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Characterization of Lipophilic Gemcitabine Prodrug−Liposomal Membrane Interaction by Differential Scanning Calorimetry

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Abstract: Gemcitabine is an anticancer agent rapidly deaminated to the inactive metabolite 2′,2′-difluorodeoxyuridine. Its stability as well as bioavailability can be increased by making prodrugs. A series of lipophilic prodrugs of gemcitabine were synthesized by linking the 4-amino group with valeroyl, lauroyl, and stearoyl linear acyl derivatives. We studied, by the differential scanning calorimetry technique, and compared the interaction of pure gemcitabine and its prodrugs with dimyristoylphosphatidylcholine and distearoylphosphatidylcholine vesicles with the aim of demonstrating if the gemcitabine prodrug is more able than the pure gemcitabine to interact with lipid vesicles employed both as model biomembranes and as carriers in the transport of antitumor drugs. These studies, carried out by static and kinetic calorimetric measurements, give evidence that the increase of the prodrug's lipophilic character improves the interaction with lipid bilayers, favoring the absorption through the lipid barriers and allowing the liposomes to work (when the prodrug is inserted inside the vesicles) as a lipophilic carrier which is able to deliver the drug near the cell surface. The use of different prodrugs modified in their lipophilic character, of different kinds of vesicles (multilamellar and unilamellar), and of different kinds of vesicles forming phospholipids permitted us to determine the better equilibrium between in-vesicle solubility and through-vesicle diffusion of the drug, important in the preformulative studies of antitumor carriers based on phospholipid formulations. Such studies suggest that the prodrug lipophilic tail should modulate the transport and the release of gemcitabine inside the cellular compartments, and the efficiency of the liposomal system is related to the length of the prodrug's acyl chain which has to match the phospholipid acyl chain allowing or retarding the migration through the lipid release device.

Keywords: Gemcitabine; lipophilic gemcitabine derivatives; liposomes; pharmacokinetics; calorimetry

Introduction

Gemcitabine (2′,2′-difluorodeoxycytidine) is a pyrimidine antimetabolite structurally and pharmacologically similar to cytarabine (Ara-C), specifically developed to extend the activity of Ara-C to nonhematological malignancies. When used in monotherapy, gemcitabine is indicated for pancreatic cancer and non-small cell lung cancer,[1] but it has also demonstrated activity against many solid tumors.[2] Gemcitabine is rapidly and extensively deaminated by cytidine deaminase in blood, liver, kidney, and other tissues to the inactive metabolite 2′,2′-difluorodeoxyuridine and excreted in the urine, showing a very short plasma half-life (8−17

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min).[1] Many different approaches have been tried to improve gemcitabine metabolic stability and, consequently, its "in vivo" cytotoxic activity. Eli Lilly patented the synthesis of saturated and monounsaturated C18 and C20 long-chain 4-(*N*)-acyl-derivatives and 5′-esters of gemcitabine. It has been shown that liposomes provide protection against rapid metabolic inactivation of drugs.[3] Nevertheless, low-molecular-weight water-soluble molecules, such as 5-fluorouridine or Ara-C, a drug strictly related to gemcitabine, rapidly diffuse through the liposome bilayers[4,5] thus limiting the shelf life and clinical utility of these liposomes. To overcome this problem, a large number of lipophilic prodrugs of 5-fluorouridine or Ara-C have been synthesized[5−7] and encapsulated in liposomes, with high efficiency. The in vivo antitumor activity of these liposomal prodrugs against a variety of tumors is generally superior to that of the pure drug.[8,9]

Recently, the synthesis and encapsulation in liposomes of a series of gemcitabine 4-(*N*)-acyl derivatives prodrugs was reported.[10] The presence of an acyl moiety in the prodrugs exerts a 2-fold action: (a) to increase the physical chemistry properties so as to make it more lipophilic and consequently more ready to be inserted in the lipid vesicles; (b) to chemically protect the amidic group in the N-4 position of cytosine that is rapidly metabolized in plasma.

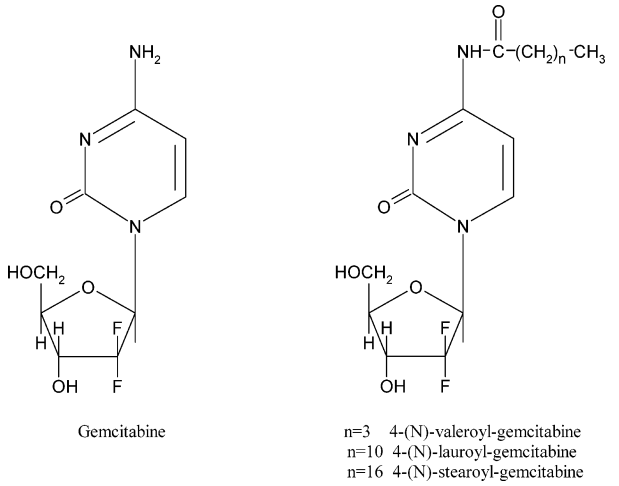

Figure 1. Gemcitabine and 4-(*N*)-acyl-gemcitabine prodrug structure.

The gemcitabine (Gem) and three of its prodrugs, 4-(*N*)-valeroyl-gemcitabine (Gem-C5), 4-(*N*)-lauroyl-gemcitabine (Gem-C12), and 4-(*N*)-stearoyl-gemcitabine (Gem-C18) (Figure 1), were here considered with the aim of evaluating the effect of the length of the acyl chain on the ability to interact with the biomembrane model in an aqueous environment.

Such interactions can be studied by differential scanning calorimetry (DSC), a nonperturbative technique largely employed to detect the effects exerted by biomolecules on the lipid bilayers of a cell-like membrane in the processes of entrapment and release inside lipid vesicles.[11,12]

Lipid membranes (multilamellar and unilamellar vesicles, MLV and LUV, are usually employed as synthetic simplified model membranes) undergo a sharp phase transition from an ordered gel-like structure to a disordered fluidlike structure. The differential scanning calorimetry technique can detect such a phase change. The presence of foreign substances dissolved in the lipid bilayer strongly affects the thermodynamic properties of the phase transition, sometimes causing variations in the phase transition temperature (T_m). The amplitude of the effect can depend on the amount of chemicals dissolved in the lipid structure.[13−18]

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In this paper, the DSC technique has been used to study the interaction of gemcitabine and three of its prodrugs, with dimyristoylphosphatidylcholine (DMPC) and distearoylphosphatidylcholine (DSPC), as well as to monitor uptake experiments as a function of the time, to gain information on the transfer of such compounds through an aqueous medium. The procedure previously described to study the interaction of similar compounds with DMPC multilamellar vesicles[19,20] has been here followed.

The calorimetric investigation of the interaction between bioactive compounds and membranes was carried out by following a "classical" method (by dissolving the bioactive compounds and the lipids in organic solvent before multilamellar vesicle preparation) that can only be considered as a way to detect the maximum interaction between a foreign molecule and the lipid bilayer. The data obtained by the "classical" method and those obtained by the transfer experiments are compared each other. The comparison gives information on the ability of a compound to permeate the aqueous layer surrounding the lipid membrane, reach the outer lipid layer, and pass through the internal layers, penetrating into a cell-like model.[19−23] In this way it is possible to have knowledge in the understanding of the penetration routes of such antitumor agents by modulating their lipophilicity. The data are compared with those obtained by using fatty acids (valeric acid, C5; lauric acid, C12, and stearic acid, C18) to better define the influence of the hydrophobic chain on the interaction of the 4-(*N*)-acyl-gemcitabine derivatives with model membranes.

Experimental Section

Materials. Gemcitabine was synthesized in our laboratory following the procedure reported in the literature.[24] The synthesis of 4-(*N*)-acyl-gemcitabine derivatives as well their characterization was reported in a previous paper by some of us.[13]

1,2-Dimyristoyl-*sn*-glycero-3-phosphatidylcholine was supplied by Genzyme Pharmaceuticals (Liestal, Switzerland). 1,2-Distearoyl-*sn*-glycero-3-phosphatidylcholine, valeric acid, lauric acid, and stearic acid were supplied by Fluka Sigma Aldrich (Switzerland).

Lipids were chromatographically pure as assessed by two-dimensional thin-layer chromatography. Lipid concentrations were determined by phosphorus analysis.[25] 100 mM phosphate buffer solution, adjusted to pH 7.4, was employed. All other reagents and solvents used were of analytical grade from Carlo Erba Reagenti (Milan, Italy).

MLV Preparation. To obtain DMPC or DSPC multilamellar vesicles, in the presence and absence of gemcitabine and 4-(*N*)-acyl-gemcitabine derivatives and pure fatty acids, chloroform stock solutions of compounds were prepared. Aliquots of the DMPC or DSPC solution were delivered in glass tubes in order to have the same amount (0.010 32 mmol) of lipid, and then aliquots of gemcitabine or gemcitabine prodrugs or fatty acid solution were added in order to have 0.00 and 0.12 molar fractions of compound with respect to the lipid. Solvents were removed under nitrogen, and the resulting film was freeze-dried to remove the residual solvents. Phosphate buffer solution (168 μL; 100 mM pH 7.4) was added to the films, to obtain liposomes. The samples were heated to 37 °C (DMPC) or 65 °C (DSPC) (temperatures above the gel−liquid crystalline phase transition happening at 24.8 °C for DMPC and 55.1 °C for DSPC) and vortexed three times for 1 min. The samples were left for 1 h at 37 or 65 °C to homogenize the liposomes and to allow the redistribution of the compounds between lipid and aqueous phases; the liposomes being in a disordered phase, the compounds' partition is favored. Aliquots of 120 μL of lipid suspension (0.007 375 mmol of lipids) were then transferred to a 160 μL DSC aluminum pan, hermetically sealed, and submitted to DSC analysis. Phospholipid phosphorus content was assessed in each liposome preparation by phosphate assay after destruction with perchloric acid.[25] Size distribution of liposomes was monitored by photon correlation spectroscopy using a Coulter model N4SD submicron particle analyzer (Coulter Electronics, Florida, USA).

LUV Preparation. To obtain LUV, MLV were repetitively (19 times) passed under moderate pressure at a

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temperature at least 5 °C above the T_m through polycarbonate membranes (pore diameter 100 nm) in an extruder system (LiposoFast Basic, Avestin Inc.).[26,27] The membrane pores are almost cylindrical, and vesicles (unilamellar or multilamellar) that are larger than the mean pore diameter are reduced in size and lamellarity during the passage through the pores, resulting in final vesicle size that corresponds to the mean size of the pores.[28,29]

Differential Scanning Calorimetry. DSC was performed by using a Mettler TA Star^e system equipped with a DSC 822^e calorimetric cell and Mettler STAR^e V 6.10 SW software. The scan heating rate employed was 2 °C/min in the temperature range 5−37 °C for DMPC and 25−65 °C for DSPC. The sensitivity was automatically chosen as the maximum possible by the calorimetric system, and the reference pan was filled with phosphate buffer solution. The calorimetric system was calibrated, in temperature and enthalpy changes, by using indium, stearic acid, and cyclohexane by following the procedure of the Mettler TA STAR^e software.

To check the reproducibility of the results, the calorimetric scan was repeated at least four times; aliquots of all samples were then extracted from calorimetric aluminum pans and used to determine, by the phosphorus assay,[25] the exact amount of phospholipids present in each sample.

Permeation Experiments. A fixed amount of powdered or liquid (valeric acid) compound (aimed at obtaining a 0.12 molar fraction with respect to the phospholipid) was placed in the bottom of the 160 μL DSC aluminum crucible, and then 120 μL of DMPC or DSPC MLV or DMPC LUV (0.007 375 mmol of phospholipids dispersed in phosphate buffer) was added. The samples, hermetically sealed in the pans, were gently shaken for 10 s and then submitted to the following calorimetric cycles: (1) a heating scan, at the rate of 2 °C/min, between 5 and 37 °C (DMPC) or between 25 and 65 °C (DSPC), to detect the interaction between the compounds and the model membrane during the first heating of the sample to a temperature higher than the T_m; (2) an isothermal period (1 h) at 37 or 65 °C to allow the compounds to be hydrated, to dissolve in the aqueous medium, and eventually to interact and permeate the lipid layers, which at the chosen temperatures (above the lipid transition temperature) are in a disordered state; (3) a cooling scan between 37 and 5 °C (DMPC) or between 65 and 25 °C (DSPC), at the rate of 4 °C/min, to bring the lipid layers back to an ordered state before restarting the heating program (step 1).

This procedure was run at least six times, and after a longer incubation time (72 h) to follow eventual variations of the transition temperature of the DMPC or DSPC calorimetric peak due to the time dependent compound−model membrane interaction.

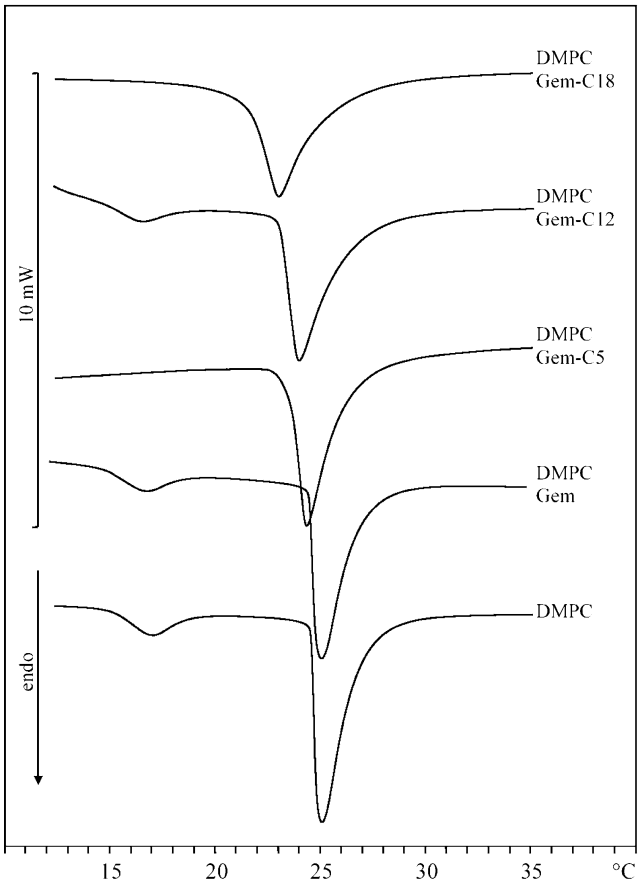

Figure 2. Calorimetric curves of DMPC MLV prepared in the absence and in the presence of gemcitabine (Gem) and its prodrugs 4-(*N*)-valeroyl-gemcitabine (Gem-C5), 4-(*N*)-lauroyl-gemcitabine (Gem-C12), and 4-(*N*)-stearoyl-gemcitabine (Gem-C18) at 0.12 molar fraction with respect to phospholipids.

Results

Gemcitabine and Gemcitabine Prodrug Interaction with DMPC MLV Prepared in Organic Phase. Pure DMPC liposomes show a sharp main peak at 24.8 °C (T_m), associated with the gel to liquid crystalline phase transition, and a pretransition peak at about 16.8 °C which reflects a change in tilt of the hydrocarbon chains.

In Figure 2 the calorimetric curves of unloaded or loaded liposomes with gemcitabine, 4-(*N*)-valeroyl-gemcitabine, 4-(*N*)-lauroyl-gemcitabine, and 4-(*N*)-stearoyl-gemcitabine at the molar fraction $X = 0.12$ are reported. Gemcitabine causes no significant change in the peak shape and transition

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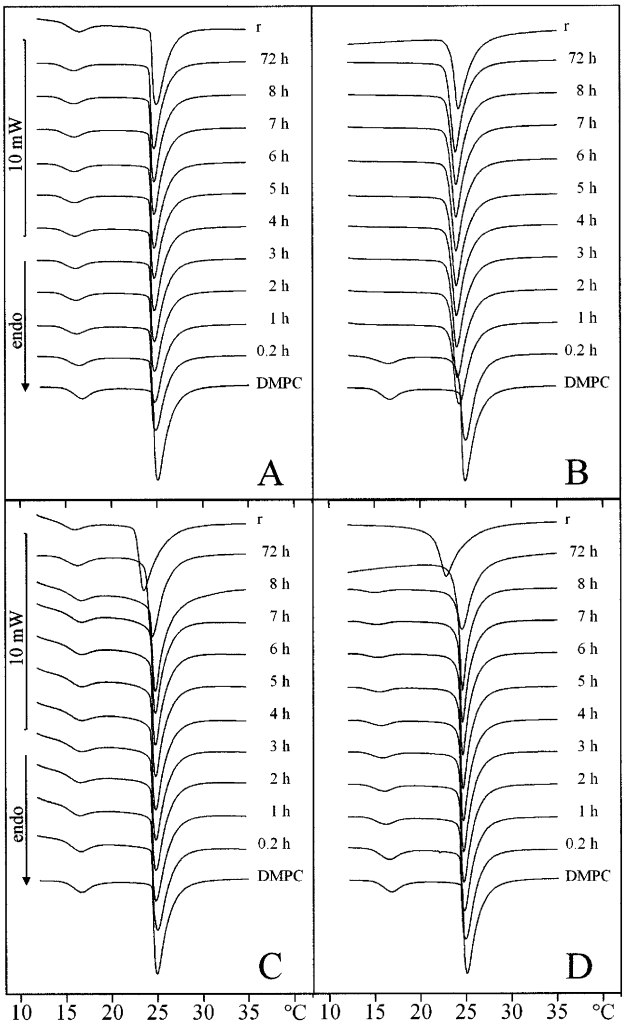

Figure 3. Calorimetric curves of DMPC MLV left in contact, at increasing incubation time and at 37 °C, with 0.12 molar fraction of (A) gemcitabine, (B) 4-(*N*)-valeroyl-gemcitabine, (C) 4-(*N*)-lauroyl-gemcitabine, and (D) 4-(*N*)-stearoyl-gemcitabine. Curve r is referred to DMPC MLV prepared in the presence of compounds (0.12 molar fraction with respect to phospholipids) and represents the maximum obtainable shift by MLV/compound interaction.

temperature. All the prodrugs shift the calorimetric curve toward lower temperatures with respect to the pure DMPC. The 4-(*N*)-stearoyl-gemcitabine exerts the greatest effect on the T_m accompanied by a broadening of the curve with the concomitant disappearance of the pretransition peak. Such effects, in the presence of 0.12 molar fraction of compounds in the aqueous dispersion, will be considered as a reference for the most complete interaction between compounds and lipids.

Gemcitabine and Gemcitabine Prodrug Interaction with DMPC MLV. Permeation Experiments. Uptake experiments were performed to study the ability of gemcitabine, its 4-(*N*)-acyl-gemcitabine derivatives, and the pure fatty acids to go through the aqueous phase surrounding the compounds, to be absorbed on the external lipid bilayer of multilamellar vesicles, and successively to be transferred into

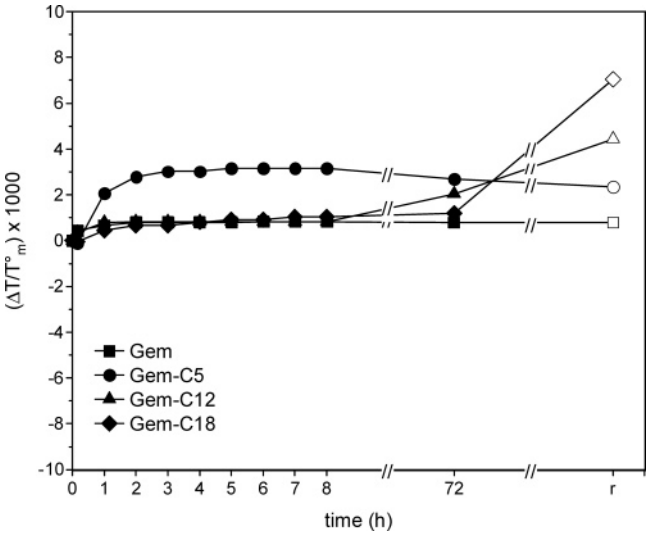

Figure 4. Transition temperature variations of DMPC MLV left in the presence of compounds at 0.12 molar fraction with respect to phospholipids. $\Delta T = T^0_m - T_m$, where T^0_m is the transition temperature of pure DMPC vesicles and T_m is the transition temperature of DMPC vesicles left in the presence of compound at increasing incubation time. The *r* values represent the effect exerted by 0.12 molar fraction of each compound on MLV, obtained starting from organic solvent solutions of phospholipids and compounds, to be considered as the maximum interaction between compounds and MLV.

the deeper lipid bilayers, with the aim of obtaining information about the influence of the fatty acid moiety on such a process. The calorimetric curves obtained by leaving a fixed amount ($X = 0.12$) of gemcitabine (or its prodrugs) in contact with DMPC multilamellar vesicles, following the procedure reported in the Permeation Experiments section, are reported in Figure 3A−D and compared with the curve of DMPC MLV prepared in the presence of each compound at a 0.12 molar fraction (curves r), as reported in the MLV Preparation section. If a full penetration of the lipid layers occurs, a calorimetric effect similar to curve r should be obtained.

It appears evident that gemcitabine (Figure 3A) leaves the transition temperature nearly unchanged. A different behavior is observable among the three prodrugs. In fact only Gem-C5 (Figure 3B) exerts an increasing effect on the lipid phase transition, as the contact time increases, causing a fast shift of the temperature toward the same value observed in curve r and a concomitant disappearance of the pretransition peak. Lauroyl (Figure 3C) and stearoyl prodrugs (Figure 3D) do not exert such a rapid and complete interaction. In fact, the calorimetric peaks remain nearly unchanged in shape and temperature, and far away from the curves r. The data appear better comparable by reporting (Figure 4) the effects of the compounds, at the fixed molar fractions present in the aqueous lipid dispersion, on the lipid transition temperature shifts as $(\Delta T \times 10^3)/T^0_m$ ($\Delta T = T^0_m - T_m$, where T^0_m is the transition temperature of pure DMPC and T_m is the transition temperature of each compound/DMPC aqueous dispersion), as a function of the contact time. It is evident that Gem-C5

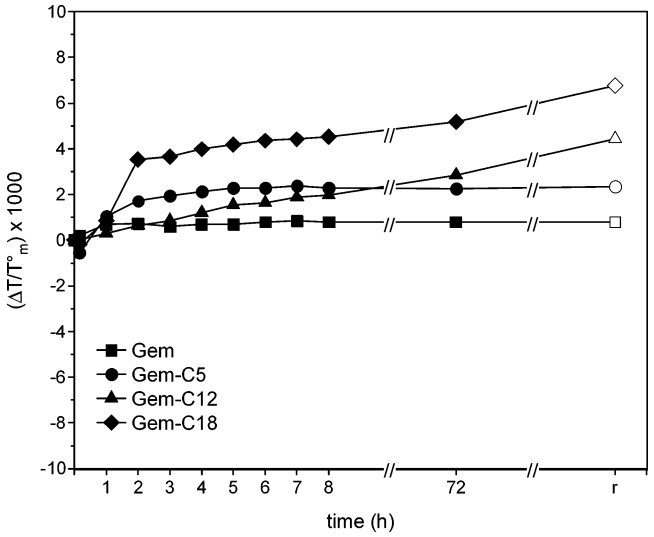

Figure 5. Transition temperature variations of DMPC LUV left in the presence of compounds at 0.12 molar fraction with respect to the phospholipids. $\Delta T = T^0_m - T_m$, where T^0_m is the transition temperature of pure DMPC vesicles and T_m is the transition temperature of DMPC vesicles left in the presence of compound at increasing incubation time. The *r* values represent the effect exerted by 0.12 molar fraction of each compound on vesicles, obtained starting from organic solvent solutions of phospholipids and compounds, to be considered as the maximum interaction between compounds and LUV.

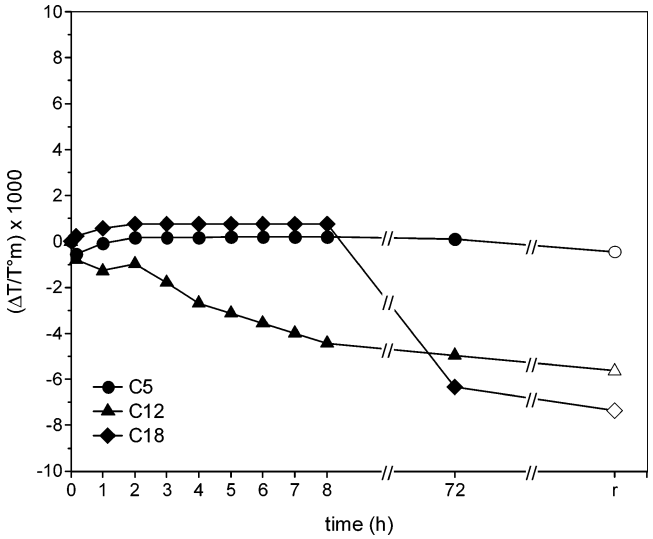

Figure 6. Transition temperature variations of DMPC MLV left in the presence of compounds at 0.12 molar fraction with respect to the phospholipids. $\Delta T = T^0_m - T_m$, where T^0_m is the transition temperature of pure DMPC vesicles and T_m is the transition temperature of DMPC vesicles left in the presence of compound at increasing incubation time. The *r* values represent the effect exerted by 0.12 molar fraction of each compound on MLV, obtained starting from organic solvent solutions of phospholipids and compounds, to be considered as the maximum interaction between compounds and MLV.

quickly reaches the maximum effect similar to that of the "organic" preparation (value *r* obtained from the curve r reported in Figure 3). Gem-C12 after a long time of contact with the MLV tends to increase the T_m shift. Gemcitabine and Gem-C18 are unable to affect the transition temperature, which is very far from that found in the case of the maximum interaction (the *r* value).

Gemcitabine and Gemcitabine Prodrug Interaction with DMPC LUV. Permeation Experiments. The calorimetric curves of DMPC LUV left in contact with gemcitabine or its prodrugs at increasing incubation time (curves not reported) show that gemcitabine does not affect the LUV transition, whereas all the prodrugs cause the shift of the peak toward lower temperature. Such a shift is more pronounced when Gem-C18 is used, which causes in addition the peak broadening. The results are compared in Figure 5, where the transition temperature variation is reported, as $\Delta T/T^0_m$, as a function of the incubation time: Gem-C5 shows a quick kinetics of interaction reaching the value *r* (maximum interaction); Gem-C12 and Gem-C18 show a tendency to slowly reach the values *r*.

Fatty Acid Interaction with DMPC MLV by Organic Phase and Permeation Experiments. Calorimetric measurements of pure DMPC MLV prepared either in the absence or in the presence of a 0.12 molar fraction of the same fatty acid used in the prodrug formulation were carried out. They demonstrate that the interaction between 4-(*N*)-acyl-gemcitabine derivatives and the lipid vesicles must be assigned to the entire prodrug molecule and not solely to the apolar fatty acid tail. Valeric acid causes no changes in the DMPC calorimetric curve, while lauric and stearic acids produce a broadening and a large shift of the main peak toward higher temperature and the disappearance of the pretransition peak (curves not reported).

The comparison of the calorimetric curves of DMPC MLV left in contact with fatty acids (0.12 molar fraction) at increasing incubation times with that of pure DMPC MLV and with those prepared in the presence of a 0.12 molar fraction of each fatty acid reveals that lauric acid interacts by broadening the curve (with a phase separation during the first incubation periods) and by shifting the peak toward higher temperatures. Stearic acid, during the first 8 h of incubation, does not modify the DMPC MLV curves, whereas after 72 h it causes peak broadening and a phase separation. Valeric acid does not modify the DMPC calorimetric curve (data not reported). The results are shown in Figure 6, where the temperature variations, $\Delta T/T^0_m$, are reported as a function of the incubation time. Valeric acid does not cause any temperature variation. Temperature variation caused by lauric acid regularly increases with the incubation time. When stearic acid is considered, the temperature variation is constant up to 8 h; thereafter it sharply increases around 72 h.

Gemcitabine and Gemcitabine Prodrug Interaction with DSPC MLV Prepared in the Organic Phase. The calorimetric curve of DSPC MLV presents a main peak, at 55.1 °C (T_m), associated with the transition from a gel to a liquid crystalline phase, a pretransition peak, at about 51.8

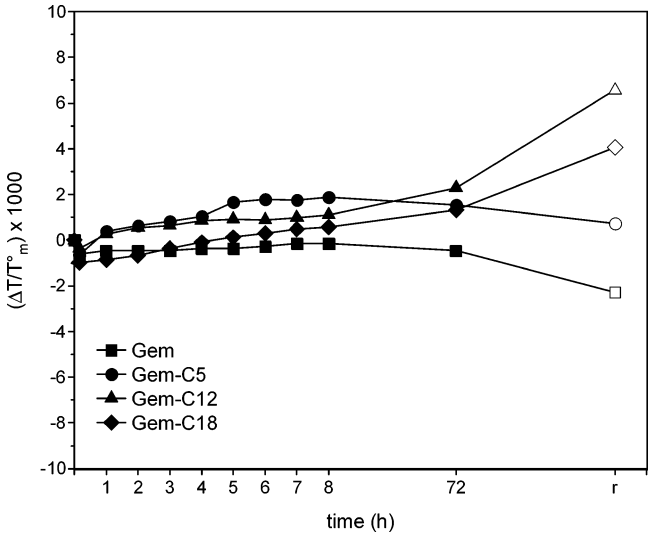

Figure 7. Transition temperature variations of DSPC MLV left in the presence of compounds at 0.12 molar fraction with respect to the phospholipids. $\Delta T = T^0_m - T_m$, where T^0_m is the transition temperature of pure DSPC vesicles and T_m is the transition temperature of DSPC vesicles left in the presence of compound at increasing incubation time. The *r* values represent the effect exerted by 0.12 molar fraction of each compound on MLV, obtained starting from organic solvent solutions of phospholipids and compounds, to be considered as the maximum interaction between the compound and MLV.

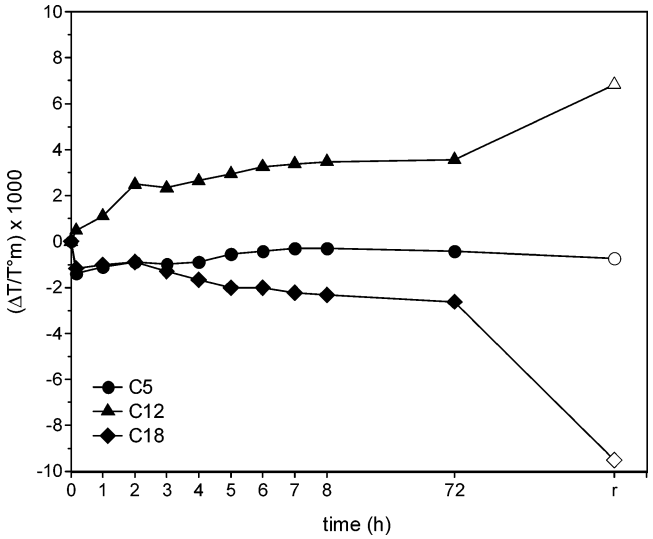

Figure 8. Transition temperature variations of DSPC MLV left in the presence of compounds at 0.12 molar fraction with respect to the phospholipids. $\Delta T = T^0_m - T_m$, where T^0_m is the transition temperature of pure DSPC vesicles and T_m is the transition temperature of DSPC vesicles left in the presence of compound at increasing incubation time. The *r* values represent the effect exerted by 0.12 molar fraction of each compound on MLV, obtained starting from organic solvent solutions of phospholipids and compounds, to be considered as the maximum interaction between the compound and MLV.

°C, related to a change in tilt of the hydrocarbon chains and a small shoulder next to the main peak. The curves of DSPC MLV prepared in the presence of 0.12 molar fractions of gemcitabine, Gem-C5, Gem-C12, and Gem-C18 show that these compounds produce different effects on the DSPC MLV phase transition (curves not reported). Gemcitabine shifts the peak toward higher temperatures without changing its shape; the valeroyl derivative negligibly affects the DSPC calorimetric peak, whereas the stearoyl derivative and, even more, the lauroyl derivative cause a large shift toward lower temperature accompanied by a peak broadening. Stearoyl and lauroyl derivatives suppress the pretransition peak and the small shoulder characteristic of the DSPC curve.

Such an interaction (in the presence of 0.12 molar fraction of the compounds in the aqueous liposomal dispersion) is considered as the highest possible between compounds and lipids in the liposomes.

Gemcitabine and Gemcitabine Prodrug Interaction with DSPC MLV. Permeation Experiments. The calorimetric curves obtained by permeation experiments using 0.12 molar fraction of gemcitabine, or its prodrugs, in the presence of DSPC multilamellar vesicles are compared with those of pure DSPC MLV and DSPC MLV prepared in the presence of 0.12 molar fraction of each compound (data not shown). The first result is that gemcitabine leaves nearly unchanged the phase transition temperature of pure DSPC; also the pretransition peak and the shape of the curves remain unchanged. Prodrugs show a different behavior. In fact, Gem-C5 increasingly shifts the temperature toward the same value observed in curve r (homogeneous dispersion of the prodrug in the lipid matrix), with a concomitant disappearance of the pretransition and a modification of the DSPC peak. Instead Gem-C12 causes only a small shift toward lower temperatures leaving the peaks nearly unchanged in shape and far from the curve r. Gem-C18 modifies both the temperature and the shape of the calorimetric peak. The data are compared each other in Figure 7, which shows the effects of the compounds, at fixed molar fraction, on the lipid transition temperature shifts, $\Delta T/T^0_m$, as a function of the contact time. Gemcitabine does not perturb the DSPC vesicles, while Gem-C5 quickly reaches the maximum effect (value *r*). Gem-C12 and Gem-C18 slowly increase the T_m shift without reaching the maximum of the interaction.

Fatty Acid Interaction with DSPC MLV by Organic Phase and Permeation Experiments. The calorimetric curves obtained by dissolving a 0.12 molar fraction of the same fatty acids present in the gemcitabine prodrug formulation in DSPC multilamellar vesicles (during their preparation in the organic phase) reveal that lauric acid and stearic acid interact with DSPC MLV (curves not reported). Both the fatty acids produce a broadening of the main peak, but lauric acid shifts the peak toward lower temperature whereas stearic acid shifts the peak toward higher temperature with a concomitant phase separation. Valeric acid is unable to modify the DSPC MLV calorimetric curves.

The calorimetric data (not reported) of the experiments carried out by leaving DSPC MLV in contact with the compounds (0.12 molar fraction) show that valeric acid

produces no detectable effects whereas lauric acid shifts the main peak toward lower temperature, changing the curve shape, which becomes very similar to the curve of MLV prepared in the presence of a 0.12 molar fraction of fatty acids. More complex is the interaction of the stearic acid: the main peak broadens, leading to a phase separation which increases with the time. The results are summarized in Figure 8, where we report $\Delta T/T^0_m$ as a function of contact time. Valeric acid causes a very small effect on transition temperature. Lauric and stearic acids cause a decrease and an increase of the transition temperature, respectively; in any case the equilibrium value *r* is never reached.

Discussion

The obtained data suggest that gemcitabine by itself is not able to perturb the employed biomembrane models (DMPC MLV and LUV and DSPC MLV). Instead, gemcitabine bound to fatty acids, to form 4-(*N*)-acyl derivatives prodrugs, shows a substantial and greater interaction with the considered biomembrane models. The entity of the interaction depends on the acyl chain length of both phospholipids and prodrugs. The DSC data demonstrate that generally all prodrugs exert a destabilizing effect on biomembranes, changing their thermotropic behavior. In particular, the prodrugs decrease the transition temperature with respect to that of pure phospholipids.

Considering the behavior of MLV prepared in the presence of prodrug and comparing the results obtained with DMPC MLV with those obtained with DSPC MLV, it is noted that Gem-C5 shows a similar effect on the two model membranes. The two remaining prodrugs show a different behavior: Gem-C12 exerts a bigger destabilizing effect on DSPC MLV, whereas Gem-C18 exerts a stronger destabilizing effect toward DMPC MLV. These results are attributable to the different length of the hydrocarbon chains present in the two membrane systems (DMPC and DSPC) with respect to the prodrug's hydrocarbon chain; in particular the bigger the chain length difference between phospholipid and prodrug, the greater the prodrug destabilizing effect. Gem-C12, possessing a hydrophobic chain similar to that of DMPC, does not destabilize the lipid bilayer as Gem-C18 does. On the other hand, Gem-C18 exerts a smaller destabilizing effect on DSPC bilayer, its hydrophobic portion being nearly equal to that of DSPC, whereas Gem-C12, whose hydrophobic chain is shorter than that of DSPC, perturbs the phospholipid bilayer.

The comparison of the results obtained by employing prodrugs with those obtained by using the fatty acids shows that the destabilizing effect exerted by the prodrug is assignable to the joint gemcitabine−fatty acid and not only to the fatty acid because they exert opposite effects on MLV. In particular, according to the literature data,[30] fatty acids alone increase the T_m of the lipid bilayer (the only exception being represented by the lauric acid) whereas prodrugs decrease it. This evidence leads us to hypothesize that the fatty acid contribution in prodrug/MLV interaction favors the insertion of gemcitabine inside the lipid bilayer.

This hypothesis is supported by the results obtained for permeation kinetics of prodrugs and fatty acids. In such conditions prodrugs reach the inner lipid bilayer of multilamellar liposomes. The rate of such interaction depends on the hydrophobic tail length of the prodrug: Gem-C5, the less lipophilic among the three prodrugs, despite showing the smallest interaction in prodrug/phospholipid MLV, possesses the fastest interaction rate; Gem-C12, possessing an intermediate lipophilicity, shows both an intermediate interaction in prodrug/phospholipid MLV and rate of transfer in kinetic experiments; Gem-C18, the most lipophilic prodrug, shows the biggest interaction with the lipid membrane and the slowest transfer rate. This is because its motion through the aqueous phase is hindered by its lipophilic character.

The interaction of gemcitabine prodrugs with LUV differs from that with MLV, being faster and more complete. Given that LUV are constituted by a single bilayer, they expose a surface much larger than that of MLV for the contact with the prodrugs. It could be the reason of the faster and more complete LUV/compound interaction. These experiments strengthen the hypothesis that the limiting step in the interaction prodrug/phospholipid bilayer is the prodrug dissolution in the aqueous medium.

The calorimetric data highlight the prodrugs' ability to interact with biomembranes. Prodrugs (apart from Gem-C5) are subjected to a slow uptake by MLV; the rate of their passage through the aqueous medium to reach the lipid vesicles is hindered by their low solubility due to their lipophilic nature.

The present study was aimed at determining the differences in the mechanism of dissolution, transport through the aqueous phase, and absorption by lipidic systems mimicking biomembranes of gemcitabine and three of its 4-(*N*)-acyl derivatives. It was evidenced, as partially reported in a previous study of some of us,[13] that the efficiency of a liposomal system is related to the length of the prodrug's acyl chain, which has to match the phospholipid acyl chain allowing or retarding the migration through the lipidic release device.

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