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Synthesis of n-squalenoyl cytarabine and evaluation of its affinity with phospholipid bilayers and monolayers

Maria Grazia Sarpietro[a], Sara Ottimo[a], Maria Chiara Giuffrida[a], Flavio Rocco[b], Maurizio Ceruti[b], Francesco Castelli[a,*]

[a] Dipartimento di Scienze Chimiche, Viale Andrea Doria 6, 95125 Catania, Italy
[b] Dipartimento di Scienza e Tecnologia del Farmaco, Via Pietro Giuria 9, 10125 Torino, Italy

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ABSTRACT

Cytarabine (1-β-D-arabinofuranosylcytosine, Ara-C), a pyrimidine nucleoside analogue, is an attractive therapeutic agent for the treatment of both acute and chronic myeloblastic leukemias. 1,1′,2-*tris*-*nor*-Squalene acid (squaleneCOOH) has been conjugated to cytarabine with the formation of the squalenoyl-cytarabine prodrug, in order to improve the drug lipophilicity and, consequently, the affinity towards the environment of biological membranes, as well as of lipophilic carriers.

The interaction of cytarabine and its prodrug with dimyristoylphosphatidylcholine (DMPC) multilamellar vesicles and monolayers has been studied by the differential scanning calorimetry and the Langmuir–Blodgett techniques. The interaction has been evaluated considering the effect of the compounds on the DMPC MLV and monolayers behaviour. The aim was to have information on the interaction of the drug and the prodrug with the biological membranes and on the possibility to use liposomes as carriers for the prodrug. The results showed an improved affinity of the prodrug with MLV and monolayers with respect to the free drug.

1. Introduction

1-β-D-Arabinofuranosylcytosine (cytarabine, Ara-C) (Fig. 1) a pyrimidine nucleoside analogue, is a therapeutic agent to treat both acute and chronic myeloblastic leukemias. In combination with other anticancer agents, it is therapeutically effective against solid tumors (Rustum and Raymakers, 1992; Grant, 1997). Its action is presumably exerted both by inhibition of DNA polymerase and by incorporation into DNA, which result in defective ligation or incomplete synthesis of DNA fragments (Graham and Whitmore, 1970a,b; Major et al., 1981). Cytarabine has a low permeability across the intestinal membrane and is rapidly deaminated to the biologically inactive 1-β-D-arabinofuranosyluracil in intestinal and hepatic cells. Thus, it has a very short plasma half-life and a very low oral bioavailability (about 20%) (Capizzi et al., 1991); as a consequence the use of complex and precise dosing schedules is needed, so its clinical utility and patient compliance are hindered.

Much effort has been devoted to the design of prodrugs of cytarabine, but few have led to desired products. For example, L-valine was introduced at the NH_2 group of cytarabine to synthesize *N*4-L-valyl-Ara-C, but the oral bioavailability was only 4% after its oral administration in rats (Cheon et al., 2006; Cheon and Han, 2007). Similar results were obtained when the long-chain fatty acids were introduced at the 5′-hydroxyl of cytarabine (Gray et al., 1972). A way to provide protection against the metabolic inactivation of drugs is the encapsulation within liposomes. Nevertheless, it has been shown that cytarabine rapidly diffuses through liposomes bilayers. A way to overcome this difficulty is the chemical modification of the drug to lipophilic prodrugs having physicochemical properties for incorporation into liposomes (Rubas et al., 1986; Tokunaga et al., 1988; Schwendener and Schott, 1992). The enhancement of the lipophilic character of drugs is a widely applied strategy also used to overcome their difficulty in entering cells.

We have conjugated cytarabine to the acyclic isoprenoid chain of squalene (squaleneCOOH) obtaining the squalenoyl-cytarabine prodrug. The squalenic moiety confers a lipophilic character to the prodrug which should render the molecule suitable to be inserted in phospholipid bilayers, such as a biological membrane or a liposomal carrier. In addition, the squalenic moiety protects the NH_2 group of cytarabine from the enzymatic attach. Following previous similar approaches (Castelli et al., 2006, 2007a; Sarpietro et al., 2009), static and kinetic experiments employing monolayers and multilamellar vesicles (MLV) made of dimyristoylphosphatidylcholine (DMPC) have been carried out to have information on the mechanisms involved in the interaction of the prodrug with the biological membranes and to have information on the use of liposomes as carrier for the prodrug.

* Corresponding author. Tel.: +39 095 221796; fax: +39 095 580138.
E-mail address: fcastelli@dipchi.unict.it (F. Castelli).

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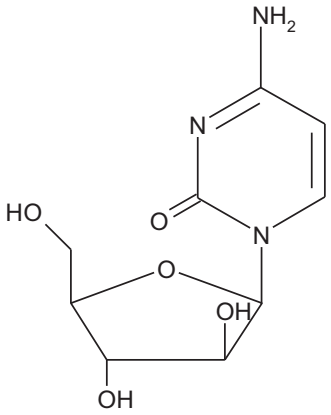


Fig. 1. Cytarabine structure.

Liposomes and monolayers have been investigated in recent years from several points of view, ranging from their usefulness as model biological membranes to their potential medical applications. In medicine, major interest has been focused on liposomes as potential carriers of biological agents both *in vivo* and *in vitro* (Pleyer et al., 1995).

The differential scanning calorimetry (DSC) has been employed with MLV, whereas the Langmuir–Blodgett (LB) technique has been used with monolayers. DMPC, structured as MLV, undergo a sharp phase transition from an ordered gel-like structure (L_β) to a disordered fluid-like structure (L_α), which is affected by the presence of foreign molecules inside the phospholipid bilayers. DSC can detect such a phase change and reveal the presence of the prodrug inside the MLV (Silvius, 1991; Bach, 1994).

Langmuir–Blodgett technique is commonly used for studying the interaction between drugs and phospholipids. Monolayers are an excellent model for studying two-dimensional ordering, with temperature and pressure being readily controlled (Kaganer et al., 1999; Brezesinski and Mohwald, 2003). The analysis of the LB results may provide important information on the organization of biological compounds in lipid membranes (Diociaiuti et al., 2002).

The results have demonstrated that the lipophilic prodrug exhibits a stronger affinity with the phospholipid monolayer and bilayers than cytarabine does and that the liposomes could be used as carrier for the prodrug.

2. Materials and methods

2.1. Chemicals

^{1}H NMR spectra were recorded on a Bruker 300 ultrashield instrument (Karlsruhe, Germany) for samples in $CDCl_3$ solution at room temperature, with Me_4Si (TMS) as internal standard. Coupling constants (J) are given in Hz. Mass spectra were recorded on a Finnigan MAT TSQ 700 spectrometer (San Jose, CA). The reactions were monitored by TLC on F_{254} silica gel precoated sheets (Merck, Damstadt, Germany); after development, the sheets were exposed to iodine vapour. Flash-column chromatography was performed on 230–400 mesh silica gel. Tetrahydrofuran was dried over sodium benzophenone ketyl. All solvents were distilled prior to flash chromatography.

Cytarabine (1-β-D-arabinofuranosylcytosine, Ara-C) (purity > 99%) was purchased from Sigma–Aldrich (Italy). Squalene (purity > 99%) was purchased from VWR (Italy). 1,1′,2-*tris-nor*-squalene aldehyde was obtained from squalene using the method previously described (Ceruti et al., 2005 and references therein). Synthetic L-α-dimyristoylphosphatidylcholine (DMPC) (purity = 99%) was obtained from Genzyme (Switzerland). Lipids were chromatographically pure as assessed by two-dimensional thin layer chromatography. Lipid concentration was determined by the phosphorous analysis (Rouser et al., 1970). 50 mM tris(hydroxymethyl)-aminomethane (Tris) (pH 7.4) was used to prepare MLV.

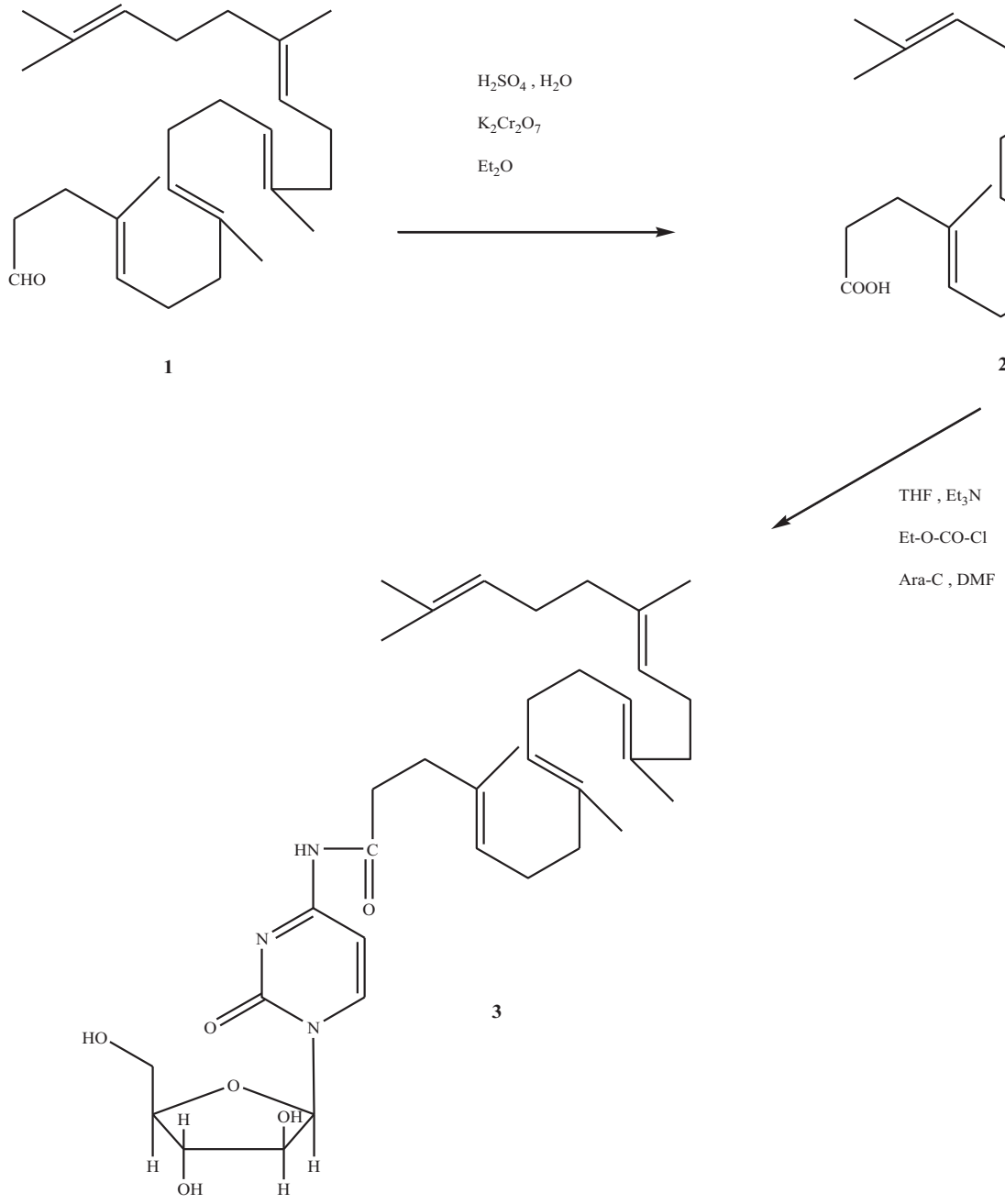
2.2. Synthesis of squalenoyl-cytarabine

2.2.1. Synthesis of 1,1′,2-tris-nor-squalene acid **2**: (4E,8E,12E,16E)-4,8,13,17,21-pentamethyl-4,8,12,16,20-docosapentaenoic acid (Scheme 1)

1,1′,2-*tris-nor*-Squalene aldehyde **1** (1.58 g, 4.12 mmol) was dissolved in diethyl ether (20 ml) at 0 °C. Separately, sulphuric acid (2.3 ml) was added at 0 °C to distilled water (20 ml) with stirring, followed by potassium dichromate (1.21 g, 4.12 mmol), to obtain chromic acid. It was then added at 0 °C within 20 min to the solution of aldehyde **1** previously prepared, and left to react for 2 h at 0 °C with stirring. The reaction mixture was extracted with diethyl ether (50 ml × 3), washed with saturated brine, dried over anhydrous sodium sulfate and evaporated *in vacuo*. The completion of the reaction was revealed by silica gel TLC with light petroleum/diethyl ether/methanol, 70:23:7. The crude product was purified by flash chromatography with light petroleum, then light petroleum/diethyl ether, 95:5 as eluant, to give 578 mg of 1,1′,2-*tris-nor*-squalene acid **2** (35% yield), as a colourless oil. ^{1}H NMR ($CDCl_3$): δ, 1.55–1.63 (m, 18 H, allylic CH_3), 1.90–2.05 (m, 16 H, allylic CH_2), 2.26 (t, 2 H, CH_2CH_2COOH), 2.38 (t, 2 H, CH_2CH_2COOH), 5.00–5.19 (m, 5 H, vinylic CH), 12.20 (broad, 1 H, COOH). MS (EI): m/z 400 (M^+, 5), 357 (3), 331 (5), 289 (3), 208 (6), 136 (3), 81 (100).

2.2.2. Synthesis of 4-[(N)-1,1′,2-tris-nor-squalenoyl]-1-β-D-(arabinofuranosyl)cytosine (squalenoyl-cytarabine) **3**: 4-[(N)-(4E,8E,12E,16E)-4,8,13,17,21-pentamethyl-4,8,12,16,20-docosapentaenoyl]-1-β-D-(arabinofuranosyl)cytosine (squalenoyl-cytarabine) (Scheme 1)

1,1′,2-*tris-nor*-Squalene acid **2** (338 mg, 0.845 mmol) was dissolved in anhydrous THF (5 ml) in a three necked flask under nitrogen, with stirring, followed by triethylamine (85.5 mg, 0.845 mmol) in anhydrous THF (2 ml) and it was cooled at 0 °C. Ethyl chloroformate (Et-O-CO-Cl) (91.7 mg, 0.845 mmol) dissolved in anhydrous THF (82 ml) was then added and left at 0 °C for 20 min, with stirring, followed by the addition of cytarabine (Ara-C) (205.52 mg, 0.845 mmol) dissolved in anhydrous warm DMF (5 ml), due to its low solubility. The reaction mixture was allowed to reach room temperature and allowed to react for 3 days, with stirring, under nitrogen. The reaction mixture was controlled by silica gel TLC with light dichloromethane/acetone, 95:5. The crude product was purified by flash chromatography with dichloromethane, then dichloromethane/acetone, 85:15 and finally dichloromethane/acetone, 75:25 as eluant, to give 296 mg of squalenoyl-cytarabine **3** (56% yield), as a colourless viscous oil. ^{1}H NMR (CD_3COCD_3): δ, 1.55–1.68 (18 H, m, allylic CH_3), 1.97–2.12 (16 H, m, allylic CH_2), 2,35 (2 H, t, $NHCOCH_2CH_2$), 2.64 (2 H, t, $NHCOCH_2$), 3.88 (2 H, m, 5′-CH_2), 4.08 (1 H, m, 4′-CH), 4.24–4.30 (2 H, m, 2′-CH and 3′-CH), 4.65–5.00 (3 H, broad peaks, OH), 5.02–5.25 (5 H, m, vinylic CH), 6.189 and 6.200 (1 H, d, J = 3.0 Hz, 1′-CH), 7.323 and 7.349 (1 H, d, J = 9.0 Hz, 5-CH), 8.187 and 8.212 (1 H, d, J = 9.0 Hz, 6-CH), 9.58–9.68 (1 H, broad peak, NHCO). ^{1}H NMR (CD_3OD): δ, 1.55–1.68 (18 H, m, allylic CH_3), 1.97–2.12 (16 H, m, allylic CH_2), 2.35 (2 H, t, $NHCOCH_2CH_2$), 2.54 (2 H, t, $NHCOCH_2$), 3.90 (2 H, m, 5′-CH_2), 4.03 (1 H, m, 4′-CH), 4.10 (1 H, m, 3′-CH), 4.250 (1 H, m, 2′-CH), 5.02–5.25 (5 H, m, vinylic CH), 6.192 and 6.205 (1 H, d, J = 3.9 Hz, 1′-CH), 7.424 and 7.449 (1 H, d, J = 7.5 Hz, 5-CH), 8.230 and 8.255 (1 H, d, J = 7.5 Hz, 6-CH). MS (CI): m/z 627 (M^+, 100), 609 (65).



Scheme 1. SqualeneCOOH and squalenoyl-cytarabine synthesis.

2.3. Differential scanning calorimetry

DSC was performed by using a Mettler TA Stare equipped with a DSC 822^e calorimetric cell and a Mettler STAR^e V 8.10 Software. The reference pan was filled with 120 μl Tris solution. The sensitivity was automatically chosen as the maximum possible by the calorimetric system. The calorimetric system was calibrated, in transition temperature and enthalpy changes, by using indium, stearic acid and cyclohexane by following the procedure of the Mettler TA STAR^e Software.

2.4. MLV preparation

MLV were prepared both in the presence and in the absence of cytarabine and squalenoyl-cytarabine at different molar fractions (0.015, 0.03, 0.045, 0.06, 0.09, 0.12). Stock solutions of DMPC, cytarabine and squalenoyl-cytarabine in chloroform/methanol (1:1, v:v) were prepared. Aliquots, containing 0.010325 mmol, of DMPC solution were distributed in glass tubes where aliquots of cytarabine or squalenoyl-cytarabine solutions, in order to have the chosen molar fraction, were added. The solvents were removed under nitrogen stream at 37 °C. The samples were, then, freeze dried to remove eventual solvents residues.168 μl of Tris solution was added; the samples were heated at 37 °C for 1 min and vortexed for 1 min, for three times and kept at 37 °C for 1 h.

2.5. MLV/compounds interaction analysis

120 μl of MLV was put into an aluminium calorimetric pan, which was hermetically sealed, and submitted to the following DSC

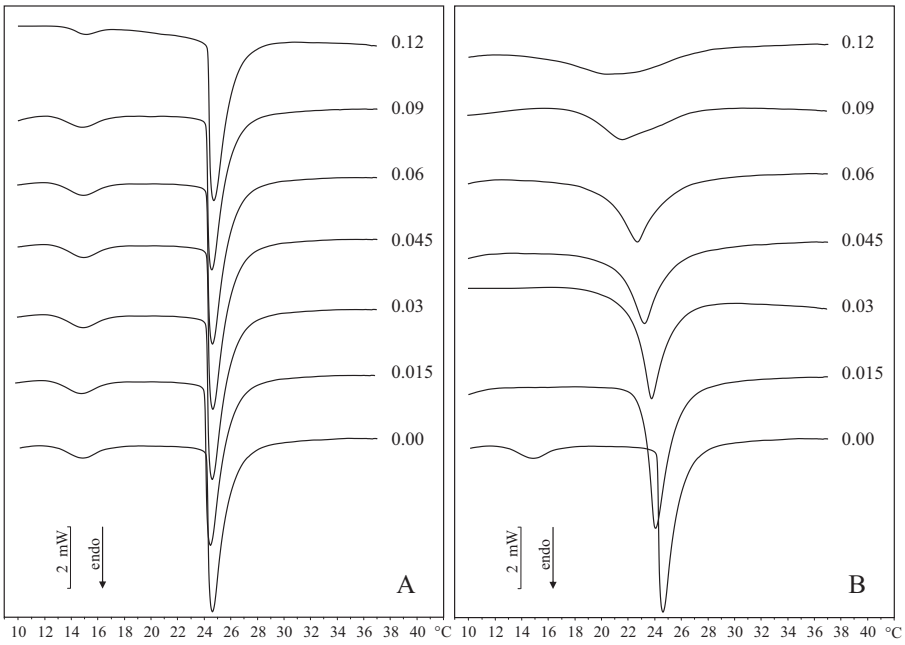


Fig. 2. Calorimetric curves in heating mode, of DMPC MLV prepared in the presence of increasing molar fractions of cytarabine (A) and squalenoyl-cytarabine (B).

analysis: (i) a heating scan from 5 to 37 °C, at the rate of 2 °C/min; (ii) a cooling scan from 37 to 5 °C, at the rate of 4 °C/min; for at least three times. Each experiment was carried out in triplicate.

2.6. Monolayer measurements

These experiments were performed by a KSV minitrough apparatus provided with a computer interface unit, an operating software, a trough (24,225 mm^2 available area) made in Teflon, two mobile barriers made in delrin. 5 mM Tris (pH 7.4) in ultrapure Millipore water with resistivity of 18.2 MΩ cm was used as subphase. Equimolar solutions (0.001 mmol/ml) of DMPC, cytarabine and squalenoyl-cytarabine in organic solvents were prepared. Mixed DMPC/compound solutions were successively prepared to obtain for each compound the following molar fractions: 0.015, 0.03, 0.045, 0.06, 0.09, 0.12, 0.25, 0.50, and 0.75. 30 μl of the mixed solutions as well as of the pure components were spread drop by drop on the surface of the subphase by a Hamilton microsyringe; after waiting 15 min (at the fixed temperature) to allow the solvent evaporation, the monolayers were compressed by the use of the two mobile barriers at the constant speed of 10 mm min^{-1}. Surface pressure vs molecular area isotherms were recorded by the Wilhelmy plate arrangement attached to a microbalance. Before spreading the sample, the subphase was checked twice by running blank experiments to be sure that no impurities were present. The experiments were performed at a subphase temperature of 10 and 37 °C which was kept constant by a cryothermostat connected to the trough. Each experiment was repeated at least three times to be sure of the results reproducibility.

2.7. Transmembrane transfer

60 μl of MLV prepared in the presence of cytarabine or squalenoyl-cytarabine at 0.12 molar fraction (loaded MLV) was put into the calorimetric pan and, then, 60 μl of MLV of pure DMPC (unloaded MLV) was added. The pan was hermetically sealed and submitted to the following DSC analysis: (i) a heating scan from 5 to 37 °C, at the rate of 2 °C/min, to detect the behaviour of the samples as soon as after their contact; (ii) a isotherm scan of 1 h at 37 °C (above the transition temperature of DMPC), to permit the compound to eventually transfer from the loaded MLV to the unloaded MLV; (iii) a cooling scan from 37 to 5 °C, at the rate of 4 °C/min, to bring the phospholipid system back to the ordered state. The three steps were repeated for at least eight times. Each experiment was carried out in triplicate.

3. Results and discussion

One of the aims of this research was to have information on the interaction of cytarabine and its squalenoyl-cytarabine prodrug with biological membranes. The second aim was to have information on the use of liposomes as possible carrier for the squalenoyl-cytarabine prodrug. The biological membranes are very intricate structures and hence their biophysical interactions with drugs are very difficult to study. For this reason, the choice of a simplified model membrane, where all experimental parameters can be accurately monitored, is of great importance in the study of the elemental processes of drug–lipid interaction and several biological membrane models have been developed. Among them, phospholipid monolayers and liposomes were used in our research (Maget-Dana, 1999; Peetla et al., 2009).

3.1. Synthesis of squalenoyl-cytarabine

1,1′,2-*tris*-*nor*-Squalene aldehyde **1** was obtained starting from squalene according to a method previously developed by us (Ceruti et al., 2005). 1,1′,2-*tris*-*nor*-Squalene acid **2** was obtained by reaction of squalene aldehyde **1** with chromic acid, prepared by reacting aqueous sulphuric acid at 0 °C with potassium dichromate, in 35% yield.

Squalenoyl-cytarabine **3** was obtained according to a previously developed method for the synthesis of squalenoyl-gemcitabine, slightly modified (Stella et al., 2004, 2005; Couvreur et al., 2006). Compound **3** was obtained by reacting squalene acid **2** with ethyl chloroformate in anhydrous THF and triethylamine, followed by addition of cytarabine, dissolved in anhydrous warm DMF, due to its very low solubility, even in warm THF. It was allowed to react for 3 days at room temperature; after standard work-up and chromatographic purification, squalenoyl-cytarabine **3** was obtained, as a colourless viscous oil, in 35% yield.

3.2. MLV/compounds interaction analysis

The calorimetric curve of pure DMPC MLV (Fig. 2) shows a pretransition peak at about 17 °C, due to the tilting of the hydrophobic chains of the phospholipid, and a transition peak at about 25 °C, characterized by a well defined enthalpy variation (ΔH), due the passage from an ordered phase (the gel phase) to a disordered phase (the liquid-crystalline phase) (Walde, 2004). Stranger molecules in the phospholipid bilayers of MLV can influence the phase transition changing the temperature (T_m) at which it occurs and/or the ΔH (Jorgensen et al., 1991; Marsh, 1996; Castelli et al., 2007b).

MLV were prepared in the presence of different amounts of cytarabine and squalenoyl-cytarabine and submitted to calorimetric analysis. The interaction of the compounds with the MLV was evaluated considering the variations of T_m, ΔH, and shape of the calorimetric curves with respect to that of DMPC MLV prepared in the absence of compounds (Fig. 2A and B). The effect of cytarabine is just a small shift of the pretransition peak towards lower temperature; whereas the transition peak remains unchanged. Squalenoyl-cytarabine exerts a strong effect both on the pretransition and on the transition peaks. In fact, the pretransition peak disappears at all the molar fractions of squalenoyl-cytarabine present in the MLV; the transition peak broadens and shifts towards lower temperature; moreover, starting from 0.09 molar fraction, a phase separation occurs which indicates a not uniform distribution of squalenoyl-cytarabine in the phospholipid bilayers and the consequent formation of compound reach and compound poor regions in the bilayers (Lambros and Rahmanb, 2004).

The transition temperature, as $\Delta T/T_m^0$, and the enthalpy change, as $\Delta\Delta H/\Delta H^0$, variations of the DMPC are shown in Fig. 3A and B as a function of the compound molar fraction in the MLV aqueous dispersion. The T_m, as well as the ΔH, linearly decreases as the molar fraction of squalenoyl-cytarabine increases. In the figure, the T_m and ΔH variations relative to cytarabine are shown also: almost flat lines are obtained. The results demonstrate that cytarabine interacts with the DMPC MLV very weakly, probably with the polar head of the phospholipids producing the small shift of the pretransition peak. This is in accord with the hydrophilic character of cytarabine which hindrances its insertion in the phospholipid bilayers. On the contrary, squalenoyl-cytarabine strongly interacts with the MLV localizing among the phospholipid molecules, probably with the squalenoyl moiety in the acyl chain region and the cytarabine moiety in the polar head region, and causing the fluidization of the bilayers and the decrease of the cooperativity of the phospholipid molecules.

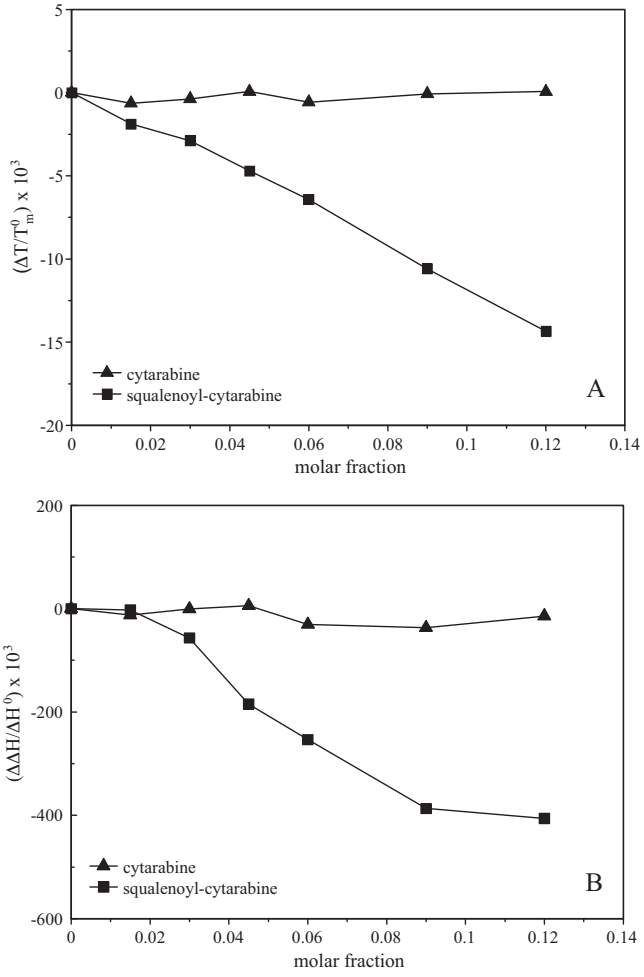


Fig. 3. Transition temperature, as $\Delta T/T_m^0$ (panel A) and enthalpy variation, as $\Delta\Delta H/\Delta H^0$ (panel B) of DMPC MLV prepared in the presence of increasing molar fractions of cytarabine and squalenoyl-cytarabine as a function of the compound molar fraction. $\Delta T = T_m - T_m^0$, where T_m is the transition temperature of DMPC MLV prepared in the presence of the compound and T_m^0 is the transition temperature of pure DMPC MLV. $\Delta\Delta H = \Delta H - \Delta H^0$, where ΔH is the enthalpy variation of MLV prepared in the presence of the compound and ΔH^0 is the enthalpy variation of pure DMPC MLV.

3.3. Monolayer measurements

Interesting results were obtained when DMPC monolayers were used. Single component monolayers of DMPC, cytarabine or squalenoyl-cytarabine and mixed monolayers of DMPC and cytarabine and of DMPC and squalenoyl-cytarabine were spread over the subphase. The effect of the compounds on the biomembrane model was obtained comparing the isotherm of pure DMPC monolayer and the isotherms of DMPC/cytarabine and DMPC/squalenoyl-cytarabine mixed monolayers. The isotherms were recorded at 10 °C (below the DMPC transition temperature) and 37 °C (above the DMPC transition temperature).

The measures carried out at 10 °C are shown in Fig. 4A and B. The isotherm of DMPC is characterized by a gaseous state from 120 to 100 Å^2, a liquid expanded (LE) state from 100 to about 70 Å^2, a liquid expanded/liquid condensed (LE/LC) transition from about 70 Å^2 to about 44 Å^2, and a liquid condensed (LC) state from about 44 Å^2 to smaller values of molecular area. Cytarabine (Fig. 4A), as expected, does not form monolayer; in fact, due to its hydrophilicity, it dissolves in the subphase. The addition of cytarabine, up to 0.12 molar fraction, does not cause important variations on the DMPC isotherm. For molar fraction higher than 0.12, the isotherms shift towards lower molecular area and the LE/LC transition becomes less evident. Squalenoyl-cytarabine (Fig. 4B) exhibits a gas state from 120 to 100 Å^2, and a LE state at lower values of molecular area. The addition of squalenoyl-cytarabine to DMPC causes the shift of the isotherms towards higher values of molecular area for all the molar fraction, with the exception of 0.75 molar fraction for which the isotherm is shifted towards lower values of molecular area. In addition, as squalenoyl-cytarabine molar fraction increases, the LE/LC transition becomes less evident and then it disappears.

In Fig. 5A and B the isotherms recorded at 37 °C are shown. At this temperature, the DMPC isotherm is characterized by a gaseous state between 120 and 110 Å^2, and an LE state for lower values of molecular area. Even at this temperature, cytarabine (Fig. 5A) does not form any monolayer, as demonstrated by the absence of isotherm. Its presence in the DMPC monolayer does not cause variation in the DMPC isotherm up to 0.12 molar fraction; at higher molar fractions, the isotherms shift towards lower values of molecular area. Squalenoyl-cytarabine isotherm (Fig. 5B) shows a gaseous state from 120 to 90 Å^2, and a LE state at lower molecular area values. The isotherms of the DMPC/squalenoyl-cytarabine mixed

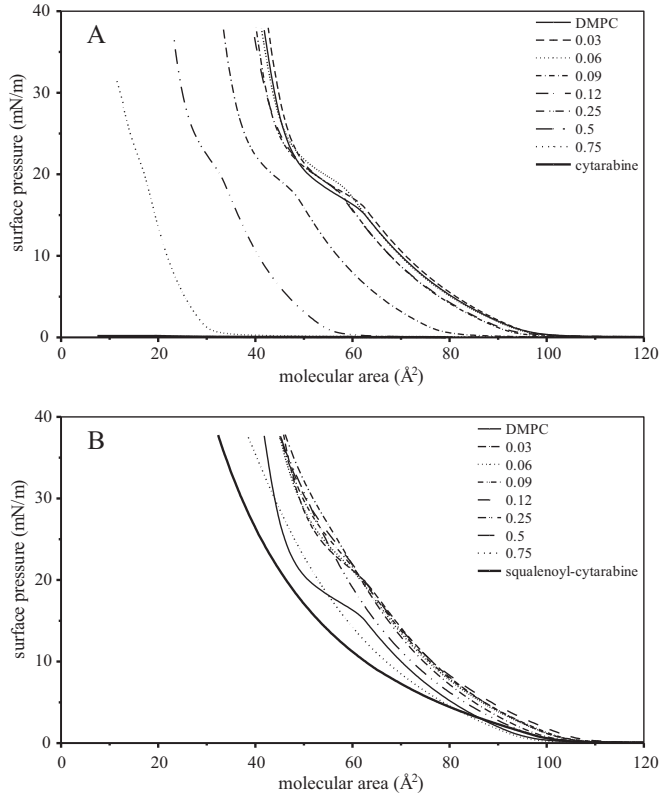


Fig. 4. Surface pressure/molecular area isotherms of the DMPC/cytarabine (A) and DMPC/squalenoyl-cytarabine (B) mixed monolayers, at the air–water interface at 10 °C.

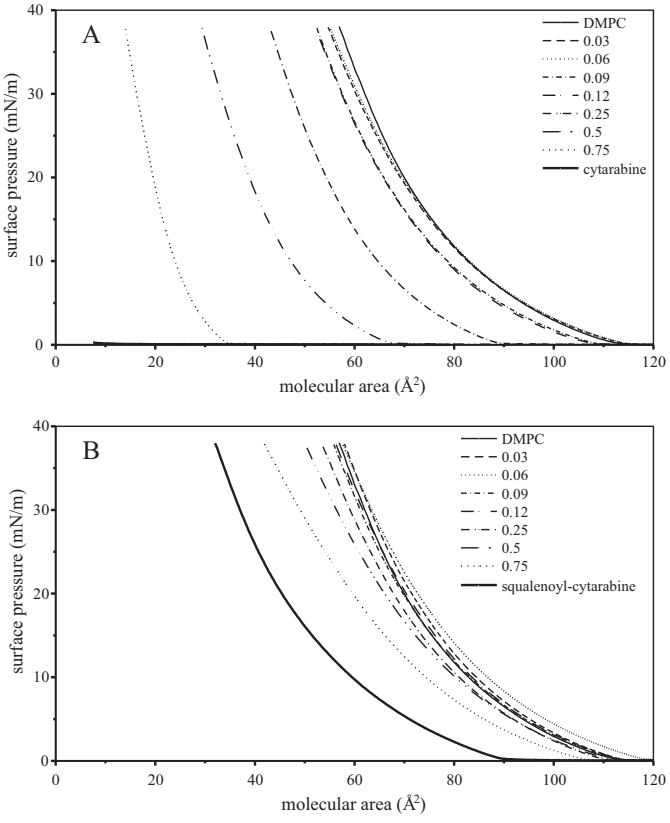


Fig. 5. Surface pressure/molecular area isotherms of the DMPC/cytarabine (A) and DMPC/squalenoyl-cytarabine (B) mixed monolayers, at the air–water interface at 37 °C.

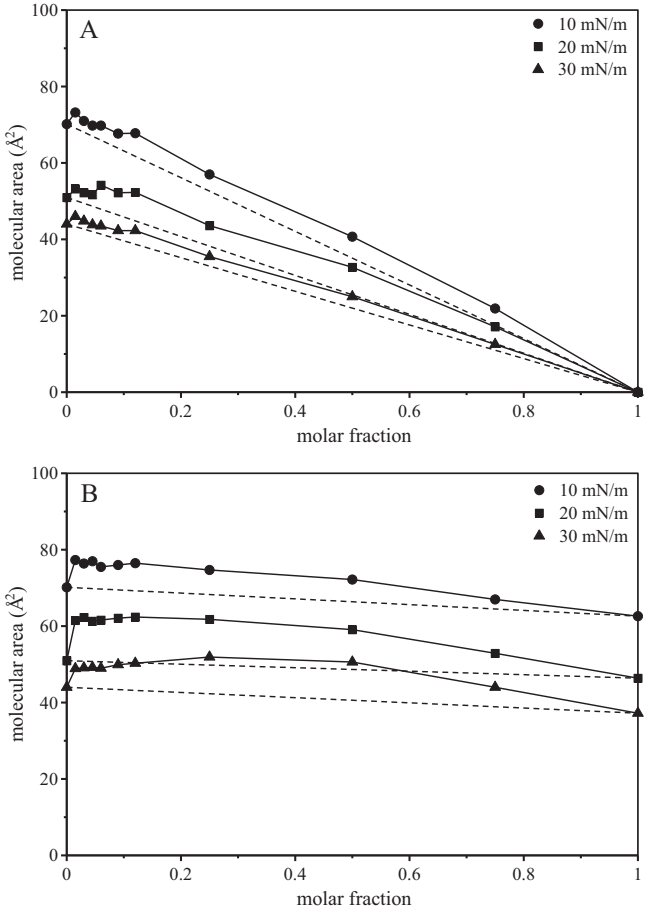


Fig. 6. Molecular area of DMPC/cytarabine (A) and DMPC/squalenoyl-cytarabine (B) mixed monolayers at the air–water interface plotted as a function of the molar fraction of compound at various values of surface pressures at 10 °C.

monolayer are shifted towards higher values of molecular area up to 0.06 molar fraction. The isotherms with 0.09 and 0.12 molar fractions of prodrug overlap the DMPC isotherm. Higher molar fractions of squalenoyl-cytarabine cause the shift of the isotherm towards lower values of molecular area.

To have information on the interaction among the molecules in a mixed monolayer, the surface pressure/molecular area isotherms were analyzed by calculating, at three different surface pressures (10, 20 and 30 mN/m), the molecular area as a function of the molar fraction of cytarabine or squalenoyl-cytarabine present in the monolayer. The mean molecular area of a two-components monolayer can be calculated by $A = A_1X_1 + (1 - X_1)A_2$, where A is the mean molecular area, X_1 is the molar fraction of component 1, and A_1 and A_2 are the areas per molecule of the pure components monolayers at the same surface pressure. Reporting in a graph A as a function of X_1, a straight line is obtained in the case the two components are either ideally mixed or complete immiscible at the air–liquid interface (Gaines, 1966; Shahgaldian and Coleman, 2003). Any deviation from the straight line indicates that the components of the monolayer are miscible and exhibit a non-ideal behaviour.

The graphs in Fig. 6A and B show the values obtained at 10 °C. Cytarabine causes a small positive deviation with respect to the ideal (straight, dashed) line for all the molar fractions and all the values of surface pressure, but less evident a 30 mN/m. Squalenoyl cytarabine exerts strong positive deviations at all the molar fractions and values of surface pressure. The values recorded at 37 °C are shown in Fig. 7A and B. Even at this temperature,

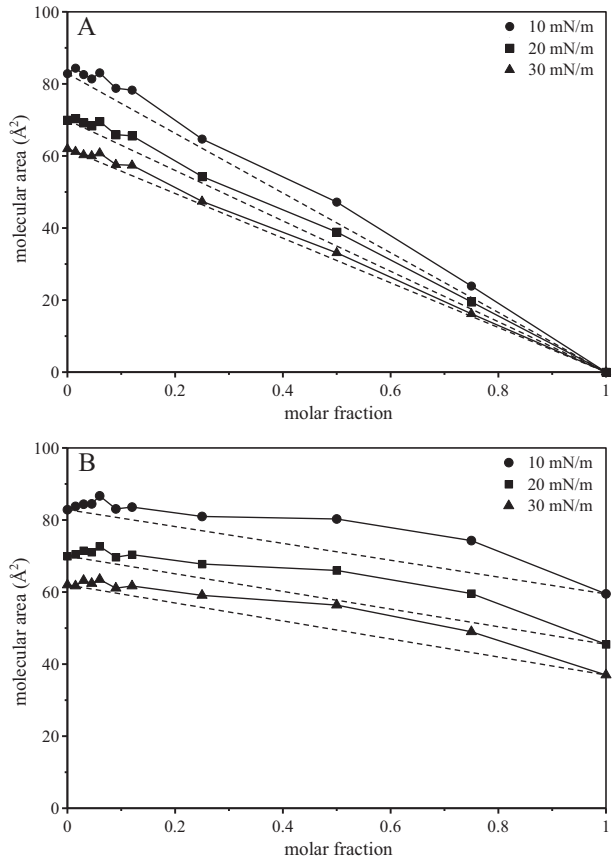


Fig. 7. Molecular area of DMPC/cytarabine (A) and DMPC/squalenoyl-cytarabine (B) mixed monolayers at the air–water interface plotted as a function of the molar fraction of compound at various values of surface pressures at 37 °C.

cytarabine causes small positive deviations which decrease and almost overlap the ideal line at 30 mN/m, whereas squalenoyl-cytarabine causes big positive deviations at all the molar fractions and surface pressures. This indicates that both cytarabine and squalenoyl-cytarabine interact with DMPC molecules in the monolayer, repulsive forces occurring among the drug or prodrug and DMPC molecules, but with quite different intensity. In fact, these forces are very weak in the case of cytarabine and very strong in the case of squalenoyl-cytarabine. In the case of cytarabine, the interaction could be limited with the polar head of the DMPC molecules. The decrease of the positive deviation at 30 mN/m could indicate the high compression squeezes the cytarabine molecules out of the DMPC monolayer, with a consequent dissolution in the subphase and the loss of interaction with the DMPC molecules. In the case of squalenoyl-cytarabine the interaction could involve, besides the polar head, also the hydrophobic chains of the phospholipids at all the surface pressures. As it is generally accepted that monolayers mimic bilayer behaviour at surface pressures between 30 and 50 mN/m (Jones and Chapman, 1995), the Langmuir–Blodgett results are in agreement with those of the calorimetric experiments. In fact from the calorimetric results it emerged a very weak interaction between cytarabine and DMPC and very strong fluidizing effect of squalenoyl-cytarabine towards the phospholipid bilayer.

3.4. Transmembrane transfer

Liposomes are widely recognized as an effective drug delivery system. Due to their unique characteristics, liposomes are employed to deliver a variety of agents such as cancer therapy drugs, antivirals, and antibiotics (Drummond et al., 1999; Allen and Cullis, 2004; Torchilin, 2005). In addition, liposomes have been investigated as non-viral vehicles for reaching targeted cells in gene therapy (Noguchi et al., 1998), and as antigen carriers for immunization (Thérien and Gruda, 1990). The liposome protects the drug from metabolism and inactivation in the plasma, and, due to size limitations in the transport of large molecules or carriers across healthy endothelium, the drug accumulates to a reduced extent in healthy tissues (Mayer et al., 1989).

We put in contact unloaded MLV (which mimic the biomembrane) and drug or prodrug loaded MLV (which mimic a loaded lipophilic carrier) and submitted the obtained sample to DSC analysis. The calorimetric curves were recorded at intervals of one hour and are shown in Fig. 8A and B. In figure, also the calorimetric curves

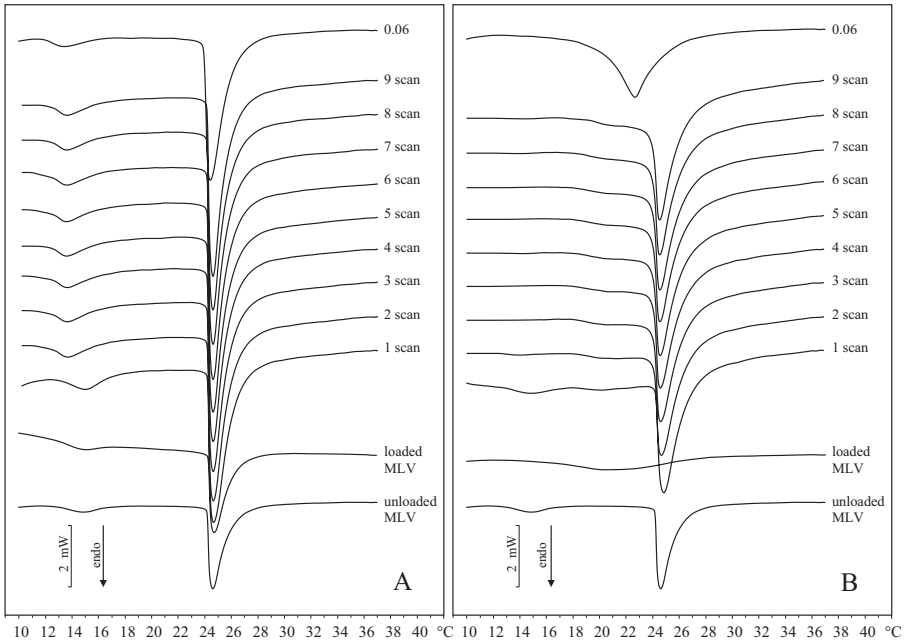


Fig. 8. Calorimetric curves, in heating mode, of cytarabine (A) or squalenoyl-cytarabine (B) loaded DMPC MLV left in contact with unloaded DMPC MLV at increasing time of incubation. The curve 0.06 belongs to DMPC MLV prepared in the presence of cytarabine or squalenoyl-cytarabine at 0.06 molar fraction.

of loaded and unloaded MLV and the curve of the MLV prepared in the presence of compound at 0.06 molar fraction are present. The latter is considered a reference curve. If loaded MLV lost the compound, the relative peak should decrease and disappear; and if the compound was absorbed by the unloaded MLV, the relative peak should broaden and move towards lower temperature approaching the reference curve. As far as cytarabine (Fig. 8A) is concerned, as expected, all the calorimetric curves present the pretransition peak and the transition peak which are not subjected to modification neither in the shape nor in the position. With regard to squalenoylcytarabine (Fig. 8B), in the calorimetric curve of the first scan, three signals are present: a pretransition peak, a broad peak corresponding to the loaded MLV and a well shaped peak corresponding to the unloaded MLV. In the successive scans, the pretransition peak decreases and disappears; whereas the broad peak and the well shaped peak remain almost unchanged meaning that loaded MLV do not lose the loaded compound.

4. Conclusion

The enhancement of the lipophilic character of cytarabine was obtained by the conjugation to the acyclic isoprenoid chain of squalene, in order to increase the affinity towards the phospholipid environment of biological membrane as well as of lipophilic carrier. The study of the interaction of the prodrug and the free drug with multilamellar vesicles and monolayers made of DMPC, showed that the prodrug has a stronger affinity both for the monolayers and the bilayers with respect to cytarabine. This is probably due to the squalene moiety which allows the molecule to insert among the phospholipid molecules. In addition, our results could give information on the use of liposomes as devices for the delivery of the encapsulated prodrug within the cell, protecting the cargos from both clearance from the blood circulation and biological degradation.

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