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Assembly of prednimustine low-density-lipoprotein complexes and their cytotoxic activity in tissue culture

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Summary. The lipophilic anticancer drug prednimustine was incorporated into model low-density-lipoprotein (m-LDL) using a novel modified method. The major steps of this procedure involve the preparation of a microemulsion containing the drug and the complexing of this emulsion with apolipoprotein B (apo B) that has been delipidated by heptane extraction. The resulting particles contained on average 338 mol prednimustine/mol apo B and exhibited a diameter that was ca. 2.5 times that of native LDL. The cellular binding, uptake, and metabolism of the complexes were found to be similar to those of native LDL. The cytotoxic activity of the complexes was monitored in vitro against T-47D breast cancer cells and normal 3T3 fibroblasts. The activity of prednimustine in m-LDL against T-47D cells after 24 h treatment was nearly 50% higher than that of the free drug, whereas in 3T3 cells the difference was relatively small. The results indicate that it is possible to target drug/m-LDL complexes to cancer cells exhibiting high LDL-receptor activity.

Introduction

A major problem in cancer chemotherapy is the lack of selectivity of antitumoral drugs. To improve the selectivity of cancer chemotherapeutics, attempts have been made to link them to carriers, e.g., hormones and antibodies, or to enclosed them in liposomes before their administration (for review see [18]). However, the in vivo administration of such drug-carrier complexes has proved to be problematic because of rapid clearance, immunological reactions, or problems with the stability of drug-carrier complexes.

Recently, low-density lipoprotein (LDL) has been considered as a carrier system for antineoplastic agents. LDL consists of an apolar core of cholesteryl esters and of triglycerides surrounded by a monolayer of phospholipids, free cholesterol, and apoprotein B (apo B). Apo B mediates the cellular uptake of LDL via a specific high-affinity receptor pathway [5]. The neutral lipid core of LDL can be replaced by exogenous neutral lipids or other suitable hydrophobic compounds showing retained biological activity [14]. The rationale for the use of LDL as a carrier for targeted delivery of antitumoral drugs is that many cancer cells express LDL-receptor activity higher than that of normal cells [20].

In a previous study in this laboratory, the successful incorporation of a lipophilic cytotoxic agent into the core of reassembled LDL was reported and the biological activity of the resulting complex was evaluated [11]. The hypothesis that a cytotoxic drug-LDL complex may be valuable as a carrier system is supported by a recent report demonstrating therapeutic effects of such preparations in vivo [21]. The present paper describes a simplified method for the incorporation of lipophilic drugs into LDL using the anticancer drug prednimustine as the drug component. The receptor-dependent uptake and the cytotoxicity of the resulting complex were determined in vitro against normal and cancer cells.

Materials and methods

Chemicals. Prednimustine, the 21-chlorambucil ester of prednisolone, and [^{3}H]-prednimustine were synthesized at Pharmacia LEO Therapeutics AB (Helsingborg, Sweden). Chlorambucil and prednisolone were supplied by Sigma Chemical Company (St. Louis, Mo.). Sodium iodide I 125 (16.4 mCi/μg, carrier-free, pH 7–11) was obtained from the Radiochemical Centre (Amersham, UK). Egg phosphatidylcholine (EPC) was obtained from Sigma Chemical Company. Triolein and cholesteryl oleate were purchased from Merck (Darmstadt, FRG). The purity of the lipids was checked by thin-layer chromatography (TLC). Triton X-100 was provided by Calbiochem (La Jolla, Calif.), and Pluronic F68 was obtained from Fluka Chemie AG (Buchs, Switzerland). All tissue-culture media were purchased from Gibco Biocult (Scotland).

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Lipoprotein. Human LDL (density, 1.019–1.063 g/ml) and lipoprotein-deficient serum (density, >1.215 g/ml) were isolated by differential density ultracentrifugation from fresh, pooled human serum using standard procedures [10]. The isolated lipoprotein showed no detectable impurity at agarose gel electrophoresis. LDL was labeled with iodine 125 by the iodine monochloride method [1]. Labeled LDL was passed through a Sephadex G-25 column and dialyzed against 0.15 M NaCl and 0.05% ethylenediaminetetraacetic acid (EDTA, pH 7.4) to remove free iodine. The lipoprotein preparations were filtered through a 0.22-μm Millipore filter and stored at 4° C in sterile ampoules.

Cell culture. Normal 3T3 fibroblasts and T-47D breast cancer 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 5% (v/v) CO_2 in air.

Preparation of prednimustine-LDL complexes. The cytotoxic drug prednimustine was incorporated into m-LDL by a modification of a previously described method [11]. The main steps in the procedure involve the preparation of a microemulsion containing the lipophilic drug and the complexing of the emulsion particles with delipidated apo B. The composition of the microemulsion particles by weight was 3 parts triolein, 1 part cholesteryl oleate and 1 part prednimustine as core components and 2.5 parts EPC, 1 part Pluronic F68, and 1 part Triton X-100 as surface components. The components were dispersed from stock solutions into vials and the solvents were evaporated under a stream of N_2 and were vacuum-desiccated overnight. Phosphate-buffered saline (PBS) was added to give the desired concentration, and the mixture was sonicated for 3 × 20 s at room temperature using an MSE sonifier equipped with a titanium microprobe.

The protein part of LDL, apo B, was delipidated by extraction with heptane as described by Vitols et al. [21]. LDL was lyophilized overnight in the presence of 25% (w/w) sucrose as a protecting agent, and the dried LDL was then extracted with 3 × 2.5 ml heptane at 4° C. The prednimustine microemulsion was added to the delipidated apo B, and the mixture was incubated at room temperature for 30 min. Before its use, the prednimustine/m-LDL preparation was dialyzed against PBS and filtered through a 0.22-μm Millipore filter.

Cellular uptake and metabolism of [^{125}I]-LDL and [^{125}I]-m-LDL. Determinations of the binding, incorporation, and degradation of native and reconstituted m-LDL were performed as described by Goldstein and Brown [5]. Cellular degradation of LDL was quantitated from an aliquot of trichloroacetic acid-soluble radioactivity of the incubation medium following the extraction of free iodine with chloroform. Incorporated LDL was determined from the radioactivity in aliquots of washed detached cells. Values for bound LDL were obtained by incubation of the cells with heparin (5 mg/ml) in PBS.

Determination of toxicity. The ability of prednimustine and prednimustine/m-LDL complexes to inhibit cell growth was measured by cell counting and by determination of [^{3}H]-thymidine incorporation. Cells were grown to the midlogarithmic phase and were refed with fresh medium on the day on which the experiment started. Free prednimustine was added to the incubation medium as an ethanol : dimethylsulfoxide (DMSO; 1 : 1, v/v) solution such that the final ethanol concentration did not exceed 0.1%.

Cell counts were done using a Coulter Counter, with the cells being dispersed in Isoton II (Coulter Electronics, Ltd., Luton, England). [^{3}H]-Thymidine incorporation was determined by a double-lable method. After the cells had adhered to the surface of the dish, 0.5 μCi L-[^{35}S]-methionine/ml was added to the medium for measurements of the relative amount of cellular protein. Prior to the experiment, fresh medium containing the same concentration of [^{35}S]-methionine was added. At 3 h prior to the end of the experiment, 1 μCi [^{3}H]-thymidine/ml was added to the culture medium. The cells were detached by trypsin treatment, and the suspension was filtered through Whatman GF/C filters and washed with ice-cold PBS. The filters were dried in an oven at 60° C for 30 min, transferred to scintillation vials, and counted for radioactivity in a 1216 Rackbeta (LKB-Wallac, Turku, Finland) scintillation counter using gate settings computed to distinguish between ^{3}H and ^{35}S.

Analytical procedures. The prednimustine concentration was measured by high-pressure liquid chromatography (HPLC) using a 25-cm Ultra Techsphere 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. During the preparative steps, the concentration of prednimustine was calculated from the radioactivity using the specific activity of [^{3}H]-prednimustine. Protein was measured by the modified Lowry method [16] using albumin as the standard. The particle size of native LDL and drug-LDL complexes was measured by quasi-elastic light scattering on a Malvern system 4700 submicron particle analyzer (Malvern Instruments, Malvern, UK).

Results and discussion

Preparation and characterization of prednimustine/m-LDL complexes

The LDL-reconstitution method for the preparation of drug/m-LDL complexes presented in this paper is a modification of previously described procedures [11, 14]. The major steps involve the preparation of a drug-containing microemulsion and a partially delipidated apo B followed by the recombination of the two components. Lipid microemulsions have long been used in this laboratory as protein-free lipoprotein models [12]. The delipidation of apo B by heptane extraction was first used by Gustafsson [6] and was subsequently modified by other investigators [9, 17].

Preliminary experiments showed that prednimustine per se does not have suitable physicochemical properties for its successful incorporation into m-LDL. The lipophilic components that constitute the core of the m-LDL particle must be in a liquid or liquid crystalline state [13]. This requirement was accomplished by mixing prednimustine with the two neutral lipids triolein and cholesteryl oleate. Except for its physicochemical properties, prednimustine is a very suitable compound for incorporation into m-LDL. It exhibits a strong lipophilic character, and the ester bond between prednisolone and its active component chlorambucil is likely to be split by esterases in the lysosomes. Prednimustine as an intact molecule seems to be inactive, and its activity is induced by the release of chlorambucil [7]. The importance of lipophilicity is stressed by the observation that in spite of its low water solubility, cholesterol undergoes rapid transfer between lipoproteins and cells [15].

To reduce the sonication time, we enhanced the emulsification properties of EPC by adding the mild "biological" detergents Pluronic F68 and Triton X-100. A satisfactory microemulsion can also be prepared without these detergents, but this requires a much longer sonication period. The reconstituted prednimustine/m-LDL particles obtained by the simplified method presented herein are essentially similar to those obtained by previously described methods [11, 14]. Special attention was paid to the phar-

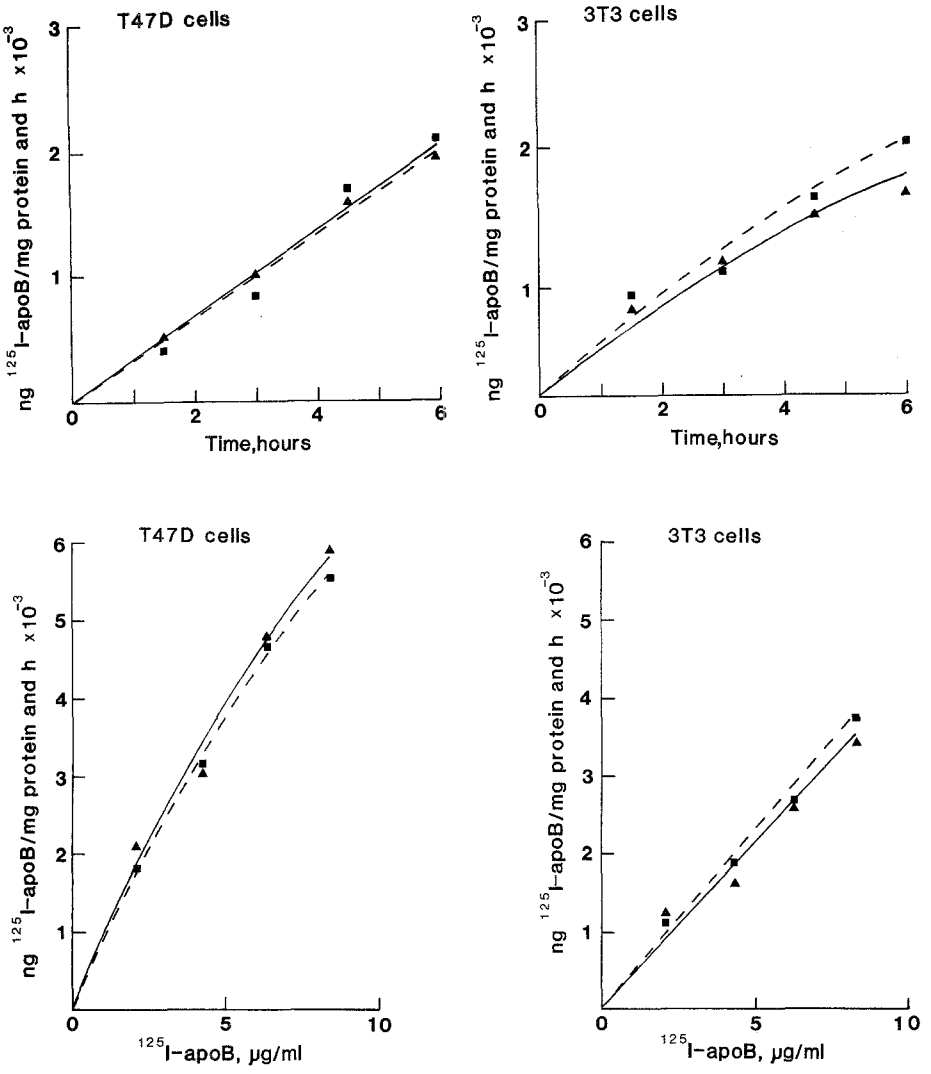

Fig. 1. Degradation of native [^{125}I]-LDL (▲) and reconstituted [^{125}I]-m-LDL (■, *broken line*) by T-47D (*left*) and 3T3 cells (*right*) as a function of time (*upper panels*) and concentration (*lower panels*). Data represent mean values for two separate experiments

maceutical feasibility in terms of properties such as stability and reproducibility.

The physicochemical stability of the product was very good, and no change in particle diameter, aggregation, or precipitation was noted, even after several months of storage at 4° C. The factor limiting the long-term storage of prednimustine/m-LDL complexes is obviously the chemical stability of the components. Apo B is known to be susceptible to degradation during storage [19], and a slow hydrolysis of prednimustine was noted (~ 1% per month), even at 4° C. In medium containing 10% serum at 37° C, the degradation of prednimustine was much faster, with a $t_{1/2}$ value of about 9 h being observed for the free drug and that of 18 h being noted for the prednimustine/m-LDL complex.

The reproducibility of the method was good as demonstrated by a small variation in the resulting particle diameter (mean, 67.7 ± 9.4 nm, n = 4). This particle diameter was larger than the 23 nm found for the native LDL. The recovery of prednimustine in the final product was 82.4% ± 6.7% (n = 6). The quantity of prednimustine incorporated into the m-LDL was 338 mol drug/mol apo B. This value was somewhat higher than those obtained by other authors for AD-32 (120 molecules) [17], *N*-(*N*-retinoyl)-L-leucyldoxorubicin-14-linoleate (100–200 molecules) [22], estramustine (143 molecules) [3], and prednimustine (163 molecules) [4].

Metabolism of [^{125}I]-LDL and [^{125}I]-m-LDL

The heptane extraction method for the delipidation of LDL has been shown in several studies to preserve the receptor-binding capacity of apo B [9, 17]. This observation was confirmed in the present study using both normal 3T3 fibroblasts and T-47D breast cancer cells. The data in Fig. 1 show that the [^{125}I]-m-LDL particles were metabolized by the cells at a rate that was essentially the same as that found for native [^{125}I]-LDL particles. Moreover, the values obtained for the binding and internalization of the lipoprotein particles were similar, typically showing a variation of <10% (data not shown). The same experiments performed using an excess of unlabeled LDL in the medium gave equal inhibition of cellular uptake for native and reconstituted LDL.

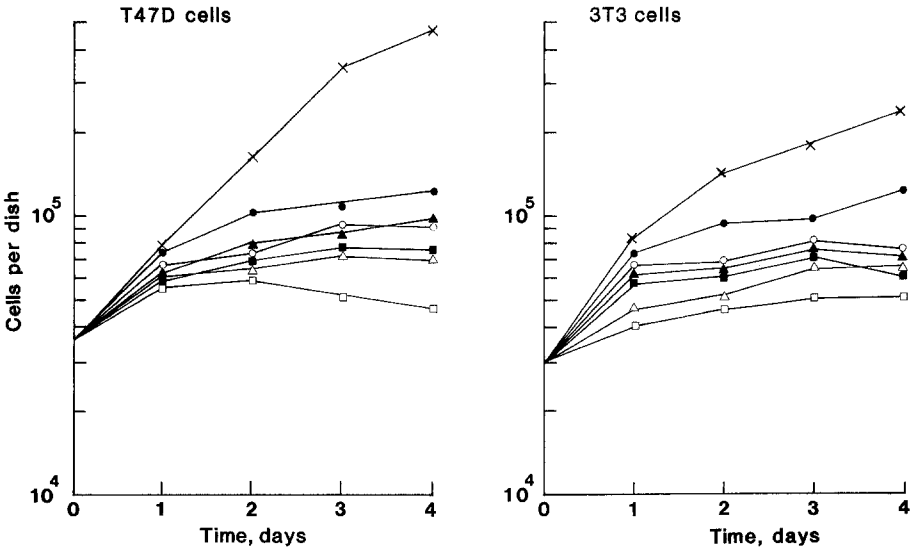

Fig. 2. Cytotoxicity of prednimustine/m-LDL complexes and prednimustine against T-47D (*left*) and 3T3 cells (*right*). The prednimustine concentrations used were 2.5 (○), 5 (△), and 10 μg/ml (□). *Open symbols* represent prednimustine/m-LDL and *filled symbols* indicate free prednimustine. *x*, Control cell growth

Cytotoxicity of prednimustine/m-LDL complexes

The growth-inhibitory effect of the prednimustine/m-LDL complexes was tested on T-47D and 3T3 cells by two methods: cell counting and [^{3}H]-thymidine incorporation. Figure 2 shows the effect of drug/m-LDL complexes on the cell number per dish as a function of time and concentration. The activity of the complexes was compared with that of free prednimustine added as a DMSO-ethanol solution.

The results demonstrated that the activity of the drug-lipoprotein complexes was higher than that of the free drug. After 4 days' treatment of the T-47D cells, the growth-inhibitory value obtained for 10 μg prednimustine/ml incorporated in m-LDL was 90%, whereas that found for the free drug was 59%. The corresponding values for 5 μg/ml were 65% vs 38%, and those for 2.5 μg/ml were 46% vs 32%. These values demonstrate that the efficacy of the drug/m-LDL complexes was about twice that of the free drug. For the 3T3 cells, the difference between the growth inhibition induced by prednimustine/m-LDL complexes and that caused by free drug was less profound at the two higher concentrations. The inhibition obtained after 4 days of incubation with drug-lipoprotein and free drug were similar at 76% vs 65% for 10 μg/ml and 59% vs 53% for 5 μg/ml but were obviously different at 47% vs 14% for 2.5 μg/ml. The results of cytotoxicity testing by cell counting thus clearly show that the efficacy of the drug/m-LDL complexes is considerably greater than that of the free drug.

The cytotoxic activity of prednimustine/m-LDL complexes as compared with that of the free drug against T-47D and 3T3 cells was further studied by measurement of [^{3}H]-thymidine incorporation into DNA combined with quantitation of [^{35}S]-methionine incorporation into cellular proteins (Fig. 3). The results agree well with those of the cell-counting experiments. After 24 h incubation concentration of 10 μg prednimustine/ml in m-LDL had reduced the incorporation of [^{3}H]-thymidine into T-47D cells to 10% of that obtained in control cells in the absence of drug. The corresponding value for the free drug was 35%. The prednimustine concentration that resulted in a 50% reduction in [^{3}H]-thymidine incorporation at 24 h was 1.6 μg/ml for the m-LDL complex and 5.1 μg/ml for the free drug. At 48 h, these values were 1.1 and 1.5 μg/ml, respectively. The experiments on the 3T3 cells showed a less apparent effect for the drug formulation. The concentrations of prednimustine that resulted in a 50% reduction in [^{3}H]-thymidine incorporation into these cells were 2.1 and 3.1 μg/ml at 24 h and 1.8 and 2.8 μg/ml at 48 h for the drug/m-LDL complex and the free drug, respectively. In congruence with the results of the cell-counting experiments, a marked preferential specificity of the prednimustine/m-LDL complex for the T-47D cells was noted.

The [^{3}H]-thymidine incorporation experiments were combined with measurements of cellular [^{35}S]-methionine uptake. The labeled amino acid was added immediately after the cells had attached to the dishes and should thus give a relative measure of the amount of protein per dish. The growth-inhibitory effect of prednimustine is also reflected in the amount of [^{35}S]-methionine incorporation (Fig. 3). In accordance with the results of thymidine incorporation, the inhibition was greater for prednimustine in m-LDL than for the free drug and was more pronounced in T-47D cells than in 3T3 cells.

However, if the amount of [^{3}H]-thymidine in the cells is put in relation to the quantity of accumulated [^{35}S]-methionine and, hence, to the relative mass of cellular protein, the efficiency of prednimustine/m-LDL complexes as compared with the free drug is less marked (Fig. 4). This was particularly the case for the 3T3 cells, in which the differences were practically negligible. For the T-47D cells at 24 h and at the highest drug concentrations, the drug/m-LDL complexes continued to exhibit approximately 50% higher activity; at 48 h there was an obvious leveling of the ratios between the two labels. It can thus be concluded that by any criteria, the cytotoxic potency of prednimustine/m-LDL complexes against T-47D breast cancer cells is higher than that of the free drug and that this difference is less pronounced in the 3T3 cells.

How can the stronger effect of prednimustine incorporated in m-LDL as compared with the free drug be ex-

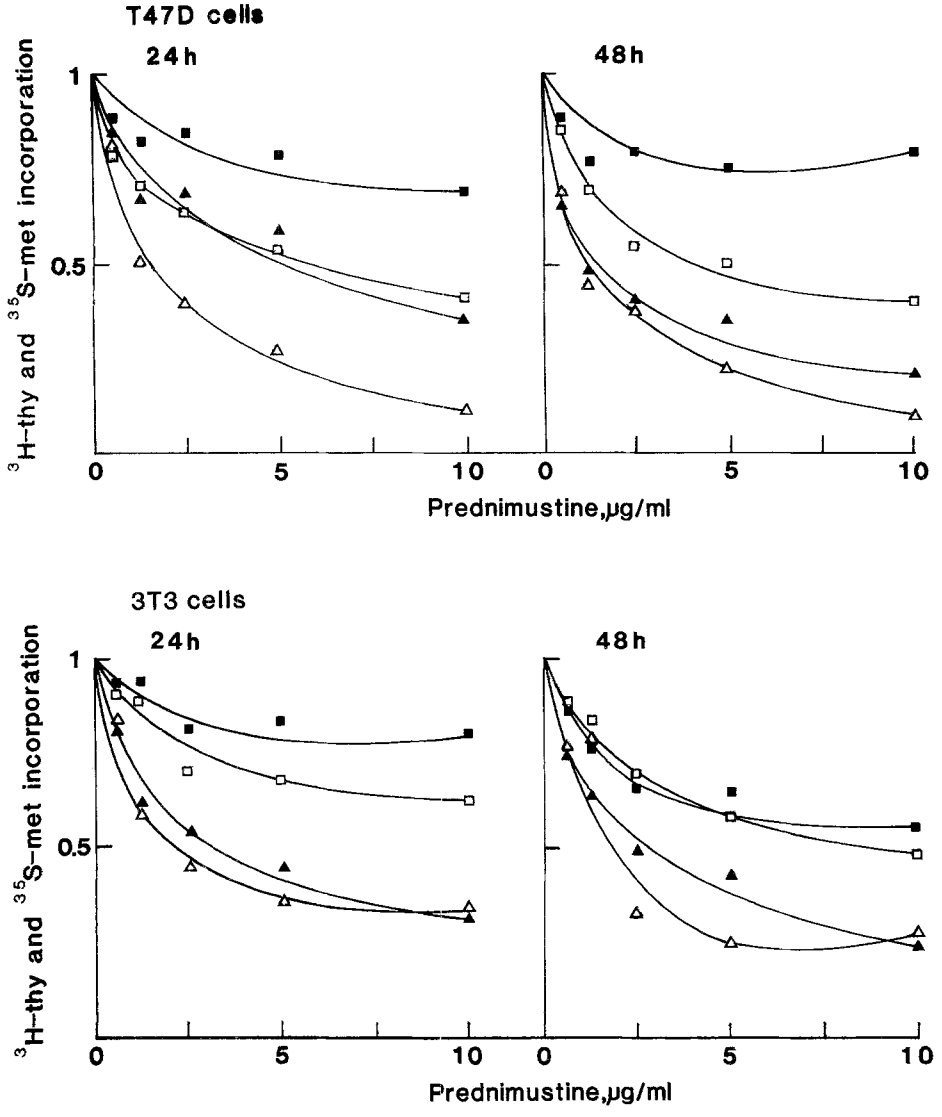

Fig. 3. Effect of prednimustine/m-LDL and prednimustine on the incorporation of [^{3}H]-thymidine ([^{3}H]-thy, △) and [^{35}S]-methionine ([^{35}S]-met, □) by T-47D (*upper panels*) and 3T3 cells (*lower panels*) after 24 (*left*) and 48 h treatment (*right*). *Open symbols* represent prednimustine/m-LDL and *filled symbols* indicate free prednimustine. Control incorporation = 1

plained. Cells that are resistant to chlorambucil have been reported to accumulate less drug than sensitive cells [8]. The rate of internalization thus seems to be a crucial factor for the effect. It might be that the incorporation of prednimustine into m-LDL increases the cellular uptake of the drug. An additional factor might involve the increased stability against degradation of the drug in m-LDL, as the $t_{1/2}$ value for prednimustine is 18 h in the drug/m-LDL complexes and 9 h as the free drug. However, after 24 h incubation in serum-containing medium, most of the prednimustine is degraded, even that incorporated in m-LDL. This might partly explain the leveling of the effects at 48 h. The conditions are further complicated by the observation that the primary active component, chlorambucil, decays quite rapidly leaving only 10% of the original drug in solution after 24 h [2].

The results presented in this report are in abvious contrast to those of Eley et al. [4]. The latter authors found that the activity of a prednimustine-LDL complex against P388 mouse leukemia cells in culture was 10 times lower than that of the free drug. One plausible explanation for this discrepancy could be differences in the properties of the cytotoxic complex arising from the different methods used to complex prednimustine with LDL. Eley et al. [4] used a modification of the method described by Masquelier et al. [17]; the final complex contained on average 163 mol prednimustine/mol LDL (vs 338 in the present study) and exhibited a particle diameter of 105 nm (vs about 68 in the present work). These authors also reported a cellular uptake for the complex that was approximately one-half that for native LDL, whereas the present study yielded similar values for the drug/m-LDL complex and the native LDL. It therefore seems likely that prednimustine is unsuitable for incorporation into LDL by the freeze-drying method used by Eley et al. [4]. Estramustine proved to have better properties in this respect, producing a drug-LDL complex exhibiting a particle diameter similar to that of native LDL [3]. However, although the cellular uptake of this complex was approximately 2.5-fold that of the native LDL, the complex was found to be about 100 times less active than the free estramustine.

The major objective of the use of targeted drug-carrier systems in cancer therapy is to increase the specificity of the drug and to decrease its toxic effect on normal cells.

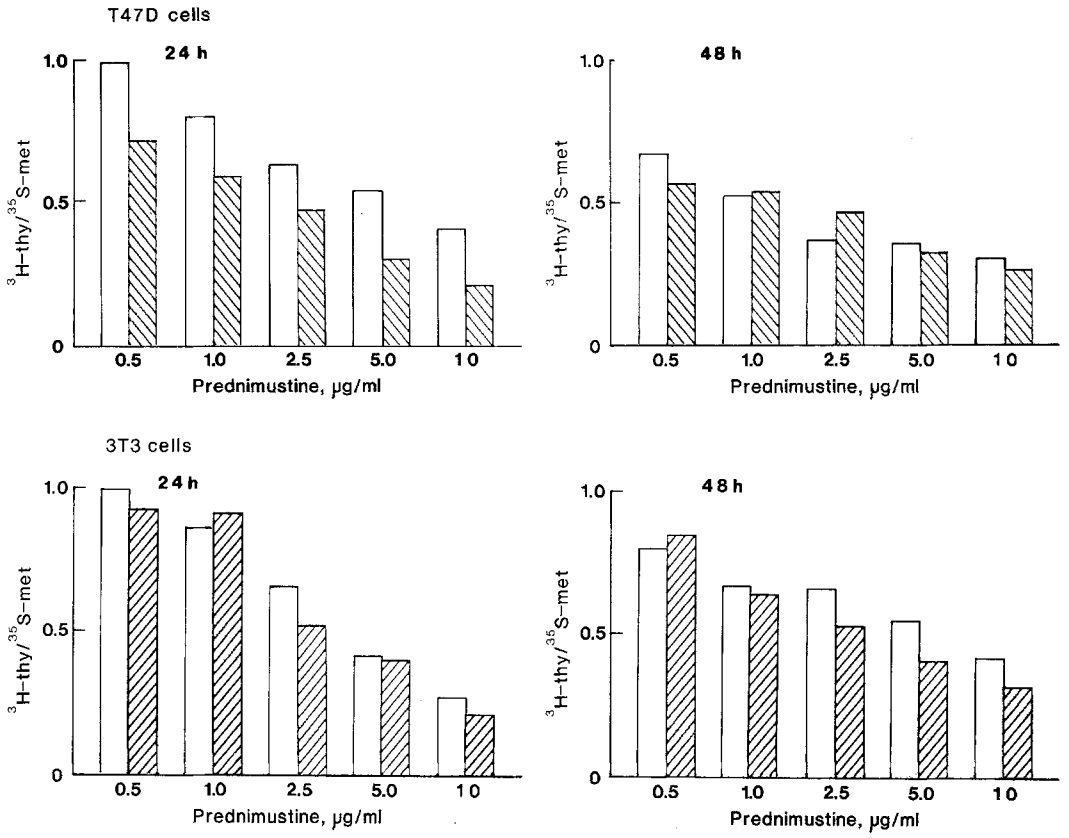

Fig. 4. Ratios between [^{3}H]-thymidine and [^{35}S]-methionine incorporated into T-47D (*upper panels*) and 3T3 cells (*lower panels*) after 24 (*left*) and 48 h treatment (*right*) with prednimustine/m-LDL (*shaded bars*) and free prednimustine (*open bars*)

The prednimustine/m-LDL complexes used in this study exerted a small but definite preferential effect on T-47D breast cancer cells as compared with that on normal 3T3 fibroblasts. This finding might offer some hope for the therapeutic efficacy of the preparation. Since normal cells take up LDL by the same mechanism used by cancer cells, only partial specificity in drug targeting via the LDL-receptor pathway can be obtained. However, as most drugs in current use are completely untargeted, even a partial success would be an important advance, especially in cancer chemotherapy, in which side effects are notoriously severe.

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