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Delivery of daunorubicin to cancer cells with decreased toxicity by association with a lipidic nanoemulsion that binds to LDL receptors

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Abstract

A lipidic nanoemulsion termed LDE concentrates in neoplastic cells after injection into the bloodstream and thus can be used as a drug carrier to tumour sites. The chemotherapeutic agent daunorubicin associates poorly with LDE; the aim of this study was to clarify whether the derivatization of daunorubicin by the attachment of an oleyl group increases the association with LDE, and to test the cytotoxicity and animal toxicity of the new preparation. The association of oleyl-daunorubicin (oDNR) to LDE showed high yield ($93 \pm 2\%$ and $84 \pm 4\%$ at 1:10 and 1:5 drug:lipid mass, respectively) and was stable for at least 20 days. Association with oDNR increased the LDE particle diameter from 42 ± 4 nm to 75 ± 6 nm. Cytotoxicity of LDE–oDNR was reduced two-fold in HL-60 and K-562 cell lines, fourteen-fold in B16 cells and nine-fold in L1210 cells when compared with commercial daunorubicin. When tested in mice, LDE–oDNR showed remarkable reduced toxicity (maximum tolerated dose > 253 μmol kg^{-1}, compared with <3 μmol kg^{-1} for commercial daunorubicin). At high doses, the cardiac tissue of LDE–oDNR-treated animals had much smaller structural lesions than with commercial daunorubicin. LDE–oDNR is therefore a promising new preparation that may offer superior tolerability compared with commercial daunorubicin.

Introduction

A cholesterol-rich nanoemulsion termed LDE that resembles the structure of the lipid portion of low-density lipoprotein (LDL) (Maranhão et al 1992, 1993) binds to LDL receptors on the cell surface. Although manufactured without protein, LDE acquires apolipoprotein (apo) E in contact with plasma; apo E is recognized by the LDL receptors, which allows internalization of the nanoemulsion by receptor-mediated endocytosis. Involvement of the LDL receptor in LDE uptake was demonstrated in in-vitro competition studies with native LDL and by poor removal of LDL from the plasma after injection in patients with familial hypercholesterolaemia (Maranhão et al 1997; Santos et al 2005). Because LDL receptors are overexpressed in most neoplastic cells (Ho et al 1978; Henriksson et al 1989; Gueddari et al 1993), LDE concentrates in neoplastic tissues and can thus be used as vehicle to carry anticancer drugs (Maranhão et al 1992, 1994). After injection into patients with breast and ovarian tumours, LDE uptake was 4–8 times greater in the tumour than in the contralateral normal tissue (Ades et al 2001; Graziani et al 2002). In comparison with the commercial formulations, the pharmacological activities of carmustine, etoposide and paclitaxel were increased when these drugs were associated with LDE. This was shown in Walker 256 tumour-bearing rats and in melanoma B16 F10-bearing mice either by greater inhibition of tumour growth or by increase in animal survival (Teixeira et al 2004; Lo Prete et al 2006). The association of these drugs with LDE markedly reduced their toxicity, as shown in animal experiments and in clinical trials in patients with advanced cancer (Maranhão et al 2002; Valduga et al 2003; Hungria et al 2004; Teixeira et al 2004; Pinheiro et al 2005; Rodrigues et al 2005; Lo Prete et al 2006).

Daunorubicin is a first-line drug for the treatment of acute myeloid leukaemia, but its usefulness is limited by marked toxicity – haematological and cardiac toxicity are frequent causes of death in daunorubicin-treated patients (Elliott 2006). A liposomal formulation of

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daunorubicin reportedly diminishes the toxicity of the drug (Alberts et al 2004), but the association with LDE would be interesting since this system has established drug-targeting properties (Maranhão et al 1993, 1994; Azevedo et al 2005; Pinheiro et al 2005). However, daunorubicin associates poorly with LDE (Dorlhiac-Llacer et al 2001) and chemical modification is therefore required to improve the association and stability of the drug in the nanoemulsion (Masquelier et al 2006).

Attachment of an oleyl group increases the lipophilicity of etoposide and paclitaxel and allows the formation of stable complexes with excellent yield of association. Furthermore, there is no loss of the anti-tumour effect after drug modification and association with LDE (Valduga et al 2003; Rodrigues et al 2005). In this study, a similar strategy was attempted for obtaining stable LDE–daunorubicin complexes with good association yield and reduced toxicity.

Material and Methods

Cell lines and animals

The human leukaemic cells K-562 and HL-60 were purchased from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). L1210 lines were generously donated by Dr M C Sogayar (Institute of Chemistry, University of São Paulo, Brazil) and the B16 cells by Dr D A Maria (Butantan Institute, São Paulo, Brazil).

Swiss mice weighing 25–30 g were housed in a temperature- and humidity-controlled room with a 12 h light–dark cycle. Water and food were provided ad libitum. All protocols were approved by the Ethics Committee of the Medical School, University of São Paulo, Brazil.

Chemicals

The following reagents were used: daunorubicin powder (Daunocin SP) from Meizler Inc. (São Paulo, Brazil); 4-dimethylaminopyridine (DMAP), *N,N′*-dicyclohexylcarbodiimide (DCC), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), triolein, cholesteryl oleate, cholesterol, egg phosphatidylcholine, oleic acid, chloroform and dichloromethane (DCM) from Sigma (St Louis, MO, USA); methanol from Merck (Darmstadt, Germany); ethanol from Vetec (Rio de Janeiro, Brazil); glutaraldehyde and osmium tetroxide from Electron Microscopy Sciences (Washington, PA, USA); araldite F CY-2205 from Maxepoxi (São Paulo, Brazil).

Equipment

HPLC was performed with a Shimadzu SPD-10 AV system (Columbia, ML, USA) equipped with a UV detector (Shimadzu), 15 cm × 4.6 mm i.d. C18 (2) column (Phenomenex, Torrance, CA, USA) and a precolumn shim-pack CLC-ODS (M) C_8 (4 cm × 1.0 mm i.d.) (Shimadzu). Methanol was used as the mobile phase, delivered at a flow rate of 1 mL min^{-1}; detection was at 230 nm.

^{1}H and ^{13}C NMR spectra were obtained in a Bruker DPX-300 spectrometer (Madison, WI, USA). All spectra were obtained in deuterated chloroform at 25°C.

Mass spectra were obtained in a Q-TOF Ultima spectrometer (Micromass, Manchester, UK) coupled to a Shimadzu LC-10A pump. Samples were analysed by electrospray ionization (ESI). ESI mass spectra were acquired using an ESI capillary voltage of 3.0 kV, *m/z* 50–2000 range and skimmer voltage of 35 V at 80°C source temperature. Microanalysis of a solid sample was done in a 2400 CHN Perkin Elmer Elemental Analyzer (Waltham, MA, USA). A 450 Branson Sonifier (Danbury, CT, USA) equipped with a 1 cm flat titanium probe was used for ultrasonic irradiation. Particle diameter was determined in a zeta potential analyzer (Brookhaven Instruments Corp. Holtsville, NY, USA). Infrared spectra were obtained in a Bomem Hartmann & Braunn (MB-Series) spectrometer (Quebec, Canada). A Sorvall OTD-Combi ultracentrifuge with TH 641 rotor was used for preparation of the nanoemulsion.

Purification of daunorubicin

The commercial formulation of daunorubicin was a hydrochloride salt in 83.4% mannitol. Daunorubicin was purified by solubilization in 10 mM Tris-HCl buffer (pH 8.0) and extracted with chloroform. The organic phase containing daunorubicin was washed with saturated sodium thiosulfate, dried with magnesium sulfate, filtered and concentrated. The purity was confirmed by HPLC and ^{1}H and ^{13}C NMR.

Synthesis of *N*-oleyl derivatized daunorubicin (oDNR)

DCC (0.178 mmol) and DMAP (0.081 mmol) were added to oleic acid (0.089 mmol) dissolved in 5.0 mL anhydrous DCM and the mixture was stirred for 30 min at room temperature. Daunorubicin (0.089 mmol) dissolved in 0.89 mL DCM was added and the mixture was then stirred for 45 min and washed with water. The organic phase was dried with magnesium sulfate, filtered and concentrated. The product was purified in a C_{18} column with 100% methanol. The purity was confirmed by HPLC, as described above. Under these conditions, the retention time of the daunorubicin derivative was 4.41 min and that of daunorubicin was 1.9 min. Figure 1 shows a simplified scheme of the reaction.

Characterization of the daunorubicin derivative

^{1}H-NMR and ^{13}C-NMR spectra of the oDNR yielded the following results:

300 MHz ^{1}H-NMR ($CDCl_3$) δ (ppm): 0.86 (3H, t, J = 6.6 Hz); 1.17–1.35 (28H, m); 1.48–1.59 (2H, m); 1.71–1.86 (2H, m); 1.91–1.99 (4H, m); 2.02 (1H, s); 2.10 (3H, t, J = 7.1 Hz); 2.28 (1H, d, J = 15 Hz); 2.39 (3H, s); 2.81 (1H, d, J = 18 Hz); 3.17 (1H, d, 18 Hz); 3.62 (1H, s); 4.03 (3H, s); 4.08–4.27 (2H, m); 5.20 (1H, s); 5.26–5.34 (2H, m); 5.46 (1H, s); 5.91 (1H, d, J = 8.6 Hz); 7.34 (1H, d, J = 8.0 Hz); 7.74 (1H, t, J = 8.0 Hz); 7.98 (1H, d, J = 8.0 Hz); 13.18 (1H, sl); 13.93 (1H, sl).

75 MHz ^{13}C-NMR ($CDCl_3$) δ (ppm): 14.07; 16.70; 22.62; 24.89; 25.65; 27.09; 27.14; 29.07; 29.16; 29.20; 29.25 (2C);

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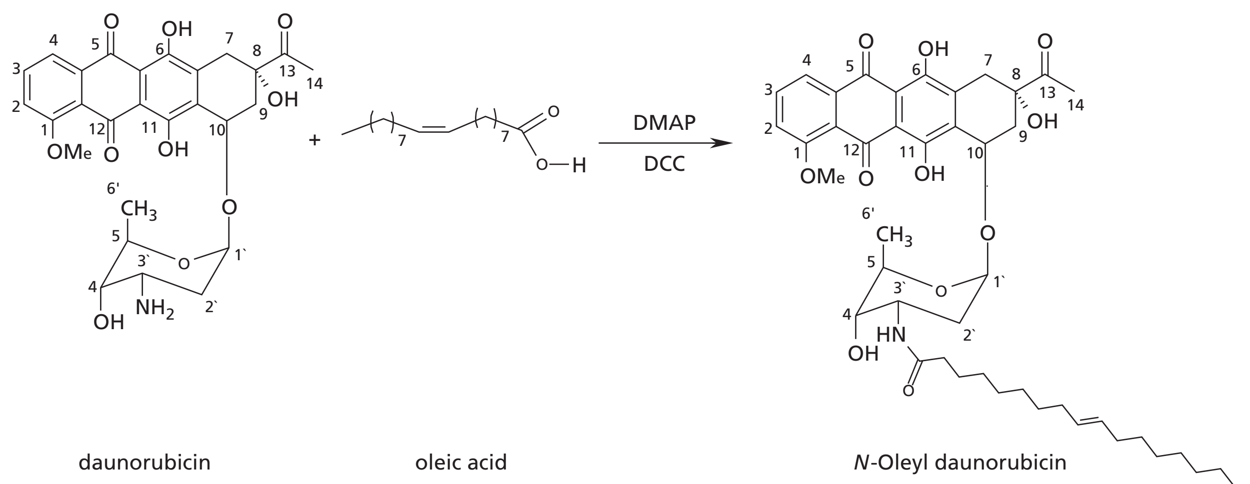

Figure 1 Simplified scheme of the reaction between daunorubicin and oleic acid in the presence of 4-dimethylaminopyridine (DMAP) and *N,N′*-dicyclohexylcarbodiimide (DCC).

29.45; 29.65; 29.69; 29.90; 31.83; 33.28; 33.31; 35.00; 36.69; 45.10; 56.54; 67.08; 69.60; 69.86; 76.57; 100.59; 111.14; 111.30; 118.29; 119.72; 120.70; 129.68; 129.90; 133.96; 134.40; 135.58; 135.62; 155.71; 156.35; 160.87; 172.54; 186.45; 186.86; 212.28.

The ESI–MS measurements were made in positive-ion mode with all samples dissolved in acetonitrile:water: trifluoroacetic acid (50:50:0.1) solution; 10 μL of the sample was injected under a flow of 20 μL min^{-1} acetonitrile/0.2% formic acid aqueous solution (1:1). The results (Figure 2) showed the molecular ion at *m/z* 792 ($C_{45}H_{61}NO_{11}$), and two main fragments with *m/z* 394 ($C_{24}H_{44}NO_3$) and 359 ($C_{19}H_{15}O_7$ + 4 hydrogens) that correspond, respectively, to the daunosamine ring with the oleyl moiety and to the

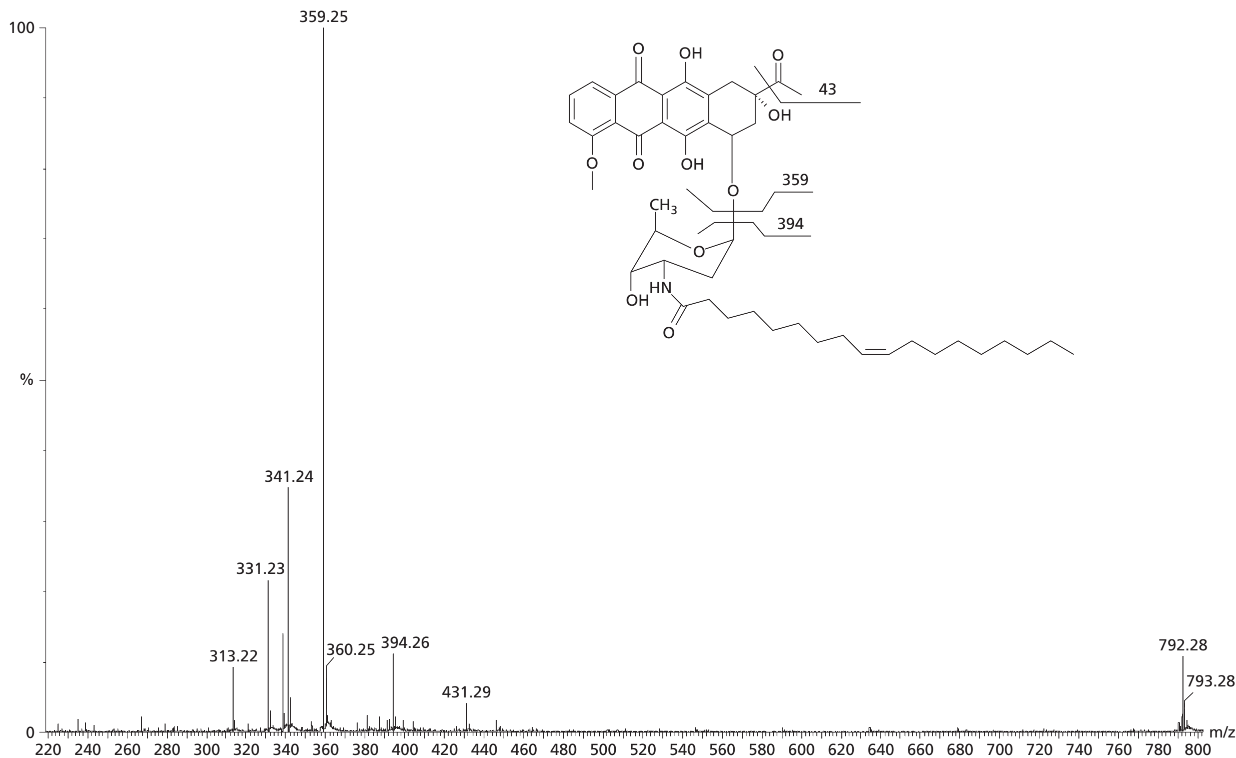

Figure 2 Representative full-scan chromatogram of *N*-oleyl daunorubicin. The sample was solubilized in acetonitrile:water:trifluoroacetic acid (50:50:0.1) solution and 10 μL of the sample was injected into the electrospray ionization (ESI) mass spectrometer (Q-TOF mass spectrometer with ESI coupled with a LC-10A bomb) under a flow of 20 μL min^{-1} acetonitrile/0.2% formic acid aqueous solution (1:1). ESI mass spectra were acquired using an ESI capillary voltage of 3.0 kV, *m/z* 50–2000 range and skimmer voltage of 35 V at 80°C source temperature.

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tetrahydrotetracenquinonic ring without the daunosamine ring and the acyl group at C13.

Infrared spectra of the daunorubicin derivative acquired in KBr presented stretching bands at 3429 cm^{-1}, 2925 cm^{-1}, 2853 cm^{-1}, 1715 cm^{-1}, 1630 cm^{-1}, 1580 cm^{-1} and 1119 cm^{-1}.

Microanalysis of oDNR solid samples

Calculated: $C_{45}H_{61}NO_{11}$, C 68.25; H 7.76; N 1.77; found: C 68.29; H 7.45; N 1.49. The melting point was 102–104°C.

Log P of the daunorubicin derivative was calculated using X LogP software, interface ALOGPS v.2.1 (Wang et al 1997, 2000; University of Bejing, China). The log P of the daunorubicin derivative was 10 times greater than that of daunorubicin (7.0 and 0.7, respectively).

Preparation of LDE

LDE was prepared according to the method described by Ginsburg et al (1982) modified by Maranhão et al (1993). A lipid mixture composed of 40 mg cholesteryl oleate, 20 mg egg phosphatidylcholine, 1 mg triolein and 0.5 mg cholesterol was dissolved in chloroform:methanol (2:1) and dispensed into vials. The mixture was dried under a nitrogen stream followed by overnight vacuum desiccation at 4°C to remove residual solvents. The dried lipids were resuspended in 10 mL 0.1 M KCl, 0.01 M Tris-HCl, pH 8.0. The suspension was sonicated using a cell disrupter with a 125 W output in the continuous operating mode, for 180 min under a nitrogen atmosphere. The temperature was kept above 52°C (the melting point of cholesteryl oleate), monitored by a thermocouple inserted in the vials during this procedure. The emulsified lipid suspension was then transferred to clean tubes for ultracentrifugation at 195 000 g (30 min) at 4°C. The top 10% of the solution, containing particles that float at background density of approximately 1.006 g mL^{-1}, was removed by aspiration with a needle. The remaining solution was adjusted to a background density of 1.22 g mL^{-1} by adding solid KBr. A second ultracentrifugation step was then performed at 195 000 g (120 min) at 4°C. The top 20–30% of the sample was collected by aspiration after attaining room temperature and was dialysed overnight in Tris-HCl buffer to remove KBr contained in solution. This LDE fraction was sterilized by passage through a 0.22 μm filter (Millipore Inc., Billerica, MA, USA). The same buffer was used in all experiments. Under the conditions described above, no increase in peroxide content occurs (determined by the TBARS method (Buege & Aust 1978)) and no additional bands resulting from lipid breakdown appear on thin-layer chromatography (unpublished data).

Association of oDNR with LDE

oDNR was dissolved in ethanol in a volume corresponding to 10% LDE (by volume). LDE was added to the solution and the preparation submitted to ultrasonic irradiation for 40 min at 40°C. The LDE-bound and unbound drug were separated by centrifugation (3000 *g* for 20 min at 4°C). The pellet of unbound drug was dissolved in methanol and the amount determined by measuring the absorbance at 230 nm using a molar absorptivity (ϵ) of 35 000 M^{-1} cm^{-1}. Samples prepared at drug:lipid proportions of 1:5 and 1:10 in mass yielded 84% and 93% incorporation, respectively. The former was used in toxicity studies.

Stability of LDE–oDNR

To verify whether oDNR may dissociate from LDE, 2 mL 3.65 mM LDE–oDNR prepared in buffer was dialysed against either 20 mL human plasma or 20 mL buffer for 24 h at 37°C under continuous agitation. Aliquots of 30 μL were taken from the external compartments containing either plasma or buffer every hour during the first 6 h and after 24 h. Samples from the plasma-containing external compartment were diluted to 1.0 mL with methanol/water (1:1 v/v) and the optical absorbance determined at 230 nm. Samples from the buffer-containing external compartment were diluted to 1.0 mL with buffer and the optical absorbance determined at 230 nm. Dialysis membranes with a pore size $\leq$2.5 nm (molecular weight cut-off 12 000 Da; D0405, Sigma) were used in all assays.

Diameter of LDE particles

Average diameter and polydispersity index of LDE particles, either alone or associated with oDNR, were estimated by laser light scattering (Koppel 1972). Samples were diluted with buffer until they reached an average count rate between 100 and 200 kcps. The average particle diameter and polydispersity index of each sample were determined at 18°C after five runs of 1 min each. Those parameters were determined shortly after preparation of LDE and LDE–oDNR and after 5, 10, 15 and 20 days during which samples were kept at 4°C.

Cell culture assays

HL-60, K-562 and L1210 leukaemia and B16 melanoma cells were cultured in RPMI or DMEM media, to which was added 10% fetal calf serum and 0.1% antibiotics (1:1 penicillin/streptomycin). All cultures were kept in a humidified incubator at 37°C with 5% (v/v) CO_2. Cell replication was performed every 2 or 3 days by transfer of 1×10^5 cells mL^{-1} to a new bottle. When the culture reached confluence, cells were seeded in 96-well plates (5×10^4 K-562 or HL-60 cells per well; 1×10^5 L1210 or B16 cells per well). The cells were incubated for 24 h with commercial daunorubicin at concentrations ranging from 0.001 to 1.0 mM or LDE–oDNR at concentrations ranging from 0.0025 to 0.5 mM. The concentration of LDE alone was in the same range as that of LDE–oDNR. Cell viability was evaluated by the MTT method (Hayon et al 2003). The absorbance of each well was read at 570 nm. The cell survival data were plotted as dose–response curves and the drug concentrations necessary to inhibit 50% cell growth (IC50) determined from these curves.

Determination of toxicity in-vivo

Stock solutions of the drug were prepared as follows. Commercial daunorubicin was dissolved to a concentration close to its solubility in saline and subsequently diluted with

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saline (1 volume of drug in 5 volumes of saline). The LDE–oDNR preparation in buffer was diluted five times with saline.

Groups of 4–10 Swiss female mice were injected i.p. with different volumes of the stock solutions so as to receive doses of commercial daunorubicin ranging between 2.2 and 105.6 μmol kg^{-1} or doses of LDE–oDNR ranging from 63 to 252 μmol kg^{-1}. Control groups received saline solution or LDE without drug at the same concentration of LDE associated with the 252 μmol kg^{-1} oDNR dose.

Animals were observed daily for 32 days and their weight was measured every 4 days. The maximum tolerated dose (MTD) was determined as the dose at which the weight loss was less than 15% (Ecobichon et al 1996). The lethal doses for 10% and 50% of the animals (LD10 and LD50) were determined from dose–response curves created using Origin v.5.0 graphics software (Microcal Software Inc. Northampton, MA, USA).

Cardiotoxicity

Two animals each received i.p. injections of 3.5 μmol kg^{-1} commercial daunorubicin or LDE–oDNR, or LDE without drug at the same LDE concentration as in 3.5 μmol kg^{-1} LDE–oDNR. The control group were injected with 0.9% saline. Stock solutions as well as solutions injected into control animals were prepared as described above. Each dose was divided in two portions and these were administered 1 week apart. One week after the last injection, the animals were killed and the hearts collected for ultrastructural analysis.

Left ventricular wall fragments of 1 mm^3 were taken and fixed in 3% glutaraldehyde diluted in 100 mM phosphate buffer, pH 7.3, at 4°C for 3 h, and then fixed in 1% osmium tetroxide diluted in 100 mM phosphate buffer, pH 7.3 at 4°C for 2 h. The fragments were washed with 0.9% saline plus 1.78% sucrose and left overnight in 0.5% uranyl acetate plus 13.3% sucrose. After this, the samples were routinely processed for inclusion in Araldite. Ultrathin sections were examined under transmission electron microscopy. A qualitative analysis was performed in each case and the most severe lesions of each group were photographed.

Statistical analysis

A descriptive analysis of the data was initially done by determining means and s.e. and then inferential analysis was performed by analysis of variance or *t*-test. The survival data were plotted as Kaplan–Meier survival curves using GraphPad Prism Statistical Software v3.0 (GraphPad Software Inc., La Jolla, CA, USA) and the curves compared using the log rank test (Matthews & Farewell 1985).

Results

Yield of oDNR association to LDE

When co-sonicated with LDE in the drug:lipid ratio of 1:5 (by mass), 84 ± 4% of the oDNR was found associated with the nanoemulsion, increasing to 93 ± 2% at the 1:10 drug: lipid ratio (data from 17 experiments).

Particle size

LDE particle diameter roughly doubled after association with oDNR. Without drug, the LDE diameter was 42 ± 4 nm. When the drug was associated at the 1:5 drug:lipid ratio, the particle diameter increased to 75 ± 6 nm ($P < 0\ 0001$ vs LDE without drug). At the 1:10 ratio, the particle diameter increased further to 99 ± 16 nm ($P = 0.0164$ vs 1:5 drug:lipid ratio). Data are means ± s.e. obtained from 5–7 experiments.

The polydispersity index obtained for the LDE solution was 0.16 ± 0.04, which defines a narrowly dispersed population of particles (Hou et al 2003). There was no change in polydispersity after drug association (0.15 ± 0.02 for the 1:10 ratio preparation and 0.15 ± 0.02 for the 1:5 ratio preparation; n = 6 for both preparations).

The diameter of the LDE–drug complex (measured every 5 days) did not vary over the 20-day observation period (data not shown).

Stability of the LDE–oDNR complex

When LDE–oDNR was dialysed against aqueous buffer or human plasma, there was no escape of the drug from the dialysis bag (as determined at 1, 2, 3, 4, 5, 6 and 24 h after the beginning of the experiment; data not shown).

Cytotoxicity in tumour cells

IC50 values extracted from dose–response curves for leukaemia and melanoma cell survival are shown in Table 1. The IC50 of LDE–oDNR was greater than that of commercial daunorubicin in all four cell lines, indicating that the cytotoxicity of daunorubicin was reduced when it is associated with the nanoemulsion.

Acute toxicity

Figure 3 shows Kaplan–Meier survival curves for the animals treated with LDE–oDNR or commercial daunorubicin. The animals treated with LDE–oDNR showed greater survival time than those treated with commercial daunorubicin, regardless of the dose level. Commercial daunorubicin was lethal to all animals at doses of 3.1 μmol kg^{-1} and higher. In contrast, most animals treated with LDE–oDNR survived at doses above 252.5 μmol kg^{-1}. In control experiments in which LDE was injected into the animals, survival was 100% (data not shown).

Table 1 Drug concentrations that caused 50% inhibition of cell growth (IC50), obtained for K-562, HL-60, L1210 and B16 lines after incubation with daunorubicin, LDE–*N*-oleyl daunorubicin (LDE–oDNR) and LDE without drug

Cell line	K-562	HL-60	L1210	B16
Daunorubicin	34.70	22.80	0.07	11.90
LDE–oDNR	85.00	47.30	0.60	160.00
LDE alone	1.90	1.90	–*	1.70

Data are in 10^{-6} M for daunorubicin and LDE–oDNR and in mg mL^{-1} for LDE alone.
*IC50 was not reached in this experiment.

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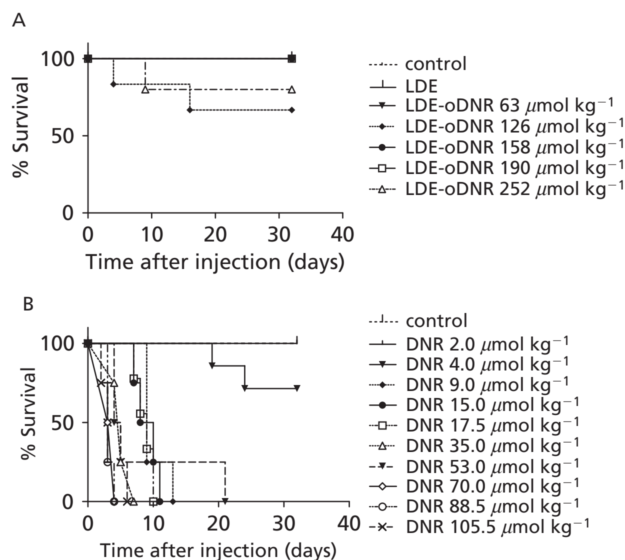

Figure 3 Kaplan–Meier survival curve for Swiss mice given (A) LDE–*N*-oleyl daunorubicin (LDE–oDNR) i.p. at doses of 63 (n = 4), 126 (n = 6), 158 (n = 4), 190 (n = 5) or 252 μmol kg^{-1} (n = 6), or (B) commercial daunorubicin (DNR) at doses of 2.0, 4.0 (n = 7), 9.0, 15.0 (n = 9), 17.5, 35.0, 53.0, 70.0, 88.5 and 105.5 μmol kg^{-1} (n = 4 for each dose level). All doses were diluted in saline before injection (1 volume of drug in 5 volumes of saline). Control groups were injected with saline or LDE particles. The animals were followed daily for 32 days after injection.

Decreased toxicity was also demonstrated by greater LD10 and LD50 values in the LDE–oDNR treated animals.

As shown in Figure 4, the animals in the two treatment groups that survived gained weight during the 32 days of observation; weight gain was greater in animals given LDE–oDNR than in those given commercial daunorubicin, indicating that the toxicity of daunorubicin was reduced in the LDE–oDNR. In control experiments in which LDE alone was injected into the animals, the weight gain was similar to that in the control group injected with saline solution (data not shown).

Table 2 shows the toxicity parameters measured in the two experimental groups. LDE–oDNR was markedly less toxic than commercial daunorubicin. In fact, toxicity was reduced to such an extent that it was not possible to measure the MTD, LD10 or LD50 accurately because this would have required the injection of a very large volume of solution that would not be tolerated by the animals.

Cardiac tissue ultrastructure

The hearts of mice injected with commercial daunorubicin (3.5 μmol kg^{-1}) showed intracellular swelling, dilation of the sarcoplasmic reticulum, foci of sarcomeric myofilament lysis plus mitochondrial cristolysis (Figure 5A). Animals injected with LDE–oDNR at the same concentration (Figure 5B) or with LDE without drug (Figure 5C) showed preserved sarcomeres and only scarce foci of mitochondrial cristolysis. Control animals injected with saline solution showed no ultrastructural lesions (Figure 5D).

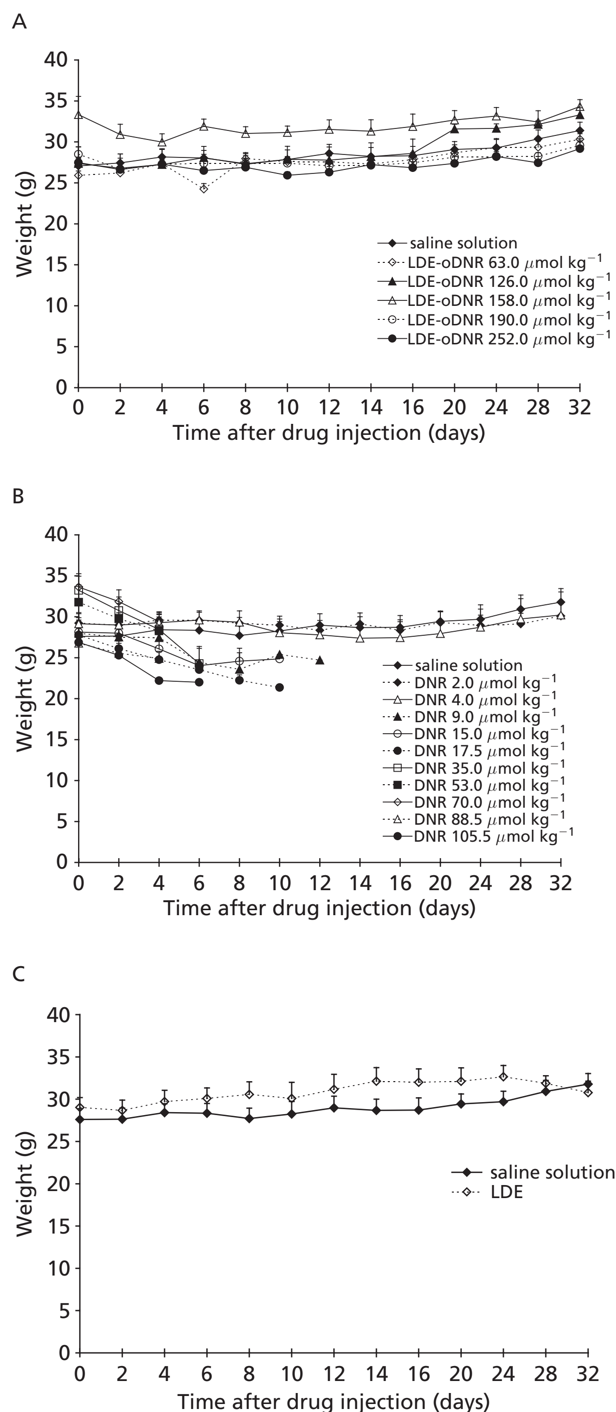

Figure 4 Weight variation of Swiss mice that received: (A) LDE–*N*-oleyl daunorubicin (LDE–oDNR) i.p. at doses of 63 (n = 4), 126 (n = 6), 158 (n = 4), 190 (n = 5) and 252 μmol kg^{-1} (n = 6); or (B) commercial daunorubicin (DNR) i.p. at doses of 2.0, 4.0 (n = 7), 9.0, 15.0 (n = 9) 17.5, 35.0, 53.0, 70.0, 88.5 and 105.5 μmol kg^{-1} doses (n = 4 for each dose level). (C) Control animals were given i.p. injections of 0.9% saline or LDE particles. All doses were diluted in saline before injection (1 volume of drug in 5 volumes of saline). The animals were followed daily for 32 days after injection.

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Table 2 Maximum tolerated dose (MTD) and lethal doses to 10% (LD10) and 50% (LD50) of the animals that received daunorubicin or LDE–*N*-oleyl daunorubicin (LDE-oDNR) i.p.

Formulation	MTD (μmol kg^{-1})	LD10 (μmol kg^{-1})	LD50 (μmol kg^{-1})
DNR	<3.1	3.1	5.6
LDE-oDNR	>252.5	>252.5	>252.5

Discussion

In this study, we demonstrate the feasibility of obtaining a stable association of oDNR with LDE. LDE–oDNR is less cytotoxic than commercial daunorubicin; toxicity in animals was remarkably diminished, such that it would be possible to administer much larger doses of the drug.

Performed as described, the association was a high-yield process, in which 93% of the drug co-sonicated with LDE became associated with the nanoemulsion when the ratio of drug:LDE lipids was increased to 1:10. At the 1:5 ratio, the association yield (84%) was satisfactory. This is important because substantial drug loss in the association process may raise the cost of the preparation. The 1:10 drug:lipid ratio offers an almost complete association yield. However, the 1:5 ratio may be of more interest, since the injection volume of the preparation is smaller and drug loss in the association process, although greater than with the 1:10 ratio, is still small.

It is worth mentioning that when drugs are added to the lipid mixture before ultrasonic irradiation, considerable amounts of drug associate with the non-LDE emulsion fraction, which is discarded after the first ultracentrifugation. Thus, this alternative manufacturing approach was not adopted in this study.

It is apparent that the association of the drug to LDE increased the particle size by 78% when the drug:lipid ratio in the mixture was 1:5, and by 136% when the ratio was 1:10. This increasing size effect was also observed in our previous studies in which carmustine (Maranhão et al 2002), etoposide (Valduga et al 2003) or paclitaxel (Rodrigues et al 2005) were associated with the nanoemulsion, but was less marked than with the current preparation. The increase in size might be ascribed to swelling of the particle core after drug association. Particle fusion after drug association, which would increase the particle size, is unlikely since the polydispersity index did not change. Particle size may be important in terms of binding to the LDL receptors. The particles originated from the 1:5 drug:lipid ratio with average diameter of 75 nm would be adequate for LDE drug targeting rather than the particles produced by the 1:10 ratio.

When dialysed against either plasma or buffer, LDE–oDNR did not release oDNR to the surrounding medium. In the dialysis against plasma, several plasma constituents such as apolipoproteins Cs can cross the dialysis membrane and

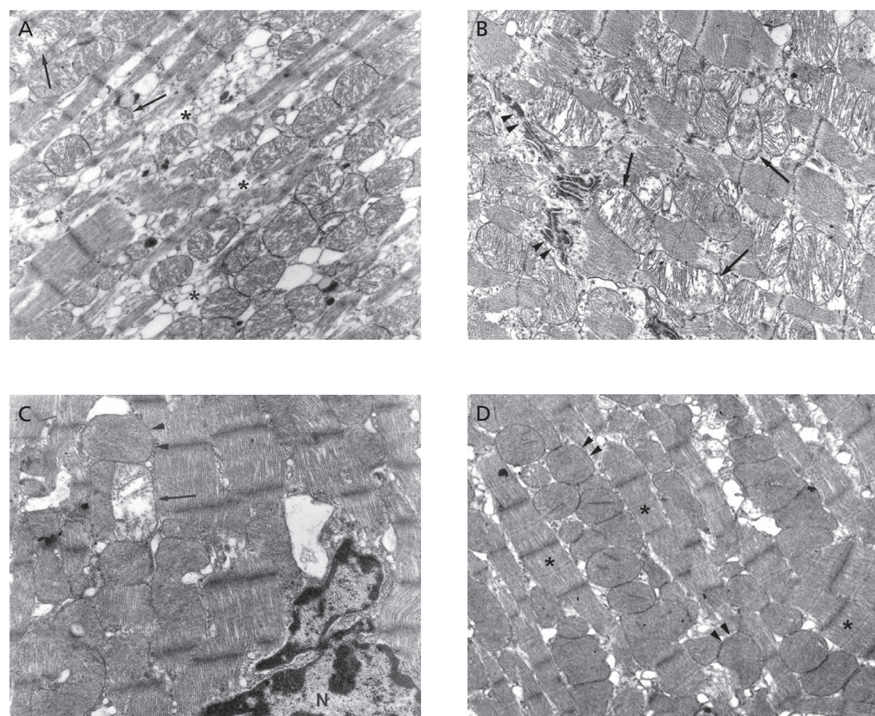

Figure 5 Cardiac tissue ultrastructure. A. Animals injected with commercial daunorubicin (3.5 μmol kg^{-1}) showed foci of sarcomeric myofilaments lysis and disintegration (asterisks) and mitochondrial cristolysis (arrows). B. Animals injected with LDE–*N*-oleyl daunorubicin at the same concentration showed preserved sarcomeres, but foci of mitochondrial cristolysis (arrows). Note preserved intercalated disk (arrowheads). C. Animals injected with LDE without drug showed preserved sarcomeres, but rare mitochondrial cristolysis (arrows). Note normal mitochondria (arrowheads) and nucleus (N). D. Control animals injected with saline presented normal sarcomeres (asterisks) and mitochondria (arrowheads). Transmission electron microscopy × 14 000 (A), × 11 100 (B), × 18 600 (C) and × 10 600 (D).

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mix with the LDE–oDNR solution, whereas lipoprotein and LDE particles do not cross the membrane. The results of the stability experiments therefore suggest that the LDE–oDNR association is stable over time. This is of great importance because stability of LDE–oDNR is a prerequisite for the drug-targeting effect.

The stability of the preparation was tested at 4°C for 20 days. The particle size measured by laser light scattering was unchanged during this period. Stability for 20 days is enough for the performance of clinical assays but stability should be tested for longer periods to verify adequacy for commercially required shelf lives. In this regard, addition of antioxidants to the LDE and other standard measures to increase long-term stability will be required.

Previous studies have shown that carmustine and derivatives of etoposide and paclitaxel are able to associate stably to LDE (Valduga et al 2003; Teixeira et al 2004; Rodrigues et al 2005) and this association markedly decreased the toxicity of these drugs in animals. The association with LDE increased the therapeutic efficiency, as shown in Walker 256 tumour-bearing rats and melanoma B16-bearing mice (Teixeira et al 2004; Rodrigues et al 2005). In clinical trials enrolling patients with advanced solid cancers, LDE reduced the toxicity of high doses of carmustine (Maranhão et al 2002) etoposide (Pinheiro et al 2005) and paclitaxel (Pires et al 2008). These previous results suggested that the development of the daunorubicin association with LDE would be interesting because daunorubicin has good antineoplastic action in haematological cancers but this is hampered by the marked toxicity of the compound, particularly cardiotoxicity. Our results show that LDE pronouncedly reduced the toxicity of the drug and, in view of our previous studies, it is reasonable to hypothesize that the use of the nanoemulsion will also improve the pharmacological action of the drug. Interestingly, in contrast with the commercial formulation, a clear dose–response relationship was not observed with LDE–oDNR – when LDE is used as vehicle, an optimal response was attained with low doses of daunorubicin.

Besides the drug-targeting effect, the diminished toxicity of LDE–oDNR can be ascribed to the new biodistribution of the drug. The liver is the main uptake site for LDE (Maranhão et al 1993), as for natural lipoproteins, because of the dense expression of LDL receptors by hepatocytes. Thus, drugs associated with LDE are predominantly taken up by the liver. Despite this fact, hepatotoxicity has not been observed in the clinical assays performed with LDE as vehicle for carmustine (Maranhão et al 2002), etoposide (Pinheiro et al 2005) or paclitaxel (Pires et al 2008). Another possible mechanism for the diminished toxicity comes from the fact that the drug is protected inside nanoemulsion particles whilst in the blood, thus avoiding direct contact with tissues and red blood cells. Finally, the association of the drug with LDE presumably increases the half-life of the drug. Although pharmacokinetics have not been documented in this study, drugs stably associated to LDE assume the plasma kinetics of the LDE particles, which is much slower than kinetics of commercial etoposide (Lo Prete et al 2006) and paclitaxel (Rodrigues et al 2005).

Conclusion

By diminishing the toxicity of daunorubicin, the association with the lipid nanoemulsion may offer a novel strategy for the use of this agent in cancer chemotherapy that should be explored in further studies.

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