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Incorporation of prednimustine into low density lipoprotein: activity against P388 cells in tissue culture

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Summary

The steroidal anticancer drug prednimustine has been incorporated into low density lipoprotein (LDL) using a lyophilisation technique. The resulting complexes contained 163 mol prednimustine/mol LDL, but were approx. 4 times larger than the native lipoprotein. The complexes retained a degree of in vitro cytotoxic activity against P388 leukaemia cells but this was 10 times less than that of the free drug. The complex was internalised by P388 cells via the LDL receptor pathway with a similar affinity for the receptor but a lower uptake and number of receptors per cell. The results demonstrate that the cytotoxicity of the particular complex is not mediated through the LDL receptor pathway and that other drug uptake mechanisms must be operative in vitro.

Introduction

The possible use of drug targeting vectors in the treatment of systemic cancer has been extensively investigated since methotrexate was first linked to globulins in 1958 (Mathé et al., 1958). Since this seminal paper much research has been conducted into the use of antibodies as drug targeting vectors (Sikora, 1988). Research has demonstrated that the ability of the antibody to bind to the target antigen on the tumour cell can be affected by a variety of processes involved in the production of antibody drug conjugates. The methods of drug attachment to the antibody have a significant effect on its antigen binding capacity (Kulkarni et al., 1981). Additionally the method used to effect drug binding may render the drug inactive if subsequent release of the drug is poor or at the extreme, non-existent (Halbert et al., 1987). A similar problem exists with the novel drug targeting vector low density lipoprotein (LDL) which has aroused interest as a possible means for targeting to tumours (Counsell and Pohland, 1982). The attachment of drugs to groups on the surface of LDL (Halbert et al., 1985) can reduce the receptor binding capacity and hence targeting utility. To circumvent this problem, drugs have been incorporated into the internal core of the LDL particle (Vitols et al., 1984). To assess if this may also affect receptor binding we have

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investigated the incorporation of the steroidal anticancer drug prednimustine into LDL. After drug incorporation the receptor dependent uptake and cytotoxicity of the complexes against the murine leukaemia P388 were measured 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 disposables were obtained from Gibco-Biocult (Paisley, U.K.), MTT from Sigma (Dorset, U.K.), and HPLC solvents from Rathburn Chemicals (Walkerburn, U.K.). Prednimustine was a gift from Leo Laboratories (Helsinborg, Sweden) and used as received.

The buffers used were: phosphate-buffered saline (PBS): 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4; pH 7.4; and Tricine buffer: 10 mM *N*-tris(hydroxymethyl)methylglycine, pH 8.4.

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 Corp., 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.

Tissue culture. P388 cells, a continuous mouse leukaemia cell line, was obtained from Flow Laboratories (Irvine, U.K.) and maintained in RPMI 1640, supplemented with 10% v/v foetal calf serum and gentamicin 0.1 μg/ml. Cells were maintained at 37°C and gassed with 2% v/v CO_2 in air.

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 ± 0.1°C, and at an angle of 90° to the incident beam. Data were treated to determine the *z*-average diffusion coefficient of the LDL and, by application of the Stokes-Einstein equation, its diameter (Halbert et al., 1985).

Preparation of prednimustine-LDL complexes. A modification of the method of Masquelier et al. (1986) was adopted. A sample of LDL (2 ml at 2.5–3.1 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. Prednimustine (5 mg/ml) dissolved in 1 ml of ethyl acetate was added, the suspension agitated for 5 min, then incubated at 4°C for a further 15 min. The ethyl acetate was removed under vacuum and the prednimustine-LDL complex solubilised by the addition of 1 ml of Tricine buffer (1 ml, 10 mM, pH 8.4) and left overnight at 4°C. The final complex was obtained and concentrated by ultrafiltration (XM100A membrane, Diaflo, Amicon Corp., U.S.A.), packed under N_2 and stored at 4°C. Prednimustine content of the complexes was measured using an HPLC technique.

HPLC analysis of prednimustine. To a sample of the prednimustine-LDL complex was added oestrone (internal standard) and the mixture extracted by the addition of ethanol (5 ml) followed by hexane (5 ml). 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 37°C; the residue was dissolved in methanol (0.5 ml). The prednimustine content was measured by a modification of the HPLC method of Newell et al. (1979). Samples were chromatographed using a 5 μm Partisil PXS 5/25 silica gel column with a mobile phase of methanol : 0.175 M acetic acid (60 : 40, v/v), and a flow rate of 1 ml/min. The eluate was monitored spectrophotometrically at 257 nm (Spectra-Physics 8450 UV detector) and quantified on a Spectra-Physics 4290 integrator. Concentrations were determined with respect to a standard curve of prednimustine.

Cytotoxicity testing. Toxicity testing was conducted by the method of Carmichael et al. (1987) with minor modifications. P388 cells harvested from log phase growth were seeded into 96-well round-bottomed plates at 2.5×10^3 cells/ml. 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 and xSLDL plates were allowed 48 h to establish cell growth, whilst in the remaining plates only 24 h was necessary. Prednimustine (dissolved in dimethyl sulphoxide) or prednimustine-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 plates were centrifuged (1200 rpm for 12 min, IEC Centra 7R), 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 the cells were centrifuged, the media removed and dimethyl sulphoxide (200 μl/well) added. The plates were gently shaken and the absorbance of the wells measured at 570 nm using a Bio-Rad 2550 EIA plate reader.

LDL-receptor binding and uptake studies. The method of Goldstein and Brown (1974) was used. P388 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 tubes at 1×10^5 cells/ml 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 cells were centrifuged (1200 rpm, 10 min, 4°C), the medium removed and the cells washed with 3 ml ice-cold buffer (150 mM NaCl, 50 mM Tris, BSA 2 mg/ml, 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 tube (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 M NaOH (15 min at 20°C) and a sample (1 ml) from each tube counted to determine the internalised ^{125}I-LDL.

Results and Discussion

The native LDL used in this study had a measured diameter of 24.7 ± 0.4 nm (mean ± S.D., $n = 4$) in agreement with previously published results (DeBlois et al., 1973). After prednimustine incorporation, the measured size of the particles increased by approx. 400% to 105 nm when they contained on average 163 ± 65 ($n = 4$) mol prednimustine/mol LDL (based on an LDL molecular weight of 2.5×10^6, and [LDL] = 5 × measured protein concentration). This increase in diameter was coupled with an increase in the measured polydispersity of the samples indicating a greater size distribution after drug incorporation. The variation in prednimustine incorporated was large as some samples accepted up to 310 mol/mol LDL with others as low as 16 mol/mol LDL. This variation was not reflected in the size measurements and no correlation could be found between increase in size and drug incorporation. Similar

size effects have been noted on the incorporation of estramustine into LDL at 143 mol/mol LDL (Eley et al., 1990). The incorporation level is comparable to values reported for other compounds. 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) determined a value of 100–200 molecules of *N*-(*N*-retinoyl)-L-leucyldoxorubicin-14-linoleate.

The internal core of LDL contains 1500 cholesterol ester molecules (Deckelbaum et al., 1977) and in all cases extraction removed 37% or approx. 560 molecules. If prednimustine was simply to substitute for the extracted cholesterol esters in the internal core then the size should reduce, since the molecular volume of prednimustine is approximately two thirds that of a cholesterol ester (cholesterol oleate used for comparison (Bondii, 1964)). The results imply that the prednimustine does not reside in the internal core of the particle, and may, due to the presence of free secondary hydroxyl groups, locate in the surface phospholipid layer similar to free cholesterol. The large increase in size therefore infers that a complex microemulsion is formed on reconstitution, in marked contrast to the incorporation of estramustine into LDL which maintains its original size (Eley et al., 1990). These results demonstrate that the incorporation of prednimustine into LDL using the lyophilisation technique dramatically alters the size and structure of the particles.

The prednimustine loaded LDL was tested in vitro to determine cytotoxicity against the P388 murine leukaemia (Fig. 1). The activity of the free drug against the cells provides an ID_{50} value in normal media of 4.5×10^{-5} M; the measured values in LPDS and xSLDL media are slightly higher at 8×10^{-4} and 10^{-5} M, respectively. The prednimustine-LDL complex is approximately ten times less active than the free drug in all the media tested. The use of LPDS media should enhance the receptor-dependent uptake of the complex into the cells and this effect will be negated by the addition of excess native LDL to the media. If the activity of the complex is mediated through the LDL receptor pathway then a greater activity

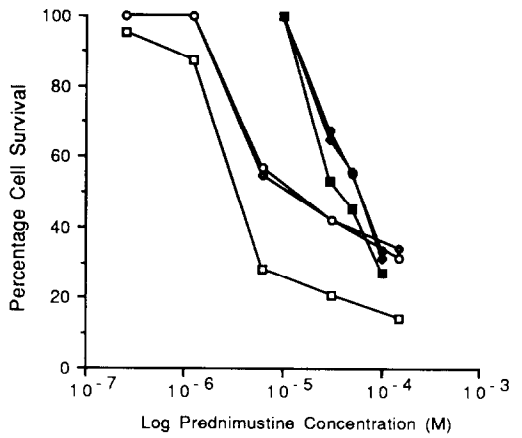

Fig. 1. Cytotoxicity of prednimustine and prednimustine-LDL complex against P388 cells in a range of media as a function of drug concentration. (■) Foetal calf serum; (◆) lipoprotein deficient serum; (●) xSLDL. (Open symbols) Free drug; (closed symbols) prednimustine-LDL complex.

should be evident in LPDS media. However, the activity of the complex in both LPDS and xSLDL media is similar and less than that in normal media.

The results for the receptor uptake experiments are presented in Fig. 2 with the calculated Scatchard plots in Fig. 3. Native LDL exhibits the classical receptor-dependent uptake profile with a calculated dissociation constant of 1.94×10^{-10} M and 1.53×10^{7} receptors per cell. These results are in broad agreement with those of Goldstein

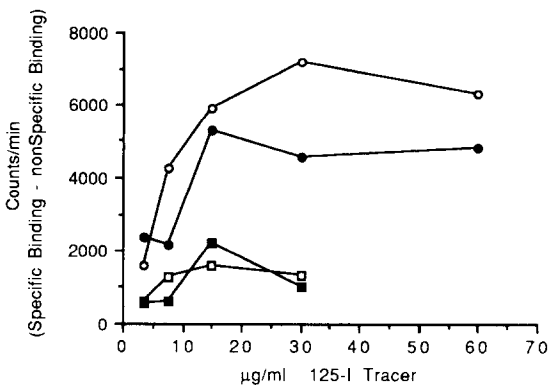

Fig. 2. Binding and internalisation of LDL and prednimustine-LDL complex to P388 cells. (■) Surface bound; (●) internalised. (Open symbols) Native LDL; (closed symbols) prednimustine-LDL complex.

and Brown (1974) for fibroblasts and similar to the values quoted for the L-Dan human lung tumour cell line (Eley et al., 1990). The complex has a slightly lower measured dissociation constant (1.8×10^{-10} M) and half the number of receptors per cell at 6.5×10^6. The internalisation data (Fig. 2) show that the complex uptake by the cells is approximately one half that of native LDL. These results indicate that the complex is taken up by P388 cells through the LDL receptor pathway with a similar dissociation constant to that of native LDL but a reduced number of receptors per cell and internalisation rate. This is in contrast to an estramustine-LDL complex which exhibited a similar cell receptor value to native LDL, a reduced affinity for the receptor but an increased internalisation (Eley et al., 1990). These results indicate that complex size is not predictive of LDL receptor function, since the estramustine complex has a similar size to native LDL whilst the prednimustine complex is very much larger. The large size of the prednimustine complex may prevent interaction with LDL receptors in coated pits thus reducing receptor numbers and cell uptake. This would imply that the receptor function of the apoprotein B remains stable in the larger particles and that the estramustine complex alters Apo B function without affecting size. Further detailed work would be required to elucidate fully the interactions occurring in the drug-LDL complex particles.

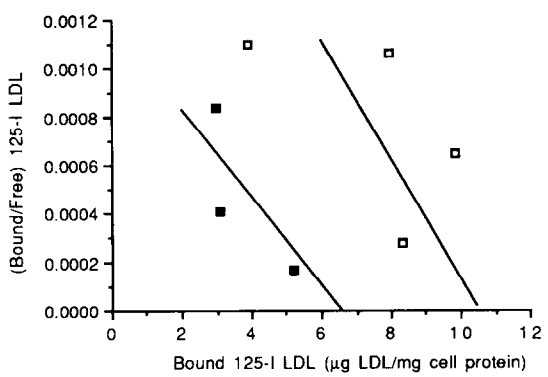

Fig. 3. Scatchard plot of cell surface binding data. (□) Native LDL; (■) prednimustine-LDL complex.

Using the internalisation data it is possible to calculate a value for the quantity of complex internalised during the cytotoxicity assay, assuming that the maximal uptake over 2 h can be extrapolated to 24 h and allowing for the difference in cell numbers present. The P388 cells in the cytotoxicity assay would internalise approx. 1.8 ng of complex every 24 h/well. This is equivalent to the uptake of 1.2×10^{-13} mol prednimustine, a value that is several orders of magnitude smaller than the lowest concentration of free drug that produces a measurable growth inhibition. Additionally, the quantity of complex used in the lowest concentration is approx. 30 μg/well; complex uptake should therefore be maximal at every concentration. If the activity of the complex was mediated through receptor-dependent uptake alone then the measured cytotoxicity should be the same at all concentrations tested. The results imply that the activity of the complex is not dependent on the LDL receptor pathway and may be due to either non-specific uptake of the complex or physicochemical transfer of prednimustine from complex to cell. Similar results have been noted with the uptake of benzo(*a*)pyrene (Remsen and Shireman, 1981), AD32 (Vitols et al., 1984) and estramustine (Eley et al., 1990) from LDL complexes into cells, and the physicochemical transfer of cholesterol from LDL to cells in vitro (Lundberg and Suominen, 1985).

The results demonstrate that prednimustine will form a complex with LDL but that the size and receptor uptake properties of the complex are markedly different from native LDL. The complex retains a degree of cytotoxic activity in vitro but this is ten times less than that of the free drug and not mediated through the LDL receptor pathway. To enhance the utility of this system it will be necessary either to improve the complex formation method or to use different drugs which possess greater activity and which can be readily incorporated into the LDL particle without disturbing its native structure and size. The former of these options is currently under investigation in this Department.

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