International Journal of Pharmaceutics 436 (2012) 135–140

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Squalenoyl prodrug of paclitaxel: Synthesis and evaluation of its incorporation in phospholipid bilayers

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ARTICLE INFO

Article history:
Received 3 May 2012
Received in revised form 11 June 2012
Accepted 12 June 2012
Available online xxx

Keywords:
Paclitaxel
Prodrug
Phospholipid bilayers
DSC
Squalenoyl–paclitaxel

ABSTRACT

1,1′,2-*Tris*norsqualenoic acid was conjugated to paclitaxel to obtain the squalenoyl–paclitaxel prodrug with the aim to improve the incorporation in phospholipid bilayers. Differential scanning calorimetry technique was employed to compare the interaction of squalenoyl–paclitaxel prodrug and free paclitaxel with phospholipid bilayers. The possibility of using lipid vesicles as carrier for the prodrug was also evaluated. An increased encapsulation into phospholipid bilayers of squalenoyl–paclitaxel with respect to the free drug was observed. The ability of lipid vesicles to retain the loaded prodrug was also observed which make this system to be considered as carrier for the prodrug.

1. Introduction

Paclitaxel (Taxol®) is an antineoplastic agent that is derived from the bark of the Pacific yew tree (*Taxus brevifolia*) (Wani et al., 1971). Paclitaxel has been used to treat ovarian carcinoma, breast carcinoma, leukemia, melanoma, prostate carcinoma etc. (Choi and Jo, 2004). Its transport and delivery is obstacolated by a very low water solubility (Vyas, 1995) then, at the present, it is formulated in a mixture of 50:50% (v/v) polyoxyethylated castor oil (Cremophor EL) and dehydrated ethanol. However, this formulation vehicle has been found to cause serious side-effects, including hypersensitivity and neurotoxicity reactions (Weiss et al., 1990; Fjallskog et al., 1993). Then, there is a continued interest in finding formulations that can be administered easily and safely. Water-soluble paclitaxel derivatives have been prepared and their activity has been investigated (Greenwald et al., 1996, 2003; Ceruti et al., 2000; Singer et al., 2003). Alternatively, paclitaxel has been encapsulated in biodegradable polymers (Mu and Feng, 2003; Liu et al., 2010; Nanda et al., 2011). Moreover, cyclodextrins (Alcaro et al., 2002) emulsion (Han et al., 2004; Constantinides et al., 2004) microspheres (De et al., 2005; Jackson et al., 2007) nanoparticles (Bhardwaj et al., 2009; Chakravarthi et al., 2010) formulation have been prepared and investigated. The incorporation of paclitaxel in vesicular carrier has been attempted (Meng et al., 2010; Paolino et al., 2012). However, the amount of paclitaxel that can be incorporated into lipid bilayers is limited (Sharma et al., 1998; Shieh et al., 1997; Balasubramanian and Straubinger, 1994). Therefore, it could be of interest to use a lipid-based prodrug of paclitaxel that could be incorporated and retained in lipid carrier preparations. Some attempt has been done to achieve this goal. 2′-Alpha-bromohexadecanoyl paclitaxel prodrug has been synthetized and incorporated in lipid systems that were found more effective than pacliataxel against a human ovarian tumor (Ahmad et al., 1999). We have exploited the conjugation of a lipophilic moiety to some drug in order to increase their affinity for lipid systems with respect to the free drug. In those researches we have utilized as lipophilic moiety 1,1′,2-*tris*norsqualenoic acid (squaleneCOOH) (Castelli et al., 2007; Sarpietro et al., 2009, 2010, 2011) a derivative of squalene, a compound widespread in nature, that is synthesized within cells and consumed as an integral part of the human diet. The prodrugs obtained showed a deep interaction with phospholipid bilayers. Following this approach, in the present research, we conjugated squaleneCOOH with paclitaxel with the aim to obtain a highly lipophilic squalenoyl–paclitaxel prodrug (Scheme 1) that can be incorporated and retained into the lipid system. The interaction of the prodrug with lipid bilayers represented

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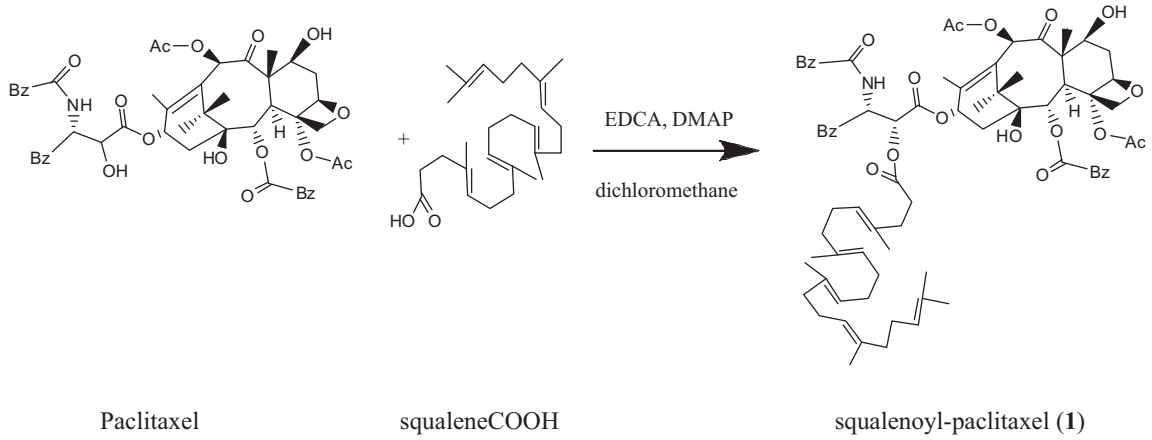
http://dx.doi.org/10.1016/j.ijpharm.2012.06.034



Scheme 1. Squalenoyl–paclitaxel synthesis.

by dimyristoylphosphatidylcholine (DMPC) multilamellar vesicles (MLV) has been investigated by differential scanning calorimetry technique that can reveal the effect caused by the insertion of "stranger" molecules in the phospholipid bilayers through the variation of the phospholipid bilayers thermotropic parameters (transition temperature, T_m, enthalpy change, ΔH) induced by the incorporated molecules. Transmembrane experiments have been also carried out to verify the ability of the prodrug to be retained in the lipid system.

2. Materials and methods

2.1. Materials

Synthetic 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC, purity 99%) was obtained from Genzyme (Switzerland). Paclitaxel was purchased from Indena (Milan, Italy), other reagents were purchased from Sigma–Aldrich (Milwaukee, WI). The NMR spectra were recorded using a NMR Bruker Avance 300 spectrometer. Elemental analyses were carried out by Redox Snc (Monza, Italy). All HPLC analyses were performed on a Merck-Hitachi L-6200 Liquid Chromatographer equipped with L5000 LC Controller (Merck, Milan, Italy) and the eluting fractions containing PTX were monitored at 227 nm using an L-4200 UV detector.

2.2. Synthesis and characterization of squalenoyl–paclitaxel

Paclitaxel (1.2 g, 1.4 mmol), dissolved in 30 ml of dichloromethane, was reacted with *N*-ethyl-*N*′-3-dimethylaminopropyl carbodiimide (0.6 equiv.), in the presence of 4-dimethylamino pyridine (0.2 equiv.) and 1,1′,2-*tris*norsqualenoic acid (0.6 equiv.) previously dissolved in DCM at room temperature. After 3 h, the reaction was stopped with water and extracted with brine. The crude mixture was purified by chromatography on SiO_2 eluted with a gradient (from 95:5 to 80:20) of dichloromethane/ethyl acetate to give the pure compound (1) (Scheme 1) (Yield 65%). TLC control dichloromethane/ethanol (97:3) Rf 0.55. The purity of squalenoyl–paclitaxel was checked by HPLC on a RP-18 reverse phase column (LiChrospher 100 RP 18e 5 μm, Merck) eluted with an acetonitrile/water mixture (40:60 and, after 5 min, gradient up to 100% acetonitrile, 20 min), elution time 19.23 min. Purity by HPLC was above 92%. Characterization: ^{1}H NMR (300 MHz, $CDCl_3$): 8.13 (d, 2H, C23, C27ArH), 7.75 (d, 2H, C39, C43 ArH), 7.62 (t, 1H, C25 ArH), 7.53–7.49 (band, 3H, C24, C26, C41 ArH), 7.43–7.35 (band, 7H, C33, C34, C35, C36, C37, C40, C42 ArH), 6.91 (d,1H, 4′NH), 6.35 (s, 1H, C(10)-H), 6.24 (m, 1H, C(13)-H), 5.99 (dd, 1H, C(3′)-H), 5.69 (d, 1H, C(2)-H), 5.53 (d, 1H, C(2′)-H), 5.20 (m, 5H, C(SQ-H)), 4.97 (d, 1H, C(5)-H), 4.44 (m, 1H, C(7)-H), 4.33 (d, 1H, C(20)-H_a), 4.20 (d, 1H, C(20)-H_b), 3.85 (d, 1H, C(3)-H), 2.55 (m, 1H, C(6)-H_a), 2.49 (s, 3H, C(29)-H), 2.45 (m, 2H, $\underline{CH_2}$–CH_2–CO SQ), 2.30 (t, 2H, CH_2–$\underline{CH_2}$–CO SQ), 2.23 (s, 3H, C(31)-H), 2.09 (s, 3H, C(18)-H), 2.00 (m, 16H, CH_2 SQ), 1.97 (m, 1H, C(6)-H_b), 1.70 (m, 1H, C(14)-H_a), 1.67 (s, 3H, C(19)-H), 1.61 (m, 18H, C(SQ)–CH_3), 1,25 (m, 1H, C(14)-H_b), 1.21 (s, 3H, C(16)-H), 1.13 (s, 3H, C(17)-H). ESI-MS calculated for $C_{74}H_{93}NO_{15}$: 1235.65. Found 1237.24 (MH+). Elemental analysis calc: C 71.88%, H 7.58%, N 1.13%; measured C 71.99%, H 7.63%, N 1.19%.

The lipophilic character of the synthesized compound was determined using a chromatographic Rm method as described by some of us (Dosio et al., 2010). Theoretical log *P* was calculated using the software ALOGPS 2.1 available on the Web site http://www.vcclab.org/lab/alogps/start.html.

2.3. Differential scanning calorimetry

Differential scanning calorimetry studies were performed using a Mettler TA STAR^e System equipped with a DSC 822^e cell and a Mettler STAR^e V8.10 software. The reference pan was filled with 120 μl of 50 mM TRIS. The calorimetric system was calibrated, in transition temperature and enthalpy changes, by using indium and palmitic acid (purity ≥ 99.95% and ≥99.5%, respectively; Fluka, Switzerland) following the procedure of the Mettler STAR^e software.

2.4. Multilamellar vesicles preparation

Multilamellar vesicles were prepared empty and loaded with compounds. Stock solutions of DMPC, paclitaxel and squalenoyl–paclitaxel were prepared in chloroform/methanol (1:1, v/v). Aliquots of DMPC solution corresponding to 0.010325 mmol were put in glass tubes and aliquots of paclitaxel or squalenoyl–paclitaxel were added to have the following molar fraction of compounds with respect to DMPC: 0.00, 0.015, 0.03, 0.045, 0.06, 0.09, 0.12. The solvents were evaporated under a nitrogen stream and the obtained films were freeze dried to eliminate solvents traces. 168 μl of 50 mM TRIS (pH 7.4) was added to the films and the samples were heated at 37 °C (temperature higher than the DMPC T_m) for 1 min and vortexed for 1 min, for three times and, then, left in a water bath at 37 °C for 60 min.

2.5. MLV/paclitaxel and MLV/squalenoyl–paclitaxel interaction

120 μl of the MLV (0.007375 mmol of DMPC) were put in a 160 μl aluminum pan which was hermetically closed and submitted to calorimetric scans, for at least three times, as follows: (i) a

heating scan from 5 to 37 °C, at 2 °C/min; (ii) a cooling scan from 37 to 5 °C, at 4 °C/min. The experiments were carried out in triplicate to be sure of the results reproducibility.

2.6. Paclitaxel and squalenoyl–paclitaxel absorption by MLV

120 μl of MLV were put in the calorimetric pan where an amount of drug or prodrug corresponding to a 0.09 molar fraction with respect the DMPC had been weighted. The pan was closed and submitted to calorimetric scans as follows: (i) a heating scan from 5 to 37 °C, at 2 °C/min; (ii) a isothermal scan of 60 min at 37 °C; (iii) a cooling scan from 37 to 5 °C, at 4 °C/min; for at least eight times.

2.7. Evaluation of liposomes as carrier of squalenoyl–paclitaxel

In this kind of experiments MLV were considered both as lipophilic carrier for paclitaxel and squalenoyl–paclitaxel and as biomembrane model. In particular compound loaded MLV were used as carrier whereas unloaded MLV were used as biomembrane model. The point of this experiment was that loaded MLV mixed with unloaded MLV at a temperature higher than the T_m can transfer the loaded compound to unloaded MLV so after several incubation periods an equilibrium between MLV could be reached. 60 μl of unloaded MLV (prepared without compound) were put in the calorimetric pan and 60 μl of loaded MLV (prepared with paclitaxel or squalenoyl–paclitaxel at 0.06 molar fractions with respect to the DMPC) were added. The pan was closed and submitted to the same calorimetric scans described in the previous section.

3. Results and discussion

We have conjugated paclitaxel with squaleneCOOH to obtain the squalenoyl–paclitaxel prodrug (Scheme 1). This derivative was obtained by linkage at the 2′-hydroxyl group of paclitaxel, as several previously synthesized taxoids (Skwarczynski et al., 2006). Derivatives obtained exploiting this position are much more accessible to enzymes and are able to undergo hydrolysis so as to release the active drug. In the reported experimental conditions the ester in 2′ position was the most relevant derivative obtained while the 7-hydroxyl reacted only after an almost complete titration of 2′ hydroxyl using a larger amount of squaleneCOOH and *N*-ethyl-*N*′-3-dimethylaminopropyl carbodiimide reagent (1–1.4 equiv.). The achieved products were clearly identified following the NMR spectra at 4.44 ppm (7 –CH–OH proton) and 4.78 ppm (2′ –CH–OH proton). Its relative lipophilicity factor (Rm) together with that of paclitaxel was evaluated. The experimental evaluation was compared with theoretical log *P* values. It was observed that squalene moiety strongly increased the lipophilicity of paclitaxel. Experimental and theoretical evaluations were in agreement (Table 1).

The interaction of the prodrug with biomembrane model was evaluated and compared with that of paclitaxel. With this aim, MLV were prepared empty and loaded with the drug or the prodrug and submitted to DSC analysis. The interaction of the compounds with MLV was evaluated comparing the calorimetric curves of the MLV with compound with that of MLV without compound (Fig. 1A and B). In fact, any compound interacting with MLV phospholipids produces a variation of the calorimetric curve of MLV; usually the variation is dependent on the amount of compound interacting with MLV. The calorimetric heating thermogram of MLV made of DMPC alone exhibits two thermal events, a lower-temperature, less energetic endotherm centered at about 17 °C, corresponding to the well characterized pretransition, and a higher-temperature, more energetic endotherm centered at about 24.8 °C, which correspond to the main or chain-melting phase transition of DMPC (Lewis et al., 1987). The incorporation of paclitaxel in the MLV

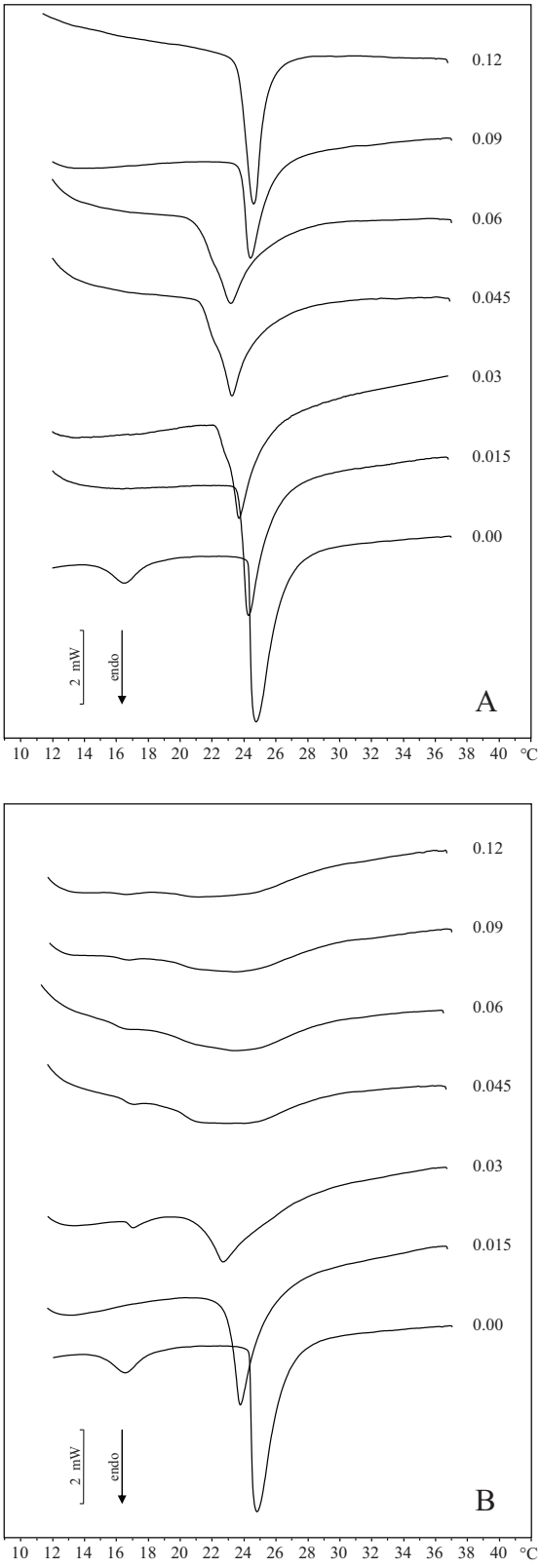


Fig. 1. Calorimetric curves, in heating mode, of MLV prepared without and with increasing molar fractions of (A) paclitaxel and (B) squalenoyl–paclitaxel.

Table 1
Theoretical and experimental characteristics of squalenoyl–paclitaxel derivative.

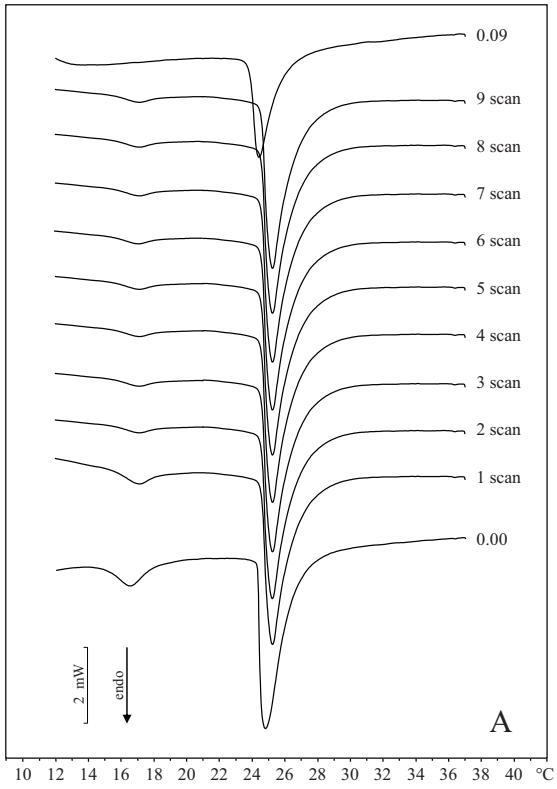
Compound	R_{m0}[a]	ALOGPS[b]	Theoretical distance Paclitaxel and squaleneCOOH (Å)[c]
Paclitaxel	8.76	3.20	
Squalenoyl–paclitaxel	14.35	7.30	2.35

[a] Lipophilicity values for paclitaxel and squalenoyl–paclitaxel derivatives was determined by reversed-phase TLC (R_{m0}).
[b] Theoretical analysis was performed with ALOGPS 2.1 (log *P*).
[c] MMFF94s software (mean distance).

produces some variation in the MLV thermogram (Fig. 1A). The pretransition is abolished; the main phase transition peak is gradually shifted toward lower temperature and broadened for molar fraction of paclitaxel up to 0.06 but turns to higher temperature and sharp for molar fraction of paclitaxel >0.06. Moreover, when the molar fraction of paclitaxel is >0.015 and <0.09, the drug produces a two-component main phase transition, with a lower temperature endotherm superimposed over a higher temperature endotherm. This characteristic of the calorimetric thermogram suggests the possible presence of distinct lipid-drug domains. The appearance of the second component in the thermogram is a consequence of a non-ideal mixing behavior, which creates a not homogeneous distribution of the drug in the bilayers. The low temperature component is attributed to regions of lipids that are rich in drug and are highly perturbed whereas the high temperature component is attributed to phospholipids domains that, being poor in drug, are less perturbed (Lohner and Prenner, 1999; Lambros and Rahman, 2004). During the phase transition the lipids do not melt independently of each other but rather as a cooperative domain. The width at half height of the main phase transition peak is a calorimetric parameter whose value is inversely proportional to the size of this cooperative unit (Marsh et al., 1977; Kanehisa and Tsong, 1978). Broadening of the main transition peak by the presence of paclitaxel <0.06 is indicative of a destabilization of the phospholipid assemblies, suggesting an intercalation of this drug within the lipid bilayer. This fact could be related to a reduction of the average number of lipid molecules, which undergo the transition as a cooperative unit. When the paclitaxel molar fraction is higher than 0.06, it does not interact with MLV probably because of the inability of DMPC to "dissolve" paclitaxel at high concentration. These results are quite in agreement with those found by Ali and coworkers (Ali et al., 2000) which studied the interaction of paclitaxel with DMPC and by Zhao et al. (2004) which studied the interaction of paclitaxel with MLV of DPPC and correlated the effect of paclitaxel on the thermotropic behavior of the phospholipid to its localization in the outer hydrophobic cooperative zone of the bilayer (the region of the C1–C8 carbon atoms of the acyl chain). In such a way, the C13 side chain of the paclitaxel is relatively hydrophobic because of the two aromatic rings and spans toward the hydrophobic tails, whereas the main taxane ring bearing substituents that have comparatively greater propensity for polar interactions, localizes next to the polar head of the phospholipids. As shown in Fig. 1B, squalenoyl–paclitaxel causes the reduction of the pretransition peak, the shift of the main transition peak toward lower temperature and its contemporary strong broadening. In addition, at molar fraction >0.03, a two-component main phase transition is evident which indicates a not homogeneous distribution of the prodrug in the bilayers and, hence the presence in the bilayer of regions of phospholipids that are rich in prodrug and perturbed and of regions of phospholipids poor in prodrug and less perturbed (Lohner and Prenner, 1999; Lambros and Rahman, 2004). The stronger interaction of squalenoyl–paclitaxel with biomembrane with respect to paclitaxel could be due to its increased lipophilicity that in turn increases the affinity for the phospholipid bilayers.

As described above, we put paclitaxel or squalenoyl–paclitaxel (molar fraction = 0.09) in contact with MLV and submitted the samples to subsequent calorimetric scans separated by isothermal (37 °C) periods of 60 min. This experiment was carried out to evaluate the capability of the drug and prodrug to migrate through the aqueous medium and subsequently be absorbed by MLV. If this occurred the calorimetric behavior of MLV should change due to the presence of the compounds within the bilayers. The calorimetric thermograms shown in Fig. 2 are compared with the calorimetric thermogram of MLV prepared without compound and with that of MLV prepared with compound at 0.09 molar fraction. The latter thermogram is used as reference as it should be obtained if the compound was absorbed by MLV. There is not evidence of variation in MLV behavior neither when paclitaxel nor when squalenoyl–paclitaxel are used which indicate the inability of the two compounds to dissolve in the aqueous medium and be absorbed by MLV, as expected given the hydrophobic nature and the water insolubility of the compounds.

Liposomes have been widely investigated for their properties as potential drug delivery systems (Gregoriadis, 1988). They have become a valuable experimental and commercially important drug delivery system, due to their biodegradability, biocompatibility and ability to entrap lipophilic and hydrophilic drugs (Torchilin, 2005). In this research MLV were used as biomembrane model as well as drug carrier; in particular, we evaluated the capability of MLV to retain the incorporated prodrug and, then, their possible use as prodrug carrier. With this aim we put prodrug loaded MLV (prodrug carrier) in contact with unloaded MLV (biomembrane model) and submitted the sample to calorimetric scans at intervals of 60 min during which the temperature was kept at 37 °C. For comparison reasons the experiment with paclitaxel was carried out too. The loaded MLV were prepared with 0.06 molar fraction of compound. This molar fraction was chosen as it exerted the highest effect on MLV, with concern to paclitaxel (see Fig. 1A). The calorimetric thermograms are compared with those of unloaded and loaded MLV which were put in contact and with that of MLV prepared with 0.03 molar fraction of drug or prodrug (reference curve) (Fig. 3A and B). If the carrier was able to hold the compound incorporated, the calorimetric thermograms should remain unchanged; if, instead, the carrier lost the compound, we should observe some variation in the calorimetric thermograms which should look like the reference thermogram. What we see in Fig. 3A, relative to paclitaxel, is only the disappearance of the pretransition peak while the main transition peak remains unchanged. With regard to squalenoyl–paclitaxel (Fig. 3B), the calorimetric thermograms show three components: the first (at about 17 °C) is attributable to the pretransition; the second (a large shoulder from about 21 to 24 °C) attributable to loaded MLV and the third (at about 24.8 °C) relative to unloaded MLV. They remain almost unchanged for all the incubation times meaning that the lipophilic carrier holds the incorporated prodrug. This means that the MLV may be used as a carrier while maintaining the drug loaded up to inside the cell.



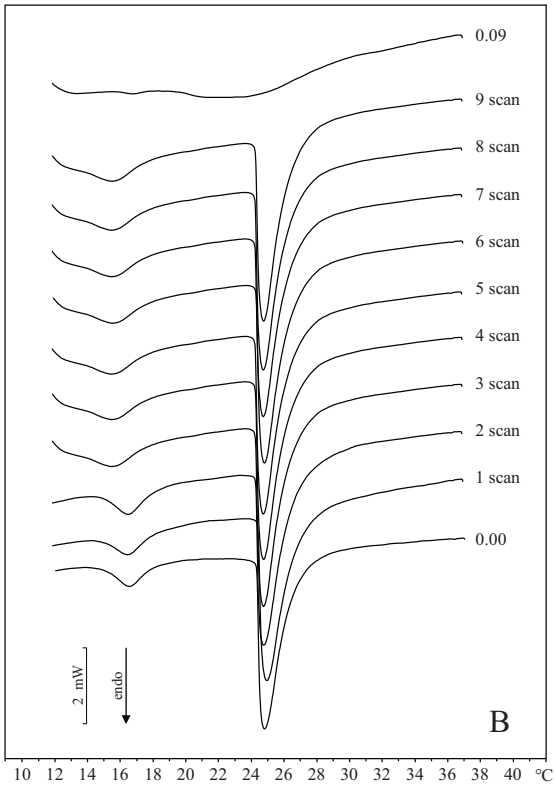
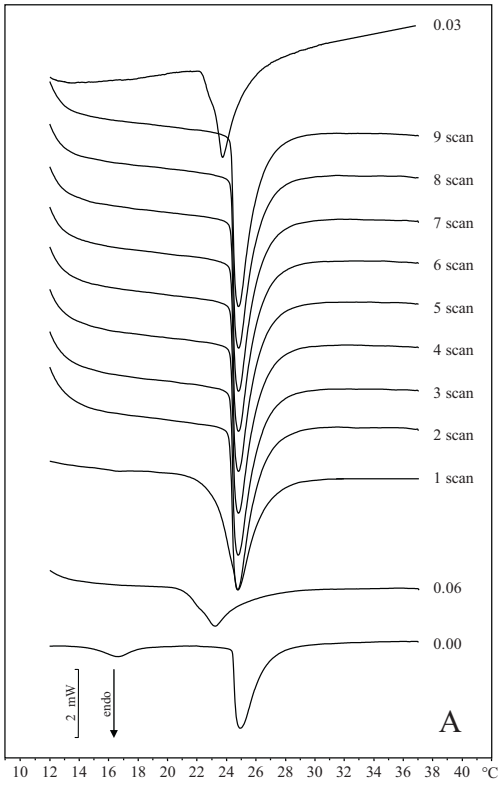


Fig. 2. Calorimetric curves, in heating mode, of MLV left in contact with (A) paclitaxel and (B) squalenoyl–paclitaxel at 0.09 molar fraction. Curve 0.00 belongs to MLV prepared without compound. Curve 0.09 belongs to MLV prepared with 0.09 molar fraction of compound.



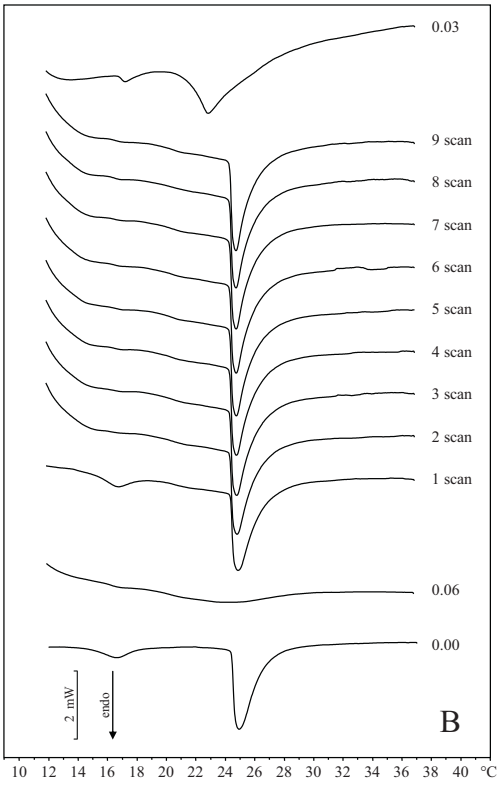


Fig. 3. Calorimetric curves, in heating mode, of MLV left in contact with (A) paclitaxel loaded MLV and (B) squalenoyl–paclitaxel loaded MLV. Loaded MLV were prepared with 0.06 molar fraction of compound. Curves are compared with curves of sample that were put in contact (curves 0.00 and 0.06) and with curve of MLV prepared with 0.03 molar fraction of compound (curve 0.03).

4. Conclusion

Starting from the evidence that only a small amount of paclitaxel can be incorporated into liposomes, we conjugated the drug to 1,1′,2-*tris*norsqualenoic acid with the aim to obtain a molecule with a stronger affinity with the phospholipid bilayers and that can, consequently, be incorporated and retained in the lipid system. The results obtained clearly indicate an improved incorporation efficiency of squalenoyl–paclitaxel with respect to paclitaxel into the liposome, probably due to its stronger lipophilic character. In addition, liposome can retain the incorporated squalenoyl–paclitaxel and hence a lipid system could be considered as a possible carrier for the prodrug.

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