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Self-assembled micelles of monosialogangliosides as nanodelivery vehicles for taxanes

Victoria Leonhard [a,b], Roxana V. Alasino [a,b], Ismael D. Bianco [a,b,c], Ariel G. Garro [a], Valeria Heredia [a], Dante M. Beltramo [a,b,d,*]

[a] Centro de Excelencia en Productos y Procesos de Córdoba (CEPROCOR), Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET)), Argentina
[c] Departamento de Ciencias Exactas, Físicas y Naturales—Universidad Nacional de La Rioja, Argentina
[d] Laboratorio de Biotecnología, Facultad de Ciencias Químicas, Universidad Católica de Córdoba, Argentina

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ABSTRACT

We demonstrate herein that taxanes (paclitaxel (Ptx) and docetaxel (Dtx)) can be spontaneously loaded into ganglioside nanomicelles. The efficiency of gangliosides to solubilize taxanes was highly dependent on their self-aggregating structure. Thus, GM3 that forms unilamellar vesicles was less efficient to solubilize taxanes than gangliosides that form micelles (i.e. GM1 and GM2). Sialic acid cyclization of GM1 by acid treatment led to an important reduction in its capacity to solubilize taxanes, as also did the replacement of the fatty acid of ceramide by a dicholoracetyl group.

Water solubility of paclitaxel (Ptx) is less than 1 μg mL^{-1} and increased up to 6.3 $mg.mL^{-1}$ upon its association with GM1 micelles. The incorporation of Ptx in GM1 reached an optimum at GM1/Ptx 20/1 molar ratio when performed at room temperature. An increase in the solubilization capacity of GM1 micelles was observed upon dehydration of their polar head group by pre-treatment at 55 °C. Loading of Ptx into the micelle induced a structural reorganization that led to an important protection of Ptx reducing its hydrolysis at alkaline pH. Diffusion of either GM1 or Ptx was restricted upon mixed-micelle formation indicating that they are kinetically more stable than pure ganglioside micelles.

X-ray powder diffraction of lyophilized GM1 micelles with Ptx showed a change in their internal structure from a crystalline state to completely amorphous. Taxane-ganglioside mixed micelles were stable in solution for at least 4 months and also upon freeze-thawing or lyophilization-solubilization cycles.

Upon mixing with human blood constituents, GM1/Ptx micelles did not induce hemolysis or platelet aggregation and were spontaneously covered with human serum albumin (HSA), which could aid in the delivery of micellar content to tumors.

In vitro antimitotic activity of GM1/Ptx mixed micelles was qualitatively equivalent to that of free drug in DMSO solution.

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1. Introduction

Paclitaxel (Ptx) is one of the most active and used anticancer agents [1,2]. Because of its poor aqueous solubility it has been formulated in 50% (v/v) polyoxyethylated castor oil (Cremophor EL®) and 50% dehydrated ethanol, which portends several toxicologic, pharmacologic, and pharmaceutical disadvantages [3–5]. In order to avoid the toxicities associated with the co-solvents required for the administration of taxanes several strategies have been evaluated [6–8]. These studies led to different formulations of Ptx associated with human serum albumin (HSA) and other polymers [9,10]. One of these (Abraxane®) has been approved in over 40 countries for the treatment of metastatic breast cancer and is under clinical trials for several tumors. However, this formulation has a very limited stability upon reconstitution in saline solution (a maximum of 8 h refrigerated at 2 °C to 8 °C) and requires the use of a high pressure homogenizer for its production [9]. Therefore, alternative formulations of Ptx are still actively sought.

There is a vast range of strategies available for drug delivery in cancer. The newer approaches to cancer treatment not only supplement the conventional chemotherapy and radiotherapy but also aim to prevent damage to the normal tissues and overcome drug resistance. At present, there is no single universal method that offers

* Corresponding author at: Centro de Excelencia en Productos y Procesos de Córdoba (CEPROCOR), Ministerio de Ciencia y Tecnología de Córdoba, Pabellón CEPROCOR, CP 5164, Santa María de Punilla, Córdoba, Argentina. Tel.: +54 3541 489651/53x143; fax: +54 3541 488181.
E-mail address: 0050640@campus1.uccor.edu.ar (D.M. Beltramo).

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stable encapsulation of most drugs, mainly because each drug requires a different approach to manage all of its properties. A very promising approach to overcome systemic toxicity is the application of drug-loaded nanosized carriers, such as liposomes, polymeric nanoparticles, dendrimers and micelles [11]. The incorporation of chemotherapeutic agents into nanosized carriers has several advantages compared to systemic chemotherapy. Low-molecular weight drugs are rapidly eliminated by liver and kidneys decreasing substantially their bioavailability, a problem that is overcome by its incorporation in a nanosized vehicle [12]. Due to their small size (≤100 nm), nanosized drug carriers are passively targeted to the tumors by the enhanced permeability and retention effect, leading to a higher drug concentration at the tumor site and decreased toxicity compared with systemic administration [13]. In addition, as stated above, hydrophobic drugs can only be administered intravenously after addition of solubilizing adjuvants like ethanol or Cremophor EL®, which is often accompanied with toxic side effects [14]. In this regard, although liposomes composed of a phospholipid bilayer are capable of encapsulating active drugs, most of them are rapidly opsonized by plasma proteins and removed by phagocytic cells [15,16]. To overcome this problem, phospholipids have been conjugated to polyethylene glycol to evade the immune system and prolong the circulation time [17]. Thus, long-circulating PEG-coated liposomes were developed for the sustained delivery of doxorubicin [18]. However, it has not yet been possible to adapt this strategy for the delivery of taxanes, mainly because under the conditions employed so far, they are rapidly extruded from the bilayer rendering unstable formulations [19,20]. In this context, a strategy to overcome the unstability of delivery vehicles of taxanes in water could be the use of micelles. The major advantages of micelles are: (a) self-assembly and (b) smaller size than liposomes (usually within a range of 5–100 nm). The major disadvantage observed so far for these systems arises mainly from the high turnover of their constituents what lends them relatively unstable upon dilution [21]. As the dynamic of micellar systems is at least partially related to their critical micellar concentration (CMC), with higher turnover of constituents frequently associated to higher CMCs, we hypothesized that amphiphilic molecules that spontaneously self-assemble into micelles with a very low CMC ($\leq 10^{-8}$ M) could be used to solubilize highly hydrophobic drugs as taxanes in water-based media. The primary goal of this study was to evaluate how effectively the hydrotropic ganglioside micelles can solubilize taxanes without the aid of any organic solvent remaining in the formulation. The results reported in this work show that gangliosides, with CMCs in the order of 10^{-10}–10^{-8} M spontaneously interact with taxanes forming water soluble structures that are relatively insensitive to dilution and allow their aqueous delivery and antimitotic activity [22].

2. Materials and methods

2.1. Materials

Paclitaxel (Ptx) and docetaxel (Dtx) were from Yunnan Smandbet Co. Ltd. (Kumming, China). Stock solutions of Ptx or Dtx were prepared by dissolving the drug in either ethanol or dimethylsulfoxide (DMSO) at a final concentration of 20 mg mL^{-1}. Flutax-1 (7-O-[*N*-(4′-fluoresceincarbonyl)-l-alanyl]taxol) was purchased from Calbiochem (San Diego, CA).

Purified monosialogangliosides GM1, GM2, GM3 and LIGA in a sodium salt form were given by Dr. P. E. A. Rodriguez. Stock solutions of purified monosialogangliosides were prepared by dissolving the gangliosides in bidistilled water at a final concentration of 250 mg mL^{-1}. The solutions were maintained at 4–8 °C for 24 h. Then they were centrifuged at 50,000*g* for 15 min and the supernatant was filtered through 0.22 μm.

All other chemicals used were of analytical grade.

2.2. Methods

2.2.1. Standard procedure for preparing mixed micelles of gangliosides and taxanes

Stock solutions of GM1, GM2, GM3 or LIGA, with a concentration of 250 mg mL^{-1}, were prepared in bidistilled water 24 h prior to use.

Stock solutions of Ptx and Dtx (50 mg mL^{-1}) were prepared in DMSO. The solutions were slowly added with gentle agitation to the solution of ganglioside micelles previously adjusted to the different conditions to be tested.

The mixtures were incubated at 4 °C for 24 h and dialyzed for 24 h at the same temperature to remove all DMSO.

When evaluating the effect of temperature on the ability of GM1 micelles to load Ptx, stock solutions of GM1 with a concentration of 250 mg mL^{-1} were incubated for 30 min at 55 °C. Next, these solutions were mixed with increasing amounts of Ptx in DMSO to reach GM1/Ptx molar ratios from 25/1 to 10/1. Drug loading was performed in two temperature conditions: 55 °C and 4 °C for 30 min before incubating the samples at 4 °C for 24 h. After incubation time, the samples were dialyzed for 24 h at 4 °C to remove the DMSO.

2.2.2. Determination of Ptx or Dtx concentration and assay validation

Ptx or Dtx concentration was measured on a Curosil B C18 column (250×3.20 mm I.D., particle size 5 μm) and a Curosil B C18 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 ambient temperature (20 °C).

Validation of the method was carried out according to FDA Guidance for Bioanalytical Method Validation. Validation values obtained using the method described above were: linear range 0.5–150 μg mL^{-1}; intra-day CV <15%, max 12.3%; inter-day CV <15%, max 14.4%; baseline noise ~0.0001 AU; LLOD 0.5 μg mL^{-1}; LLOQ 1.0 μg mL^{-1}; accuracy 100.2 ± 7.8%; recovery 101.7 ± 6.1%.

To use the validated HPLC assay to determine the taxane loading capacity and efficiency Ptx or Dtx was extracted from micelles with 10 volumes of ethyl acetate. Then, samples were centrifuged at 2500 rpm for 5 min, the organic layer was transferred to a clean tube and evaporated to dryness at 40 °C. The dried residue was solubilized in 1 volume of ethanol.

2.2.3. Determination of ganglioside concentration and assay validation

Ganglioside concentrations were measured by the modified colorimetric resorcinol assay wherein determining the content of sialic acid [23]. Briefly, 1 mL of resorcinol reagent and was added to 1 mL of the samples and heated to 100 °C for 15 min. (Resorcinol reagent: 2 mg of resorcinol powder dissolved in 0.1 mL of bidistilled water + 0.8 mL of 37.9% (w/v) HCl + 2.5 μL of 0.1 M $CuSO_4$ + bidistilled water amount sufficient to reach 1 mL). Then, the samples were allowed to cool and the chromophore developed was extracted with 2.5 mL of *n*-butyl acetate: *n*-butanol (85/15 by vol). After centrifugation at 2500 rpm for 5 min, the supernatants were removed and measured spectrophotometrically at 580 nm.

Validation values obtained using the method described above were: linear range 0.5–2 μmol mL^{-1}; intra-day CV <15%, max 10.5%; inter-day CV <15%, max 13.1%; accuracy 100.1 ± 6.7%; recovery 100.9 ± 7.8%.

2.2.4. Physical stability of GM1/Ptx mixed micelles

2.2.4.1. Dialysis assay. Solutions of pure GM1 and GM1/Ptx mixed micelles, with a GM1 concentration of 250 mg mL^{-1}, underwent extensive dialysis for 72 h using dialysis membranes with a cut off of 14 kDa to ensure the outlet of the monomers during the assay. Aliquots of both dialysates were taken at 0, 24, 48 and 72 h and total amount of GM1 and Ptx was determined as described above.

2.2.4.2. Centrifugation assays. GM1/Ptx mixed micelles (20/1 mol/mol) were centrifuged at 25,000, 50,000 or 100,000*g* for 1 h at 20 °C in an XL-90 ultracentrifuge (Beckman Coulter Inc., USA). Immediately after centrifugation, GM1 and Ptx concentrations were determined on the supernatant as described above.

2.2.5. Effect of temperature on the stability of mixed micelles

GM1/Ptx mixed micelles (20/1 mol/mol) were stored for 4 months in different temperature conditions: 4, 25 and 37 °C. Aliquots of samples were taken at various time periods and quantified the amount of soluble Ptx by HPLC.

2.2.6. Effect of freeze-thawing cycles and lyophilization

GM1/Ptx mixed micelles (20/1 mol/mol) were frozen at −80 °C for 24 h. Subsequently the samples were allowed to reach room temperature and then centrifuged at 15,000*g* for 10 min before measuring the concentrations of Ptx and GM1 that remained soluble.

Lyophilized mixed micelles were dissolved in their initial volume and filtered through a 0.22 μm pore before measuring soluble Ptx and GM1.

2.2.7. Structural characterization of GM1/Ptx mixed micelles

2.2.7.1. X-ray diffraction. GM1/Ptx mixed micelles (20/1 mol/mol) were lyophilized and then analyzed in a Bruker D8-Advance with Cu anode X-ray diffractometer. GM1 and Ptx powders were used as controls.

2.2.7.2. Chromatographic analysis. Samples and controls were run on an Åkta Explorer 100 system (GE Healthcare) fitted with a Superdex 200 column, previously equilibrated with 50 mM phosphate buffer (pH 7.0) and 150 mM NaCl at a rate of 0.4 mL min^{-1}. The elution profile was followed using a UV-detector at 227 and 280 nm. Ganglioside levels were quantified as described above.

2.2.7.3. Electron microscopy. The morphology of micelles was detected using a JEOL JEM-1200 EX II transmission electron microscope at a magnification of 250 K.

Samples were prepared by loading 50 μL in a carbon grid, allowed to stand for 5 min and then dried. Then the sample excess was washed out with distilled water. Carbon grids were then incubated with 50 μL of 10% uranil acetate solution for 1 min and then dried.

2.2.8. Chemical stability of Ptx in ganglioside micelles

To evaluate the effect of alkaline pH on the stability of Ptx incorporated into the micelle of GM1, GM1/Ptx (20/1 mol/mol) mixed micelles were incubated at pH 10. Aliquots of samples were taken at various time periods and quantified the amount of soluble Ptx by HPLC. A solution of paclitaxel in ethanol 50% was used as a control.

2.2.9. Interaction of GM1/Ptx mixed micelles with blood components

Purified human red blood cells (RBC) at 4.0×10^{6} cells mm^{-3} were incubated at room temperature during 6 h with 1.5 $mg.mL^{-1}$ Ptx as GM1/Ptx (20/1 mol/mol) mixed micelles. Aliquots of samples were taken at various time periods and quantified the amount of Ptx in the supernatant of RBC by HPLC and the percentage of hemolysis measuring free hemoglobin by UV-visible spectrophotometry at 540 nm.

Purified human platelets were incubated with 10% volume of samples with GM1 at 1.13 mg mL^{-1} and Ptx at 25 μg mL^{-1} as GM1/Ptx (20/1 mol/mol) mixed micelles for 1 h at 37 °C and under constant agitation to avoid spontaneous platelet aggregation. Type 1 collagen (1 mg mL^{-1}) was used as positive control for platelet aggregation. Also a solution of paclitaxel in DMSO was used as control of pure drug effect on platelets.

2.2.10. Interaction of GM1/Ptx micelles with human plasma

GM1/Ptx mixed micelles (20/1 mol/mol) were incubated with human plasma at 37 °C for 1 to 4 h. Samples were studied by chromatographic analysis performed as mentioned above.

2.2.11. Characterization of the cellular uptake of Ptx by fluorescent microscopy

NIH 3 T3 cells (mouse embryonic fibroblast) were cultured at 37 °C with 5% CO_2 in MEM medium supplemented with 10% fetal bovine serum (GIBCO) and 2 mM L glutamine.

Ptx and GM1/Ptx (20/1 mol/mol) mixed micelles with 1% of Flu-tax were diluted in MEM at a Ptx final concentration of 10 μg mL^{-1}.

NIH 3T3 cells monolayers at 70% confluence were incubated with each sample for 15, 30 and 60 min at 37 °C. Then, cells were washed out with PBS and the incorporated drug was observed by fluorescent microscopy.

2.2.12. In vitro cytotoxicity of GM1/Ptx mixed micelles on tumoral and non-tumoral cell lines

HeLa (Human epithelial carcinoma of cervix), Hep2 (Human epithelial carcinoma of larynx), Vero (African green monkey epithelial kidney) and MA (monkey epithelial kidney) cell lines were incubated until 90% confluence in MEM medium supplemented with 10% of fetal bovine serum (NATOCOR, Córdoba, Argentina) at 37 °C with 5% CO_2. Cell monolayers were incubated during 24 h with increasing concentration of GM1, Ptx and GM1/Ptx (20/1 mol/mol) micelles. The amounts of surviving cells after incubation were evaluated by an MTT staining assay. The IC50 values, or the concentrations of various preparations at which the cell growth inhibition was 50% compared to untreated control cells, were estimated (or extrapolated) from the dose–response curves.

3. Results

3.1. Solubilization of Ptx in ganglioside micelles

Gangliosides are double-tailed anionic glycosphingolipids with a complex polar headgroup formed by several sugar units. When dissolved most of them form micelles in water above a very low CMC [24,25]. Therefore, we hypothesized that these micelles could display some detergent behavior and thus dissolve hydrophobic drugs. Generally speaking, gangliosides share the same hydrophobic portion with different headgroups, so the geometrical packing properties can be qualitatively attributed to the hydrophilic moiety. In fact, the steric requirements due to the geometric hindrances of the head groups determine that different gangliosides inside an aggregate require an interfacial area large enough to provide a place in the hydrophilic layer to host the oligosaccharide chain and its hydration water. As a general rule, the larger the polar head group, the smaller the self-assembled aggregates and the lower the aggregation numbers. In order to explore if the capacity to solubilize taxanes has any dependence on the self-assembled structure of the ganglioside we evaluated a series of monosialogangliosides (GM1, GM2 and GM3) on their capacity to solubilize Ptx. Fig. 1A shows that the solubility of Ptx was maximum when it was dissolved into micelle forming gangliosides (GM1 and GM2) while was significantly lower when it was mixed with GM3 that spontaneously forms unilamellar vesicles.

As shown in Fig. 1B, GM1 micelles (250 mg mL^{-1}) dissolve Dtx and Ptx up to 6.0 and 6.3 mg mL^{-1}, respectively, which represent an increase in water solubility of about 6000 times. Under these conditions, the addition of taxanes led to the formation of optically clear solutions. Upon increasing taxane concentration the amount that remained in solution progressively decreased suggesting that after saturating the micelles, the excess of taxane unstabilizes the mixed micelles. In agreement with this finding, it was observed that the maximum loading capacity of GM1 is around 20/1 GM1/taxane molar ratio (Fig. 1A).

In the same context, modification in the structure of the oligosaccharide chain of GM1 by lactonization of its sialic acid led to an important reduction (~40%) in its capacity to interact and solubilize Ptx (data not shown). However, it was found that the ability to load

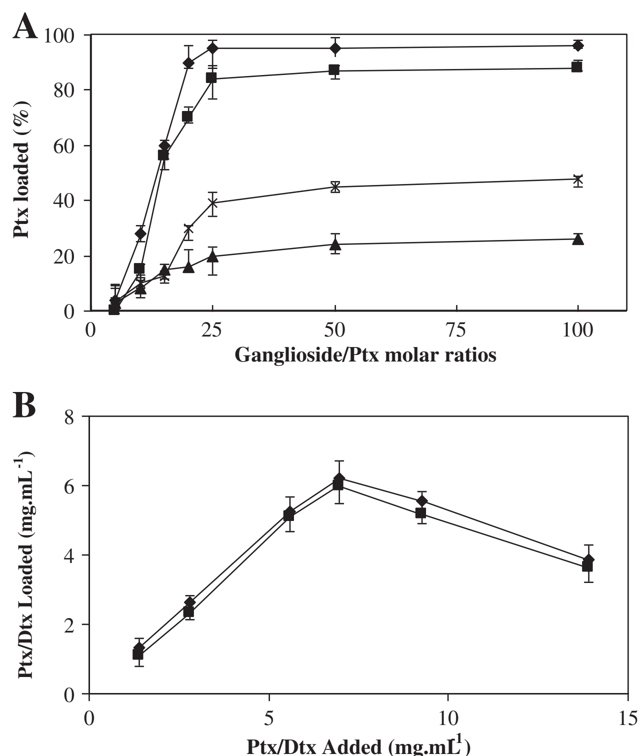


Fig. 1. (A) Loading of Ptx into Gangliosides micelles at different Ganglioside/Ptx molar ratios: 5/1; 10/1; 15/1; 20/1; 25/1; 50/1; 100/1. GM1 (—◆—), GM2 (—■—), GM3 (—▲—) and LIGA (—✱—).Error bars indicate the SD of the mean (n = 3). (B) Ability of GM1 micelles (250 mg mL^{-1}) to solubilise taxanes. The loading of Ptx (—◆—) and Dtx (—■—) was done at 4 °C for 24 h. Error bars indicate the SD of the mean (n = 3).

taxanes was not dependent on the presence of a net charge in the sialic acid of GM1 micelle because neither its titration by a pH reduction from 7 to 2 nor its counter ion affected its ability to load taxanes (data not shown).

In order to explore on the influence of the hydrophobic domain of the micelles on their capacity to solubilize taxanes we reduced the length of the fatty acid (stearic acid) bound to ceramide in GM1 by a dichloroacetyl group (obtaining what is known as LIGA). These molecules form almost spherical micelles with a mean molecular mass of 102 kDa, which is about one-fifth of the molecular weight of natural GM1 micelles [26]. The profile of Ptx solubility as a function of ganglioside concentration showed that the incorporation of Ptx in LIGA micelles was around 50% lower than in GM1 micelles (Fig. 1A).

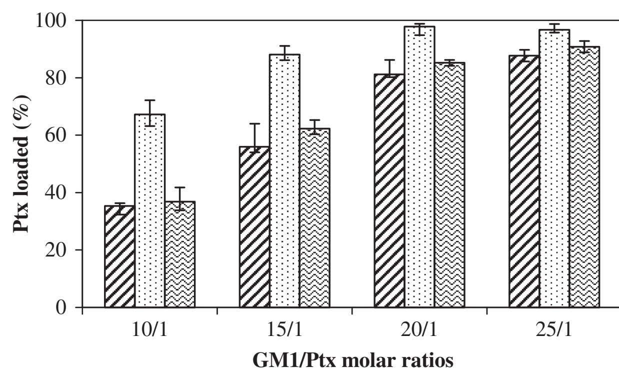


Fig. 2. Effect of temperature on the ability of GM1 to solubilize Ptx. Ptx loaded at 4 °C, 55 °C and loaded at 4 °C after a pre-incubation of GM1 at 55 °C during 30 min and then cooled at 4 °C, as described under Materials and Methods. Error bars indicate the SD of the mean (n = 3).

3.2. Effect of incubation temperature on taxane solubilization by GM1

It is known that ganglioside micelles present a bistable behavior between two states that are due to a conformational change which involves the oligosaccharide chain and is triggered by some external agent like temperature [27]. These two states are characterized by different aggregative properties and a different degree of hydration [28,29]. Therefore, in order to further characterize the dependence of the solubilization of taxanes by the structural properties of ganglioside micelles we proceeded to study the effect of temperature on the interaction between taxanes and GM1. It is known that incubation of GM1 at 55 °C induces a dehydration of the oligosaccharide chain producing a significant shrinkage in the hydrophilic region of the ganglioside micelle that is accompanied by a slight expansion of the hydrophobic region. As shown in Fig. 2, pre-incubation of GM1 micelles at 55 °C led to an important increase in its capacity (on a molar base ratio) to solubilize Ptx. This phenomenon is reversible [30,31], therefore when the samples of GM1 pre-incubated at 55 °C where cooled in an ice bath before Ptx addition the capacity to solubilize the drug returned to similar values to those found at 4 °C (Fig. 2).

3.3. Physical stability of Ptx-GM1 mixed micelles

One of the major problems with micellar systems as drug delivery vehicles is the unstability upon dilution that has been mainly associated to high CMCs and fast equilibrium between monomers and micelles [32–34]. Although gangliosides have lower CMCs than most micelle forming amphiphiles, the high turnover of their monomer constituents could still lead to stability problems upon dilution [35]. In agreement with this observation, we found a reduction of around 24% of the total content of GM1 upon its dialysis during 72 h (Table 1). Interestingly, the incorporation of Ptx into GM1 micelles led to the formation of mixed micelles that are kinetically more stable than pure GM1 micelles because the reduction in either GM1 or Ptx after extensive dialysis of GM1/Ptx micelles was only 5% (Table 1).

Another problem that has been frequently observed with solvent-free drug delivery systems of taxanes is their unstability in solution (i.e. Abraxane® is stable only for 8 h after reconstitution). Therefore, although the results of the dialysis experiments suggested a high aqueous stability of the micelles, the next step in this investigation was to determine the stability in solution of the different mixed micelles than can be formed between taxanes and GM1. As shown in Table 2, more than 90% of Ptx remained in solution after centrifugation up to 100,000 g of mixed micelles prepared at 4 °C (see Materials and Methods for details). In agreement with this result, it was also observed that the amount of Ptx that remains soluble in solution was not affected when stored for at least 4 months at 4 °C. As expected, raising the temperature to 25 and 37 °C was a factor that turned the system more hydrophilic and led to a progressive reduction of the amount of Ptx that remains in solution

Table 1
Effect of extensive dialysis in the stability of GM1 and GM1/Ptx: 20/1 molar ratio micelles.

Time of dialysis (h)	GM1 remaining (%)		Ptx remaining (%)
	GM1 Micelles	GM1/Ptx Micelles	GM1/Ptx Micelles
0	100	100	100
24	98	97	96
48	96	96	95
72	76	95	95

Note: Solutions of GM1 and GM1/Ptx mixed micelles, with initial concentrations of 250 mg mL^{-1} GM1 and 6.3 mg mL^{-1} Ptx, underwent extensive dialysis against bidistilled water for 72 h using dialysis membranes with a cut off of 14 kDa. Aliquots of both dialysates were taken at 0, 24, 48 and 72 h and total amount of GM1 and Ptx were determined by colorimetric resorcinol assay and HPLC respectively.

(Table 3). On the other hand, at least 96% of GM1/Ptx mixed micelles were resolubilized after freeze-thawing cycles or after lyophilization (data not shown).

3.4. Structural characterization of GM1/Ptx mixed micelles

It is known that GM1 micelles can exist in two stable average dimensions indicating that GM1 molecules can self-aggregate in different packing geometries [27]. The fact that most of the molecules of mixed micelles with taxanes remained associated after extensive dialysis clearly indicates that their dynamic is different from that of pure GM1 micelles. It was therefore of interest to explore whether the incorporation of Ptx in GM1 micelles produces a structural change of the aggregates. X-ray powder diffraction of lyophilized GM1 showed two major peaks that indicate the presence of ordered crystalline domains (Fig. 3A). The incorporation of Ptx into the micelles abolished the crystalline structure that is observed in pure GM1 (Fig. 3D). A simple mixture of the powders was run as a control in order to show that the observed effect was not due to a simple dilution of the ganglioside present in the sample (Fig. 3C). As the major component of the diffraction pattern of pure ganglioside powders is due to the fatty acid tail structure, this result suggests that, as expected, upon its association with GM1, Ptx is located in the hydrophobic domain of the micelles disrupting its internal crystalline structure.

In order to gain further insight into the structural characteristics of the mixed micelles formed between Ptx and GM1, we prepared GM1/Ptx micelles using different molar ratios and run them on a size exclusion chromatography column. As shown in Fig. 4, as the molar ratio of Ptx was increased the elution profile was shifted to higher volumes indicating a reduction in the average hydrodynamic radius of the mixed micelles. Under these conditions, pure GM1 micelles eluted at a volume equivalent to that of a globular protein with an average molecular mass of around 365 kDa and GM1/Ptx (20/1) mixed micelles with that of a 280 kDa protein (Fig. 4). In agreement with the results of the dialysis experiments it was observed that the peak that corresponds to GM1 monomers disappeared upon addition of Ptx (Fig. 4).

Water solutions of GM1/Ptx mixed micelles were optically clear (O.D. at 600 nm $\leq$ 0.100). This impaired the use of dynamic light scattering to characterize their average size. Therefore, transmission electron microscopy (TEM) was used in order to evaluate the form and size distribution of GM1 micelles loaded with Ptx (Fig. 5). The electron micrography of pure GM1 micelles showed two populations, one with an average diameter of 9–10 nm and other with 27–28 nm. Upon Ptx incorporation a single population of micelles with average diameter of 9–12 nm was observed (Fig. 5B).

As mentioned above, it is known that GM1 micelles undergo a structural change with increasing temperature to 55 °C, where the oligosaccharide chains are dehydrated producing a significant shrinkage in the hydrophilic region of the ganglioside micelle that is accompanied by a slight expansion of the hydrophobic region [30,31]. Fig. 5C shows that this warming shifts the balance between the two populations of GM1 micelles to the smaller population of 9–10 nm.

Table 2
Effect of centrifugation in the stability of GM1 and GM1/Ptx micelles.

Centrifugation (g)	GM1/Ptx micelles (molar ratios)			
	100/1	50/1	25/1	20/1
	Soluble Ptx (%)			
25,000	100	100	97	97
50,000	99	100	94	95
100,000	99	99	93	93

Note: Ptx were determined by HPLC on the supernatant, next to the centrifugation of the GM1/Ptx mixed micelles at 25,000; 50,000 and 100,000g for 1 h at 20 °C.

Table 3
Effect of storage temperature in the stability of GM1/Ptx (20/1 mol/mol) mixed micelles.

Time (days)	Soluble Ptx (%)		
	4 °C	25 °C	37 °C
0	100	100	100
8	96	100	94
17	95	90	94
42	95	91	87
113	95	90	86

Note: GM1/Ptx mixed micelles (20/1 mol/mol) were stored for 4 months in different temperature conditions: 4, 25 and 37 °C. Aliquots of samples were taken at various time periods and quantified the amount of soluble Ptx by HPLC.

3.5. Chemical stability of Ptx in ganglioside micelles

Structural characterization studies suggest that upon its association with GM1, Ptx is located in the hydrophobic core of the micelles and induce a reduction in their dynamics. As water hardly penetrates the hydrophobic core of ganglioside micelles we hypothesized that the incorporation of Ptx in GM1 micelles could protect it from hydrolysis at extreme pH conditions. In agreement with our speculation, it was observed that more than 60% of free Ptx was hydrolyzed in 5 h at pH 10 while almost no degradation was observed when Ptx was associated with GM1 micelles (Fig. 6).

3.6. Interaction of GM1/Ptx mixed micelles with blood components

The final purpose of drug delivery systems of taxanes is their use as parenteral vehicles. From these, intra venous is the main route for the administration of taxanes. Therefore, the following step in this study was to evaluate the interactions between GM1/Ptx micelles and human blood components. The incubation of GM1/Ptx micelles with red blood cells (RBC) led to a slight reduction of Ptx in the supernatant that was attributable to the association of micelles with RBC membranes (data not shown). Interestingly, almost no hemolysis ($\leq$3%) was detected after the exposure of RBC to 1.5 mg mL^{-1} Ptx as GM1/Ptx micelles.

We have previously shown that gangliosides do not induce platelet aggregation and exert an inhibitory role on their activation by different effectors [36]. It was therefore not surprising to find that mixed micelles of Ptx with GM1 did not induce platelet aggregation (data not shown).

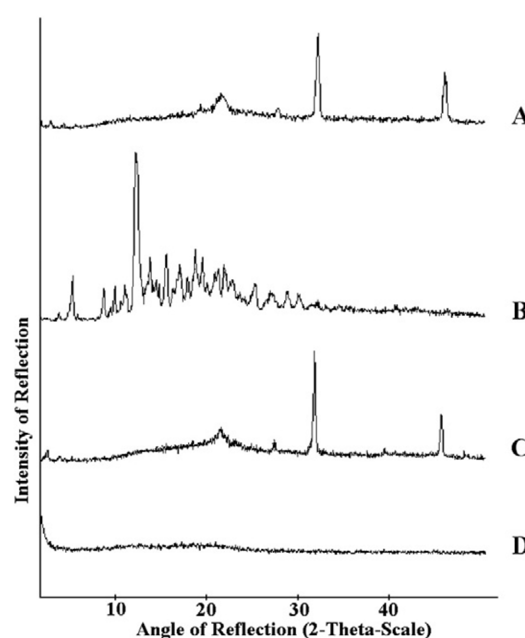


Fig. 3. X-ray diffraction patterns of : (A) GM1 powder, (B) Ptx powder, (C) mix of GM1 and Ptx powders and, (D) lyophilized GM1/Ptx (20/1) micelles.

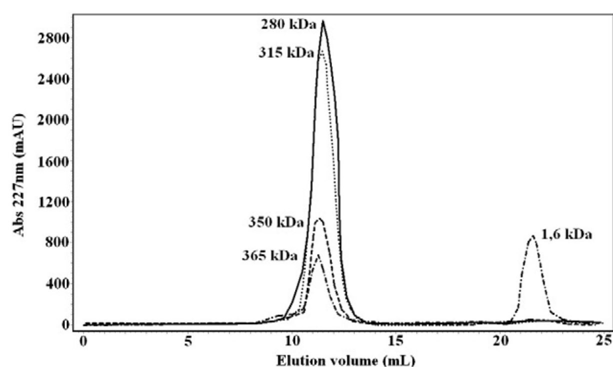


Fig. 4. Size-exclusion chromatographic patterns of GM1 micelles with different amounts of Ptx. Chromatography on Superdex 200® of GM1(–·–·) and GM1/Ptx molar ratios:100/1(– – –); 50/1 (··········) and 20/1 (——).

When GM1/Ptx micelles were incubated with human plasma at 37 °C and then run on a size exclusion chromatography column a marked reduction was observed in the peak that corresponds to human serum albumin (HSA) while the other major peaks remained unchanged (Fig. 7). A new peak appeared, eluting with a hydrodynamic radius corresponding to a globular protein of around 375 kDa (Fig. 7). This peak increased in size as a function of incubation time of GM1/Ptx micelles with human plasma (Fig. 7). Interestingly, polyacrilamide gel electrophoresis (SDS-PAGE), HPLC and TLC analysis of the samples eluting in the new peak revealed the presence of HSA, Ptx and GM1 indicating a physical association of HSA with GM1/Ptx mixed micelles (data not shown).

3.7. Characterization of the cellular uptake and biological activity of Ptx

We used a fluorescently labelled derivative of Ptx (Flu-Tax) in order to assess whether its cellular uptake is affected by its incorporation into GM1 micelles. As shown in Fig. 8, the kinetic profile of Ptx delivery from GM1 to a fibroblast cell line was similar to that from a control solution in DMSO.

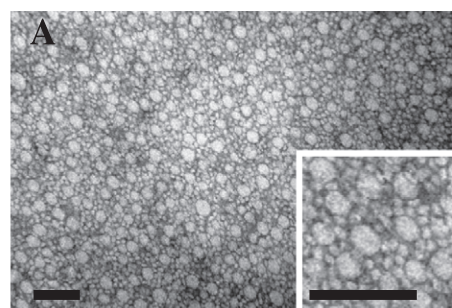
In agreement with this finding, we also observed a similar cytotoxic activity of Ptx incorporated into GM1 micelles compared with a control solution in DMSO on tumoral (Hep-2 and HeLa) and non-tumoral (Vero and MA) cells. Fig. 9 shows the cytotoxic effect of Ptx on Hep-2 (Fig. 9A) and Vero cells (Fig. 9B). The results obtained for HeLa and MA cells were similar to those obtained for Hep-2 and Vero respectively (data not shown).

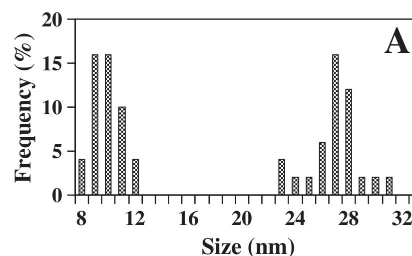
As expected, GM1 displayed almost no cytotoxic activity (Fig. 9). These results clearly indicate that Ptx can be released from ganglioside micelles into the cells where it binds to the cytoskeleton blocking the mitotic activity.

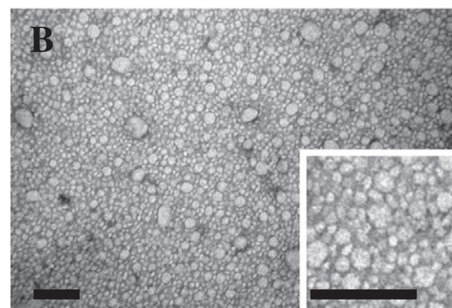
4. Discussion

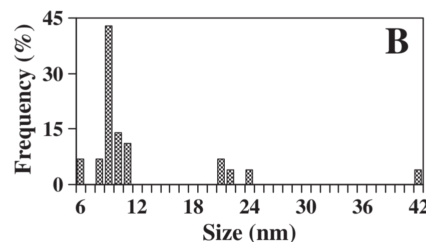
Glycosphingolipids, and especially the sialic-acid containing gangliosides occur ubiquitously in the outer layer of eukaryotic cells [37]. Gangliosides have been involved in a variety of surface phenomena, such as signal transduction, cell-cell and cell-matrix interactions [38,39]. From the structural point of view, these lipids are marked amphiphiles with several sugar rings in their polar head groups and are therefore found in the outer layer of cell membranes [40]. In aqueous solutions most of them self-assemble spontaneously as micelles with a very low CMC (~10^{-10}–10^{-8} M) and aggregation number in the order of hundreds [24,35]. These properties suggested that we consider them as promising candidates for drug delivery platforms for hydrophobic molecules.

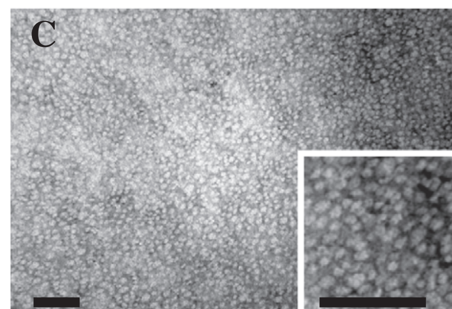
The results reported herein show that ganglioside micelles are suitable to carry large amounts of hydrophobic oncological drugs











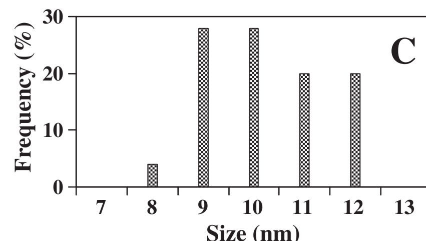


Fig. 5. Electron micrography and frequency histograms of GM1 (A), GM1 heated at 55 °C (B), and GM1/Ptx (20/1 molar ratio) mixed micelles (C). Size bar: 100 nm.

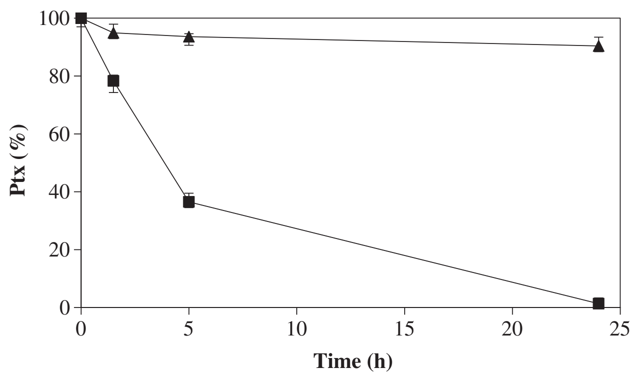


Fig. 6. Effect of pH 10 on Ptx stability. GM1/Ptx 20/1 (▲) and Ptx (50% EtOH) (■). Error bars indicate the SD of the mean (n = 3).

like taxanes in aqueous solution where the drug is spontaneously loaded without any complex process.

The three major representatives of the family of monosialogangliosides are GM1, GM2 and GM3. Structurally speaking, these lipids share the same hydrophobic chain but differ in the number of sugar rings in their hydrophilic portion, with one sialic acid and four, three and two neutral saccharides respectively. From the structural point of view both the hydrophobic/hydrophilic balance of the ganglioside micelles and their chemical composition seem to play an important role in their capacity to solubilize taxanes. Thus, when dissolved in water, GM1 and GM2 form micelles of around 300 and 450 monomers, while GM3 forms unilamellar vesicles with around 14,000 monomers [37]. As expected from these results, the maximum solubilization was attained with micelle forming gangliosides. In this context, GM1 seems to form the optimum micellar structures for taxane loading because either bigger micelles of GM2 or smaller micelles of LIGA were less efficient to dissolve the drugs. Electron micrography of pure GM1 micelles showed that GM1 self aggregates in water as two populations with 9 and 27 nm of mean diameters that are highly dynamic (Fig. 5). This study also shows that taxanes are preferably incorporated into 9 nm micelles that are dynamically stabilized. Therefore, upon taxane incorporation the population of micelles with 27 nm of mean diameter gradually tends to disappear and the newly formed mixed-micelles are stable upon dilution. These results and those from X-ray diffraction suggest that the incorporation of taxanes induces a disruption in the internal micellar nanostructure that displaces the equilibrium towards the smaller aggregate thus leading to a reduction in the CMC and a greater stability of the mixed micelles as compared to micelles of pure monosialoganglioside.

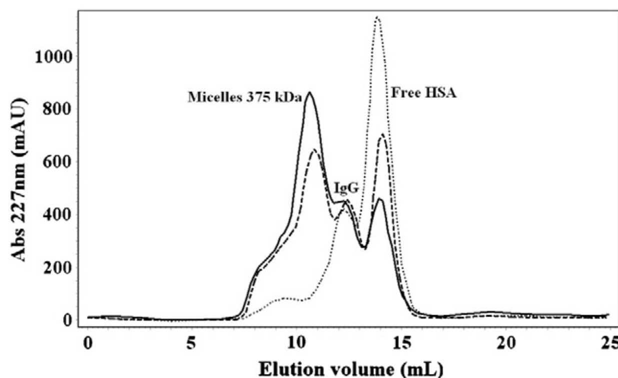


Fig. 7. Size-exclusion chromatographic patterns of: human plasma (·········) and GM1/Ptx: 20/1 micelles incubated with human plasma at 37 °C pH 7 for 1 h (- - -) and 4 h (——).

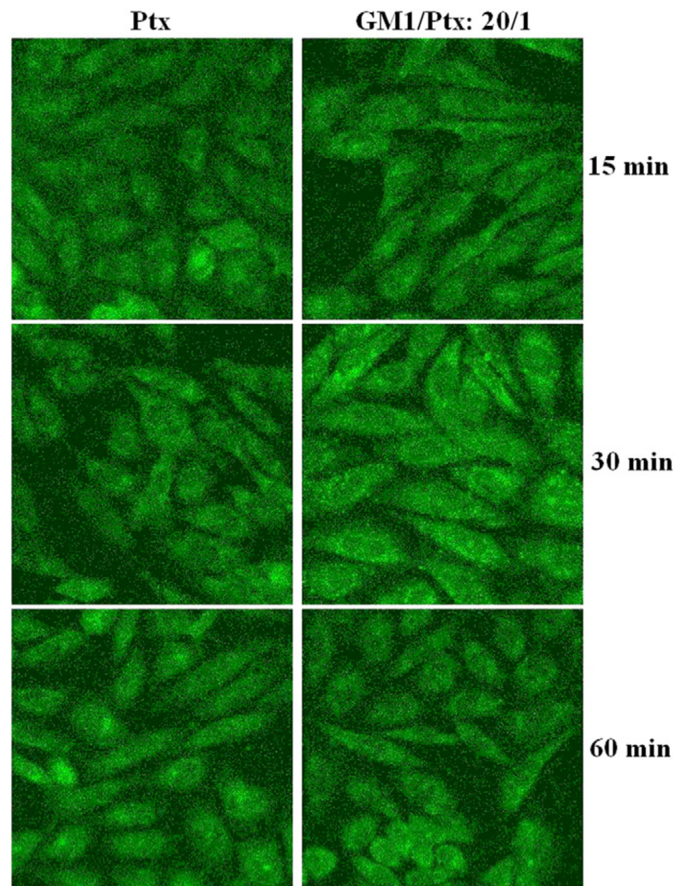


Fig. 8. Cellular uptake of Ptx from a solution in DMSO, and from GM1/Ptx 20/1 mixed micelles at 15, 30 and 60 min.

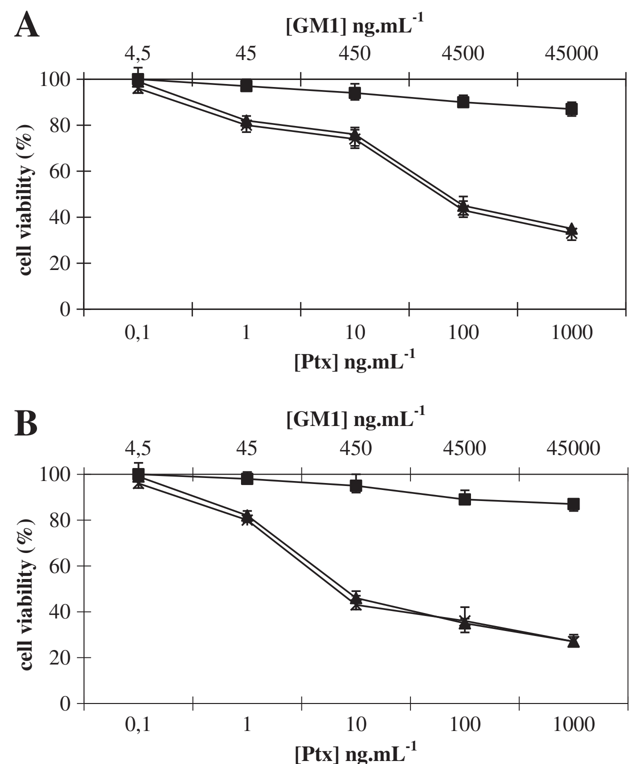


Fig. 9. *In-vitro* cytotoxic effects of GM1 (■), Ptx (▲) and GM1/Ptx: 20/1 (✳) on: (A) Vero and (B) Hep-2 cells. Error bars indicate the SD of the mean ($n = 3$).

As could be expected, dehydrating the polar head group by preheating the micelles at 55 °C increased their loading capacity. However, a reduction in the polarity of the head group of GM1 by titration of the sialic acid did not have any effect and its lactonization reduced its loading capacity.

It is important to stress the fact that there is a saturating limit of taxane to ganglioside in the mixed micelles (around 1/20 on a molar base) and that once the ganglioside micelles are saturated with taxane, its excess unstabilizes the mixed micelles with a resultant precipitation of most of the drug (see Fig. 1B from right to left sense). As stated above, the loading capacity of GM1 micelles to solubilize taxanes may be increased by a temperature-induced dehydration of the polar head group. However, as this dehydration is readily reversed by a reduction of temperature, this proved not to be a good option for the effective solubilization of high amounts of taxanes.

In spite of good colloidal and drug-solubilizing properties, the low drug loading efficiency and use of organic solvents for drug loading are still significant concerns in the polymeric micelle formulations developed so far as delivery systems of poorly soluble drugs [41,42]. In this regard, the incorporation of taxanes into GM1 micelles proved to be a platform that allows increasing their water solubility up to 6 mg mL^{-1} which is in agreement to the concentrations required for their therapeutic application and presence in the commercially approved formulations of Ptx (Taxol® and Abraxane®) and Dtx (Taxotere®).

Research on drug delivery systems has greatly advanced; however, the ability to achieve high targeting efficiency at the tumor site and associated cells remains as a significant challenge for the development of micelle-mediated drug delivery systems. In this context, it has been shown that albumin binding to the gp60 receptor of endothelial cells could promote transcytosis to the interstitial space near the tumor, where its interaction with the tumor secreted protein SPARC (secreted protein, acidic and rich in cysteine) may result in intratumoral accumulation of albumin-bound paclitaxel [10,43]. The results of this study show that upon interaction with blood GM1/Ptx mixed micelles do not display cellular toxicity and form ternary complexes with albumin which could improve the delivery of their content to tumors. In this connection, *in vitro* studies showed that GM1/Ptx mixed micelles interact and release their content to normal (Vero and MA) and tumoral (Hep-2 and HeLa) cells with IC50s of around 100 ng mL^{-1} and 10 ng mL^{-1}, respectively, suggesting that there could also be some preferential uptake by tumoral cells as compared to normal ones. It is also important to remark that these IC50s are similar to those obtained with other delivery systems of taxanes [44,45].

Recent studies have addressed the issue that gangliosides and cholesterol could be involved in the formation of senile plaques of amyloid β-peptide [46]. On the other side, GM1 was shown to increase the viability of PC12 cells exposed to hydrogen peroxide and amyloid β-peptide [47]. In this context, as the role of GM1 in amyloid β-protein precursor processing is not yet defined, further studies should be performed to determine if the amounts of GM1 required for the delivery of the therapeutic doses of taxanes could have any impact in amyloid β-peptide formation.

5. Conclusions

In this study, a novel self-assembled nanomicellar system for the aqueous delivery of taxanes has been disclosed [22]. Our results show that:

- Aqueous solutions of ganglioside micelles spontaneously load taxanes increasing their water solubility up to 6000 fold from around 1 μg mL^{-1} to around 6 mg mL^{-1} allowing to attain therapeutically required concentrations.
- GM1/taxane micelles can easily be prepared without the use of high-pressure homogenization.
- Mixed micelles of taxanes and GM1 are stable in aqueous solution without organic solvent for at least 4 months when kept refrigerated.
- Loading of the taxane into GM1 micelles induces a structural reorganization that leads to an important protection of Ptx reducing its hydrolysis at alkaline pH.
- Diffusion of either GM1 or taxane is restricted upon mixed-micelle formation leading to structures that are stable upon dilution, freeze-thawing cycles and lyophilization.
- Upon incubation with blood components the mixed micelles do not induce hemolysis or platelet aggregation.
- The incorporation of taxanes in ganglioside micelles does not impair their uptake and biological effect in cell cultures.

Altogether, the physico-chemical properties and the good biocompatibility of the system open the possibility to use this platform for the design of novel nanocarriers for hydrophobic or amphipathic active pharmaceutical ingredients.

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References

[1] M.C. Wani, H.L. Taylor, M.E. Wall, P. Coggon, A.T. McPhail, Plant antitumor agents VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*, J. Am. Chem. Soc. 93 (1971) 2325–2327.
[2] E.K. Rowinsky, R.C. Donehower, Paclitaxel (Taxol), N. Engl. J. Med. 332 (1995) 1004–1014.
[3] R.T. Liggins, W.L. Hunger, H.M. Burt, Solid-state characterization of paclitaxel, J. Pharm. Sci. 86 (1997) 1458–1463.
[4] E.K. Rowinsky, The taxanes: dosing and scheduling considerations, Oncology 11 (1997) 7–19.
[5] F.A. Greco, T.M. Hainsworth, One-hour paclitaxel infusions: a review of safety and efficacy, Cancer J. Sci. Am. 5 (1999) 179–191.
[6] S.M. Moghimi, A.C. Hunter, J.C. Murray, Long-circulating and target-specific nanoparticles: theory to practice, Pharmacol. Rev. 53 (2001) 283–318.
[7] M.L. Adams, A. Lavasanifar, G.S. Kwon, Amphiphilic block copolymers for drug delivery, J. Pharm. Sci. 92 (2003) 1343–1355.
[8] Z.G. Gao, A.N. Lukyanov, A. Singhal, V.P. Torchilin, Diacyllipid-polymer micelles as nanocarriers for poorly soluble anticancer drugs, Nano Lett. 2 (2002) 979–982.
[9] N.K. Ibrahim, N. Desai, S. Legha, P. Soon-Shiong, R.L. Theriault, E. Rivera, B. Esmaeli, S.E. Ring, A. Bedikian, G.N. Hortobagyi, J.A. Ellerhorst, Phase I and pharmacokinetic study of ABI-007, a Cremophor-free, protein-stabilized, nanoparticle formulation of paclitaxel, Clin. Cancer Res. 8 (2002) 1038–1044.
[10] W.J. Gradishar, Albumin-bound paclitaxel: a next-generation taxane, Expert Opin. Pharmacother. 7 (2006) 1041–1053.
[11] B. Mishra, B.B. Patel, S. Tiwari, Colloidal nanocarriers: a review on formulation technology, types and applications toward targeted drug delivery, Nanomedicine 6 (2010) 9–24.
[12] Y. Matsumura, Poly (amino acid) micelle nanocarriers in preclinical and clinical studies, Adv. Drug Deliv. Rev. 60 (2008) 899–914.
[13] H. Maeda, J. Wu, T. Sawa, Y. Matsumura, K. Hori, Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review, J. Control. Release 65 (2000) 271–284.
[14] R.B. Weiss, R.C. Donehower, P.H. Wiernik, T. Ohnuma, R.J. Gralla, D.L. Trump, J.R. Baker Jr., D.A. Van Echo, D.D. Von Hoff, B. Leyland-Jones, Hypersensitivity reactions from taxol, J. Clin. Oncol. 8 (1990) 1263–1268.
[15] R. Kunstfeld, G. Wickenhauser, U. Michaelis, M. Teifel, W. Umek, K. Naujoks, K. Wolff, P. Petzelbauer, Paclitaxel encapsulated in cationic liposomes diminishes tumor angiogenesis and melanoma growth in a “humanized” SCID mouse model, J. Invest. Dermatol. 120 (2003) 476–482.
[16] D.C. Drummond, O. Meyer, K. Hong, D.B. Kirpotin, D. Papahadjopoulos, Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors, Pharmacol. Rev. 51 (1999) 691–743.
[17] D. Papahadjopoulos, T.M. Allen, A. Gabizon, E. Mayhew, K. Matthay, S.K. Huang, K.D. Lee, M.C. Woodle, D.D. Lasic, C. Redemann, Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy, Proc. Natl. Acad. Sci. 88 (1991) 11460–11464.

[18] J.A. O'Shaughnessy, Pegylated liposomal doxorubicin in the treatment of breast cancer, Clin. Breast Cancer 4 (2003) 318–328.

[19] A. Sharma, R.M. Straubinger, Novel taxol formulations: preparation and characterization of taxol-containing liposomes, Pharm. Res. 11 (1994) 889–896.

[20] R.M. Straubinger, A. Sharma, M. Murray, E. Mayhew, Novel taxol formulation: taxol-containing liposomes, J. Natl. Cancer Inst. Monogr. 15 (1993) 69–78.

[21] V.P. Torchilin, Micellar nanocarriers: pharmaceutical perspectives, Pharm. Res. 24 (2007) 1–16.

[22] V. Leonhard, D.M. Beltramo, R.V. Alasino, I.D. Bianco, Water-soluble pharmaceutical composition comprising at least one therapeutically active substance having hydrophobic properties and at least one compound selected from among sialoglycosphingolipids, glycosphingolipids or a mixture of sialoglycosphingolipids and glycosphingolipids, 2011. WO 2011/113981 A1.

[23] T. Miettinen, I.T. Takki-Luukkainen, Use of butyl acetate in determination of sialic acid, Acta Chem. Scand. 13 (1959) 856–858.

[24] B. Ulrich-Bott, H. Wiegandt, Micellar properties of glycosphingolipids in aqueous media, J. Lipid Res. 25 (1984) 1233–1245.

[25] S. Sonnino, L. Cantú, M. Corti, D. Acquotti, B. Venerando, Aggregative properties of gangliosides in solution, Chem. Phys. Lipids 71 (1994) 21–45.

[26] S. Sonnino, L. Cantú, M. Corti, D. Acquotti, G. Kirschner, G. Tettamanti, Aggregation properties of semisynthetic GM1 ganglioside ($II^3Neu5AcGgOse_4Cer$) containing an acetyl group as acyl moiety, Chem. Phys. Lipids 56 (1) (1990) 49–57.

[27] P. Brocca, L. Cantú, M. Corti, E. Del Favero, A. Raudino, Collective phenomena in confined micellar systems of gangliosides, Physica A 304 (2002) 117–190.

[28] L. Cantú, M. Corti, E. Del Favero, E. Muller, A. Raudino, S. Sonnino, Thermal hysteresis in ganglioside micelles investigated by differential scanning calorimetry and light-scattering, Langmuir 15 (1999) 4975–4980.

[29] D. Orthaber, O. Glatter, Time and temperature dependent aggregation behaviour of the ganglioside GM1 in aqueous solution, Chem. Phys. Lipids 92 (1) (1998) 53–62.

[30] M. Hirai, T. Takizawa, Intensive extrusion and occlusion of water in ganglioside micelles with thermal reversibility, Biophys. J. 74 (1998) 3010–3014.

[31] T. Hayakawa, M. Hirai, Hydration and thermal reversibility of glycolipids depending on sugar chains, Eur. Biophys. J. 31 (1) (2002) 62–72.

[32] V.P. Torchilin, Micellar nanocarriers: pharmaceutical perspectives, Pharmacol. Res. 24 (1) (2007).

[33] G. Gaucher, M.H. Dufresne, V.P. Sant, N. Kang, D. Maysinger, J.C. Leroux, Block copolymer micelles: preparation, characterization and application in drug delivery, J. Control. Release 109 (2005) 169–188.

[34] A.N. Lukyanov, V.P. Torchilin, Micelles from lipid derivatives of water-soluble polymers as delivery systems for poorly soluble drugs, Adv. Drug Deliv. Rev. 56 (2004) 1273–1289.

[35] S. Formisano, M.L. Johnson, G. Lee, S.M. Aloj, H. Edelhoch, Critical micelle concentrations of gangliosides, Biochemistry 18 (6) (1979).

[36] H.A. Guglielmone, J.J. Daniele, I.D. Bianco, E.J. Fernandez, G.D. Fidelio, Inhibition of human platelet aggregation by gangliosides, Thromb. Res. 98 (1) (2000) 51–57.

[37] S. Sonnino, L. Mauri, V. Chigorno, A. Prinetti, Gangliosides as components of lipid membrane domains, Glycobiology 17 (1) (2006) 1R–13R.

[38] C.R. Partington, J.W. Daly, Effect of gangliosides on adenylate cyclase activity in rat cerebral cortical membranes, Mol. Pharmacol. 15 (1979) 484–491.

[39] E.G. Bremer, S. Hakomori, D.F. Bowen-Pope, E. Raines, R. Ross, Ganglioside-mediated modulation of cell growth, growth factor binding, and receptor phosphorylation, J. Biol. Chem. 259 (1984) 6818–6825.

[40] D. Acquotti, S. Sonnino, Use of nuclear magnetic resonance spectroscopy in evaluation of ganglioside structure, conformation and dynamics, Methods Enzymol. 312 (2000) 247–272.

[41] K.M. Huh, S.C. Lee, Y.W. Cho, J. Lee, J.H. Jeong, K. Park, Hydrotropic polymer micelle system for delivery of paclitaxel, J. Control. Release 101 (1) (2005) 59–68.

[42] J. Lee, S.C. Lee, G. Acharya, C.J. Chang, K. Park, Hydrotropic solubilization of paclitaxel: analysis of chemical structures for hydrotropic property, Pharm. Res. 20 (7) (2003) 1022–1030.

[43] M. Fukunaga-Kalabis, M. Herlyn, Unraveling mysteries of the multifunctional protein SPARC, J. Invest. Dermatol. 127 (2007) 2497–2498.

[44] J. Wang, D. Mongayt, V.P. Torchilin, Polymeric micelles for delivery of poorly soluble drugs: preparation and anticancer activity *in vitro* of paclitaxel incorporated into mixed micelles based on poly(ethylene glycol)-lipid conjugate and positively charged lipids, J. Drug Target. 13 (1) (2005) 73–80.

[45] F. Danhier, N. Magotteaux, B. Ucakar, N. Lecouturier, M. Brewster, V. Préat, Novel self-assembling PEG-p-(CL-co-TMC) polymeric micelles as safe and effective delivery system for Paclitaxel, Eur. J. Pharm. Biopharm. 73 (2009) 230–238.

[46] Q. Zha, Y. Ruan, T. Hartmann, K. Beyreuther, D. Zhang, GM1 ganglioside regulates the proteolysis of amyloid precursor protein, Mol. Psychiatry 9 (2004) 946–952.

[47] T.V. Sokolova, I.O. Zakharova, V.V. Furaev, M.P. Rychova, N.F. Avrova, Neuroprotective effect of ganglioside GM1 on the cytotoxic action of hydrogen peroxide and amyloid β-peptide in PC12 cells, Neurochem. Res. 32 (2007) 1302–1313.