Biochemical Pharmacology, Vol. 40, No. 2, pp. 203–212, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00
Pergamon Press plc

COMPARATIVE STUDY OF THE INCORPORATION OF ELLIPTICINE-ESTERS INTO LOW DENSITY LIPOPROTEIN (LDL) AND SELECTIVE CELL UPTAKE OF DRUG–LDL COMPLEX VIA THE LDL RECEPTOR PATHWAY *IN VITRO**

MEHDI SAMADI-BABOLI,† GILLES FAVRE,‡ JEAN BERNADOU,§ DANIELLE BERG‡ and GEORGES SOULA‖

† Laboratoire de Biochimie, Faculté des Sciences Pharmaceutiques, 35 Chemin des Maraichers, 31062 Toulouse Cedex; ‡ Departement de Biologie Clinique du Centre Claudius-Regaud, 20–24 Rue du pont St-Pierre, 31052 Toulouse Cedex; and § Laboratoire de Chimie de Coordination du CNRS, 205 Route de Narbonne, 31077 Toulouse Cedex, France

(*Received* 4 *September* 1989; *accepted* 23 *January* 1990)

Abstract—Esters of elliptinium with stearic (ST-NME), palmitic (PAL-NME) or oleic (OL-NME) acids, a series of lipophilic derivatives of ellipticine, were synthetized, in order to evaluate their incorporation into Low Density Lipoprotein (LDL). Among the three derivatives, OL-NME shows the most potent incorporation (83 μg/mg protein LDL) compared to ST-NME (37 μg/mg protein LDL) and PAL-NME (58 μg/mg protein LDL). The size of OL-NME-LDL was determined by size distribution particles, showing their homogeneity compared to native LDL. When culture normal human fibroblasts were incubated with [^{125}I]LDL incorporated drug, they bound to the LDL receptor with the same affinity as native LDL and were internalized and degraded intracellularly. The presence of excess native LDL inhibited the cellular uptake and degradation of [^{125}I]drug-LDL. We have used [^{125}I]acetyl-LDL as a probe for a binding site on macrophages that mediated the uptake and degradation of chemically altered or denatured LDL. Mouse peritoneal macrophages were shown to take up and degrade [^{125}I]acetyl-LDL at rates that were greater than those for the uptake and degradation of native [^{125}I]LDL and [^{125}I]drug-LDL. The *in vitro* cytotoxic test on L1210 murine leukemic cells demonstrated that the complex was cytotoxic and was more effective than the free drug. This cytotoxic activity of the drug–LDL complex depends on the LDL high affinity receptor since the addition of native LDL reduces the killing power. In contrast, methylated LDL, which does not bind to the LDL receptor, has no effect on it. We conclude that it is possible to incorporate a large amount of cytotoxic drug into LDL without modifying their cellular metabolism via the high affinity LDL receptor pathway. It indicates also that the delivery of lipophilic drugs using LDL might provide distinct advantages over the use of synthetic carriers.

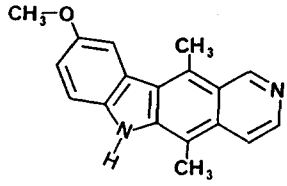
Elliptinium acetate (9-OH-NME¶) is an antineoplastic agent derived from ellipticine, a natural alkaloid, by a hydroxyl function in position 9 and a methyl group on the nitrogen in position 2 (Fig. 1). This drug is highly cytotoxic against L1210 cell [1] and is currently used in the treatment of metastatic breast cancer [2, 3]. Ellipticine derivatives intercalate into DNA and altered the catalytic activity of topoisomerase II *in vitro* [4] but the cytotoxicity of these drugs has been associated with the presence of a hydroxyl function in a position 9 [1]. In fact, 9-OH-NME is easily oxidized by peroxidase [5, 6] *in vitro*, with hydrogen peroxide as electron acceptor, to yield a quinoneimine (9-oxo-NME) able to form covalent adducts with different nucleophiles. Rat and human metabolic studies have revealed a 10-(*S*)-conjugate of 9-OH-NME [7, 8], suggesting that 9-OH-NME can also be oxidized *in vivo*. Moreover for the family of ellipticine derivatives, other mechanisms have been proposed and the role of metabolic activation in cellular toxicity of these drugs is still discussed [9, 10].

The toxicity of such drugs has limited the effectiveness of treatment. The delivery of such agents specifically to neoplastic target cells would improve the therapeutic index by increasing tumor cell destruction and decreasing adverse effects. Cancer cells that are replicating continuously require large amounts of cholesterol for the synthesis of the cell membrane. Cholesterol must be supplied either by *de novo* synthesis from 2-carbon units or by assimilation and degradation of low-density lipoprotein (LDL) from plasma. Cancer cells were found to metabolize LDL at a higher rate than that seen in non-neoplastic cells [11]. So LDL might prove to be a useful discriminatory vehicle for the delivery of

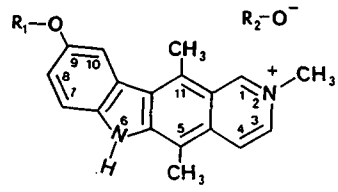
* This work was supported in part by Federation Nationale des Centres de Lutte Contre le Cancer and Comité Departementaux (Region Midi-Pyrénées) de la Ligue Nationale de Lutte Contre le Cancer.

‖ Author to whom the correspondence and requests for reprints should be sent: Professeur G. Soula, Departement de Biologie Clinique, Centre Claudius-Regaud, 20–24 Rue du Pont St-Pierre, 31052-Toulouse Cedex, France.

¶ Abbreviations used: 9-OH-NME, 9-hydroxy-N^2-methyl ellipticinium acetate; 9-O-MeE, 9-methoxy-ellipticine; ST-NME, 9-stearoyloxy-N^2-methyl ellipticinium stearate; PAL-NME, 9-palmitoyloxy-N^2-methyl ellipticinium palmitate, OL-NME, 9-oleoyloxy-N^2-methyl ellipticinium oleate; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; HPLC, high performance liquid chromatography.



9-OMe-E



9-OH-NME	$R_1 = H, R_2 = CH_3CO$-
PAL-NME	$R_1 = R_2 = CH_3(CH_2)_{14}CO$-
ST-NME	$R_1 = R_2 = CH_3(CH_2)_{16}CO$-
OL-NME	$R_1 = R_2 =$ $CH_3(CH_2)_7CH{=}CH(CH_2)_7CO$-

Fig. 1. Structure of ellipticine derivatives.

chemotherapeutic drugs [12] and radionucleotides to tumors [13].

In a recent paper, we have reported a technique to prepare 9-methoxy-ellipticine–LDL complex [14] and its efficiency on the cytotoxicity of cancer cell lines (L1210 and P388) *in vitro* compared to the free drug (see reference for discussion about advantages of this technique compared with other methods). However, the low incorporation rate of the drug limited us in the process of investigating the *in vivo* studies.

In order to increase the drug amount into LDL, we report the data concerning esters of ellipticine, which have been synthetized, and studied for their incorporation into LDL. We have investigated the interaction of drug–LDL with LDL–receptor of human fibroblasts and mouse peritoneal macrophages in monolayer culture and its *in vitro* cytotoxicity on L1210 cultured cells.

MATERIALS AND METHODS

Materials

Cholesteryl-oleate and dimirystoylphosphatidyl choline (DMPC) were purchased from Sigma (France) and were judged 99% pure and used without further purification, palmitic anhydride (97% pure), stearic anhydride (98% pure) and oleic anhydride (99% pure) were obtained from Aldrich-Chemical (France). 9-Hydroxy-N^2-methyl ellipticinium acetate was kindly provided by Sanofi Group (France).

Millex-0.45 μm filter was from Millipore S.A., methanol was chromatographic grade (Merck, France). Ammonium hexafluorophosphate, ammonium acetate and glacial acetic acid were obtained from Merck. Sodium iodine-125 was obtained from Amersham France (Les Ullis, France).

Fetal calf serum (FCS), RPMI 1640, phosphate buffer saline (PBS), penicillin, streptomycin and glutamin were obtained from Intermed (Strasbourg, France). T75 flasks, 1.9 cm^2 multiwell dishes and culture tubes were from NUNC Laboratories (U.S.A.).

L1210 and murine leukemic cells were obtained from the National Cancer Institute (Bethesda, MD, U.S.A.) and kept by Laboratoire de Pharmacologie et Toxicologie Fondamentale du CNRS (Toulouse, France). Human skin fibroblasts were a generous gift from Dr Soleilhavoup (Faculté de Médecine, Toulouse, France).

Methods

Esters of fatty acids with 9-hydroxy-N^2-methyl-ellipticinium acetate. These esters were prepared by a modification of the procedure described by Guthrie *et al.* for acylation of 9-hydroxy-ellipticine [15].

9-Palmitoyloxy-N^2-methyl-ellipticinium palmitate (PAL-NME). 9-OH-NME (134 mg, 0.4 mmol) and Na_2CO_3 (47 mg, 0.44 mmol) were successively added to a stirred solution at 60° of palmitic anhydride (396 mg, 0.8 mmol) in 16 mL of absolute ethanol. After 1 hr, the solvent was evaporated at 50° under reduced pressure, then the reaction mixture was dissolved in 40 mL $CHCl_3$ and washed with 40 mL of aqueous Na_2CO_3 in the presence of 10 mL ethanol. The chloroformic layer was dried (Na_2SO_4), concentrated and precipitated with diethyl ether. The dried solid was crystallized from EtOH-H_2O (1:1) to give 200 mg (65% yield) of the yellow crystalline ester Pal-NME.

NMR ($CDCl_3$) δ 0.86 (t, 2 × 3 H, J = 6.0 Hz, $C\underline{H}_3$-CH_2), 1.24 (br s, 2 × 24 H, CH_3-($C\underline{H}_2$)$_{12}$), 1.86 (m, 2 × 2 H, $C\underline{H}_2$-CH_2-CO), 2.47 (t, 2 H, J = 7.6 Hz, $C\underline{H}_2$-COO^-), 2.71 (t, 2 H, J = 7.6 Hz, $C\underline{H}_2$-COOAr), 2.85 (br s, 2 × 3 H, Me_5 and Me_{11}), 4.20 (s, 3 H, Me_2), 7.18 (d, 1 H, J = 7.1 Hz, H_3), 7.20 (dd, 1 H, J = 8.6 and 1.9 Hz, H_8), 7.48 (d, 1 H, J = 1.9 Hz, H_{10}), 7.59 (d, 1 H, J = 7.1 Hz, H_4), 7.74 (d, 1 H, J = 8.6 Hz, H_7), 10.10 (s, 1 H, H_1).

UV-visible (CH_3OH) λ = 425 nm ($\varepsilon_M \times 10^{-3}$ = 5 M^{-1} cm^{-1}), 374 (6), 358 (6), 309 (63), 252 (27), 242 (sh).

HPLC data: RRT = relative retention time defined as Rt of ester derivative/Rt of 9-OH-NME = 1.73 (see conditions of HPLC determination in the section "assay of the ellipticine esters").

9-Stearoyloxy-N^2-methyl-ellipticinium stearate (ST-NME). This ester was prepared by the procedure described above. The crystallization from EtOH-H_2O gave 198 mg (60% yield) of the yellow ester ST-NME.

NMR ($CDCl_3$) δ 0.86 (m, 2 × 3 H, $C\underline{H}_3$-CH_2), 1.24 (br s, 2 × 28 H, CH_3-($C\underline{H}_2$)$_{14}$), 1.68 (m, 2H,

$C\underline{H}_2$-CH_2-COO^-), 1.88 (m, 2H, $C\underline{H}_2$-CH_2-COOAr), 2.38 (t, 2H, J = 7.6 Hz, CH_2-COO^-), 2.71 (t, 2H, J = 7.5 Hz, CH_2-COOAr), 2.79 (s, 3H, Me_5 or Me_{11}), 2.82 (s, 3H, Me_5 or Me_{11}), 4.17 (s, 3H, Me_2), 7.20 (dd, 1H, J = 8.6 and 2.0 Hz, H_8), 7.21 (d, 1H, J = 7.2 Hz, H_3), 7.50 (d, 1H, J = 2.0 Hz, H_{10}), 7.57 (d, 1H, J = 7.2 Hz, H_4), 7.71 (d, 1H, J = 8.6 Hz, H_7), 9.89 (s, 1H, H_1).

UV-visible (CH_3OH) $\lambda = 430$ nm ($\varepsilon_M \times 10^{-3} = 4\ M^{-1}\ cm^{-1}$), 374 (5), 356 (5), 308 (56), 250 (21.5), 242 (sh).

HPLC data: RRT = 2.0.

9-Oleoyloxy-N^2-methyl-ellipticinium oleate (OL-NME). The reaction was worked up as in the previous examples from 0.4 mmol (134 mg) of 9-OH-NME and 0.8 mmol of oleic anhydride (438 mg). Final precipitation from EtOH-H_2O afforded 263 mg (71% yield) of a yellow past of OL-NME.

NMR ($CDCl_3$) δ 0.86 (m, 2 × 3 H, $C\underline{H}_3$-CH_2), 1.25 to 1.40 (m, 2 × 20 H, CH_3-$(C\underline{H}_2)_6$) and $(C\underline{H}_2)_4$-CH_2-CH_2-CO, 1.88 (m, 2 × 2 H, $C\underline{H}_2$-CH_2-CO), 2.04 (m, 2 × 4 H, $C\underline{H}_2$-CH=CH—$C\underline{H}_2$, 2.49 (t, 2H, J = 7.8 Hz, CH_2-COO^-), 2.71 (t, 2H, J = 7.6 Hz, CH_2-COOAr), 2.85 (s, 3H, Me_5 or Me_{11}), 2.87 (s, 3H, Me_5 or Me_{11}), 4.20 (s, 3H, Me_2), 5.36 (m, 2 × 2 H, CH=CH), 7.17 (d, 1H, J = 7.1 Hz, H_3), 7.20 (dd, 1H, J = 8.6 and 2.1 Hz, H_8), 7.47 (d, 1H, J = 2.1 Hz, H_{10}), 7.59 (d, 1H, J = 7.1 Hz, H_4), 7.73 (d, 1H, J = 8.6 Hz, H_7), 10.14 (s, 1H, H_1).

UV-visible (CH_3OH) $\lambda = 430$ nm ($\varepsilon_M \times 10^{-3} = 4\ M^{-1}\ cm^{-1}$), 374 (5), 356 (5), 308 (62), 250 (24.5), 240 (sh).

HPLC data: RRT = 1.85.

Preparation of LDL

LDL was separated from human plasma by rate-zonal ultracentrifugation by the method of Patsch *et al.* [16] using a Kontron TGA-65 ultracentrifuge and Ti-14 zone rotor (Kontron Instruments, France). The lipoprotein from 50 mL of human plasma was isolated from other plasma constituents by flotation through a linear sodium bromide gradient, density range 1.0 to 1.4 kg/L, at 40,000 rpm and at 10° for 110 min. Eluant from the rotor was collected in fractions of 14 mL and the optical density at 280 nm monitored using a 2138 UVICORD S Ultraviolet Absorbance monitor (LKB, Instruments France).

The peak fractions containing LDL were pooled and concentrated by pressure filtration in an Amicon ultraconcentration cell using a XM100A filter (Amicon corp, France). Excess sodium-bromide was removed by washing with working buffer (0.15 M NaCl, 0.03 mM EDTA, pH 8.1) and reconcentrating, several times or by exhaustive dialysis against working buffer.

Lipoprotein purity was assayed by 1% agarose gel electrophoresis and by immunochemical analysis. LDL migrated as a single band on agarose and did not react with anti-apo AI and anti-apo AII. Positive reactions were obtained with anti-apo B. The LDL were determined as protein by the assay of Lowry *et al.* [17] using bovine serum albumin as standard.

The LDL were iodinated by the iodine monochloride method of McFarlane modified by Bilheimer *et al.* [18]. The LDL were acetylated with repeated addition of acetic anhydride as described by Goldstein *et al.* [19] or methylated with the repeated addition of formaldehyde as described by Weisgraber *et al.* [20].

HPLC assay of elliptinium-esters

An aliquot (200 μL) of LDL–drug complex and native LDL were spiked with 25 μL of *N*2-propyl-9-hydroxy-ellipticinium (9-OH-NPE) as internal standard (final concentration 1.25 μg/mL). All the drug and internal standard were extracted twice with ethylacetate (2 × 1 mL) after the addition of 100 μL of ammonium hexafluorophosphate (final concentration 250 mM) as contre-ions and (2 × 3 mL) acetate buffer 0.5 M, pH 5.5.

The mixture was centrifuged twice at 2000 rpm for 10 min. The organic phase was aspirated and dried under a stream of nitrogen. The residue was redissolved in 200 μL of the mobile phase (methanol : water 75 : 25 with 5 mM acetate buffer adjusted to pH 5.5 with glacial acetic acid). A Waters CN μ Bondapak reversed-phase column (30 × 3.9 mm i.d.) was used. The assay was carried out at 313 nm. This technique allows us to quantitatively determine the two drugs (9-OH-NME and OL-NME) at one and the same time.

Preparation of elliptinium-esters–LDL complex

Each elliptinium-ester (PAL-NME, ST-NME, OL-NME) was incorporated into LDL using the fusion technique between drug-containing microemulsions and LDL.

The preparation of the microemulsion containing each elliptinium-ester was carried out according to Parks *et al.* [21] and Craig *et al.* [22] with a few modifications concerning the molar proportion of each component: 2 mg of both DMPC and elliptinium-esters were added to 1 mg cholesteryl oleate in chloroform. The solvent was evaporated and the residue was dissolved in 200 μL of dry isopropanol and injected into 3 mL of rapidly vortexing phosphate buffer with the entire system kept at 46° throughout the procedure.

In order to incorporate elliptinium-esters into LDL, 5–6 mg LDL (2 mg/mL) were incubated with equal volume of elliptinium-esters containing microemulsion in LDL buffer for 5 hr at 37° according to Parks *et al.* [21]. Drug–LDL was separated from the microemulsion by discontinuous sucrose gradient from 15 to 4%. Ultracentrifugation was accomplished using a Beckman SW-40 rotor for 2 hr at 40,000 rpm and 4° in a Beckman Model L8-70 ultracentrifuge. After the separation by ultracentrifugation, two bands were noticed. The bands were aspirated from the top of the centrifuge tubes and subjected to extensive dialysis for 24 hr against LDL buffer to remove sucrose and sterilized by filtration through a 0.45 nm millipore filter. The identification of the LDL band was accomplished by a protein assay. The elliptinium-ester content of the LDL fraction increased as a function of the incubation time of the mixture. A maximum is obtained for 5 hr of incubation.

Size distribution particle (S.D.P.)

The S.D.P. program analyses the correlation of

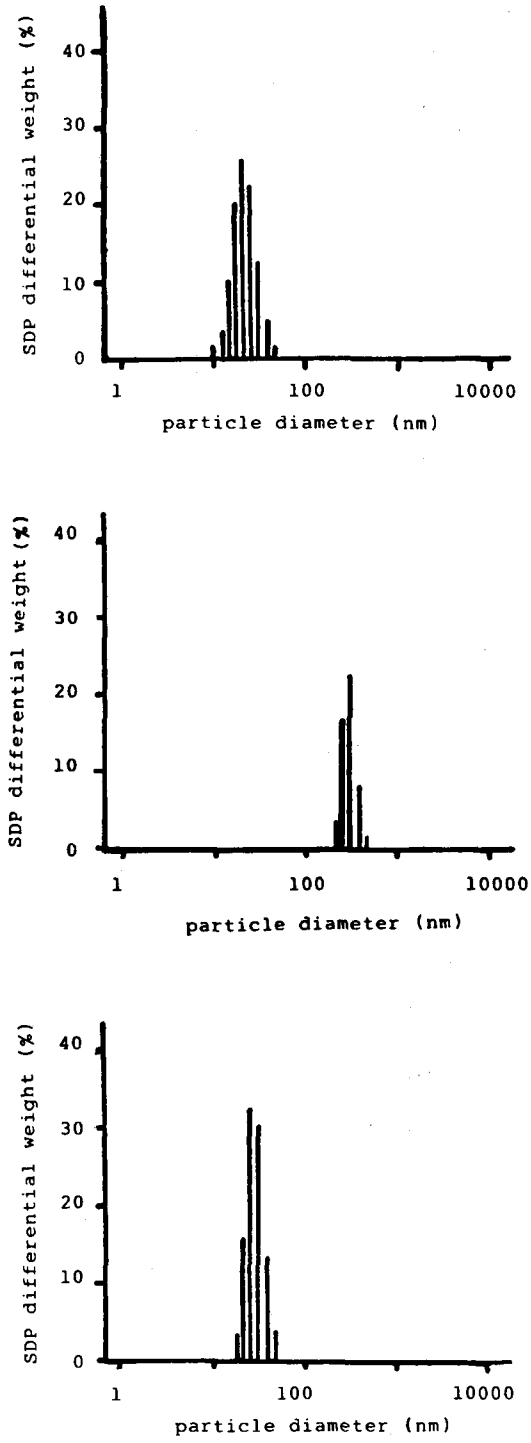


Fig. 2. Size distribution of native LDL microemulsions and OL-NME–LDL complexes. (A) LDL was separated from human plasma by rate-zonal ultracentrifugation. Two mL of native LDL (1 mg lipid/mL) were placed in the measurement cuvette. The mean diameter and size distribution was determined: size (nm) = 22.1, SD (nm) = 8, amount = 100%. (B) Microemulsions prepared by dimyristoylphosphatidylcholine and cholesteryl oleate and OL-NME: size (nm) = 299, SD (nm) = 54, amount = 100%. (C) OL-NME–LDL complexes were separated from microemulsions by discontinuous sucrose gradient from 15% to 4%: size (nm) = 25.4, SD (nm) = 7, amount = 100%.

function data and presents the particle size distribution as either differential or cumulative distributions (by photo intensity) against particle diameter. The size of samples was estimated by laser light scattering, with a monochromatic laser ray diffusion counter (Nanosizer, coultronics, Margency, France). The samples were diluted to approx 1 mg lipid/mL and 2-mL samples were placed in the measurement cuvette. The mean diameter and size distribution were determined by the instrument.

Cell culture

L1210 cells and fibroblasts were grown in RPMI 1640 and MEM, respectively, with 10% (v/v) fetal calf serum (FCS), 2 mM glutamine, 10 I.U./mL streptomycin and 100 I.U./mL penicillin. The cells were maintained in a humidified incubator (5% CO_2) at 37°.

For all experiments, the fibroblasts from the stock flasks were dissociated with 0.05% Trypsin, 0.05% EDTA and were seeded (day 0) at a concentration of 5×10^4 cells per dish into 40 mm dishes containing 2 mL of the above growth medium with 10% FCS. On day 2 when the cells were at a density of 2×10^5 cells per dish, the medium was removed and the cellular monolayer was washed once with 2 mL of Dulbecco's phosphate-buffered saline (PBS) (0.9% NaCl) at 37°, following which 2 mL of fresh medium containing 5% (v/v) lipoprotein-deficient human serum (LPDS) (final concentration of protein: 5 mg/mL) were added for 2 days before the assay to induce maximum apo-B,E (LDL) receptor expression.

Monolayer cultures of mouse peritoneal macrophages were prepared as described previously [23]. Briefly, peritoneal cells from unstimulated females Swiss mice (25–30 g) were harvested in PBS, collected by centrifugation, and plated on 40 mm dishes (3×10^6 cells/dish) in DMEM containing 10% (v/v) fetal calf serum and penicillin, streptomycin, and glutamine as above. After 2 hr of incubation in the 37° incubator, the monolayer was washed three times with warm PBS to remove nonadherent cells and then incubated overnight in the 10% FCS medium unless otherwise indicated.

Intracellular uptake of free drug and drug–LDL complex

In a series of experiments, the cellular uptake of OL-NME–LDL complex, OL-NME and 9-OH-NME for L1210 cells was studied. Typical experiments contained in a total volume of 1 mL of RPMI 1640 supplemented with 5% LPDS, 10^6 cells and an amount of each drug corresponding 2×10^{-8} M. After varying times of incubation (30 min to 4 hr) at 37°, the cells were washed twice with ice-cold PBS and harvested by centrifugation. OL-NME and 9-OH-NME were then extracted from the cell pellet and measured by HPLC as described before. Drug levels were expressed as mol/10^6 cells.

Binding, internalization and degradation assays

The assays were performed at 37° for 4 hr with the indicated concentrations of [^{125}I]drug–LDL or [^{125}I]acetyl-LDL, native [^{125}I]LDL, alone or with 40-fold excess of the respective unlabeled lipoprotein

using the methods of Brown and Goldstein [24]. The degradation was determined from the amount of trichloroacetic acid-soluble radioactivity in the incubation medium after extraction of free iodine with chloroform. To measure [^{125}I]lipoprotein binding to fibroblasts, the cells were washed three times with 2 mL of PBS without bovine serum albumin (BSA) and twice with 1 mL of PBS that contained BSA (2 mg/mL). The LDL were detached by incubation for 30 min with a solution containing heparin (5 mg/mL) in PBS. After standard wash, the cells were dissolved by incubation at 24° for 24 hr in 0.6 mL of 0.1 N NaOH. Aliquots were taken for determination of protein and radioactivity (incorporated lipoprotein). The total cellular content of [^{125}I]lipoprotein in mouse macrophage monolayers was measured as above, except for the heparin-release step that was omitted.

ID_{50} *assay*

L1210 cells were usually grown in T75 flasks and the experiments were performed in culture tubes by harvesting cells in exponential growth phase, plating out into the culture tubes at a density of 6×10^3 cells/mL. The drug or the drug–LDL complexes were added at indicated concentration in triplicate tubes. After 5 hr incubation at 37°, the cells were centrifuged at 200 *g* for 10 min and washed twice with 1 mL of PBS (0.9% NaCl). The cells were then grown in RPMI medium supplemented with 10% FCS.

Cells were sampled after 6 days and counted (three counts/tubes), after dilution, in a coulter counter (Coultronic Model Z.M). Six tubes in each experiment were left drug-free as a control. The mean of the cell count value from the control tube was taken to represent 100% of cell growth and the counts of other tubes were expressed relative to this result. A plot of percentage cell growth against log (concentration M) was constructed for each of the drug and drug–LDL complexes studied. From these curves, the dose which corresponds to 50% inhibition of cell growth was calculated for each drug.

RESULTS

Incorporation of elliptinium-esters

We have compared the incorporation rate of the synthetized elliptinium-esters into LDL. The incorporation was performed by a technique which consisted of a fusion of microemulsions containing drug with LDL for 5 hr at 37°. In contrast to the previously described technique [14], the drug–LDL complex was separated from microemulsions containing drug by a one step ultracentrifugation in a discontinuous sucrose gradient from 15 to 4%. This technique allows a rapid drug–LDL separation with a protein recovery of 80%.

All of the three elliptinium-esters allow a higher incorporation rate than 9-MeOE (Table 1), the ellipticine derivative use for previous studies [14]. However, we can notice significant differences in the incorporation rate of elliptinium-esters as a function of the nature of the esterified fatty acid. The rate of incorporation ranged from 37 μg/mg protein LDL with ST-NME to 83 μg/mg protein LDL with OL-NME corresponding an average of 70 molecules of OL-NME incorporated by LDL particle (assuming 515 kDa for Apolipoprotein B molecular weight). Consequently the latter experiments have been performed with OL-NME–LDL complexes.

The OL-NME–LDL particles were subjected to analytical 1% polyacrylamide gel electrophoresis. They migrated as a single band and presented the same electrophoretic migration as native LDL. Immunoreactivity against anti-apo B antisera was similar to those observed with native LDL.

The mean diameter and the size distribution of OL-NME–LDL compared to native LDL have then been determined. Figure 2 shows that OL-NME–LDL was of equal size to native LDL, since microemulsions containing OL-NME were a greater size.

As a test of stability of the OL-NME–LDL complex, the hydrolysis of the elliptinium-ester was studied in human serum at 37°, 2 mL of OL-NME–LDL (300 μg/mL) was stored at 37° in 4 mL of human serum. At the indicated time, aliquots were removed and assayed for 9-OH-NME and OL-NME by HPLC. Degradation of the complex under this condition is shown in Table 2. During the first 24 hr of the experiment the esters decayed slowly and reached saturation to 72 hr, leaving more than 90% of the original OL-NME in the solution. In parallel, the 9-OH-NME which is the hydrolysis product of OL-NME appears in the solution. These results demonstrated the good stability of OL-NME–LDL complex in the serum.

OL-NME–LDL metabolism via the LDL-receptor pathway

As a test of the biological activity of the drug–LDL complex, the cellular metabolism of [^{125}I]OL-NME–LDL was studied in cultured fibroblasts. Specific binding, internalization and degradation after incubation of normal skin fibroblasts with increasing concentrations (0–250 μg protein LDL/mL) of [^{125}I]OL-NME–LDL complex are shown in Fig. 3. For binding and internalization, saturation curves were obtained. The maximal binding and uptake of [^{125}I]LDL–OL-NME as a function of concentration was achieved at a concentration of 150 μg of [^{125}I]OL-NME–LDL protein per mL (Fig. 3). Likewise, the rate of degradation was linear with respect to the [^{125}I]OL-NME–LDL complex concentration. These results are in agreement with previous conclusions concerning the cellular metabolism of native LDL by human fibroblasts [25, 26].

The presence of an increasing concentration of native LDL, inhibited the cellular binding, internalization and degradation of the OL-NME–LDL (15 μg/mL) (Fig. 4). In contrast, methylated LDL, which does not bind to the LDL receptor, did not affect the cellular metabolism of [^{125}I]OL-NME–LDL (data not shown). This indicates that the [^{125}I]OL-NME–LDL complex retained LDL receptor-recognizing ability.

OL-NME–LDL complex uptake and degradation by mouse peritoneal macrophages

In order to demonstrate that the OL-NME–LDL complex has not become denatured or chemically

Table 1. Comparison of the incorporation of ellipticine derivatives into LDL by fusion technique between drug-containing microemulsions and LDL

	9-OMe-E	ST-NME	PAL-NME	OL-NME
μg/mg protein LDL	2	37 ± 2	58 ± 1.5	83 ± 2
Molecules/LDL particle	5	32	55	70

Table 2. Stability of elliptinium oleate within LDL

Time (hr)	OL-NME–LDL	
	Free elliptinium (μg/mL plasma)	Oleate-elliptinium (μg/mL plasma)
0	0	90 ± 1.4
24	3.48 ± 1.2	85.5 ± 1.1
48	5.2 ± 0.82	82.5 ± 1.8
72	5.48 ± 1.1	82 ± 0.6

The drug–LDL complex containing elliptinium oleate was stored into plasma at 37°. Aliquots were removed at the times shown for drug quantitation by HPLC.

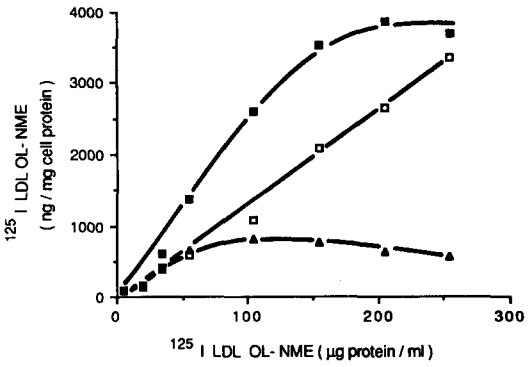


Fig. 3. Binding, uptake and degradation of [^{125}I]OL-NME by normal skin fibroblasts. Cells were seeded in dishes as described in Materials and Methods. At the beginning of the experiment, the medium was replaced with 1 mL of medium MEM containing 5% LPDS and the indicated concentrations of [^{125}I]OL-NME–LDL (186 cpm/ng protein LDL) either in the absence or presence of 40-fold excess of OL-NME–LDL. After incubation at 37° for 4 hr, the medium from each dish was removed. Its content of acid-soluble material (□) was measured, and the amount of [^{125}I]lipoprotein bound to the cells (▲) and cellular uptake (■) were determined. The results were calculated by subtracting the cpm in the presence of OL-NME–LDL from that obtained in the absence of OL-NME–LDL. The values are representative of three experiments and are the mean of duplicate dishes from one experiment.

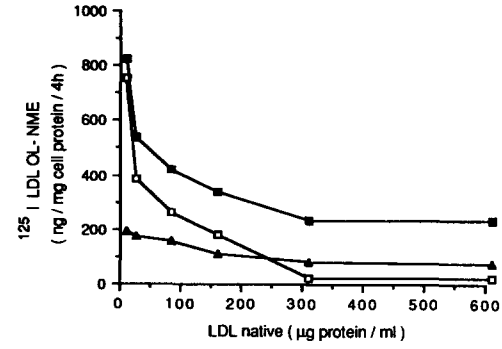


Fig. 4. Competition assays between [^{125}I]OL-NME–LDL and native LDL. Normal skin fibroblasts were seeded as described in Materials and Methods. Each dish received 15 μg of [^{125}I]OL-NME–LDL together with the amount of native LDL (non radioactive) shown on the abscissa. After 4 hr of incubation at 37°, the medium was removed and the ^{125}I-labeled acid-soluble content (□), the cellular uptake (■) and the [^{125}I]lipoprotein bound to the cells (▲) determined. Each point represents the mean triplicate determination.

modified by the incorporation process of drug within LDL, we used [^{125}I]acetyl-LDL as a model of chemically modified LDL.

The data in Fig. 5 shows that the OL-NME–LDL particles are taken up and degraded by the freshly isolated peritoneal macrophages at a rate similar to that of native LDL. Moreover, [^{125}I]acetyl-LDL was taken up and degraded by the cells three times more than native LDL or OL-NME–LDL complex.

This data suggest that OL-NME–LDL complex presents similar metabolism with native LDL and are not recognized as chemically modified by the mouse peritoneal macrophages.

Cellular accumulation of OL-NME in L1210 cells

OL-NME–LDL was stable in the culture medium at 37° for 4 hr with no evidence of complex dissociation. Cellular uptake of 9-OH-NME proceeded rapidly and approached equilibrium by approximately 30 min. In contrast, the cellular uptake of OL-NME proceeded slowly and to a lesser extent than 9-OH-NME. It is of note that no 9-OH-NME, the hydrolysis product of OL-NME, appeared during the 4 hr of experiments. When OL-NME is delivered to the cells via the LDL complex, the rate of incorporation of the OL-NME is higher than the rate observed with the free drug. On the other hand we notice a high intracellular level of 9-OH-NME.

This indicated that the degree of intracellular of OL-NME metabolism differed markedly regardless of whether free drug or the complex was used.

Cytotoxic activity of OL-NME–LDL complex

To demonstrate the possibility of the growth inhibitory effect of OL-NME–LDL complex, L1210

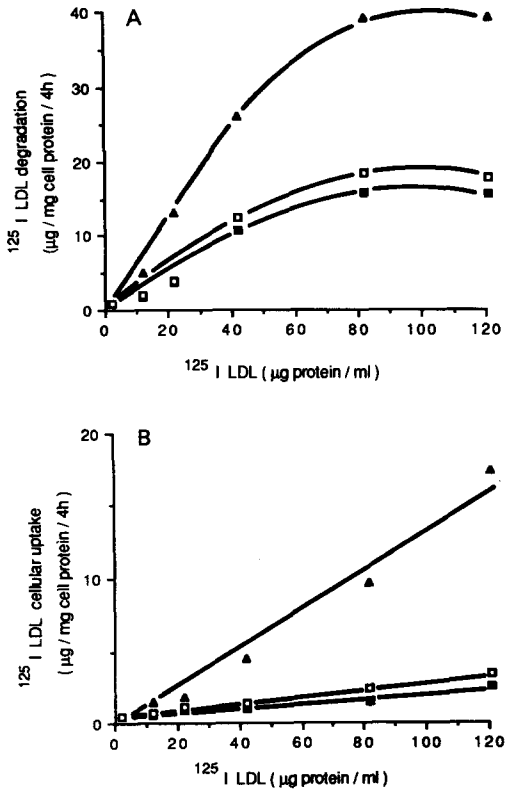


Fig. 5. Degradation (A) and accumulation (B) of [^{125}I]native LDL, [^{125}I]acetyl-LDL and [^{125}I]OL-NME–LDL by mouse peritoneal macrophages. Cells were incubated with 1 mL of DEME medium containing the indicated concentrations of either [^{125}I]native LDL (159 cpm/ng protein LDL) (□), or [^{125}I]OL-NME–LDL (153 cpm/ng protein LDL) (■), or [^{125}I]acetyl-LDL (157 cpm/ng protein LDL) (▲), for 4 hr at 37°. The amount of ^{125}I-labeled acid-soluble material in the medium (A), and the amount of [^{125}I]lipoprotein in the cells (B) were determined (see Materials and Methods) in duplicate dishes. Results shown are representative of two experiments.

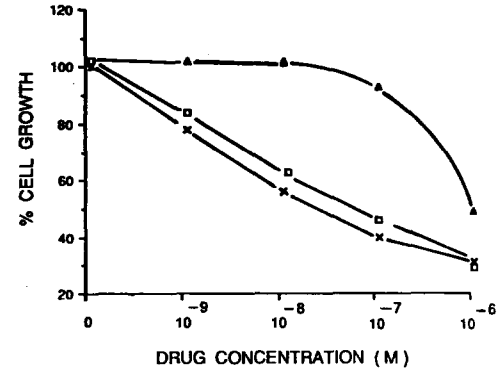


Fig. 6. L1210 cells growth inhibition assay with OL-NME–LDL compared to free OL-NME and free OH-NME. The cells (6000 cells/mL) were incubated in 5% LPDS supplemented medium with various concentrations of OL-NME within LDL complex (□), free OL-NME (▲), and free 9-OH-NME (×). The cells were sampled after 5 hr incubation at 37°, followed by washing with PBS, and incubated for 6 days and counted. Each point is the mean of triplicate tubes in two experiments.

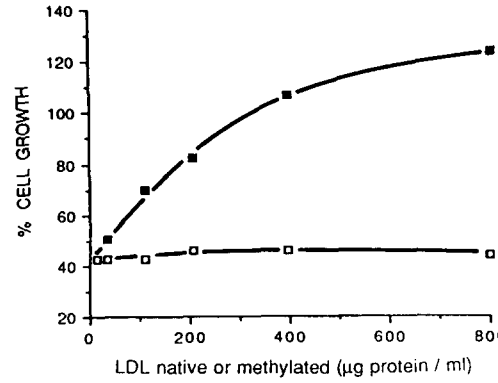


Fig. 7. Cytotoxic effect of the OL-NME–LDL complex in the presence of increasing quantities of native LDL or methylated LDL. L1210 cells were incubated at 37° with OL-NME–LDL (19 μg protein LDL) and increasing quantities of native LDL (■), or methylated-LDL (□). The cells were sampled after 5 hr incubation and followed by washing twice with PBS and counted after 6 days incubation as described Fig. 5.

cells were incubated with 9-OH-NME, free OL-NME and LDL containing OL-NME (Fig. 6). OL-NME–LDL was more effective than OL-NME, whereas the native 9-OH-NME was as effective as the OL-NME–LDL complex.

The ID_{50} of the drug fell from 10^{-6} to 5×10^{-8} M when transported within LDL, illustrating the ability of the lipoprotein to potentiate the action of this drug. The native LDL in the same LDL protein concentration range, had no effect on the cell growth. When we added increasing quantities of LDL to LPDS supplemented medium which contains the OL-NME–LDL complex (19 μg/protein LDL) (Fig. 7), the cytotoxic effect was continuously reduced and reached about 120% level of cell growth. In the same conditions, increasing quantities of methylated LDL, which were unable to bind themselves to the LDL receptor, had no effect on the drug–LDL complex cytotoxic effect.

This data supports the hypothesis of a receptor mediated cytotoxicity of OL-NME–LDL complex.

DISCUSSION

The LDL particle provides several sites for the insertion of drug molecules, depending on their lipid solubility. The core is by far the best site, since chemically sensitive drugs sequestered there would be well protected from serum hydrolases and from the potentially damaging effects of an aqueous environment. Furthermore, the core has the capacity to store a large number of drug molecules. However, only very hydrophobic agents are suitable for inclusion in this domain. A second site for drug intercalation is the phospholipid monolayer. Agents which have both polar and lipid soluble components may partition here, between the apolar core and the

aqueous environment, but in this location, they are less well protected from serum hydrolases and water and, further, may be able to stimulate the immune system to remove the drug–LDL complex from the circulation.

In order to approach the natural structure of the lipoprotein core, we report the synthesis of lipophilic elliptinium derivatives which can be incorporated into LDL. It was not possible to incorporate 9-OH-NME, since immediate aggregation of LDL occurred at several concentrations of the drug.

Our attempts focused on linking a fatty acyl chain to the OH-9 position of elliptinium, and neutralizing the permanent charge on nitrogen-2 position with the same free fatty acid as counter-ion. So we formed a pair of ion (9-acyl-elliptinium/free fatty acid), globally neutral, able to be inserted into the LDL lipid core. Among the three elliptinium-esters used, the OL-NME was the most potent and led to the highest incorporation rate (83 μg OL-NME/mg protein LDL). These data indicate that the nature of the fatty acid is of importance for the incorporation rate of drug into LDL. It is of interest to notice that oleic acid is the major fatty acid of the cholesterol-ester in the LDL lipid core.

At storage at 37° in the presence of human serum, a low portion of the drug within LDL complex was degraded over the first 24 hr. Thereafter hydrolysis was much less significant and remained constant over the 72 hr of the test. This suggests the possibility that OL-NME molecules are located both on the surface and in the core of the LDL particle. Assuming that this deduction is correct, core-located drug molecules would be protected from the aqueous environment and from the serum hydrolases.

The incorporation level obtained was close to 70 molecules/LDL particles which represent about 10% of the cholesterol ester molecule content of the LDL lipid core. It is likely that changes in the content of the core of a lipoprotein can affect the chemical and physical properties of the lipoprotein surface [27–31] thereby modifying the interaction of apolipoprotein B with the LDL receptor. For example, conformational changes in apolipoprotein B around the receptor-binding domain or an unmasking or exposure of lysine residues that may be important in receptor binding might result from modification of the lipoprotein core. It is possible that such conformational changes might expose more lysine on the surface of the lipoprotein, thereby permitting more electrostatic interaction with negatively charged regions of the ligand-binding domain of the LDL receptor.

When the elliptinium-esters were incorporated into dimyristoylphosphatidylcholine stabilized microemulsions and the latter fused with LDL [14] the physical properties of the drug–LDL complexes were identical to native LDL. The data of size distribution particles shows that the complex is of equal size as native LDL and that the presence of microemulsions, of greater size, was not observed, assuming the purity of the complexes. On the other hand the OL-NME LDL retain identical electrophoretic mobility on 1% agarose gel electrophoresis and immunological properties against an anti apolipoprotein B antibody, as native LDL (data not shown).

Then, the OL-NME–LDL particles are essentially identical to native LDL as their ability to be specifically incorporated and degraded by normal human skin fibroblasts and mouse peritoneal macrophages. [^{125}I]Acetyl-LDL were used as a probe for a binding site on macrophages that mediated the uptake and degradation of chemically altered or denatured LDL. In addition to LDL receptors, macrophages possess scavenger receptor that recognize certain modified forms of LDL [32].

The present study is a supplementary finding, showing that apo B of the LDL retains its native conformation when the drug is incorporated by the procedure described above. This suggests that the clearance of an adequately prepared OL-NME–LDL by macrophages could be similar to that of native LDL. The LDL receptor-mediated endocytosis of cytotoxic drug–LDL might provide distinct advantages over the trapping of antineoplastic drugs in liposomes. The main reasons for this statement are that liposomes are subjected to destruction by blood components, primarily by lipoproteins [33] and to a fast clearance from the circulation by the reticulo-endothelial system [34].

In this study, we have selected, as the experimental materials, the L1210 mouse leukemia cells who obey to the quantitative growth laws in mice as well as in *in vitro* culture [35]. It has also been previously reported that 9-hydroxy-N^2-methyl ellipticinium inhibits the *in vitro* growth of these cells [4].

In the experiment showing the growth-inhibitory effect of OL-NME–LDL complex, free OL-NME and 9-OH-NME on the L1210 cells, we have observed no difference in the cytotoxic effect between OL-NME–LDL complex and free 9-OH-NME. In contrast, OL-NME was less cytotoxic. Investigations reported in the literature suggest that the quaternarization of nitrogen in the pyridinic ring (ellipticinium series) does not modify markedly either the cytotoxicities of the drugs or the slope of their dose–activity relationship [36]. These observations suggest that the positive charge of the ellipticinium derivatives does not modify their permeation when compared to the ellipticine derivatives and is not directly involved in the target–drug interaction. They also indicate that the improvement of the therapeutic efficiency of the ellipticinium compounds as compared to other ellipticines is very probably due more to favourable pharmacokinetics and biodisposition parameters rather than to a higher killing efficiency towards the malignant cells. As already described, the occurrence of a substitution in the 9-position seems to be critical in order to improve the cytotoxic and antitumor effects of ellipticines [10]. These observations explain that the low cytotoxicity of OL-NME is related to the low metabolic intracellular transformation of OL-NME. When OL-NME is incorporated within LDL, the drug–LDL complexes were degraded in lysosomes with subsequent release of the 9-OH-NME, illustrating the importance of the 9-OH substitution with respect to the occurrence of cytotoxic properties and the ability of the lipoprotein to potentiate the action of this cytotoxic drug.

Table 3. Intracellular levels of 9-OH-NME and OL-NME (±SD, N = 4) following treatment of L1210 cells with identical concentration (1.2×10^{-5}M) of 9-OH-NME, OL-NME and OL-NME–LDL

Time (min)	Free 9-OH-NME	Free OL-NME		OL-NME–LDL	
	Intracellular drug mol/10^6 cells ($\times 10^8$)	Intracellular drug mol/10^6 cells ($\times 10^8$)		Intracellular drug mol/10^6 cells ($\times 10^8$)	
	9-OH-NME	9-OH-NME	OL-NME	9-OH-NME	OL-NME
0	0	0	0	0	0
30	0.48 ± 0.5	0	0.097 ± 0.03	0.038 ± 0.03	0.094 ± 0.02
60	0.44 ± 0.67	0	0.105 ± 0.03	0.057 ± 0.01	0.111 ± 0.05
120	0.45 ± 0.3	0	0.112 ± 0.01	0.075 ± 0.03	0.123 ± 0.03
180	0.45 ± 07	0	0.134 ± 0.02	0.098 ± 0.02	0.167 ± 0.07
240	0.435 ± 0.6	0.003	0.135 ± 0.04	0.119 ± 0.04	0.181 ± 0.03

This present study demonstrates that cytotoxic drugs can be modified, incorporated into LDL and selectively delivered to cells by LDL receptors *in vitro*. It also demonstrates the possibility of LDL to be carriers of cytotoxic derivatives, acting as a prodrug which could be activated intracellularly by the lysosomes enzyme activity. Studies are now in progress to investigate the *in vivo* fate of the OL-NME–LDL complex and to see if the drug can be targeted to malignant cells via the high affinity LDL receptor pathway *in vivo*.

Acknowledgements—Sanofi company is gratefully acknowledged for providing 9-O-MeE and 9-OH-NME. We are also grateful to Dr Pierre Canal and Dr Bernard Meunier for fruitful discussions.

REFERENCES

1. Paoletti C, Cros S, Dat-Xuong N, Lecointe P and Moisand A, Comparative cytotoxic and antitumoral effects of ellipticine derivatives on mouse L1210 leukemia. *Chem Biol Interact* **25**: 45–58, 1979.
2. Paoletti C, Le Pecq JB, Dat-Xuong N, Juret P, Garnier H, Amiel JL and Rouesse J, Antitumor activity pharmacology and toxicity of ellipticinium and 9-hydroxy derivatives: preliminary clinical trials of 2-methyl-9-hydroxyellipticinium (NSC 264137). *Cancer Res* **74**: 107–123, 1980.
3. Dodion P, Rozencweig M, Nicaise C, Piccart M, Cumps E, Crespeign N, Kisner D and Kenis Y, Phase I clinical study of 9-hydroxy-2*N*-methyl ellipticinium acetate (NSC 264137) administered at a 5-day i.v. schedule. *Eur J Cancer Clin Oncol* **18**: 519–522, 1982.
4. Auclair C, Pierre A, Voisin E, Pepin O, Cros S, Colas C, Saucier JM, Verschuere B, Gros P and Paoletti C, Physiochemical and pharmacological properties of the antitumor ellipticine derivative 2-(diethylamino-2-ethyl)9-hydroxy ellipticinium-chloride, HCl. *Cancer Res* **47**: 6254–6261, 1987.
5. Auclair C and Paoletti C, Bioactivation of the antitumor drugs 9-hydroxy ellipticine and derivatives by a peroxidase-hydrogen peroxide system. *J Med Chem* **24**: 289–295, 1981.
6. Bernadou J, Meunier G, Paoletti C and Meunier B, *o*-Quinone formation in the biochemical oxidation of the antitumor drug N^2-methyl-9-hydroxy ellipticinium acetate. *J Med Chem* **26**: 574–579, 1983.
7. Maftouh M, Monsarrat B, Rao RC, Meunier B and Paoletti C, Identification of the glucuronide and glutathione conjugates of the antitumor drug N^2-methyl-9-hydroxy-ellipticinium acetate (Celiptium). *Drug Metab Dispos* **12**: 111–119, 1984.
8. Bernadou J, Monsarrat B, Roche H, Armand JP, Paoletti C and Meunier B, Evidence for electrophilic properties of N^2-methyl-9-hydroxy ellipticinium acetate (Celiptium) from human biliary metabolites. *Cancer Chemother Pharmacol* **14**: 37–39, 1985.
9. Meunier G, DeMontauzon D, Bernadou J, Grassy G, Bonnafous M, Cros S and Meunier B, The biooxidation of cytotoxic ellipticine derivatives: a key to structure–activity relationships studies. *Mol Pharmacol* **33**: 93–102, 1988.
10. Auclair Ch, Multimodal action of antitumor agents on DNA: the ellipticine series. *Arch Biochem Biophys* **259**: 1–14, 1987.
11. Gal D, MacDonald PC, Porter J and Simpson ER, Cholesterol metabolism in cancer cells in monolayer culture. III. Low density lipoprotein metabolism. *Int J Cancer* **28**: 315–319, 1981.
12. Shaw JM, Shaw KV, Yanovich S, Iwanik M and Futch WS, Delivery of lipophilic drugs using lipoproteins. *Ann NY Acad Sci* **507**: 252–271, 1988.
13. Gal D, Ohashi M, MacDonald PC, Buchsbaum HJ and Simpson ER, Low density lipoprotein as a potential vehicle for chemotherapeutic agents and radionucleotides in the management of gynecologie neoplasms. *Am J Obstet Gynecol* **139**: 877–885, 1981.
14. Samadi-Baboli M, Favre G, Blancy E and Soula G, Preparation of low density lipoprotein-9-methoxy-ellipticin complex and its cytotoxic effect against L1210 and P388 leukemic cells *in vitro*. *Eur J Cancer Clin Oncol* **25**: 233–241, 1989.
15. Guthrie RW, Brossi A, Mennona FA, Mullin JG, Kierstead RW, Grunberg E, Ellipticine derivatives. *J Med Chem* **18**: 755–760, 1975.
16. Patsch JR, Sailer S, Kostner G, Sandhofer F, Holasek A and Braunsteiner H, Separation of the main lipoprotein classes from human plasma by rate-zonal ultracentrifugation. *J Lipid Res* **15**: 356, 1974.
17. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
18. Belheimer OH, Eisenberg S and Levy RI, The metabolism of very low density lipoprotein. I. Preliminary *in vitro* and *in vivo* observation. *Biochim Biophys Acta* **260**: 212–221, 1972.
19. Goldstein JL, Ho YK, Basu SK and Brown MS, Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein producing massive cholesterol deposition. *Proc Natl Acad Sci USA* **76**: 333–337, 1979.
20. Weisgraber KH, Innerarity T and Mahley RW, Role of the lysine residues of plasma lipoproteins in high affinity binding to cell surface receptors on human fibroblasts. *J Biol Chem* **253**: 9053–9062, 1978.
21. Parks JS, Martin JA, Johnson FL, Rudel LL, Fusion

of low density lipoproteins with cholesterol ester phospholipid microemulsions. *J Biol Chem* **260**: 3155–3163, 1985.
22. Craig IF, Via DP, Sherrill BC, Sklar LA, Mantulin WW, Gotto AM Jr and Smith LC, Incorporation of defined cholesteryl esters into lipoproteins using cholesteryl ester-rich microemulsions. *J Biol Chem* **257**: 330–335, 1982.
23. Koo C, Wernette-Hammond ME and Innerarity TL, Uptake of canine β-very low density lipoproteins by mouse peritoneal macrophages is mediated by a low density lipoprotein receptor. *J Biol Chem* **261**: 11194–11201, 1986.
24. Brown MS and Goldstein JL, Analysis of a strain of human fibroblasts with a defect in the internalization of receptor bound low density lipoprotein. *Cell* **9**: 663–674, 1976.
25. Bruce R, Carr MD, Richard-Parker JR, The binding and metabolism of Low-density lipoprotein by skin fibroblasts of fetuses and new borns with anencephaly. *Am J Obstet Gynecol* **157**: 351–354, 1987.
26. Brown M and Goldstein JL, Regulation of the activity of the Low-density Lipoprotein receptor in human fibroblasts. *J Biol Chem* **6**: 307–316, 1975.
27. Hale JE and Schroeder FJ, Differential scanning calorimetry and fluorescence probe investigations of very low density lipoprotein from the isolated perfused rat liver. *J Lipid Res* **22**: 838–851, 1981.
28. Kleinmany Y, Hypolipidemic therapy modulates expression of apolipoprotein B epitopes on low density lipoproteins. Studies in mid to moderate hypertriglyceridemic patints. *J Lipid Res* **28**: 540–548, 1987.
29. Steinbrecher U, Oxidation of human low density lipoprotein results in derivatization of lysine residues of apolipoprotein B by lipide peroxid decomposition products. *J Biol Chem* **262**: 3603–3608, 1987.
30. Chait A, Eisenberg S, Steinmetz A, Albers JJ and Bierman EL, Low density lipoprotein modified by lipid transfer protein have altered biological activity. *Biochim Biophys Acta* **795**: 314–325, 1984.
31. Aviram M, Bierman EL and Chait A, Modification of low density lipoprotein by lipoprotein lipase or hepatic lipase induces enhanced uptake and cholesterol accumulation in cells. *J Biol Chem* **263**: 15416–15422, 1988.
32. Brown MS and Goldstein JL, Lipoprotein metabolism in the macrophages: implication for cholesterol deposition in atherosclerosis. *Annu Rev Biochem* **52**: 223–261, 1983.
33. Kirby C, Clarke J and Gregoriades G, The effect of the cholesterol content of small unilamellar liposomes on their stability *in vivo* and *in vitro*. *Biochem J* **186**: 591–598, 1980.
34. Abra RM and Hunt CA, Liposome disposition *in vivo*. III. Dose and vesicle-size effects. *Biochim Biophys Acta* **666**: 493–503, 1981.
35. Le Pecq JB, Gosse Ch, Dat-Xuong N and Paoletti C, Deux nouveaux derivés antitumoraux: L'hydroxy-9-methyl-2-elliptinium et l'hydroxy-9-dimethyl-2,6-elliptinium. Action sur la leucemie L1210 de la souris. *C R Acad Sci, Ser D* **281**: 1365, 1975.
36. Paoletti C, Cros S, Dat-Xuong N, Lecointe P and Moisand A, Comparative cytotoxic and antitumoral effects of ellipticine derivatives on mouse L1210 leukemia. *Chem Biol Interact* **25**: 45–58, 1979.