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Evaluation of methods for complexing prednimustine to low density lipoprotein

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Summary

Many cancer cells express a higher receptor-mediated uptake of low-density lipoprotein (LDL) than normal cells. LDL has been proposed as a carrier for anticancer agents in order to improve the selectivity of cancer chemotherapeutics. In this study two reassembly and one transfer method for preparation of prednimustine-LDL complex were evaluated. All three methods gave stable complexes between drug and lipoprotein, but only the reassembly methods gave satisfactory incorporation of drug. The binding, uptake, and metabolism of the drug-LDL conjugates by T-47D breast cancer cells and GM 2000 LDL-receptor negative fibroblasts showed similar values for all three types of complexes and native LDL. Native LDL was able to compete for the cellular uptake of ^{125}I-labelled drug-LDL complexes as well as for the ^{125}I-labelled native LDL, strongly suggesting a LDL-receptor mediated uptake. The cytotoxicity of the complexes prepared by the reassembly methods was tested on cultured T-47D cells. The preparations showed high and similar activities with ID_{50} values near 2 μg/ml while the free drug gave a value of 5.1 μg/ml under the same incubation conditions. The results are discussed in terms of important factors for the successful conjugation of drugs with LDL.

Introduction

A major problem associated with chemotherapy of cancer is the lack of selectivity of antineoplastic drugs. A fascinating strategy to improve the therapeutic response is to target drug-carrier conjugates on neoplastic cells. Recent advances in cell biology and immunology have formed promising rationales for targeted drug-carrier systems utilizing, e.g., cellular receptors and monoclonal antibodies (Poznansky and Juliano, 1984). A candidate for targeted drug delivery, which has been paid recent attention, is LDL (De Smidt and Van Berkel, 1990). LDL is a spherical particle (diameter 23 nm) with a core of neutral lipids (cholesteryl esters, triglycerides) surrounded by a monolayer of phospholipids, cholesterol, and protein. Cellular LDL receptors bind the protein component apolipoprotein B (apoB), LDL is then internalized through receptor-mediated endocytosis and delivered to the lysosomes where the components are hydrolyzed (Goldstein and Brown, 1977). Interest in LDL as a drug carrier has been triggered by the discovery that many cancer cells express higher LDL receptor activity

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than corresponding normal cells; for example, epidermoid cervical carcinoma (EC-50) cells, metabolize LDL at a rate 50 times faster than non-neoplastic gynecologic tissue (Gal et al., 1981). The LDL particle can also be modified in such ways that it is recognized by receptors other than the classical LDL receptor; e.g., acetylated LDL is rapidly taken up by endothelial liver cells (Nagelkerke et al., 1983) and lactosylated LDL is effectively catabolized by hepatocytes (Bijsterbosch and Van Berkel, 1990).

The objective of the present study is to compare different techniques for conjugation of drugs with LDL. The methods can be divided into three groups; transfer methods, reassembly methods, and covalent binding. A severe problem associated with the use of drug-LDL complexes is unspecific cellular uptake of drug as a result of physical transfer (Vitols et al., 1984). A solution to this problem is to use highly lipophilic, non-exchangeable, compounds. This approach limits the available procedures mainly to the reassembly methods. Among these, two alternative protocols have been developed, both involving extraction of LDL neutral lipids. The delipidated apoB is then reconstituted with the drug dissolved in an organic solvent (Krieger et al., 1978) or in the form of a microemulsion (Lundberg, 1987). The delipidation methods are efficient and particles with high amounts of incorporated drug can be prepared. However, the physiological behaviour of the reconstituted LDL is more problematic, since the extraction step may modify apoB. The transfer methods are potentially milder to the protein structure, but the efficiency of the incorporation is doubtful. The facilitated transfer method has proven to be successful for endogenous molecules, but the specificity of the transfer protein limits its use with foreign molecules like drugs (De Smidt and Van Berkel, 1990). The covalent binding method yields a stable product, but the attachment of the drug to groups on the surface of LDL reduces the receptor binding capacity and hence targeting utility (Halbert et al., 1985).

In a previous study from this laboratory, the cytotoxic activity of prednimustine-LDL complexes against T-47D breast cancer cells was shown to be nearly 50% higher than that of the free drug (Lundberg, 1992). However, in a recent paper, Eley et al. (1990) found a much lower activity of complexes prepared by a modified Krieger method. The goal of this study was to clarify this obvious contradiction and to extend our knowledge about factors governing the formation of active drug-LDL complexes.

Materials and Methods

Chemicals

Prednimustine, the 21-chlorambucil ester of prednisolone, was synthesized at Pharmacia LEO Therapeutics AB (Helsingborg, Sweden). Sodium [^{125}I]iodide (16.3 mCi/μg, carrier free, pH 7–11) was obained from the Radiochemical Centre (Amersham, U.K.). High purity egg phosphatidylcholine (EPC) was obtained from Sigma Chemical Co. Triolein was purchased from Merck (Darmstadt, Germany) and polysorbate 80 from Fluka Chemie AG (Buchs, Switzerland). All tissue-culture media were purchased from Gibco Biocult (Paisley, U.K.).

Lipoprotein

Human LDL (density, 1.019–1.063 g/ml) was isolated by differential density ultracentrifugation from fresh, pooled serum using standard procedures (Lindgren et al., 1972). The purity of the isolated lipoprotein was checked by agarose gel electrophoresis. LDL was labelled with ^{125}I by the iodine monochloride method (Bilheimer et al., 1972). The lipoprotein preparations were filtered through a 0.22 μm Millipore filter and stored at 4°C in sterile ampoules.

Cell culture

T-47D breast cancer cells and GM 2000 LDL-receptor negative cells were grown in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 0.1 mM nonessential amino acid solution, 0.08% (w/w) sodium bicarbonate, streptomycin (10 μg/ml), penicillin (10 μg/ml), and 10% (v/v) fetal calf serum. Cells were maintained at 37°C and gassed with 6% (v/v) CO_2 in air. Cells were detached from the culture flasks using 0.25% trypsin in balanced salt solution.

Cellular uptake and metabolism of [^{125}I]LDL and drug-[^{125}I]LDL

Determination of binding, incorporation, and degradation of native LDL and drug-LDL complexes was performed essentially as described by Brown and Goldstein (1976). The degradation was calculated from the amount of trichloroacetic acid-soluble radioactivity in the incubation medium after extraction of free iodine with chloroform. Values of bound LDL and drug-LDL complexes were obtained by incubation of the cells with a solution of heparin (5 mg/ml) in PBS at 4°C. The dishes were then washed three times with cold PBS and the cells were detached by gentle scraping. The cells were dissolved in 0.1 N NaOH to quantify protein and internalized LDL and drug-LDL complexes.

Preparation of drug-LDL complexes

Three different methods were used for complexing of drugs to LDL, two reassembly procedures denoted method A (Krieger et al., 1978) and method B (Lundberg, 1992), and a transfer method, method C (Craig et al., 1982). The two reassembly methods involved extraction of LDL with heptane originally described by Gustafson (1965). In short, LDL (0.5 mg of protein) in siliconized glass tubes was dialyzed against 0.3 mM sodium EDTA, lyophilized in the presence of potato starch (starch/protein 12:1), and the neutral lipids were removed by two extractions with 5 ml of heptane at −10°C. Regarding method A, the heptane-extracted LDL was then reconstituted by addition of 0.5 mg prednimustine and 1.5 mg triolein dissolved in 100 μl of heptane-ethyl ether (1:1 v/v). The tube was incubated for 1 h at −10°C, the solvents were evaporated at 0°C, and the reconstituted LDL was solubilized by incubation in PBS for 12 h at 4°C. Starch and unincorporated drug were then removed by two centrifugation steps, 2000 × *g* for 10 min and 10000 × *g* for 20 min, at 4°C.

The main steps in method B involve the preparation of a miroemulsion containing the lipophilic drug and the conjugation of the emulsion particles with delipidated LDL. The delipidation of LDL was performed by heptane extraction in the same way as for method A. The composition of the microemulsion was 0.5 mg prednimustine, 1.5 mg triolein, 1 mg EPC, and 0.4 mg polysorbate 80. The components were dispersed from stock solutions into vials and after evaporation of the solvent under a stream of nitrogen the vials were vacuum-desiccated overnight. PBS was added to the vials and the mixture was sonicated for 3 × 20 s at 30°C using a MSE sonifier equipped with a titanium microprobe. The resulting drug microemulsion was added to the delipidated apo B, and the mixture was incubated at room temperature for 30 min.

The main steps of method C are the preparation of a drug-microemulsion by an injection technique and incubation of the resulting preparation with LDL in the presence of whole plasma or lipoprotein-deficient plasma. The microemulsion components, 1 mg each of the components prednimustine, cholesteryl oleate and dimyristoylphosphatidylcholine, in dry 2-propanol solution maintained at 55°C were injected into a rapidly vortexing solution of PBS at 18°C. The 2-propanol was subsequently removed from the solution by centrifugation through a buffer-depleted Sephadex G-50 column. LDL was then fused with the microemulsion by incubation at 37°C for 5 h. In some initial experiments the incubation was performed with and without 20% lipoprotein deficient plasma with essentially the same result for both procedures. In further experiments, LPDS was omitted from the reaction mixture. After completion of incubation, LDL with incorporated drug was obtained by flotation of the drug-containing microemulsion and isolation of LDL at a density of 1.019–1.063. After completion of drug-LDL conjugation the preparations were dialyzed against PBS, filtered through a 0.22 μm Millipore filter, and stored in sterile ampoules at 4°C.

Determination of cytotoxicity

The ability of drug-LDL complexes to inhibit cell growth was measured by determination of [^{3}H]thymidine incorporation. Cells were grown to the mid-logarithmic phase and incubation was initiated by adding fresh medium and the indicated concentration of drug-LDL complex or free drug dissolved in ethanol:DMSO (1:1 v/v). At 3

h prior to the end of the experiment, 1 μCi [^{3}H]thymidine/ml was added to the culture medium. After 24 h the incubation was terminated by discarding the medium and washing the cells with ice-cold PBS. The cells were detached by trypsin treatment, and the cell suspension was filtered through Whatman GF/C filters and washed with cold PBS. The filters were dried in an oven at 50°C for 30 min, transferred to scintillation vials, and counted for radiactivity in a 1216 Rackbeta scintillation counter (LKB-Wallac, Turku, Finland).

Analytical procedures

The prednimustine concentration was measured by high-pressure liquid chromatography (HPLC) using a 25 cm Ultra Techspere 5-ODS column (HPLC Technology Ltd) eluted with methanol:0.1 M acetic acid (80:20, v/v). The liquid chromatograph consisted of a Spectroflow 400 solvent delivery system and a 757 absorbance detector (Kratos Analytical Instruments) coupled to a Schimadzu C-R3A Chromatopac integrator. The particle size of native and drug-LDL complexes was measured by quasi-elastic light scattering (distribution of mass) on a Malvern Autosizer (Malvern Instruments, Malvern, U.K.). Protein concentration was determined by a modified Lowry method (Markwell et al., 1978) using bovine serum albumin as standard. Alternatively, protein concentration in drug-LDL complexes was calculated from radioactivity using the specific activity of [^{125}I]LDL.

Results

Three methods for complexation of the antineoplastic drug prednimustine to LDL were evaluated on the basis of quantitative association, in vitro receptor-dependent cellular uptake and cytotoxic activity. The conjugation of drug and LDL was performed essentially as described in the original papers by Krieger et al. (1978) (method A), Lundberg (1992) (method B), and Craig et al. (1982) (method C). However, the use of serum in the transfer method (method C) was abandoned since its presence did not increase the incorporation of prednimustine. The physical characteristics of the drug-LDL complexes have been reported in previous papers and hence this study was limited to measurements of particle size, solution stability, and recoveries of drug and protein. Three batches of native human LDL were used and each was sujected to the three complexation methods. The native human LDL had a particle diameter of 23.5 ± 0.3 nm (mean $\pm$ SD, $n = 3$). The drug-LDL complexes, regardless of preparation method, all showed an increase in particle diameter with values of 44.2 ± 16.5, 55.8 ± 3.8, and 28.7 ± 4.2 nm, for method A, B, and C, respectively. The stability of the complexes was very good and no significant aggregation or change in particle diameter were noted during storage of sterile preparations at 4°C for several months. In practice the preparations were used within 1 week after they were made. The recoveries of protein were good for methods A and B with values of 83.2 ± 8.3 and 89.1 ± 8.6%, respectively, while that of method C was considerably lower at 61.7 ± 11.3%. The recoveries of drug were much lower than those for protein and were measured to 16.2 ± 2.8, 25.0 ± 3.0, and 3.6 ± 0.8% for method A, B, and C, respectively. Eley et al. (1990) employed a modification of method A, using a very high drug to LDL ratio and sucrose for protection of the structure of apoB. This method showed a poor recovery of drug, perhaps as a result of weak extraction of neutral lipids from LDL. A control of the extraction procedure showed an extraction efficiency of only about 10%.

A basic condition for the successful use of drug-LDL conjugates as a site-specific drug delivery system is that the receptor-binding property of apoB is preserved. The biological activity of reassembled LDL particles was studied in vitro by the classical methods of Brown and Goldstein (1976). The results with T-47D cells, which possess LDL receptors, are shown in Fig. 1. The values for binding, uptake, and degradation of ^{125}I-labelled apoB are very similar for all three types of complexes and native LDL. The cellular uptake of native LDL was approx. 400 ng/mg cell protein per h. In order to further study the specificity of cellular uptake, the incorporation of

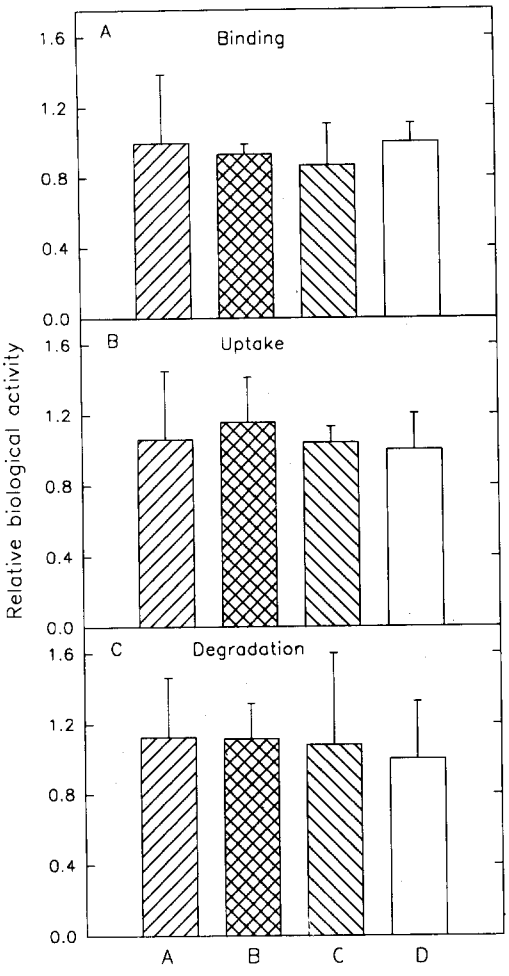

Fig. 1. Relative values for binding, uptake, and degradation by T-47D cells of drug-[^{125}I]LDL complexes compared to those of native [^{125}I]LDL. (A–C) Represent methods A–C, respectively, and (D) native LDL (mean ± SD, $n = 5$).

^{125}I-labelled drug-LDL complexes and native LDL into T-47D cells was measured in the presence and absence of excess unlabelled LDL. The data presented in Fig. 2 show that native LDL competes to a similar degree with all three types of drug-LDL conjugates as well as with labelled native LDL itself. This fact strongly supports the concept that reassembled LDL particles are taken up by the LDL-receptor pathway (Goldstein and Brown, 1977).

The specificity of the binding and uptake of drug-LDL complexes as compared to native LDL was further studied by incubation with receptor-negative GM 2000 fibroblasts (Fig. 3). None of the conjugates showed any excessive binding or uptake compared to native LDL, which supports the conclusion that the LDL-receptor pathway is the major mechanism for cellular uptake of drug-LDL conjugates. The LDL uptake value for the GM 2000 cells was about 50 ng/mg cell protein per h. Regarding degradation, conjugates prepared by methods A and C demonstrate considerably higher relative values than method B and native LDL, but the absolute values were very low for all preparations.

The cytotoxic activity of prednimustine-LDL complexes towards T-47D cells was studied by measurement of [^{3}H]thymidine incorporation into DNA (Fig. 4). Complexes prepared by methods A and B showed a very similar cytotoxic effect with 50% inhibition (ID_{50}) at a concentration of around 2 μg/ml. The procedure used by Eley et al. (1990) gave complexes with a somewhat greater cytotoxic effect with an ID_{50} value of 1.8 μg/ml. The ID_{50} value of the free drug was 5.1 μg/ml under the same incubation conditions. The incorporation of drug into LDL by method C was regarded as too low to allow a meaningful test of the cytotoxic effect.

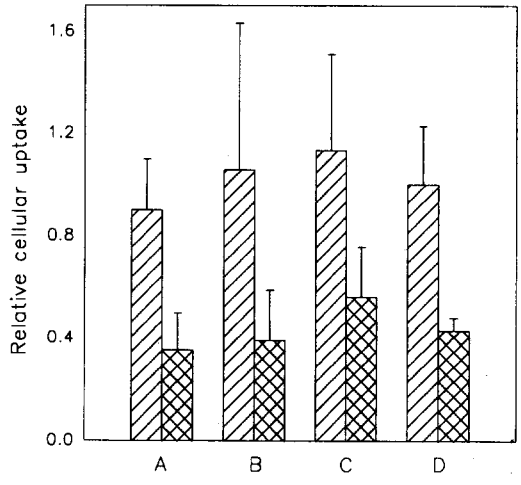

Fig. 2. Ability of an ten-fold excess of native LDL to compete for the uptake by T-47D cells with drug-[^{125}I]LDL complexes prepared by methods A (A), B (B) and C (C) in relation to native [^{125}I]LDL (D). Stacked bars represent values without and hatched bars values with excess unlabelled LDL (mean ± SD, $n = 4$).

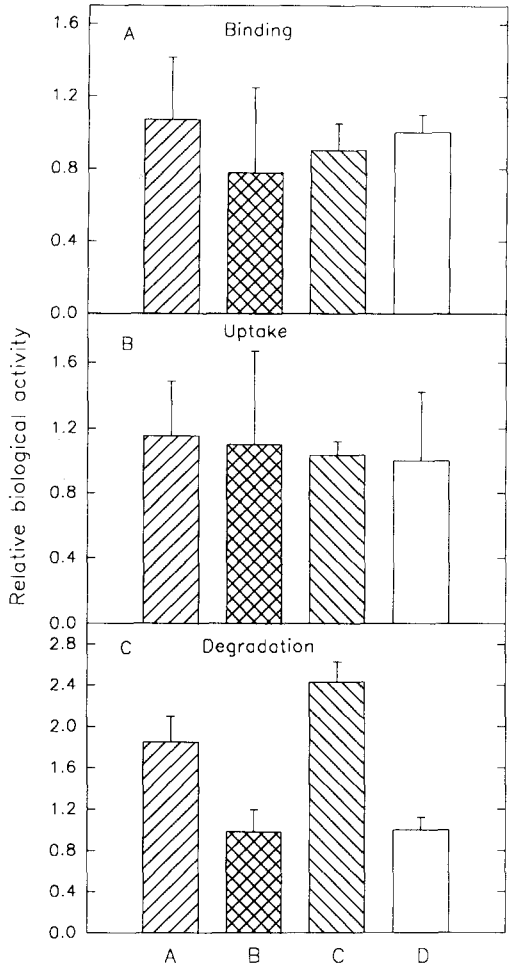

Fig. 3. Relative values for binding, uptake, and degradation by LDL-receptor negative GM 2000 fibroblasts of drug-[^{125}I]LDL complex. (A–C) Denote values for complex prepared by methods A–C, respectively, and (D) shows native [^{125}I]LDL for comparison (mean ± SD, $n = 4$).

Discussion

The results presented in this study support the findings presented in earlier papers that drug-LDL complexes represent good delivery systems for lipophilic drugs (Lundberg, 1987, 1992). The ID_{50} value of about 2 μg/ml for the complexes is considerably lower than that of 5.1 μg/ml obtained for the free drug. In contrast to these results, Eley et al. (1990) reported that the prednimustine-LDL complex is approx. 10 times less active than the free drug. However, preparations made by the same protocol in the present study showed in fact somewhat better activity than the two other methods tested (Fig. 4). An obvious difference is, however, that Eley et al. (1990) used P388 murine leukemia cells for cytotoxicity testing, while this study employed T-47D breast cancer cells. It has been demonstrated that P388 cells have a very low lipoprotein uptake (Lombardi et al., 1989). It is thus tempting to interpret the diverging results as an expression of the different LDL-receptor activity of the cells used for cytotoxicity testing. The conclusion is that, since the specificity of the drug-LDL method depends on the LDL-receptor activity, care should be taken to use cells expressing many LDL-receptors. The supply of cancer cells demonstrating high LDL-receptor activity is abundant, which has been demonstrated by several authors (for a review, see Peterson, 1991).

A factor which must be considered regarding the reassembly methods is the efficiency of neutral lipid extraction from the core of the lipoprotein. The delipidation of LDL, stabilized with potato starch, by cold heptane has been shown to remove neutral lipids almost completely (Krieger et al., 1978). This observation was confirmed in this study. Masquelier et al. (1986) modified this

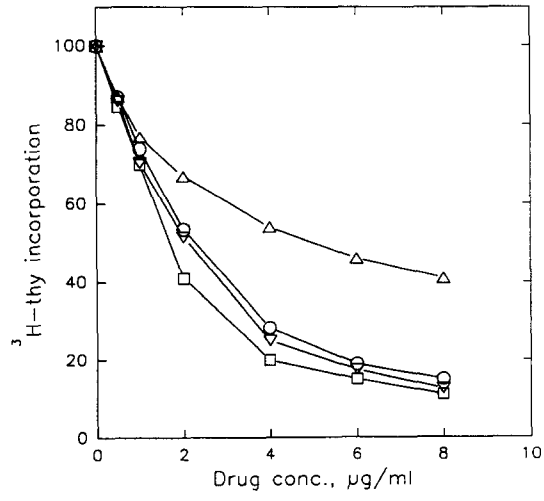

Fig. 4. Cytotoxicity of prednimustine-LDL complexes prepared by method A (○), B (▽), and that used by Eley et al. (1990) (□) compared to that of the free drug (△).

procedure and used sucrose as stabilizing agent. This modified Krieger method resulted in more monodisperse particles and an improved in vivo behaviour (Vitols et al., 1990). A plausible explanation for these effects could be that the remaining neutral lipids help to stabilize apoB and enhance the reassembly of lipoprotein particles. However, one should bear in mind that incomplete delipidation may also reduce the incorporation of drug. Therefore, it is important to check the extraction efficiency before proceeding to the reassembly step.

A considerable problem regarding the application of the drug-LDL method is the tendency of many drugs to leak out from the complex. This drawback can be illustrated by the behaviour of the natural lipids cholesterol and cholesteryl esters. Cholesterol belongs to the water-insoluble, polar lipids (it has a polar hydroxyl group) and will orient itself mainly in the surface monolayer of a LDL particle. In spite of its low water solubility, cholesterol will undergo rapid spontaneous transfer (leak out) from LDL to cells (Lundberg and Suominen, 1985). Cholesteryl esters, on the other hand, have no polar group and will reside in the oil core of LDL. The spontaneous transfer of cholesteryl esters is very slow (Ekman and Lundberg, 1987). A much slower rate of transfer than for cholesterol is also shown by phosphatidylcholine, which is anchored with two fatty-acid chains in the surface monolayer (Ekman and Lundberg, 1987). Thus, two structural features of a drug which lead to the formation of stable drug-LDL complexes are a nonpolar nature, e.g., for cholesteryl esters or a double-chain amphiphatic structure, such as for phosphatidylcholine.

However, a nonpolar and lipophilic nature of a drug is insufficient as such to make a drug suitable for incorporation into LDL. Another factor which is also very important is the physicochemical properties of the drug. It has been clearly demonstrated that irrespective of whether the drug is complexed with apoB in the form of a solution in organic solvent (Krieger et al., 1978) or as a microemulsion (Lundberg, 1987), its physical state should be liquid or liquid crystalline. Krieger et al. (1979) concluded that heptane-extracted LDL can be reconstituted with a wide variety of hydrophobic molecules. However, it was not possible to incorporate cholesteryl, glyceryl, and methyl esters of saturated fatty acids with high melting points while the corresponding low-melting-point unsaturated esters offered no problem. Regarding the preparation of appropriate drug microemulsions, not only the physicochemical state but also the microviscosity of the compound is an important factor. This fact has been demonstrated with cholesteryl oleate and triolein as model substances. Triolein with lower microviscosity than cholesteryl oleate yields a finer emulsion. However, the properties of the cholesteryl ester can be improved by addition of triolein to the ester sample (Lundberg, 1991). The same procedure was used in this study. Prednimustine is a solid powder at body temperature with a melting point as high as 165°C. In order to improve the physicochemical properties it was solubilised in triolein in both methods A and B.

The facilitated transfer method (method C) utilizing the cholesteryl ester transfer protein in serum is potentially a very mild method. It has also been shown to result in efficient incorporation of cholesteryl ester into LDL (Blomhoff et al., 1984). Since prednimustine shows structural resemblance to the natural substrate for the transfer protein, it was considered important to test this method. However, the result was very disappointing regarding the recovery of drug. The same result was reached by by De Smidt and Van Berkel (1990) for incorporation of dioleoyl-FdUrd. The conclusion is that the high degree of structure specificity of the transfer protein limits the general use of the facilitated transfer method for complexing drugs to LDL. The other two methods proved to be comparable regarding both cellular uptake and cytotoxic activity. In favour of method A is the higher drug recovery, which might be an important factor when dealing with expensive drugs. It is worth noting that with all three complexation methods the sizes of the resulting particles are greater than that of native LDL. This factor could possibly change the rate of internalization (St. Clair et al., 1980), but no systematic connection between particle size and cellular uptake was noted in this study.

The use of the LDL particle as a drug carrier in cancer chemotherapy may help to solve at least two problems; LDL can be used to solubilize lipophilic drugs and more importantly to increase the drug concentration in malignant cells expressing a higher number of LDL receptors than normal cells. However, it is not possible to achieve absolute selectivity since normal cells also have LDL receptors. This problem can partly be overcome by downregulating the LDL receptors in the liver and the adrenals (Hynds et al., 1984). A prerequisite to the application of LDL as a drug carrier is the development of functional drug-LDL particles that express in vivo behaviour similar to that of native LDL. In fact, recent animal in vivo studies of drug-LDL complexes indicate that positive therapeutic effects can be obtained (De Smidt and Van Berkel, 1990; Vitols et al., 1990). Furthermore, a pioneering human study has been presented (Filipowska et al., 1992). However, the development of new cytotoxic drugs specially designed for incorporation into LDL would greatly enhance the progress in this field. For large-scale clinical use, it might be necessary to employ artificial lipoprotein (neo-lipoproteins). Such lipoprotein particles can be assembled from suitable lipids and genetically engineered apoproteins.

Acknowledgments

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