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Review Article

Evaluation in melanoma-bearing mice of an etoposide derivative associated to a cholesterol-rich nanoemulsion

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Abstract

A cholesterol-rich nanoemulsion (LDE) may be used as a vehicle to target antineoplastic drugs against cancer cells. The association of an etoposide derivative to LDE is stable and retains the cytotoxic activity of etoposide. We have evaluated the toxicity and antitumoral action of this new preparation in-vivo. Melanoma-bearing mice and control mice were administered LDE-etoposide oleate or commercial etoposide, either with or without radioactive labelling. The maximum tolerated dose (MTD), tissue distribution, plasma decay curves, pharmacokinetic parameters and antitumoral activity were determined. Association to LDE drastically reduced the drug toxicity, since MTD was approximately five-fold greater than in commercial etoposide. LDE-etoposide oleate was concentrated four-fold in the tumour compared with the normal adjacent tissues, was removed faster from plasma in tumour-bearing mice than in controls, and remained in the bloodstream longer than commercial etoposide. The tumour growth inhibition rate and survival were greater in animals treated with LDE-etoposide oleate compared with commercial etoposide. However, increasing the dose from 17 to 85 μM kg^{-1} did not result in further improvement of the antitumour action. The incorporation of etoposide oleate to LDE resulted in markedly reduced toxicity and superior antitumoral activity. LDE-etoposide oleate is a promising new weapon for cancer treatment.

Introduction

In previous studies we have shown that a cholesterol-rich nanoemulsion, a liquid-in-liquid system termed LDE, is taken up by the low-density lipoprotein (LDL) receptors after injection into the bloodstream (Maranhão et al 1994). As most cancer cells show upregulation of LDL receptors, LDE can be used as a vehicle to direct antineoplastic drugs to those cells (Ades et al 2001; Graziani et al 2002). We showed that carmustine and derivatized compounds of etoposide and paclitaxel were able to form a stable association to LDE (Maranhão et al 2002; Valduga et al 2004; Rodrigues et al 2005), and this association markedly decreased the toxicity of carmustine and paclitaxel to animals. Moreover, the therapeutic efficiency of those drugs was increased by association with LDE, as shown in Walker 256 bearing rats and melanoma B16 bearing mice (Teixeira et al 2004; Rodrigues et al 2005). In a dose-escalating study enrolling patients with advanced solid cancers (Hungria et al 2004), we observed that the association with LDE resulted in a marked reduction of the side effects of carmustine, even at very high doses. In multiple myeloma patients treated with LDE-carmustine at 180 mg m^{-2} body surface carmustine dose, it was also possible to document the antitumoral efficiency of the preparation. During the treatment there was a substantial reduction in the plasma levels of the disease marker γ-globulin and in all seven treated patients there was reduction in pain and improvement of the haemoglobin concentration. Side effects were virtually absent in all treatment cycles.

Etoposide is poorly incorporated into nanoemulsions, and so its lipophilicity was increased by derivatizing the agent with an oleyl group. This resulted in excellent association yield and the formed LDE-etoposide oleate complex was stable for

approximately two weeks at 4°C. LDE-etoposide oleate appears as a lightly cloudy aqueous solution. Association to LDE reduced the lethality of etoposide to mice without diminishing the cytotoxicity (Valduga et al 2004).

In this study, we have conducted a comparison between LDE-etoposide oleate and the commercial presentation of etoposide to clarify whether the new preparation was superior regarding toxicity, pharmacokinetics and antitumour efficacy.

Materials and Methods

Materials

Etoposide was purchased from Calbiochem (CA), oleic acid, ethyl acetate, chloroform, triethylamine, dichloromethane, triolein, cholesteryl oleate, cholesterol and phosphatidylcholine were purchased from Sigma Chemical Company (St Louis, MO, USA). Methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). Commercial etoposide was provided by Quiral Química (Juiz de Fora, Brazil). Each 1 mL of commercial formulation consisted of a solution of 20 mg etoposide, 2 mg citric acid, 30 mg benzyl alcohol, 80 mg modified Polysorbate 80 (Tween 80), 650 mg polyethylene glycol 300 and 30.5% (v/v) alcohol. Etoposide oleate was obtained by the attachment of an oleoyl group to etoposide, and [^{3}H]etoposide oleate was prepared by addition of [^{3}H]etoposide to the etoposide before the reaction with oleoyl chloride (Lundberg 1994). [^{14}C]Cholesteryl oleate used to label the emulsion was purchased from Amersham (Amersham, UK) and [^{3}H]etoposide (dissolved in ethanol) from Moravek (Brea, CA, USA). The murine B16F10 melanoma cell line was obtained from the American Type Culture Collection. C57BL/6J mice were purchased from the Central Animal Care Unit at Instituto Butantan. Females aged 8–12 weeks were used in all experiments.

All the animal experiments reported in this study were approved by the Animal Ethics Committee of the University of São Paulo Medical School Hospital.

Preparation of LDE

LDE was prepared from a lipid mixture composed of 40 mg cholesteryl oleate, 20 mg egg phosphatidylcholine, 1 mg triolein and 0.5 mg cholesterol. All components were mixed and the solvent evaporated. Emulsification of lipids was made by ultrasonic irradiation for 3 h in 10 mL aqueous media, and the procedure of two-step ultracentrifugation of the crude emulsion with density adjustment by addition of KBr to obtain LDE nanoemulsion was carried out by the method described by Ginsburg et al (1982) modified by Maranhão et al (1993). LDE was dialysed against 0.9% NaCl solution. The plasma kinetic and biodistribution experiments required radioactive labelling, and so [^{14}C]cholesteryl oleate was added to the starting lipid mixture.

Prepared as described, LDE had approximately 64% phospholipids, 33% cholesteryl ester, 1% unesterified cholesterol and 2% triacylglycerols (Maranhão et al 1994).

Association of etoposide oleate to LDE

LDE (1 mL) was added over 6.0 mg etoposide oleate dissolved in 100 μL ethanol. In those conditions, the drug precipitated as a very slight powder and the dissolution into LDE was facilitated. The solution was sonicated for 40 min at 55°C using a Branson Sonifier 450 (Danbury, CT, USA), with a 1-cm flat titanium probe. LDE-etoposide oleate was centrifuged (3000 *g*) for 45 min to separate the free etoposide oleate (pellet) from the association LDE-etoposide oleate (supernatant). The pellet was dissolved in 1 mL methanol and quantified by HPLC to give the final yield of association by diminishing the amount of free drug from the total added at the beginning of the procedure. LDE-etoposide oleate was then passed through a 0.22-μm pore polycarbonate filter and kept at 4°C until use. The final LDE-etoposide oleate preparation had a mean diameter of 65 nm ($\pm$ 10), polydispersity of 0.2 and maximum loading of etoposide oleate of 98% (5.88 g drug mL^{-1} LDE) from the total mass of drug added at the proportion 1:5 drug/nanoemulsion total lipids (w/w). For the plasma kinetic and biodistribution experiments that required radioactive labelling, [^{3}H]etoposide oleate was added to the starting lipid mixture.

Maximum tolerated dose (MTD)

MTD for LDE-etoposide oleate and commercial etoposide formulations was determined in groups of eight female C57BL/6J mice. LDE-etoposide oleate or commercial etoposide was administered intraperitoneally in a single dose ranging from 176 to 293 μmol kg^{-1} and from 43 to 128 μmol kg^{-1}, respectively. The maximum administered volume was 2 mL. Intraperitoneal injection was used rather than the intravenous route due to the great preparation volumes required by the experiment. Drug effects were investigated by observation of body weight changes and survival rates over 15 days. MTD was defined as the allowance of an average body weight loss of 15% initial weight that caused neither death due to toxic effects nor marked changes in the general signs within two weeks after administration (Zhang et al 1997).

Inoculation of B16 melanoma cells in mice

Murine B16F10 variants of the B16 melanoma cell line originating from C57BL/6J mice were used in the experiments. Cells were cultured in RPMI-1640 medium supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 1 mM sodium pyruvate, streptomycin (50 μg mL^{-1}) and penicillin (50 IU mL^{-1}). Cells were detached from plates with trypsin and 0.2% versene. After trypsin inactivation with 10% FCS, viable cells were counted by trypan blue dye exclusion. For tumour transfer 5×10^4 cells suspended in 100 μL phosphate buffer saline (PBS) were injected subcutaneously into the

flank regions of mice. Ten days after inoculation the tumours became macroscopically apparent.

Cell cycle distribution

Mice were inoculated with B16 melanoma cells as described above. On day 14, when the tumour implanted in the animals reached a volume of 200–500 mm^3, mice were randomly allocated to three groups of eight animals. The animals were intraperitoneally injected with 0.05 μmol etoposide oleate associated to LDE, 0.05 μmol commercial etoposide or 1 mL 0.9% NaCl solution. After 24 h, the tumours were excised, and chopped into small pieces, washed with PBS and resuspended in buffer (375 μL trypsin 0.03 $g\,L^{-1}$, 10 mM Tris pH 8.0). After 15-min incubation at room temperature, the neutralization solution (trypsin inhibitor 0.5 $g\,L^{-1}$, RNase A 0.1 $g\,L^{-1}$ and spermine 1.2 $g\,L^{-1}$) was added, and the incubation continued for an additional 15 min. The pelleted cells were resuspended in 0.3 mL PBS and fixed by addition of ice-cold ethanol (70%). Before analysis, cells were incubated with 18 $\mu g\,mL^{-1}$ propidium iodide solution and incubated in the dark for 30 min. Flow cytometry analysis was performed on a FACScan flow cytometry system (Scalibur-Becton Dickinson, San Jose, CA, USA). The DNA content in the cell cycle phases was analysed by Cell-Quest software and by the Mod-fit software cell.

Plasma kinetics of LDE-etoposide oleate and commercial etoposide

[^{14}C]LDE-[^{3}H]etoposide oleate (100 μL; 1.2 kBq, 3 mg LDE total lipids and 0.7 μmol etoposide oleate) or commercial [^{3}H]etoposide (1.2 kBq, 0.7 μmol drug) was injected as a single dose into the retro-orbital venous plexus of B16 melanoma-bearing mice (tumour volume 60–100 mm^3) and control mice. Approximately 500 μL blood was collected from the retro-orbital venous plexus at pre-established intervals over 24 h. Plasma was separated by 15-min centrifugation (3000 *g*) and the radioactivity was counted in a scintillation solution. The fractional clearance rate (FCR) of the LDE-etoposide oleate and commercial etoposide was calculated according to the method described by Matthews (1957). In this method a_1, a_2, b_1 and b_2 were estimated from biexponential curves obtained from the remaining radioactivity found in plasma after injection, fitted by least squares procedure, as $y = (a_1.e^{-b_1 t}) + (a_2.e^{-b_2 t})$, where y represents the radioactivity plasma decay.

The pharmacokinetic parameters of etoposide oleate associated with LDE and of commercial etoposide were calculated using a multi-compartmental model by means of computer software (PK Solutions, Ashland, OH, USA). The log plasma concentration vs time curves were fitted by biexponential equations and the half-life (t½) calculated by dividing 0.693 by the rate constant for each phase. The total area under the plasma concentration–time curve (AUC) was calculated using the linear trapezoid method with extrapolation to infinity. Total plasma clearance was calculated by dividing the dose by the AUC. The volume of distribution at steady state (Vd_{ss}) was estimated graphically from trapezoidal total area measurements.

Biodistribution of LDE, LDE-etoposide oleate and commercial etoposide in B16 melanoma-bearing mice

[^{14}C]LDE (100 μL; 1.2 kBq, 3 mg total lipids) or [^{14}C]LDE-[^{3}H]-etoposide oleate (1.2 kBq, 3 mg LDE total lipids and 0.7 μmol drug) or commercial [^{3}H]-etoposide etoposide (1.2 kBq, 0.7 μmol drug) was injected intravenously as a single dose in groups of ten melanoma-bearing mice (tumour volume 60–100 mm^3). The animals were kept in individual cages for 24 h, after which time the animals were killed. Tissue samples of 200–250 mg of skin, tumour, liver, spleen, lung and kidney were collected and kept in cold 0.9% NaCl solution. Lipids and drugs were extracted (Folch et al 1957) with chloroform/methanol (2:1, v/v), concentrated by nitrogen flow. The residue was re-suspended in 7 mL scintillation solution (Ultima Gold XR, Meriden, CT, USA) and the radioactivity was measured with a Packard 1600 TR model Liquid Scintillation Analyser.

Antitumour activity of LDE-etoposide oleate and commercial etoposide

Melanoma-bearing mice were allocated to four groups of 18 animals. Treatment was started on day 11, when the tumour volume reached 60–100 mm^3. The dose was adjusted by weight. On days 11, 13 and 15 counted from the day of tumour inoculation, each group was intraperitoneally administered a bolus injection of one of the following preparations: LDE-etoposide oleate 17 or 85 $\mu mol\,kg^{1}$ (equimolar doses of 10 and 50 mg kg^{-1} etoposide, respectively), commercial etoposide 17 $\mu mol\,kg^{-1}$, or 0.9% NaCl solution (control group). The intraperitoneal route was used due to the large volumes of the injected preparations.

The tumour sizes were measured four times a week using a calipers-like instrument. The size measurement was converted to tumour volume by the equation: tumour volume = (length × $width^2$)/2. Observation of the tumour volume ceased on day 25, but survival was monitored over 45 days. The antitumour activity was assessed according to the guidelines established by the National Cancer Institute (Plowman et al 1997).

Statistical analysis

The Mann–Whitney test was used for FCR data analysis. The analysis of variance test was used to analyse cell cycle, AUC of growth tumour inhibition, plasma radioactivity decay curves and MTD curves. The survival time plotting (Kaplan–Meier test) and survival comparison between groups were made using the Graph Pad Prism statistical software. All the values were expressed as means ± s.e.m. In all analyses, $P < 0.05$ was considered statistically significant.

Results

Maximum tolerated dose of LDE-etoposide oleate and commercial etoposide

Figure 1 shows the body weight variation of the animals under a single dose treatment with either LDE-etoposide oleate (A) or commercial etoposide (B) at three escalating dose levels. At the initial five days of the 15-day observation period, there was weight loss in all treatment groups. Thereafter, the animals treated with LDE-etoposide oleate at all dose levels tended to progressively recover the initial weight, that was attained towards the end of the observation period. Regarding the commercial etoposide-treated groups, the two smaller dose levels progressively gained weight after day 5, but at the end of the observation they were still 3% below the initial weight (43 μmol, $P = 0.422$; 85 μmol, $P = 0.871$). Moreover, at 85 μmol kg^{-1} three of the eight animals died. In the largest dose level group (128 μmol kg^{-1}) the weight loss was markedly greater than in the other groups in the first five days and all the animals died at day 6. As calculated from Figure 1, the MTD of LDE-etoposide oleate (235 μmol kg^{-1}) was approximately five-fold that of the commercial etoposide (43 μmol kg^{-1}).

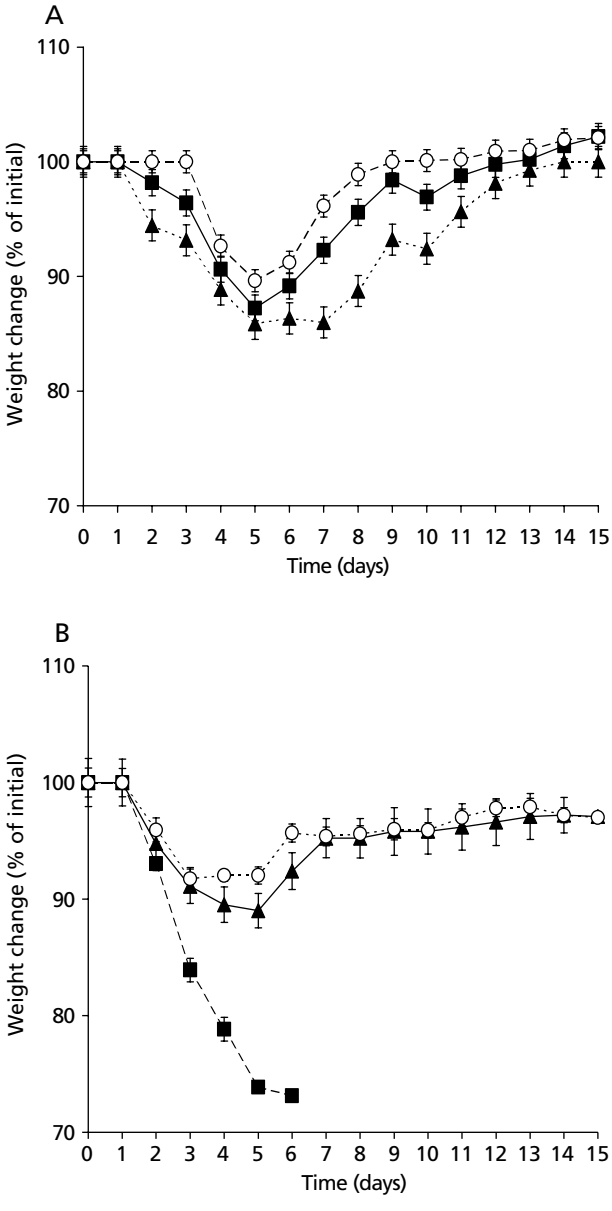

Figure 1 Changes in body weight after a single (i.p.) injection of etoposide formulations. A. LDE-etoposide oleate at 176 (○), 235 (■) or 293 μmol kg^{-1} (▲), $P = 0.0274$. B. Commercial etoposide at 43 (○), 85 (▲) or 128 μmol kg^{-1} (■), $P = 0.0007$. The animals were observed over 15 days. Each group comprised eight animals.

Cell cycle distribution

Cell cycle distribution was assessed in B16 tumour-bearing mice 24 h after the injection of LDE-etoposide oleate or commercial etoposide. The relative volume of cells in each phase of the cell cycle was estimated from the overall DNA content. In comparison with the nontreated animals, after one single dose LDE-etoposide oleate and commercial etoposide enhanced the volume of B16 cells by 95% in the G_0/G_1 phase. In contrast, both treatments reduced the volume of cells in the G_2/M and S phase by an average 2%. Figure 2 shows three representative scans obtained for the treatments with the two etoposide preparations and the control experiments.

Plasma kinetics of LDE-etoposide oleate and commercial etoposide in control and B16 melanoma-bearing mice

Figure 3 shows the plasma decay curves obtained in control (A) and in tumour-bearing (B) mice, of the LDE-etoposide oleate labelled with [^{3}H]etoposide oleate and [^{14}C] cholesteryl oleate, and commercial etoposide labelled with [^{3}H]etoposide ($n = 10$). The FCR of [^{14}C]cholesteryl oleate, the LDE label, was greater in tumour-bearing than in control mice (mean value $\pm$ s.e.m, $B = 0.156 \pm 0.022$ and $A = 0.248 \pm 0.034$ h^{-1}, $P = 0.03$). In control mice, the FCR of the etoposide oleate associated with LDE did not differ from that of the commercial etoposide (0.156 ± 0.024 and 0.264 ± 0.055 h^{-1}, respectively, $P = 0.071$). In contrast, in the tumour-bearing animals, the FCR of the etoposide oleate associated with LDE was greater than that of the commercial etoposide (0.304 ± 0.019 and 0.151 ± 0.025 h^{-1}, respectively, $P = 0.004$).

Table 1 summarizes the pharmacokinetic parameters obtained in control mice of etoposide oleate when associated with LDE. Derivatization and association with LDE resulted in remarkable changes in the pharmacokinetics of the drug characterized by two-fold half-lives and AUC, and 1.7-times smaller clearance rates compared with commercial etoposide.

Biodistribution of LDE, LDE-etoposide oleate and commercial etoposide in B16 melanoma-bearing mice

Figure 4 shows the uptake of LDE labelled with [^{14}C]cholesteryl oleate, of LDE associated with etoposide oleate labelled with both [^{14}C]cholesteryl oleate and [^{3}H]etoposide oleate and of commercial etoposide labelled with [^{3}H]etoposide in the tumour-bearing mice 24 h after the LDE injection. For each of the analysed tissues, there were no differences in uptake between LDE and the etoposide oleate associated to the nanoemulsion. Also, there were no

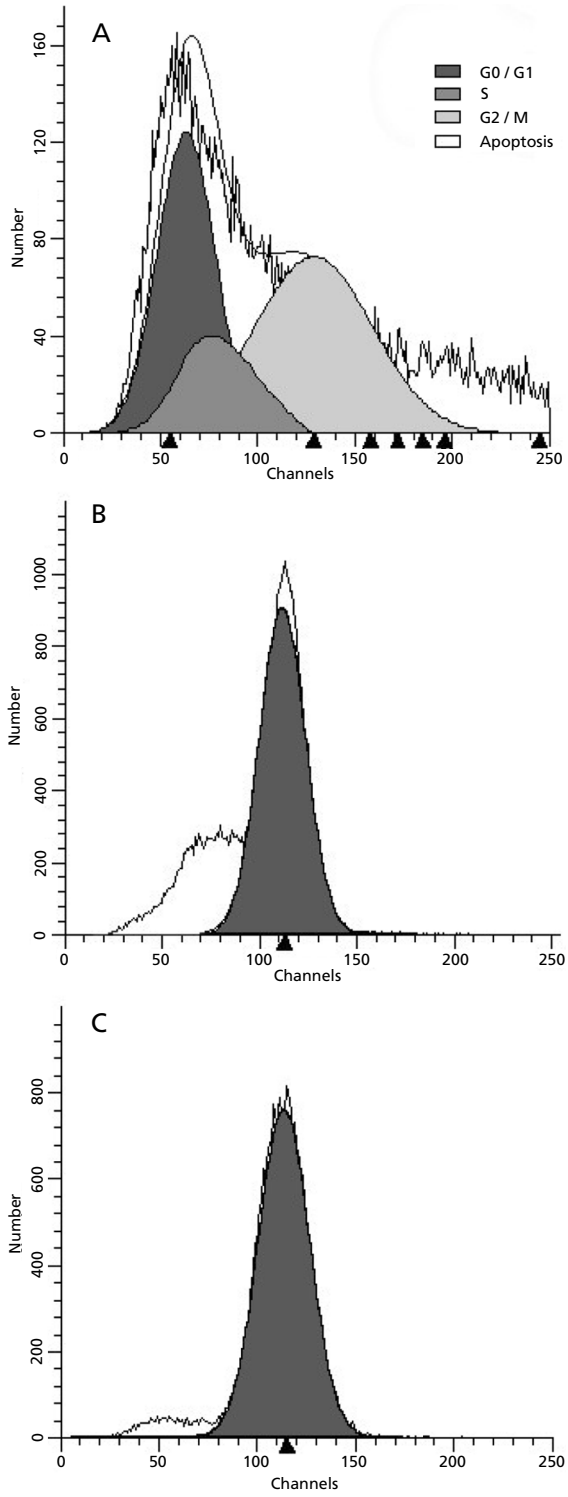

Figure 2 Flow cytometric analysis performed on B16 melanoma-bearing mice inoculated with B16 melanoma cells. The mice were injected with 0.05 μmol LDE-etoposide oleate, commercial etoposide or 0.9% NaCl (without treatment). This is a representative scan obtained from (A) control, (B) LDE-etoposide oleate and (C) commercial etoposide experiments. Each group comprised eight animals.

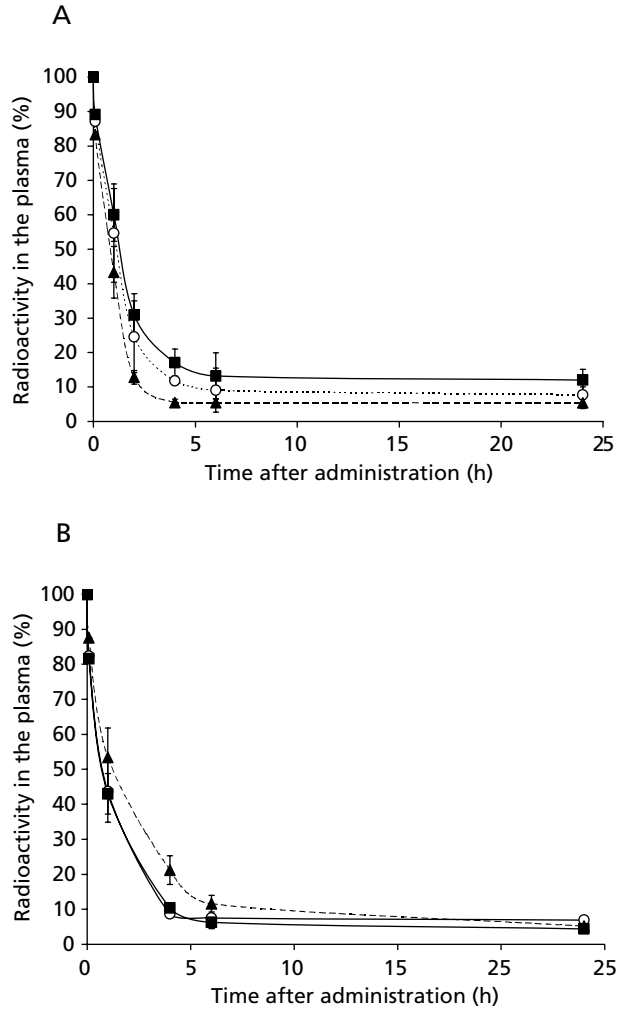

Figure 3 Comparison of the curves of removal from plasma of the LDE-etoposide oleate labelled with [^{3}H]etoposide oleate (○) and [^{14}C]cholesteryl oleate (■), and commercial etoposide labelled with [^{3}H]etoposide (▲), injected as an intravenous single dose into (A) control mice and (B) tumour-bearing mice. Plasma samples were taken over 24 h for radioactive counting in scintillation vials. Results are presented as means ± s.e.m. (bars). Each group comprised ten animals. $P = 0.8301$ and $P = 0.9476$, comparison by analysis of variance among the three curves in A and in B, respectively.

differences in the uptake of the labelled LDE associated with the drug and the uptake of the nanoemulsion alone for the different tissues.

Tumour uptake of the etoposide oleate associated with LDE was approximately four-fold compared with the surrounding skin tissue. The liver showed the greatest uptake of LDE and the associated drug, followed by the spleen and the tumour. The mean tumour uptake of LDE and the associated etoposide oleate was approximately one-third that of the uptake by the liver. In contrast, the uptake of the commercial etoposide by the tumour was clearly no greater than that of the surrounding skin tissue and was only half that of the etoposide oleate associated to LDE.

Table 1 Pharmacokinetic parameters obtained in control mice after intravenous administration of LDE-etoposide oleate or commercial etoposide

	Dose (μmol)	$t^{1/2\beta}$[a](min)	AUC[b] (μg min mL^{-1})	CL[c] (mL min^{-1})	Vd_{ss}[d] (mL)
Two-compartment open model:					
LDE-etoposide oleate[1]	2	43.2 ± 7.9	12604.7 ± 3394.6	0.75 ± 0.2	2.0 ± 0.4
Commercial etoposide[2]	2	21.7 ± 2.8	5380.3 ± 1339.0	1.33 ± 0.2	2.8 ± 0.2

[1]Etoposide oleate associated to LDE injected in control mice. [2]Commercial etoposide injected in control mice. [a]$t\,1/2_{\beta}$, elimination half-life. [b]AUC, area under the plasma concentration–time curve. [c]CL, total body clearance. [d]Vd_{ss}, volume of distribution at steady state. All values are represented as mean value $\pm$ s.e.m. Each group comprised ten animals.

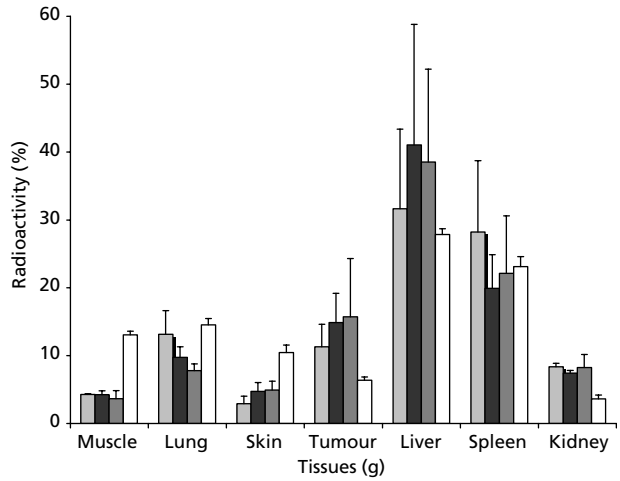

Figure 4 Tissue and organ biodistribution in B16 melanoma-bearing mice of LDE-etoposide oleate doubly labelled with [^{14}C]cholesteryl oleate (black) and [^{3}H]etoposide oleate (grey), of LDE alone labelled with [^{14}C]cholesteryl oleate (dark grey), and of commercial etoposide labelled with [^{3}H]etoposide (white), 18 h after a single intraperitoneal bolus injection. Radioactivity was measured by liquid scintillation. For the determination of the amount of radioactivity, the measured values were corrected for 1 g tissue or organ mass. Results are presented as means + s.e.m. (bars). Each group comprised ten animals.

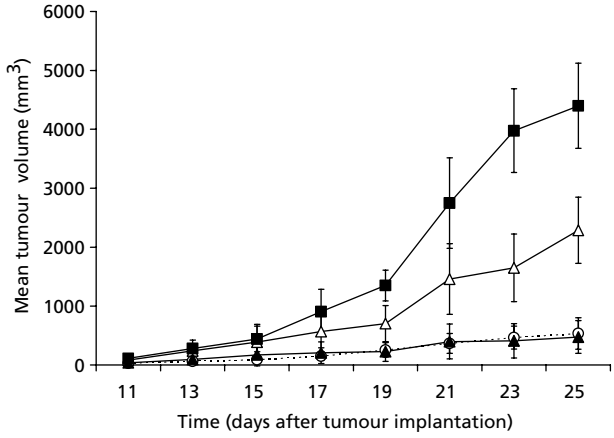

Figure 5 Effect of LDE-etoposide oleate on tumour growth of B16F10 tumour-bearing mice. B16 melanoma cells (5×10^4) were subcutaneously injected in the dorsal region of B57CL/6 mice. On days 11, 13 and 15 as counted from the initial inoculation day, LDE-etoposide oleate 17 (○) or 85 μmol kg^{-1} (▲), commercial etoposide 17 μmol kg^{-1} (△) or 0.9% NaCl (■) were intraperitoneally injected. Tumour volumes were monitored by periodic calipers measurements and are presented as means $\pm$ s.e.m. (bars). Each group comprised eighteen animals.

Antitumour activity of LDE-etoposide oleate and commercial etoposide

Figure 5 shows the effects on tumour growth of LDE-etoposide oleate and of the commercial etoposide. At equivalent dose (17 μmol kg^{-1}), the antitumour effect of LDE-etoposide oleate was pronouncedly greater compared with the commercial etoposide ($P = 0.0326$). Interestingly, when the etoposide oleate dose was increased from 17 to 85 μmol kg^{-1} in the LDE-etoposide oleate preparation, there was no improvement of the antitumoral effect ($P = 0.9265$).

Figure 6 shows the Kaplan–Meier survival curves of tumour-bearing mice treated with LDE-etoposide oleate, commercial etoposide or 0.9% NaCl solution. As observed at day 34, when all the 0.9% NaCl solution-treated animals had died, the best results (67% survival rate) were achieved by LDE-etoposide oleate treatment at the greater dose (85 μmol kg^{-1}), followed by LDE-etoposide oleate at the smaller dose (50% survival rate). The

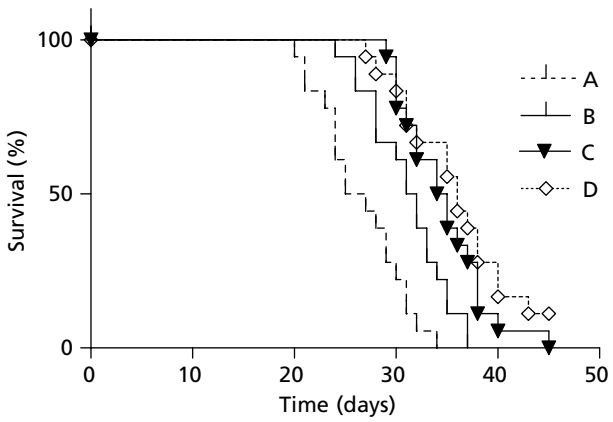

Figure 6 Effect of LDE-etoposide oleate on survival rate. B16F10 tumour-bearing mice were treated with (A) 0.9% NaCl, (B) commercial etoposide 17 μmol kg^{-1}, (C) LDE-etoposide 17 μmol kg^{-1} or (D) 85 μmol kg^{-1}. The survival rates were calculated daily over 45 days. The results were plotted using Kaplan–Meier test and are presented as mean. Each group comprised eighteen animals.

worst result (22% survival only) was observed in the group treated with the commercial preparation.

It was clear that the antitumour LDE-etoposide oleate action at both dose levels was superior compared with that of the commercial etoposide, as inferred by the tumour growth delay and the tumour average volume parameters.

Discussion

The association with LDE improved etoposide as an antineoplastic agent because it reduced the toxicity and increased the antitumour activity of the drug.

Previously in acute toxicity studies, we showed (Valduga et al 2004) that association of etoposide oleate to LDE reduced the median lethal dose of the drug by five-times: LD50 (50% lethal dose) of LDE-etoposide oleate was $315\,mg\,kg^{-1}$ as compared with $58\,mg\,kg^{-1}$ found for commercial etoposide. In this study, the toxicity experiments were extended for determination of the MTD and based on this parameter it was suggested that the reduction of the toxicity by LDE was in the order of fivefold. The weight variation, another important toxicity parameter, also supported the assumption that the use of LDE drastically reduced toxicity: the severe weight loss observed under commercial etoposide treatment was abolished in the LDE-etoposide oleate-treated animals. The great attenuation of the toxicity could be ascribed to the new biodistribution of the drug determined by its association to the nanoemulsion that tended to concentrate in the liver and in the tumour (Maranhão et al 1993; Rodrigues et al 2005). The reduced toxicity of LDE-etoposide oleate compared with commercial etoposide could be ascribed also to the fact that the vehicle used in the commercial preparation was not devoid of adverse effects (O'Dwyer & Weiss 1984), whereas LDE was virtually atoxic.

The flow cytometric measurement of DNA content showed that LDE-etoposide oleate arrested the cells in the G_0/G_1 phase of the cell cycle, as did commercial etoposide (Figure 2). The predominant action of etoposide on G_0/G_1 had been reported by Sugimoto et al (1998). The cell populations on S and G_2/M phases have a greater content of topoisomerase II (Zhou et al 1999) that was the target for etoposide. This accounted for the effect of commercial etoposide on those cycle phases and the LDE-etoposide oleate followed this trend, indicating that the intracellular mechanism of action of the drug was not changed by association with LDE. Moreover, in this study, after 24-h exposure, the diminution of the volume of tumour cells in those cycle phases and the increase in number of apoptotic cells were indeed an indication that LDE-etoposide oleate decreased the tumour proliferative activity.

The B16 tumour-bearing mice model was chosen for our experiments because those cells show the overexpression of LDL receptors (Verluis et al 1996; Rodrigues et al 2005) that makes ground for the use of LDE as a drug targeting vehicle. Therefore, the increased LDE removal from plasma in the tumour-bearing mice (Figure 3) could be ascribed to the increased uptake of the nanoemulsion by the melanoma cells overexpressing those receptors.

Previously, we showed that, when dialysed against plasma, etoposide oleate did not dissociate from the nanoemulsion over time (Valduga et al 2004). In this study, the finding that the decay curves and removal rates of [^{3}H]etoposide oleate and of the [^{14}C]cholesteryl oleate of LDE were similar (Figure 3) confirmed the lack of dissociation of the drug from the nanoemulsion. This is of paramount importance because the stability of the association LDE-etoposide oleate in the bloodstream is a pre-requisite for the drug-targeting effect.

It was noteworthy that when associated with LDE, etoposide oleate showed greater plasma half-life and AUC than the commercial formulation (Table 1). This was interesting because the duration of exposure of neoplastic cells to etoposide is important for achieving the maximal antitumour activity (Drewinko & Barlogie 1976; Wolff et al 1987; Slevin et al 1989). Recently, we showed that etoposide oleate was taken up together with LDE by NCI-H292 cells (Valduga et al 2004). This is confirmed here in-vivo, since uptake of the two components of the complex, the drug and the nanoemulsion, by the tumour were not different. Also, the fact that the unassociated nanoemulsion had biodistribution similar to that of LDE-etoposide oleate (Figure 4) indicated that the presence of the drug did not affect the biological properties of the nanoemulsion, especially the ability to concentrate in the tumour. It is important to point out that when the drug was associated to LDE its concentration increased twofold compared with the commercial preparation, confirming the drug targeting ability of the nanoemulsion.

The superior antitumour action of LDE-etoposide oleate over the commercial preparation was clearly documented by the reduction of tumour growth and the increase in survival rates. It is interesting, however, that when the dose of the drug associated to LDE was increased from 17 to $85\,\mu mol\,kg^{-1}$ no further improvement in the tumour regression was seen, but the survival of the animals treated with the $85\,\mu mol\,kg^{-1}$ was increased. This suggested that the larger dose could be more active, despite an apparent lack of additional effect on tumour regression. Another possibility was that the diminished toxicity contributed to the prolonged survival. The saturation of LDL receptors (Brown & Goldstein 1986) of the tumour cells by the nanoemulsion particles or downregulation of those receptors by the entry of increasing amounts of LDE cholesterol into the cells may eventually account for the lack of dose–response pattern in the tumour regression experiment.

In this study, different doses and routes of administration were used. This was to optimize the experiments, but did not influence the overall results because the comparisons were systematically performed in the same conditions. Regarding tumour size, this was similar in all the experiments with the exception of those utilized in cell cycle experiments, wherein a larger tumour mass was required.

In conclusion, the association of etoposide oleate to LDE resulted in a preparation with less toxicity and greater antitumour activity, as tested in-vivo. Ongoing research in cancer patients is necessary as the new preparation has a clear potential for clinical use.

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