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Use of a cholesterol-rich microemulsion that binds to low-density lipoprotein receptors as vehicle for etoposide

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Abstract

A cholesterol-rich microemulsion (LDE) that binds to low-density lipoprotein (LDL) receptors is selectively taken up by malignant cells that overexpress those receptors and may be used as vehicle for antineoplastic agents. This study aimed to develop the association of etoposide with LDE. It was firstly observed that etoposide poorly associates with the microemulsion, therefore the experiments were performed with a lipophilic fatty acid derivative of the drug. The association of etoposide oleate with LDE was almost 100% and was tested for physical and chemical stability, as well as for cellular uptake, toxicity in mice and cytotoxic activity against a neoplastic cell line (NCI-H292). Uptake and cytotoxic activity of LDE-etoposide oleate by NCI-H292 cells was mediated by LDL receptors. The anti-proliferative activity of LDE-etoposide oleate against the neoplastic cells was smaller than that of etoposide oleate (IC50 (drug concentration required to inhibit 50% of the cell growth) = 0.48 and 0.19 mM, respectively). This difference, however, can be ascribed to the activity of the commercially used vehicle and not the drug itself because when this vehicle was added to the cultures with LDE-etoposide oleate, the IC50 decreased. On the other hand, the tolerability of LDE-etoposide oleate to mice was remarkable, such that its lethal dose (LD50) was about five-fold that of the commercial formulation (LD50 = 315 and 58 mg kg^{-1}, respectively). In conclusion, LDE-etoposide oleate association is stable and the cytostatic activity of the drug is preserved while its toxicity to animals is small. By diminishing the side effects and directing etoposide to neoplastic tissues, LDE may be regarded as an advance in chemotherapy with this drug.

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Introduction

In previous studies (Maranhão et al 1994), we showed that an artificially made microemulsion, termed LDE, had the ability to concentrate in neoplastic cells after injection into the bloodstream. LDE is composed of quasispherical nanoparticles basically formed by a monolayer of phosphatidylcholine surrounding a core of cholesteryl esters. Small amounts of unesterified cholesterol and triglycerides are also present. LDE mimics the lipidic portion of low-density lipoprotein (LDL) and in contact with plasma it acquires apolipoproteins from the circulating native lipoproteins. LDE is then removed from the circulation into the body tissues by LDL receptors present on the cell membrane. One of the proteins acquired by the microemulsion, apolipoprotein E (apo E), is recognised by the LDL receptors and enables the LDE particles to bind to the receptors. LDE is then internalised into the cytoplasm via the LDL-receptor-mediated endocytosis. Because most cancer cells show LDL receptor upregulation, LDE may target those cells and eventually may specifically deliver to them chemotherapeutic agents loaded into the microemulsion particles (Maranhão et al 1993).

In recent studies, direct evidence was provided of the selective LDE uptake by neoplastic tissues in patients with ovarian and breast carcinoma (Ades et al 2001; Graziani et al 2002). Previously, it had been suggested that in patients with acute myelocytic leukaemia those leukaemia cells also take up the microemulsion. It was also shown in patients with advanced cancers that the association of carmustine with LDE markedly diminishes the

toxicity of the drug (Maranhão et al 2002). Therefore, LDE may be, indeed, a promising addition to the weaponry of drug targeting vehicles for cancer therapeutics.

In this study, the development of the association with LDE of etoposide, one of the most commonly used antineoplasic agents, was attempted. Etoposide is a semisynthetic derivative of podophyllotoxin and is a potent inhibitor of topoisomerase II. During the study, it turned out to be necessary to increase the lipophilicity of the drug to facilitate its incorporation into the microemulsion. This was achieved by attaching an oleyl group to etoposide. The resulting preparation, LDE-etoposide oleate, was stable and consistently diminished the toxic effects of the drug without altering its in-vitro cytotoxicity.

Materials and Methods

Materials

Crystalline etoposide was purchased from Calbiochem (CA). Dimethylsulfoxide (DMSO), 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), oleic acid, ethyl acetate, chloroform, triethylamine, dichloromethane, triolein, cholesteryl oleate, cholesterol and phosphatidylcholine were purchased from Sigma Chemical Company (St Louis, MO) and methanol and acetonitrile from Merck (Darmstadt, Germany). The commercial etoposide and its vehicle were provided by Quiral Química (Juiz de Fora, Brazil). Each 1 mL of the used commercial formulation consisted of 20 mg etoposide, 2 mg citric acid, 30 mg benzyl alcohol, 80 mg of modified polysorbate 80 (Tween 80), 650 mg of polyethylene glycol 300 and 30.5% (v/v) of alcohol. Etoposide oleate and *cis*-etoposide oleate were synthesized as described below. [^{3}H]Etoposide oleate was prepared by addition of [^{3}H]etoposide to the etoposide before the reaction with oleyl chloride. [^{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). The small lung carcinoma NCI-H292 cell line was purchased from Adolfo Lutz Institute (São Paulo, Brazil) and female isogenic mice B57BCL6 from Butantan Institute (São Paulo, Brazil).

Etoposide oleate and *cis*-etoposide oleate synthesis

Etoposide was modified following the method described by Lundberg (1994). Briefly, oleyl chloride, prepared using oxalyl chloride and oleic acid, was quickly added to a solution of etoposide and triethylamine in dry acetonitrile. The mixture was stirred at room temperature for 45 min and then purified.

cis-Etoposide oleate was prepared by a modification of a known procedure for the conversion of podophyllotoxin to picropodophyllotoxin (Strife & Jardine 1980). Etoposide oleate was converted to its *cis*-isomer by addition of sodium acetate in ethanol (75 °C). The product was then extracted and purified.

^{1}H and ^{13}C NMR used to characterize the structure of the products were performed using a Bruker DPX-300 instrument. The purity was confirmed by thin-layer chromatography (TLC), and high-performance liquid chromatography (HPLC) in a Shimadzu SPD-10AV.

Preparation of LDE, LDE-etoposide and LDE-etoposide oleate

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. Emulsification of lipids by prolonged ultrasonic irradiation in aqueous media and the procedure of two-step ultracentrifugation of the crude emulsion with density adjustment by addition of KBr to obtain LDE microemulsion was carried out by the method of Ginsburg et al (1982) modified by Maranhão et al (1993). LDE was dialysed and passed through a 0.22-μm pore filter. Etoposide and etoposide oleate were associated to LDE by solubilizing 6.0 mg etoposide or etoposide oleate powder in 10% final volume ethanol and by addition of 1.0 mL of emulsion to the etoposide or etoposide oleate in ethanol solution. The solution was then sonicated for 40 min at 55 °C using a Branson Sonifier 450 (Danbury, CT).

Separation of non-associated etoposide or etoposide oleate

Separation of non-associated etoposide or etoposide oleate was performed according to two different procedures. In the first, 200-μL samples were passed through Stracta cartridges (Phenomenex, CA), eluted with 3.0 mL of PBS and collected in a tube containing 100 μL of 20% (v/v) Triton X-100 in water. The non-associated drug was eluted with 2×1.5 mL methanol (Bellott et al 2001). The LDE-etoposide or LDE-etoposide oleate fractions recovered in Triton X-100 were extracted with 3×2.0 mL chloroform. The organic phase was concentrated and reconstituted in 1.0 mL methanol before analysis.

In the second procedure, the solutions were centrifuged at 3000 rev min^{-1} for 35 min. After this procedure the LDE-etoposide or etoposide oleate fraction was found in the supernatant while non-associated etoposide or etoposide oleate precipitated.

The LDE-associated and the non-associated drug fractions obtained by the procedures described above were quantified either by radioactive labelling with addition of 2.5 μCi [^{3}H]etoposide oleate or by HPLC. The drug chemical stability was analysed by HPLC.

Calibration curve for etoposide and etoposide oleate

HPLC analysis of etoposide, *cis*-etoposide, etoposide oleate and *cis*-etoposide oleate was performed using a Shimadzu Model SPD-10AV instrument equipped with a UV detector at 229 nm, 15 cm $\times$ 4.6 mm i.d. shim-pack column CLC-ODS (M) C_{18} (Shimadzu, Columbia, ML)

and a 4 cm × 10 mm i.d. pre-column with shim-pack CLC-ODS (M) C_{18} (Shimadzu, Columbia, ML). Etoposide and etoposide oleate were dissolved in methanol in concentrations ranging from 0.025 to 1.0 $\mu g\,\mu L^{-1}$. Standard curves were generated by plotting the peak area of etoposide or etoposide oleate in the UV detector against the drug concentration tested.

The *cis*-etoposide and *cis*-etoposide oleate were quantified by the relationship between the percent area of *trans*-etoposide or *trans*-etoposide oleate and their respective *cis* form (Zou et al 2001). The samples were analysed in duplicate by HPLC using an isocratic solvent system consisting of water–acetonitrile–acetic acid (68:30:2) at a flow-rate of 1.0 mL min^{-1}. All solvents were clarified with a 0.5 μm MF-Millipore filter (Millipore Co., MA).

LDE-etoposide oleate particle size

The size of LDE-etoposide oleate and LDE particles were measured using a Zeta Potential Analyzer (Brookhaven Instruments, Holtsville, NY). All samples were maintained at 4 °C and were diluted and filtered immediately before diameter measurement.

Stability of LDE-etoposide oleate

Double-labelled [^{14}C]LDE-[^{3}H]etoposide oleate was dialysed against plasma to evaluate the dissociation of drug from the emulsion particle. The experiment was performed with 1.0 mL of [^{14}C]LDE-[^{3}H]etoposide oleate into a dialysis tubing (Seamless cellulose tubing; Sigma Chemical Co., St Louis, MO) into a tube containing 20 mL of plasma under magnetic stirring, for 24 h at 37 °C. Samples (5 μL) of [^{14}C]LDE-[^{3}H]etoposide oleate were collected at 0.5, 1, 2, 3, 4, 5, 6, 20 and 24 h. The radioactivity was measured in a liquid scintillation solution.

Cell growth inhibition experiments

The small-cell lung carcinoma cells (NCI-H292) were grown in RPMI 1640 medium supplemented with streptomycin (50 $\mu g\,mL^{-1}$), penicillin (50 IU mL^{-1}) and 10% (v/v) foetal calf serum (FCS). Cells were maintained at 37 °C in a humidified incubator with 5% (v/v) CO_2. For the experiments, the cells were detached by trypsin treatment and distributed into 96-well culture plates. After 24 h incubation, LDE-etoposide oleate, etoposide oleate (dissolved in the same vehicle as commercial etoposide) and LDE-etoposide oleate with vehicle and their respective controls (commercial etoposide, LDE and vehicle) were added to the incubated cells. The final drugs concentrations were 0.03–0.6 mM. After 48 h incubation the medium was removed and the number of living tumour cells were determined by the colorimetric MTT assay (Ruben & Neubauer 1987). The cell viability of each well was expressed as survival index.

The 50% inhibitory concentration (IC50) was determined as the drug concentration required to inhibit 50% of the cell growth.

LDE-etoposide oleate uptake by neoplastic cells

The cellular uptake of etoposide oleate was determined by incubation of NCI-H292 cells with increasing amounts of [^{14}C]LDE-[^{3}H]etoposide oleate. Cells (10^6/mL) were plated on 35-mm Petri dishes. After 24 h incubation with 10% lipoprotein-deficient serum (LPDS), increasing amounts of LDE-etoposide oleate labelled with [^{14}C]cholesteryl oleate and [^{3}H]etoposide oleate (0.1 μCi each) were added to the plates for 4 h incubation at 37 °C. The cells were then washed three times with cold PBS plus BSA and twice with PBS at 37 °C, harvested and centrifuged at 14 000 rev min^{-1} for 15 min and 500 μL of NaOH 0.5 M was added to the pellet to disrupt the cells. The radioactivity was measured in a liquid scintillation solution.

Competition between [^{14}C]LDE-[^{3}H]etoposide oleate and native LDL

NCI-H292 viable cells (10^6) were incubated for 24 h in RPMI 1640 enriched with 10% LPDS. After this period, 80 μg of LDE labelled with [^{14}C]cholesteryl oleate and [^{3}H]etoposide oleate and increasing amounts of human LDL were added to the plates in duplicate and incubated for 4 h at 37 °C. The cells were washed, harvested and the radioactivity was measured as above.

Toxicity to mice of LDE-etoposide oleate

Acute dose toxicity experiments were performed using C57BCL6 mice weighing roughly 20 g. LDE-etoposide oleate and commercial etoposide were administered intraperitoneally in a single dose or multiple doses of 100–500 mg kg^{-1} and 25–150 mg kg^{-1}, respectively, and LDE control was also administered. The mice were allocated to groups of five and LDE-etoposide oleate doses up to 200 mg kg^{-1} were given in a two-day schedule. Observation of survival and weight changes were taken daily over a 60-day period. Lethal doses (LD) were determined by simple interpolation. Studies were approved by the Animal Ethics Committee of the University of São Paulo.

Statistical analysis

The IC50 data, expressed as mean ± standard error of the mean, were analysed for statistically significant differences by the Kruskal–Wallis test followed by the post-hoc Dunn's test. The curve points of the dialysis experiments were compared by one-way analysis of variance. For the other data, Mann–Whitney *U*-test was used. In all analyses, difference of $P < 0.05$ was considered statistically significant.

Results

Association of etoposide with LDE

Only about 50% of the amount of etoposide that was incubated under ultrasonic irradiation with LDE became associated with the emulsion. Due to this low association yield,

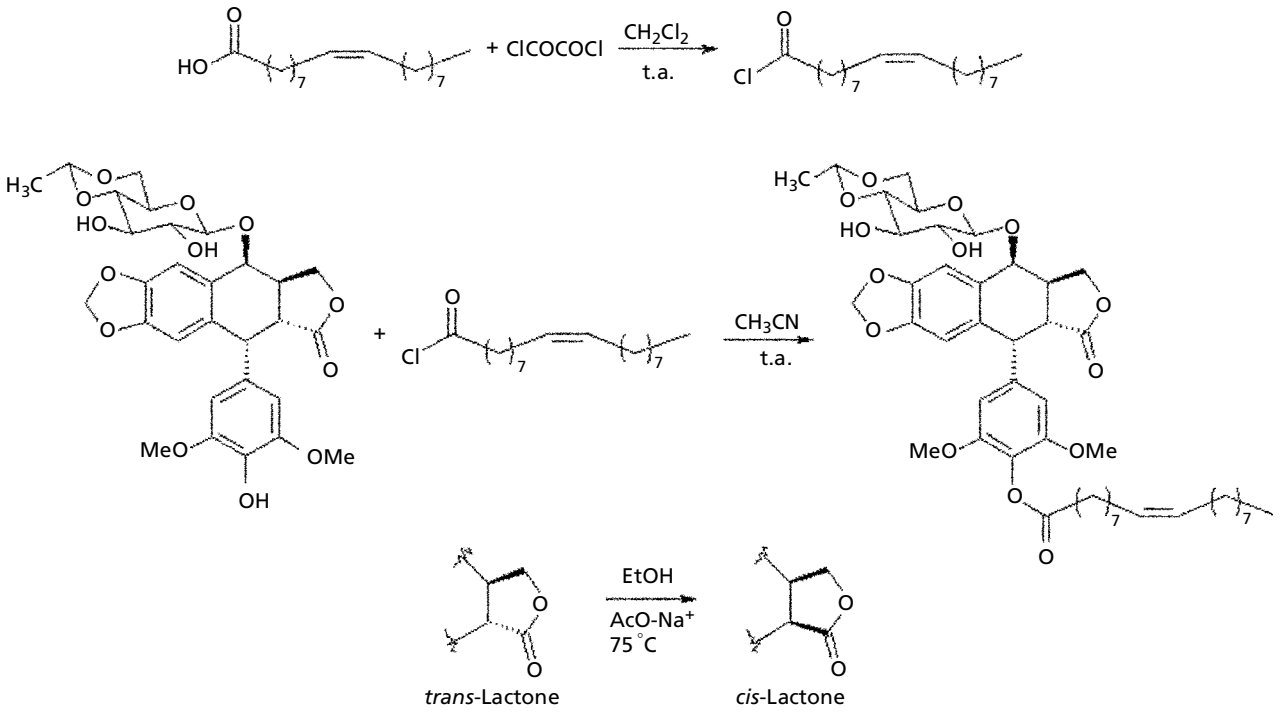

Figure 1 Synthesis of etoposide oleate and isomerization of *trans*- to *cis*-lactone of etoposide oleate.

we proceeded to a modification of the etoposide molecule, aiming to increase its lipophilicity. An oleyl group was attached to etoposide by a sterification reaction following the general scheme shown in Figure 1. This reaction produced 85% etoposide oleate. With the strategy described above, when etoposide oleate was associated with LDE, under the same conditions used for non-esterified etoposide, the yield of the association increased to nearly 98%.

As evaluated by HPLC, the association with LDE strongly protected the isomerization of *trans*-etoposide oleate. Compared with the approximately 50% conversion to the inactive *cis* form measured with the non-associated drug, when the drug was associated with the emulsion at most 3% of the *cis* form was found (Figure 2).

Regarding the physical status of the emulsion particles, the association with etoposide oleate resulted in a 40% increase in the particle diameter that reached about 65 nm, as monitored by light scattering. The LDE-etoposide preparation is perfectly suitable for injection into the bloodstream.

Figure 3 shows the in-vitro kinetics of the dissociation of etoposide oleate from LDE against plasma. In this experiment, performed over 24 h, different proportions of LDE to drug (w/w) were tested. It is apparent that most of the dissociation occurred over the first 5 h of the experiment and thereafter the dissociation pronouncedly decreased. The ratio of drug dissociation was 55% for the proportion of mass LDE:drug 5:1, 47% for 10:1 and 60% for 20:1, in the first 5 h, but the differences between the curves were not statistically significant.

Cell growth inhibition

Figure 4 shows the cytostatic activity against NCI-H292 tumour cell line of etoposide and etoposide oleate, both in the commercial etoposide vehicle and LDE-etoposide oleate. When etoposide oleate was associated with LDE its IC50 value was higher than that for etoposide oleate ($P = 0.0043$) and etoposide ($P = 0.0038$). However, when the etoposide oleate and etoposide dose–response curves are compared, it is clear that the chemical modification of the drug does not affect its cytostatic activity ($P = 0.7648$).

The vehicle of the commercial etoposide had a strong cytotoxic activity per-se, in contrast with LDE alone, which was harmless to the cells (Figure 5). This assumption is confirmed in Figure 6 where it is shown that the addition of the commercial etoposide vehicle further increases the cytotoxicity of LDE-etoposide oleate ($P = 0.9439$). Therefore, the greater cytostatic activity of commercial etoposide compared with LDE-etoposide oleate can be ascribed to the vehicle. Table 1 shows the IC50 values calculated for the different formulations of the drug. The LDE-etoposide oleate IC50 was roughly half that of the commercial etoposide and of the etoposide oleate with the vehicle of the commercial formulation. It can also be noted that the difference in cytostatic activity between the LDE-etoposide oleate and the commercial etoposide was abolished when the vehicle of the commercial preparation was added to the incubates of LDE-etoposide oleate with the cells.

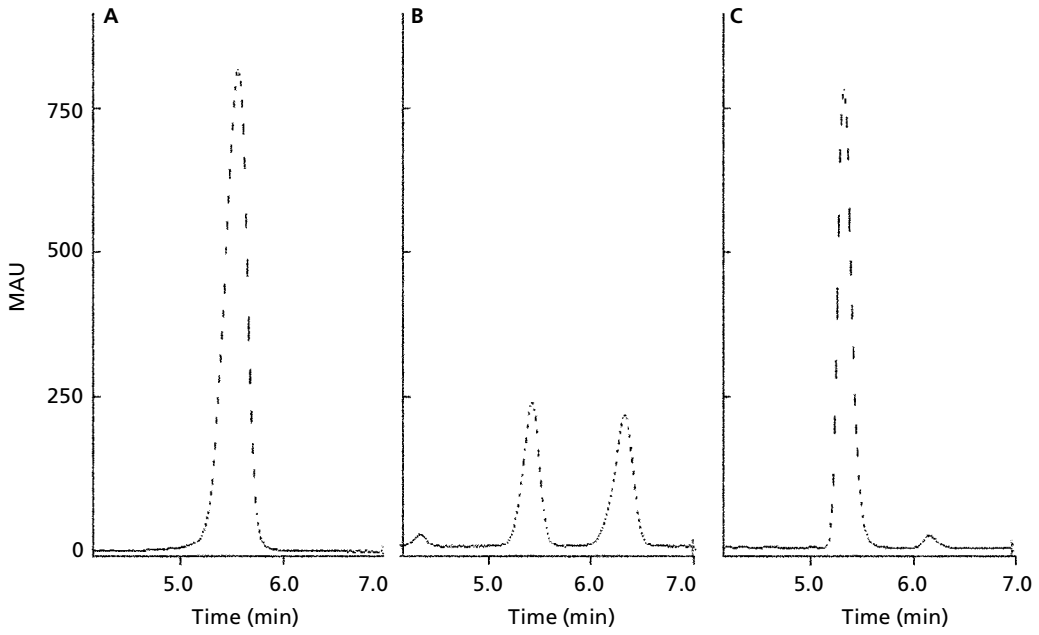

Figure 2 HPLC analysis spectra of *trans*-etoposide oleate used as a standard (5.56 min) (A), etoposide oleate dissociated from the emulsion, approximately 1:1 (w/w) *trans*- and *cis*-etoposide oleate (5.5 and 6.3 min, respectively) (B) and etoposide oleate associated with the emulsion (*trans*-:*cis*-etoposide oleate at ratio 97:3) (C).

LDE-etoposide oleate uptake by neoplastic cells

The uptake of LDE alone labelled with [^{14}C]cholesteryl oleate by the NCI-H292 cells was proportional to the mass of the microemulsion incubated with the cells until it reached a plateau at 7.6 mg mL^{-1} (Figure 7). Figure 8 shows the uptake by the cells of LDE associated with etoposide oleate labelled with both [^{14}C]cholesteryl oleate and [^{3}H]etoposide oleate. It is apparent that the saturation curve of LDE-etoposide oleate is not different to that of LDE alone ($P = 0.5166$). This indicates that the addition of the drug does not affect the interaction of the microemulsion with the cell uptake mechanisms and that the drug is taken up by the cells in conjunction with LDE. It is also clear that the uptake curves of the [^{14}C]cholesteryl oleate and of the [^{3}H]etoposide oleate of the emulsion-drug association are identical (Figure 8), indicating that both labels are simultaneously taken up by the neoplastic cells ($P = 0.5166$).

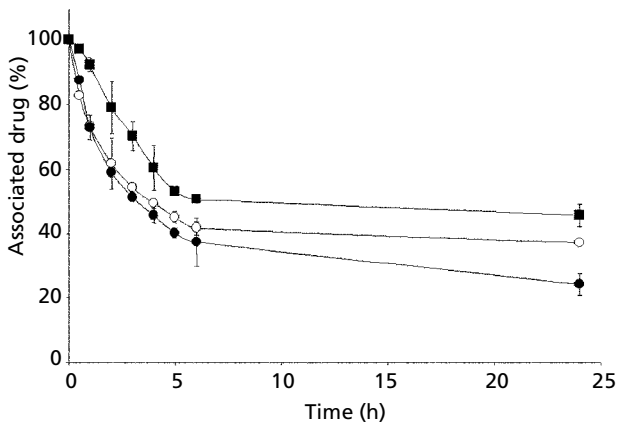

Figure 3 Dialysis of 1.0 mL of LDE-etoposide oleate against 20 mL of human plasma. [^{14}C]LDE-[^{3}H]etoposide oleate was prepared with different ratio (5:1 (○), 10:1 (■) and 20:1 (●)) of total mass of lipids:mass of drug (each 1.0 mL of emulsion contains 30 mg of total lipids). Results are presented means ± s.e.m. of three experiments performed in triplicate.

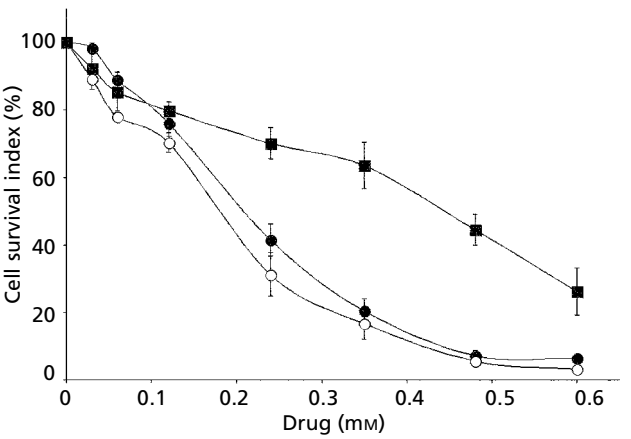

Figure 4 Effects on NCI-H292 cell survival of increasing amounts of commercial etoposide (●), etoposide oleate dissolved in the same vehicle of commercial preparation (○) and LDE-etoposide oleate (■). Drug concentration varies from 0.03 to 0.6 mM. Cells were incubated for 48 h at 37 °C. Results are presented as means ± s.e.m. of three experiments performed in triplicate.

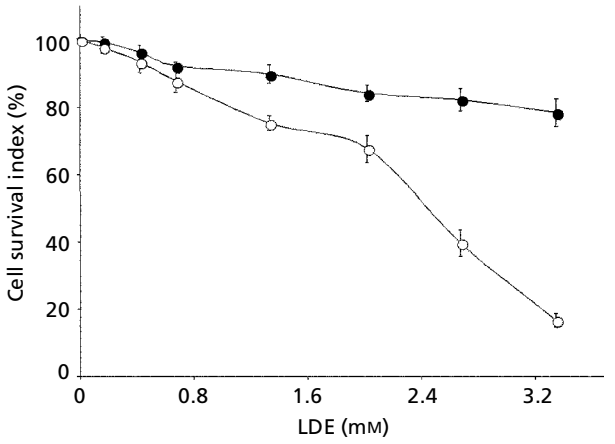

Figure 5 Effects on NCI-H292 cell survival of increasing amounts of LDE (●) and etoposide vehicle (○). The amount of LDE and vehicle corresponded to those of the association LDE-etoposide and commercial preparation, respectively. Cells were incubated for 48 h at 37 °C. Results are presented as means ± s.e.m. of three experiments performed in triplicate.

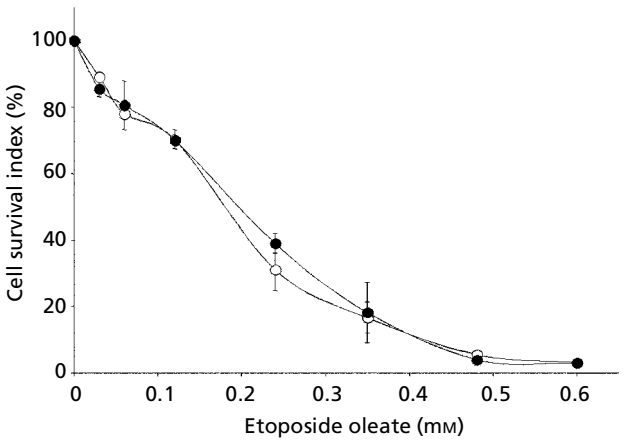

Figure 6 Effects on NCI-H292 cell survival of increasing amounts of etoposide oleate in etoposide vehicle (○) and LDE-etoposide oleate plus etoposide vehicle (●). Drug concentration varies from 0.03 to 0.6 mM. The amount of LDE and vehicle corresponded to those of the association LDE-etoposide and commercial preparation, respectively. Cells were incubated for 48 h at 37 °C. Results are presented as means ± s.e.m. of three experiments performed in triplicate.

Competition between [^{14}C]LDE-[^{3}H]etoposide oleate and native LDL

Because the uptake of LDE-etoposide oleate label decreased as it was challenged by increased amounts of native LDL in the incubates (Figure 9), it can be stated that LDE-etoposide oleate and the native lipoprotein compete for the same receptor uptake mechanisms.

Toxicity to mice of LDE-etoposide oleate

Table 2 shows the results of the acute toxicity experiments with LDE-etoposide oleate and commercial etoposide performed in mice. The association with LDE led to a remarkable reduction of the drug toxicity, estimated by the LDE in the experimental mice groups (LDE-etoposide oleate $LD50 = 315\,mg\,kg^{-1}$; commercial etoposide $LD50 = 58\,mg\,kg^{-1}$). LDE alone was used as a control; to attain the LD10, LD50 and LD90, very large volumes of the emulsion preparation were administered, so that the death rate could be attributed to hypervolaemia rather than to toxicity of the emulsion lipids.

Table 1 Cytostatic effects upon the NCI-H292 neoplastic cell line of LDE-etoposide oleate, commercial etoposide, etoposide oleate and LDE-etoposide oleate in the commercial vehicle, as well as the effects of LDE and the commercial vehicle as used without drugs.

Preparation tested	IC50 (mM)
LDE-etoposide oleate	0.48 (0.07)
Commercial etoposide	0.18 (0.02)
Etoposide oleate + vehicle of the commercial preparation	0.19 (0.01)
LDE-etoposide oleate + vehicle of the commercial preparation	0.19 (0.03)
LDE alone[a]	> 3.5
Vehicle of the commercial preparation alone[a]	2.5 (0.05)

IC50 = dose producing 50% inhibition of cell growth. Values are means of at least three experiments performed in triplicate with s.e.m. in brackets. [a]Etoposide-free solutions – the same LDE-etoposide oleate and commercial etoposide formulation without the drug.

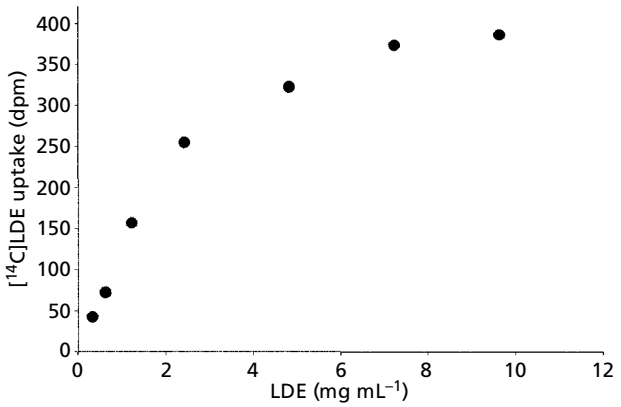

Figure 7 Uptake by NCI-H292 of LDE labelled with [^{14}C]cholesteryl ester. Cells were incubated with increasing amounts of LDE or LDE-etoposide oleate for 4 h at 37 °C in 4 mL of RPMI. Results are presented as means ± s.e.m. of three experiments.

Discussion

In this study, it was shown that etoposide poorly associates with LDE, but with the addition of an oleyl group to the molecule to increase lipophilicity the association

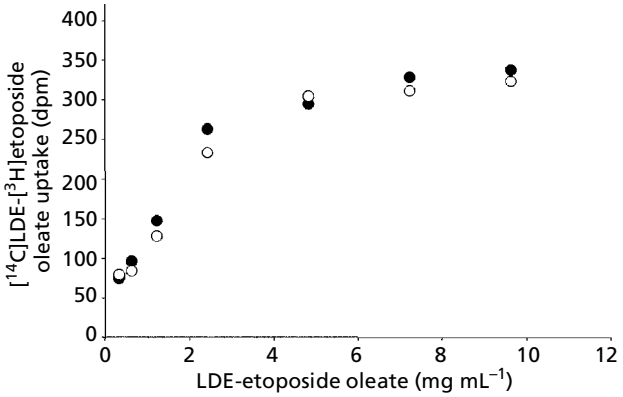

Figure 8 Uptake of LDE-etoposide oleate doubly labelled with [^{3}H]etoposide oleate (●) and [^{14}C]cholesteryl ester (○). Cells were incubated with increasing amounts of LDE or LDE-etoposide oleate for 4 h at 37 °C, in 4 mL of RPMI. Results are presented as means ± s.e.m. of three experiments.

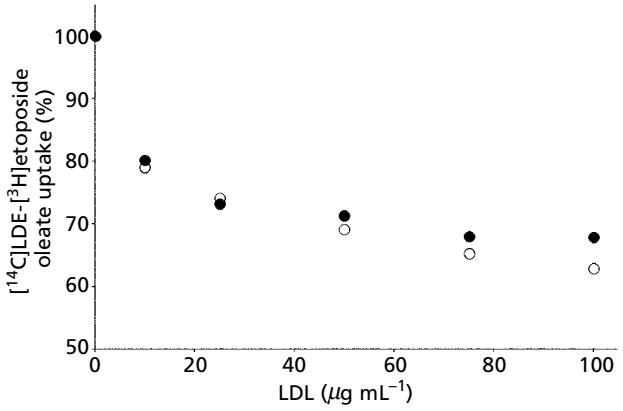

Figure 9 Effect of human LDL addition on the uptake by NCI-H292 of LDE-etoposide oleate labelled with [^{3}H]etoposide oleate (●) and [^{14}C]cholesteryl ester (○). Cells were incubated with 10 μg LDE-etoposide oleate doubly labelled with [^{3}H]etoposide oleate and [^{14}C]cholesteryl ester in increasing concentrations of human LDL, for 4 h at 37 °C, in 4 mL of RPMI. Results are presented as means ± s.e.m. of three experiments.

approaches 100% yield at a 5:1 total lipid-to-drug ratio. It was also shown that the pharmacological action of the transformed drug is preserved and the association LDE-etoposide oleate is stable. The strong lipophilicity of etoposide has been a major pharmaceutical challenge and the commercially used vehicles have often been implicated in hypotension, anaphylaxis, bronchospasm and other adverse effects (O'Dwyer et al 1985). In this regard, the LDE system offers a non-toxic vehicle for chemotherapeutic agents, as clearly shown in the in-vitro experiments performed here.

Another potentially advantageous aspect of using LDE as a vehicle for etoposide refers to the protective effect of the microemulsion, as documented here, against the conversion of this chiral drug to its inactive *cis*-lactone form. Although the presence of citric acid in the formulation of the commercial etoposide vehicle lowers the pH, thus preventing the conversion of the stored drug, when diluted in plasma this protection may vanish. In contrast, the emulsion constantly isolates the drug from the plasma until its final delivery into the cell cytoplasm. In fact, in a previous study it was observed that association with LDE protects carmustine against degradation (Maranhão et al 2002).

Table 2 Acute lethal toxicity in mice of LDE-etoposide oleate, commercial etoposide and LDE.

Drug/vehicle	LD10	LD50	LD90
LDE-etoposide oleate	183	315	464
Commercial etoposide	39	58	72
LDE[a]	400	> 420	> 420

LD10, LD50 and LD90 are the doses producing deaths of 10, 50 and 90% of mice, respectively. Doses are presented as mg kg^{-1} of body weight. [a]The amount of LDE was equal to that of LDE in LDE-etoposide oleate.

The marked reduction of etoposide cytotoxicity by the association with LDE was not achieved at the expense of pharmacological activity. In fact, it was shown that the greater cytotoxicity of the commercial preparation compared with that of LDE-etoposide oleate was clearly due to the cytotoxicity of the vehicle and not to the drug itself (Tsujino et al 1999). This was demonstrated in the experiments wherein the commercial vehicle was tested alone or added to the LDE-etoposide oleate incubates with the neoplastic cells. Similarly, in our previous study dealing with the incorporation of paclitaxel into LDE (Rodrigues et al 2002), the commercial vehicle Cremophor, that is cytotoxic, when added to LDE-paclitaxel in the incubates with the cells elicited a further increase in cytotoxicity. It is important to point out, however, that the cytotoxicity of the vehicle is restricted to the in-vitro experiments. The vehicle has no considerable in-vivo antitumoricidal effect.

In contrast with vehicles that only dissolve the drug in aqueous media, LDE is a consistent drug-targeting system that has ability to concentrate chemotherapeutic agents in neoplastic tissues. In this study, the in-vitro cell uptake and the competition experiments strongly suggest that the LDL receptor endocytosis is, indeed, the entry pathway of the drug into the cell when it is associated with LDE. It was previously documented by our group that LDE is taken-up five-fold and ten-fold more by breast carcinoma and ovarian carcinoma, respectively, compared with the normal breast and ovarian tissue. LDL receptor upregulation, the mechanism that allows the concentration of LDE in the tumour cells, was shown not only in those neoplasias but in several others, such as endometrial carcinoma, uterine carcinoma (Rudling et al 1986), gall bladder cancer (Ueyama et al 1990) and prostate carcinomas (Henriksson et al 1989). Therefore, LDE bears the advantage of being not

only a non-toxic vehicle but also a powerful device to selectively carry the drugs to their site of action. As shown here in the experiments with dialysis against plasma, most of the etoposide oleate does not dissociate from the microemulsion over time. Therefore, it can be assumed that most of the drug is retained in the microemulsion particles until its removal from the circulation and internalization by the cells.

Another important issue is the remarkable diminution of etoposide oleate toxicity when incorporated with LDE, as tested in mice. In fact, in drug carrier systems such as liposomes and other emulsion preparations (Souza et al 1993; Allen 1998) other than LDE, this drug side effect attenuation is frequently observed and is probably related to the new drug biodistribution created by the inclusion into those vehicles.

Conclusion

This study shows that etoposide oleate stably associates with LDE at almost 100% proportion, in the conditions used. LDE-etoposide oleate diminishes the toxicity but not the in-vitro therapeutic action of the drug. It is then a very promising preparation for the weaponry of anticancer chemotherapy and further studies are currently being undertaken in our laboratory.

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