Characteristics of Association of Oleoyl Derivatives of 5-Fluorodeoxyuridine and Methotrexate with Low-Density Lipoproteins (LDL)

P. Chris de Smidt[1] and Theo J. C. van Berkel[1,2]

Received July 11, 1991; accepted October 7, 1991

In order[1] to prepare cytotoxic drug-low density lipoprotein (LDL) particles, we have synthesized lipophilic prodrugs of two broad-spectrum antineoplastic agents, methotrexate (MTX) and 5-fluorodeoxyuridine (FUdR), by coupling them to oleic acid. ^{3}H-Labeled prodrugs were incorporated in ^{125}I-LDL carriers, allowing the study of both drug and carrier simultaneously. Utilizing the dry film procedure, monooleoyl FUdR showed the highest incorporation efficiency with over 40% of the initial drug associated with LDL. The prepared prodrug–LDL solution was found to be a single complex of 30 molecules of prodrug per LDL particle when examined by agarose gel electrophoresis and density gradient ultracentrifugation. No unbound $FUdROl_1$ could be recovered, indicating that all dissolved prodrug associates with LDL. After incubation of $FUdROl_1$ with human serum, 22% of the compound associates with lipoproteins and 78% was recovered in the albumin-containing fraction. When human serum was pretreated with oleic acid in order to saturate albumin's fatty acids binding sites, a strong decrease (from 78 to 22%) in association to the albumin-containing fraction was observed, accompanied by a threefold increase (from 22 to 67%) in lipoprotein association. It is concluded that existing hydrophilic antineoplastic agents can be successfully modified in order to achieve association with LDL. This may ultimately lead to an increased delivery of cytotoxic drugs to tumor cells.

KEY WORDS: 5-fluorodeoxyuridine (FUdR); methotrexate (MTX); low-density lipoprotein (LDL); high-density lipoproteins (HDL); phosphate-buffered saline (PBS); monooleoyl FUdR ($FUdROl_1$).

INTRODUCTION

The LDL receptor pathway can be considered an attractive possibility for the specific delivery of antineoplastic agents to their target tissues (1–3). A variety of tumor cell types, *in vitro* (4,5) as well as *in vivo* (6,7), expresses high amounts of LDL receptors on their cell membranes. By active internalization of LDL these rapidly dividing cells acquire the cholesterol necessary for the stability of cell membranes. The LDL receptor pathway (8) can potentially introduce large amounts of LDL-associated drugs into the cell.

Essential for a successful delivery of a compound via this mechanism is an adequate incorporation into LDL. Only lipophilic drugs or prodrugs such as the anthracycline AD-32 (9), "compound 25" (10), or oleoyl-ellipticine (11) were reported to be incorporated into LDL. The degree of lipophilicity was assumed to be an important factor for drug incorporation into LDL.

Water-insoluble drugs, however, require rigorous procedures for LDL incorporation, e.g., the delipidation-reconstitution method, which can introduce hundreds of drug molecules per LDL but impairs the physiological recognition of the particle (12).

Drugs with a mixed hydrophobic/hydrophilic character such as cyclosporin A (13) and Tris-gal-chol (14) possess a natural affinity for lipoproteins and can spontaneously associate with lipoprotein particles.

In the present investigation, we have derivatized two broad-spectrum antineoplastic drugs, methotrexate and 5-fluorodeoxyuridine (FUdR; floxuridine) with oleic acid, in order to study the possibilities for nondestructive incorporation into LDL. All syntheses were performed with ^{3}H-labeled antineoplastic drugs, which facilitated the determination of incorporation efficiency and possible redistribution.

We have compared the spontaneous association of dioleylmethotrexate ($MTXOl_2$), monooleoylfloxuridine ($FUdROl_1$), and dioleoylfloxuridine ($FUdROl_2$) with LDL, as analyzed by agarose gel electrophoresis and density ultracentrifugation. Further, their affinity for lipoproteins is compared with their binding to albumin.

MATERIALS AND METHODS

Materials

^{125}I, sodium salt (98.5% pure), was obtained from Amersham, UK. [3′,5′,7-^{3}H]Methotrexate (sodium salt, 98.5% pure) was obtained from Amersham. 5-[6-^{3}H]FUdR (99.9% pure, No. NET 774) was from New England Nuclear, Boston, MA. Agarose was purchased from Sigma Chemical Co., St. Louis, MO. Potassium bromide was purchased from J. T. Baker, Deventer, The Netherlands. 5-Fluorodeoxyuridine was kindly provided by Hoffmann-LaRoche Ltd., Mijdrecht, The Netherlands. Methotrexate was a gift from Pharmachemie, Haarlem, The Netherlands. [^{3}H]Dioleylmethotrexate was synthesized by the neutral esterification of methotrexate with alkylhalide and cesium carbonate according to the method of Rosowsky and Yu (15). The procedure as described by Nishizawa and Casida (16), utilizing oleoylchloride, was followed to convert [^{3}H]FUdR to [^{3}H]monooleoyl FUdR and [^{3}H]dioleoyl FUdR. Purity of the obtained lipophilic prodrugs was 98% as assessed by thin-layer chromatography (TLC) and nuclear magnetic resonance (NMR).

Lipoproteins

LDL was isolated from human plasma at density $1.024 < d < 1.055$ g/ml by two repetitive centrifugations according to the method of Redgrave *et al.* (17) as described previously (14). The LDL preparation contained solely apolipoprotein B (99.97%, MW 514,000), and no degradation products were noticeable when checked by electrophoresis in sodium dodecyl sulfate gels. Radioiodination of LDL was done accord-

[1] Division of Biopharmaceutics, Center for Bio-Pharmaceutical Sciences, Sylvius Laboratory, University of Leiden, P.O. Box 9503, 2300 RA Leiden, The Netherlands.
[2] To whom correspondence should be addressed.

0724-8741/92/0400-0565$06.50/0

ing to the ^{125}I-iodine monochloride method described by Bilheimer *et al.* (18).

Incorporation of Lipophilic Prodrugs in LDL

[^{3}H]Monooleoylfloxuridine (150 μl, 32 μg, and 63 nmol, dissolved in toluene) was added to a glass tube. The drug was coated on the surface of the tube by evaporation under N_2. After complete evaporation of all solvent, 200 μl of a solution of ^{125}I-LDL (260 μg LDL protein, in phosphate-buffered saline (PBS)–1 m*M* EDTA, pH 7.4) was added. The mixture was incubated for 4 hr at 37°C under N_2 while shaking continuously.

For the experiment described in Fig. 1, 25 nmol of $MTXOl_2$, $FUdROl_1$, and $FUdROl_2$ was coated onto the surface of a glass tube and, after extensive evaporation under N_2, incubated with 200 μl of a solution of LDL (520 μg protein, 1 nmol LDL protein, in PBS–1 m*M* EDTA, pH 7.4). The mixture was incubated for 4 hr at 37°C under N_2 while shaking continuously. Samples of the incubation mixture were then analyzed by agarose gel electrophoresis as described below.

The log *P* of the lipophilic prodrugs was determined according to a modification of the procedure described by Yeh and Higuchi (19) and Hobbelen *et al.* (20). Forty nanomoles of the ^{3}H-labeled compound was coated on 4-ml stoppered glass vials. Octanol, 1.0 ml, and PBS–1 m*M* EDTA, pH 7.4, 1.0 ml, were added and the samples were incubated on a Luckham Multimix MM1 Rollerbank for 24 hr at room temperature. Samples of octanol and aqueous phase were then assayed for ^{3}H radioactivity utilizing a Packard 1500 Tri-Carb liquid scintillation analyzer.

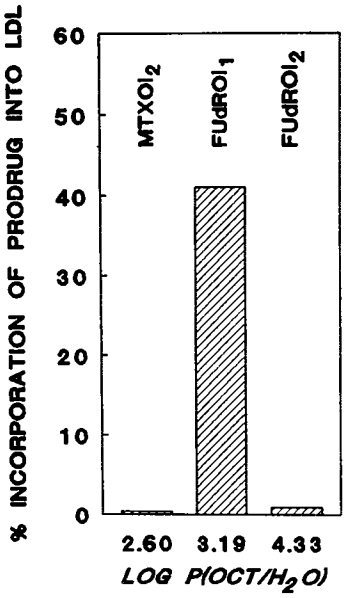

Fig. 1. Efficiency of incorporation of lipophilic derivatives of MTX and FUdR into LDL in relation to the octanol/water distribution. ^{3}H-Labeled prodrugs (25 nmol) were incubated with a solution of 1 nmol of LDL according to the dry film procedure (21). After incubation for 4 hr at 37°C, samples were subjected to agarose gel electrophoresis. The LDL-containing segments in the gels were excised and counted for radioactivity. In addition, the octanol/water distribution of the prodrugs was determined (expressed as log *P*).

Agarose Gel Electrophoresis

Aliquots of ^{3}H-drug–^{125}I-LDL complexes or ^{3}H-$FUdROl_1$–human serum albumin (HSA) preparations were subjected to electrophoresis in agarose gels at pH 8.8 (Tris–hippuric acid buffer). After electrophoresis, the gel was cut into segments, which were then treated with 750 μl of soluene. After 20 hr, 10.0 ml scintillation cocktail was added and the samples were assayed for ^{3}H radioactivity (^{3}H-$FUdROl_1$–HSA preparation) or ^{3}H and ^{125}I radioactivity utilizing a Packard 1500 Tri-Carb liquid scintillation analyzer equipped with software validated for ^{3}H–^{125}I-double-labeled samples.

Distribution over Serum (Lipo)Proteins—Density Gradient Ultracentrifugations

[^{3}H]Monooleoylfloxuridine (20 μl, 4.2 μg, dissolved in toluene) was added to a glass tube. After coating of the compound on the surface after extensive evaporation under N_2, 1.0 ml of human serum was added and the solution was incubated for 4 hr at 37°C under N_2 while shaking continuously.

For the redistribution studies, $FUdROl_1$–LDL particles were prepared as described under Incorporation of Lipophilic Prodrugs in LDL. Twenty microliters of this solution was then added to either 500 μl human serum or 500 μl human serum that was preincubated with 3 mg oleic acid. The solutions were subsequently incubated for 4 hr at 37°C under N_2 while shaking continuously.

After the incubations were completed, the samples were transferred to polyallomer centrifuge tubes. Thereafter, 1106 mg of solid KBr and PBS–1 m*M* EDTA, pH 7.4, was used for the complete dissolution of KBr to a final volume of 4.0 ml. Consecutive layers of 3.0 ml of KBr solution (1.063, 1.019, and 1.006 g/ml, respectively) were then added. After centrifugation, 500 μl samples, starting from the bottom of the tube, were taken and the gradient was subdivided according to density. Three hundred microliters of the fractions was mixed with 5.0 ml of scintillation cocktail and the samples were assayed for radioactivity.

RESULTS

Incorporation of Lipophilic Prodrugs into LDL

The efficiency of incorporation of lipophilic prodrugs for LDL was investigated by determining their spontaneous incorporation from a drug-coated glass tube. Twenty-five nanomoles of ^{3}H-labeled prodrug was coated onto a glass vial and subsequently incubated at 37°C with a solution containing 1 nmol of LDL in buffer. The resulting solution was then subjected to agarose gel electrophoresis in order to separate potentially solubilized LDL-unbound prodrugs from LDL-associated prodrugs. The LDL-containing fraction was then removed from the agarose gel and counted for radioactivity. Less than 2% of $MTXOl_2$ and $FUdROl_2$ became associated with LDL, while this value was over 40% for $FUdROl_1$ (Fig. 1). A determination of the hydrophobicity of the three prodrugs (octanol/water distribution) indicates increasing hydrophobicity from dioleylmethotrexate to monooleoyl FUdR and dioleoyl FUdR.

Agarose Gel Electrophoresis

Because of the low incorporation of $MTXOl_2$ and $FUdROl_2$ into LDL compared to the excellent association of $FUdROl_1$, we have further analyzed the LDL-association properties of this compound.

After incubation of ^{3}H-$FUdROl_1$ with ^{125}I-LDL at 37°C for 4 hr, the drug–LDL-containing solution was subjected to agarose gel electrophoresis. Figure 2A shows that both ^{3}H and ^{125}I radioactivities were recovered at the same position in the gel. As a control, ^{3}H-$FUdROl_1$ was incubated with human serum albumin and analyzed by the same technique. ^{3}H Radioactivity was now recovered at a distinct position. Control ^{125}I-LDL migrated to a position 2.75 cm from the starting spot (Fig. 2C), which is similar to the $FUdROl_1$–LDL complex.

Density Ultracentrifugation

^{3}H-$FUdROl_1$/^{125}I-LDL solutions were analyzed by density-gradient ultracentrifugation in order to assess if the incorporated drug changed the density of LDL. Upon density-gradient ultracentrifugation, both ^{3}H and ^{125}I radioactivities were recovered in the fractions of the LDL density range between 1.019 and 1.063 g/ml (Fig. 3A).

In order to analyze the distribution of $FUdROl_1$ in serum, the compound was incubated with 1.0 ml of freshly prepared human serum for 4 hr at 37°C. The solution was then analyzed by density-gradient ultracentrifugation (Fig. 3B). Approximately 78% of $FUdROl_1$ was recovered in the fraction at density >1.21 g/ml. Twenty-two percent had distributed over the lipoprotein-containing fractions.

When preformed drug–LDL complexes are incubated with human serum, a density ultracentrifugation pattern similar to that in Fig. 3B is observed (Fig. 4A). Pretreatment of human serum with oleic acid and subsequent incubation with preformed $FUdROl_1$–LDL particles result in a significant decrease (from 78 to 33%) in radioactivity in the fraction at density >1.21 g/ml. The HDL- and LDL-containing fractions show a threefold increase in ^{3}H activity, while the VLDL/chylomicron-containing fractions ($d < 1.006$) do not show any significant change in radioactivity.

DISCUSSION

We have derivatized two broad-spectrum antineoplastic agents, FUdR and MTX, with oleic acid side chains. Oleic acid was chosen as "LDL anchor," as previous investigations have reported successful incorporation of "compound 25" (10) and a lipophilic doxorubicin derivative (6), both compounds that are coupled to oleyl side chains. The synthesis of oleyl derivatives of two drugs allowed identification of possible structural requirements that may be favorable for incorporation.

Of the three prodrugs investigated, the monooleoyl derivative of FUdR clearly had the highest incorporation efficiency. The method used to measure the incorporation efficiency is based on the dry film procedure described earlier by Shaw *et al.* (21). In this experimental setup, both water solubility and affinity for the lipoprotein will determine the final outcome. For a compound that is too water insoluble, as is the case with $FUdROl_2$ [log P(oct/H_2O) = 4.33], the initial partitioning from the glass surface in the aqueous phase may become the rate-limiting step. The data suggest that log P (oct/H_2O) gives only a limited indication about the incorporation efficiency. Structural requirements clearly

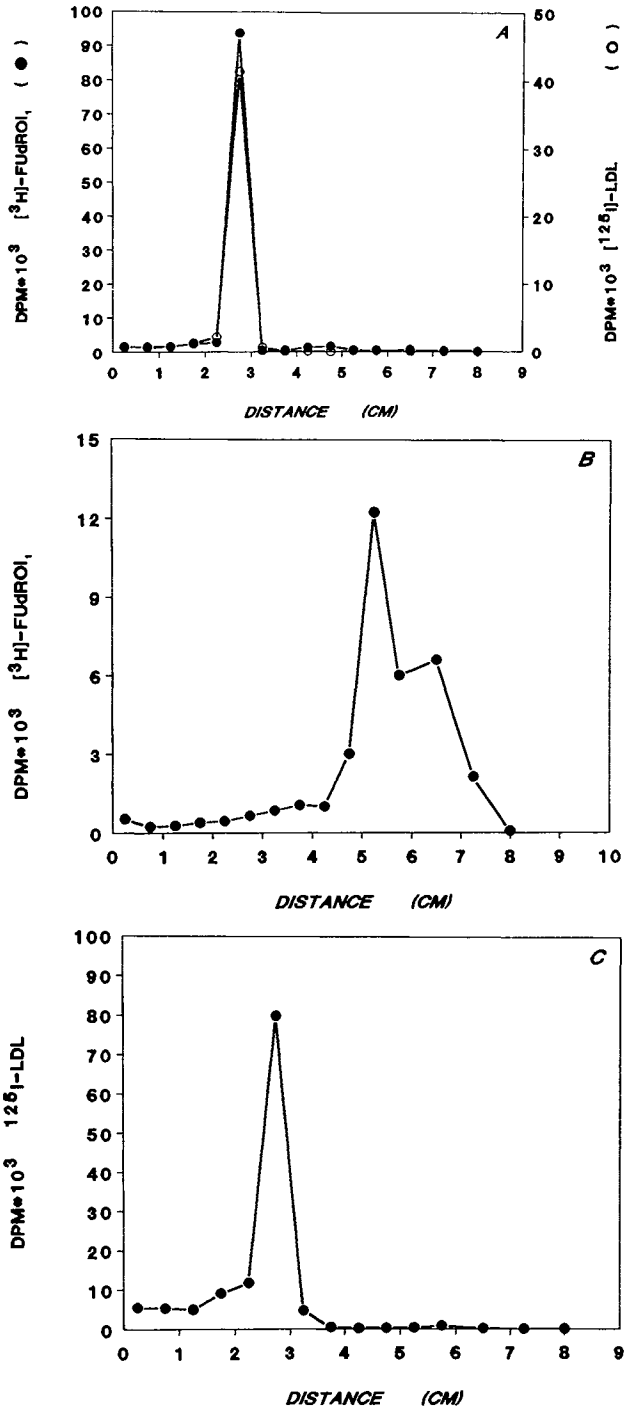

Fig. 2. Agarose gel electrophoresis of $FUdROl_1$–LDL solutions compared to albumin-bound $FUdROl_1$ or native LDL. Thirty-two micrograms of [^{3}H]$FUdROl_1$, coated on a glass vial, was incubated with 260 μg of ^{125}I-LDL (A) or with 6.8 mg of human serum albumin (B) for 4 hr at 37°C. (C) Native LDL. After agarose gel electrophoresis, the gels were cut into segments which were then assayed for ^{3}H and/or ^{125}I radioactivity.

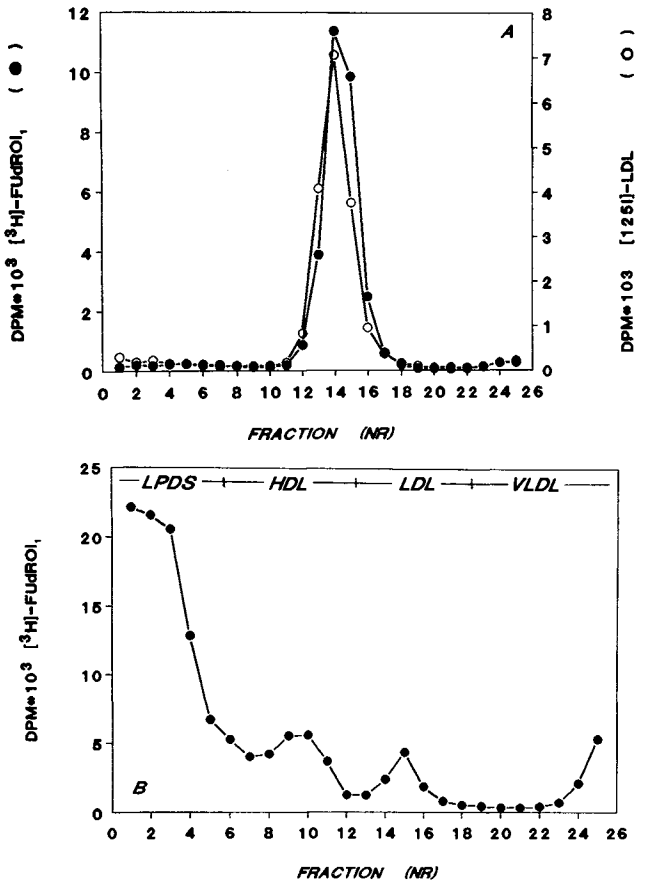

Fig. 3. Density ultracentrifugation of solutions of $FUdROl_1$ that were incubated with LDL (A) or human serum (B). [^{3}H]$FUdROl_1$ was incubated with LDL or human serum according to the dry film method. After density ultracentrifugation, tubes were subdivided according to density and assayed for radioactivity. LPDS, lipoprotein-deficient serum; VLDL, very low-density lipoprotein-containing fraction.

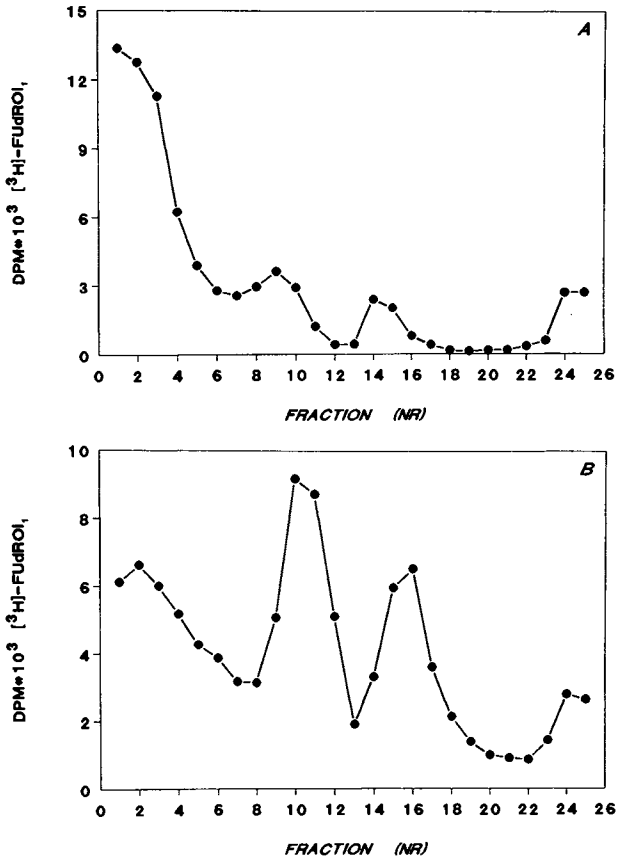

Fig. 4. Effect of the preincubation of human serum with oleic acid on the association of $FUdROl_1$ with serum (lipo)proteins. Preformed ^{3}H-$FUdROl_1$–LDL particles were incubated for 4 hr at 37°C with 0.5 ml of human serum (A) or with 0.5 ml of human serum that was preincubated with 3 mg of oleic acid (B). After density ultracentrifugation, the tubes were subdivided according to density and assayed for radioactivity.

play a role since dioleyl MTX, which has a superior water solubility, has a significantly lower incorporation efficiency than $FUdROl_1$.

All further experiments were focused on $FUdROl_1$, as this was the compound with the most favorable incorporation efficiency. Synthesis of this compound was carried out with ^{3}H-labeled FUdR precursors, allowing to prepare double-labeled drug–LDL carrier particles by incubating ^{3}H-$FUdROl_1$ with ^{125}I-LDL.

Two independent physicochemical methods were chosen to analyze ^{3}H-$FUdROl_1$–LDL solutions in order to examine the purity of the complex. When ^{3}H-$FUdROl_1$–^{125}I-LDL solutions were analyzed by agarose gel electrophoresis, a single ^{3}H/^{125}I peak was recovered in the gel with the same relative electrophoretic mobility as native LDL. No ^{3}H radioactivity was located in the gel other than at the LDL position. This indicates that all $FUdROl_1$ that partitions from the glass vial into the aqueous phase associates with LDL. The control ^{3}H-$FUdROl_1$–albumin preparation was found to migrate to a clearly different position in the agarose gels.

Analysis of ^{3}H-$FUdROl_1$–^{125}I-LDL solutions by density ultracentrifugation also indicated that a single drug–LDL particle had formed, with no unbound drug present in the solution. The drug–LDL particles had an average incorporation of 30 molecules of $FUdROl_1$ per LDL particle. Initial efforts to measure the effect of incorporation of $FUdROl_1$ on the size of the lipoproteins by a light-scattering method (Malvern system 4700 C particle analyzer) indicate that the drug–LDL particle has the same size as native LDL (23 nm).

In order to examine the distribution of $FUdROl_1$ over the different serum (lipo)proteins, ^{3}H-$FUdROl_1$ was incubated with human serum (dry film method) and the mixture was subsequently analyzed by density gradient ultracentrifugation. Seventy-eight percent of the compound was recovered in the lipoprotein-deficient fraction (LPDS) of serum, and 22% associated with HDL, LDL, and VLDL/chylomicrons. As albumin, which is one of the main proteins in the LPDS fraction, is known to bind a variety of endogenous and exogenous compounds, we tried to examine if albumin was possibly responsible for the high association of $FUdROl_1$. Albumin is known to possess high-affinity sites for fatty acids (22). After saturation of these binding sites by pretreatment of human serum with a solution of oleic acid and subsequent incubation with ^{3}H-$FUdROl_1$, a significant decrease (from 78 to 33%) in the amount of $FUdROl_1$ recovered in the albumin density fraction was observed. This was

accompanied by a strong increase (from 22 to 67%) in the amount of $FUdROl_1$ associated with HDL and LDL. The data suggest that an equilibrium exists between $FUdROl_1$ bound to fatty acid binding sites on albumin and lipoproteins present in serum. This equilibrium can be influenced by compounds that compete for the fatty acid binding sites on albumin.

It is concluded that existing antineoplastic drugs can be modified in order to increase their affinity for lipoproteins. Further modifications are now being explored that will increase and stabilize incorporation into LDL, resulting in a favorable delivery of the compound into tumor cells.

REFERENCES

1. R. E. Counsell and R. C. Pohland. Lipoproteins as potential site-specific delivery systems for diagnostic and therapeutic agents. *J. Med. Chem.* 25:1115–1120 (1982).
2. A. Catapano. Transport of cytotoxic compounds to cells via the LDL pathway. *Med. Sci. Res.* 15:411–413 (1987).
3. M. Krieger, M. S. Brown, J. R. Faust, and J. L. Goldstein. Replacement of endogenous cholesteryl esters of low density lipoprotein with exogenous cholesteryl linoleate. *J. Biol. Chem.* 253:4093–4101 (1978).
4. D. Gal, M. Ohashi, P. C. MacDonald, H. J. Bucksbaum, and E. R. Simpson. Low density lipoprotein as a potential vehicle for chemotherapeutic agents and radionucleotides in the management of gynecologic neoplasms. *Am. J. Obst. Gynecol.* 133:877–885 (1981).
5. D. Gal, P. C. MacDonald, J. C. Porter, and E. R. Simpson. Cholesterol metabolism in cancer cells in monolayer culture. III. LDL metabolism. *Int. J. Cancer* 28:315–319 (1981).
6. S. Vitols, M. Bjorkholm, G. Gahrton, and C. Peterson. *Lancet* 2:1150–1154 (1985).
7. G. Norata, G. Canti, L. Ricci, A. Nicolin, E. Trezzi, and A. L. Catapano. In vivo asimilation of LDL by a fibrosarcoma tumour cell line in mice. *Cancer Lett.* 25:203–208 (1984).
8. M. S. Brown and J. L. Goldstein. A receptor-mediated pathway for cholesterol homeostasis. *Science* 232:34–47 (1986).
9. S. G. Vitols, G. Gahrton, and C. Peterson. Significance of the LDL receptor pathway for the in vitro accumulation of AD-32 incorporated into LDL in normal and leukemic white blood cells. *Cancer Treat. Rep.* 68:515–520 (1984).
10. R. A. Firestone, J. M. Pisano, J. R. Falck, M. M. McPhaul, and M. Krieger. Selective delivery of cytotoxic compounds to cells by the LDL pathway. *J. Med. Chem.* 27:1037–1043 (1984).
11. M. Samadi-Baboli, G. Favre, J. Bernadou, D. Berg, and G. Soula. Comparative study of the incorporation of ellipticine-esters into low-density lipoprotein (LDL) and selective cell uptake of drug-LDL via the LDL receptor pathway in vitro. *Biochem. Pharmacol.* 40:203–212 (1990).
12. P. C. de Smidt and Th. J. C. van Berkel. Prolonged serum half-life of antineoplastic drugs by incorporation into the low density lipoprotein. *Cancer Res.* 50:7476–7483 (1990).
13. M. Lemaire and J. P. Tillement. Role of lipoproteins and erythrocytes in the in vitro binding and distribution of cyclosporin A in the blood. *J. Pharm. Pharmacol.* 34:715–718 (1982).
14. Th. J. C. van Berkel, J. K. Kruijt, H. H. Spanjer, J. F. Nagelkerke, L. Harkes, and H. J. M. Kempen. The effect of a water-soluble tris-galactoside-terminated cholesterol derivative on the fate of low density lipoproteins and liposomes. *J. Biol. Chem.* 260:2694–2699 (1985).
15. A. Rosowsky and C.-S. Yu. New methods of neutral esterification of methotrexate and related compounds. *Dev. Biochem.* 4:273–277 (1978).
16. Y. Nishizawa and J. E. Casida. 3′,5′-Diesters of 5-fluoro-2′-deoxyuridine: Synthesis and biological activity. *Biochem. Pharmacol.* 14:1605–1619 (1965).
17. T. G. Redgrave, D. C. K. Roberts, and C. E. West. Separation of plasma lipoproteins by density gradient ultracentrifugation. *Anal. Biochem.* 65:42–49 (1975).
18. D. W. Bilheimer, S. Eisenberg, and R. I. Levy. The metabolism of very low density lipoproteins. I. Preliminary in vitro and in vivo observations. *Biochim. Biophys. Acta* 280:212–221 (1972).
19. K. C. Yeh and W. I. Higuchi. Oil-water distribution of p-alkylpyridines. *J. Pharm. Sci.* 65:80–85 (1976).
20. P. M. J. Hobbelen, A. Coert, J. A. A. Geelen, and J. van der Vies. Interactions of steroids with serum lipoproteins. *Biochem. Pharmacol.* 24:165–172 (1975).
21. J. M. Shaw, K. V. Shaw, S. Yanovich, W. S. Futch, A. Rosowsky, and L. B. Schook. Delivery of lipophilic drugs using lipoproteins. *Ann. N.Y. Acad. Sci.* 507:252–271 (1987).
22. D. S. Goodman. The interaction of human serum albumin with long-chain fatty acid anions. *J. Am. Chem. Soc.* 80:3892–3898 (1958).