Polyamines in the Surface of Lipid Micelles Improve the Cellular Uptake of Antitumoral Agents

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Abstract:

Purpose: The ability of spermidine to increase the selectivity of anticancer agents has been studied extensively. In this research we report the combination of this polyamine and GM1 ganglioside micelles and characterize their behavior for drug delivery.

Methods: Dynamic light scattering and electron microscopy were used to characterize size and morphology of micelles. Zeta potential was determined using a Nano-zeta potential analyzer. Size-exclusion chromatography was used to separate different populations. Cytotoxic effect of micelles was evaluated on Hep2 cell line.

Results: Covalent conjugation of spermidine to gangliosides produces a clear reduction of the electronegative *z* potential of micelle surface. DLS analysis shows no significant differences between both micelles, while TEM image shows a smaller size of Spermidine-GM1.

These modified micelles load hydrophilic or hydrophobic antitumor drugs and conjugation does not affect the stability of micelles/drug in solution. Spermidine-GM1/Doxo micelles show faster drug release into cells as compared with GM1/Doxo micelles; however, no evidence can be found for the participation of the polyamine transport system in the uptake of modified micelles.

Conclusion: While Spermidine-GM1 and GM1 micelles show similar physical properties, spermidine modified micelles are most efficient to release drugs, making this an interesting alternative to consider for drug delivery.

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Keywords: Spermidine-GM1 micelles, polyamines, drug targeting, cancer therapy.

1. INTRODUCTION

The great progresses made on the understanding of the deep mechanisms of cancer have not achieved equal degree of improvement in their treatment, which remains one of the major problems in human health. In this sense, chemotherapy is one of the most used therapies even though many serious side effects derive from a number of factors including toxicity and lack of tissue specificity. Some of these treatments involve the use of nanoparticles, including liposomes, polymers, dendrimers and micelles, as carriers of chemotherapeutic agents. These strategies are based on the small size and high loading capacity of drugs, their longer residence time, greater tissue permeability and especially because these structures seem to reduce side effects; however, the most important factor, i.e., specificity of action, remains particularly low. Many ligands have been studied in order to improve the specificity of delivery of these transporters on the basis of their specific interaction with receptors of interest; some of the molecules studied include proteins, antibodies, peptides, aptamers and small-molecule compounds. Polyamines belong to this last group of small-molecule compounds as they are highly charged aliphatic polycations of low molecular weight, widely distributed in all living cells. The three major polyamines in mammalian cells are putrescine (PUT), spermidine (SPD) and spermine (SPM) [1]. In recent decades, interest in natural polyamines has increased due to their participation in a number of cellular processes. The concentration of polyamines is maintained within the very narrow range; their decrease inhibits cell proliferation, whereas their excess leads to toxic effects. Polyamines are strictly regulated by a complex network of biosynthesis, catabolism and transport mechanisms that are still not well known in normal cells [2-3]; yet, the polyamine metabolism is often poorly regulated during the growth of various neoplasic cells. For example, the presence of high concentrations of N 1-(3- 2 aminopropyl)-1, 4-butanediamine (spermidine, 1) in rapidly proliferating cells [4] has been reported. This higher concentration of polyamines plays, at all stages, a central role in the development of cancer, which, from the beginning, maintains the phenotype transformed [5-7]. One

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mechanism by which cancer cells increase intracellular concentrations of polyamines involves increasing preformed polyamine uptake from the extracellular environment through polyamine transport system (PTS) [2, 8-10]. Previous reports describe the use of these PTS for cellular internalization of molecules conjugated to polyamine backbone [11-13]. This model of conjugation of drugs to polyamines to enhance cytotoxic activity has been widely described in recent decades. Binding of a chemotherapeutic agent to spermidine could improve their activity, either by increasing absorption (through polyamine feedback systems) or simply by competition of polyamines for their specific union to the cells. On the basis of the above evidence, here we evaluate the superficial covalent coupling of polyamines to the nanocarrier-loaded antitumor drugs as a new cancer chemotherapeutic alternative. Recently, we described a highly anionic micellar nanostructure composed of sialoglycosphingolipids (GM1), capable of delivering hydrophobic (paclitaxel) and hydrophilic (doxorubicin) drugs with high efficiency [14, 15]. We also found that GM1/drug micelles interact with albumin (HSA) to give GM1/ drug /HSA complexes [16].

Here, we modify the negative sialic acid of GM1 molecules through its binding to the central nitrogen of spermidine to make a Spermidine-GM1- micelle. Porter *et al*. [17] demonstrated that spermidine molecule can be considerably modified at N4 position and still be accumulated by the polyamine uptake system. In this paper, we describe the physical, chemical, functional and biological characterization of these Spermidine-GM1 modified micelles.

2. MATERIALS AND METHODS

2.1. Materials

Paclitaxel (Ptx) solutions at 50 mg mL -1 (from Yunnan Smandbet Co. Ltd.) were prepared in dimethylsulfoxide (DMSO).

Spermidine ≥ 98% purity and 1-[3-Dimethylamino-propyl]-3- ethyl carbodiimidemethiodide was purchased from Sigma-Aldrich. Doxorubicin (Doxo) was a gift from Nanox S.A. Stock solutions of Doxo were prepared in physiologic solution at 6 mg mL-1. Purified monosialo-gangliosides GM1 were a gift from Dr. P. Rodriguez. Gangliosides were dissolved at 250 mg mL −1 in bidistilled water, as we previously described [10]. The solutions were centrifuged for 15 min at 50,000g after 24 h at 4-8 °C and the supernatant was filtered through 0.22 μm.

Superdex-G-200 columns were purchased? from GE Healthcare (Buckinghamshire, UK); all other analytical-grade reagents were purchased from Merck (Darmstadt, Germany) and used as received.

2.2. Methods

2.2.1. Conjugation of Spermidine to GM1 Micelles

The conjugation was carried out using surface activation method described in previous studies [18]. 20 mg ml-1 GM1 solutions were prepared in MES buffer 4 pH 4.5 24 h before use; the solution was then incubated with a 5- to 10-fold excess of 1-[3- Dimethylaminopropyl]-3-ethylcarbodiimide methiodide (EDC methiodide) for 60 minutes at 25 ° C. After that, 1- to 10-fold molar excess of spermidine solution was added to the activated GM1 and incubated for another 2 h at 25 °C. The free spermidine was separated from that conjugated to GM1 by dialysis against buffer PBS (pH 7. 4), for 24 h at the same temperature. The amount of conjugated spermidine was determined by measuring total nitrogen following the method of Dumas in a LECO FP-2000nitrogen analyzer (Leco Instruments GmbH, Monchengladbach, Germany, UST-ID). The nitrogen from the spermidine was calculated from the difference between Spermidine-GM1 dialyzed micelles and GM1 alone. Finally, the concentration of spermidine was obtained by extrapolating the nitrogen content with the concentration of known standard curve of spermidine solution.

2.2.2. Ptx Encapsulation and Loading Efficiency

To determine the loading capability of Spermidine-GM1 micelles, we loaded increasing concentrations of Ptx in these micelles using the same procedure described in previous work [14]. Briefly, 10 mg mL−1 micelles were incubated with Ptx in DMSO at molar ratios from 50/1 to 1/1 under stirring for 30 min at 20 ° C. After 24 h at 4 ° C, samples were dialyzed to DMSO removal. Ptx concentration was determined by chromatography as we previously described [14].

Ptx concentration was measured on a Curosil B C18 column (250×3.20 mm I.D., particle size 5 μm) and a Curosil BC18 guard column (30×4.60 mm I.D., particle size 5 μm) supplied by Phenomenex. 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 20 °C. Validation of the method was carried out according to FDA Guidance for Bioanalytical Method Validation.

2.2.3. Doxo Encapsulation and Loading Efficiency

The Doxo loading capacity of the micelles was determined by mixing 10 mg.mL−1 Spermidine-GM1 micelles with the drug in physiological solution to obtain micelle:drug molar ratios from 50 /1 to 1 /1. The general procedure was as described in previous work [15]. Briefly, after 30 min at 20 °C, the mixtures were maintained at 4 °C for 24 h. The samples were then dialyzed to free Doxo removal. The efficiency of doxorubicin loading in Spermidine-GM1 micelles was determined by comparison with the absorbance of known standard curve of Doxorubicin solution, at 485 nm corresponding to λ max.

2.2.4. Physicochemical Characterization of Spermidine-GM1 Micelles

2.2.4.1. Particle Size and Distribution

Particle size and dispersity of Spermidine-GM1 micelles were measured by Dynamic light scattering (DLS, DelsaTM Nano, Beckman Coulter Instruments). Micelles were diluted with PBS buffer pH 7 and filtrated before measurement.

2.2.4.2. Electron Microscopy (TEM)

A JEOL JEM-1200 EX II transmission electron microscope was used to identify the morphology of Spermidine-GM1 micelles according to the same procedure as described in previous work [14]. Samples loaded in carbon grid and negatively? stained with uranyl acetate were observed at a magnification of 250 K.

2.2.4.3. Determination of Micellar Surface Charge

The zeta potential of GM1, Spermidine-GM1- and Spermidine-GM1/Ptx was determined using a DelsaTM Nano zeta potential analyzer (Beckman Coulter Instruments,) at room temperature. The samples were prepared by diluting the micelle solutions with PBS buffer pH 7. Zeta potential was determined by triplicate and the results were automatically calculated by the analyzer.

2.2.4.4. Size-Exclusion Chromatography (SEC)

After clarification by centrifugation, different samples of micelles were analyzed using a Superdex 200 column equilibrated with phosphate saline buffer at a rate 0.4 mL min-1 on an Akta Explorer 100 system (GE Healthcare). UV-visible detector at 227 and 485 nm was used to follow the elution profile.

2.2.5. In Vitro Cytotoxicity Spermidine-GM1/Drug Micelles

Hep2 (Human epidermoid carcinoma of larynx) and HeLa (Human epithelial carcinoma of cervix) cell lines were grown in MEM medium supplemented with 10% of fetal bovine serum. Cell monolayers grown to confluence in 96-well plates were incubated with increasing concentrations of GM1/Doxo or Spermidine-GM1/Doxo micelles for different times. After 24 h, cells were washed three times with phosphate-buffered solution and the amount of surviving cells was measured by a crystal violet staining assay. Briefly, cells were stained with 0.4% crystal violet solution in methanol for 15 min and carefully washed with distilled water. Quantitative analysis (colorimetric evaluation of fixed cells) was performed by absorbance measurements in an automated plate reader (Bio-Rad, CA, USA) at 620 nm. The effect on cell growth was calculated as the difference in absorbance in the presence and absence of samples. The results represent mean values of three measurements and their respective deviations.

Ptx assays were carried out using the same protocol as described for Doxo Controls of Spermidine GM1 and Spermidine-GM1 micelles were performed to exclude the effect of vehicle components.

3. RESULTS

3.1. Evaluation of Spermidine Conjugated to GM1 Micelles

Spermidine-GM1 micelles were prepared using a simple two-step method as mentioned above. Briefly, the activation of sialic acid of GM1 was performed with carbodiimide; an excess of spermidine was then added to the conjugation. This procedure led to the formation of milky-white micellar aggregates, reversible by the addition of ionic strength (> 100 mM), leading to the formation of optically clear complexes (O.D. at 600 nm). According to the measurements of total nitrogen in Spermidine-GM1 micelle in relation to GM1, spermidine was conjugated with about 25-30% of GM1 molecules. Higher concentrations of spermidine or crosslinking agent did not improve this percentage, suggesting that this is the maximum binding capacity of spermidine to the micelle.

3.2. Characterization of Spermidine-GM1 Micelles

The physicochemical properties of Spermidine-GM1 micelles in comparison with GM1, including their mean diameter, polydispersity index, morphology and *z* potential, were evaluated. We also studied whether the properties of GM1 micelle to load and release antitumor drugs into the cells were affected by conjugation with spermidine.

Particle size analysis by dynamic light scattering of Spermidine-GM1 micelles showed a homogeneous population distribution with an average hydrodynamic radius of 11.8 nm (PI < 0.1) (Table **1**). This value is slightly lower to that found for micelles of GM1 alone. Similarly the polydispersity of these new micelles was substantially lower, suggesting a more homogeneous size.

Table 1. Physicochemical characteristics of GM1 and Spermidine-GM1 micelles.

Parameters	Micelles	
	GM1	Spermidine-GM1
MD (nm)	15± 3	11,8 ± 3
PI	0,19 ± 0,04	0,098 ± 0,002
ζ (mV)	-7.35 ± 0.96	-0.14 ± 0.97

After appropriate dilution of the different micelles obtained, mean diameter (MD) and polydispersity index (PI) and ζ potential were determined by DELSA as described in Section 2.

Similar results were obtained through observation by transmission electron microscopy (TEM); Fig. (**1**) shows, in both types of micelles, a single population of spherical structures with a diameter ranging from 11 to 15 nm homogeneous medium.

According to the determinations of *z* potential of Spermidine-GM1 micelles, it was observed, as expected, a clear decrease from that obtained with unmodified GM1 micelles, from -7,35 for GM1 to -0,14 for Spermidine-GM1 (Table **1**). This result agrees with previous data showing that not all sialic groups are covalently bound to spermidine. It also suggests that cationic molecules were evidently incorporated in the micelle surface area. With these results we define the Spermidine-GM1 micelles such as zwitterionic micelle.

3.3. Loading of Drugs in Spermidine-GM1 Micelles

Previously, we have shown that GM1 micelles are capable of spontaneously loading hydrophobic antitumor drugs, forming water-soluble stable structures that could be used for drug delivery. In the same context, it was noted that the modification of the oligosaccharide portion of GM1 by

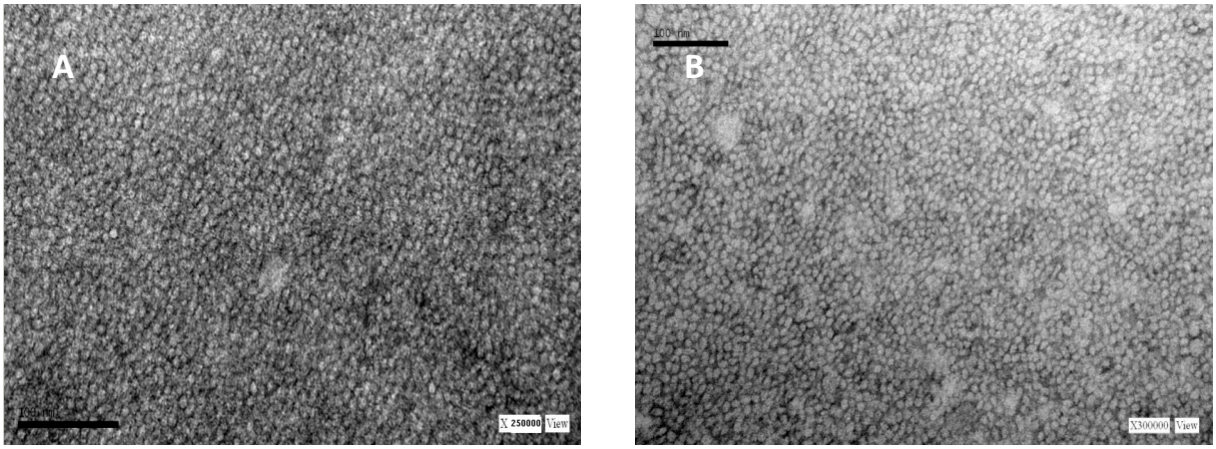

Fig. (1). Electron micrography of GM1 (**A**) and Spermidine-GM1 (**B**). Size bar: 100 nm.

lactonization of its sialic acid led to a significant reduction (~ 40%) in their ability to solubilize Ptx. On the basis of the above results, here we have assessed the ability of GM1 micelles modified by conjugation of spermidine to the sialic acid of the polar head group to load Ptx and Doxo. Then we have compared the chromatographic behavior of Spermidine-GM1/Ptx with that of GM1/Ptx micelles.

Fig. (**2**) shows that the loading profile of Ptx and Doxo into Spermidine-GM1 micelles is quite similar to that previously described for GM1 (Fig. **2A**, **B**). HPLC studies confirmed that the maximum loading capacity of Ptx is about 5 /1 molar ratio Spermidine- GM1/ Ptx (molar ratio of GM1/drug), reaching about 2.2 mg/ml of Ptx. Under these conditions, aqueous solutions of Spermidine-GM1/Ptx obtained remain stable and optically transparent (600 nm≤0.100 DO) at least for 4 months under refrigeration at 8° C. In order to gain a better understanding of the structural features of Spermidine-GM1 micelles, we ran GM1/Doxo and Spermidine-GM1/Doxo (10/1 molar ratio) on a size-exclusion chromatography column.

As shown in Fig. (**3**), the chromatographic elution profile of Spermidine -GM1 micelles undergoes a shift to higher elution volume with respect to GM1 micelles, indicating a decrease in its average hydrodynamic radius. Similar results were obtained when the loading drug was Ptx. In addition, to evaluate the effect of Doxo concentration into Spermidine-GM1 micelles on the stability of the complex, we analyzed, by size-exclusion chromatography, the behavior of different molar ratios of Spermidine-GM1/Doxo. Fig. (**4**) shows that, as the molar ratio of Spermidine-GM1/Doxo decreases from 10:1 to 1:1, which means an increase of Doxo in the complex, a co-elution of the drug with Spermidine-GM1 micelles is observed, clearly indicating a physical association between them. However, at a molar ratio of 2,5:1, a new

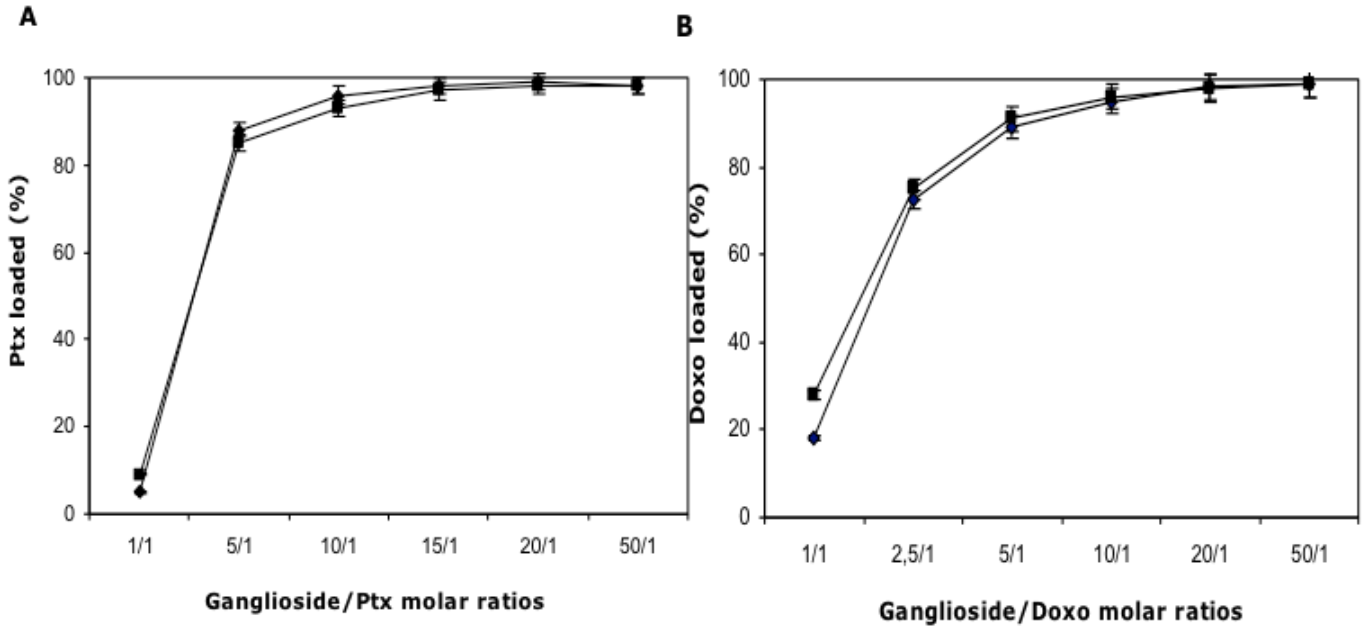

Fig. (2). (**A**) Loading of Ptx into GM1 (-□-) and Spermidine-GM1 (-■-) 1 /1; 5 /1; 10 /1; 15 /1; 20 /1 and 50/1 molar ratios of micelle /Ptx. (**B**) Loading of Doxo into GM1 micelles at: 1 /1; 2,5/1; 5/1; 1/10; 20/1 and 1/50 molar ratios. GM1 20 mg/ml (-□-) and Spermidine-GM1 20 mg/ml (-■-). Error bars represent the SD of three replicates (n=3).

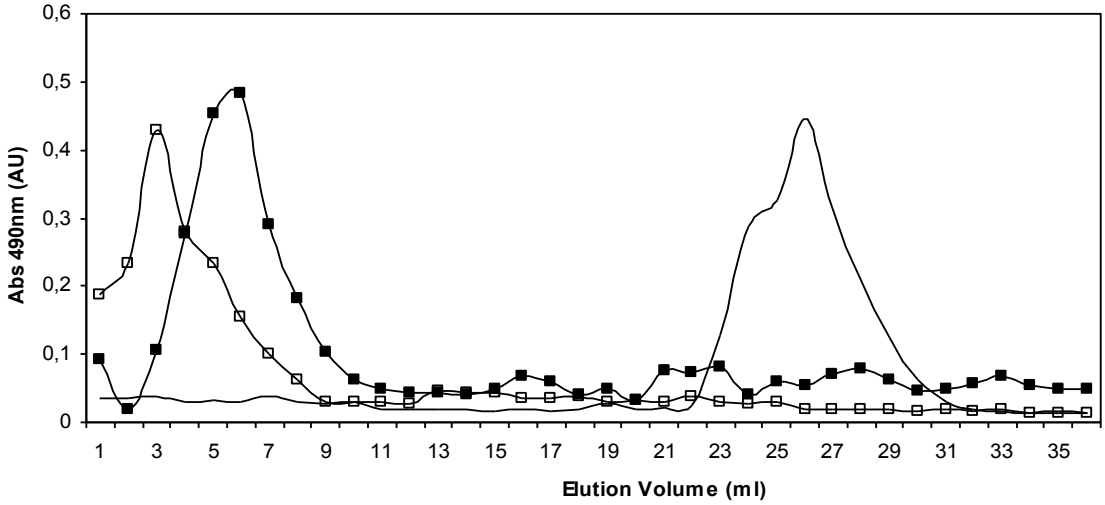

Fig. (3). Patterns of the chromatographic size-exclusion of GM1 and Spermidine-GM1 micelles with Doxo (10/1 ratio molar). Superdex 200® chromatography of Doxo (---), GM1/Doxo (-□-) and Spermidine-GM1/ /Doxo (-■-) micelles incubated at pH 7 and 20°C.

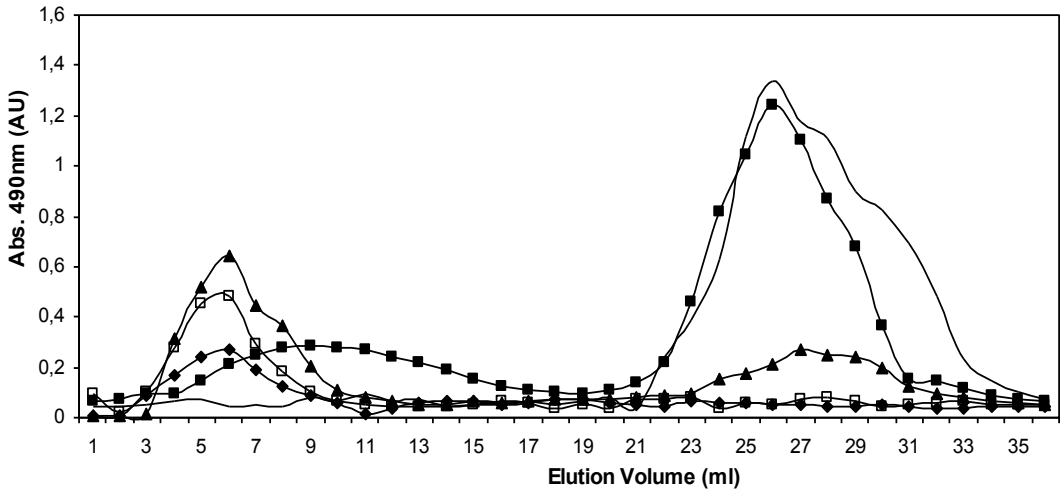

Fig. (4). Size-exclusion chromatographic patterns of Spermidine -GM1 micelles with different amounts of Doxo. Chromatography on Superdex 200® of Doxo (---) and Spermidine-GM1/Doxo at: 10/1(-♦-); 5/1 (-□-); 2.5/1 (-▲-) and 1/1 (-■-) molar ratios.

peak overlapping with the elution profile of Doxo appears, suggesting that the system starts to saturate. By increasing the concentration of Doxo to reach a 1:1 molar ratio, the behavior changes drastically, since the peak of Spermidine-GM1/Doxo appears to shift to low molecular weights with less Doxo content; most of drug begins to elute as free Doxo. The spectrophotometric quantification determined that the maximum loading capacity was about 5/1 molar ratio, which represents a concentration of 0.7 mg/ml of Doxo in 10 mg/ml of GM1/Spermidine.

In all cases, during the storage period of 4 months at 8 °C, Spermidine-GM1/drug micelles remained stable and optically transparent (600 nm≤0.100 DO), with no precipitate, drug release or alteration of physicochemical properties such as size and zeta potential.

3.4. *In Vitro* Biological Activity of Doxo in Spermidine-GM1/Doxo Micelles

Doxo loaded in GM1 micelles has been previously shown to have an effect on cell cultures similar to that of free Doxo [15]. Hence, we have evaluated whether the superficial modification of the micelle with spermidine produces any change in the biological activity of the drug on cell culture. We evaluated the effect of Spermidine-GM1/Doxo, as compared to that already described for GM1/Doxo, on Hep-2 and HeLa cell lines.

Fig. (**5**) shows the viability of Hep-2 cells after incubation with GM1/Doxo and Spermidine-GM1/Doxo micelles for 30, 60, 90 and 120 min at 10, 25 and 50 µg/ml of Doxo. As expected, cell viability decreased proportionally with the increase in drug concentration and exposure time. However, it was also observed that cells incubated with Spermidine-GM1 / Doxo had a larger decrease in cell viability than that incubated with GM1 micelles/Doxo in all conditions tested. This difference was documented in a narrow time and concentration range; [semicolon] no different activities between these two micelles were observed with longer incubation times or a higher Doxo concentration (data not shown), suggesting kinetic differences between the two micelles. The results obtained with HeLa were similar to those found for Hep-2 (data not shown).

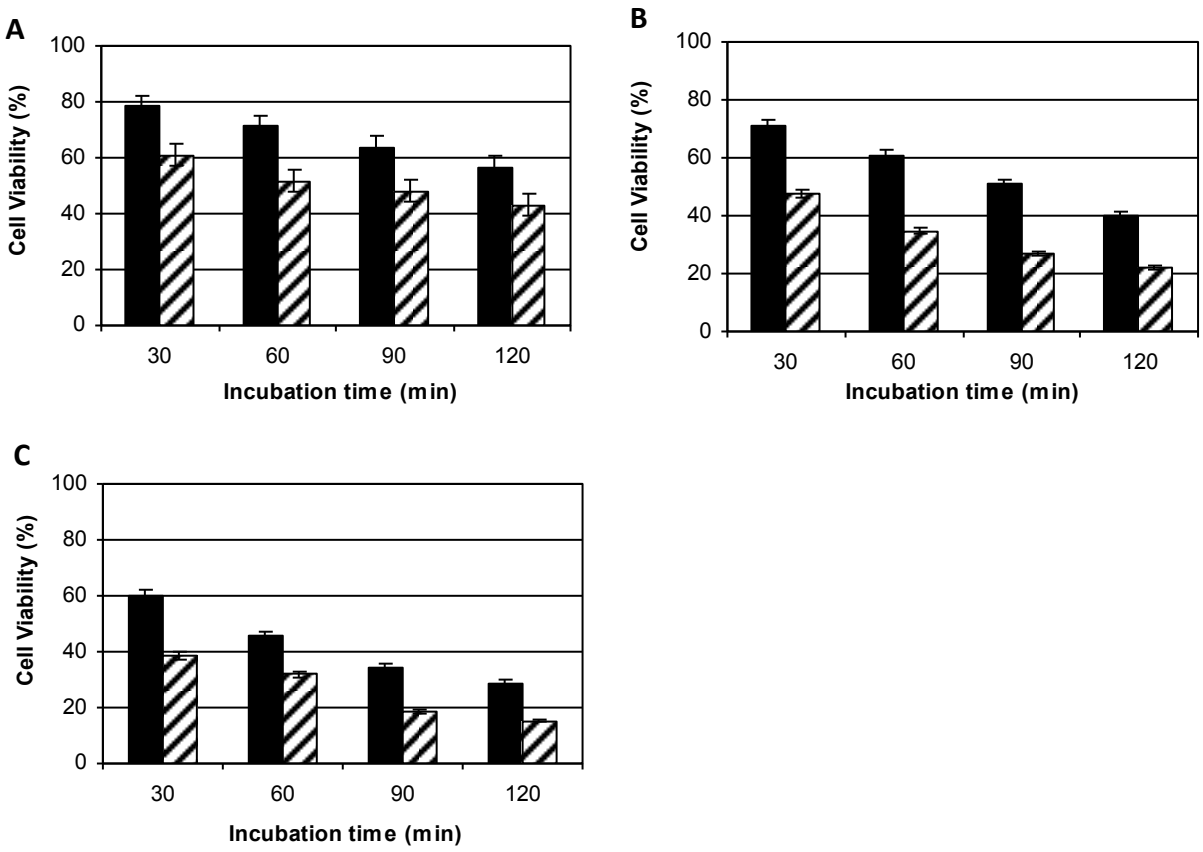

Fig. (5). *In vitro* effect of Doxo from GM1/Doxo and Spermidine-GM1/Doxo micelles at 30, 60, 90 and 120 min on Hep-2 cells: (**A**) Doxo 10µg/ml, (**B**) Doxo 25µg/ml, (**C**) Doxo 50µg/ml. GM1/Doxo (■) and Spermidine-GM1/Doxo (▨). Error bars indicate the SD of the mean (n = 3).

Moreover, toxicity controls performed with each of the vehicle components showed that neither spermidine nor GM1 or Spermidine-GM1 micelles exhibit activity on cells in the test conditions.

Several reports describe that the polyamine uptake system (PUS) is an energy-dependent process that involves a saturable carrier-mediated diffusion system [2, 9, 10]. Considering these reports, we evaluated whether GM1 micelle modified with spermidine uses some active uptake system that can be saturated by pre-incubation of the monolayers with the corresponding ligand. Furthermore, we also evaluated the dependence of the uptake process on incubation temperature, for which tests were performed at two temperature conditions, 37 and 4 °C.

To determine whether any active transport systems mediated by receptors exist, cell monolayers were pre-saturated with spermidine, Spermidine-GM1 or GM1 before contact with the micelles of Spermidine-GM1/Doxo. The results showed that pretreatment for 30 to 60 minutes did not modify cell cytotoxic effect of the drug compared to that of cells with no pretreatment (Fig. **6**); thus we had no evidence of saturable spermidine uptake mechanism in this system.

Fig. (**7**) shows the viability of Hep-2 cells after incubation with Spermidine-GM1/Doxo micelles for 30, 60, 90 and 120 min at 10 and 50 µg/ml of Doxo. Cytotoxicity curves obtained in the two temperature conditions evaluated show no significant differences in activity, suggesting that the incorporation of Spermidine-GM1/Doxo micelle does not occur through an active polyamine uptake system.

On the other hand, trials made with Ptx show similar results to those described for Doxo (data not shown).

4. DISCUSSION

Polyamines are ubiquitous polycations strictly controlled by biosynthetic and catabolic pathways that regulate their intracellular levels. They are essential for cell growth, differentiation and survival of? all mechanisms closely linked to cancer, which explains the increased attention given to these molecules and their metabolic pathways in the last times. In addition, polyamines have also been highly studied for gene therapy due to their natural condition of DNA complexing [19-26]. The use of polyamines for the delivery of cytotoxic agents is presented as a promising option to improve current oncological treatments. Many reports have described a greater effectiveness of the compounds conjugated to polyamines; yet none refers to the binding of spermidine to the vehicles. The objective of this study was to determine whether covalent attachment of spermidine to GM1 micelle surface improves absorption of antitumor agents.

The results show that spermidine binds about 25-30% of the gangliosides of the micelle. This limited binding of spermidine to GM1 molecules can be ascribed to steric hindrance on the external surface of the micelle. On the other

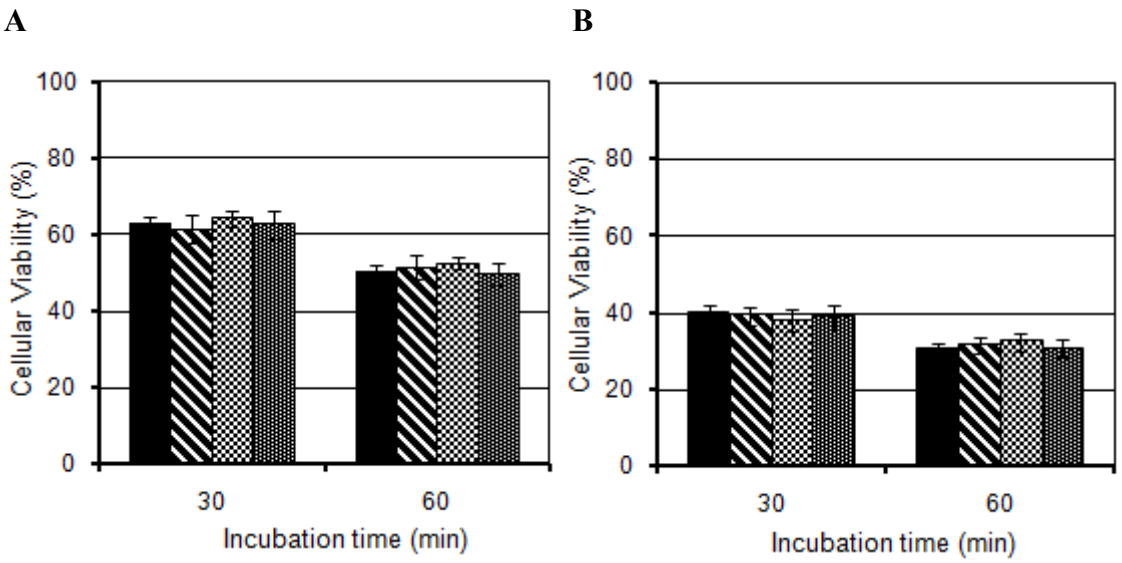

Fig. (6). Hep-2 cells were pre-treated for 60 min with 10 mg/ml Spermidine, Spermidine-GM1 micelles and GM1 before incubation with Doxo in Spermidine-GM1/Doxo micelles at 10µg/ml (**A**) and 50 µg/ml (**B**) of Doxo for 30 and 60 min. Pre-treatment: with MEM as a control (■), with GM1 (■), Spermidine (▩) and Spermidine-GM1 micelles (■).

hand, these data correlate with the z potential, showing a marked reduction of the electronegative micelle surface, from -7,35 in Spermidine-GM1 micelle to - 0,14 in GM1 micelle. This change in the net charge of the micelle surface after binding to spermidine determines the self-aggregation of the micelles in low ionic strength media, caused by the simultaneous presence of positive and negative charges on the micelle surface that interact electrostatically. This effect is reverted by the addition of 100 mM of NaCl.

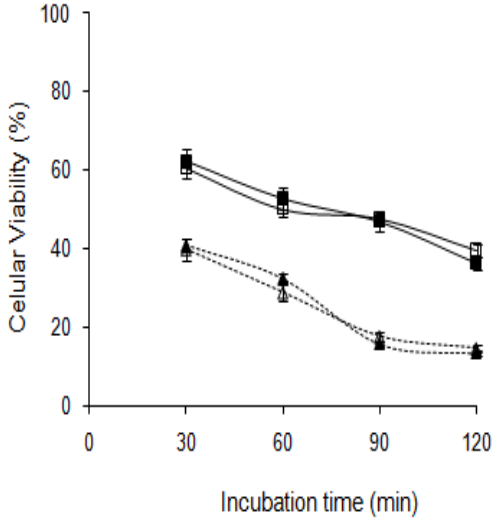

Fig. (7). *In vitro* effect of Doxo from Spermidine-GM1/Doxo micelles at 30, 60, 90 and 120 min on Hep-2 cells under two temperature conditions: 37°C and 4°C and 10 and 50 µg/ml of Doxo.

The analysis of micelle size by TEM shows slight but no significant differences between GM1 and Spermidine-GM1 micelles. Although Dynamic light scattering (DLS) studies show similar results in both cases, the polydispersity index reveals a more homogeneous population of Spermidine-GM1 micelles. Finally, size-exclusion chromatography (SEC) shows a clear shift in Spermidine-GM1 micelles in the elution profiles, indicating a smaller size of Spermidine-GM1 micelles than that of GM1 micelles.

Accordingly, it should be noted that the basis of measurement in each of these methods is completely different; therefore, the shift found by SEC could be attributed to a delay in the passage of Spermidine-GM1 micelles as a greater interaction of these micelles with the resin of the column, as resulting from the change in the net surface charge of the nanostructures. It should also be noted that the size of these structures is at the limit of detection of DLS.

The evaluation of the potential of these new Spermidine-GM1 micelles to load hydrophilic or hydrophobic antitumor drugs showed no differences from unmodified GM1 micelles, since loading Ptx or Doxo was similar to that found for GM1 micelles. These results demonstrate that spermidine conjugation does not affect the ability of GM1 micelles to spontaneously load oncologic drugs or the stability of Spermidine-GM1/drug complexes in solution.

Finally, the biological effect of Spermidine-GM1/Doxo micelles shows kinetic differences as compared to GM1/Doxo micelles in a narrow rate of concentration and short incubation time.

5. CONCLUSION

These results are in agreement with those reported by other authors in relation to different drugs conjugated to spermidine [27-29]. Thus, the conjugation of spermidine to the carrier behaves in a way similar to that of Spermidine-drugs conjugation, as described in numerous publications [12, 13, 27-29]. However, the results showed no evidence involving the participation of the polyamine transport system in the uptake of modified micelles. This increase in uptake rate may be ascribed to reasons including adsorption to binding sites in the anionic cell surface and *via* some nonspecific mechanism such as pinocytosis.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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