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ORIGINAL ARTICLE

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Improvement of paclitaxel therapeutic index by derivatization and association to a cholesterol-rich microemulsion: in vitro and in vivo studies

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Abstract A cholesterol-rich microemulsion or nanoparticle termed LDE concentrates in cancer tissues after injection into the bloodstream. Here the cytotoxicity, pharmacokinetics, toxicity to animals and therapeutic action of a paclitaxel lipophilic derivative associated to LDE is compared with those of the commercial paclitaxel. Results show that LDE-paclitaxel oleate is stable. The cytostatic activity of the drug in the complex is diminished compared with the commercial paclitaxel due to the cytotoxicity of the vehicle Cremophor EL used in the commercial formulation. Competition experiments in neoplastic cultured cells show that paclitaxel oleate and LDE are internalized together by the LDL receptor pathway. LDE-paclitaxel oleate arrests the G_2/M phase of cell cycle, similarly to commercial paclitaxel. Tolerability to mice is remarkable, such that the lethal dose (LD_{50}) was ninefold greater than that of the commercial formulation (LD_{50} = 326 μM and 37 μM, respectively). LDE concentrates paclitaxel oleate in the tumor roughly fourfold relative to the normal adjacent tissues. At equimolar doses, the association of paclitaxel oleate with LDE results in remarkable changes in the drug pharmacokinetic parameters when compared to commercial paclitaxel ($t_{1/2}$ = 218 min and 184 min, AUC = 1,334 μg h/ml and 707 μg h/ml and CL = 0.125 ml/min and 0.236 ml/min, respectively). Finally, the therapeutic efficacy of the complex is pronouncedly greater than that of the commercial paclitaxel, as indicated by the reduction in tumor growth, increase in survival rates and % cure of treated mice. In conclusion, LDE-paclitaxel oleate is a stable complex and compared with paclitaxel toxicity is considerably reduced and activity is enhanced, which may lead to improved therapeutic index in clinical use.

Keywords Nanoparticles · Paclitaxel · Emulsions · Cholesterol · Low-density lipoprotein receptors · Cancer treatment · Drug targeting

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Introduction

After being injected into the blood stream, a cholesterol-rich microemulsion or nanoparticle termed LDE is taken up by the low-density lipoprotein (LDL) receptors in the plasma membrane and is internalized into the cells. We previously showed that LDE can concentrate in leukemia cells or in solid tumors such as ovarian and breast carcinomas [1, 9, 16] that overexpress those receptors. Therefore, LDE may serve as a vehicle to chemotherapeutic agents directed against neoplastic tissues. Recently, we reported that the antineoplastic agent paclitaxel can be associated at high rates with LDE without loss of cytotoxicity [21]. However, we subsequently observed that the LDE-paclitaxel complex tends to dissociate in the bloodstream after injection into mice (unpublished data).

The most remarkable clinical antitumor activity of paclitaxel has been in advanced ovarian and breast cancers, resulting in the worldwide regulatory approval of the agent in both cancers. The drug is commercially available as a 1/1 mixture with a derivative castor oil named Cremophor EL and ethanol which is rarely

responsible for severe life-threatening hypersensitivity reactions in patients [27]. Because LDE is atoxic and has a further drug targeting ability demonstrated in either ovarian or breast carcinomas, it should be an attractive candidate vehicle to improve the paclitaxel therapeutic index.

An usual strategy to render chemotherapeutic agents more stable when associated to LDL or artificial lipid emulsions has been to attach lipophilic groups to their molecules. In this respect, Lundberg et al. [14] have recently shown that a lipophilic derivative of paclitaxel in a lipid o/w emulsion covered with a hydrophilic polymer improved the pharmacokinetic parameters of paclitaxel. On the other hand, we showed that the stability of the LDE-etoposide complex is also improved when this antineoplastic agent is attached with an oleoyl group [24].

In this study, the modification strategy was attempted to enhance the stability of the LDE-paclitaxel oleate complex by attaching an oleoyl group to the drug. Compared with commercial paclitaxel, the resulting preparation showed higher therapeutic index that was achieved by diminution of the toxicity and increase of the anticancer action.

Materials and methods

Materials

Crystalline paclitaxel was purchased from Calbiochem (CA) dimethlysulfoxide (DMSO), 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), derivative of castor oil (Cremophor EL), triolein, cholesteryl oleate, cholesterol, oleic acid, ethyl acetate, chloroform, triethylamine, dichloromethane, and phosphatidylcholine were purchased from Sigma (St Louis, MO), methanol and acetonitrile from Merck (Darmstadt, Germany). [3H]-paclitaxel with the tritium at the *m*-position and *p*-position of the aromatic ring, at the 10′-position and 2′-position of the taxane ring and 3′-position of the side chain was purchased from Moravek (Brea, CA). The clinically approved formulation of paclitaxel, Taxol was obtained from Bristol-Myers Squibb Co (1 ml contains 6 mg of paclitaxel, 527 mg of Cremophor EL, 49.7% v/v dehydrated alcohol, USP). The human small lung cell carcinoma NCI H292 cell line was purchased from Adolfo Lutz Institute (São Paulo, Brazil). 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.

Paclitaxel oleate synthesis and characterization

Paclitaxel was modified following the method described by Lundberg [14]. Briefly, oleoyl chloride, prepared by use of oxalyl chloride and oleic acid, was quickly added to a solution of paclitaxel and triethylamine in dry acetonitrile. The mixture was stirred at room temperature for 45 min and the product was then extracted and purified. When necessary, trace amounts of [^{3}H]-paclitaxel were added to the initial solution. The purity and the structure of the product were characterized by [^{1}H] and [^{13}C] NMR using a Bruker DPX-300 instrument and by liquid chromatography coupled to tandem mass spectrometry with electrospray ionization (LC-ESI/MS/MS) using a Shimadzu Model LC-10AD high-pressure liquid chromatograph (HPLC) equipped with a UV detector at 227 nm, 15 cm × 4.6 mm Luna C8 [2] (Phenomenex, Torrance, CA), and a 4 cm × 10 mm I.D. pre-column with shim-pack CLC-ODS (M) C_{18} (Shimadzu, Columbia, ML) coupled to a mass spectrometer triple quadrupole Quattro II (Micromass, Manchester, UK). The LC–MS–MS experiments were performed while scanning from m/z 500 to 1,300 at a scan rate of 2 s/scan. The samples were analyzed in duplicate by HPLC using an isocratic solvent system constituted of acetonitrile–methanol (90:10) at a flow-rate of 1.0 ml/min.

Preparation of LDE

In brief, LDE was prepared from a lipid mixture composed of 40 mg cholesteryl oleate, 20 mg egg phosphaditylcholine, 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 described previously [7] modified by Maranhão [15]. LDE was dialyzed against saline solution and passed through 0.22 μm filter for the experiments. When necessary, trace amounts of [^{14}C]-cholesteryl ester were added to the initial solution.

Association of paclitaxel oleate to LDE

Paclitaxel oleate was incorporated into LDE by solubilization of paclitaxel oleate in ethanol and by adding it into the emulsion. The solution was then sonicated for 30 min at 70°C using a Branson Sonifier 450 (Danbury, CT), equipped with a 1 cm flat titanium probe. LDE-paclitaxel oleate was centrifuged at 3,000 rpm for 15 min to separate the unbound paclitaxel oleate. LDE-paclitaxel oleate was then passed through 0.22 μm pore polycarbonate filter and kept at 4°C until it was used. When necessary [^{3}H]-paclitaxel oleate were added to the initial solution. The yield of each batch was assayed before the use. LDE-paclitaxel oleate was always prepared at the same day of the experiments.

Stability of the drug-LDE complex in vitro

Stability of LDE-paclitaxel oleate was tested by membrane dialysis (12,000 MW cut-off) against human plasma and Tris–HCl solution pH 7.4. One milliliter of labeled [^{14}C]-cholesteryl oleate-LDE:[^{3}H]-paclitaxel oleate was dialyzed against 20 ml of plasma and Tris–HCl solution. Samples of 5 μl were collected from the dialysis bag at 0.03–168 h interval and placed separately into vials with 7 ml scintillation solution "Hisafe" (Perkin Elmer, Loughborough, England). The radioactivity was measured by liquid scintillation spectrometry with a Packard 1600 TR model Liquid Scintillation Analyzer.

Incubation of LDE-paclitaxel oleate with human plasma

[^{14}C]-cholesteryl oleate-LDE:[^{3}H]-paclitaxel oleate was incubated with human plasma for 1 h at 37°C. Following incubation, plasma was separated in its lipoprotein and lipoprotein-deficient plasma (LPDP) fractions by density gradient ultracentrifugation and the percentage of the LDE and of the paclitaxel oleate label recovered in each fraction was determined by radioactivity counting as previously described.

Cell growth inhibition

The NCI H292 cells were maintained in RPMI 1640 medium supplemented with antibiotics and 10% (v/v) fetal calf serum (FCS) at 37°C in a humidified incubator with 5% (v/v) CO_2. Prior the experiments, the cells were harvested from the culture and distributed into 96-well culture plates at 10^5 cells/well. After 24 h incubation, serial dilutions of a Cremophor/EL stock solution of paclitaxel oleate and paclitaxel and LDE-paclitaxel oleate were added to the wells in triplicate. The final concentrations of paclitaxel or paclitaxel oleate (0.003–3 μM) and LDE-paclitaxel oleate (0.0001–10 μM) were used. The cells were left in the incubator for further 72 h, at the end of this period the medium was removed and the number of living tumor cells were determined by the colorimetric MTT assay. The 50% inhibitory concentration (IC_{50}) was determined as the drug concentration required to inhibit 50% of the cell growth.

LDE-paclitaxel oleate uptake by neoplastic cells

The cellular uptake of paclitaxel oleate was determined by incubation of NCI H292 cells with increasing amounts of [^{14}C]-cholesteryl oleate-LDE-[^{3}H]-paclitaxel oleate. 10^6 viable cells were platted on 35 mm Petri dishes. On the next day, the medium was replaced by other containing 10% lipoprotein-deficient serum (LPDS). On the third day, increasing amounts of [^{14}C]-cholesteryl oleate-LDE-[^{3}H]-paclitaxel oleate (0.005–400 μg/ml) were added to the plates in duplicate and incubated for 4 h 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 rpm for 15 min and 200 μl NaOH 0.1 M was added to the pellet to disrupt the cell pellet under vortex mixing. The samples were placed separately into vials with scintillation solution and the radioactivity was measured by liquid scintillation spectrometry.

Competition between LDE-paclitaxel oleate and native LDL

NCI H292 viable cells (10^6) were incubated during 24 h in RPMI 1640 containing antibiotics, supplemented with 10% LPDS. After this period, 200 μg/ml of [^{14}C]-cholesteryl oleate-LDE-[^{3}H]-paclitaxel oleate and increasing amounts of human LDL (50–400 μg/ml) were added to the plates in duplicate and incubated for 4 h 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 rpm for 15 min; 200 μl of NaOH 0.1 M were added to the pellet to disrupt the cell pellet under vortex mixing before radioactivity measurement.

LDE-paclitaxel oleate toxicity to mice

Toxicity experiments were performed using groups of five C57BL/6J mice weighing roughly 20 g. LDE-paclitaxel oleate or commercial paclitaxel were administered i.p. in a single or multiple doses ranging from 90 μM/kg to 450 μM/kg and 12 μM/kg to 120 μM/kg, respectively. The same volume of LDE alone and Cremophor EL/ethanol were also administered as a control. Survival and weight changes were observed daily over a 60-day period. Lethal doses (LD) were determined by simple interpolation. Rapid death occurring after bolus administration was rejected, only that manifested more slowly, as a result of drug effects on GI and/or bone marrow was measured. The maximum tolerated dose (MTD) was defined as the allowance of a median body weight loss of 15% of the control and causes neither death due toxic effects nor remarkable changes in the general signs within 1 week after administration. All the animal experiments reported in this study were approved by the Animal Ethics Committee of the University of São Paulo.

Inoculation of the B16 melanoma cells in mice

Murine B16F10 (H2b) variant 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% FCS, 2 mM-L-glutamine, 1 mM sodium pyruvate and antibiotics. Cell suspensions 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

tumor transfer 5×10^4 cells suspended in 100 µl of PBS were injected subcutaneously into the flank regions of mice. Ten days after inoculation the tumors became macroscopically apparent.

Immunohistochemistry for LDL receptors in the B16 melanoma cells

The presence of LDL receptors in the melanoma cell lineage that was inoculated in mice was documented by the immunoperoxidase technique. In summary, silane-coated glass microscope smears used in the preparation of melanoma cell film were immediately fixed in 95% ethanol for 10 min and equilibrated in PBS prior to immunohistochemistry. The procedure involves the application to cell smears of the following antibody sequence: mouse IgG2ak (LP02) antihuman LDL receptor at 2.5 µg/ml for 18 h at 4°C; DAKO LSAB^R System (Carpinteria, USA), followed by biotinylated link antibody and streptavidin-HRP, for 30 min each. After each step the smears were washed in PBS (pH 7.6) and, finally, the peroxidase activity was revealed using diaminobenzidine (40 mg/100 ml) and H_2O_2 (6 µl/ml) [(substrate-chromogen solution)] by 5–10 min. Smears were counterstained with haematoxylin solution. A positive tissue control for the LDL receptor was provided by performing the immunoperoxidase reaction in human liver sections. Negative control smears were performed in the same cell line by incubating an adjacent section with an irrelevant murine IgG monoclonal antibody.

Flow cytometry DNA analysis

B16 melanoma cells were incubated with 0.05 µM LDE-paclitaxel oleate, commercial paclitaxel and controls for 24 h. Following the treatment, the cells were harvested, washed with PBS and resuspended in 375 µl trypsin 0.03 g/l, 10 mM Tris (pH 8.0). After 15 min incubation at room temperature, the neutralization solution (tripsin inhibitor 0.5 g/l, RNase A 0.1 g/l and spermine 1.2 g/l) was added and incubation continued for 15 min. Pelleted cells were resuspended in 0.3 ml PBS and fixed by addition of ice-cold ethanol (70%). Prior to analysis, cells were incubated with 18 µg/ml 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). The DNA content in the cell cycle phases (G_0/G_1, S and G_2/M) was analyzed by the Cell-Quest software and by the Mod-fit software cell.

Biodistribution of LDE and LDE-paclitaxel oleate in B16 melanoma-bearing mice

One hundred microliter of LDE (1.2 kBq, 0.3 mg total lipids) labeled with [^{14}C]-cholesteryl oleate or LDE-paclitaxel oleate (1.2 kBq, 0.3 mg total lipids and 0.06 mg drug) labeled with [^{3}H]-paclitaxel oleate and [^{14}C]-cholesteryl oleate were injected i.p. as a single bolus in groups of five C57BL/6J mice. The animals were kept in individual cages for 18 h, when they were sacrificed and tissues samples of 200–250 mg of skin, tumor, liver, brain, spleen, lung, testicle, kidney, heart and intestine (animals treated with LDE alone) and skin, liver and tumor (animals treated with LDE-paclitaxel oleate) were collected and kept in cold saline solution prior to lipid extraction with chloroform/methanol (2:1 v/v). The extracted lipids and drug were concentrated and measured by liquid scintillation spectrometry.

Plasma kinetics of double-labeled LDE-paclitaxel oleate and commercial paclitaxel in mice

To verify whether the derivatization and association of paclitaxel to LDE would alter the pharmacokinetics of the commercially available drug, the pharmacokinetic profile of LDE-paclitaxel oleate and of commercial paclitaxel were determined in control mice. LDE-paclitaxel oleate labeled with [^{3}H]-paclitaxel oleate and [^{14}C]-cholesteryl oleate (100 µl total volume, 1.2 kBq, 0.3 mg total lipids and 0.06 mg drug) and commercial [^{3}H]-paclitaxel (0.08 mg) were injected as a single bolus into the retro-orbital venous plexus of five mice for determination of the plasma decaying curves. Blood samples were collected at pre-established intervals during 24 h. Plasma was separated by a 15 min centrifugation (3,000g) and the radioactivity was counted in a scintillation solution. The fractional clearance rate (FCR) of the [^{14}C]-cholesteryl oleate-LDE, [^{3}H]-paclitaxel oleate and [^{3}H]-paclitaxel was calculated according to the method described by Matthews [18], where 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 $y = \left(a_1\,e^{-b_1 t}\right) + \left(a_2\,e^{-b_2 t}\right)$ where y represents the radioactivity plasma decay.

The pharmacokinetic parameters of paclitaxel oleate associated with LDE and commercial paclitaxel were calculated using a multicompartmental model by means of a computer software (PK Solutions, Ashland, OH). The log plasma concentration versus time curves were fitted by biexponential equations and the half-life ($t_{1/2}$) calculated by dividing 0.693 by the rate constant for each phase. The total 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 was estimated graphically from trapezoidal total area measurements.

Antitumor activity

Mice were inoculated with B16 melanoma cells as described above. Treatments were started on day 11,

when the tumor implanted in the animals reached a 60–100 mm^3 volume. The mice were randomly allocated to groups of eight animals. On days 11, 14, 19 as counted from the initial inoculation day, each group was injected i.p. with one of the following preparations: LDE-paclitaxel oleate at two dose levels, 17.5 mg/kg and 70.3 mg/kg (equimolar doses with 15 mg/kg and 60 mg/kg of paclitaxel, respectively), commercial paclitaxel at 15 mg/kg; 0.9% saline solution or LDE alone. These last two were control groups.

The tumor sizes were measured three times a week using a calliper-like instrument during the experiment. The size measurement was converted to tumor weight by the equation: tumor weight = (length^2 × width)/2. The experiment was ended on day 34, but the survival of the animals in each experimental group was monitored over 180 days. The antitumor activity was assessed according to the guidelines established by the National Cancer Institute [19].

Statistical analysis

The differences in the cell survival and cell uptake curves were evaluated by the unpaired Student's *t* test. The ANOVA variance test was used to compare the results obtained from the flow cytometry DNA analysis and to compare the AUC obtained from the growth tumor inhibition curve. The Mann–Whitney test was used for FCR data analysis. All the values were expressed as means ± SEM. In all analysis, $p < 0.05$ was considered statistically significant. The survival time plotting (Kaplan–Meyer test) and survival comparison between groups were carried out using the Graph Pad Prism statistical software.

Results

Paclitaxel oleate synthesis, characterization and association to LDE

Figure 1 shows the full scan mass spectra of the paclitaxel oleate product ions $[M + H]^+$. The peaks of the core fragments appear at *m*/*z* 551 and at *m*/*z* 854 whereas the whole molecule peak appears at *m*/*z* 1,118. As estimated by HPLC, the yield of the esterification reaction was roughly 90%. The amount of paclitaxel oleate associated to LDE by co-sonication was 85%. It follows that each ml of the emulsion (30 mg of emulsion total lipids) solubilizes 4.8–5.4 mg paclitaxel oleate.

Stability of the LDE-paclitaxel oleate complex in vitro

Figure 2 shows the results of the experiment wherein the LDE-paclitaxel oleate was dialyzed for 168 h against either plasma or Tris buffer. It is clear that during the first 24 h, only negligible dissociation of the complex occurred regardless plasma or Tris buffer was used in the experiment. In the ensuing 24–168 h, however, there was some dissociation against plasma, which was less intense for the Tris solution.

Incubation of LDE-paclitaxel oleate with human plasma

After incubation with plasma, 97.8% of the LDE radioactive label and 98.4% of the paclitaxel oleate label were found at the lipoprotein-containing plasma fraction whereas 2.2% and 1.6% of the two labels, respectively, were found at the lipoprotein-deficient fraction. Therefore, only residual amounts of drug leaks out of

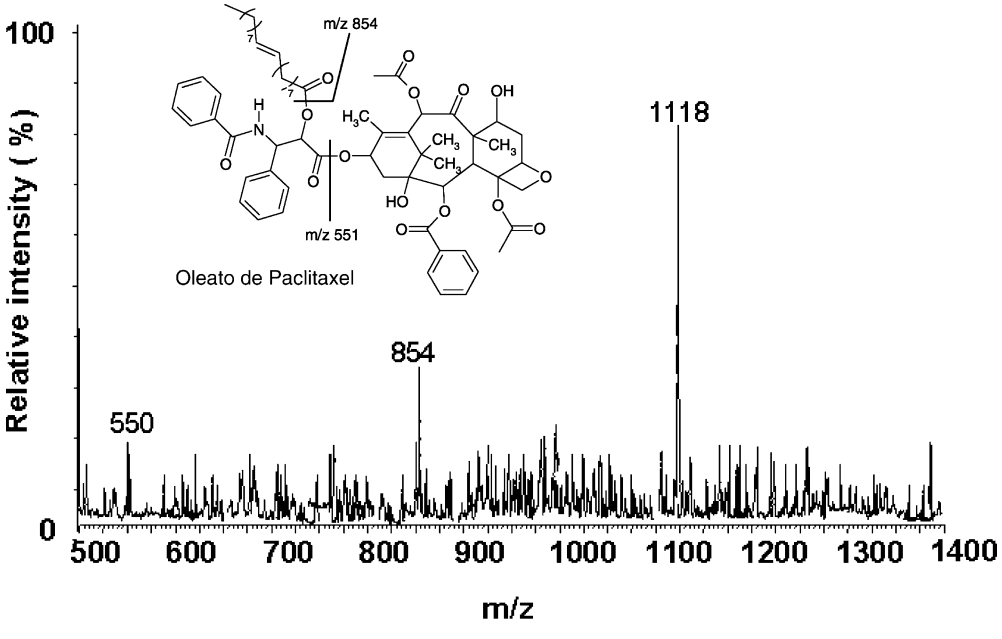

Fig. 1 Representative full scan chromatogram (*m*/*z* 500–1,400) of paclitaxel oleate analyzed by LC-ESI/MS/MS (liquid chromatography coupled to tandem mass spectrometry with electrospray ionization) at a scan rate of 2 s/scan. The samples were analysed in duplicate by HPLC using an isocratic solvent system constituted of acetonitrile–methanol (90:10) at a flow-rate of 1.0 ml/min

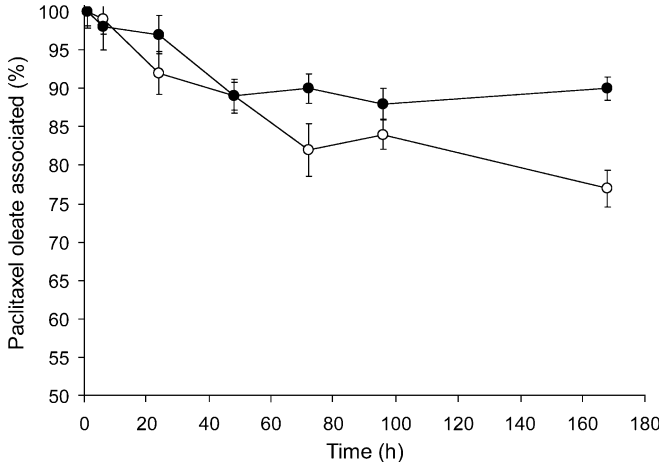

Fig. 2 Dialysis of 1.0 ml of LDE-paclitaxel oleate doubly labeled with [^{14}C] cholesteryl ester-LDE and [^{3}H]-paclitaxel oleate against 20 ml of human plasma (*open circle*) and Tris–HCl buffer (*filled circle*) at 0.03–168 h interval. The radioactivity were measured by liquid scintillation. Results are presented means ± SEM (bars) of three experiments

the microemulsion and is found in the fraction that contains mainly albumin, globulins and α-glycoprotein.

Cell growth inhibition

Figure 3a shows the dose–response curves of the cytostatic activity of paclitaxel oleate and of paclitaxel both using Cremophor as solubilizing agent. It is clear that the chemical modification of the drug does not affect the cytostatic activity of the drug ($p = 0.523$). The cytostatic index of paclitaxel oleate (IC_{50} = 0.11 μM) and paclitaxel (IC_{50} = 0.11 μM) calculated from the curves are similar.

Figure 3b shows the cytostatic activity curves of LDE-paclitaxel oleate, paclitaxel oleate solubilized in Cremophor and, finally of LDE-paclitaxel oleate with addition of Cremophor. This last experiment was performed to discriminate the additive cytotoxicity of Cremophor. LDE-paclitaxel oleate has a clear-cut lower cytotoxicity than paclitaxel oleate solubilized in Cremophor (IC_{50} = 1.00 μM and 0.09 μM, respectively, $p = 0.015$). However, when Cremophor is added to LDE-paclitaxel oleate incubates, the cytotoxicity increases (IC_{50} = 0.15 μM, $p = 0.756$), so that the IC_{50} approaches to that of paclitaxel oleate solubilized in Cremophor. Therefore, the greater cytotoxicity of the commercial formulation and of the paclitaxel oleate solubilized in Cremophor can be ascribed to the presence of Cremophor.

LDE-paclitaxel uptake by neoplastic cells

Figure 4a shows that when NCI H292 cells were incubated with increasing amounts of LDE-paclitaxel oleate labeled with [^{3}H]-paclitaxel oleate and [^{14}C]-cholesteryl

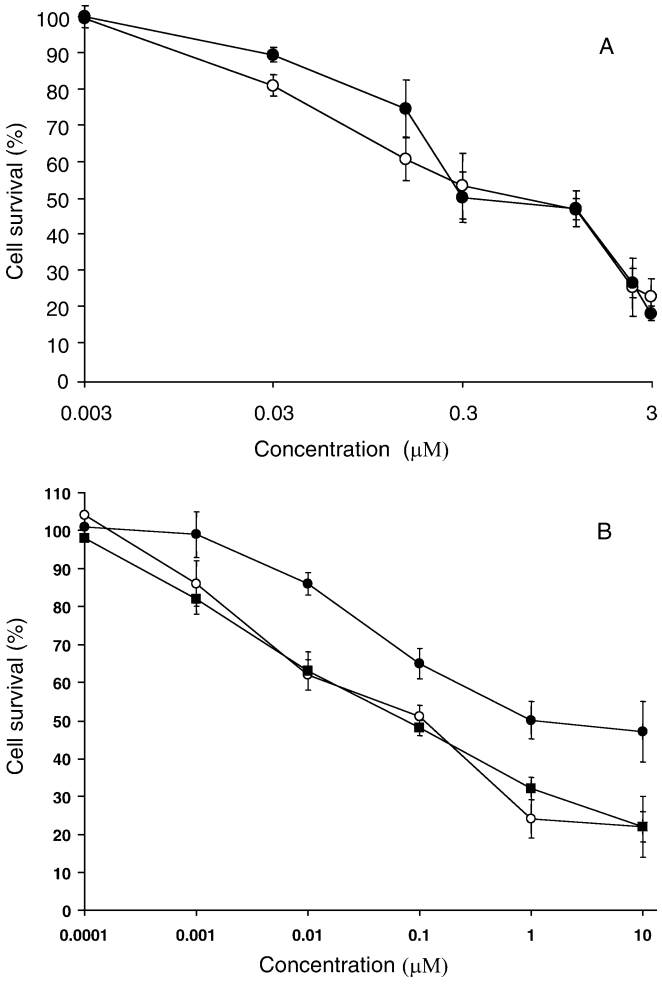

Fig. 3 Effects on NCI-H292 cells survival of serial dilutions of a Cremophor/EL stock solution of **a** paclitaxel oleate (*filled circle*) and paclitaxel (*open circle*). **b** Effects on survival of LDE-paclitaxel oleate with (*open circle*) or without (*filled circle*) the addition of Cremophor EL/ethanol and commercial paclitaxel (*filled square*). Cells were incubated for 72 h at 37°C. Results are presented as means ± SEM (bars) of three experiments performed in triplicate

oleate, there was a proportionally increasing uptake of the two labels and the two uptake curves are similar ($p = 0.2717$). This indicates that both paclitaxel and cholesteryl oleate components of the complex are simultaneously internalized into the cells.

Figure 4b shows that the addition of increasing amounts of native human LDL to the incubates of LDE-paclitaxel oleate and the malignant cells leads to a progressive diminution of cell uptake of both [^{3}H]-paclitaxel oleate and [^{14}C]-cholesteryl oleate contained in LDE. This indicates that when LDE uptake is diminished by the competition of native LDL the cell uptake of the drug associated to the microemulsion is also proportionally diminished.

LDE-paclitaxel oleate toxicity to mice

Table 1 shows the results of the toxicity experiments with LDE-paclitaxel oleate and commercial paclitaxel

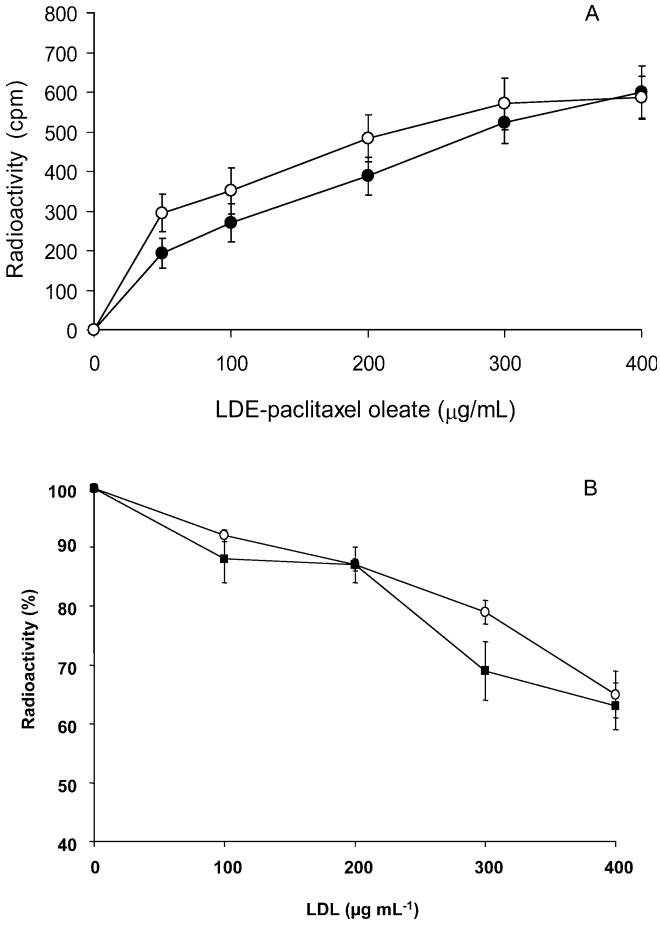

Fig. 4 Uptake by cultivated NCI-H292 cells of increasing amounts of **a** LDE-paclitaxel oleate doubly labeled with [^{14}C]-cholesteryl ester (*filled circle*) and [^{3}H]-paclitaxel oleate (*open circle*). **b** Effect of the addition of increasing amounts of human LDL (50–400 μg/ml) on the uptake by NCI H292 cells of LDE-paclitaxel oleate doubly labeled with [^{14}C]-cholesteryl ester (*filled square*) and [^{3}H]-paclitaxel oleate (*open circle*). The concentration of LDE-paclitaxel oleate was constant corresponding to 200 μg/ml. Cells were incubated for 4 h at 37°C. Results are presented as means ± SEM (bars) of three experiments performed in triplicate

performed in mice. The association with LDE lead to a remarkable reduction of the drug toxicity, compared to the commercial drug (LDE-paclitaxel oleate LD_{50} = 326 μM/kg; commercial paclitaxel LD_{50} = 37 μM/kg). When comparing the toxicity of both vehicles, Cremophor EL was found to be much more toxic than LDE. The estimated MTD of LDE-paclitaxel oleate was ten-fold higher than the commercial paclitaxel (178 μM/kg and 18 μM/kg, respectively).

Table 1 Lethal toxicity in mice of LDE-paclitaxel oleate, commercial paclitaxel and their respective vehicles

Formulation	Paclitaxel dose (μM/kg)			
	DL_{10}	DL_{50}	DL_{90}	MTD
LDE-paclitaxel oleate	290	373	>376	178
LDE[a]	400	>420	>420	ND[b]
Commercial paclitaxel	18	37.5	40	18
Cremophor EL[c]	34	76	89	ND[b]

[a]The volume of LDE injected was equal to that of the corresponding mass of LDE of LDE-paclitaxel oleate.
[b]Not measured.
[c]The volume of Cremophor EL injected was equal to that of corresponding mass of Cremophor EL of commercial paclitaxel.

Immunohistochemistry of LDL receptors in melanoma B16 cells

Figure 5 shows the presence of LDL receptors in B16F10 melanoma cells documented by the intense immunoperoxidase reaction observed in the upper micrograph. The micrograph below shows the negative reaction run in B16F10 cells through the incubation with an irrelevant murine monoclonal antibody.

Flow cytometry DNA analysis

To investigate whether G_2/M arrest can be induced by LDE-paclitaxel oleate, 24 h incubates of the complex or the commercial paclitaxel formulation with B16F10 cells were performed. Those experiments were performed twice and in triplicates. Figure 6 shows that within the first 24 h incubation, LDE-paclitaxel oleate increased

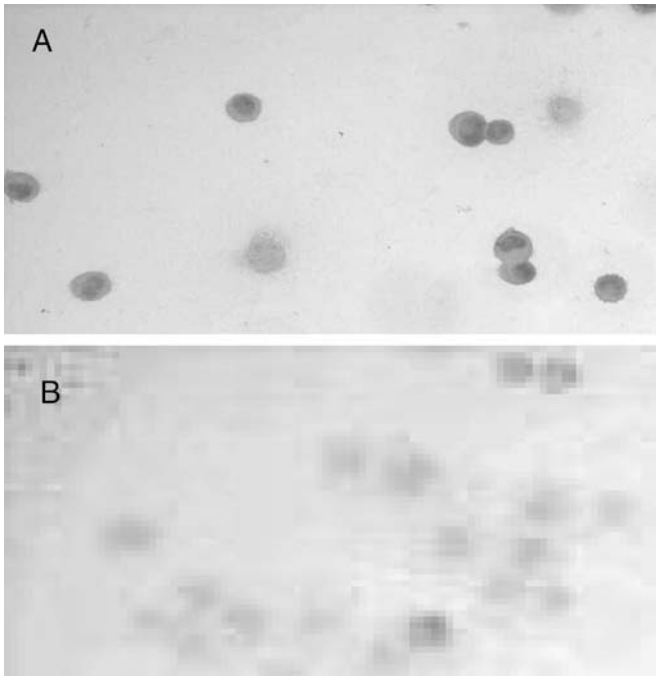

Fig. 5 Immunoperoxidase reaction of B16 melanoma cell smears for LDL receptors. H_2O_2 plus DAB was used as chromogen substrate and hematoxylin solution for counterstaining **a** positive reaction, **b** negative reaction, adjacent section was incubated with an irrelevant murine IgG monoclonal antibody. After washing and fixation, cells were observed under light microscopy ×400

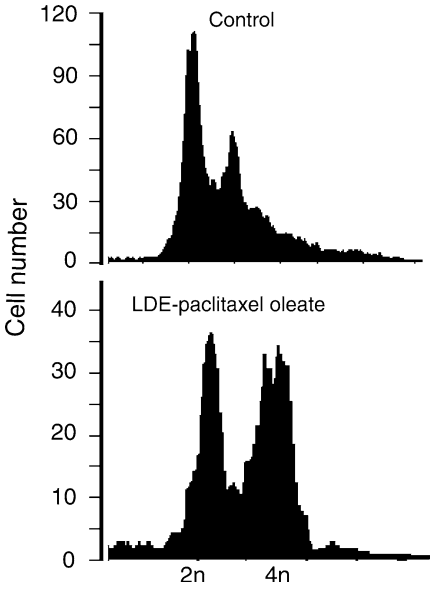

Fig. 6 Flow cytometric analysis performed on B16F10 melanoma cells treated with 0.05 μM LDE-paclitaxel oleate for 24 h and respective control (without treatment) before fixation and propidium iodide staining. This is a representative scan of three similar experiments

the relative proportion of the cells at the S and G_2/M phase.

Biodistribution of LDE and LDE-paclitaxel oleate in tumor-bearing mice

Figure 7a shows the uptake of LDE not associated with the drug and labeled with [^{14}C]-cholesteryl oleate by the tumor and by several tissues in the melanoma B16F10-bearing mice 18 h following single i.p. bolus injection of the microemulsion. The tumor tissue was second only to the hepatic tissue regarding the uptake of the LDE label. The tumor uptake was roughly 60% of the hepatic uptake (tumor/liver uptake ratio = 0.6 ± 0.2).

Figure 7b shows the uptake of LDE-paclitaxel oleate, doubly labeled with [^{14}C]-cholesteryl oleate and [^{3}H]-paclitaxel oleate by the tumor, liver and skin. The association with the drug does not change the tumor/liver uptake ratio of the emulsion [^{14}C]-cholesteryl oleate (0.6 ± 0.2). It is also clear that the drug was taken-up by the liver and the tumor together with LDE because the uptake ratio of [^{3}H]-paclitaxel oleate (0.6 ± 0.4) is not different from that of the [^{14}C]-cholesteryl oleate. It is worthwhile to point out that LDE concentrates paclitaxel oleate in the tumor roughly fourfold relative to the adjacent skin.

Plasma kinetics of double-labeled LDE-paclitaxel oleate and commercial paclitaxel in mice

Figure 8a shows the plasma decay curve of the LDE-paclitaxel oleate labeled with both [^{3}H]-paclitaxel oleate and [^{14}C]-cholesteryl oleate following single i.v. bolus injection in control mice. The FCR's of the LDE and paclitaxel oleate labels are not different from each other (0.078 ± 0.051 h^{-1} and 0.042 ± 0.022 h^{-1}, respectively, $p = 0.088$).

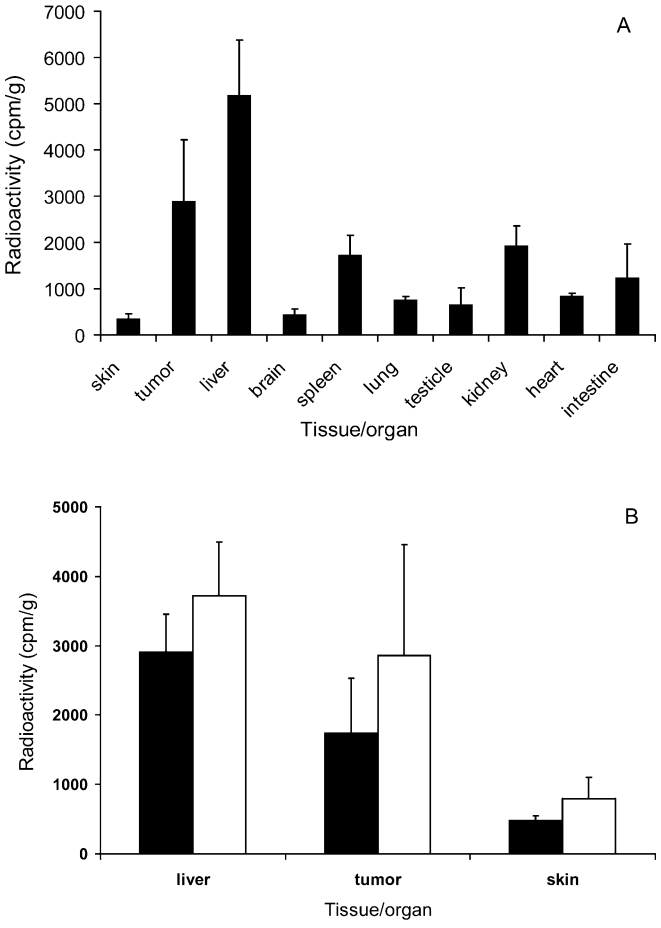

Fig. 7 Tissue and organs biodistribution in B16 melanoma bearing mice of **a** labeled [^{14}C] cholesteryl ester-LDE alone and **b** LDE-paclitaxel oleate doubly labeled with [^{3}H]-paclitaxel oleate (*open bars*) and [^{14}C] cholesteryl ester-LDE (*filled bars*) following 18 h of single i.p. bolus injection. Radioactivity was measured by liquid scintillation. For the determination of the radioactivity amount, the measured values were corrected for 1 g of tissue or organs mass. Results are presented as means ± SEM (bars). Each group comprises eight animals

Figure 8b shows the plasma decay curve of label [^{3}H]-paclitaxel and [^{3}H]-paclitaxel oleate associated to LDE following i.v. bolus injection in control mice. The FCR's of the commercial paclitaxel is greater than paclitaxel oleate associated to LDE (0.134 ± 0.047 h^{-1} and 0.042 ± 0.022 h^{-1}, respectively, $p = 0.032$).

Table 2 summarizes the pharmacokinetic parameters of paclitaxel oleate associated to LDE and of commercial paclitaxel. Derivatization and association with LDE results in remarkable changes in the pharmacokinetics of the drug characterized by pronouncedly longer half-lives and AUC and twofold smaller plasma clearance rates compared to commercial paclitaxel.

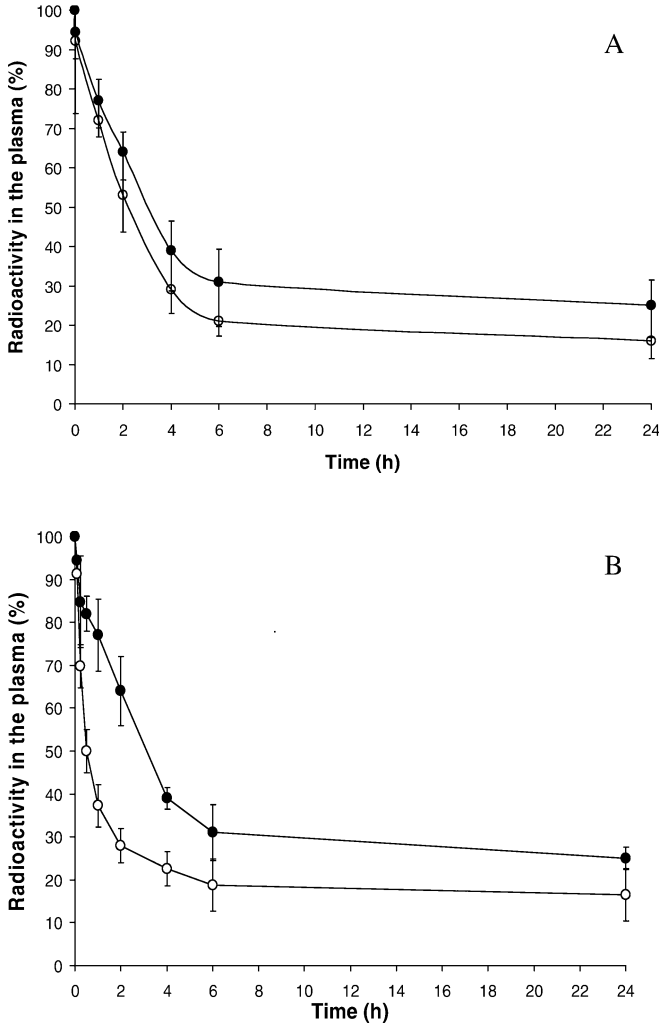

Fig. 8 Comparison of the curves of removal from plasma of LDE-paclitaxel oleate and commercial paclitaxel, injected as a i.v. bolus into mice. **a** Plasma decay curve of doubly labeled with [^{3}H] paclitaxel oleate (*filled circle*) and [^{14}C] cholesteryl ester-LDE (*open circle*). **b** Plasma decay curve of LDE-paclitaxel oleate labeled with [^{3}H]-paclitaxel oleate (*filled circle*) and commercial labeled [^{3}H]-paclitaxel (*open circle*). Plasma samples were taken over 24 h, for radioactive counting in scintillation vials. Results are presented as means ± SD (bars). Each group comprises eight animals

Table 2 Paclitaxel pharmacokinetic parameters observed in control mice following i.v. bolus injection of LDE-paclitaxel oleate and commercial paclitaxel

	Dose (mg/Kg)	$t_{1/2\beta}$ (min)	AUC (μg h/ml)	CL (ml/min)	*Vss* (ml/Kg)
Two-compartment open model					
Paclitaxel oleate[a]	5.2[b]	218	1334	0.125	39.3
Commercial paclitaxel[c]	4	184	707	0.236	62.7

[a]Paclitaxel oleate associated to LDE injected in control mice.
[b]The paclitaxel oleate 5.2 mg/kg dose is an equimolar dose of 4 mg/kg of paclitaxel.
[c]Commercial paclitaxel injected in control mice.

Therapeutic action of LDE-paclitaxel oleate and commercial paclitaxel

Figure 9a shows the 31 day follow-up effects of LDE-paclitaxel oleate on tumor growth, as observed at two dose levels (17.5 mg/kg and 70.3 mg/kg, equimolar doses with 15 mg/kg and 60 mg/kg of paclitaxel, respectively), compared with the effect of the commercial paclitaxel formulation at the MTD (15 mg/kg). The antitumor effect of LDE-paclitaxel oleate at both doses was greater than that of commercial paclitaxel ($p < 0.01$). It is worthwhile to point out that the increase from 17.5 mg/kg to 70.3 mg/kg LDE-paclitaxel oleate did not induce any additional antitumor effect ($p > 0.05$).

Figure 9b shows the Kaplan–Meyer survival curves of mice treated with LDE-paclitaxel oleate at doses 17.5 mg/kg and 70.3 mg/kg, commercial paclitaxel at

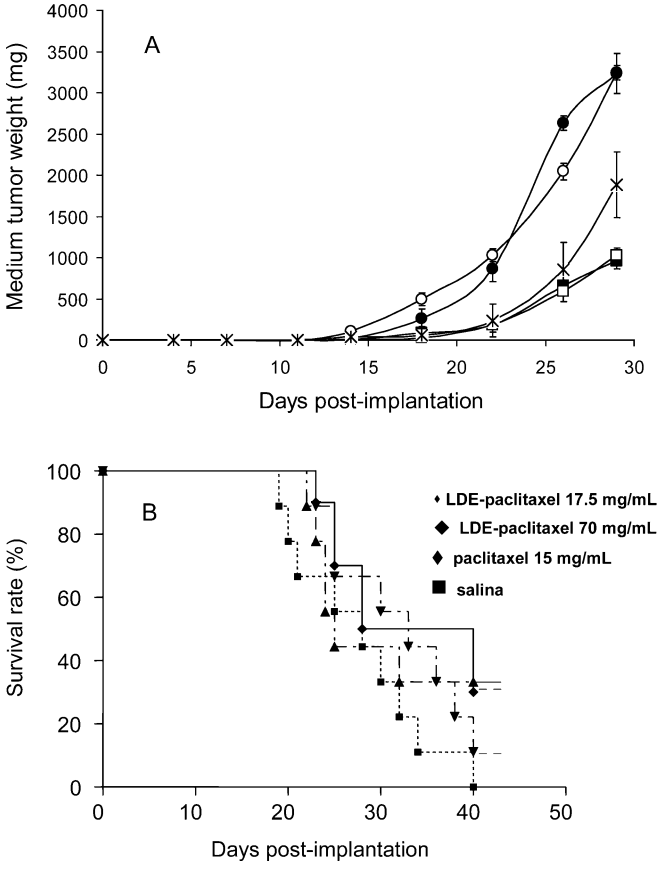

Fig. 9 Effect of LDE-paclitaxel oleate on tumor growth and survival rate of B16F10 tumor bearing mice. **a** 5×10^4 cells B16 melanoma cells were subcutaneously injected in dorsal region of B57CL/6 mice (8 mice per group). On days 11, 14, 19 as counted from the initial inoculation day, LDE-paclitaxel oleate 17.5 mg/kg (*filled diamond*) and 70 mg/kg (*open square*), 15 mg/kg commercial paclitaxel (*cross mark*) and respective controls, saline (*open circle*) and LDE (*filled circle*) were i.p. injected. Tumor volumes were monitored by periodic callipers measurements and presented as means ± SEM (bars). **b** Percent B16 melanoma bearing mice survival in response to the treatment as a function of time. The survival rates were calculated daily and the experiment was terminated when all the mice of control group died (at day 40)

dose 15 mg/kg and of the controls treated with saline solution, as observed over 40 days. The survival of the controls was 0% on day 40. In the group treated with commercial paclitaxel the survival increased to 15%. The best survival rates, however, were achieved by the LDE-paclitaxel oleate treatment, where the 40-day survival increased to 25% with 17.5 mg/kg and further to 33% with the 70.3 mg/kg dose.

Regarding the total tumor regression or cure, it was achieved in one out of eight mice at each LDE-paclitaxel oleate dose levels, whereas there was not tumor regression among the animals treated with commercial paclitaxel.

Discussion

This study shows that the derivatization of paclitaxel and association to LDE results in a preparation with a superior therapeutic index. Attachment of fatty acids to paclitaxel is a simple and easily performed procedure that allows excellent yield of association to LDE. It was previously reported [14] that upon internalization into the cells enzymatic hydrolysis of the fatty esters occurs and the drug is reversed to its original structure. Because paclitaxel oleate is inactive, the experiments performed here showing that in the LDE-paclitaxel oleate complex the activity of the drug is preserved strongly suggests that this intracellular hydrolysis also occurs with the new preparation. Furthermore, fatty acid coupling renders the drug stable in the complex with LDE, as demonstrated in the experiments in which dialysis of the doubly labeled complex against both plasma and Tris solution were performed. In this regard, as shown by the dialysis experiments, there was indeed virtual lack of dissociation of paclitaxel from LDE over 24 h. As indicated by the plasma kinetics of LDE-paclitaxel oleate, this period of time is enough for most part of the complex to disappear from the plasma compartment.

The use of paclitaxel radioactively labeled in the taxane ring allows assessment of the stability of the association of the drug with LDE, as shown in other emulsion systems [14], although radioactive labeling does not monitor the appearance of metabolites of the drug. Nonetheless, the experiments in which doubly labeled LDE-paclitaxel oleate was incubated with plasma that was subsequently fractionated by ultracentrifugation, as well as in the in vitro and in vivo kinetic studies clearly show that dissociation of the drug from the microemulsion does not occur at appreciable rates. Thus, it is conceivable that a substantial degradation of the drug while in the plasma compartment is not likely to take place.

The growth inhibitory assays comparing paclitaxel oleate to commercial paclitaxel showed that attaching of the oleoyl group does not result in diminution of the cytotoxic activity. This suggests that after internalization into the cell the drug is hydrolyzed and reversed to its original structure because attaching a fatty acid to the paclitaxel 2′-position inactivates the drug. When paclitaxel oleate was associated to LDE, the growth inhibitory effect was diminished. This is ascribed to the substitution of LDE for the paclitaxel vehicle Cremophor EL, because when Cremophor EL was added to incubates of LDE-paclitaxel oleate with cancer cells, the cytotoxic effects increased and equaled that of the commercial formulation. Indeed, the Cremophor EL cytotoxicity per se has been reported by others [3, 20].

The flow cytometry experiments showed that LDE-paclitaxel oleate, like paclitaxel, arrests the cells in the G_2/M phase of the cell cycle after 24 h. These actions have been classically associated with the paclitaxel molecule [26] and are thus maintained when the drug is modified and associated with LDE. This is indeed, an indication, that paclitaxel oleate is converted to paclitaxel or other active molecule after its internalisation.

We recently showed that LDE uptake by NCI H292 and RPMI B226 cancer cells is mediated by the LDL receptor-mediated endocytosis [12, 17]. An important issue is whether the association of paclitaxel oleate to LDE would affect this uptake mechanism. In this respect, when neoplastic cells were incubated with LDE-paclitaxel oleate labeled with [^{14}C]-Cholesteryl oleate-LDE and [^{3}H]-paclitaxel oleate, the cell uptake of the two labels was simultaneous and was dislocated to the same extent by the addition of native LDL. This supports the assumption that paclitaxel oleate and LDE are internalized together by the LDL receptor pathway.

The lack of dissociation of paclitaxel from LDE is shown here by the similar removal rates of both drug and LDE labels after injection of the complex into the animals. This observation is very important because the stability of the association in the bloodstream is a prerequisite for the drug-targeting effect. When associated with LDE, paclitaxel showed greater plasma half-life and AUC and lower systemic clearance than those for commercial paclitaxel. This is an interesting finding favoring the pharmacological action of the drug, because the antitumoral efficiency of paclitaxel depends rather on the duration of cell exposure to the drug than on the concentration of the drug [8].

B16 melanoma animal model was chosen to test the antitumoral action of LDE-paclitaxel oleate because we were able to confirm the presence of LDL receptors in the tumor cells by the immunoperoxidase reaction technique. Moreover, it is a fast-growing tumor that has been commonly used in early screening of several anticancer agents. It is noteworthy that the LDE uptake ratio between the melanoma and the liver we found in this study is similar to that described in literature when the native LDL was injected in B16 melanoma bearing mice [25]. Other important issue regards our finding that the association of the drug to LDE does not alter the tumor/liver uptake rate of the [^{14}C]-cholesteryl ester of the microemulsion. Furthermore, the tumor/liver uptake ratio of the labeled drug associated to the microemulsion is rigorously equiva-

lent to that of the LDE [^{14}C]-cholesteryl ester. Those findings support the assumption that the association with the drug does not alter the biological properties of the microemulsion that allow the binding to the receptors and that paclitaxel is indeed delivered to the cells together with LDE.

Similarly to non-modified paclitaxel associated with LDE [21], LDE-paclitaxel oleate dramatically reduced the toxicity compared with the commercial formulation. Because these experiments were performed in control rather than in tumor mice, the toxicity reduction was not due to the drug targeting effect of LDE that we documented in this study. At least two major causes for this great reduction in toxicity can be raised: firstly, the new biodistribution for the drug created by the association with LDE. Although we did not document this fact here, it is possible that LDE-paclitaxel oleate becomes less concentrated in the bone marrow than the commercial formulation, thus diminishing myelotoxicity that is one of the main paclitaxel side effects. Secondly, substitution of the virtually non-toxic LDE for Cremophor EL, a vehicle that is commonly associated with severe hypersensitivity reactions [27] might have as well influenced the toxicity reduction. Indeed, we documented here a strong toxicity to the animals of Cremophor EL as administered alone, in agreement with the study by Sharma [22]. Attenuation of toxicity was reported previously in control animals with association of paclitaxel with other drug carrier systems [2, 22].

Besides the reduction of toxicity, our data also indicate that the derivatization and association with LDE greatly increases the antitumor activity of paclitaxel. This assumption is supported by the comparison of LDE-paclitaxel oleate with commercial paclitaxel in all the parameters of therapeutic response to one treatment cycle that were evaluated in this study: tumor growth delay, median tumor weight, median survival and % of cure. B16 melanoma is a fast growing, highly metastatic tumor. By the time the primary tumor reaches 5 mm, micrometastases are already present in the mice lungs [25]. Previously, the cure of B16 melanoma bearing mice using paclitaxel had been observed only when commercial paclitaxel was associated with other treatments such as immunomodulators or exposition to electric field [4, 13]. Therefore, the 12% cure obtained with LDE-paclitaxel at both dose levels, contrasting with 0% with commercial paclitaxel are particularly meaningful regarding the antitumoral efficiency of the new preparation.

It is remarkable in our results that no additional therapeutic effect was gained beyond the 17.5 mg/kg dose of paclitaxel oleate associated with LDE. Lack of effect of higher doses can be ascribed to experimental conditions such as saturation of the LDL receptors of the tumor by the amount of LDE-paclitaxel particles administered in a short interval. The fact that, in this study, greater therapeutic responses were not achieved by substantially augmenting the drug dose should, however, be born in mind in the planning of future clinical studies of LDE-paclitaxel.

It is conceivable that the drug targeting effect afforded by the association with LDE is lost in cancers in which the LDL receptor overexpression does not occur. However, it is remarkable LDL uptake was enhanced in several neoplastic disease, such as breast cancer [5], bronchogenic carcinoma [10], gallbladder [23], metastatic prostate carcinoma [11] and myeloproliferative diseases [6], so that the potential of LDE as vehicle may cover a great variety of malignant tumors with great populational incidence.

In conclusion, our study in control and tumor-bearing mice indicates that the association of a paclitaxel derivative to LDE results in a preparation with superior antitumor activity. The drug targeting properties of the microemulsion is clearly seen, the remarkably lower toxicity and the increase in antitumoral activity when compared with commercial paclitaxel may lead to the improvement in the therapeutic index in clinical use. Therefore, LDE-paclitaxel oleate may be a strong new weapon and it is currently being tested in a clinical trial.

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