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Original article

Plasma stability and cytotoxicity of lipophilic daunorubicin derivatives incorporated into low density lipoproteins

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Abstract – The selective targeting of antineoplastic drugs to tumours by incorporation in low density lipoproteins (LDL) is an attractive possibility if the drug–LDL complex remains stable in the circulation and is taken up by the tumour. In previous studies we have shown that vincristine– and N-trifluoroacetyladriamycin-14-valerate–LDL complexes were unstable in vivo. We synthesized five N-substituted lipophilic derivatives of daunorubicin and studied their incorporation into LDL. Three out of five daunorubicin derivatives incorporated successfully into LDL. In vitro these complexes were more cytotoxic towards LDL receptor positive Chinese hamster ovary cells than LDL receptor negative cells. Non-specific cytotoxicity was explained by slow dissociation of the drug–LDL complex in plasma. Our results underline the importance of careful studies of plasma stability when investigating lipoproteins and other carriers in drug targeting. © 2000 Éditions scientifiques et médicales Elsevier SAS

low-density lipoprotein / daunorubicin derivatives / drug carrier / tumour therapy

1. Introduction

In cancer chemotherapy efficacy is limited by the low selectivity of the antineoplastic drugs used, leading to severe side-effects such as bone marrow aplasia. In order to achieve a more selective treatment we are investigating the possibilities of targeting cytotoxic drugs to tumour cells using low density lipoproteins (LDL) as carriers, since many types of cancer cells have elevated receptor mediated uptake of LDL compared to the corresponding normal cells [1–4]. Cells acquire cholesterol by de novo synthesis and by internalising LDL, the major cholesterol-carrying lipoprotein, via receptor-mediated endocytosis. Our aim is to use the LDL receptor pathway to more selectively concentrate antitumoural drugs in the tumour cells. We have developed a method to incorporate cytotoxic agents into LDL without interfering with the in vitro and in vivo properties of the lipoprotein [5].

To achieve an effective targeting, a drug–LDL complex should fulfil two criteria: a high degree of incorporation of the drug into LDL and stability of the drug–LDL complex in blood. A major problem in drug targeting using LDL as a carrier is the construction of a drug–LDL complex that has a satisfactory in vivo behaviour. Previous studies have shown that vincristine could be incorporated at a high yield into LDL, with a molar ratio between 100 and 200 (moles drug per mole LDL). The complex was, however, unstable in blood in vivo. AD-32 and paclitaxel could be incorporated into LDL at a lower molar ratio, 40–60, and were also unstable in plasma. When cholesteryl-linoleate was incorporated into LDL by the same method, at a ratio similar to paclitaxel and AD-32–LDL, the complex was stable in the blood as judged by dialysis and mice autoradiography experiments (manuscript in preparation).

It has been described that the structure of the lipophilic substances is important in order to allow a good incor-

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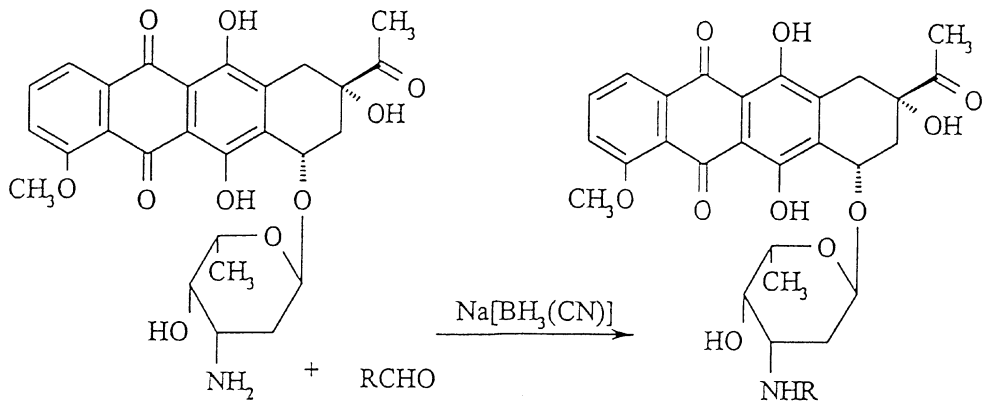
Abbreviations: AD-32: N-trifluoroacetyladriamycin-14-valerate; CHO: Chinese hamster ovary; DNR: daunorubicin; DOX: doxorubicin; LDL: low density lipoprotein; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.



where

comp. 1 R= CH_2-C_6H_4-$OCH_2C_6H_5$-3

comp. 2 R=CH_2-C_6H_3-$(OCH_2C_6H_5)_2$-3,4

comp. 3 R=$(CH_2)_{15}CH_3$

comp. 4 R=$(CH_2)_4$COO-cholesteryl-3

Figure 1. Structures of daunorubicin derivatives.

poration into LDL [6, 7]. By studying the incorporation of various cholesteryl esters and other esters of long chain fatty acids into LDL these authors concluded that the protein or phospholipid components of LDL interact with long chain hydrocarbons containing one or more double bond such as oleyl, retinyl, or cholesteryl esters. However, the stability of LDL complexes in plasma or in vivo has not been studied extensively. It was reported that a complex obtained by mixing LDL with dietylenetriaminepentaacetic acid-bis(stearylamide) was stable in plasma in vitro [8]. The purpose was to use LDL, labelled with this lipophilic chelator, as a potential radiopharmaceutical for tumour imaging. A lipophilic prodrug of daunorubicin incorporated into apolipoprotein E-exposing liposomes was recently also shown to be stable in vitro in serum and in vivo in mice [9].

The objective of our investigation was to synthesize lipophilic derivatives of daunorubicin in order to study their incorporation into LDL and to study the in vitro stability of the drug–LDL complexes in blood. We synthesized four N-substituted derivatives of daunorubicin with lipophilic alkyl, aryl- and cholesteryl- substituents. The free amino function of anthracyclines, like daunorubicin, plays a key role in the activity of the drug but it has been suggested that 3'-N-alkylation of the parent anthracycline decreases cardiotoxicity of the product while preserving some antitumour activity [10, 11]. We also prepared a C-14 1,4-dihydroisonicotinoil derivative of daunorubicin. These derivatives were incorporated into LDL by the sucrose method [5]. The plasma stability of the complexes was studied using a dialysis method and we studied cytotoxicity towards normal and mutant (LDL–receptor negative) Chinese hamster ovary (CHO) cell lines by the MTT method [12].

2. Chemistry

We describe the synthesis of five compounds: N-(3-benzyloxybenzyl)-daunorubicin hydrochloride dihydrate (**1**), N-(3,4-dibenzyloxybenzyl)-daunorubicin (**2**), N-hexadecyldaunorubicin hydrochloride (**3**), N-(3-cholesteryloxycarbonyl-(4'-butyl))-daunorubicin (**4**) and 14-(2,6-dimethyl-3,5-diethoxycarbonyl-1,4-dihydroisonicotinoyl)-doxorubicin (**5**). The structures of these derivatives are illustrated in *figure 1*.

For the introduction of a substituent into the 3' position we chose the method of reductive alkylation that has already been used for some N- and N,N-di- substituted daunorubicin and doxorubicin derivatives containing short and intermediate (up to C10) alkyl groups, as well as benzyl and morpholine moieties [10, 11]. The reaction takes advantage of formation of a Shiff base between the anthracycline's amino group and an aldehyde, which is in

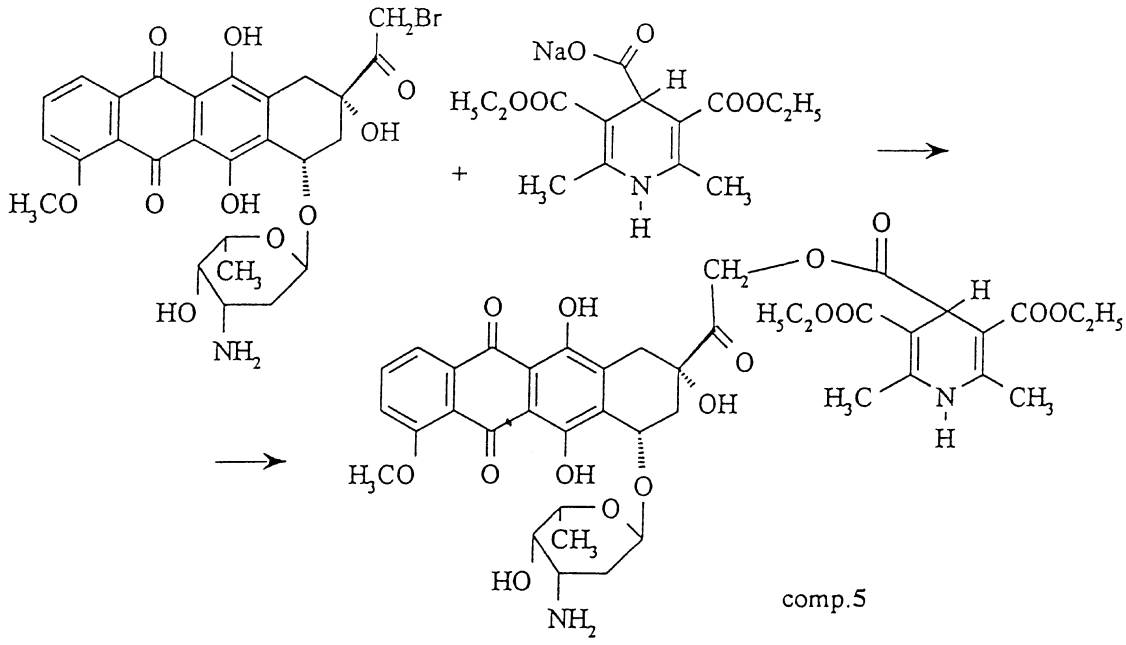


Figure 2. Synthesis of **5**.

situ reduced to alkyl amine. In order to investigate the possibility of reductive N-alkylation to introduce larger lipophilic substituents, we chose hexadecanal (palmitic aldehyde, synthesized from 1-bromohexadecane according to the procedure described in [10]), 3-benzyloxybenzaldehyde and 3,4-dibenzyloxybenzaldehyde (Aldrich).

In order to obtain N-alkylated doxorubicin containing the cholesterol moiety (**4**) (distanced from the sugar moiety in order to eliminate sterical influence), we had to prepare the ω-aldehyde of a cholesterol ester. Using a procedure similar to that in [13] we produced cholesteryl 5-bromovalerate. To oxidize the bromo ester into the corresponding aldehyde we tried to oxidize with dimethyl sulfoxide [14]. However, the method did not lead to the expected aldehyde. Then we tried the oxidation with pyridine N-oxide, according to [15]. The desired cholesteryl 5-oxovalerate was isolated from the product mixture with 18% yield.

The synthesis of **5** is described in *figure 2*. We thought that the great lipophilicity of the 1,4-dihydropyridine moiety could facilitate the incorporation into LDL [16]. Some 1,4-dihydropyridine derivatives are also good scavengers of hydroxyl radicals [17]. The cardiotoxicity caused by anthracyclines has been suggested to be caused by the overproduction of hydroxyl radicals [18]. So, the liberation of a 1,4-dihydropyridine residue by enzymatic hydrolysis of the ester bound at C-14 could result in additional benefit.

3. Results

3.1. Incorporation of the anthracycline derivatives

The results of the incorporation of compounds **1**, **2** and **5** into LDL are presented in *table I*: The incorporation of compounds **3** and **4** into LDL failed. The drug yields were generally low, around 20% or less, except for **5**. The highest molar ratio, 154 moles of drug per mole lipoprotein, was obtained with **5**, when using 1 mg of drug for 2 mg of LDL. At 1 mg of drug for 10 mg LDL, an LDL yield of 96% was obtained but the molar ratio was reduced to 53. The incorporation of compound **2** was less successful, with a lower drug yield, 5–15% and a molar ratio of 30. A molar ratio between 62 and 79 was obtained for compound **1.**

3.2. Stability of drug–LDL complexes: dialysis against PBS and human plasma at 37 °C

As reference, we studied the stability of AD-32–LDL by dialysis against human plasma. As illustrated in *figure 3*, AD-32 slowly leaked into the plasma chamber of the dialysis device where it was detected after 80 min

Table I. Yields and molar ratios obtained after incorporation of the DNR derivatives into LDL.

Compound	Mg	LDL (mg)	Drug yield[a]	LDL yield[a]	MR
1	2	10	30	60	79
1	1	2	10	63	62
2	2	10	15	75	32
2	1	2	5.5	76	28
5	1	10	65	96	53
5	1	2	23.5	60	154
AD-32	2.5	10	25	77	71
AD-32	1	10	40	77	46

[a] In %; [b]moles of drug per mole LDL.

together with a more polar metabolite, AD-41, N-trifluoroacetyl-adriamycin. A second metabolite was detected after 140 min. After 10 h the sum of AD-32 and its metabolites reached 8.5% of the AD-32 present in LDL at time zero. When AD-32–LDL was dialysed against PBS, neither AD-32 nor AD-41 were found in PBS. The LDL was unable to pass the membrane as determined by radioactivity measurements (data not shown).

The dialysis of the complex **1**–LDL against PBS is illustrated in *figure 4a*. Compound **1** wasn't dialysing out of the LDL complex. After about 5 h, two metabolites of compound **1** were found in the PBS chamber and less than 5% of the **1** present originally in the LDL complex was detected after 12 h. When **1**–LDL was dialysed against plasma (*figure 4b*) the same metabolites were also detected. The major substance leaking out in plasma was compound **1** which reached almost 5% after 12 h. Another minor metabolite (m3) was also detected (less than 1% after 12 h).

The dialysis of **2**–LDL against PBS is illustrated in *figure 5*. In the LDL complex chamber, compound **2** was

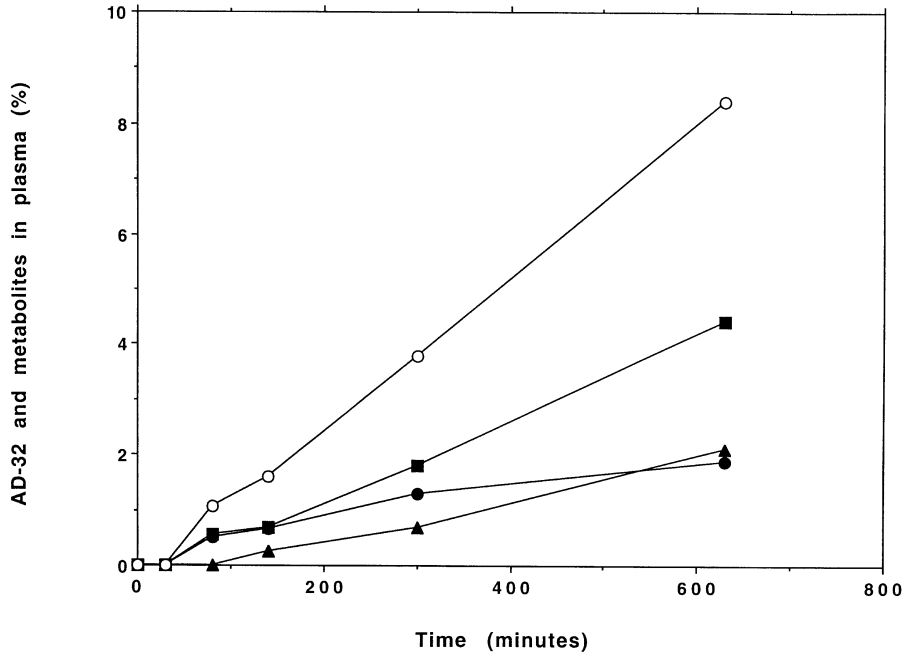


Figure 3. Dialysis of AD32–LDL against plasma. One millilitre of AD-32–LDL complex was dialysed against 1 mL of plasma as described in Experimental protocols. AD-32 (●) and its metabolites, AD-41 (■), m2 (▲) and their sum (○) were measured by HPLC in plasma as a function of time (see Experimental protocols) and expressed as percent of the initial value of AD-32.

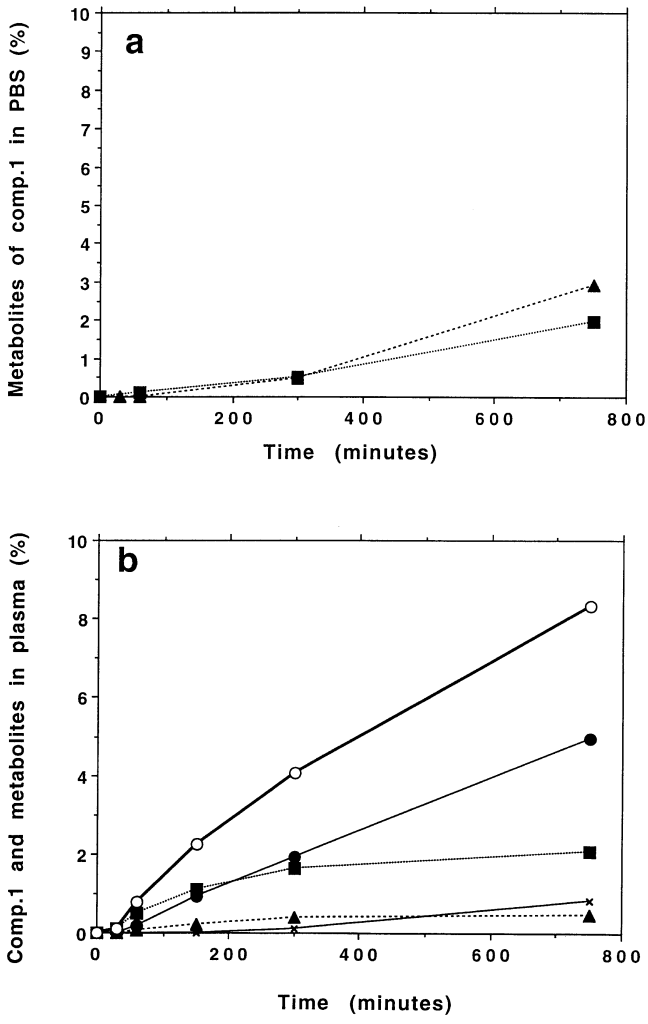


Figure 4. Dialysis of **1**–LDL against PBS (**a**) and plasma (**b**). The **1**–LDL complex was dialysed as described in Experimental protocols. **1** (●), its metabolites m1 (■), m2 (▲), m3 (**x**) and their sum (○) were measured by HPLC in plasma or PBS as a function of time and expressed as percent of the initial value of **1**.

rapidly degraded. Some fluorescent metabolites could be detected after 5 h, but they were also degraded into non-fluorescent substances. Neither compound **2** or its metabolites could be detected in the PBS chamber.

Figure 6a shows the recovery of **2** and its metabolites in the LDL complex chamber, during dialysis of **2**–LDL against plasma: compound **2** was much more stable in this case. After 30 min 20% of compound **2** had disappeared and the remaining was leaking slowly out of the chamber. Two more polar metabolites were also detected increasing slowly with time. The leakage of **2** into the plasma chamber is shown in *figure 6b*: after 12 h, only 0.5% of **2** could be detected, but the sum of the metabolites exceeded 11%.

When **5**–LDL was dialysed against plasma, only compound **5** was detected in the plasma chamber after 11 h, representing 7.5% of the initial amount present in the LDL complex (data not shown).

3.3. In vitro cytotoxicity of the **1**–, **2**– *and* **5**–*LDL complexes towards normal and mutant (LDL receptor-negative) Chinese hamster ovary cells (CHO cells)*

As illustrated in *figure 7*, the cytotoxicity of the 3 complexes **1**–, **2**– and **5**–LDL was studied at two different concentrations of LDL. All three complexes were slightly more cytoxic for cells expressing the LDL receptor.

4. Discussion

Of the five anthracycline derivatives studied, compounds **3** and **4** could not be incorporated into LDL by our method. Studies published earlier by Krieger et al. stressed the importance of the structure of the substance to be incorporated in order to achieve a good incorporation [6]. Our derivative **4** presents such a favourable structure but it could not be incorporated with our method. Our incorporation method uses sucrose to protect LDL during freezing and freeze-drying. The protection of LDL by sucrose during the freeze-drying step is so efficient that it almost totally hinders the extraction of the lipids of LDL by heptane. By this method, the incorporation of a substance into LDL is not really a lipid substitution but a lipid addition. In the case of compound **4** the incorporation failure could possibly be attributed to steric hindrance due to the size of the cholesteryl group. We have however no rational explanation for the failure of incorporation of compound **3**, the hexadecyl derivative of DNR.

In order to check the stability of the LDL complexes, we studied the dialysis of the complexes against PBS and plasma. As reference, we studied AD-32–LDL, a complex which is unstable in plasma as judged from clearance studies in leukaemic patients.

AD-32–LDL was stable when dialysed against PBS. However when the complex was dialysed against plasma, AD-32 leaked slowly into the plasma chamber where it was rapidly metabolized into AD-41 and another more polar metabolite, probably the C-14 reduced form of AD-41. This leakage in plasma can be attributed to an association with other lipoproteins since we know that plasma lipoproteins, particularly HDL, compete with

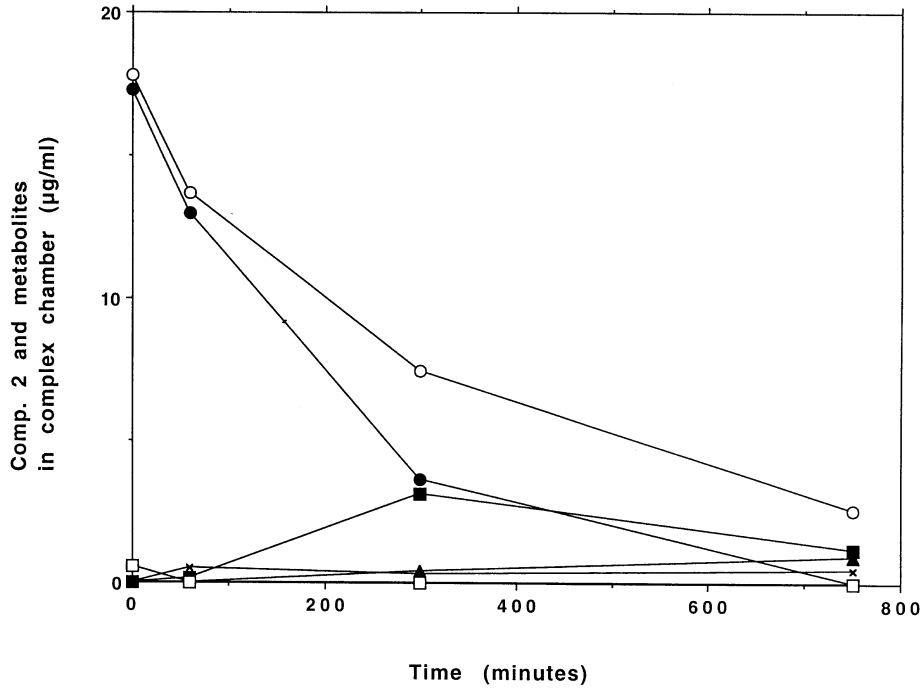


Figure 5. Dialysis of **2**–LDL against PBS. The **2**–LDL complex was dialysed as described in Experimental protocols. **2** (●), its metabolites m1 (■), m2 (▲), m3 (**x**), m4 (□) and their sum (○) were measured by HPLC in the complex chamber as a function of time and expressed as concentrations in µg/mL.

LDL to complex AD-32 during an incubation in plasma as measured after isolation of the lipoproteins by sequential ultra-centrifugation (data not shown).

The dialysis patterns obtained with the three **1**–, **2**–, **5**–LDL complexes were similar to that of AD-32–LDL; each drug dialysed slowly into the plasma chamber where it was metabolized (except for **5**). Moreover, the complexes were cytotoxic towards CHO receptor negative cells even if the cytotoxicity was greater towards receptor positive cells. It seems obvious to conclude that the three complexes studied did not fulfil the criteria for plasma stability required to obtain an effective targeting.

At this point it is difficult to conclude that it is possible to use the sucrose method to incorporate a lipophilic cytotoxic derivative into LDL so that it will not leak out in the plasma. We know that after incorporation of cholesteryl linoleate into LDL, a stable complex is obtained. Indeed, cholesteryl linoleate does not dialyse into plasma and presents a plasma clearance similar to LDL after intravenous injection of cholesteryl-linoleate–LDL in rabbits (to be published elsewhere).

The stability of LDL complexes in plasma has been studied by some groups. Jasanada et al. succeeded in incorporating a lipophilic anchor (bis-stearylamide) bound to an In^{3+} chelator into LDL and the complex obtained was stable in vitro in plasma [8]. Their stability test is based on the precipitation of LDL by addition of heparin to the plasma samples. They did not specify if the incubation was done at 37 °C, but if this was the case, bis-stearylamide is surely an interesting lipophilic anchor. Another interesting lipophilic anchor, 3α-O-(oleoyl)-5β-cholanic acid was recently described by Versluis et al. [9]. It was coupled to daunorubicin through a lysosomally degradable peptidic spacer-arm. The complex obtained by co-sonication of this lipophilic derivative of daunorubicin and apolipoprotein E-exposing liposomes was also relatively stable in blood, as 70% of the initially incorporated drug was still associated to the liposomes 4 h after an intravenous injection in mice.

Our results underline the importance of a careful study of plasma stability when investigating LDL or lipid emulsions as carriers in drug targeting. The dialysis against plasma is a simple and rapid test to check plasma stability.

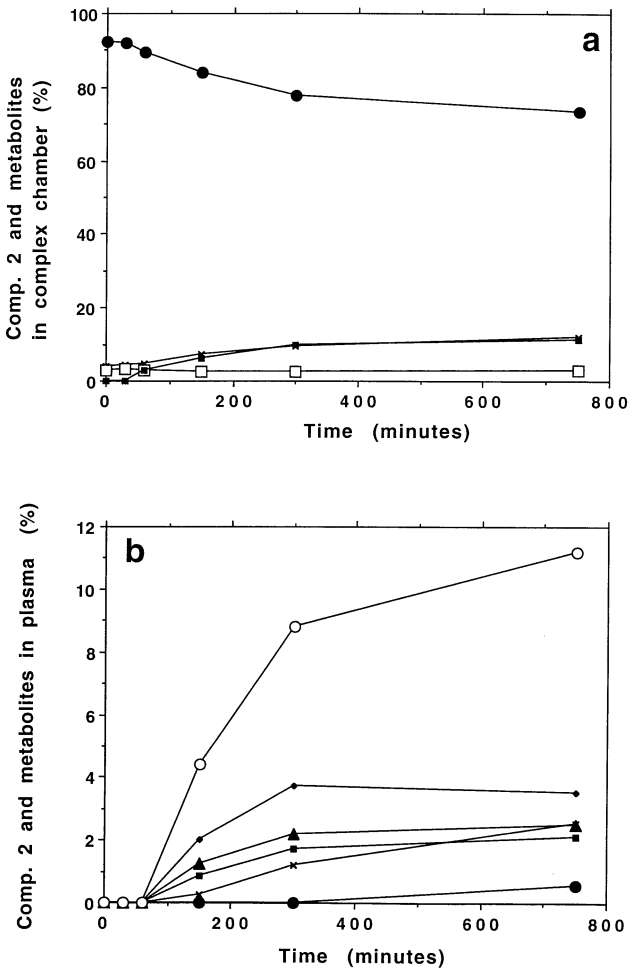


Figure 6. Dialysis of **2**–LDL plasma. The **2**–LDL complex was dialysed as described in experimental protocols. **2** (●), its metabolites m2 (▲), m3 (**x**), m4 (□), m5 (■), m6 (◆) and their sum (○) were measured by HPLC in the complex (**a**) and in plasma chambers (**b**) as a function of time and expressed as percent of the initial quantity of **2**.

5. Experimental protocols

All glassware was baked at 89–100 °C for a minimum of 2 h before being used. ^{1}H-NMR spectra were obtained with an NMR spectrophotometer Brucker-90/DS at 90 MHz. Chemical shifts are reported in parts per million (δ scale), employing tetramethylsilane as internal standard and using the following abbreviations: singlet (s), doublet (d), triplet (t), multiplet (m). All reagents and solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI).

*5.1. N-(3-benzyloxybenzyl)-, N-(3,4-dibenzyloxybenzyl)- and N-hexadecyldaunorubicin (***1***, **2** *and* **3***)*

Daunorubicin hydrochloride (0.100 g, 0.18 mmol) and the corresponding aldehyde (3.6 mmol) in 5.5 mL of a mixture of CH_3CN and water (3:1) were mixed (the palmitic aldehyde was dissolved in 0.1 mL of $CHCl_3$, then a mixture of 7 mL of 1,2-dimethoxyethane and 0.5 mL water was added; after the addition of daunorubicin, methanol was added dropwise under vigorous stirring until the mixture was almost homogenous). Thereafter $Na[BH_3(CN)]$ (18 mg, 0.28 mmol) was added and the mixture was stirred for 30 min. Water (7 mL) was finally added and the solution was extracted with 3 × 5 mL of $CHCl_3$. The pooled $CHCl_3$ extracts were washed with water, dried over Na_2SO_4 and the solvent was evaporated. The residue, most of which was the excess aldehyde, contained, as determined by TLC, a mixture of eight–nine anthracycline derivatives: daunorubicin was almost completely consumed and there was one obvious major product. The mixture was purified by chromatography over a Silasorb 600 (10 μ, Chemapol, Czech Republic) column (26 × 150 mm). Compound **1** was eluted with $CHCl_3$ (to remove the excess aldehyde) then with a $CHCl_3/CH_3OH$ mixture (15:1). Compound **2** was eluted with $CHCl_3$ then with a $CHCl_3/CH_3OH$ mixture in a volume ratio of first 15:1, then 7:1. Compound **3** was eluted with a $CHCl_3$/hexane mixture in a volume ratio 1:1 first, then 3:1 and finally with $CHCl_3$ alone. The major product was collected. Since the chromatographic bands of this complex mixture overlapped partly, part of the crude product required repeated purification by column or thin layer chromatography. This resulted in a 3–10% yield of the corresponding daunorubicin derivatives. Hydrochlorides of compounds **1** and **3** were prepared by dissolving amorphous substance in $CHCl_3$, adding an equimolar amount of 1 M methanolic HCl and stirring the mixture for a few minutes. The solvent was evaporated under reduced pressure, the residue was dissolved in methanol, the solution was dried over Na_2SO_4 and evaporated again. The structures of the products were confirmed by ^{1}H-NMR spectrum and elemental analysis data.

1: ^{1}H-NMR ($CDCl_3$), δ: 13.95, 13.15 (2H, s, 6,11-OH); 8.00 (1H, d (7 Hz), 1-H); 7.77 (1H, t (7 Hz), 2-H); 7.36 (1H, d (7 Hz), 3-H); 7.30 (ovl. $CHCl_3$, s, arom. N-substituent); 7.08, 7.00, 6.82 (3H, m, arom. N-substituent); 5.55 (1H, m, 1'-H); 5.21 (1H, m, 7-He); 4.92 (2H, s, 3-PhCH_2O); 4.60 (1H, br., 9-OH); 4.17, 3.95 (2H, d (AB, 13 Hz), NCH_2Ph non-equiv.); 4.13, 4.10 (2H, m, 5'-H, 3'-H); 4.04 (3H, s, 4-OCH_3); 4.00 (1H, br., 4'-OH); 3.33 (1H, d (6 Hz), 4'-H); 3.16, 2.90 (2H, d (AB,

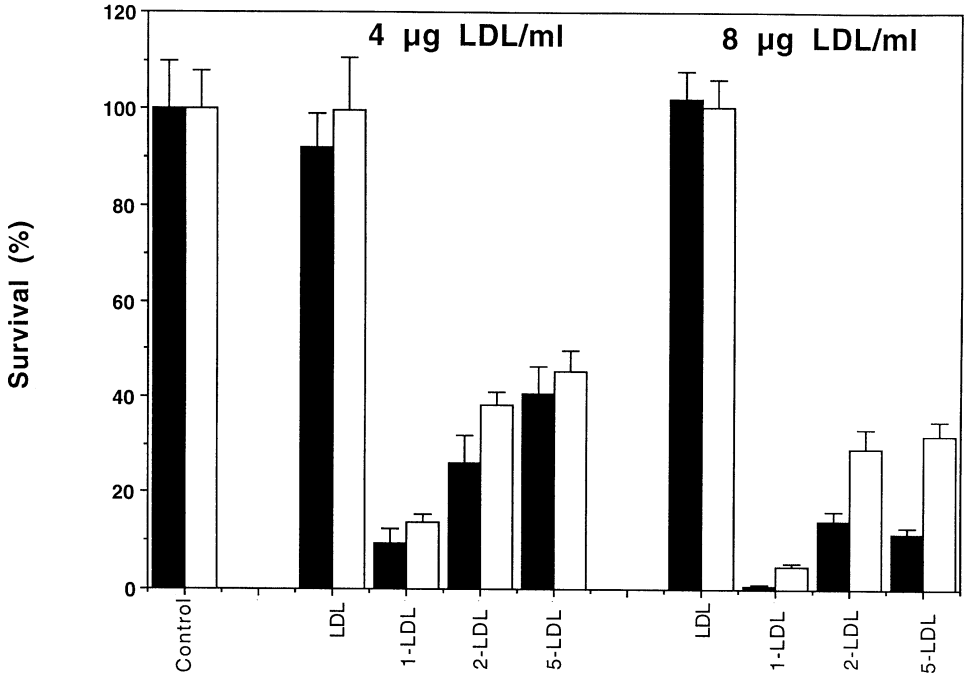


Figure 7. Cytotoxicity of the LDL complexes towards CHO receptor positive and negative cells. The cytotoxicity of LDL, **1**–LDL, **2**–LDL and **5**–LDL towards CHO receptor positive (filled bars) and receptor negative cells (empty bars) was determined by the MTT method following incubation at two LDL concentrations (4 and 8 µg/mL) for 96 h as described in Experimental protocols and the results were expressed as percentage of the absorbance of the control cells incubated with vehicle only. Values are means ± SEM of six incubations.

18 Hz), 10-He, 10-Ha); 2.36 (3H, s, 14-CH_3); 2.25, 2.08 (2H, d (AB, 15 Hz), 8-He, 8-Ha); 1.8 (1H, m, 2'-H); 1.28 (3H, d (6 Hz), 5'-CH_3). Calcd. (C, H, N %): 61.84, 5.82, 1.76. Found (C, H, N %): 61.17, 5.55, 1.81.

2: ^{1}H-NMR: ($CDCl_3$), δ: 14.00 (2H, b.s, 6,11-OH); 7.95 (1H, d (7 Hz)., 1-H); 7.70 (1H, t (7 Hz), 2-H); 7.35–7.18 (11H, m, ovl. $CHCl_3$, 3-H and 2x(C_6H_5)); 6.86 and 6.73 (3H, two s, C_6H_3); 5.50 (1H, m, 1'-H); 5.23 (1H, m, 7-He); 5.04 and 5.00 (4H, two s, 2x(OCH_2Ph)); 4.52 (1H, b.s, 9-OH); 4.02 (3H, s, 4-OCH_3); 4.20–3.90 (2H, m, 5'-H, 3'-H); 3.62 (3H, m, ovl. NCH_2 and 4'-OH); 3.21 (1H, d (20 Hz), 10-He); 2.85 (2H, d (20 Hz), ovl. 10-Ha and 4'-H); 2.39 (3H, s, 14-CH_3); 2.30 and 2.03 (2H, two d (15 Hz), 8-He, 8-Ha); 1.74 (1H, m, 2'-H); 1.29 (3H, d (6 Hz), 5'-CH_3).

3: ^{1}H-NMR ($CDCl_3$), δ: 13.95, 13.15 (2H, s, 6,11-OH); 8.00 (1H, d (7 Hz), 1-H); 7.77 (1H, t (7 Hz), 2-H); 7.36 (1H, d (7 Hz), 3-H); 5.55 (1H, m, 1'-H); 5.21 (1H, m, 7-He); 4.80 (1H, br., 9-OH); 4.13, 4.10 (2H, m, 5'-H, 3'-H); 4.04 (3H, s, 4-OCH_3); 4.0 (1H, br., 4'-OH); 3.75 (2H, t (5 Hz), NCH_2); 3.33 (1H, m, 4'-H); 3.25, 2.83 (2H, d (AB, 18 Hz), 10-He, 10-Ha); 2.39 (3H, s, 14-CH_3); the higher fields poorly resolved due to the hexadecyl substituent and hydrocarbon contamination. Calcd. (C, H, N %): 65.50, 7.93, 1.78. Found (C, H, N %): 68.89, 9.68, 1.10.

*5.2. N-[3-Cholesteryloxycarbonyl-(4'-butyl)]-daunorubicin (**4**)*

5.2.1. Cholesteryl 5-bromovalerate

Cholesterol (3.09 g, 8.0 mmol) and 5-bromovaleric acid (1.81 g, 10 mmol) were reacted in 30 mL of refluxing benzene in the presence of *p*-toluenesulfonic acid (0.26 g, 1.5 mmol) for 3 h with separation of the produced water from the benzene condensate (Dean-Stark trap). The reaction mixture was extracted three times with aqueous $NaHCO_3$ (1%) and three times with water. The organic phase was dried over K_2CO_3 and the solvent was evaporated. The obtained light yellow oil was recrystallized from ethanol to give 2.40 g (55%) of white crystals, m.p.: 96–98 °C. ^{1}H-NMR ($CDCl_3$), δ: 5.24 (1H, d (4 Hz), 6-CH); 4.60 (1H, br., 3-CH); 3.40 (2H, t (6 Hz), CH_2Br).

Calcd. (C, H, N %): 69.92, 9.72, 14.50. Found (C, H, N %):70.50, 9.90, 13.22.

5.2.2. Cholesteryl 5-oxovalerate

Cholesteryl-5-bromovalerate (3.0 g, 5.4 mmol), pyridine N-oxide (1.04 g, 10.9 mmol) and $NaHCO_3$ (0.92 g, 10.9 mmol) were refluxed for 4 h in toluene (7 mL) under argon. The reaction mixture was poured into water (15 mL) and extracted with ether. The organic phase was washed with water and dried over Na_2SO_4. The solvent was evaporated, the oily residue was purified by column chromatography over Silasorb 600 (10 μ) with $CHCl_3$ as the eluent. The product was obtained as 0.47 g (18%) of light yellow oil that slowly crystallizes; m.p.: 90–92 °C. ^{1}H-NMR ($CDCl_3$), δ: 9.72 (1H, t (1.5 Hz), CHO); 5.24 (1H, d (4 Hz), 6-CH); 4.60 (1H, br, 3-CH).

*5.2.3. N-[3-Cholesteryloxycarbonyl-(4'-butyl)]-daunorubicin (**4**)*

To cholesteryl-5-oxovalerate (1.79 g, 3.6 mmol) dissolved in 1 mL of $CHCl_3$, was added daunorubicin hydrochloride (100 mg, 0.18 mmol), dissolved in 5 mL of a mixture of CH_3CN and water (3:1) under vigorous stirring. CH_3OH was then added dropwise until the mixture was almost homogeneous and the stirring was continued for 15 min. Thereafter $Na[BH_3(CN)]$ (18 mg 0.28 mmol) was added and the mixture was stirred for 30 min. After addition of water (7 mL), the solution was extracted with $CHCl_3$ (3 × 5 mL); the pooled $CHCl_3$ extracts were washed with water, dried over Na_2SO_4 and the solvent was evaporated. The residue, most of which was the excess aldehyde, contained, as determined by TLC, a mixture of seven–eight red coloured spots. Daunorubicine was almost completely consumed, and there was one obvious major product. The mixture was separated by column chromatography over Silica gel 60 (0.04–0.063 mm, Fluka), with a $CHCl_3$/hexane mixture (1:2) as the eluent, then with $CHCl_3$ alone and finally with different mixtures of $CHCl_3/CH_3OH$ (15:1; 7:1; 3:1). The major product was collected. Since the chromatographic bands of this complex mixture overlapped partly, a part of the crude product required repeated purification by column or thin layer chromatography. This resulted in 35 mg of N-[3-cholesteryloxycarbonyl-(4'-butyl)]-daunorubicin (**4**). ^{1}H-NMR ($CDCl_3$), δ: 14.05 and 13.35 (2H, two s, 6,11-OH); 8.00 (1H, d (7 Hz), 1-H); 7.75 (1H, t (7 Hz), 2-H); 7.40 (1H, d (7 Hz), 3-H); 5.55 (1H, m, 1'-H); 5.30 (3H, m, ovl. 7-He and 6″-H(hol) and NH); 4.75 (1H, s, 9-OH); 4.80–4.40 (2H, m, ovl. 3″-H(hol) and 5'-H); 4.10 (3H, s, 4-OCH_3); 4.10–3.90 (2H, m, ovl. 3'-H and 4'-OH); 3.60 (1H, m, 4'-H); 3.25 and 2.95 (2H, two d (18 Hz), 10-He, 10-Ha); 2.42 (3H, s, 14-CH_3); the higher fields poorly resolved due to the cholesteryl substituent and hydrocarbon contamination: 2.60–0.70 (59H, m, ovl. 5'-CH_3, 2'-H, 8-H, Hol, $(CH_2)_4$).

*5.3. 14-(2,6-Dimethyl-3,5-diethoxycarbonyl-1,4-dihydroisonicotinoyl)-daunorubicin (**5**)*

A mixture of 14-bromodaunorubicin hydrochloride [19] (0.065 g, 0.1 mmol) and sodium 2,6-dimethyl-3,5-diethoxycarbonyl-1,4-dihydroisonicotinate (0.5 g, 1.6 mmol) in 60 mL of anhydrous acetone was stirred at refluxing temperature for 3.5 h, then filtered and evaporated. The residue was suspended in 15 mL aqueous $NaHCO_3$ (1%), the solid was filtered, washed with water and dried. The HPLC profile showed that the residue contained the desired product (60%) and 14-bromodaunorubicin (5%). The mixture was purified by thin layer chromatography (Kieselgel 60, Merck) using a mobile phase of $CHCl_3$–CH_3OH–H_2O in a volume ratio of 13:6:1. This resulted in 10 mg of compound **5**.

^{1}H-NMR ($CDCl_3$), δ: 13.60 (2H, two s, 6,11-OH); 7.95 (1H, d (7 Hz), 1-H); 7.70 (1H, t (7 Hz), 2-H); 7.40 (1H, d, 3-H); 6.00 (1H, s, N''-H); 5.50 (3H, m, 1'-H); 5.25 (1H, m, 7-He); 5.15 (2H, s, 14-CH_2); 5.00 (1H, s, 4″-H); 4.70 (1H, b.s, 9-OH); 4.22 (4H, q (7 Hz) 3'', 5''- CH_2); 4.05 (3H, s, 4-OCH_3); 4.40–3.80 (3H, m, 5'-H, 3'-H, 4'-OH); 3.65 (1H, m, 4'-H); 3.15 and 2.85 (2H, two d (20 Hz), 10-He, 10-Ha); 2.40 (6H, s, 2'', 6''-CH_3); 2.30 and 2.03 (2H, two d (15 Hz), 8He, 8Ha); 1.74 (1H, m, 2'H); 1.30 (9H, m, ovl. 5'-CH_3 and 3'', 5''-CH_3).

5.4. Incorporation of daunorubicin derivatives into LDL

Human LDL (density 1.02–1.063 g.mL^{-1}) was isolated from plasma from healthy blood donors by sequential ultra-centrifugation as described by Havel et al. [20]. The LDL was labelled by reaction with ^{14}C-sucrose as described by Langer et al. and Pittman et al. [21, 22].

The six anthracycline derivatives were incorporated into LDL as previously described for the cytotoxic drug N-trifluoroacetyladriamycin-14-valerate (AD-32) with minor modifications [5]. In order to allow the quantification of the lipoprotein, traces of ^{14}C-LDL were added giving a final specific activity: 0.1 dpm per ng protein. Briefly, after dialysis against 0.3 mM NaEDTA pH 7.4, LDL (at 5 mg/mL) was transferred to a glass tube containing 10 mg sucrose/mg LDL (40% sucrose solution). The solution was rapidly frozen in liquid nitrogen and lyophilized overnight. The anthracycline derivative, dissolved in methylene chloride, was added to the dried LDL and the mixture was incubated for 1 h at room temperature. The solvent was then evaporated under

nitrogen and the LDL complex was reconstituted by addition of 100 µL of phosphate buffered saline, pH 7.4 per mg of LDL. The preparation was finally filtered through a Sephadex G-25 column (PD-10 columns, Pharmacia Biotech Sweden), then through a 0.22 µm sterile filter and stored in the dark at 4 °C.

Drug content of LDL was determined by high performance liquid chromatography using a phenyl-µBondapak column (3.9 × 150 mm, 5 µm, Waters Associates, Milford, MA) eluted with acetonitrile and 0.2% ammonium format pH 4 (60:40, v/v) at a flow rate of 1.0 mL/min. The drug was quantified using a Gilson Model FL-1B fluorescence spectrophotometer.

The radioactivity of ^{14}C-labelled LDL was measured on an LKB Wallac 1217 liquid scintillation counter using Ecoscint A as scintillation solution (National Diagnostics, Atlanta, USA). Protein content was measured by the method of Lowry et al. using bovine serum albumin as standard.

5.5. Stability test

One millilitre of LDL–drug complex was dialysed against 1.0 mL of human plasma, alternatively PBS, in a Plexiglas device containing two chambers separated by a dialysis membrane (Spectra/Por membrane, MWCO: 12–14 000, Spectrum Medical Industries, Inc., Houston). The dialysis was performed at 37 °C in a shaking water bath. Aliquots were removed at different times and analysed for drug and protein content. The human plasma used in these experiments was obtained freshly from healthy blood donors and was frozen. A control dialysis performed with fresh plasma did not show any differences.

5.6. In vitro cytotoxicity on normal and mutant (LDL receptor-negative) CHO cells

Normal and mutant CHO cells were grown in monolayer culture. They were maintained in a humidified incubator (5% CO_2, 95% air) at 37 °C in a growth medium consisting of RPMI-1640 supplemented with antibiotics (100 IU of penicillin + 100 mg of streptomycin/mL), L-glutamine (1%), and 10% foetal calf serum. For experiments, confluent cell monolayers were detached with a solution containing 0.05% trypsin and 0.02% EDTA and seeded in growth medium on day 0 at a concentration of 4 500 cells/mL (100 µL) into 96 well round-bottomed microtitre plates. The complex was added the next day in 10 µL PBS. On day 4, 10 µL of an MTT (Sigma) solution (5 mg/mL) was added and the plates were incubated at 37 °C for 4 h; 100 µL of 10% SDS in 10 mM HCl were added and incubated for 24 h at 37 °C. The absorbances were read by a microplate spectrophotometer (Labsystem Multiscan RC) at 540 nm with a reference at 640 nm in order to quantify the proliferation of the cells [12].

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References

[1] Gal D., MacDonald P.C., Porter J.C., Simpson E.R., Int. J. Cancer 28 (1981) 315–319.

[2] Ho Y.K., Smith R.G., Brown M.S., Goldstein J.L., Blood 52 (1978) 1099–1114.

[3] Vitols S., Angelin B., Ericsson S., Gahrton G., Juliusson G., Masquelier M. et al., Proc. Natl. Acad. Sci. 87 (1990) 2598–2602.

[4] Vitols S., Gahrton G., Ost A., Peterson C., Blood 63 (1984) 1186–1193.

[5] Masquelier M., Vitols S., Peterson C., Cancer Research 46 (1986) 3842.

[6] Krieger M., McPhaul M.J., Goldstein J.L., Brown M.S., J. Biol. Chem. 254 (1979) 3845–3853.

[7] Firestone R.A., Pisano J.M., Falck J.R., McPhaul M.M., Krieger M., J. Med. Chem. 27 (1984) 1037–1043.

[8] Jasanada F., Urizzi P., Souchard J.P., Le G.F., Favre G., Nepveu F., Bioconjug. Chem. 7 (1996) 72–81.

[9] Versluis A.J., Rump E.T., Rensen P.C., Van Berkel T.J., Bijsterbosch M.K., J. Pharmacol. Exp. Ther. 289 (1999) 1–7.

[10] Acton E.M., Anthracyclines: Current Status and New Developments, Academic Press, 1980, pp. 15–25.

[11] Tong G.L., Henry D.W., Acton E.M., J. Med. Chem. 22 (1979) 36–39.

[12] Mosmann T.J., Immunol. Meth. 65 (1983) 55–63.

[13] Satawari K., Mukai T., Tsukinuki K., Kamenosono S., Jap. Pat. 78 80059 CA (1975) 112202.

[14] Dave P., Byun H.S., Engel R., Synth. Commun. 16 (1986) 1343–1346.

[15] Stowell J.C., J. Org. Chem. 35 (1970) 244–245.

[16] Shatz V.D., Muhametshina V.G., Tirzite D.Y., Tirzit G.D., Dubur G.Y., Khimikofarmatevt. Zhurnal 4 (1985) 482–486.

[17] Tirzit G.D., Kazush E.Y., Dubur G.Y., Chem. Heterocycl. Comp. 4 (1992) 435–437.

[18] Pratt W.B., Ruddon R.W., Ensminger W.D., Maybaum J., The Anticancer Drugs, University Press, N.Y., Oxford, 1994.

[19] Arcamone F., Franceschi G., Penco S., Belgium Patent 731 (1969) 398.

[20] Havel R.J., Eder H.A., Bragdon J.H., J. Clin. Invest. 34 (1955) 1345–1353.

[21] Langer T., Strober W., Levy R.I., J. Clin. Invest. 51 (1972) 1528–1536.

[22] Pittman R.C., Attie A.D., Carew T.E., Steinberg D., Biochim. Biophys. Acta 710 (1982) 7–14.