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Journal of Colloid and Interface Science 313 (2007) 363–368
JOURNAL OF Colloid and Interface Science
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Note

Interaction of lipophilic gemcitabine prodrugs with biomembrane models studied by Langmuir–Blodgett technique

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Received 11 January 2007; accepted 6 April 2007
Available online 13 April 2007

Abstract

The stability and bioavailability of anticancer agents, such as gemcitabine, can be increased by forming prodrugs. Gemcitabine is rapidly deaminated to the inactive metabolite (2′,2′-difluorodeoxyuridine), thus to improve its stability a series of increasingly lipophilic gemcitabine prodrugs linked through the 4-amino group to valeroyl, lauroyl, and stearoyl acyl chains were synthesized. Studies of monolayer properties are important to improve understanding of biological phenomena involving lipid/gemcitabine or lipid/gemcitabine derivative interactions. The interfacial behavior of monolayers constituted by DMPC plus gemcitabine or lipophilic gemcitabine prodrugs at increasing molar fractions was studied at the air/water interface at temperatures below (10 °C) and above (37 °C) the lipid phase transition. The effect of the hydrophobic chain length of gemcitabine derivatives on the isotherm of pure DMPC was investigated by surface tension measurement, and the results are reported as molar fractions as a function of mean molecular area per molecule. The results show that the compounds interact with DMPC producing mixed monolayers that are subject to an expansion effect, depending on the prodrug chain length. The results give useful hints of the interaction of these prodrugs with biological membranes and increase knowledge on the incorporation site of such compounds, as a function of their lipophilicity, in a lipid carrier; they may lead to improved liposomal formulation design.

Keywords: Gemcitabine; Lipophilic gemcitabine derivatives; Dimyristoylphosphatidylcholine; Langmuir–Blodgett

1. Introduction

Gemcitabine (2′,2′-difluoro-2′-deoxycytidine), a pyrimidine antimetabolite [1], is known to be active against a variety of solid tumors, and is well tolerated in clinical trials [2–4].

One disadvantage of gemcitabine is its rapid and extensive deamination to its inactive metabolite 2′,2′-difluorodeoxyuridine by cytidine deaminase in the blood, liver, kidney and other tissues [5] and its subsequent excretion in the urine; the plasma half-life is very short (8–17 min). An approach to improve gemcitabine's metabolic stability and the related cytotoxic activity is to protect the amide group by forming prodrugs and/or by incorporating the prodrugs into lipid vesicles. In order to understand the therapeutic action of any drug it is very important to know its interaction with the biomembrane lipids.

Differential scanning calorimetry (DSC) has recently been employed to study the interaction of gemcitabine and three of its acylic prodrugs, 4-(*N*)-valeroyl-gemcitabine (Gem-C5), 4-(*N*)-lauroyl-gemcitabine (Gem-C12) and 4-(*N*)-stearoyl-gemcitabine (Gem-C18) (Scheme 1) with biomembrane models (phospholipid multilamellar vesicles) in order to evaluate the effect of acyl chain length on the prodrugs/lipid interaction [6]. This study used dimyristoylphosphatidylcholine (DMPC) monolayers as biomembrane model and the Langmuir–Blodgett (LB) technique to obtain additional information over that offered by the calorimetric technique.

The Langmuir–Blodgett technique, which uses phospholipid monolayers, is one of the commonest ways to study the interaction between drugs and phospholipids. Monolay-

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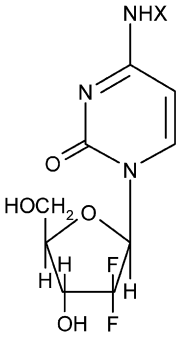
0021-9797/$ – see front matter
doi:10.1016/j.jcis.2007.04.018



X = H gemcitabine

X = $CO(CH_2)_nCH_3$
n = 3 4-(*N*)-valeroyl-gemcitabine
n = 10 4-(*N*)-lauroyl-gemcitabine
n = 16 4-(*N*)-stearoyl-gemcitabine

Scheme 1. Gemcitabine and 4-*N*-acyl-gemcitabine prodrugs structures.

ers are an excellent model to study two-dimensional ordering, with two thermodynamical variables, temperature and pressure, being readily controlled [7–9]. The physico-chemical analysis of the LB results may provide important information on the organization of biological compounds in the lipid membrane.

The film-balance method enables phase diagrams of phospholipids to be obtained; these are generally in the form of surface pressure/mean molecular area ($\pi/\text{Å}^2$) isotherm curves. The phospholipids are spread over an aqueous subphase, providing monomolecular distribution and the subsequent variation of the available area per molecule. If the monolayer is submitted to compression, the molecules' surface distribution changes and they are forced to go from a "gaseous" or "liquid expanded" (LE) phase at low density to a "liquid condensed" (LC) phase at a higher density and, successively, to a "solid condensed" phase [10]. In multicomponent monolayers spread over a liquid, the two dimensional miscibility of the components is a significant problem. Conclusions about the mixing process of two pure monolayers can be made by comparing surface pressure-area isotherms of mixed and pure films. Results provide indications on the compounds' ability to dissolve in the phospholipid molecules used as model membrane. The interactions enable hypothesis to be made about the intake and collocation of the compounds within the membrane [11–13].

The Langmuir–Blodgett technique has been applied to studying the interaction of phospholipid membranes with antitumor drugs including doxorubicin and paclitaxel. Paclitaxel has been found to be miscible in the lipid monolayer and a repulsive interaction between paclitaxel and the lipid was concluded to exist. It has been recognized that differences in phospholipid molecular structure, such as lipid chain length, chain unsaturation and head group type, as well as the presence of cholesterol, may profoundly affect drug–membrane interactions; paclitaxel affects membrane morphology and stability more easily when phospholipids with shorter chains are present [14–19].

2. Experimental

2.1. Materials

Gemcitabine was synthesized in our laboratory as described in [20]. The 4-(*N*)-acyl-gemcitabine derivatives were synthesized and characterized as reported elsewhere [21].

1,2-Dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC) was supplied by Genzyme Pharmaceuticals (Liestal, Switzerland). All reagents and solvents used were of analytical grade.

2.2. Langmuir film balance

A film balance apparatus, KSV Langmuir minitrough (KSV, Instruments Ltd., Finland), was used. It includes a trough (24,225 mm^2 available area for the monolayer formation) coated with polytetrafluoroethylene (Teflon) and surrounded by a water jacket providing temperature control, and two mechanically-coupled barriers of hydrophilic Delrin. The film pressure at the air/water interface was measured using the Wilhelmy plate arrangement attached to a microbalance. A platinum plate was suspended for film pressure measurements. 5 mM phosphate buffer (pH 7.4) in ultrapure Millipore water with resistance of 18.2 MΩ was used as subphase. Surface purity of monolayers was checked by closing and opening the barriers and ensuring that surface pressure readings did not differ by more than ±0.1 mN/m. The KSV system was checked using stearic acid [22].

2.3. Mixed monolayer surface pressure/molecular area isotherms

DMPC, gemcitabine and 4-(*N*)-acyl-gemcitabine conjugates were prepared at equimolar concentrations in chloroform (Aldrich, 99.9%). Appropriate volumes of DMPC and gemcitabine or 4-(*N*)-acyl-gemcitabine derivatives were then mixed to form DMPC/gemcitabine and DMPC/4-(*N*)-acyl-gemcitabine derivatives at 0.015, 0.03, 0.045, 0.06, 0.09, 0.12, 0.25, 0.50, and 0.75 molar fractions. Aliquots of about 30 μl of the above mixtures, as well as of solutions of the pure components were spread drop by drop onto the aqueous subphase using a Hamilton syringe. After waiting 15 min for solvent evaporation, the films were linearly compressed with the two mobile barriers at a rate of 10 (mN/m)/min and the surface pressure vs molecular area isotherms were recorded. The experiments were performed at subphase temperatures of 10 and 37 °C, (temperatures below and above the phase transition temperature of DMPC), to allow considerations both at physiological temperature (37 °C) at which DMPC is in a disordered state, and at a temperature (10 °C) at which the DMPC is in an ordered state. The effect of a foreign compound dissolved in the ordered lipid structure can then be amplified giving more information on its packing in the lipid matrix and on the consequent loss of lipid cooperativity. Each experiment was repeated at least three times to obtain reproducible results.

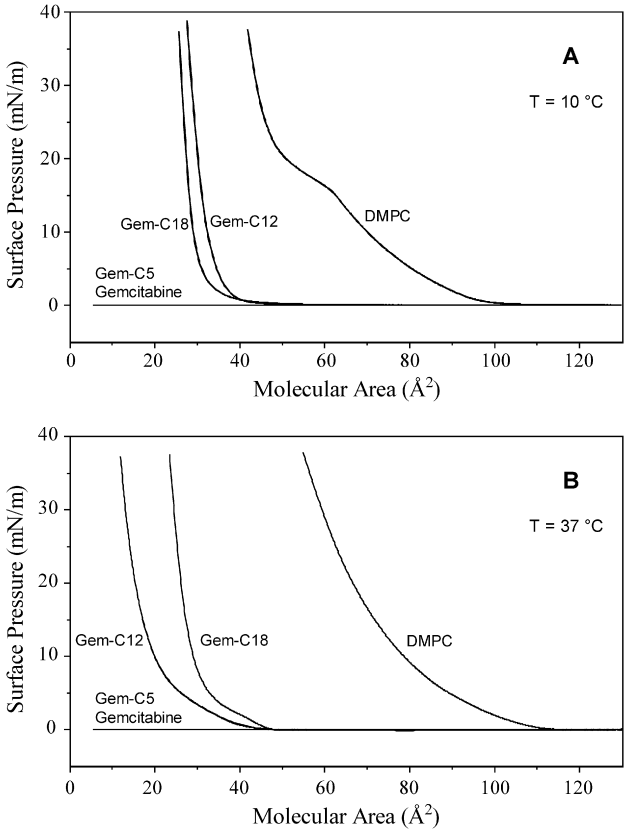


Fig. 1. Surface pressure/molecular area isotherms of the monolayers of pure DMPC, gemcitabine, and 4-(*N*)-acyl-gemcitabine conjugates at the air/water interface at (A) 10 °C and (B) 37 °C.

3. Results and discussion

3.1. DMPC, gemcitabine and gemcitabine derivatives monolayers

The compression surface pressure-area per molecule (mN/m-Å^2) isotherms of pure compounds are plotted in Fig. 1. We will analyze the isotherms recorded at 10 °C first (Fig. 1A). The DMPC isotherm shows characteristic features: a gaseous phase for areas larger than 100 Å^2, a LE phase at 100 < Å^2 < 70, a LE to LC lipid phase transition between 70 and 45 Å^2 and a LC phase for areas lower than 45 Å^2 [23,24]. Gemcitabine does not form a monolayer and even at high concentrations no isotherm is observable. No isotherms are visible for Gem-C5. This compound, probably because of its hydrophilic character, either does not form monolayers or remains in a gaseous state even at high compression. Gem-C12 exists in a gaseous phase for areas larger than 50 Å^2, whereas for areas lower than 50 Å^2 it exists in a LC phase. Gem-C18 isotherms indicate that the compound is in a LC phase for areas lower than 35 Å^2, whereas in larger areas it is in a gaseous phase. With regard to the results obtained at 37 °C (Fig. 1B), the DMPC isotherm curve is shifted toward higher area per molecule values than at 10 °C. In addition it also shows a gaseous (130–110 Å^2) and a LE (below 110 Å^2) state with no evidence of the LE/LC transition. Gem-C5, likewise is unable to form a monolayer at 37 °C. Gem-C12 exists in a gaseous state in areas larger than 45 Å^2, in a LE state between 20 and 45 Å^2, and in a LC state for lower areas. The behavior of Gem-C18 at 37 °C is similar to that observed at 10 °C but the LE/LC transition occurs between 45 and 35 Å^2. The isotherm of Gem-C18 is steeper than that of Gem-C12, which may depend on the tilt angle of the prodrug at the air water interface. The longer the acyl chain of the prodrug, the more hydrophobic it becomes and the less likely it is to be in the liquid expanded state with the chains tilted at the interface [25].

3.2. Mixed monolayers of DMPC and gemcitabine or gemcitabine derivatives

Mixtures of DMPC and each of the studied compounds, prepared at compounds molar fractions ranging from 0.015 to 0.75, were spread at the air/water interface and their isotherms recorded. We compared the isotherms of the DMPC/Gem and DMPC/Gem-derivative mixtures with that of pure DMPC and evaluate the change produced in the pure DMPC isotherm by the compound (data not shown).

Gemcitabine: at 10 °C and 0.015 molar fraction it causes a shift toward higher molecular areas, while higher molar fractions shift the isotherm toward lower areas. In addition, the DMPC LE/LC phase transition is also shifted toward higher value of surface pressure, and becomes less marked. At 37 °C, at low molar fraction (0.015 and 0.03) of gemcitabine the isotherms are shifted toward larger areas, whereas at higher molar fractions of gemcitabine the isotherms are shifted toward lower areas.

Gem-C5: at 10 °C, 0.015 and 0.03 Gem-C5 molar fractions cause the isotherms to shift toward larger molecular areas, whereas higher molar fractions cause the isotherm to move toward lower molecular area. This does not occur at 37 °C where the isotherm is shifted toward lower areas as the Gem-C5 concentration increases.

Gem-C12: at 10 °C, isotherm shape and molecular area are almost unchanged at molar fractions of Gem-C12 ranging from 0.015 to 0.12; higher molar fractions cause the isotherm shift toward lower area together with a gradual disappearance of the LE/LC phase transition which is shifted toward higher value of surface pressure. At 37 °C, similarly to what occurs at 10 °C, low Gem-C12 molar fractions do not cause substantial change of the isotherm, whereas high molar fractions (0.5, 0.75) cause the isotherm to shift toward lower molecular area.

Gem-C18: the behavior of the DMPC isotherm in the presence of Gem-C18, at 10 °C, is very similar to that occurring with Gem-C12: there is no significant change with 0.015–0.12 molar fractions and a shift toward lower molecular areas at higher molar fractions. As the Gem-C18 molar fraction increases, the LE/LC phase transition gradually shifts toward higher values of surface pressure, at the same time becoming less evident; it disappears entirely at 0.75 molar fraction and isotherm shows only a liquid condensed phase region. At 37 °C, all the DMPC/Gem-C18 mixture isotherms are shifted toward lower molecular areas. In the isotherm at 0.75 molar fraction a LE/LC transition occurs between 55 and 45 Å^2.

Correlations between mean molecular area and molar fraction of compounds mixed with phospholipids have been reported, giving information on molecular distribution, miscibility and interaction among molecules contained in mixed monolayers [26]. The mean molecular area of a two components monolayer is calculated as $A = A_1X_1 + (1 - X_1)A_2$; where A is the mean molecular area, X_1 is the molar fraction of a component, and A_1 and A_2 are the areas of the two pure components at the same surface pressure. Graphs of A as a function of X_1 are linear if either the components are completely immiscible or they possess an ideal miscibility [22]. Any deviation from the straight line indicates an interaction between the molecules.

Fig. 2 shows the area per molecule as a function of the gemcitabine or gemcitabine-prodrug molar fraction at 10 °C and at pressure of 10 (before the LE/LC transition), 20 (just after the LE/LC transition) and 35 mN/m (at high compression); experimental values are shown in solid lines and ideal ones in dotted lines.

Gemcitabine: Fig. 2A shows the area per molecule as a function of the gemcitabine molar fraction in the DMPC/gemcitabine mixtures at 10 °C. With respect to the ideal line, at 10 and 20 mN/m, small molar fractions give a small positive deviation, whereas for high molar fractions experimental values coincide with ideal values. At 35 mN/m, experimental values coincide with ideal values up to 0.25 molar fraction, after which negative deviations occur. This shows a repulsive interaction between the molecules which disappears at the highest pressure value employed due to the high compression. At 37 °C, and pressures of 10 and 20 mN/m, there is generally a positive deviation which becomes less marked at high molar fractions; at 35 mN/m, as the molar fraction increases, positive deviation still occurs, followed by coincidence with the ideal line and lastly a negative deviation above 0.75 (Fig. 2B).

Gem-C5: at 10 °C (Fig. 2C), at all surface pressures tested, there is a slight positive deviation from the ideal line at low (0.015–0.06) and high molar fractions (0.5 and 0.75), whereas at intermediate molar fractions the experimental values coincide with the ideal values. At 37 °C (Fig. 2D), a negative deviation occurs for molar fractions between 0.015 and 0.25 and a positive deviation at the 0.5 molar fraction; the 0.75 molar fraction induces a positive deviation at 10 and 20 mN/m and a negative deviation at 35 mN/m. These results indicate that Gem-C5, at 10 °C, exerts a small expansion effect on DMPC meaning that there are repulsive forces between the molecules. At 37 °C, the negative and positive deviations that occur, respectively, at low and high Gem-C5 molar fractions, indicate, respectively, attractive and repulsive forces. This effect could be explained by assuming that: (1) Gem-C5, due to its partially hydrophilic character, localizes parallel to the subphase surface, causing the DMPC molecules to move away; this localization is favored by low temperatures, when DMPC is in an ordered state; (2) Gem-C5's hydrophobic tail is parallel to the DMPC hydrophobic chains; however, its length being shorter than that of the DMPC chains, the interaction among phospholipid chains decreases.

Gem-C12: the behavior of DMPC/Gem-C12 mixtures is quite different. At 10 °C (Fig. 2E), a positive deviation occurs at 10 mN/m for all molar fractions, apart from 0.75, which produces a negative deviation. At 20 mN/m, the deviation is negative, positive and then again negative on passing from lower to higher molar fraction. At 35 mN/m and molar fractions between 0.015 and 0.12, experimental values coincide with ideal values, whereas at higher molar fractions the deviation is negative. At 37 °C (Fig. 2F) and at 10 and 20 mN/m, there is a positive deviation for all molar fractions; at 35 mN/m, small negative and positive deviations alternate. At both temperatures and at 10 and 20 mN/m, Gem-C12 exerts an expanding effect which is more pronounced at 37 °C. At 35 mN/m, the molecules occupy a lower area than the ideal one, probably because the stronger compression makes the attractive forces dominate.

Gem-C18: at 10 °C (Fig. 2G), a positive deviation is seen at 10 and 20 mN/m, the deviation being more pronounced at 20 mN/m. At a surface pressure of 35 mN/m, and at low prodrug molar fractions, there is a very slight positive deviation, whereas at high molar fractions experimental values coincide with ideal values. At 37 °C (Fig. 2H), 10 mN/m and low molar fractions of Gem-C18, a small negative deviation occurs, while at high molar fractions the deviation becomes positive. At 20 mN/m experimental values coincide with the ideal ones and at 35 mN/m, there is a small negative deviation.

The relationship between actual molecular occupied area and molar fraction of the compounds studied is not linear, thus the compounds and DMPC were postulated to be miscible, and to show non-ideal mixed behavior in the monolayer at the air/water interface.

We found that gemcitabine and Gem-C5 did not show any isotherm, whereas Gem-C12 and Gem-C18, because of their long hydrophobic tails, showed well-defined isotherms. Gem-C12, with a shorter hydrophobic tail, is in a well-defined LC phase only at 10 °C, whereas at 37 °C there is also a LE phase because at this temperature the thermal motion overcomes the hydrophobic forces. The Gem-C18 isotherm indicates there is an LC state at 10 °C, whereas at 37 °C there is also a LE/LC transition.

Comparing the results, it is apparent that gem-derivatives in mixed monolayers substantially exert an expansion effect, which is particularly strong in the case of Gem-C12. Gem-C5, although it possesses a much shorter hydrophobic tail than C-18, exerts a similar effect: Gem-C5, with a much shorter chain than DMPC, enables the DMPC chains to interact with each other. Gem-C18, possessing a longer chain than DMPC, interacts with the phospholipid chains; the slight expansion effect might be due to the long chain tilting.

The more pronounced expanding effect on DMPC molecules that is caused by Gem-C12, compared to that of Gem-C18 at 37 °C and at 10 and 20 mN/m, could be due to the fact that at this temperature and surface pressure Gem-C12 is still in a LE phase, occupying a larger area than Gem-C18.

Gem-C12, with its 12-carbon tail, gives a smaller contribution to the hydrophobic forces than do DMPC chains, thus permitting the molecule to occupy a larger area.

From our previous DSC study, we learned that gemcitabine is unable to perturb (penetrate) a lipid multilayer (monolayer); moreover, when bound to fatty acids, to form 4-(*N*)-acyl deriva-

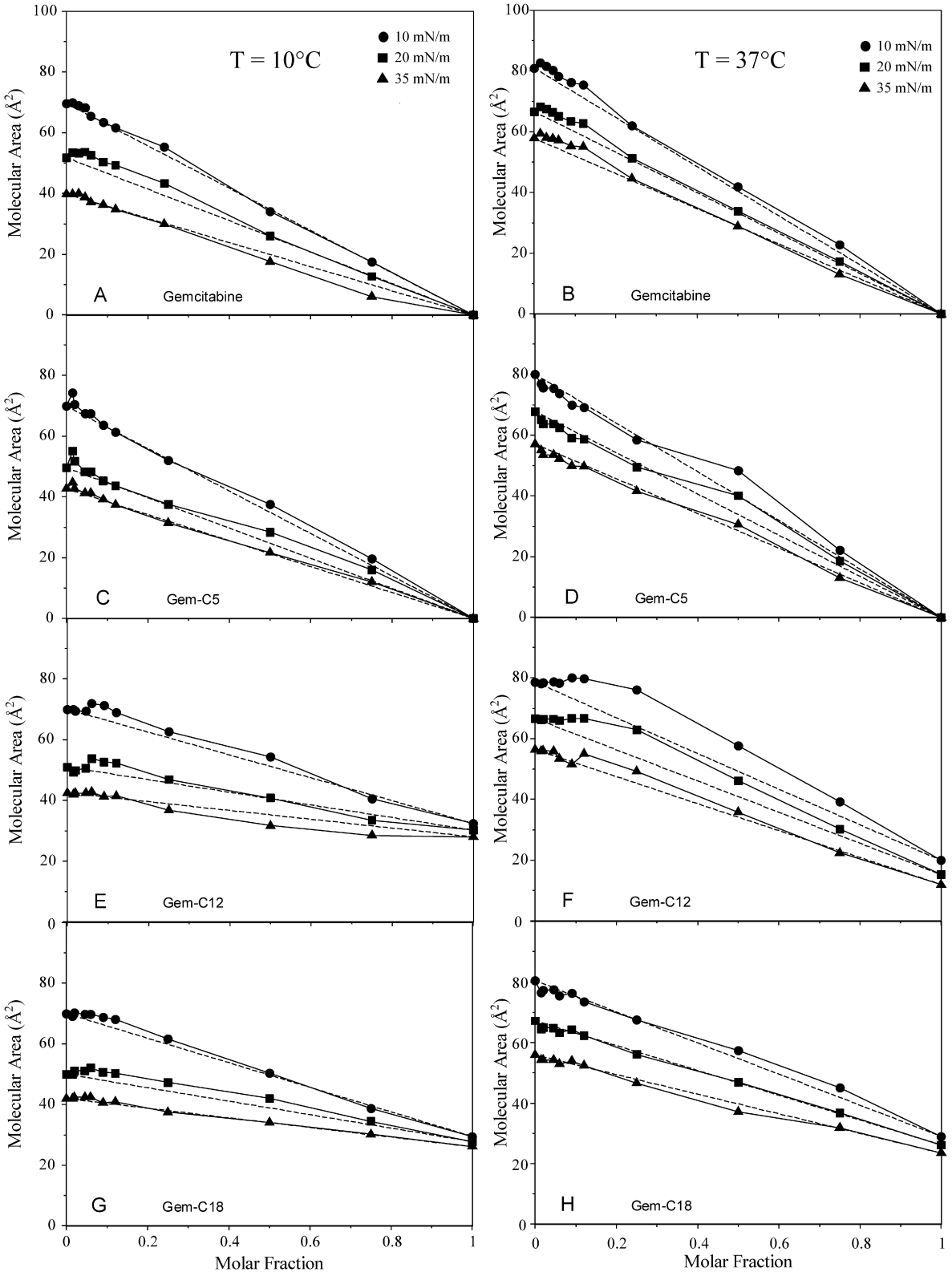


Fig. 2. Molecular area of the mixed monolayers of DMPC and gemcitabine or gemcitabine prodrug at the air/water interface plotted as a function of the molar fraction of gemcitabine or its prodrug at various surface pressure. (A) DMPC/gemcitabine 10 °C; (B) DMPC/gemcitabine 37 °C; (C) DMPC/Gem-C5 10 °C; (D) DMPC/Gem-C5 37 °C; (E) DMPC/Gem-C12 10 °C; (F) DMPC/Gem-C12 37 °C; (G) DMPC/Gem-C18 10 °C; (H) DMPC/Gem-C18 37 °C.

tives prodrugs, it interacts more strongly with the biomembrane models, the extent of interaction depending on the acyl chain length of phospholipid and prodrug. The DSC results showed that all prodrugs generally exerted a destabilizing effect on the lipid bilayer [6]. The present data are in agreement with the previously study [6] since the compounds substantially exert

an expansion effect on the mixed monolayers. Conjugation of gemcitabine with short, medium, and long-acyl side chain offers a set of molecular tools that could be helpful to evaluate the effects of the increasing lipophilicity on their interaction with biological membranes. In addition, these findings may be of use for optimizing liposomal formulations of gemcitabine prodrugs for use in anticancer treatment.

Acknowledgment

We thank the MIUR (PRIN 2006) for financial support.

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