N*-hydroxysuccinimide ester, 94731-67-6; retinoic acid, 302-79-4.

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