Cancer Chemother Pharmacol (2002) 49: 487–498
DOI 10.1007/s00280-002-0437-3

ORIGINAL ARTICLE

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Association of carmustine with a lipid emulsion: in vitro, in vivo and preliminary studies in cancer patients

Received: 11 May 2001 / Accepted: 25 January 2002 / Published online: 20 March 2002

Abstract *Purpose:* We had previously shown in acute leukemia and in breast and ovary carcinoma patients that a cholesterol-rich emulsion (LDE) that binds to receptors for low-density lipoprotein (LDL) may concentrate in neoplastic tissues. In this study, the potential of LDE as a carrier for anticancer drugs was investigated. *Methods:* LDE was associated with carmustine, and the cytotoxicity of the LDE-carmustine complex was studied in a neoplastic cell line and its biodistribution was studied in mice. The plasma kinetics of the complex and its uptake by tumor and normal tissue were determined in cancer patients. Finally, an exploratory clinical study to determine the toxicity profile of LDE-carmustine at escalating dose levels was conducted in 42 advanced cancer patients refractory to conventional chemotherapy. *Results:* Carmustine formed a stable association with LDE. The pharmacological action of carmustine, as tested in cancer cells, was not diminished by association with LDE compared with the free drug and was indeed mediated by the LDL receptor. The biodistribution in mice and plasma kinetics in patients of the emulsion were not changed by association of the drug. The uptake of LDE-carmustine by tumor was severalfold greater than the uptake by the corresponding normal tissue. Finally, patients treated with LDE-carmustine showed negligible side effects even at very high dose levels. *Conclusions:* Association with LDE preserves the cytotoxicity of carmustine and markedly diminishes its side effects.

Keywords Emulsions · Carmustine · Cancer chemotherapy · Low-density lipoprotein receptors · Cholesterol

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Introduction

In a previous study [1], we had proposed a novel approach for cancer treatment consisting of the use of a lipid emulsion referred as LDE as a vehicle to direct chemotherapeutic agents to neoplastic cells. That proposal was based on the observation that LDE binds to low-density lipoprotein (LDL) receptors [1, 2, 3]. Because LDL receptors are upregulated in cancer cells [4], we hypothesized that the association of a chemotherapeutic agent with LDE would concentrate the agent in the neoplastic tissue after intravenous injection of the complex. Thus, delivery of the drug to the tumor would be increased while normal tissues and organs would be less damaged by the chemotherapy. The hypothesis that LDE would target neoplastic cells is supported by observations in acute myelogenous leukemia patients [1]. Subsequently, we acquired nuclear medicine images of ^{99m}Tc-LDE injected into breast cancer patients, and showed that the emulsion concentrates in the tumor and at metastatic sites [5]. More recently, we have shown that ^{14}C-cholesteryl oleate-labeled LDE injected into ovary carcinoma patients before oophorectomy is taken up by the tumor tissue fragments at rates tenfold greater than by the normal contralateral ovary and by benign ovarian tumor tissue [6].

LDE structurally resembles the lipid portion of native LDL. Although LDE does not contain apoB100, the protein that binds LDL to LDL receptors [7], in contact with plasma LDE may acquire

apoE, a 34-kDa 299 amino acid protein present in HDL, VLDL and chylomicrons. ApoE, as well as other exchangeable apolipoproteins, may shift from native lipoproteins to LDE particles. Because apoE can also be recognized by LDL receptors [7, 8] it enables LDE to be taken-up by the cells via the LDL receptor-mediated pathway [1, 2, 3, 9]. Indeed, because apoE binds to LDL receptors with more strength than apoB, the affinity of LDE for the receptors is greater than that of native LDL [10].

LDL receptor upregulation in cancer may be related to the great demand for lipids for the synthesis of new membranes required by neoplastic cell proliferation. The increase in LDL receptor activity is intense, reaching up to 100-fold that of normal cells in acute myelogenous leukemia [4]. Thus the potential of LDL as a drug carrier was realized early after the original description of the increased receptor function [11, 12, 13, 14, 15] together with the demonstration that solid tumors also exhibit receptor upregulation [12]. However, the native lipoprotein is difficult to obtain and handle, so that its introduction into clinical practice would be rather problematic. In contrast, LDE is manufactured from materials supplied by the chemical industry and may be produced on a large scale.

In this study, to test the feasibility of using LDE as a drug carrier in cancer treatment the chemotherapeutic agent carmustine was associated with the emulsion. This lipophilic nitrosourea, used in cancer treatment since the 1970s, was chosen to start these studies because in our preliminary screening the carmustine product information indicated easy and substantial associability with the emulsion. Furthermore, this drug has a characteristic and well-defined myelotoxicity that is the main limitation to its use [16]. Thus, blood counts would furnish a reliable parameter to evaluate the effect of association with LDE upon the toxicity of an antineoplastic drug. The cytotoxicity of LDE-carmustine was tested in a neoplastic cell line and its biodistribution was investigated in mice. The kinetics of the complex in the plasma was determined in cancer patients, as well as the uptake by tumor in relation to corresponding normal tissue. Furthermore, a pilot exploratory study was undertaken to determine the toxicity profile LDE-carmustine given in escalating doses in cancer patients. The results showed that LDE was able to minimize the side effects of the antineoplastic drug.

Material and methods

Study subjects

Consecutive patients from the outpatient clinics of the University of São Paulo Medical School Hospital were enrolled in one of the three different study protocols, as follows:

- LDE and LDE-carmustine plasma kinetics: one patient with breast carcinoma and one with colon carcinoma.
- LDE-carmustine uptake by tumor and normal tissue: two patients with ovary and one with breast carcinoma.
- LDE-carmustine treatment pilot study: 42 advanced cancer patients who had already been treated but were refractory to standard conventional chemotherapeutic. For at least 4 weeks prior to study entry they did not receive chemotherapy or radiotherapy. The inclusion criteria were as follows: Karnofsky performance status >80 on entry; white blood cell count more than 3500/mm^3 and platelet count more than 100,000/mm^3; satisfactory renal and hepatic function; and no active infection. Their median age was 52 years (range 29 to 78 years). The diagnoses of the patients are shown in Table 1.

The experimental protocols were approved by the Ethics Committee of the University of São Paulo Medical School Hospital and by the National Council of Health of the Brazilian Ministry of Health. Informed consent was obtained from each participant. The safety of the radioactive dose in the experiments in which labeled lipids were intravenously injected into the patients was ensured according to the regulations of the International Commission on Radiological Protection (ICRP) [17], as described in our previous study [18].

Preparation of LDE

LDE used in the experiments described in this study was prepared from lipid mixtures composed of 65% phosphatidyl choline, 32.5% cholesteryl oleate, 1.6% triolein and 0.8% unesterified cholesterol submitted to prolonged ultrasonication in aqueous medium followed by a two-step ultracentrifugation procedure according to the method of Ginsburg et al. [19] as modified by Maranhão et al. [2]. The preparations were sterilized by passage through a 0.2-μm filter.

Table 1. Diagnosis of the cancer patients treated with LDE-carmustine

Primary tumor	Metastatic sites	No. of patients
Adrenal gland carcinoma	Lung	1
Bladder cancer	Peritoneum	1
	Lung	2
Breast cancer	Liver	2
	Bone	7
	Lung	1
	Bone and lung	3
	Liver and lung	1
	Bone and liver	1
	Bone, liver and lung	2
	Locally advanced disease	1
Colon cancer	Liver	2
Hypernephroma	Lung	1
	Lung, bone and adrenal	1
Laryngeal cancer	Lung	2
Lung adenocarcinoma	Pericardium	1
	Lateral metastases	2
Lung small cell carcinoma	Liver and brain	1
Melanoma	Lung	1
	Bone, liver and lung	1
Non-Hodgkin's disease	Pleural extension	1
Osteosarcoma	Lung	1
Prostate	Bone	1
Rectal cancer	Liver	1
Stomach cancer	Retroperitoneum carcinomatosis	1
Stomach cancer	Liver	2
Unknown primary carcinoma	Lymph nodes, lung and adrenal	1

Association of LDE with carmustine

Carmustine was associated with LDE by cosonication of the drug with the emulsion at a ratio of 1:3 (w/w) for 5 min at 27°C using the flat tip (1 cm) of a Branson Cell Disrupter model 450 (Danbury, Ct.) at an output of 20 W under a stream of nitrogen. To determine the amount of carmustine associated with the emulsion, samples were submitted to gel filtration on a Sephadex G-25 column (PD 10 column, Pharmacia, Uppsala, Sweden) and the absorbance of the effluent measured at 230 nm. The size of the emulsion particles associated with carmustine (milligrams LDE total lipid per milligram carmustine) according to the above-described procedure and that of non-associated LDE were measured by quasielastic laser light scattering using a Brookhaven submicron particle analyzer (Holtsville, N.Y.).

Stability of LDE-carmustine complex

LDE-carmustine complex was dissolved in chloroform/methanol (2:1 v/v), dried under a stream of nitrogen and the residue resuspended in ethanol. The samples were submitted to HPLC analysis using an ODS column (4.6×150 mm; Shimadzu, Kyoto, Japan), eluted with a gradient of 25% to 75% solvent B over 20 min (*solvent A* H_3PO_4 0.01% v/v, *solvent B* acetonitrile 90% v/v in solvent A), and the absorbance of the effluent measured at 230 nm [20]. The experiment was performed with freshly prepared LDE-carmustine complex and repeated daily for up to 2 days.

LDL receptors in a neoplastic cell line: immunohistochemical detection

To prepare for the studies of LDE uptake by neoplastic cells, the presence of LDL receptors was investigated in NCI-H-292 mucoepidermoid lung carcinoma cells. LDL receptors in NCI-H292 cell smears were stained by the immunoperoxidase technique. In summary, this procedure involved applying to cell smears the following sequence: mouse IgG2ak (LP02) anti-human LDL receptor (1:40) for 18 h at 4°C; biotinylated secondary antibody for 30 min; and finally streptavidin-HRP (DAKO LSABR peroxidase system) for 30 min. After each step the smears were washed with phosphate-buffered saline (PBS) and, finally, the peroxidase activity was determined using diaminobenzidine and H_2O_2 (substrate-chromogen solution) for 5–10 min. Smears were counterstained with hematoxylin solution. A positive tissue control for LDL receptor was provided by performing the immunoperoxidase reaction in human liver sections. Negative control smears were performed in the same cell line by omitting the primary antibody.

LDE uptake by tumor cells

These experiments were performed again using NCI-H-292 cells. The cells were plated in 5-mm dishes (10^5 cells/dish) in RPMI-1640 medium containing 10% fetal calf serum (FCS) for 24 h. The next day, the medium was replaced with medium containing 10% lipoprotein deficient serum (LPDS) prepared as described elsewhere [21]. On the third day, the subconfluent cells were allocated to incubation experiments performed in duplicate over 5 h at 37°C, with increasing amounts (0.01 to 1.0 mg/ml) of LDE total lipid labeled with ^{3}H-cholesteryl oleate ether. The cells were then washed three times with cold PBS plus bovine serum albumin and twice with PBS at 37°C followed by the addition of 1.0 ml 0.05% trypsin and incubated on a shaker for 15 min at 37°C. The cell pellets were removed from the dishes, transferred to Eppendorf tubes and centrifuged for 15 min at 14,000 rpm. The supernatant was removed and 0.5 *M* NaOH was added to disrupt the cell pellets under vortex mixing. Radioactivity was measured in liquid scintillation vials (the scintillation solution comprising PPO/DM-POPOP/Triton X-100/toluene, 5 g/0.5 g/333 ml/667 ml) using a Packard 1600 TR spectrometer (Meridien, Ct.) and the protein concentration was determined by the method of Lowry et al. [22]. To ascertain that a chemotherapeutic agent could be internalized together with LDE, the uptake by NCI-H-292 cells of increasing amounts of ^{3}H-paclitaxel associated with LDE labeled with ^{14}C-cholesteryl oleate was measured according to the procedure described above. Paclitaxel was also associated with LDE by incubation with ultrasonication.

To determine the effects of different LDL receptor activities on LDE uptake by NCI-H-292 cells, cell uptake was measured as described above in medium containing LPDS and compared with that in medium containing 10% FCS. In this case, the medium were replaced 2 days before incubation with LDE.

Competition between LDE and LDL for uptake by tumor cells

This experiment was performed with NCI-H-292 cells as described above, but with the addition of increasing amounts of native human LDL to each culture dish. The concentration of LDE labeled with ^{3}H-cholesteryl ether was kept constant at 0.2 mg/ml in each dish. The concentrations of native LDL ranged from 0.01 to 1.0 mg/ml.

LDE-carmustine cytotoxicity

NCI-H-292 cells were grown in RPMI-1640 medium with 10% FCS and plated in flat-bottomed 96-well plates (Corning, Acton, Mass.) for 48 h in an incubator under a CO_2-enriched atmosphere at 37°C. The subconfluent cells were further incubated with carmustine, free or associated with LDE. The experiments were performed in triplicate using different incubation periods (12, 24, 36 and 48 h) and with increasing carmustine doses (5 to 500 μg/ml). After the incubation period, the wells were washed with RPMI-1640 medium in order to remove carmustine or carmustine-LDE, and left for an additional 48 h in RPMI-1640 medium. The number of viable cells was then estimated by the MTT [3-(4,5- dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] assay [23]. The results are expressed as the percentage of surviving cells where 100% corresponds to the number of viable cells in control experiments in which NCI-H-292 cells were incubated with LDE not associated with drug.

LDE-carmustine cytotoxicity at 4°C and 37°C

This experiment was performed to clarify whether LDE-carmustine cytotoxicity is a result of entry of the drug into the cell by the receptor-mediated pathway which is inactive at 4°C [21]. At that temperature binding to the membrane receptors may occur but not internalization into the cytoplasm. NCI-H-292 cells were grown, plated and incubated according to the procedures described above except that 1 h before the addition of the drug and during the 3-h incubation one plate was kept at 4°C and another at 37°C. The carmustine dose, free or associated with LDE, was 200 μg/ml in each well. The postincubation procedure and the MTT cytotoxicity assay were performed as described above.

LDE-carmustine cytotoxicity in the presence of LDL

This experiment was also designed to determine whether LDE-carmustine cytotoxicity is mediated by internalization of the drug through the LDL receptor pathway, together with the emulsion. In this case, the introduction into the incubates of native LDL would block the receptor pathway. Native LDL, obtained from the blood of healthy normolipidemic donors by sequential ultracentrifugation [24], was incubated with the cells together with LDE-carmustine or with the free drug. H-292 cells were grown as described previously. The RPMI-1640 medium containing 10% FCS was replaced 24 h before the assay with medium containing 10% LPDS. The subconfluent cells were then further incubated in triplicate for 3 h at 37°C with 200 μg/ml carmustine, free or associated with LDE, and in the presence of native LDL (100 μg/ml LDL cholesterol).

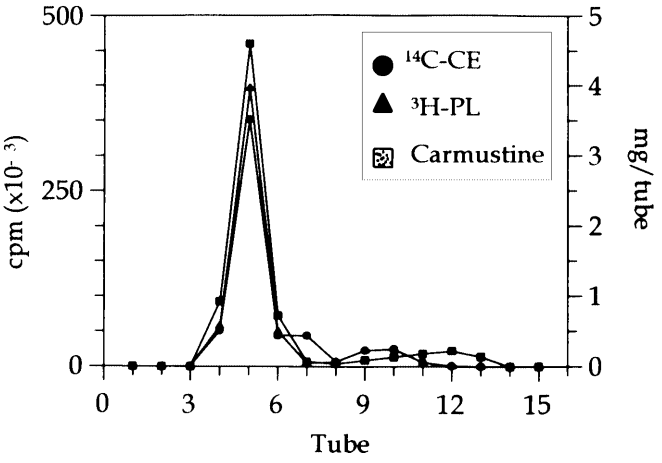

Fig. 1. Elution profile of LDE-carmustine. Carmustine was associated with LDE by cosonication at a ratio of 1:3 (w/w). To determine the amount of carmustine associated with the emulsion, samples were submitted to gel filtration. The emulsion phospholipids and cholesteryl esters were radioactively labeled and their amounts in each sequential effluent flask determined by radioactive counting. The amount of carmustine in each flask was determined by spectrophotometry at 230 nm

Another experiment was performed to determine the dose-response curve of native LDL upon cell survival under LDE-carmustine treatment. Increasing amounts of native LDL (5 to 100 μg/ml) were added to incubates of H-292 cells with 200 μg/ml carmustine as LDE-carmustine. After incubation, the cells were washed twice with PBS and RPMI and 10% FCS was added followed by a 48-h incubation at 37°C.

LDE-carmustine biodistribution in mice

For comparison of the biodistribution of LDE-carmustine with that of LDE alone, LDE without carmustine and the LDE-carmustine complex both labeled with ^{3}H-cholesteryl oleate ether were separately injected into the tail vein of two groups of ten BalbC male mice each weighing about 20 g. After 12 h the animals were killed by cervical dislocation and brain, lung, liver, spleen and kidney were removed. Tissue lipids were extracted [25] with chloroform/methanol (2:1 v/v) and lipid classes were separated by thin layer chromatography (TLC) in the solvent system hexane/diethylether/acetic acid (70:30:1 v/v/v). TLC plate bands corresponding to cholesteryl ester were scraped into vials and radioactivity was measured in a scintillation solution as described above.

LDE-carmustine toxicity in mice

LDE-carmustine at carmustine doses of 12.5, 25, 50, 100 and 150 mg/kg was injected intraperitoneally into five consecutive groups of 12 female BalbC mice. Animals were kept under a 12/12 h light/dark cycle. Food and water were available ad libitum. After 5 days they were killed by cervical dislocation.

LDE-carmustine plasma kinetics in patients

To determine whether association with carmustine would change LDE kinetics in plasma, the disappearance of LDE-carmustine was compared with that of LDE alone. LDE-carmustine labeled with ^{14}C-cholesteryl oleate and LDE labeled with ^{3}H-cholesteryl oleate were simultaneously injected as a bolus into two cancer patients. Blood samples were taken at pre-established times after injection. Determination of the disappearance curves of the labeled lipids of both LDE-carmustine and LDE alone was performed as described previously [1].

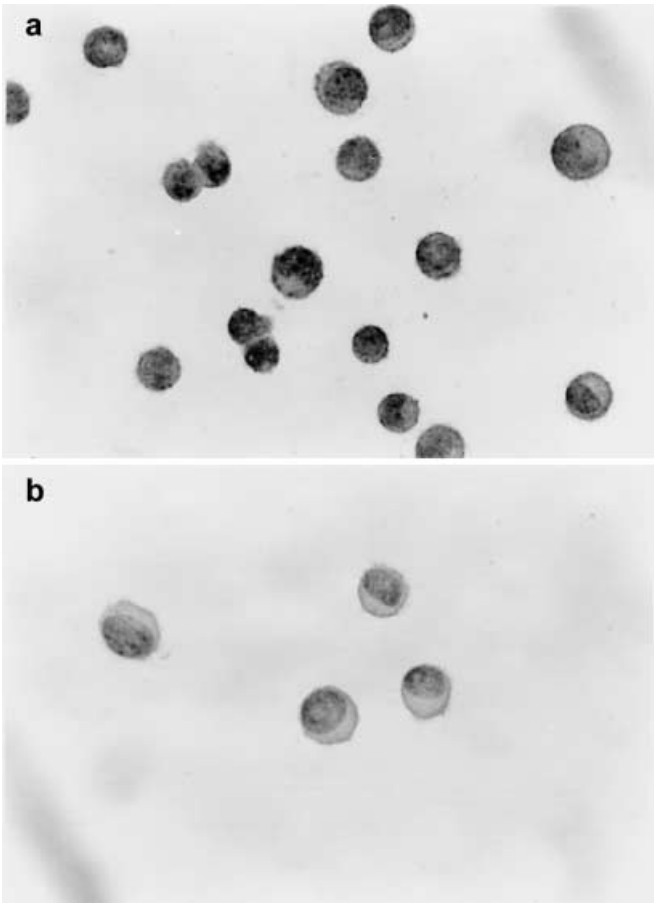

Fig. 2a, b. Immunoperoxidase reaction of NCI-H292 cell smears for LDL receptors. H_2O_2 plus DAB was used as substrate and hematoxylin solution for counterstaining (**a** positive reaction, **b** negative reaction omitting the primary antibody). Light microscopy ×400

LDE-carmustine uptake by tumor and normal tissues

For direct determination of whether LDE-carmustine is preferentially taken up by tumor tissue, LDE labeled with ^{14}C-cholesteryl oleate and associated with carmustine was injected intravenously 24 h before surgery into three cancer patients, two being submitted to total mastectomy and one to panhisterectomy. Total radioactivity of 222 kBq was injected into each patient. After excision of the tissues during surgery, fragments of tumor and normal mammary tissue from the first two patients or of the contralateral normal ovary from the third patient were collected. Anatomopathological examination was performed in the tissue fragments which were then submitted to lipid extraction followed by separation of lipid classes by TLC and radioactive counting as described above.

Pilot clinical study of the LDE-carmustine toxicity profile

LDE-carmustine at 25% escalating carmustine doses was given to two groups of six patients at the two initial dose levels and to three groups of ten patients at the three subsequent dose levels. The starting dose was 150 mg/m^2 body surface, that is the dose of free carmustine routinely utilized in clinical practice. LDE-carmustine was given intravenously over 2 h every 4 weeks. The flask

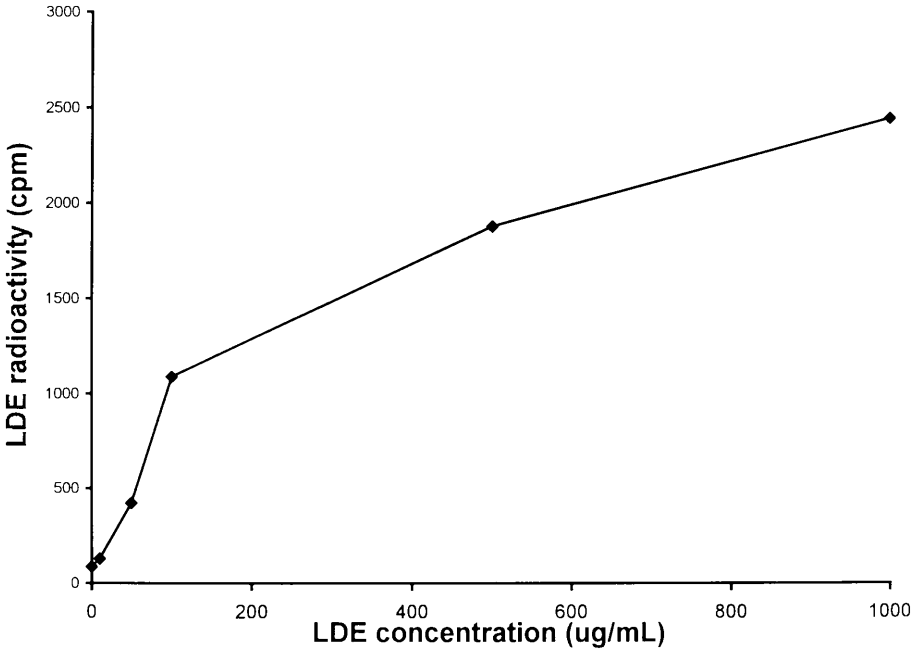

Fig. 3. LDE uptake by NCI H-292 tumor cells. Subconfluent cells were incubated in triplicate for 5 h at 37°C with increasing amounts (0.01 to 1.0 mg/ml) of LDE labeled with ^{3}H-cholesteryl oleate ether. After trypsinization and centrifugation, the washed cell pellets were disrupted with 0.5 *M* NaOH and the radioactivity was measured in liquid scintillation solution

containing the solution was protected from light. The patients received 8 mg ondansetron in 50 ml 0.9% saline with 5% glucose before infusion of the LDE-carmustine.

The protocol design required that patients be immediately removed from the study on diagnosis of disease progression. Such assessments were carried out every 3 weeks. The patients would also be dropped from the study on the appearance of grade 3 or 4 clinical or laboratory toxicity. Histories were taken, physical examination carried out, and transaminases, alkaline phosphatase, total bilirubin, urea and creatinine determined at baseline and at least monthly. Complete blood counts and differential counts were determined at baseline and weekly. Toxicities were graded according to the World Health Organization (WHO) criteria.

Results

Association of carmustine with LDE and carmustine stability

Figure 1 shows the elution profile of the phospholipids, cholesterol ester and carmustine of the LDE-carmustine complex. The elution profiles of the emulsion lipids were similar to that of the drug. Under the conditions for incorporation used in this study (1:3 carmustine/emulsion total lipid, w/w, coincubated under ultrasonication) about 80% became associated with the emulsion ($81 \pm 4\%$, mean $\pm$ SD calculated from five incorporation experiments, range 77–85%) as calculated by the amount of the components of the complex in each elution flask.

Carmustine incorporated into LDE was stable at 4°C for up to 2 days from the time of preparation, as determined from six different LDE-carmustine preparations analyzed by HPLC. As measured by light scattering, the diameter of the emulsion particles increased by 10% after incorporation of carmustine.

LDL receptors in NCI-H292 cells

An intense immunoperoxidase reaction was observed in H-292 cells indicating the presence of LDL receptors (Fig. 2a). Omitting the primary antibody resulted in a negative reaction in H-292 cells confirming the specificity of the reaction (Fig. 2b).

LDE uptake by neoplastic cells and competition with LDL

^{3}H-Cholesteryl-ether-labeled LDE incubated in increasing amounts with H-292 cells was taken up by the cells in a typical dose-saturating curve (Fig. 3). H-292 cells incubated with increasing amounts of LDE-paclitaxel labeled with both ^{3}H-paclitaxel and ^{14}C-cholesteryl oleate showed a proportional increase in the uptake of both labels (Fig. 4). This indicates that paclitaxel was internalized into the cells together with the emulsion. Replacement of FCS by LPDS resulted in marked enhancement of LDE uptake by the tumor cells (Fig. 5). The addition to H-292 cells of increasing amounts of native LDL progressively decreased the uptake of ^{3}H-cholesteryl-ether-labeled LDE (Fig. 6).

LDE-carmustine cytotoxicity

Figure 7 shows the results of the experiments in which H-292 tumor cells were incubated with carmustine alone or associated with LDE for various periods of time. The dose-response curves of the free drug were similar to those obtained with the LDE-carmustine complex. Therefore, the cell killing capacity of the drug was preserved when complexed with LDE.

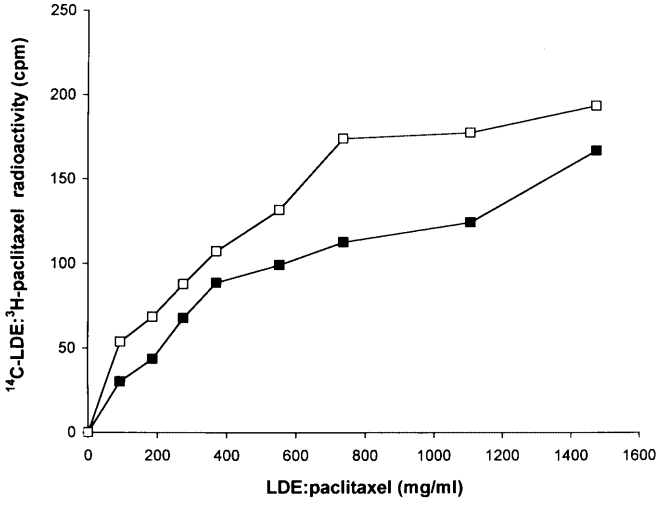

Fig. 4. Uptake by NCI-H-292 cells of ^{3}H-paclitaxel associated with ^{14}C-cholesteryl oleate-labeled LDE. Subconfluent cells were incubated in triplicate for 5 h at 37°C with increasing amounts (0.1 to 1.5 mg/ml) of LDE-paclitaxel. After trypsinization and centrifugation, the washed cell pellets were disrupted with 0.5 *M* NaOH and the radioactivity was measured in liquid scintillation solution

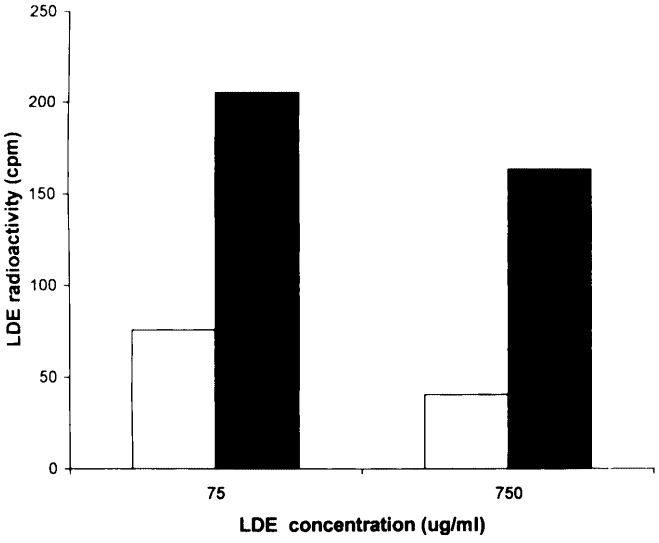

Fig. 5. LDE uptake by NCI H-292 tumor cells after exposure to 10% FCS (*white columns*) and to lipoprotein-deficient serum, LPDS (*black columns*). After a 24-h exposure and 2 days before incubation with LDE the medium containing either FCS or LPDS was replaced. Subconfluent cells were incubated in triplicate for 5 h at 37°C with 75 or 750 μg/ml of LDE labeled with ^{3}H-cholesteryl oleate ether. After trypsinization and centrifugation, the washed cell pellets were disrupted with 0.5 *M* NaOH and the radioactivity was measured in liquid scintillation solution

Role of the LDL receptor in LDE-carmustine cytotoxicity

Survival of H-292 cells was considerably increased by incubation with LDE-carmustine at 4°C compared with incubation at 37°C (Fig. 8). On the other hand, free carmustine cytotoxicity was much less affected by the change in temperature. When 100 μg/ml native LDL was added to the cells with LDE-carmustine, cell survival was increased more than twice (Fig. 9). In contrast, the addition of LDL did not affect the cytotoxicity of the free drug. Figure 10 shows the dose-response effect of native LDL on the survival of cells treated with LDE-carmustine. The addition of increasing amounts of native LDL to H-292 cells with LDE-carmustine progressively increased the cell survival rate.

Biodistribution of LDE and of LDE-carmustine in mice

Figure 11 shows the biodistribution of LDE in mice after administration of LDE-carmustine or LDE alone, obtained 24 h after injection of the emulsion preparations labeled with ^{3}H-cholesteryl oleate ether into two animal groups. The uptake of the emulsion label by liver, spleen, kidney, lung and brain was similar between LDE and LDE-carmustine. Thus, the association of carmustine with LDE did not alter the biodistribution profile of the emulsion. The liver was the main uptake organ for both LDE and LDE-carmustine.

LDE-carmustine toxicity in mice

No deaths among mice were recorded during the observation period after the administration of LDE-carmustine at any of the carmustine dose levels. In this study, it was not possible to achieve lethal doses since administration of higher LDE-carmustine doses would have meant the injection of excessive volumes of solution that would have precluded the accomplishment of the experiments.

LDE and LDE-carmustine plasma decay curve

As observed in two patients, the decay curve of LDE labeled with ^{3}H-cholesteryl oleate was virtually identical to that of the LDE-carmustine complex labeled with ^{14}C-cholesteryl oleate (Fig. 12). Therefore, the association of carmustine with LDE did not alter the kinetic behavior of the emulsion in the plasma.

LDE-carmustine uptake by tumor and normal tissues

Table 2 shows the amount of radioactivity from the labeled LDE-carmustine complex taken up by tumor tissue 12 h after injection of the complex into three cancer patients for comparison with the uptake by the corresponding normal tissues. In the two patients with mammary adenocarcinoma the amount of LDE-carmustine taken up by the tumor was 13-fold and 5-fold more than that taken up by the normal mammary tissue in the two patients respectively. In the patient with ovary adenocarcinoma the amount of LDE-carmustine radioactive label was 3-fold more in the tumor than in the normal tissue.

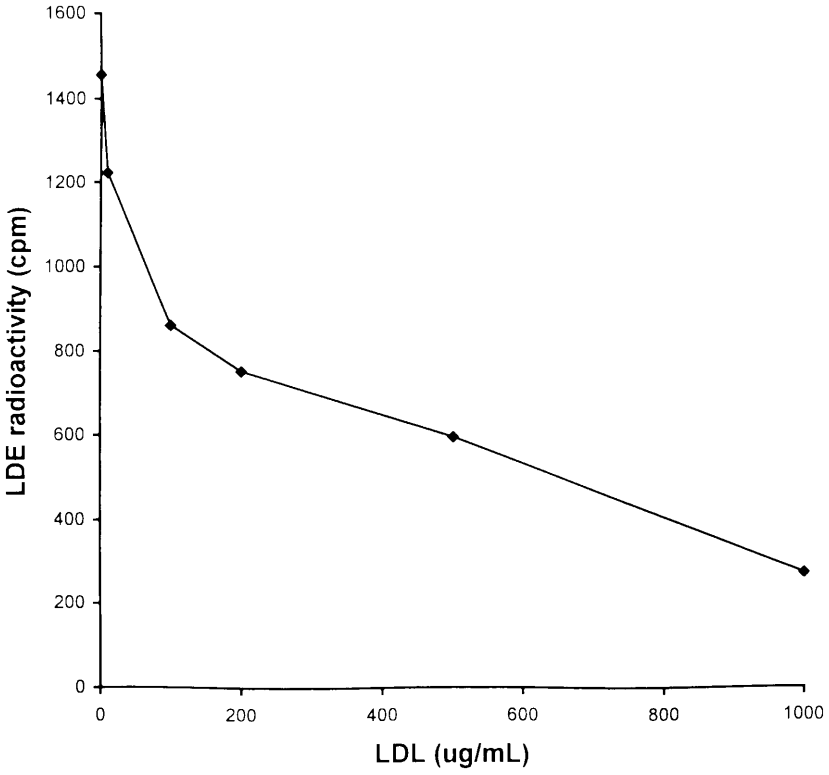

Fig. 6. Competition between LDE and native LDL for uptake by NCI-H-292 cells. Cells were incubated in the presence of increasing concentrations of native human LDL ranging from 0.01 to 1.0 mg/ml. The concentration of LDE labeled with ^{3}H-cholesteryl ether was fixed at 0.2 mg/ml in each culture dish

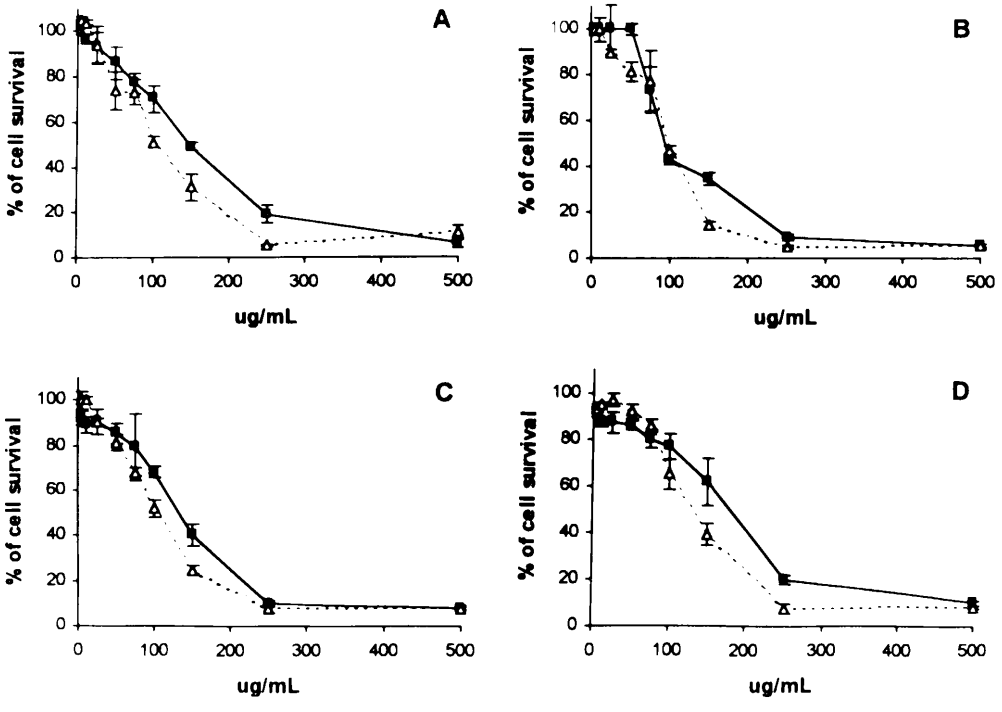

Fig. 7A–D. Cytotoxicity of free carmustine (*open triangles*) and LDE-associated carmustine (*black squares*). Cells were incubated with increasing doses (5 to 500 μg/ml) of free carmustine or LDE-carmustine for 12 h (**A**), 24 h (**B**), 36 h (**C**) and 48 h (**D**) at 37°C. After washing with RPMI-1640 medium to remove the drug, cells were kept for an additional 48 h in RPMI-1460 medium. Viable cells were then estimated by the MTT assay and their numbers expressed as a percentage in relation to surviving cells compared to control experiments in which LDE was incubated without the drug

LDE-carmustine toxicity in cancer patients

As shown in Table 3, five escalating dose levels were evaluated in this exploratory clinical study. Six patients were studied at 150 and 190 mg/m^2 and ten patients at 240, 300 and 350 mg/m^2. A total of 128 cycles were analyzed. LDE-carmustine showed no or minimal toxicity at all six dose levels. WHO toxicity grade 2 was observed in only two patients. One was receiving 300 mg/m^2 carmustine and had grade 2 toxicity in AST level on one treatment course. The other was receiving 350 mg/m^2 and had grade 2 toxicity exclusively in alkaline phosphatase level on two consecutive treatment courses. Grade 3 or 4 toxicities were not reported.

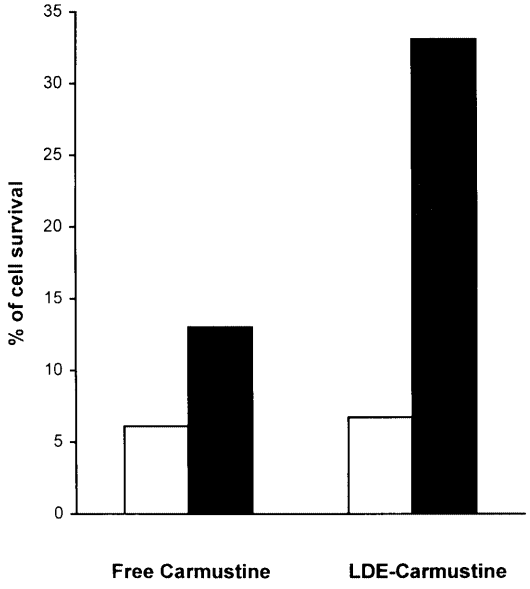

Fig. 8. Cytotoxicity of LDE-carmustine and free carmustine at 4°C (*black columns*) and 37°C (*white columns*). Columns represent percentage cell survival (100% survival corresponds to viable cells of control experiments in which cells were incubated without the drug). Viable cells were estimated by the MTT assay

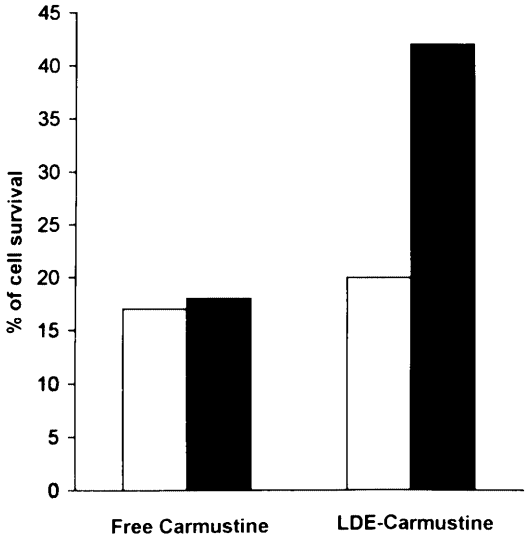

Fig. 9. Cytotoxicity of LDE-carmustine and free carmustine in the presence (*black columns*) or absence (*white columns*) of 100 μg/ml native LDL (100 μg/ml total cholesterol). Columns represent percentage cell survival (100% survival corresponds to viable cells of control experiments in which cells were incubated without the drug)

Mucositis, diarrhea, arterial hypertension, and psychotic, sensorial and neuromotor disturbances were not observed in the overall treatment cycles (not shown).

Discussion

Because we have previously shown that LDE concentrates in solid tumors of breast and ovary cancer, it is reasonable to assume that the emulsion may concentrate in the several neoplasias in which LDL receptor upregulation has previously been documented, such as glioma [26], breast cancer [27], myeloproliferative diseases [28], prostate carcinoma [29], gallbladder cancer [30, 31], endometrial adenocarcinoma [12], uterine cervical adenocarcinoma [12] and lung carcinoma [32].

Emulsions similar to that used in the current and in our previous studies have been well characterized elsewhere [19, 33]. They are constituted of stable homogeneous spherical particles with a cholesteryl ester core surrounded by a phospholipid monolayer. Because none of the LDE components is immunogenic and the emulsion is merely a structural analog of a lipoprotein existing in the plasma, the organism could not conceivably be harmed by its administration. However, it was still possible that the association of carmustine with LDE could change the structure of the emulsion in such a way as to adversely affect the adsorption of circulating apolipoproteins and binding to the receptors. In this respect, a 10% swelling of LDE particles was detected by light scattering measurements. Nonetheless, enough data were gathered in this study to show that the association with the drug did not change the biological properties of the emulsion. First, the disappearance curve of LDE from plasma in subjects was identical to that of the LDE-carmustine complex. If the presence of the drug in LDE had changed the ability of the emulsion to bind to the LDL receptors, the LDE plasma decay curve would certainly have been altered. Second, the finding that LDE-carmustine and LDE alone had similar biodistributions in mice also indicates that association with the drug did not substantially change the uptake of the emulsion by the receptor mechanisms.

Whether carmustine was damaged by the process of association involving cosonication of the drug with LDE was also investigated in this study. The HPLC spectra obtained suggested that the chemical state of carmustine was preserved and it remained stable for longer than the duration of the cell, animal and patient experiments. This suggests that an eventual reduction in toxicity of LDE-carmustine compared with the free drug could not be ascribed to an alteration in the chemical status of the drug by the process of complex formation.

In this study, the immunoperoxidase reaction technique demonstrated the intense presence of LDL receptors in the cell smears, and it was also shown that the mucoepidermoid lung carcinoma cell line (NCI-H-292) took up LDE in a dose-saturating manner that resembled that of native LDL. The internalization of LDE via LDL receptor was confirmed by enhancement in the uptake of the emulsion when cells were exposed to LPDS medium. This is a classical maneuver to increase the expression of LDL receptors. Thus the increased LDE uptake implies receptor-mediated internalization. Moreover, when paclitaxel was incorporated into LDE, it was internalized into the tumor cells together with the emulsion, as shown in the double-labeled

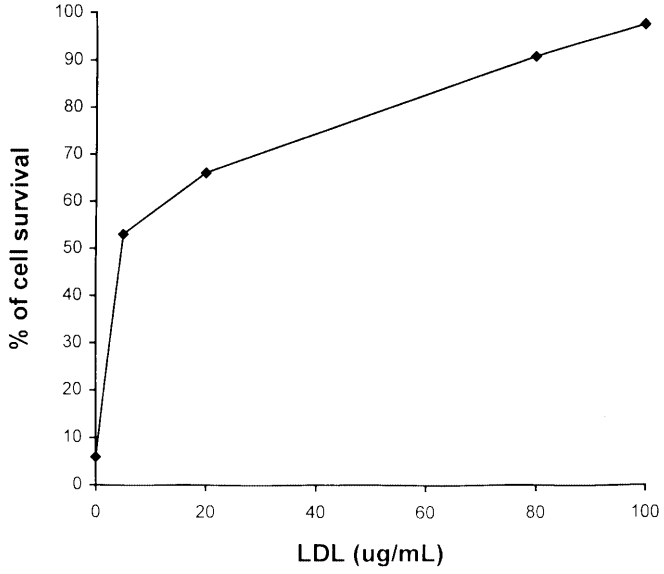

Fig. 10. Cytotoxicity of LDE-carmustine in the presence of native LDL. The carmustine dose in each dish was 200 μg/ml. H-292 cells were incubated with increasing amounts of native LDL (5–100 μg/ml total cholesterol). Cell survival (viable cells expressed as a percentage of those in the absence of drug) was estimated by the MTT assay

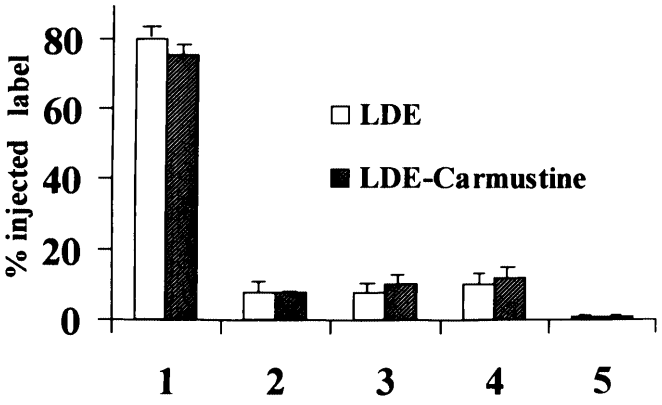

Fig. 11. Biodistribution of LDE without carmustine and of the LDE-carmustine complex in mice. Uptake by liver (*1*), spleen (*2*), kidney (*3*), lung (*4*) and brain (*5*). LDE and LDE-carmustine both labeled with ^{3}H-cholesteryl oleate ether were separately injected into the tail vein of two groups of ten BalbC male mice. After 12 h the animals were killed and the organs excised for lipid extraction and determination of radioactivity uptake

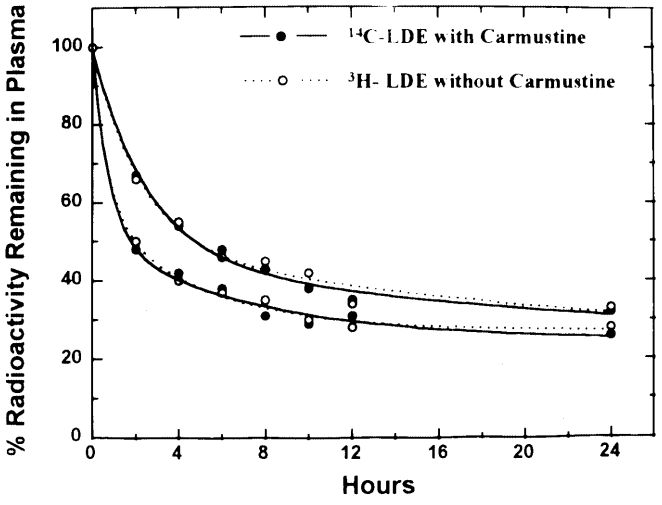

Fig. 12. Decay curves of ^{3}H-cholesteryl oleate-labeled LDE and ^{14}C-cholesteryl oleate-labeled LDE-carmustine injected intravenously simultaneously as a bolus into two breast cancer patients. Radioactivity was measured in a scintillation solution from plasma samples collected at the indicated times after injection

LDE-paclitaxel cell uptake experiments. Radioactive paclitaxel was used instead of carmustine because its greater stability offered better measurement conditions during the long (5-h) cell incubation period.

LDE internalization by NCI-H-292 via LDL receptors was further supported by the competition experiments in which LDE uptake by the neoplastic cells was disrupted by the addition of increasing amounts of native LDL. These experiments showed that native LDL and the emulsion particles competed for the same receptor mechanisms, conceivably the LDL receptors, in this neoplastic cell line. These experiments provided the rationale for the use of H-292 cells in the subsequent LDE-carmustine cytotoxicity experiments, along with the responsiveness to this drug.

Following association with LDE the chemotherapeutic agent presumably enters the cytoplasm via a pathway different from that of the free drug, with conceivably different intracellular compartmentalization, and an important question is therefore whether cytotoxicity is preserved when the drug is complexed with LDE. In this respect, the results of the experiments in which carmustine, free or associated with LDE, was incubated with H-292 cells indicate that the killing capacity of the drug was not diminished by association with LDE. This was expected because LDE was internalized into the cells by the same receptor-mediated endocytosis mechanisms involved in the take up of native LDL, and anticancer agents associated with the native lipoprotein preserve their cytotoxicity [13, 30, 34, 35].

The question as to whether the cytotoxicity of LDE-carmustine is in fact mediated via the LDL receptor endocytic pathway was addressed in two experiments. This is an important issue because the LDE-carmustine cytotoxicity observed here could well have been due to dissociation of the drug from the emulsion in the incubates. The dissociated carmustine could then freely cross the cellular membrane and exert its cytotoxicity. In the first experiment, in incubations performed at 4°C binding to the receptors could occur but the process of lipoprotein internalization via receptor is blocked at this temperature [21]. As expected, the cytotoxicity of LDE-carmustine was inhibited but not the cytotoxicity of the unassociated drug. In a second experiment designed to clarify this issue in which LDE was incubated with neoplastic cells in the presence of increasing amounts of LDL, the uptake of the emulsion by the cells was

Table 2. LDE-carmustine uptake by the tumor and the corresponding normal tissue. LDE-carmustine labeled with ^{14}C-cholesteryl oleate was injected 12 h before surgery and the amounts of radioactivity in fragments of the tumor and of normal mammary or ovarian tissue were quantified (expressed as counts per minute per gram of tissue) in a scintillation solution after lipid extraction and separation of lipid classes by thin layer chromatography

Patient age (years)	Diagnosis	LDE-carmustine uptake (cpm/g tissue)		Tumor/normal
		Tumor	Normal	
68	Ductal mammary adenocarcinoma	1439	112	12.8
70	Ductal mammary adenocarcinoma	1284	277	4.6
72	Mucinous ovary adenocarcinoma	1594	546	2.9

Table 3. Toxicity (WHO grade[a]) of the carmustine-LDE complex administered intravenously as a 2-h infusion every 4 weeks at escalating doses to 42 cancer patients. The values are the percentage of cycles showing that toxicity. The numbers of treatment cycles analyzed at each dose level were 16, 20, 28, 35 and 29 cycles, respectively

	Carmustine dose (mg/m^2 body surface)									
	150		190		240		300		350	
	Grade 1	Grade 2	Grade 1	Grade 2	Grade 1	Grade 2	Grade 1	Grade 2	Grade 1	Grade 2
Nausea	25	0	0	0	18	0	14	0	14	0
Vomiting	13	0	20	0	0	0	0	0	24	0
Local pain	31	0	60	0	14	0	40	0	59	0
Arterial hypertension	0	0	0	0	0	0	3	0	7	0
Fever	0	0	0	0	0	0	0	0	0	0
Dyspnea	0	0	0	0	0	0	0	0	0	0
Alopecia	0	0	0	0	0	0	3	0	0	0
Anemia	0	0	0	0	0	0	3	0	17	0
Leukopenia	0	0	0	0	0	0	3	0	24	0
Thrombocytopenia	0	0	0	0	7	0	6	0	0	0
Hepatic										
AST	0	0	0	0	14	0	6	3	17	0
ALP	0	0	0	0	0	0	6	0	34	0
Bilirubin	0	0	0	0	0	0	6	0	5	0
Renal										
Urea	0	0	0	0	0	0	6	0	0	0
Creatinine	0	0	0	0	0	0	0	0	0	0
Alkaline phosphatase	0	0	10	0	10	0	20	0	34	7

progressively inhibited by occupation of the receptors by the competing lipoprotein. As expected, the toxicity of LDE-carmustine progressively decreased with increasing amounts of added lipoprotein. In contrast, the cytotoxicity of carmustine alone, that freely crosses the cell membrane, was unaffected by the addition of LDL to the incubates. These results strongly favor the assertion that, when associated with LDE, carmustine does indeed exert its pharmacological action by entering the cell through the receptor-mediated pathway.

The results of the experiments in which LDE-carmustine uptake was measured in fragments of tumor and the corresponding normal tissue of cancer patients confirmed that, in a similar manner to LDE alone, the complex was preferentially taken up by the tumor while avoiding the normal tissues. It would be desirable if carmustine associated with LDE could also be labeled so that the uptake of drug carried in LDE could be estimated directly. This was not attempted because the rapid degradation of carmustine would prevent this measurement. However, the steroid ring of cholesteryl esters is not prone to degradation in the body, allowing the use of the radioactive compound as a label of the tissue uptake of the complex.

LDE-carmustine toxicity to mice was remarkably low when compared with that of the free drug described in the literature. The DL_{50} of free carmustine is in the range of 25–30 mg/kg body weight, whereas no deaths occurred with doses of carmustine associated with LDE up to 150 mg/kg.

In our preliminary clinical approach, the 42 patients treated with LDE-carmustine showed remarkable tolerance of the complex, from lower dose ranges to doses much higher than the standard doses used in conventional carmustine monochemotherapy (150–200 mg/m^2 body surface) which also uses shorter treatment times (6 weeks in conventional chemotherapy). Because we had already attained a very high dose level at which the

ability of our laboratory set-up to produce the complex for patient injection was challenged, the study was discontinued at the 350 mg/m^2 dose level despite the fact that patients still showed excellent tolerance of the drug at this dose level. It is worth mentioning that LDE can potentially be produced by other techniques such as high-pressure homogenization and extrusion that may allow the large-scale manufacture of the emulsion.

The major toxic effects of carmustine are usually leukopenia and thrombocytopenia. Thrombocytopenia occurs 3 to 5 weeks after a dose with a low period of 1 to 2 weeks duration. Leukopenia occurs 4 to 6 weeks after a dose with a low period of similar duration [16, 36]. In our study hematological toxicity resulting from LDE-carmustine administration was virtually absent. Clinical and laboratory monitoring also excluded nephrotoxicity, hepatotoxicity, and pulmonary fibrosis that eventually occur in nitrosourea-treated patients [37]. Lack of significant hepatotoxicity is of particular interest since the liver is the major organ of uptake of LDE-carmustine, as shown by the biodistribution experiments performed in the rat, as it is of natural LDL.

The diminution of the side effects of carmustine by association with LDE may not be solely attributed to the new biodistribution with the presumed tropism for cancer cells and diminished entry into normal tissues. Carmustine is a very reactive alkylating agent that attacks many biological targets such as proteins and nucleic acids, hence its toxicity. By diminishing direct contact with those targets in the body, association of carmustine with LDE may offer other mechanisms by which toxicity is diminished. In this regard, it has been shown that the addition of native lipoproteins to aqueous medium stabilizes the nitrosoureas against degradation [38]. Spectrophotometric assays of carmustine degradation have shown that the half-life of the drug increases more than twice when associated with LDE at 37°C. At room temperature LDE-carmustine is still cytotoxic for up to 120 h, whereas free carmustine loses all cytotoxicity within 24 h (Melo et al., unpublished results).

A limitation of this study was that only the plasma kinetics of the lipid (cholesteryl ester) component of the LDE-carmustine complex was determined. Investigation of the kinetics of the carmustine component compared to those of the emulsion cholesteryl ester would allow a determination of whether the drug remains associated with the emulsion while in the plasma compartment or whether some dissociation occurs, despite the poor solubility of the drug in aqueous medium. This issue will be addressed as part of an ongoing formal phase I study in which the pharmacokinetics of the drug associated with the emulsion and the maximal tolerated dose of LDE-carmustine will be determined in cancer patients.

In conclusion, association of an antineoplastic drug with LDE preserves cytotoxicity and improves the tolerability in cancer patients, so that very high doses can be administered without major side effects. Association with LDE may increase the usefulness of antineoplastic drugs in cancer treatment.

Acknowledgements The authors are indebted to Mr. Renato Barboza for expert technical assistance. This study was supported by FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo; grant nos. 95/0591-9 and 99/01229-2). R.C.M. holds a research award from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), Brasilia, Brazil.

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