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

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Abstract

A cholesterol-rich emulsion (LDE) is taken up by malignant cells which over-express low-density lipoprotein (LDL) receptors and thus may be used as a carrier for drugs directed against neoplastic cells. In this study, we associated the antineoplastic agent paclitaxel to LDE and analysed the new formulation's incorporation efficiency, chemical and physical stability, cellular uptake and cytostatic activity against a neoplastic cell line and the acute toxicity to rats. A paclitaxel incorporation efficiency of approximately 75% was achieved when paclitaxel was mixed with LDE at a 6:1 lipid-to-drug molar ratio. The association of paclitaxel with LDE increased by 54% the mean diameter of the emulsion particles but did not damage the paclitaxel chemical structure as analysed by HPLC. Results from gradient ultracentrifugation and Sephadex G25 gel filtration indicated that the binding of the drug to the emulsion was stable. It was shown that the cellular uptake and the cytotoxic activity of LDE-paclitaxel by a neoplastic cell line (NCI-H292 cells) was indeed mediated by the LDL receptors. The anti-proliferative activity of LDE-paclitaxel against NCI-H292 cells was less than that of a commercial paclitaxel preparation (50% inhibitory concentration, IC50 = 2.60 and 0.45 μM, respectively). This difference, however, can be ascribed to the in-vitro anti-proliferative activity of the commercial paclitaxel vehicle Cremophor EL; when Cremophor EL was added to the cultures with LDE-paclitaxel, the IC50 value was reduced to 0.45 μM, attaining that of the commercial paclitaxel preparation. The tolerability of LDE-paclitaxel in rats was remarkable, such that its lethal dose (LD50) was ten-fold greater than that of the commercial formulation (LD50 = 324 and 31.8 mg kg^{-1}, respectively). Therefore, LDE-paclitaxel association is stable and the cytostatic activity of the drug is preserved while its toxicity to rats is small. By diminishing the side effects and directing paclitaxel to neoplastic tissues, LDE may be useful as adjuvant in chemotherapy with this drug.

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Introduction

We have previously reported that an artificial emulsion resembling the lipid structure of low-density lipoprotein (LDL), which we denominated LDE, has the ability to bind to LDL receptors (Maranhão et al 1993). LDE, which is basically composed of a cholesteryl ester core surrounded by a phospholipid monolayer, is made without protein. When injected into the plasma circulation it acquires circulating apolipoproteins (apo), such as apo E, from the native lipoproteins. The LDL receptor recognizes apo E that can couple the LDE particles to the receptor, allowing internalization of the emulsion into the cytoplasm. Because LDL receptors are upregulated in several cancer lines, LDE may concentrate in the neoplastic

tissues (Maranhão et al 1994). LDL receptor upregulation is intense, reaching up to a 100-fold increase in the receptor expression of normal cells as originally described by Ho et al (1978) in acute myelogenous leukaemia. Taking advantage of this unique drug-targeting mechanism, the emulsion can thus be used as a carrier to shuttle chemotherapeutic agents directed against neoplastic cells, presumably decreasing the side effects of those drugs. Because paclitaxel is a lipophilic drug, we evaluated this chemotherapeutic agent as a candidate for association with LDE. Paclitaxel has been shown to be active against highly frequent cancers such as ovarian, colon, breast and non-small cell lung carcinomas. Paclitaxel has also been under clinical investigation for a broad range of human cancers, including gastric, cervical, head and neck cancer and melanoma (Rowinsky et al 1990). The drug is commercially available as a 1:1 mixture with a polyoxyethylated castor oil (Cremophor EL) and ethanol (Taxol). Cremophor EL, however, may cause severe life-threatening hypersensitivity reactions in patients (Weiss et al 1990) such as hypotension, tachycardia and dyspnoea, as well as hyperlipidaemia. Due to those vehicle side effects, premedication with corticosteroids and antihistamines is a standard procedure for administration of the drug. Therefore, substitution of LDE for Cremophor EL would be doubly advantageous: firstly because the emulsion lacks toxicity and is not immunogenic; secondly, because LDE is endowed with tropism for neoplastic cells with receptor upregulation, it may direct the drug to its site of action.

Materials and Methods

Materials

Crystalline paclitaxel was purchased from Calbiochem (CA). Dimethyl sulfoxide (DMSO), 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), polyethoxylated castor oil (Cremophor EL), triolein, cholesteryl oleate, cholesterol and phosphatidylcholine were purchased from Sigma (St Louis, MO). The clinically approved formulation of paclitaxel, Taxol, was obtained from Bristol-Myers Squibb Co (1 mL contained 6 mg of paclitaxel, 527 mg of Cremophor EL and 49.7% v/v dehydrated alcohol, USP). The small cell lung carcinoma NCI H292 cell line was purchased from Adolfo Lutz Institute (São Paulo, Brazil).

Preparation of LDE-paclitaxel

In brief, 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 described previously (Ginsburg et al 1982), modified by Maranhão et al (Maranhão 1993). LDE was dialysed against saline solution and passed through a 0.22-μm pore filter for the experiments. Paclitaxel was incorporated into LDE by hydrating 34 mg lyophilized paclitaxel powder in 1200 μL ethanol until its complete dissolution and by adding the emulsion (2800 μL) to the paclitaxel–ethanol solution. 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.

Separation of LDE-paclitaxel from the unbound drug

Separation of unbound paclitaxel from paclitaxel incorporated into LDE was performed according to three different procedures. In the first procedure, the solution was centrifuged at 3000 rev min^{-1} for 15 min, after which the LDE-paclitaxel fraction is found in the supernatant while free paclitaxel precipitates. In the second procedure, LDE-paclitaxel density was adjusted to a final density of 1.14 by addition of solid KBr. The tubes were centrifuged at 35000 rev min^{-1} for 24 h. After this procedure, the LDE-paclitaxel fraction stands at the tube top. In the third procedure, 1 mL LDE-paclitaxel was placed on a Sephadex G25 column equilibrated with NaCl 0.9% solution. Thereafter the column was washed with a saline–DMSO solution at a flow rate of about 60 mL h^{-1}. Fractions of 1 mL containing the incorporated or the unbound drug were then collected. The LDE component was labelled with [^{14}C]cholesterol and the paclitaxel component was labelled with [^{3}H]paclitaxel.

The amount of the drug incorporated into LDE and of the non-associated drug, as obtained by the three different separation procedures, was quantified by radioactive labelling with addition of 2.5 nCi [^{3}H] paclitaxel.

LDE-paclitaxel particle size and stability

The size of the LDE-paclitaxel particles was measured using a Zeta Potential Analyzer – Brookhaven Instruments Corporation (Holtscille-NY). All samples were maintained at 4°C and were diluted and filtered immediately before the diameter measurement. To evaluate the stability of the preparation, the mean diameters

of the LDE and LDE-paclitaxel particles were measured as a function of time up to 30 days.

LDE-paclitaxel stability was also evaluated by visual observation using a conventional light microscope (Nikon TMS). Samples were diluted in NaCl 0.9% solution to give paclitaxel concentrations of 0.3, 0.6, 0.9 and 1.2 mg mL^{-1} and maintained at 4°C, at room temperature or at 37°C. Crystal formation was evaluated daily for a 30-day period.

Chemical stability of paclitaxel when incorporated into LDE

The stability of paclitaxel associated with the emulsion was analysed by reverse-phase HPLC (Sharma et al 1994). In brief, paclitaxel was extracted from the LDE-paclitaxel fraction and from the free paclitaxel fraction immediately before the experiment. An isocratic solvent system of 70% methanol and 30% water was used as mobile phase. The flow rate was maintained constant at 2 mL min^{-1} and paclitaxel was detected by absorbance at 227 nm. A Shimadzu Shim-pack CLS-ODS column (6 × 150) and a C_{18} guard column were used for analysis.

Cell growth inhibition

The small cell lung carcinoma cells (NCI H292) were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum at 37°C in a humidified incubator with 5% (v/v) CO_2. The cells used in the assays were in the mid-logarithmic growth phase. For the experiments, the cells were harvested from the culture and distributed into 96-well culture plates. After 24 h incubation, LDE-paclitaxel and commercial paclitaxel were added to the wells in triplicate. We used the final paclitaxel concentration of 0.0003, 0.003, 0.03, 0.3, 3 and 30 μM. The cells were left in the incubator for a further 120 h, according to the procedure described by Reinecke et al (1997) and at the end of this period the medium was removed and the number of living tumour cells were determined by the colorimetric MTT assay. The cell viability of each well (expressed as survival index) was determined using equation 1.

$$\text{Survival index (\%)} = [(\text{Absorbance of test} - \text{Absorbance of blank})/(\text{Absorbance of control} - \text{Absorbance of blank})] \times 100 \quad (1)$$

The 50% inhibitory concentration (IC50) was determined as the drug concentration required to inhibit 50% of the cell growth. Control experiments using Cremophor EL–ethanol and LDE were also performed.

Cytostatic activity of LDE-paclitaxel at 4°C and 37°C

This experiment was performed to clarify whether the cytotoxicity of paclitaxel associated with LDE was consequent to the entry of the LDE-paclitaxel into the cell by the receptor-mediated pathway that is inactive at 4°C (Brown & Goldstein 1986). At 4°C, binding to the membrane receptors may occur but not internalization into the cytoplasm. NCI-H292 cells were grown, plated and incubated according to the procedures described above, except that one of the parallel plated plates was placed in the refrigerator (4°C), 1 h before addition of the drug and during the ensuing 4-h incubation period. The paclitaxel concentrations in the incubates were 0.0003, 0.003, 0.03, 0.3, 3 and 30 μM. After this 4-h incubation period, the medium was replaced by RPMI supplemented with 10% v/v fetal calf serum and the cells were placed in the incubator (37°C) for a further 24 h. The number of living tumour cells was then determined by the colorimetric MTT assay.

LDE-paclitaxel uptake by neoplastic cells

The cellular uptake of paclitaxel was determined by incubation of NCI H292 cells with increasing amounts of ^{14}C-LDE-^{3}H-paclitaxel. Viable cells (10^6) were plated on 35-mm Petri dishes. The next day, the medium was replaced by one containing 10% lipoprotein-deficient serum (LPDS). On the third day, increasing amounts of LDE-paclitaxel labelled with [^{14}C]cholesteryl oleate and [^{3}H]paclitaxel (0.1 μCi each) were added to the plates in duplicate for a 4-h incubation at 37°C. The cells were then washed three times with cold phosphate-buffered saline (PBS) plus bovine serum albumin (BSA) and twice with PBS at 37°C, harvested and centrifuged at 14000 rev min^{-1} for 15 min, and 200 μL of NaOH was added to the pellet to disrupt the cell pellet under vortex mixing. The radioactivity was measured in a liquid scintillation solution.

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

NCI H292 viable cells (10^6) were incubated for 24 h in RPMI 1640 containing 100 U mL^{-1} of benzylpenicillin sodium and 5 μg mL^{-1} of gentamicin, supplemented with 10% LPDS. Then, 10 μg of LDE labelled with [^{14}C]cholesteryl oleate and [^{3}H]paclitaxel and increasing amounts of human LDL were added to the plates in duplicate, and plates were 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 14000 rev min^{-1} for 15 min; 200 μL of NaOH was added to the pellet to disrupt the cell pellet under vortex mixing before radioactivity measurement.

Toxicity of LDE-paclitaxel in rats

Acute dose–toxicity experiments were performed using Wistar rats weighing roughly 100 g. LDE-paclitaxel, commercially available paclitaxel and the respective controls (LDE or Cremophor EL–ethanol alone) were administered intraperitoneally in single or multiple doses in the range 5–400 mg kg^{-1}. The rats were divided into groups of five and paclitaxel doses up to 200 mg kg^{-1} were given in a two-day schedule. Observation of the survival rate and weight change was made daily over a 60-day period. Lethal doses were determined by simple interpolation. Studies were approved by the Animal Ethics Committee of the Faculty of Pharmaceutic Science of the University of São Paulo and performed in accordance with their guidelines.

Statistical analysis

The differences in the cell survival curves and in the cell uptake curves were evaluated by the unpaired Student's *t*-test, since the distributions were normal and variances were homogenous. Curves were analysed by their individual data points. In all analyses, a difference of $P < 0.05$ was considered statistically significant.

Results

Separation of LDE-paclitaxel from the unbound drug and incorporation efficiency

In the elution profile of LDE-paclitaxel obtained by gel filtration it was observed that the labelled emulsion eluted as a single peak (from elution flasks 3–5) while the labelled paclitaxel eluted as two distinct peaks. The first peak, from elution flasks 3–5, eluted together with the emulsion. The second peak, from flasks 14–16, corresponded to the unassociated fraction of the drug. Under the association conditions described above, most of the paclitaxel became associated with LDE. As estimated by the specific activity of the emulsion lipid and paclitaxel measured in the elution flasks, 70–75% of the drug was associated with LDE. It thus resulted that each millilitre of the emulsion (30 mg of emulsion total lipids) solubilized 5.4–6 mg of paclitaxel. This value was independently confirmed by another approach, wherein LDE-paclitaxel was submitted to ultracentrifugation in a density gradient (data not shown).

LDE-paclitaxel particle size and stability

The particle size of LDE-paclitaxel and LDE alone was monitored over a 30-day storage period at 4°C. The association of paclitaxel with LDE increased, by 54%, the mean diameter of the emulsion particles. The size of both LDE and LDE-paclitaxel particles was unchanged until the 8th storage day; hence it apparently decreased until reaching 82% of the initial diameter, in the case of LDE and 88% in the case of LDE-paclitaxel, as observed on the 30th storage day. Observation by light microscopy of the LDE and LDE-paclitaxel stored samples showed that there were no crystal precipitates or other gross changes during the initial 8-day storage period.

Chemical stability of paclitaxel when associated with LDE

Paclitaxel associated with LDE was extracted from the emulsion lipids and analysed by reverse-phase HPLC. The retention time and shape of the HPLC peak of paclitaxel extracted from the emulsion were similar to that of non-associated paclitaxel (not shown). This indicates that association with LDE by the procedure we used does not damage the chemical structure of the drug.

Cell growth inhibition

Figure 1 shows the cytostatic activity of LDE-paclitaxel and of the commercial paclitaxel formulation as tested in the NCI H292 tumour cell line, as well as the cytostatic activity of the respective vehicles, namely LDE and Cremophor EL. In Figure 1A, it is shown that the addition to the cell cultures of increasing amounts of both LDE-paclitaxel and the commercial paclitaxel formulation produced decreased cell survival rates, in typical dose–response curves. Although there was an apparent trend towards a greater growth-inhibitory effect of the commercial paclitaxel than LDE-paclitaxel, this difference was not statistically significant ($P = 0.3187$). In Figure 1B it is shown that whereas LDE alone had virtually no effect upon the cell survival rate, Cremophor EL, the vehicle of the commercial paclitaxel preparation, markedly decreased the cell survival. Interestingly, when Cremophor EL was added to incubates of LDE-paclitaxel with the neoplastic cells, the reduction

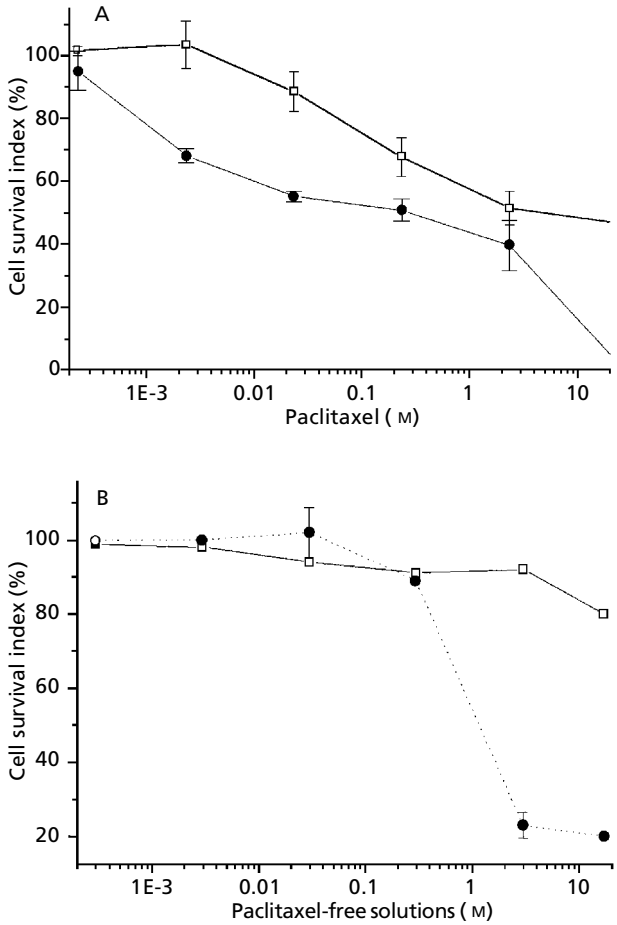

Figure 1 Effects on NCI H292 cell survival of increasing amounts of: A. LDE-paclitaxel (□), a commercial paclitaxel preparation (●) and B. respective vehicles LDE (□) and Cremophor EL (●). The paclitaxel-free solutions had the same composition (minus the paclitaxel) as the LDE-paclitaxel and commercial paclitaxel preparation, respectively. Cells were incubated for 120 h at 37°C. Results are means±s.e.m. of three experiments performed in triplicate.

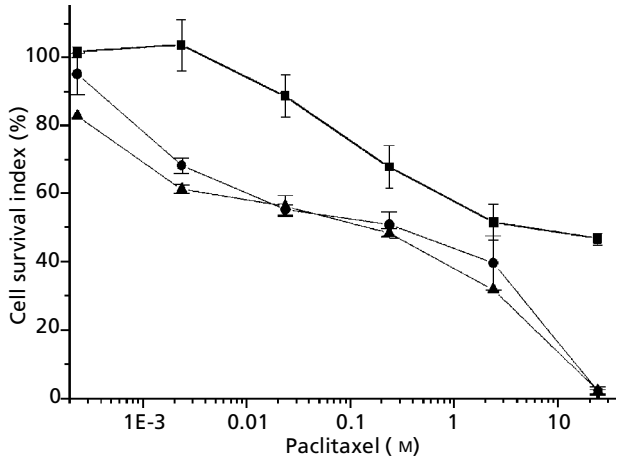

Figure 2 Effect on cell survival of the addition of increasing amounts of Cremophor EL to the incubates of NCI H292 cells with LDE-paclitaxel (●) compared with the incubates with commercial paclitaxel (▲) and LDE-paclitaxel (■) alone. Cells were incubated for 120 h at 37°C. Results are presented as means±s.e.m. of three experiments performed in triplicate.

Table 1 Cytostatic effects on NCI H292 neoplastic cell line of LDE-paclitaxel, commercial paclitaxel preparation, and of the respective vehicles, LDE and Cremophor, alone.

Drug/vehicle	IC50 (μM)
LDE-paclitaxel	2.60
LDE-paclitaxel+Cremophor EL	0.45
LDE[a]	>30.00
Commercial paclitaxel (paclitaxel+Cremophor EL)	0.45
Cremophor EL[a]	1.85

IC50 = dose producing 50% inhibition of cell growth. Values are means±s.e.m. of at least three experiments performed in triplicate. [a]LDE and Cremophor EL were diluted in ethanol before incubation with cells.

of the cell viability was such that it approached that of commercial paclitaxel (Figure 2). Therefore, it appears that the greater cytostatic activity of commercial paclitaxel compared with LDE-paclitaxel can be largely attributed to the additional growth-inhibitory activity of its vehicle.

Table 1 shows the IC50 values calculated for both paclitaxel formulations. The LDE-paclitaxel IC50 value was roughly six-fold higher than that of the commercial paclitaxel formulation (2.60 μM and 0.45 μM, respectively). The cytostatic difference between the two preparations was, however, abolished when Cremophor EL was added to the incubates of LDE-paclitaxel with the cells (IC50 = 0.45 μM).

Figure 3 shows that, when LDE-paclitaxel was incubated with tumour cells at 4°C, the cell viability was considerably increased compared with the experiment performed at 37°C (P = 0.0479). In contrast the cytostatic activity of the commercial paclitaxel preparation was not affected by the low temperature condition.

LDE-paclitaxel uptake by neoplastic cells

When NCI H292 cells were incubated with increasing amounts of LDE-paclitaxel labelled with [^{3}H]paclitaxel and [^{14}C]cholesteryl oleate, there was a proportionally

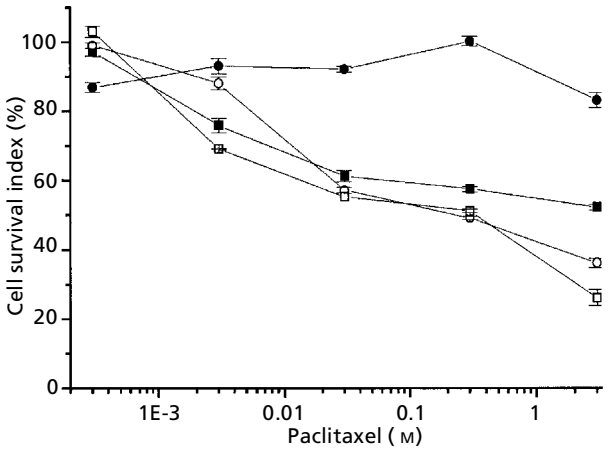

Figure 3 Effects on NCI H292 cell survival of increasing amounts of LDE-paclitaxel at 4°C (●) and 37°C (■), compared with the commercial paclitaxel preparation at 4°C (□) and 37°C (○) during 4 h incubation. Results are means ± s.e.m. of three experiments performed in triplicate.

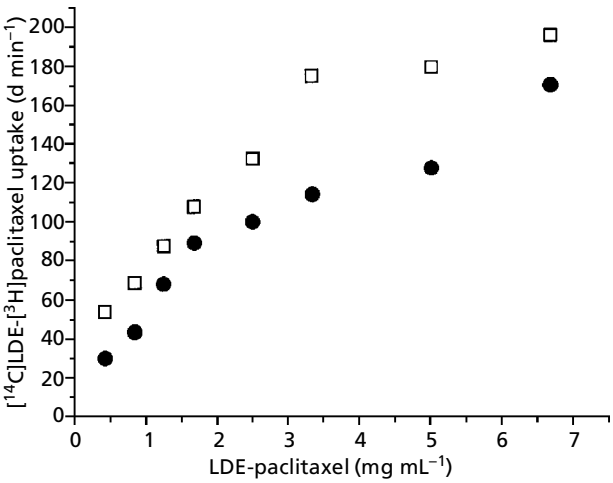

Figure 4 Uptake by NCI H292 cells of LDE-paclitaxel labelled with [^{3}H]paclitaxel (□) and [^{14}C]cholesteryl ester (●). Cells were incubated with increasing amounts of the doubly labelled LDE for 4 h at 37°C, in 4 mL of RPMI. Cells were then washed, harvested and disrupted and radioactivity was measured in a scintillation solution. The experiment was performed in triplicate.

increasing uptake of the two labels (Figure 4). The uptake curves were similar (P = 0.2717). This indicates that both paclitaxel and cholesteryl oleate components of the emulsion particles were taken up together by the malignant cells.

Incubates of LDE-paclitaxel and NCI H292 cells in the presence of increasing amounts of native human LDL showed proportionally diminished uptake of both

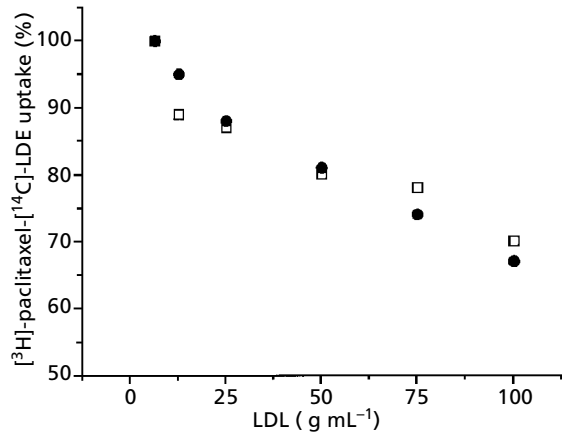

Figure 5 Effect of human LDL addition on the uptake by NCI H292 of LDE-paclitaxel labelled with [^{3}H]paclitaxel (□) and [^{14}C]cholesteryl ester (●). Cells were incubated with 10 μg LDE-paclitaxel doubly labelled with [^{3}H]paclitaxel and [^{14}C]cholesteryl ester and increasing concentrations of human LDL, for 4 h at 37°C, in 4 mL of RPMI. Cells were then washed, harvested and disrupted and radioactivity was measured in a scintillation solution. The experiment was performed in triplicate.

labelled emulsion components, [^{3}H]paclitaxel and [^{14}C] cholesteryl oleate (Figure 5). This indicates that when LDE uptake by the cells is progressively inhibited by native LDL because of the competition by the same receptor mechanisms, the cell uptake of the paclitaxel component is also proportionally inhibited.

Toxicity of LDE-paclitaxel in rats

Table 2 shows the results of the acute toxicity experiments, in rats, with LDE-paclitaxel and the commercial paclitaxel preparation. The association with LDE led to a remarkable reduction of the drug's toxicity, estimated by the lethal dose and the maximum tolerated dose

Table 2 Acute lethal toxicity in rats of LDE-paclitaxel, commercial paclitaxel preparation, and their respective vehicles.

Drug/vehicle	LD10	LD50	LD90
LDE-paclitaxel	274.0	324.0	> 336.0
LDE[a]	400.0	> 420.0	> 420.0
Commercial paclitaxel	25.0	31.8	34.4
Cremophor EL[2]	76.2	82.5	88.8

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

(MTD) in the experimental rat groups (LDE-paclitaxel LD50 = 324 mg kg^{-1}; commercial paclitaxel LD50 = 31.8 mg kg^{-1}; LDE-paclitaxel MTD $\cong$ 110 mg kg^{-1}; commercial paclitaxel $\cong$ 20 mg kg^{-1}).

Discussion

In this study, we showed that association with LDE greatly reduced the toxicity of paclitaxel in rats. Great toxicity, which results in a low therapeutic index, is one of the major drawbacks in cancer chemotherapy. In addition to the inherent side effects of paclitaxel, Cremophor EL, the vehicle used for its solubilization in aqueous media, conveys important hypersensitivity reactions. Those reactions aggravate the toxicity to patients. In an effort to circumvent the toxicity of this vehicle, chemical modifications of the paclitaxel molecule to improve solubilization have been attempted (Deutsch et al 1989). Acquisition of other vehicles such as liposomes (Sharma et al 1993), polymeric micellae (Zhang et al 1997), lipid emulsions (Lundberg 1996) and microspheres (Whang et al 1997) were other attempted strategies to solubilize this highly hydrophobic molecule.

The use of LDE, as proposed in this study, would offer additional advantages over all those other formulations because the emulsion not only solubilizes paclitaxel but also has the ability to direct the drug to its site of action. The mechanism behind the drug-targeting properties of the emulsion offers a remarkable specificity for trapping of an LDE-associated drug by cancer cells. The expression of LDL receptors in malignant cells may reach as much as 100 fold that of normal cells of the same line, as originally documented in acute lymphocytic leukaemia (Ho et al 1978). Over-expression of LDL receptors was found in solid tumours for which paclitaxel is frequently used, such as endometrial adenocarcinoma, uterine cell carcinoma, breast cancer (Rudling et al 1986), gallbladder cancer (Ueyama et al 1990) and metastatic prostate carcinoma (Henriksson et al 1989). Recently, we injected radioactively labelled LDE into patients who were about to be submitted to total oophorectomy, and verified that the uptake of the emulsion by ovarian carcinoma, a condition wherein paclitaxel is a first-line treatment drug, was on average ten-fold that of normal ovarian tissue (Ades et al 2001). Of note is the fact that the liver is the major uptake organ for LDE, as it is for native lipoprotein (Maranhão et al 1993). Therefore, hepatotoxicity should be of special concern when LDE is used as vehicle for chemotherapeutic agents.

The 70–75% LDE incorporation efficiency of the total paclitaxel amount mixed with the emulsion obtained by the technique employed in this study is, in fact, a good yield with view to the feasibility of future preclinical studies.

Emulsions are thermodynamically unstable systems that may lose physical stability even over short storage periods. Therefore, an important question regarding LDE-paclitaxel practical use is how long the preparation remains stable. In this respect, the eight-day physical and chemical stability period of LDE-paclitaxel assures a reasonable condition for the logistics and planning of clinical studies. This stability period may eventually be prolonged by other measures, such as addition of antioxidants or lyophilization.

The growth-inhibitory assays of LDE-paclitaxel show a clear-cut dose–response curve that is typical of antineoplastic compounds. Nonetheless, when the LDE-paclitaxel dose–response curve is compared with those of commercial paclitaxel, the growth-inhibitory effect of the new formulation seems smaller than that of the commercial paclitaxel, although this difference was not statistically significant. This fact led us to investigate whether this possible anti-proliferative difference could be ascribed to the substitution of LDE for Cremophor EL as paclitaxel vehicle. The experiments show clearly that Cremophor EL itself bears a dose-dependent cytostatic activity, a finding that has been reported by others (Csóka et al 1997; Reinecke et al 1997). In this respect, the greater cytostatic activity achieved when Cremophor EL was added to the LDE-paclitaxel and cancer-cell incubates, compared with commercial paclitaxel, is particularly elucidative. It is worthwhile pointing out that, contrary to what occurs in cell culture systems, the tumour concentration of Cremophor EL attained in the treatment of cancer patients is, by far, smaller than that required to achieve an antitumoral effect, due to its low volume of distribution (Sparreboom et al 1998).

An important issue in our study is whether or not the cytostatic effect of LDE-paclitaxel is mediated by the LDL receptor endocytic pathway. The results of the cytostatic assay with neoplastic cells performed at 4°C and 37°C with both LDE-paclitaxel and the commercial drug clearly indicate that LDE-paclitaxel internalization via LDL receptors is, indeed, the route that allows the new preparation to exert its effect on cell cultures. As expected, at low temperature, a condition in which internalization by receptor endocytosis of native LDL and extension of LDE is inhibited, LDE-paclitaxel was ineffective against malignant cell growth, presumably because the emulsion particles were not internalized into the cytoplasm. In contrast, the cytostatic action of the commercial paclitaxel preparation which entered

the cell by non-receptor pathways was not diminished at 4°C.

The issue of whether the action of paclitaxel in association with LDE was indeed mediated by entry into the cells through LDL receptors was endorsed by experiments involving the incubation of LDE-paclitaxel labelled with [^{14}C]LDE and [^{3}H]paclitaxel with NCI H292 cells. The fact that the cell uptake of the labelled paclitaxel was simultaneous with that of the labelled cholesteryl esters, and was equally dislocated by the presence in the incubates of human LDL, is strong and direct evidence that paclitaxel was indeed internalized by this pathway.

Another important issue is the remarkable reduction of paclitaxel toxicity when incorporated with LDE, as tested in rats. In drug carrier systems other than LDE, such as liposomes and other emulsion preparations (Souza et al 1993), this drug-side-effect attenuation is frequently observed and is probably related to the new drug biodistribution created by inclusion into those vehicles. Concerning commercially available paclitaxel, as already mentioned, Cremophor EL per-se has its own side effects that add-up those of paclitaxel.

Conclusion

In the procedure used here, paclitaxel showed a high incorporation rate into LDE. LDE-paclitaxel is stable and the cytostatic activity of the drug is preserved. The toxicity of this preparation in animals is remarkably small. Thus, LDE-paclitaxel shows interesting features that encourage further studies to ascertain its possible advantages in cancer chemotherapy.

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