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The incorporation of estramustine into low density lipoprotein and its activity in tissue culture

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Summary

The incorporation of estramustine into low density lipoprotein (LDL) using a freeze drying technique has been investigated. Complexes of LDL and estramustine containing on average 143 molecules of drug in each LDL particle were produced and the size of the particle increased slightly after drug incorporation. The cytotoxic activity of the complex was measured in vitro against P388 and L-Dan cell lines. The complex was approx. 100-times less active than the free drug in normal media; however, activity increased in media supplemented with lipoprotein-deficient serum. Receptor uptake studies demonstrated that the complex was taken up by the L-Dan cells via the LDL receptor pathway, similar to native LDL. The uptake studies, however, demonstrated that the measured cytotoxic activity was only partly due to uptake via receptors and that non-specific uptake of the complex or drug played a major role. The results indicate that it is possible to incorporate steroidal drugs into LDL and to retain both receptor dependent uptake and cytotoxic activity after drug incorporation, but that highly efficacious drugs may be required to obtain maximal cytotoxic benefit.

Introduction

Cancer chemotherapy has been beset with the problem of lack of drug selectivity and, in order to circumvent this, several strategies involving 'drug targeting' systems have been explored. For example, cancer chemotherapeutic agents have been linked to antibodies (Lee and Hwang, 1979), DNA (Trouet et al., 1979), incorporated into liposomes (Perez-Soler et al., 1989) and into non-ionic surfactant vesicles (Rogerson et al., 1988). However, the application of these systems has been less than encouraging due to problems such as uptake by the reticuloendothelial system, stability, and the problems with the lack of access to extravascular sites (Tomlinson, 1987).

Recently, low density lipoprotein (LDL) has been considered as a novel endogenous carrier system that may be able to circumvent some of the problems listed above (Gal et al., 1981; Halbert et al., 1985). It has been shown that human cells express surface receptors for LDL which bind the LDL before the particle is endocytosed and degraded in the lysosomes (Brown and Goldstein,

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1986). Studies on patients with acute myelogenous leukaemia indicate that freshly isolated leukaemic cells from peripheral blood have higher LDL receptor activities than normal mononuclear cells (Ho et al., 1978). Gal et al. (1981) demonstrated in vitro that the metabolism of LDL in EC-50 cervical carcinoma cells was 50 times greater than in non-neoplastic tissue. These results and others have stimulated research into the possible use of LDL as a drug targeting vehicle in cancer chemotherapy. In the present work the steroidal anticancer drug estramustine has been incorporated into LDL and the effect of incorporation on the diameter of the particle, cytotoxic activity and receptor binding have been studied in vitro.

Materials and Methods

Materials

All reagents and buffer salts were of Analar grade and purchased from BDH (U.K.). Tissue culture media and materials were obtained from Gibco-Biocult (Paisley, U.K.), MTT from Sigma (Dorset, U.K.) and HPLC solvents from Rathburn Chemicals (Walkerburn, U.K.). Estramustine was a gift from Leo Laboratories (Helsinborg, Sweden) and used as received.

Buffers

The following buffers were used: phosphate-buffered saline (PBS): 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4; Tricine buffer: 10 mM *N*-Tris(hydroxymethyl)-methylglycine, pH 8.4; and glycine buffer, 0.1 M glycine, 0.1 M NaCl adjusted to pH 10.5 with 0.1 M NaOH.

Low density lipoprotein was isolated from a normolipidaemic venous blood sample obtained from adults after overnight fasting. The plasma was adjusted to a density of 1.3 g/ml with NaBr and the LDL extracted by rate zonal ultracentrifugation (Patsch et al., 1974) using a Beckman L5-65 ultracentrifuge with a T14 zonal rotor. The LDL fractions were pooled, concentrated by ultrafiltration (XM100A membrane, Diaflo, Amicon, U.S.A.), sterilised by filtration (0.22 μm Millipore), packed under N_2 and stored at 4° C.

Lipoprotein-deficient serum (LPDS) was prepared from normolipidaemic plasma by adjusting to a density of 1.235 g/ml using KBr and centrifuging in a Beckman 50Ti rotor at 45000 rpm for 48 h at 10° C. The top lipoprotein fraction was discarded, the remaining material pooled, dialysed against PBS, sterilised by filtration (0.22 μm Millipore), and stored at 4° C.

Methods

Measurement of LDL

The protein content of LDL was determined using a modified Lowry method in which 0.1% w/v sodium dodecyl sulphate was added to the copper tartrate alkali solution (Craig et al., 1981). Human serum albumin was used as a standard. Cholesterol was measured using a Boehringer Mannheim cholesterol diagnostic kit based on the method of Siedel et al. (1981).

Measurement of LDL diameter

LDL diameter was determined by photon correlation spectroscopy (PCS) using a Malvern Instruments (U.K.) Model 7027 correlator with 60 channels in conjunction with an He/Ne laser (Linconix) operating at 632.8 nm with a power output of 10 mW. All samples were filtered (0.22 μm), measured at 25 $\pm$ 0.1° C, and at an angle of 90° to the incident beam. Data were treated to determine the LDL diffusion coefficient and, by application of the Stoke's Einstein equation, particle size (Halbert et al., 1985).

Preparation of estramustine-LDL complexes

A modification of the method of Masquelier et al. (1986) was adopted. A sample of LDL (2 ml at 2.9–6.15 mg/ml) in PBS containing 0.73 M sucrose was frozen in liquid N_2 and lyophilised overnight (Edwards EF-4 Modulyo Freeze Dryer). The residue was pulverised and extracted three times with *n*-heptane (5 ml) at 4° C by vortexing then centrifuging at 2000 rpm for 10 min (Mistral-3000). Residual *n*-heptane was removed by evaporation in an ice-bath using a stream of N_2. Estramustine dissolved in 1 ml of anhydrous diethyl ether was added, the suspension agitated for 5 min, then incubated at 4° C for a further 15 min. The diethyl ether was removed under vacuum and the

estramustine-LDL complex solubilised by the addition of 1 ml of Tricine buffer and left overnight at 4°C. The complex was passed down a PD-10 chromatography column (Sephadex G-25M, Pharmacia), eluted with PBS, filtered (0.22 μm), packed under N_2 and stored at 4°C. Estramustine content of the complexes was measured using an HPLC technique.

HPLC analysis of estramustine

To a sample of the LDL-estramustine complex was added β-oestradiol (internal standard) and the mixture extracted by the addition of 5 ml of ethanol followed by 5 ml of hexane. The hexane layer was removed and the sample extracted further with two 5-ml aliquots of hexane. The hexane fractions were pooled and evaporated under N_2 at 40°C; the residue was resuspended by the addition of 0.2 ml of ethanol followed by 0.3 ml of hexane. The estramustine content was measured by a modification of the HPLC method of Brooks and Dixon (1980). Samples were chromatographed using a 5 μm Partisil PXS 5/25 silica gel column with a mobile phase of 5% v/v ethanol in *n*-hexane, flow rate 1 ml/min, the eluate was monitored spectraphotometrically at 276.5 nm (LDC III Spectramonitor) and quantified on a Shimadzu CR3A chromatopac integrator. Concentrations were determined with respect to a standard curve of estramustine.

Tissue culture

L-Dan, a squamous carcinoma (derived from human lung epithelium) cell line which forms a continuous monolayer culture, was obtained from existing stocks at the Department of Medical Oncology, University of Glasgow, U.K. P-388 cells, a continuous mouse leukaemia cell line, were obtained from Flow Labs, Irvine, U.K. L-Dan cells were maintained in a 50 : 50 mixture of Hams F10 : Dulbecco's Minimum Eagle's medium and P-388 in RPMI 1640, both were supplemented with 10% v/v foetal calf serum and 0.1 μg/ml gentamicin. Cells were maintained at 37°C and gassed with 2% v/v CO_2 in air.

Cytotoxicity testing

Toxicity testing was conducted by the method of Carmichael et al. (1987) with minor modifications. L-Dan cells harvested from log-phase growth were seeded into 96-well flat-bottomed plates at 5×10^3 cells/well. Three types of media were employed, standard media as listed above, media containing LPDS instead of foetal calf serum and the latter media supplemented with extra LDL (xSLDL) at a level of 1 mg/ml. The LPDS plates were allowed 72 h to establish cell growth; in the remaining plates only 24 h was necessary. Estramustine or estramustine/LDL complex was then added to the plates at decreasing concentrations, one column of wells was used for each concentration and a duplicate plate was also set up. After 24 h drug exposure the cells were washed and fresh media added; this was repeated every 24 h until the control cells reached confluence. At this point MTT (3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide; 50 μl of a 9.65 mM solution in PBS) was added to each well and the cells incubated in the dark for 4 h at 37°C. After incubation dimethyl sulphoxide (200 μl/well) was added, the plates gently shaken and the absorbance of the well measured at 570 nm using a Bio-Rad 2550 EIA plate reader. Tests using the P-388 cells were conducted in the same manner but with the following alterations. Cells were seeded in round-bottomed well plates at 2.5×10^3 cells/well and drug added after 48 h growth in LPDS and xSLDL and 24 h in normal media. Centrifugation was required at each change of media (1200 rpm for 12 min, IEC Centra 7R) and glycine buffer (25 μl/well) was added after the addition of dimethyl sulphoxide.

LDL-receptor binding and uptake studies (L-Dan cells only)

The method of Goldstein and Brown (1974) was used. L-Dan cells were grown in 75-ml flasks containing standard medium and in LPDS medium for the final 48 h. The cells were then harvested, seeded into 6-ml petri dishes at 1×10^6 cells/dish and incubated (37°C) with various concentrations of ^{125}I-LDL (prepared by the method of Bilheimer et al. (1972), as modified by Goldstein and Brown (1974)) in the presence or absence of excess unlabelled LDL. After 2 h the medium was removed and the cells washed with 3 ml ice-cold buffer (150 mM NaCl, 50 mM Tris, 2 mg/ml BSA; pH 7.4)

three times. The cells were then washed in 3 ml of ice-cold heparin buffer (50 mM NaCl, 10 mM Hepes, 1600 μM heparin; pH 7.4), and left to stand in 2 ml of this buffer at 4°C for 1 h. A sample of each well (1 ml) was counted for the determination of ^{125}I-LDL released from the cell surface. The remaining cells were then dissolved in 0.1 N NaOH (15 min at 20°C) and a sample (1 ml) from each well counted to determine the internalised ^{125}I-LDL.

Results and Discussion

The LDL used in this study had an average diameter of 23.9 ± 1.21 nm (mean ± SD, $n = 6$) in agreement with previously published results (Tucker and Florence, 1983). After estramustine incorporation, the average LDL diameter increased by approx. 3.8% to 24.8 nm, a statistically significant feature (paired t-test, $P = 0.0005$) that was evident in every sample tested. This increase in diameter was accompanied by an increase in polydispersity, indicating a more heterogeneous sample after drug incorporation. The average quantity of estramustine incorporated was 143 ± 84 mol/mol LDL (mean ± SD, $n = 9$; based on a molecular weight for LDL of 2.5×10^6 and [LDL] = 5 × measured protein concentration; Halbert et al. (1985)) with a large standard deviation as some samples accepted up to 300 mol/mol LDL with others as low as 36. This difference in incorporation was not evident in the individual PCS results as there was no trend of increasing size with increasing drug incorporation, although all samples did increase slightly in size.

This latter result implies that the incorporation of estramustine does not greatly upset LDL structure. LDL contains approx. 1500 molecules of cholesteryl ester in its internal core (Deckelbaum et al., 1977), and the extraction procedure removed on average only 50% of the core content. Since the molecular volume of estramustine is about one third less than that of a cholesteryl ester (cholesteryl oleate used for comparison (Bondii, 1964)), the addition of up to 150 molecules of estramustine to the core after the removal of 700 molecules of cholesteryl ester should result in a reduction in size, if the estramustine locates in the same portion of the LDL. The size, however, increases slightly and this may reflect the fact that the estramustine resides in the particle in a different configuration when compared with a cholesteryl ester; possibly the free secondary alcoholic group of the estramustine may align the molecule in the surface phospholipid layer (similar to free cholesterol) rather than in the particle's core. The average incorporation value is nevertheless similar to that reported for other drugs using the same incorporation technique. Masquelier et al. (1986), for example, report an incorporation value of 120 molecules of AD-32 (a lipophilic doxorubicin derivative) and Vitols et al. (1985) report a value of 100–200 molecules of *N*-(*N*-retinoyl)-L-leucyl-doxorubicin 14-linoleate.

The estramustine-loaded LDL conjugates were tested in vitro to determine their cytotoxic activity and receptor-dependent uptake, with the results for the cytotoxicity studies being presented in Figs 1 and 2. The activity of the free drug in normal media against L-Dan and P388 cells is comparable with published data and shows 50% inhibition (ID_{50}) at a concentration of around 1×10^{-5} M, slightly higher than the reported value of 0.5×10^{-5} M against HeLa cells (Tew et al., 1983). In LPDS and xSLDL media the free drug has slightly lower ID_{50} values than in normal media. The

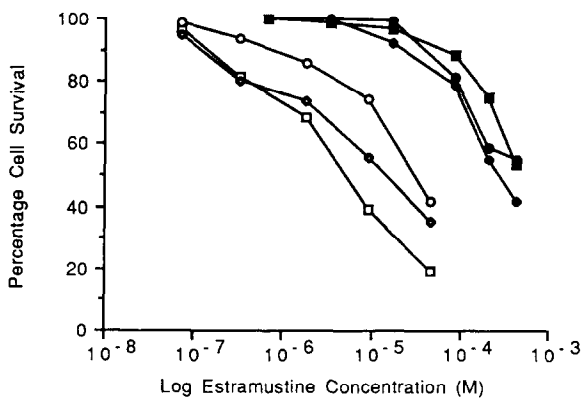

Fig. 1. Cytotoxicity of estramustine and estramustine/LDL complex against L-Dan. (■) Foetal calf serum; (◆) lipoproteins-deficient serum; (●) xSLDL (open symbols, free drug; closed symbols, estramustine/LDL complex). Mean ($n = 3$ experiments); SD values smaller than symbols.

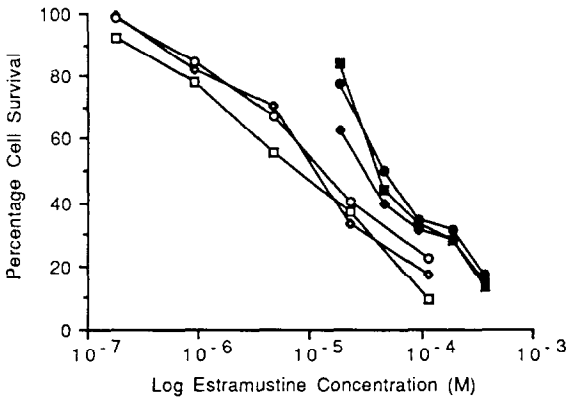

Fig. 2. Cytotoxicity of estramustine and estramustine/LDL complex against P388. For symbols and experimental details, see Fig. 1.

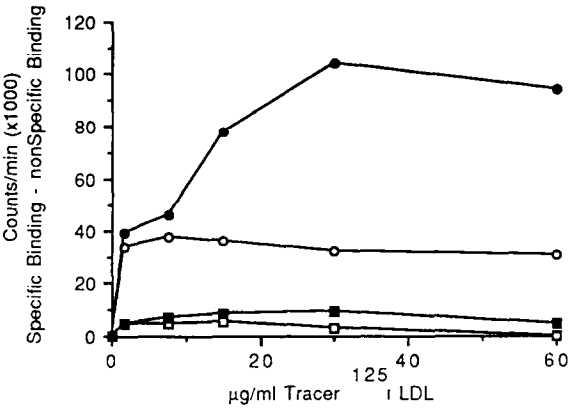

Fig. 3. Binding and internalisation of LDL and estramustine/LDL complex to L-Dan cells. (■) Surface bound; (●) internalised (open symbols, native LDL; closed symbols, estramustine/LDL complex).

LDL/estramustine complex is approx. 100-times less active than the free drug in normal media vs L-Dan cells, however, against P388 cells the reduction in activity is less at approx. 10 times. The use of LPDS media should enhance the receptor-dependent uptake of the LDL into the cells and this effect will be negated by the addition of excess LDL to the culture medium. In both the cell lines tested, the use of LPDS media increases the toxicity of the complex by a small but significant factor, and this effect is partially (L-Dan) or totally (P388) reversed by the use of extra LDL.

The results for the receptor binding and uptake studies in L-Dan cells are presented in Figs 3 and 4. Native LDL exhibits the classic receptor-dependent uptake profile (Fig. 3) with a calculated dissociation constant of 6.8×10^{-9} M (from Fig. 4) and 1.7×10^{7} receptors/cell. These values are slightly greater than those quoted originally by Goldstein and Brown (1974) for fibroblasts, but are in agreement with literature values for neoplastic tissue (Gal et al., 1981). The complex exhibits a greater degree of surface binding and internalisation than native LDL, with the internalisation being increased approx. 2.5 times (Fig. 3). The calculated dissociation constant for receptor binding is lower ($K_d = 2.2 \times 10^{-10}$ M) than that of native LDL, whilst retaining approximately the same number of receptor sites (1.2×10^{7}/cell). These results indicate that the complex can be taken up by L-Dan cells via the LDL receptor pathway and that uptake is greater compared to native LDL, but affinity for the receptor is reduced. This indicates that even small changes in particles after drug incorporation can affect the receptor-dependent uptake of the complex when compared with native LDL.

Using the data presented for the uptake experiments, it is possible to evaluate the maximum amount of complex taken up by the L-Dan cells during the 24 h drug/complex exposure in the cytotoxicity assay, assuming that the measured rate over 2 h can be extrapolated to 24 h. This calculation produces a maximal value of 1.2 µg of complex internalised every 24 h/well (based on

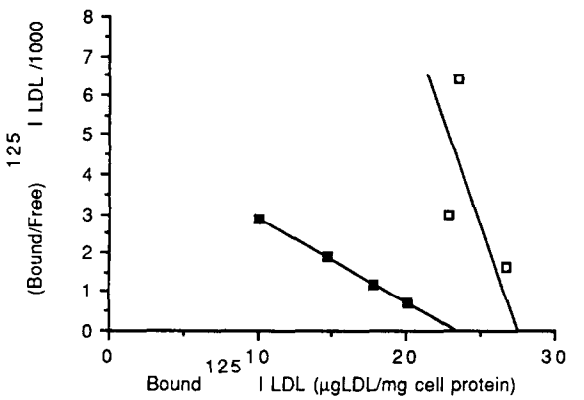

Fig. 4. Scatchard plot of cell surface binding data. (■) Native LDL; (□) estramustine/LDL complex.

the maximum measured uptake of complex, 500 ng/mg cell protein, and allowing for the variation in cell numbers between the two experiments) approximately equivalent to a total uptake of 2×10^{-7} M estramustine. The quantity of complex internalised is less than the total amount of complex present in the cytotoxicity experiments even at the lowest concentration tested of 10^{-6} M (1.2 μg taken up vs approx. 18 μg present). If the activity of the complex was mediated entirely through receptor-dependent uptake, then the measured activity should be the same at each concentration tested since the uptake process would be saturated and therefore maximal. However, the cytotoxicity increases with increasing concentration, implying that the activity of the complex is mediated through routes other than the LDL receptor pathway. The calculated number of complex particles present at 10^{-6} M concentration is 1.7×10^{7}/cell, non-specific uptake due to the presence of a large number of particles therefore being possible, especially at the higher concentrations. The activity of the complex is probably therefore largely due to non-specific uptake or access of estramustine to the cell by some other non-specific route. This feature has been reported before for the uptake of benzo(*a*)pyrene from LDL into cells (Remsen and Shireman, 1981), and an LDL/AD32 drug complex (Vitols et al., 1984). It has also been demonstrated that cholesterol can transfer physically between LDL and cells (Lundberg and Suominen, 1985) using a non-receptor-dependent mechanism.

The results imply that for the LDL/estramustine complex to be active via the receptor pathway a higher drug loading would be required. If, however, the entire core of the particle could be replaced by estramustine (1500 molecules of estramustine) then the maximum amount delivered over 24 h would still only be 1.4×10^{-6} M, which is insufficient to obtain a reasonable effect based on the cytotoxicity data for the free drug in this cell system. The use of LDL as a drug targeting vector will therefore require a highly efficacious drug, able to incorporate into the particle maximally without upsetting its structural integrity or receptor-dependent uptake. Further studies will be required to provide greater information on which drugs would meet this requirement and provide good cytotoxic efficacy once incorporated.

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