

***In vitro* cytotoxicity of the LDE:daunorubicin complex in acute myelogenous leukemia blast cells**

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Abstract

Acute myelogenous leukemia (AML) blast cells show high-affinity degradation of low-density lipoprotein (LDL), suggesting an increased expression of cellular LDL receptors. LDE is a lipid microemulsion easily synthesized *in vitro* which is known to mimic the metabolic pathway of LDL. We used LDE as a carrier for daunorubicin and assayed the cytotoxicity of the complex using AML blast cells since RT-PCR analysis showed that AML cells express LDL receptor mRNA. The LDE:daunorubicin complex killed 46.7% of blast cells and 20.2% of normal bone marrow cells ($P < 0.001$; Student *t*-test). Moreover, this complex destroyed AML blast cells as efficiently as free daunorubicin. Thus, LDE might be a suitable carrier of chemotherapeutic agents targeting these drugs to neoplastic cells and protecting normal tissues.

Key words

- Cytotoxicity
- Acute myelogenous leukemia
- Daunorubicin
- Low-density lipoprotein
- LDE complex
- RT-PCR

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Introduction

Low-density lipoprotein (LDL) is the lipoprotein that carries most of the cholesterol contained in the plasma compartment and is removed from the circulation into cells by specific receptors on the cell surface (1). The receptors recognize apolipoprotein (apo) B100, which is the only protein moiety of LDL particles, and apolipoprotein E (apo E) (2). LDL degradation is up-regulated in several cancer cell lines (3), probably because of the great demand of lipids for synthesis of new membranes required by cell proliferation in neoplasia. As originally described by Ho et al. (4) in 1978, LDL degradation reaches

up to one hundred-fold that of normal cells in acute myelogenous leukemia (AML) blast cells. It was then demonstrated that LDL could be used as a drug carrier to target neoplastic cells, while avoiding normal tissues and organs (5). Internalization of LDL-containing chemotherapeutic agents into neoplastic cells resulting in accumulation of the drug in the cytoplasm was in fact demonstrated in several *in vitro* studies (6-9). Individuals with ovarian and endometrial cancer receiving weekly doses of vincristine complexed with LDL did not show any of the side effects commonly associated with the drug (10). Although this result demonstrates that a chemotherapeutic agent encapsulated in LDL

can be safely given to cancer patients, the lipoprotein is still difficult to obtain and handle, so that its introduction in clinical practice is rather problematic. Moreover, the use of a human blood product carries the risk of transmission of infectious diseases.

In 1982, Ginsburg et al. (11) synthesized *in vitro* a microemulsion resembling the lipid portion of LDL. This emulsion (LDE) is composed of phospholipids and cholesteryl esters. When the microemulsion is incubated with high-density lipoproteins or injected into the bloodstream, several exchangeable apolipoproteins shift from the native lipoproteins to the artificial emulsion (12) including apo E. Apo E may permit LDE binding to the LDL receptor (rLDL) (13) and/or rLDL-related protein (14). Several experimental observations on humans and animals have indicated that LDE may be taken up by rLDL and/or by receptors that may recognize apo E (12,15,16).

The objective of the present study was to determine whether daunorubicin complexed with LDE can also be taken up by neoplastic cells and kill them. This microemulsion was labeled with ^{125}I and incubated with K562 cells (a cell line derived from chronic myelogenous leukemia blast cells) and membrane-bound and internalized particles were monitored. Cytotoxicity experiments were performed using the LDE:daunorubicin complex incubated with AML blast cells and normal bone marrow cells.

Material and Methods

Subjects

Seven normal bone marrow donors (4 males and 3 females aged 10-40 years) and seven patients with AML (4 males and 3 females aged 15-50 years) participated in the study. The AML patients showed 50 to 92% blast cells in peripheral blood as diagnosed by cytological, cytochemical and immunophenotyping analysis.

Cells

Blast cells were obtained from AML patients after hemolysis of whole peripheral blood with 1 mM NH_4CO_3 and 144 mM NH_4Cl for 10 min at 8°C. Control cells were obtained from the bone marrow of normal marrow donors. Cell viability was >98% as evaluated by the Trypan blue exclusion method. The K562 cell line (17) obtained from a chronic myelogenous leukemia patient was a gift from Dr. R.R. Brentani, Ludwig Institute of Cancer Research, São Paulo, SP, Brazil. The cells were kept in RPMI 1640 (Gibco-BRL, Gaithersburg, MD, USA) containing 10% FCS (Gibco-BRL) in a 5% CO_2 atmosphere at 37°C, and were preserved in liquid nitrogen until the time for use.

Preparation of the emulsion

The emulsion was prepared as described by Maranhão et al. (15). The apo E necessary for binding to the receptor was obtained from FCS (2). LDE was sterilized by passage through a 0.2- μm filter and was used in the experiments described below. The LDE emulsion was stable at 4°C for four weeks.

Preparation of the LDE:daunorubicin complex

Two to five milligrams of daunorubicin (Sigma, St. Louis, MO, USA) was diluted in methanol, dried under a nitrogen stream and resuspended in 6 to 15 mg (total lipids) of LDE solution. The mixture was sonicated for 5-10 min using a Branson Cell Disrupter model 450 equipped with a flat tip, with a 20-watt output in the "continuous" operating mode under an N_2 atmosphere, with the temperature kept below 30°C. After sonication the complex was submitted to gel filtration using a PD-10 (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) column equilibrated and eluted with 0.15 M NaCl. The effluent was monitored by absorbance

at 480 nm. The complex was recovered in the void volume. The amount of daunorubicin incorporated into LDE was estimated by extracting a sample with methanol:chloroform (1:3, v:v), drying under a nitrogen stream, resuspending the residue with 0.15 M NaCl, and absorbance reading at 480 nm. The concentration was calculated using a standard curve. Under the conditions used in this experiment the proportion of daunorubicin to LDE in the complex was 10 nmol/mg of LDE. In all experiments the LDE: daunorubicin complex used was freshly prepared and purified by gel filtration.

LDE labeling

The emulsion was labeled with ^{125}I in 1 M PBS, pH 9.0 (18), and purified by gel filtration through a PD-10 column equilibrated and developed with the same buffer. About 90% of the label was found in the phospholipid fraction after 20% TCA precipitation and thin-layer chromatography. The mean specific activity was 40,000 cpm/ μg .

LDE binding and uptake

To determine ideal conditions for LDE uptake, 10^6 K562 cells were incubated with 0.1-10 $\mu\text{g}/\text{ml}$ (total lipid concentration) of ^{125}I -LDE for 3 h at either 37°C or 4°C, in 1 ml of RPMI 1640 containing 10% FCS. After incubation, the cells were washed three times with PBS. To evaluate the plasma cell-bound ^{125}I -LDE, the cells were treated with 2 mg/ml trypsin for 15 min at 37°C and centrifuged and the supernatant was counted in a gamma-counter (COBRA II, Auto-Gamma, Packard, Canberra, Australia). The cells were then lysed with 0.1 M NaOH and the incorporated ^{125}I -LDE was determined as described above.

Cytotoxicity of the LDE:daunorubicin complex

Blast or normal bone marrow cells (10^6

cells) were incubated with 0.1-2.4 nM of either the LDE:daunorubicin complex or free daunorubicin for 3 h at 37°C. After incubation, the cells were washed three times with PBS, resuspended in RPMI 1640 without phenol red, incubated with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide thiazolyl blue) for 4 h at 37°C and washed three times with the same medium to remove free MTT (19). The cells were then lysed with 10% SDS (Sigma) and 0.01 N HCl and the incorporated MTT was determined by absorbance at 540 nm using an ELISA reader (Microwell System, Organon Teknika, Boxtel, Holland). The apparent sensitivity ($\log \text{EC}_{50}$) to the LDE: daunorubicin complex was calculated using the Graph Pad InPlot™ software (Graph Pad Software, Inc, v.4.00).

RNA extraction and RT-PCR analysis

Total cellular RNA was extracted from 10^7 AML or normal bone marrow cells using Trizol (Gibco-BRL) according to manufacturer instructions. First-strand cDNA was prepared by incubation of 3 μg of total RNA (pool of three samples) with Superscript II reverse transcriptase (Gibco-BRL) and random hexamers at 42°C for 50 min. Four micrograms of normal bone marrow cDNA or 1 μg of AML blast cell cDNA was amplified by PCR using 0.5 U ampli Taq DNA polymerase (Gibco-BRL) and either β -actin primers (sense: 5'-ATCAT GTTTGAGACCTTCAACAC-3'; antisense: 5'-TCTGCGCAAGTTAGGTTTTGTC-3') or LDL primers (sense: 5'-TTGTTGGCTGAAA ACCTACTGTCCC-3'; antisense: 5'-CAAGGCCGCGAGGTCTCAGGA-3'). To confirm the identity of the rLDL PCR product, a second round of amplification was performed using nested primers (sense: 5'-CAATGTCTC AACAAGCTCTG-3'; antisense: 5'-TCTGTC TCGAGGGGTAGCTG-3') in order to obtain a 273-bp segment. Cycle conditions were 94°C, 4 min, 35 step cycles (94°C, 45 s; 55°C, 1 min 30 s, and 72°C, 1 min 30 s) and

72°C, 10 min. Fifteen microliters of the PCR products was electrophoresed on 1% agarose gel containing ethidium bromide, and photographs were taken. Samples obtained by omitting cDNA in the PCR mixture were used as negative controls to detect possible DNA contamination.

Statistical analysis

Differences in cell viability were assessed using the Student *t*-test (20). Two-tailed *P* values below 0.05 were considered significant.

Figure 1. A, RT-PCR analysis of low-density lipoprotein (LDL) mRNA in normal bone marrow and acute myelogenous leukemia (AML) blast cells. Lane 1: 100-bp ladder; lanes 2 and 3: 4 µg of normal bone marrow cDNA amplified using β -actin (lane 2) or LDL receptor (lane 3) primers, respectively; lanes 4 and 5: 1 µg of AML blast cell cDNA amplified using β -actin (lane 4) or LDL receptor primers (lane 5), respectively. B, The second round of PCR amplification analysis. Lane 1: 100-bp ladder; lane 2: 273-bp segment obtained using nested primers; lane 3: negative control.

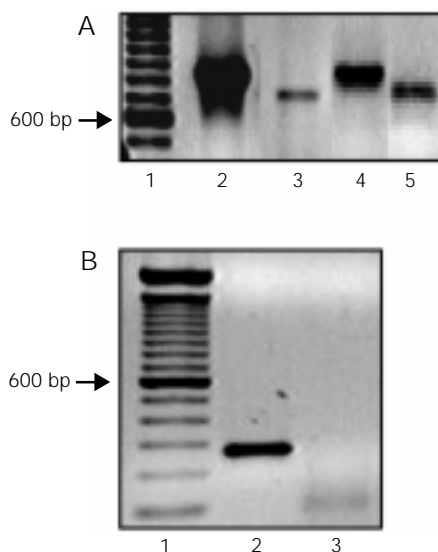
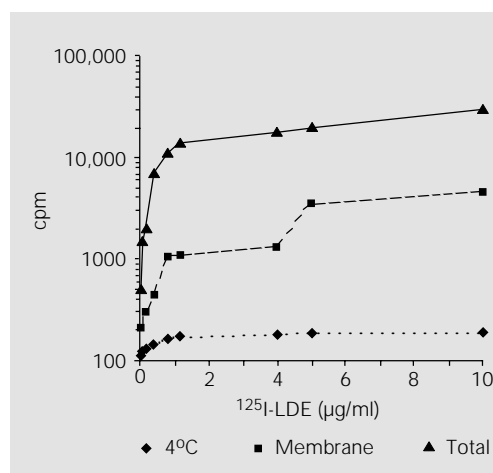


Figure 2. 125 I-LDE binding and uptake by K562 cells. Cells (10^6) were incubated with 0.10-10 µg/ml (lipid concentration) 125 I-LDE for 4 h in RPMI 1640 containing 10% FCS at 37°C or 4°C (dotted line). After incubation, the cells were washed and treated with trypsin and centrifuged and the supernatant radioactivity (membrane-bound emulsion; dashed line) was measured with a gamma-counter. The cells were then lysed and the incorporated radioactivity (solid line) was determined. Each point represents the mean of 2 experiments.



Results

Transcription of the LDL receptor

RT-PCR had shown that the expression of rLDL is higher in AML blast cells than in normal bone marrow cells, compared to β -actin expression (Figure 1A). The second round of PCR amplification confirmed the identity of the rLDL-amplified product obtained from the first (Figure 1B).

LDE uptake by K562 cells

Figure 2 illustrates the binding (dashed line) and the uptake (solid line) of 125 I-LDE by K562 cells. At 4°C (dotted line) there was no uptake. Under the conditions used, 90% of 125 I-LDE was found in the cytoplasm, indicating that the emulsion entered the cell by the endocytic pathway.

Cytotoxicity of the LDE:daunorubicin complex and free daunorubicin

Figure 3A shows the cytotoxicity on normal bone marrow and blast cells of the LDE:daunorubicin complex. A greater cytotoxic effect of the LDE:daunorubicin complex was observed on blast cells than on normal bone marrow cells ($P < 0.001$; Figure 3A). This difference was not observed when the cells were incubated with free daunorubicin (Figure 3B). The apparent sensitivity ($\log EC_{50}$) to the LDE:daunorubicin complex did not differ between blast cells (-9.60 ± 0.04) and normal bone marrow cells (-9.42 ± 0.08).

Discussion

One of the major problems associated with the chemotherapy of cancer is the lack of specificity of the drugs, which may give rise to a variety of side effects. A theoretical possibility of reducing the toxicity and improving the therapeutic response is to target

the cancer cells using a carrier. LDL was proposed as an endogenous carrier in several *in vitro* studies (6-10,13,21) and proved to prevent side effects in one *in vivo* study (7). Nevertheless, the routine use of LDL might be difficult due to the problems of its purification.

An alternative to the use of LDL would be an artificial microemulsion (LDE) which resembles its lipid portion (11). Several experimental observations indicate that LDE may be taken up by rLDL and/or by other receptors that may recognize apo E: a) LDE clearance is accelerated in rats pretreated with 17 α -ethynylestradiol, which increases rLDL expression (12), b) the LDE biodistribution in humans is very similar to that of LDL (16), c) LDE removal from plasma is increased in patients presenting AML as occurs with native LDL (3,4,16) and after remission of AML, LDE clearance in the patients approaches normal values (16), and d) *in vitro* experiments with normal leukocytes have shown that LDE competes with LDL for the same receptor (16).

In the present study, we demonstrate that it is possible to incorporate a cytotoxic anthracycline drug into LDE. The LDE:daunorubicin complex obtained by co-sonication of the drug with LDE possibly is due to the interaction of the ring structure present in the daunorubicin molecule that might interact with the interface between the core, composed of cholesterol esters and triglycerides, and the surface, composed of phospholipids and cholesterol. The incorporation procedure is reproducible and a typical preparation contains 10 nmol of drug per mg of LDE, suggesting a limited number of domains for interaction. Since only a small percentage of the initial drug added (0.7%) was found inside highly purified LDE, daunorubicin should be utilized in preparing the complex for therapeutic use in humans or animals. The low incorporation of daunorubicin into LDE observed here was not due to LDE particle characteristics since the same

rate of incorporation has been observed using LDL particles (6).

The aim of the present study was to analyze the cytotoxicity of the LDE:daunorubicin complex on myelogenous blast cells and normal bone marrow hematopoietic cells. Comparison of the cytotoxicity of the LDE:daunorubicin complex against normal bone marrow and AML blast cells clearly showed a lower cytotoxic effect on the former. The RT-PCR results indicate that in AML blast cells there is a higher transcription of rLDL than in normal bone marrow cells. Furthermore, the apparent affinity (EC_{50}) of both cell types for the LDE:daunorubicin complex did not differ, suggesting that both cells express the same receptor population. In addition, the difference in maximal response between normal and blast cells suggests a higher expression of the receptor respon-

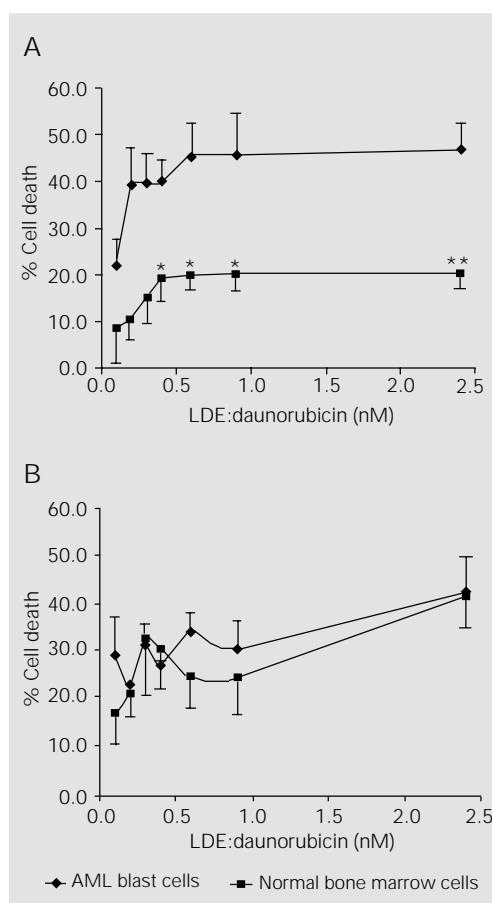


Figure 3. A, Cytotoxicity of the LDE:daunorubicin complex against normal bone marrow cells (squares) and acute myelogenous leukemia (AML) blast cells (lozenges). The points represent the mean \pm SEM of 7-15 experiments. *P<0.05 and **P<0.001 compared to normal bone marrow cells (Student t-test). B, Cytotoxicity of free daunorubicin against normal bone marrow cells (squares) and AML blast cells (lozenges). The points represent the mean \pm SEM of 7-17 experiments.

sible for the uptake of LDE in AML. Taken together, these results indicate that the LDE:daunorubicin complex may use the rLDL endocytic pathway to target AML blast cells. However, the involvement of other receptors cannot be ruled out since it has been shown that cell surface receptors such as rLDL-related protein (14,22) can also mediate specific high affinity degradation of ^{125}I -labeled LDL.

The complexed drug is as efficient as the free drug in killing blast cells. These results suggest that incorporation does not alter the cytotoxicity of daunorubicin or the receptor binding of LDE. Iwanik et al. (6) showed that the LDL:daunorubicin complex accumulates in P388 leukemia cells, indicating that the incorporation of the drug does not affect LDL binding to the receptor. The in-

cubation of free daunorubicin with blast and normal bone marrow cells confirmed the lack of specificity of the free drug.

The LDE:daunorubicin complex has been shown to be effective in protecting normal cells from the toxic effects of the free drug. Highly lipophilic compounds with promising cytotoxic effects *in vitro* have never reached clinical trials because of difficulties in finding a suitable nontoxic solvent. Our results suggest that LDE might be an interesting and simple delivery system to administer these compounds to tumor cells exhibiting high rLDL expression.

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