ABSTRACT

Objective(s): The role of lipoproteins (LDL) as active molecules with preferential tumor interaction, but limited drug delivery capacity, has been previously reported. On the other hand, in a previous report, we demonstrated the high capacity of monosialogangliosides (GM1) micelles as drug transporters.

Materials and Methods: In this work, GM1 was loaded with high doses of oncologic drugs such Paclitaxel or Doxorubicin and binded to LDL lipoproteins to form GM1-drug-LDL water-soluble complex. Evidence suggests that both, hydrophobic and electrostatic forces, participate in the interaction, regulated by conditions such as pH, temperature and ionic strength.

Results: Results of DLS and TEM show that GM1-LDL complexes are considerably larger than the sum of their individual compounds, with a high charge of electronegative surface (-55.9 mV). In addition, the cytotoxic effect on cell cultures is greater when drugs are contained in GM1-LDL complexes than when loaded in GM1 micelles.

Conclusion: The results suggest the participation of active energy-dependent mechanism in the uptake of GM1-LDL drug, probably linked to the LDL receptor by the tumor cells. However, we could not confirm that the transport through LDL receptors is the only one that participates in the cellular uptake of the micelles.

Keywords: GM1-LDL, Micelles, Nanodelivery, Oncological drugs, lipoproteins

INTRODUCTION

One of the potential active targeting strategies explored in cancer therapy implies using lipoproteins (VLDL, LDL and HDL) due to their preferential uptake by specific lipoprotein receptors overexpressed in tumor cells [1-5]. This is because rapidly dividing tumor cells have greater need for cholesterol than normal cells. Advantages of lipoproteins include biocompatibility, relatively long plasma half-life (2-3 days) and slow elimination by the reticuloendothelial system (RES system). The small particle size of lipoproteins allows extravasations from the intravascular compartment to the adjacent tissues; their internal core is an ideal domain for the transport of highly hydrophobic drugs.

In this sense, the lipoproteins mostly studied as vehicles for cytotoxic drug delivery are LDL [6-7], which contain a single large protein component, apolipoprotein B100 (550kDa, 4536 residues of amino acids), covering approximately 40% of its surface. This protein is responsible for recognition and binding to specific high-affinity cellular receptors, RLDL. Similarly, LDL has also been evaluated as a method of diagnosis and monitoring by the incorporation of contrast agents or fluorescent probes [8-9]. However, although numerous studies have shown promising results, the practical usefulness of lipoproteins as drug nanocarriers is hampered by factors such as low loading capacity, especially in amphiphilic drugs, and storage for short periods before aggregation and degradation processes that compromise the...
stability of the sample. In addition, it has been shown that the incorporation of drugs in LDL can induce subtle modifications in the structure, modifying their behavior in vivo [9-10]. However, related literature describes that LDL has sites that recognize specific glycol-conjugate carbohydrate sequences that allow interaction with gangliosides [11-14]. These studies also report that the incorporation of sialic acid into the surface of LDL, through binding of gangliosides, significantly modulates the absorption of lipoproteins in certain cells. Based on these data, we considered the possibility of combining both functionalities in the same system, linking LDL lipoproteins to GM1 micelles loaded with Ptx, whose efficiency in the transport and release of oncological drugs has already been demonstrated. [15-16]. Thus, we evaluated the influence of different physicochemical variables such as temperature, pH and ionic strength in the GM1-LDL interaction. We characterized the structures generated and their physicochemical properties. Additionally, we analyzed the biological activity and in vitro toxicity of the drugs loaded in the GM1-LDL complexes, in comparison with those loaded in pure GM1 micelles.

**MATERIALS AND METHODS**

**Materials**

Units of 250ml of human plasma with non-reactive serology from the Instituto de Hematología y Hemoterapia, Universidad Nacional de Córdoba, were used for purification of lipoproteins.

The quantitative cholesterol in plasma was determined with the reagents Enzymatic Method Colestat, Wiener Lab. and Precipitating Reagent Wiener Lab. for HDL-Cholesterol.

Stock solutions of purified pig GM1 monosialogangliosides, supplied by TRB Pharma S.A., were prepared in bidistilled water at a final concentration of 250 mg ml-1 and maintained/kept at 4–8 °C for 24 h before being centrifuged at 50,000g for 15 min. The supernatant was finally filtered through 0.22 μm.

Paclitaxel (Ptx), from Yunnan Smandbet Co. Ltd. (Kunming, China), was prepared by dissolving in dimethylsulfoxide (DMSO) at a final concentration of 50 mg mL-1.

Doxorubicin (Doxo) was purchased from Sigma (St. Louis, MO, USA) and stock solutions were prepared in physiologic solution at 6 mg mL-1.

All other analytical-grade reagents were purchased from Merck (Darmstadt, Germany) and used as received.

**Cell lines**

The following continuous cell lines were used: human laryngeal epithelioma cells (Hep-2) and human cervical epithelioma cells (HeLa).

For cell cultures, MEM was supplemented with irradiated fetal bovine serum (NATOCOR, Córdoba, Argentina) at 1 or 10% (v / v) according to maintenance or growth conditions, respectively.

**METHODS**

**Purification of LDL lipoprotein fraction**

Purification of LDL lipoprotein fraction was performed according to the method described by Burstein et al [17] for the selective isolation of human serum lipoproteins by precipitation with polyanions and divalent cations. Briefly, 25 ml of 4% sodium phosphotungstate (NaPhT) and 6.25 ml of 2M magnesium chloride (MgCl2) were added to 250 ml of human plasma (final concentrations: NaPhT 0.4%, MgCl20.05 M). Under this condition, LDL and VLDL fractions precipitated immediately, completely and selectively. The precipitate was removed by a first centrifugation during 10 min at 6,000 g, and then, LDL fraction was separated from VLDL by a second centrifugation at 100,000 g in buffer density around 1.006. The fraction of LDL isolated in TE buffer (10 mM Tris-HCl; 1 mM EDTA) was further precipitated with 4% NaPhT (final concentration: 0.4%) and 2 M MgCl2(final concentration 0.05 M), washed and resuspended in TE buffer with 150mM NaCl. To remove impurities, the suspension was extensively dialyzed against the same TE buffer with 150 mM NaCl and immediately filtered through a membrane of 0.45 μm pore size (Millipore). As a result, a clear yellow solution of concentrated lipoproteins was obtained. Highly concentrated solution of isolated lipoproteins remained clear even after prolongeddialysis. Finally, DMSO (final volume: 10%) was added and fractionated into aliquots of 1.5 ml before being stored in a freezer at -80 °C. Purity was analyzed by agarose gel electrophoresis and concentration was determined by enzymatic method.

**Determination of the purified LDL concentration**

The concentration of LDL was determined by measuring the concentration of cholesterol with the enzymatic method (Colestat)
of Wiener Lab, using cholesterol as a standard.

Briefly, 10 μl of concentrated LDL was added to 1 ml of cholesterol oxidase / peroxidase enzyme reagent and incubated for 5 min in a water bath at 37 °C or 20 min at room temperature (25 °C) before being read at 505 nm. In addition, two tubes were processed, one with 10 ml of cholesterol standard (E) and another with water as the reaction blank. The final concentration of LDL was obtained by applying the following formula:

\[ \text{Cholesterol (g/l): D x f} \text{ where f: 2 (g/l). E-1.} \]

**Preparation of GM1-LDL complexes**

Solutions of 10 and 20 mg·mL\(^{-1}\) GM1 were prepared 24 h prior to use by diluting the filtered GM1 stock solution into double-distilled water. Aliquots of purified LDL were thawed and dialyzed exhaustively against water for 24 h prior to use. Micelles (GM1) were then incubated with dialyzed LDL at different pHs (3, 5.5, 7.4 and 10), temperatures (4, 20, 37 and 55 °C) and ionic strength (up to 1 M NaCl). Results of these assays were analyzed by electrophoresis, turbidimetry and absorption spectrophotometry using merocyanine 540 (MC 540).

**Quantification of LDL associated with GM1 micelles**

The amount of LDL bound to GM1 micelles was determined by absorption at 560 nm of the lipophilic dye merocyanine 540 (MC 540), previously incorporated into the structure of the lipoprotein. MC 540 is a lipophilic probe widely used to investigate membrane properties such as phospholipid packing and phase transition. After being added to a suspension of liposomes or lipoproteins, it was partitioned and incorporated into the bilayers of phospholipids or lipid domains. Solutions of 10 mg·mL\(^{-1}\) GM1 micelles were mixed with LDL suspensions with the dye incorporated and incubated for 4 h at 37 °C. Then, 10% trichloroacetic acid (TCA) was added to precipitate the protein fraction of lipoprotein and centrifuged (10 min, 10000 \(g\)). The absorbance of MC 540 was measured in the supernatants and compared to those of the controls.

**Characterization of GM1-LDL complexes**

**Agarose gel electrophoresis**

The electrophoretic mobility of native LDL, GM1 and GM1-LDL complexes in agarose gels was analyzed using human plasma as a control. Samples were preincubated with Sudan-Black B, a lipid dye solubilized in 30% ethanol for 20 min at room temperature. Then, sucrose (40% (w / v)) was added, mixed, and 15 μl of each sample was loaded on a 0.7% (w / v) agarose gel in Tris-Acetate-EDTA (TAE) (pH 8). The run was performed at 100 V for 60 min and the mobility was visualized by staining.

**Particle size and size distribution**

Average particle size, size distribution (PDI) and zeta potential of GM1-LDL complexes were measured by laser light scattering (LLS, Delsa TM Nano, Beckman Coulter Instruments). Samples were diluted in PBS buffer pH 7.4 and filtered prior to measurement. Size and distribution data were analyzed by Delsa Nano Beckman Coulter software (version 2.2) provided by the manufacturer with the CONTIN method of analysis. All measurements were done in triplicate in three independent sample batches.

**Transmission Electron Microscopy (TEM)**

A JEOL JEM-1200 EX II transmission electron microscope at a magnification of 300,000x was used to establish the morphology of GM1-LDL complexes. 50 μl of the complex was loaded onto a carbon grid, incubated for 5 min and then dried. GM1 sample was also processed. The grids were incubated with 50 μl of a 10% uranyl acetate solution for 1 min, dried and finally carried under a microscope for observation.

**Drug incorporation into GM1-LDL complexes**

To assess whether LDL binding to GM1 micelles affected the loading capacity of the micelle, we evaluated the load of increasing amounts of Ptx or Doxo in GM-LDL complexes of different w/w ratios, to reach GM1-drug molar ratios from 50/1 to 1/1. Drug loading was performed at 20 °C for 30 min and maintained at that temperature for 24 h. After incubation time, they were dialyzed against water at 20°C for 24 h to separate free drugs. The influence of the loading order of the drug and LDL was also analyzed. The incorporated drug was quantified as described for Ptx or Doxo.

**Determination of Ptx concentration**

Paclitaxel concentration was measured on a Curosil B C18 column (250×3.20 mmID, particle size 5 μm) and a Curosil BC18 guard column (30×4.60 mm ID, particle size 5 μm) supplied by Phenomenex, as previously described [15].
The mobile phase was 60% (v/v) acetonitrile and 40% (v/v) bidistilled water. Flow rate was 0.7 mL min\(^{-1}\) and the eluent was monitored at 227 nm. Chromatography was performed at ambient temperature (20°C). Validation of the method was carried out according to FDA Guidance for Bioanalytical Method Validation.

**Determination of Doxo concentration**

Doxo concentration was determined by absorbance at 490 nm using a calibration curve performed with a standard solution of Doxo in physiologic solution, as described by Abraham et al. [18].

**In vitro cytotoxicity of GM1-LDL-drug complexes**

Cytotoxicity assays were performed using Hep-2, HeLa and Vero cells cultured in MEM medium supplemented with fetal bovine serum at 37 °C with 5% CO\(_2\). Monolayers grown to 98% confluence in 96-well plates were incubated with increasing concentrations of GM1-Doxo or GM1-LDL-Doxo complexes at different times after which the medium was replaced for free medium and incubated for 24 hr at 37 °C. Finally, cells were washed three times with PBS buffer and the number of surviving cells was determined by a crystal violet staining test. For this, cells were fixed with 10% formaldehyde and stained with a solution of 0.1% crystal violet in methanol. Cells were then washed with water and the crystal violet was solubilized with 10% acetic acid; the Abs of the resulting solution was measured at a wavelength of 620 nm. Quantitative analysis (colorimetric assay of the fixed cells) was performed by absorbance measurements on an automated plate reader (Bio-Rad., CA, USA) at 620 nm. The % cell viability was calculated and compared between samples. Untreated cells incubated with medium were taken as a negative control, 100% cell survival. The results represent mean values of three measurements and their respective deviations.

Temperature assays were performed similarly, incubating cells at 4 and 37 °C for 45 min, before adding samples to evaluate.

Ptx assays were performed as in Doxo but, unlike the latter, monolayers were incubated with Ptx, GM1-Ptx or GM1-LDL-Ptx for 24 h. In vitro cytotoxicity studies were quantitatively analyzed by crystal violet assay. Cytotoxicity controls were also performed with GM1 micelles and LDL alone to exclude the effect of individual components.

**Effect of lyophilization**

GM1-LDL complexes (ratio 10:1 w/w) loaded with Doxo at a GM1-Doxo ratio 10:1 (mol/mol) or Ptx at a GM1-Ptx ratio 5:1 (mol/mol) were frozen at -80 °C for 24 h, then lyophilized and stored at 4 °C for 120 days. Afterwards, they were resuspended to initial volume with bidistilled water and diluted in culture medium until reaching the working concentration of the drug. In that condition, its cytotoxic effect was evaluated using the crystal...
violet assay as previously described. The result was compared to that produced by the same formulations prepared on the day of the assay.

RESULTS
Interaction of GM1 micelles with LDL lipoproteins
In order to obtain the better conditions for GM1-LDL interaction, we started evaluating the influence of physicochemical variables such as temperature, pH and ionic strength. Fig 1A shows the effect of temperature from 4 to 55 °C on GM1-LDL interaction. It can be seen that the clearest solutions were achieved by preparing GM1-LDL mixtures between 37 and 55 °C, a condition in which the solutions obtained were slightly opalescent (<OD at 600 nm), whereas samples prepared in the range of 4 to 20 °C were highly turbid. When the samples were clarified by centrifugation or filtration with 0.22 μm, the mixtures prepared in the range of 4 to 20 °C were precipitated or retained in the filter; however, samples prepared at temperatures between 37 and 55 °C remained soluble and slightly opalescent, without significant changes in turbidity, which reflects the formation of soluble low-molecular-weight complexes under these temperature conditions. These results highlight temperature as a determining factor in the interaction between GM1 and LDL lipoproteins, conditioning the structure of the complexes formed. This probably responds to changes in the aggregation properties of some of the molecules involved, causing internal reorganizations in the structure.

After establishing the optimal temperature range for the GM1-LDL interaction, we evaluated the effect of pH. Incubations at pH 3, 5.5, 7.4 and 10 showed that the optimal range of interaction is between 5.5 and 7.4, where GM1-LDL complex generates slightly opalescent and more stable solutions. At pH 3 and 10 the mixtures were more turbid, with the presence of visible aggregates. When mixtures prepared at different pHs were centrifuged at 10,000 g and the supernatants were analyzed by electrophoresis on agarose gels, we can see that the electrophoretic mobility of GM1-LDL mixtures at 5.5 and 7.4 shows the higher binding capacity than that of pH 3, while at pH 10 no migration bands were seen, indicating the absence of GM1 and LDL in the supernatant, Fig 1B.

Finally, we evaluated the influence of ionic strength on the GM1-LDL complex. Thus, we observed that the incubation of LDL with increasing amounts of NaCl (up to 1 M) increases turbidity and the precipitation of lipoproteins. However, the addition of increasing amounts of NaCl to GM1-LDL micelles did not produce precipitation or modified the initial turbidity of the solution. This result demonstrates that the binding of LDL to GM1 produces a more stabilized complex and, on the other hand, suggests that both hydrophobic and electrostatic interactions are involved in the formation of the GM1-LDL complex, although the hydrophobic interaction seems to be more important [11,14].

Next, we determined the maximum amount of LDL that binds to GM1 micelles and the stability of this complex. To do this, we used the lipophilic dye, merocyanine 540; the stability of GM1-LDL complex was evaluated by its solubility in 10% TCA. It is well known that lipoproteins (LDL) are full precipitated by addition of 10% TCA. LDLs were stained with MC 540 and then incubated with increasing amounts of GM1. GM1-LDL complex were finally treated with 10% TCA to precipitate free LDL. Fig 1C shows that, as expected, control of lipoproteins precipitates completely with the addition of TCA; however, as the concentration of GM1 increases, a progressive amount of LDL remains in solution, demonstrating that solubility of complex appears to be only related to the presence of GM1. These results also indicate that the optimal association between GM1 and LDL is found in the ratio GM1-LDL 10:1 (w / w). The results demonstrate that LDL remains soluble after treatment with TCA due to its interaction with GM1 micelles.

Table 1. Physical characteristics of LDL, GM1 micelles and GM1-LDL complexes. GM1 micelles: 10mg.ml−1, GM1-LDL mass ratio 20/1 (w/w). MD: Mean Diameter, PDI: Polydispersity Index, SD: Standard Deviation. * Bibliography data

<table>
<thead>
<tr>
<th>Sample</th>
<th>MD (nm)</th>
<th>PDI</th>
<th>Zeta Potential (mV)</th>
<th>SD</th>
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<td>GM1 micelles</td>
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<td>-8.6</td>
<td>1.5</td>
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<tr>
<td>*LDL</td>
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<td>0.299</td>
<td>-25.2</td>
<td>1.0</td>
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<tr>
<td>GM1 micelles-LDL</td>
<td>105.7</td>
<td>0.317</td>
<td>-55.91</td>
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Physicochemical characterization of GM1-LDL complexes
GM1 micelles with the maximum amount of LDL bound were selected to determine mean...
diameter, PDI and zeta potential of GM1-LDL complexes. Table 1 shows that GM1-LDL particles have an average diameter of 105.7 ± 4.2 with a PDI of 0.317 ± 0.009, a much larger size than the sum of the individual molecules. The relatively high PDI indicates a non-uniform population distribution. Zeta potential of GM1-LDL complexes evidenced a marked increase in surface electronegativity, which, as in the case of particle size, was greater than the sum of its individual components.

The micrographs by TEM also confirm a non-uniform size distribution of GM1-LDL complexes (data not shown). Yet, unlike DLS results, particle diameters do not exceed 100nm.

Table 2. Incorporation of Ptx into GM1 micelles previously incubated with LDL and with GM1 micelles before binding to LDL at different GM1-LDL mass ratios. GM1-Ptx 5/1 (w/w).

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<td>40/1</td>
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The hydrophobic drug Ptx can be incorporated in a soluble form into the GM1-LDL complex onto GM1 micelles before binding to LDL with the same efficiency as that seen in pure GM1 micelles (Table 2).

However, a significant difference was observed in the stability of the complexes depending on the order of preparation. When Ptx was incorporated into the already formed GM1-LDL complex, it remained soluble only for 48 h at 37°C; when Ptx was loaded into GM1 micelles before incubation with LDL, the complex was stable for more than 15 days at 37°C without affecting the amount of bound LDL.

Results of loading the hydrophilic drug Doxo in the GM1-LDL complex were substantially different from those obtained with Ptx At low Doxo concentrations (molar ratio GM1-Doxo 40/1 to 10/1), only one peak of GM1-LDL-Doxo was seen, showing that all the Doxo added was associated with the GM1-LDL complex (Fig 2).

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**Loading of GM1-LDL complex with oncological drugs**

After selecting of best conditions to form GM1-LDL complexes, we evaluated its capability to incorporate drugs such as Paclitaxel (Ptx) or Doxorubicin (Doxo).

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However, at higher concentrations of Doxo (molar ratio GM1-Doxo 5/1 or 2.5/1), the exclusion chromatography shows the presence of two peaks, one corresponding to the GM1-LDL-Doxo complex and another of low molecular weight of free Doxo. These results are significantly different from those found in Doxo loading in GM1 micelles, where the degree of encapsulation exceeds 95% in the molar ratio GM1-Dox 5/1 or 2.5 / 1, which could indicate that LDls occupy domains shared with Doxo in the micelle of GM1, affecting the loading capacity of the drug.

**In vitro cytotoxic effect of GM1-LDL-drug complexes**

The in vitro cytotoxic effect of GM1-LDL-Doxo and GM1-LDL-Ptx complexes was evaluated in contrast with the same drugs loaded in GM1 micelles using Hep-2 line cells. Fig 3A shows that GM1-LDL-Doxo appeared to be more effective than GM1-Doxo, while nototoxicity was observed for control of GM1-LDL complex. These results seem to be linked to the presence of LDL, which affects the uptake of formulations.

Like Doxo, the cytotoxic effect of Ptx loaded on GM1-LDL-Ptx complex on Hep-2 cell was higher than that of GM1-Ptx, demonstrating the influence of LDL on the uptake of the complex (Fig 3B). Controls of empty micelles of GM showed no toxic effect on cell cultures.

Similar results were obtained with Doxo and Ptx on HeLa cells. Both drugs showed greater cytotoxicity when transported by GM1-LDL-drug complexes, reinforcing the fact that the presence of LDL in the complex modifies the uptake of GM1 micelles and that it is not a specific effect for Hep-2 cell line only (data not shown).

The better activity of drugs in GM1-LDL over pure GM1 micelles could be attributed, at least, by two mechanisms, via LDL-receptor and via direct interaction of GM1-Drug with cell membranes previously reported [15-16].

In order to inquire about the participation of active energy-dependent mechanisms, or another one related to LDL receptor, involved in the uptake of complexes, we compared the cytotoxicity of GM1-Doxo and GM1-LDL-Doxo after incubation with Hep-2 cells at 4 and 37 °C to determine whether the process involved in uptake of GM1-LDL-Doxo was the same as that described before for GM1-Doxo [16].

On the other hand, to evaluate the contribution of the LDL receptor in the improvement of micelle absorption, we evaluated the effect of pre-saturating the LDL cellular receptors before carrying out cytotoxicity tests. Thus, after incubating cells with an excess of LDL, we exposed them to increasing concentrations of Doxo in the GM1-Doxo-LDL complex at 4 or 37 °C for different periods of time. Significant differences in cell death were observed between the two temperature conditions; cell viability decreased as temperature increased, it being much higher at 37 °C than at 4°C. This result suggests that the uptake of GM1-LDL-Doxo micelles could be mediated by active transport mechanisms linked to the cellular uptake of the complex (Fig 4A).

On the other hand, when we compared cell viability for GM1-Doxo and GM1-LDL-Doxo, it can be seen that at 37 °C, at equivalent drug concentrations, differences can also be found in the cellular viability of both, the cytotoxicity observed for the GM1-LDL-Doxo complex being greater, Fig 4B. This result highlights/underlines the presence of another active mechanism, probably linked to the presence of the LDL receptor.

To gain insight into the mechanism, considering the possibility of an active LDL receptor that controls this interaction, we pre-incubated Hep-2 cells with an excess of free LDL for 3 hr. Then, we removed the excess of LDL and evaluated the cytotoxic behavior of both GM1-Doxo and GM1-LDL-Doxo. Results shown in Fig 6 reveal a significant increase in the cytotoxic activity of the GM1-Doxo system, matching now that produced by the GM1-LDL-Doxo complex. This could result from the binding of the GM1-Doxo complex with
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the LDL bound to LDL-R to form the GM1-LDL-Doxo complex, which would facilitate the uptake of the GM1-Doxo system (Fig 5).

Fig 5. In vitro cytotoxic effect of GM1-Doxo and GM1-LDL-Doxo at 1, 2 and 3 h of incubation on Hep-2 cells. For GM1-Doxo assays, Hep-2 cells were preincubated for 2 h with an excess of LDL and then removed. Controls of GM1-Doxo (Doxo: 25μg.ml-1) and GM1-LDL-Doxo (Doxo: 25μg.ml-1), GM1-Doxo preincubated with 0.24 mg.ml-1LDL, GM1-LDL-Doxo preincubated with 0.24 mg.ml-1 LDL. [Doxo] effect of: 25μg.ml-1. Error bars indicate the DS of the mean (n =5)

Taken together, these results indicate that under favorable conditions (37° C and adequate medium), LDL can bind to the GM1-Doxo complex and facilitate its uptake. Although the exact mechanism involved cannot be confirmed, the evidence strongly suggests the participation of LDL-R in the uptake of the GM1-LDL-Doxo complex.

Finally, we analyzed the stability and the activity of the GM1-LDL-Doxo and GM1-LDL-Ptx against the lyophilization and resuspension procedure. Samples of both formulations were lyophilized and stored for 4 months at room temperature, then solubilized in saline or 5% glucose. Solubility and cytotoxic effects were finally evaluated. The formulations remained stable at least during 2 months at 25°C after solubilization and their cytotoxic activity underwent no modification, showing that complexes can be stored in a solid state for long periods and then solubilized without altering their physicochemical and biological properties.

DISCUSSION

The results described that the spontaneous interaction between GM1 micelles and LDL to form soluble complexes of GM1-LDL is highly dependent on conditions such as temperature, pH and ionic strength. Interaction is favored as temperature increases; the most favorable condition seems to be 37 °C and 50 °C resulting in the greatest amount of GM1-LDL complex formed. However, at 50 °C the stability of the complex is reduced, due to a fast aggregation mechanism. This probably occurs due to structural changes in the apo-lipoproteins that affect their functionality more than changes in GM1 micelles.

Better pH conditions for the interaction take place in the 5.5 and 7.5 range, since more extreme pH (3 and 10) favors the aggregation and formation of insoluble complexes. Likewise, the increase of ionic strength in the medium produces greater turbidity and precipitation of the mixtures, mainly by aggregation of lipoproteins, unless GM1-LDL interaction was previously done in water or at a low salt concentration. Altogether, these results are in agreement with those that report that hydrophobic interactions appear to be more important than electrostatic interactions in the formation of these ganglioside-lipoprotein complexes [15-16].

Results described in Fig 3 show that the maximum binding capability of GM1 to LDL was 10/1 (w/w), suggesting the participation of several GM1 molecules in the formation of GM1-LDL complexes. This agrees with other studies that demonstrate that large amounts of gangliosides can be associated with LDL and that the complexes formed are about 2 or 3 times larger than the native LDL particles [14]. In addition, they suggest that sialic acid molecules of negatively charged gangliosides are distributed on the surface of the lipoprotein when associated, whereas most non-polar groups are contained in the interior [13]. DLS and TEM studies revealed that GM1-LDL complexes were composed of an heterogeneous population with an average diameter of 105.7nm, approximately four times greater than that of native LDL and 10 times that of GM1 alone. The high electronegative surface charge of GM1-LDL complexes (~55.9 mV), a well-known condition that induces strong electrostatic repulsions, probably contributes to the increased stability observed and the resistance to flocculation and precipitation of the complex in comparison to native LDL.

Moreover, the interaction of GM1 with LDL to render GM1-LDL complex produces a substantial
increase in the stability of LDL molecule, since no further precipitation occurs by TCA. This high stability of GM1-LDL could be a beneficial condition for the drugs loaded in this complex.

After selecting the most favorable conditions for stabilized GM1-LDL complexes, we assessed whether these complexes could maintain the ability of GM1 micelles to load cancer drugs. LDL has proven to be an interesting option to consider due to its preferential location at sites of overexpression of its receptor, as in some tumor cells [19, 20].

In this context, we evaluate the possibility of adding the loading capacity of GM1 micelles with preferential accumulation of LDL in the tumor site. Hence, it is important to confirm whether GM1-LDL complexes retain their ability to load hydrophobic or hydrophilic drugs such as Ptx and Doxo. Previously, we showed that GM1 micelles can load Ptx up to a molar ratio of 5/1 GM1-Ptx [15]; the GM1-LDL complex described in this work maintains that Ptx loading capacity. However, it is particularly important to follow a specific order in the preparation to obtain stable aqueous GM1-LDL-Ptx complexes. GM1 micelles loaded first with Ptx and then with LDL maintain their stability for more than 15 days. Yet, when Ptx is loaded into previously formed GM1-LDL complexes, the stability of GM1-LDL-Ptx micelles lasts only 48 h. These results could indicate that the drug is located in different domains according to the availability of the micelle, or that the binding of the micelle to lipoproteins generates either some kind of barrier to the sites where the Ptx is naturally located, or some distortion of the structure that determines sites of less compatibility with the drug.

On the other hand, the load of hydrophilic drug Doxo onto GM1-LDL shows rapid saturation at low concentrations (GM1-Doxo molar ratio 40/1 to 10/1), since at higher concentrations (GM1-Doxo molar ratio 5/1 to 1/1), the elution profile shows the presence of a second peak corresponding to the free Doxo. This lower binding capacity demonstrates a partial influence of LDL on the loading of Doxo into GM1-LDL.

Finally, in vitro cytotoxicity studies revealed that LDL bound to GM1 micelles modifies the cellular uptake of Doxo and Ptx, increasing the death of tumor cells. For Doxo, this effect was seen even with lower concentrations at prolonged incubation times, which would allow reducing drug dosage, minimizing damage to healthy tissues. In addition, the participation of an active energy-dependent transport mechanism involved in the uptake of the GM1-LDL-Doxo complex was evidenced, complementary to that of the GM1-Doxo complex. Moreover, results showing that preincubation of cells with LDL before addition of GM1-Doxo increases cytotoxicity at the same level as that found in GM1-Doxo-LDL reinforces the idea that LDL participates in the uptake of the GM1-Doxo system, facilitating its absorption probably via LDL-receptor. However, the results obtained do not allow establishing the intervention of the LDL-receptor as a unique factor to improve the uptake of the complexes. Evidence shows that in B16 and HepG2 tumor cells, the LDL-R activity is not affected or is only partially affected by LDL levels in the medium [21-24]. However, these results can also be attributed to another uptake mechanism, independent of LDL-R, as reported in different studies [25-28].

Note that in vitro studies were generally performed with relatively short incubation times as GM1 micelles rapidly fuse with cells, thus any change or difference in the effect sought occurred at that time. Nevertheless, an in vivo potential advantage of the GM1-LDL complex is that LDL has a prolonged circulating half-life (2-3 days) and slow elimination by the SRE system, which would improve the biodistribution of the GM1-drug complex and achieve longer residence time to reach the target. Furthermore, in vivo studies, it has been demonstrated that the expression of LDL receptors in the liver, spleen and adrenal glands can be efficiently and selectively reduced by dietary supplements such as bile acids, or drugs such as corticosteroids, which could favor the uptake of GM1-drug-LDL nanocarrier in tumoral cells [2, 7].

Our results described here show the presence of two nanocarriers with different capability in the same complex. GM1 acts as a first carrier for Ptx; LDL then acts as a second carrier to drive GM1-Ptx to the over expressed LDL receptor in cancer cells.

**CONCLUSIONS**

*The results presented in this paper allow us to conclude that:*

- GM1 micelles are able to interact with LDL lipoproteins, forming binary stable GM1-LDL complexes.

GM1-LDL complexes are capable of loading Ptx and Doxo, giving rise to ternary GM1-LDL-Ptx
or GM1-LDL-Doxo complexes, although a specific order of preparation must be followed to obtain/ 
form stable complexes with a similar capacity to 
that of GM1 micelles.

The presence of LDL in the GM1-Drug-LDL 
complex modifies drug uptake (Doxo, Ptx) in Hep- 
2 and HeLa tumor cells, increasing cell death.

Evidence shows the participation of an active 
transport system linked to LDL in the drug uptake 
by tumor cells.

However, it was not possible to establish 
clearly the role of LDL-R.

In addition, LDL could improve the in vivo 
permanence time of the GM1-Drug complex, thus 
the use of LDL lipoproteins as ligands to improve 
the selective target of the GM1-Drug micelles 
administered intravenously seems promising.

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