Available online at www.sciencedirect.com
SCIENCE DIRECT®
Thermochimica Acta 426 (2005) 163–171
thermochimica acta
www.elsevier.com/locate/tca

Lipophilic conjugates of methotrexate with glucosyl-lipoamino acids: calorimetric study of the interaction with a biomembrane model

Rosario Pignatello*, Loredana Di Guardo, Antonina Puleo, Giovanni Puglisi

Dipartimento di Scienze Farmaceutiche, Università di Catania, Città Universitaria, Viale A. Doria 6, I-95125 Catania, Italy

Received 31 March 2004; received in revised form 2 July 2004; accepted 29 July 2004
Available online 8 September 2004

Abstract

The thermotropic effects of two series of lipophilic conjugates of the anticancer drug methotrexate with glucosyl- or pentaacetyl-glucosyl-lipoamino acid residues (GL-MTX) on a biological membrane model made up of dimyristoylphosphatidylcholine (DMPC) were investigated. Changes in the alkyl chain length of the lipoamino acid residues and in the free or acetylated state of the sugar moiety modulated the overall lipophilic/hydrophilic balance of the compounds and their solubility in aqueous media, thus also affecting their biological activity profile.

The calorimetric (DSC) analysis of their interaction with DMPC multilamellar vesicles showed that when GL-MTX conjugates were mixed with DMPC during vesicle preparation, they were able to affect the packing order of the phospholipid bilayers in a concentration-dependent way.

On the contrary, by incubating the conjugates with pre-formed empty DMPC liposomes at 37 °C, these derivatives were unable to penetrate the bilayers, most probably because of the presence of the two sugar residues. This behavior was correlated with the low biological activity observed in vitro for these conjugates, with respect to a corresponding series of MTX-LAA conjugates.

Keywords: Methotrexate; Lipoamino acids; Lipophilicity; DMPC; Differential scanning calorimetry; Biomembrane model; Membrane interaction

1. Introduction

In a series of studies concerning the production of lipophilic derivatives and prodrugs of the anticancer drug methotrexate (4-amino-10-methylfolic acid, MTX), we recently described some mono- and α,γ-bis(conjugates) of the drug with lipoamino acid residues with varying alkyl chain length and lipophilicity [1,2].

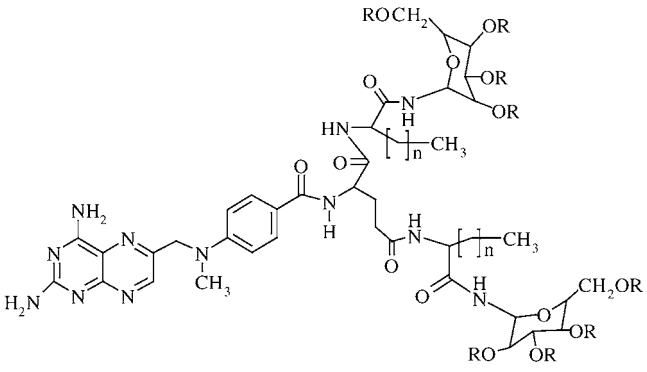
With such drugs, structurally related to the "non-classical" antifolates, the possibility arises of increasing the passive (positive) entrance of the drug into cells, over-coming one of the forms of resistance commonly shown by many tumor cell lines toward MTX in humans. Such a 'transport resistance' is due to a defective efficiency of the low-affinity, high-capacity active carrier (RFC) used by the cells for the uptake of physiologically circulating reduced folate cofactors and antifolate drugs [3].

These amide- and ester MTX-LAA conjugates were tested against two lines of human lymphoblastoid cells, a wild type (CCRF-CEM) and a subline (CEM/MTX) resistant to MTX because of a defective active transport of the drug inside cells [4]. Many conjugates confirmed the validity of the initial hypothesis: in fact, even if they were often less active with respect to the parent drug against the wild cancer cells, they did, however, keep the same order of activity against the transport-resistant cell subline, against which MTX was 200-fold less active. Therefore, a physico-chemical optimization of the molecular structure of these conjugates would probably increase their therapeutic potential, considering that most of them were still active, and in some cases even more active than MTX, against a main target enzyme of this drug, i.e., dihydrofolate reductase (DHFR) [1,2].

In fact, the linkage of LAA residues in the glutamate moiety of the MTX molecule, as well as increasing the

* Corresponding author. Tel.: +39 0957384021; fax: +39 095222239.
E-mail address: r.pignatello@unict.it (R. Pignatello).

0040-6031/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.tca.2004.07.017



1: R = $OCOCH_3$
2: R = H
a: n=7; **b**: n= 11; **c**: n=15

Fig. 1. General structure of GL-MTX conjugates **1** and **2**.

lipophilicity and the 'membrane-like character' of the resulting conjugates [5], at the same time it strongly affected their solubility in biological and experimental media, with the possibility of masking the real activity of the tested compounds.

In the light of this finding, we started two parallel studies, starting from the MTX-LAA conjugates already described in [1]: on the one hand, we evaluated the efficacy of shortening the length of the alkyl side chain in the LAA residue [6]. On the other hand, we tried to modulate the lipophilicity of the above conjugates by linking a glucose residue to the LAA moiety. Such a strategy can, in fact, significantly improve the bioavailability and activity of drugs [7,8]. Thus, a series of α,γ-bis(conjugates) of MTX with glucosyl-LAA residue (GL-MTX, compounds **1a**–**c** and **2a**–**c**, Fig. 1) was prepared and characterized, and their in vitro inhibitory activity was assessed against bovine liver DHFR, and CCRF-CEM and CEM/MTX cell line cultures [9].

The biological evaluation of these compounds showed a reduced activity compared to the former MTX-LAA conjugates, in particular, when the glucosyl residue was present in the peri-acetylated form (compounds **1a**–**c**). To gain a better insight into these findings, in the present work we tested the GL-MTX conjugates for their ability to interact with a biomembrane model, consisting of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) multilamellar liposomes (MLV). Together with the related simpler model represented by unilamellar liposomes, these aggregates of neutral (zwitterionic), amphipatic phospholipids gave useful information about the ability of a drug molecule to interact with and penetrate through phospholipid bilayers resembling a cell membrane, often giving a good correlation with the in vitro or in vivo biological data of such drugs [10–14].

The differential scanning calorimetry (DSC) technique was used to determine the thermotropic behavior of DMPC liposomes in the presence of various molar fractions of MTX and of conjugates of both series; moreover, DSC kinetic experiments were carried out to mimic the phenomena occurring in vivo during the interaction of an extraneous molecule with cells.

2. Materials and methods

2.1. Chemicals

DMPC was purchased from Genzyme Pharmaceuticals (Postfach, Switzerland). Reactants and solvents were of analytical grade or higher. The GL-MTX conjugates were prepared as described elsewhere [9].

2.2. Preparation of the liposomal suspensions

MLV were obtained by the classical thin-layer evaporation technique. DMPC or drug–DMPC mixtures at the different molar fractions (X_0 = 0.01–0.09) were dissolved in 1 ml of a $CHCl_3$/MeOH mixture, 9:1, v/v, to obtain a final lipid concentration of 5 mg ml^{-1}. The solvent was evaporated off under a nitrogen flow to obtain dry thin lipid films along the vial

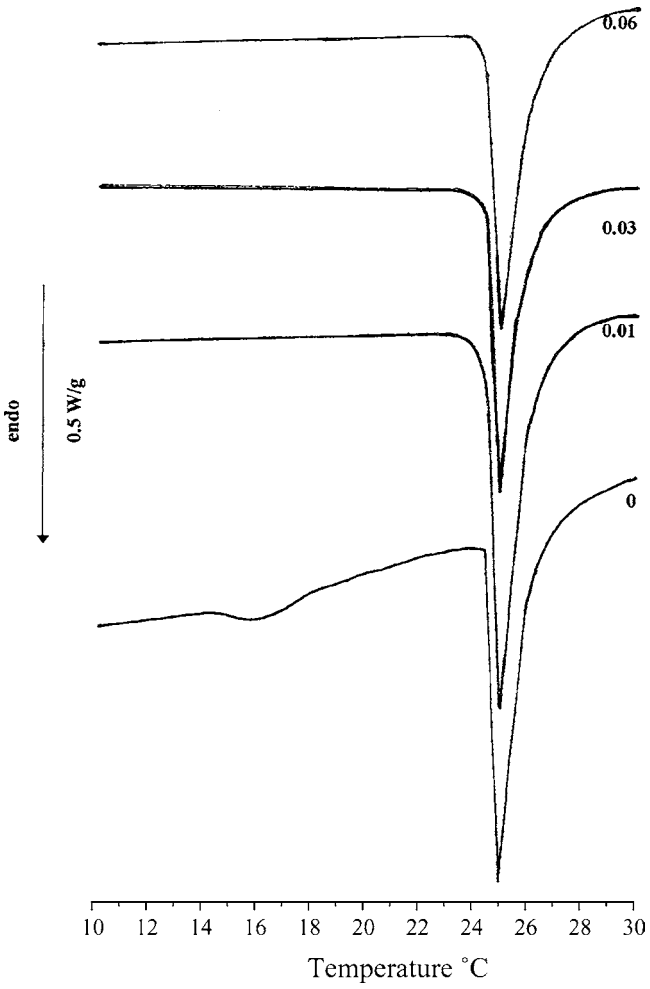


Fig. 2. DSC curves of DMPC liposomes in the presence of increasing molar fractions of conjugate **1a**.

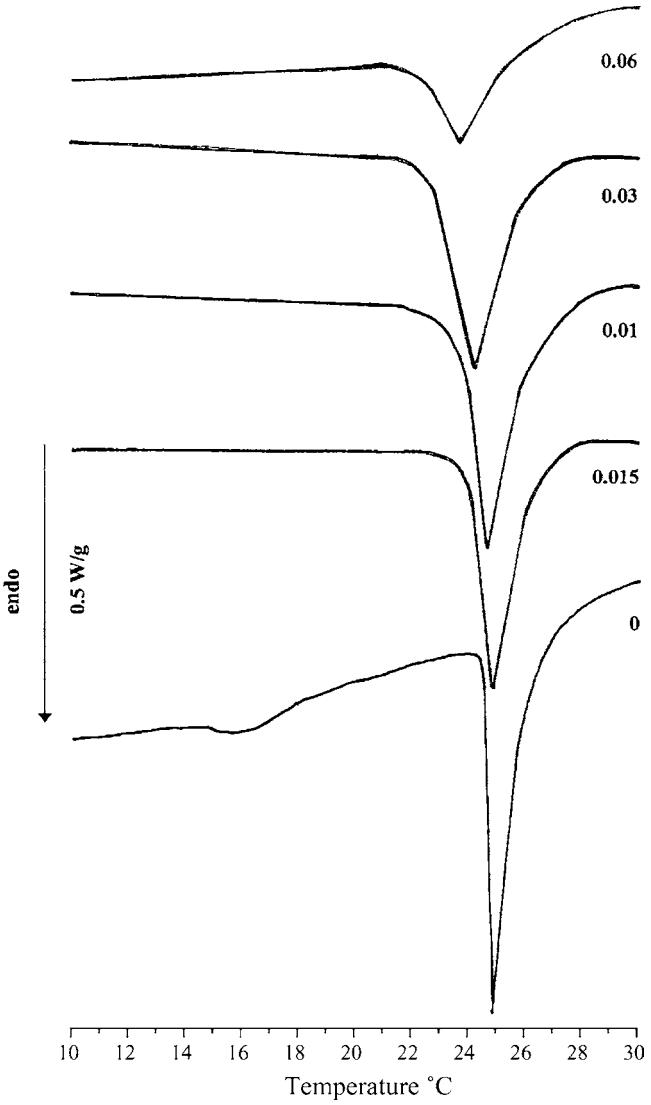


Fig. 3. DSC curves of DMPC liposomes in the presence of increasing molar fractions of conjugate **1b**.

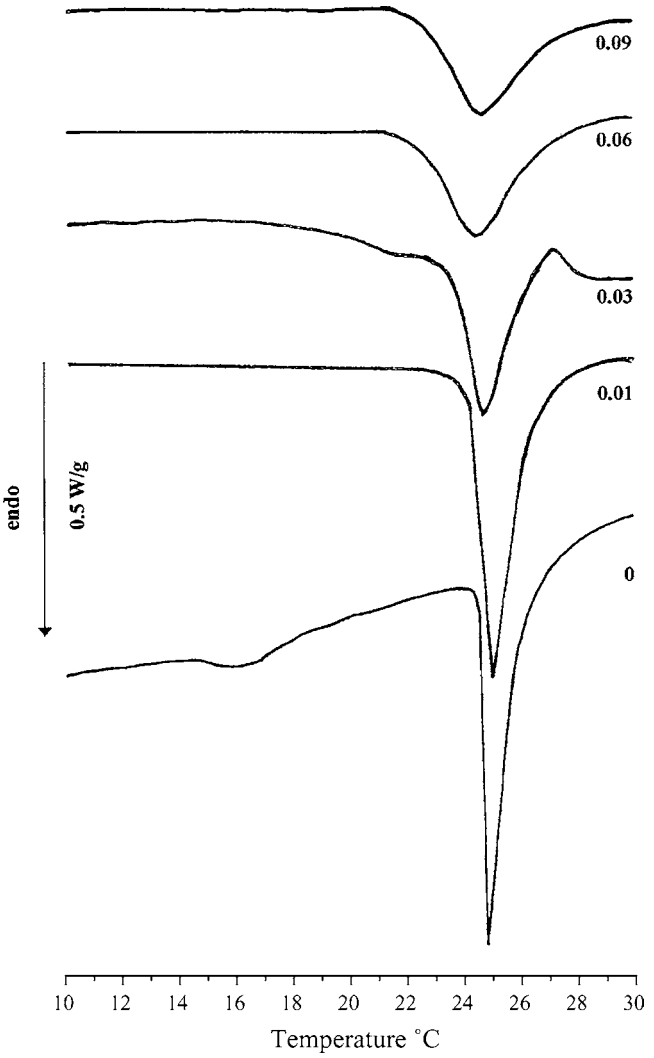


Fig. 4. DSC curves of DMPC liposomes in the presence of increasing molar fractions of conjugate **1c**.

walls. The samples were kept at 30 °C under vacuum (Büchi T-50 oven) for 6–8 h to eliminate traces of the solvent. The hydration of phospholipid films was made by adding an isotonic phosphate buffer solution (PBS, pH 7.4) and warming at 40–45 °C for 3 min, followed by a vortex mixing for a further 3 min. The entire cycle was repeated three times, and the suspensions were then left for 2 h at room temperature to allow annealing of the vesicles and reaching the equilibrium between the aqueous and lipid phases.

2.3. Calorimetric experiments

Calorimetric experiments were performed with a Mettler DSC 12E calorimeter, connected to a Haake D8-G thermocryostat. The detector consisted of a Mettler Pt100 sensor, with a thermometric sensitivity of 56 μV/°C with a calorimetric sensitivity of about 3 μV/mW, and a background noise level of less than 60 nV (<20 μV). Every DSC scan showed an accuracy of ±0.4 °C, with a reproducibility and resolution of 0.1 °C.

For the DSC runs, each sample was sealed in a 40 μl aluminum pan, while a pan containing 40 μl of PBS, pH 7.4, was used as the reference. Each sample was submitted to three cycles of analysis (in heating and cooling mode), at a scan rate of 2 °C min^{-1} in the 5–30 °C range.

2.4. DSC kinetic experiments

To evaluate the interaction of the drug with DMPC bilayers as a function of time, a weighed amount of each conjugate was placed at the bottom of a 40 μl aluminum pan and covered with the required amount of a DMPC liposome suspension, to obtain 0.06 drug molar fraction. Once sealed, the pan was submitted to repeated heating and cooling cycles in the calorimeter oven (between 5 and 37 °C), at a scan rate of 2 °C min^{-1}, to allow the migration of the drug into the phospholipid bilayers.

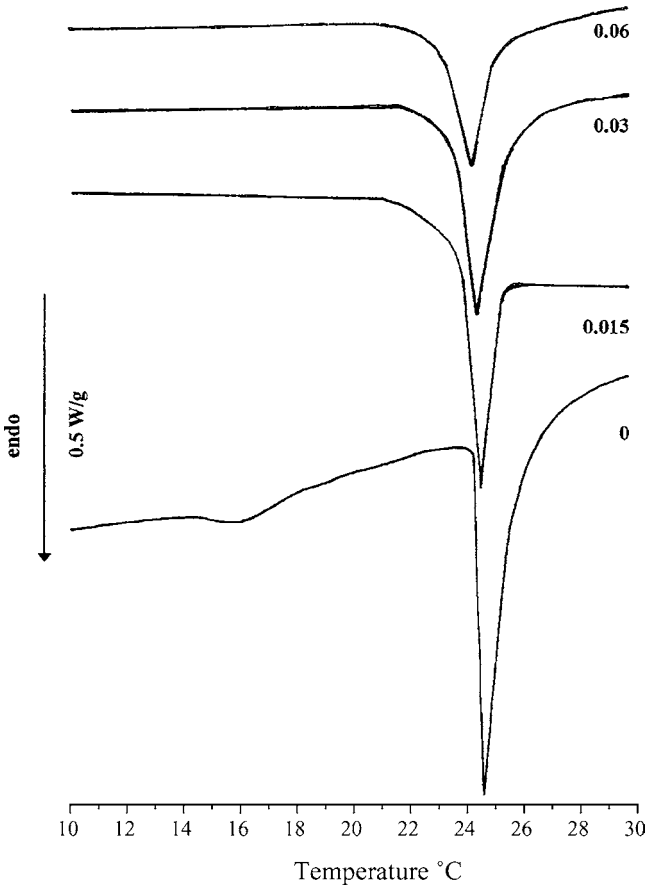


Fig. 5. DSC curves of DMPC liposomes in the presence of increasing molar fractions of conjugate **2a**.

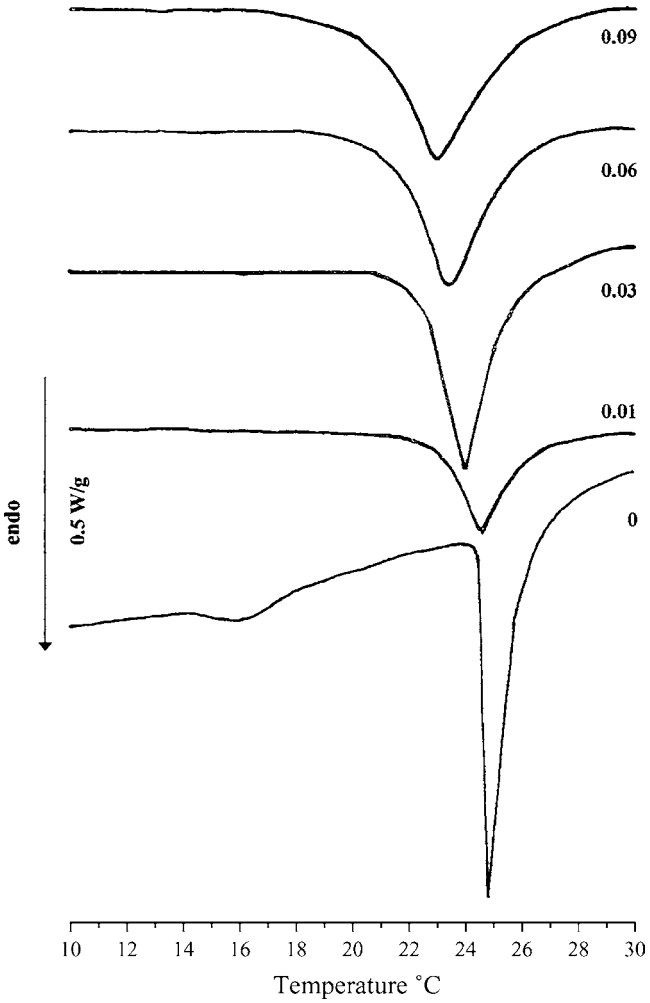


Fig. 6. DSC curves of DMPC liposomes in the presence of increasing molar fractions of conjugate **2b**.

2.5. DSC analysis

A Mettler TA89A system software (version 4.0) was used to evaluate the data from each DSC run, i.e., transition peak temperature (T_m), enthalpy changes as a function of heating (ΔH), and the full width at half height of endotherm peaks ($\Delta T_{1/2}$). Further mathematical analysis was carried out on a PC using the Origin 7 SR2 software (OriginLab Corporation, Northampton, MA, USA), by which multiple peak curve fitting, subtraction of *y*-offset, and correction for time-based drift were made.

3. Results and discussion

For the present study two series of MTX conjugates with LAA having different alkyl side chain lengths (decyl-, compound **a**; tetradecyl-, compound **b**; octadecyl-, compound **c**), linked to an glucosylamino residue in a peri-acetylated (compounds **1a**–**c**) or free form (compounds **2a**–**c**) were used (Fig. 1). The resulting structural changes gave drug conjugates with a variable lipophilicity and solubility, which can affect their ability and depth of interaction with, and penetration through, tumor cell membranes.

With the aim of gaining predictive information of such features, these conjugates were included in MLVs made of a pure phospholipid and the corresponding thermotropic behavior was investigated using DSC techniques. The effects of increasing molar fractions of conjugates **1a**–**c** and **2a**–**c** are shown in Figs. 2–7 and summarized in Table 1 and Fig. 8.

The analysis of calorimetric data suggested that, within each series of acetylated (**1**) or not acetylated compounds (**2**), increasing of the alkyl chain of the amino acid residue led to a deeper interaction with phospholipid bilayers, with a consequent shifting of the T_m to lower temperatures and, mainly, of the transition enthalpy changes associated to it (ΔH), with respect to plain DMPC liposome vesicles (cf. Figs. 2, 4 and 6 versus Figs. 3, 5 and 7).

Fig. 8 shows that each pair of conjugates sharing the same side alkyl chain (e.g., compounds **1a** and **2a**, or compounds **1b** and **2b**) exerted a similar thermotropic effect upon DMPC bilayers, more evident for compounds **2a**–**c** which contain a free glucose residue. This suggests that the length of the aliphatic chain of the LAA moiety plays a predominant role, more than the free or acetylated state of the

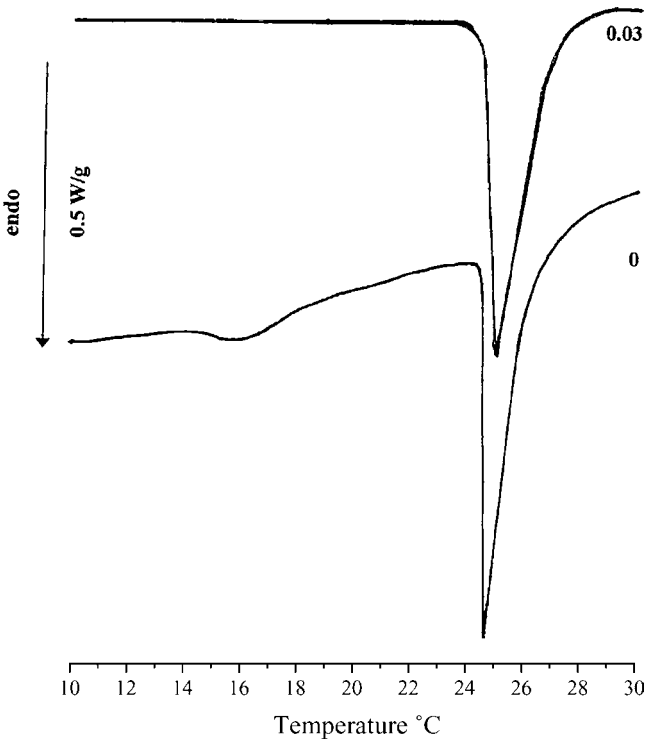


Fig. 7. DSC curves of DMPC liposomes in the presence of increasing molar fractions of conjugate **2c**.

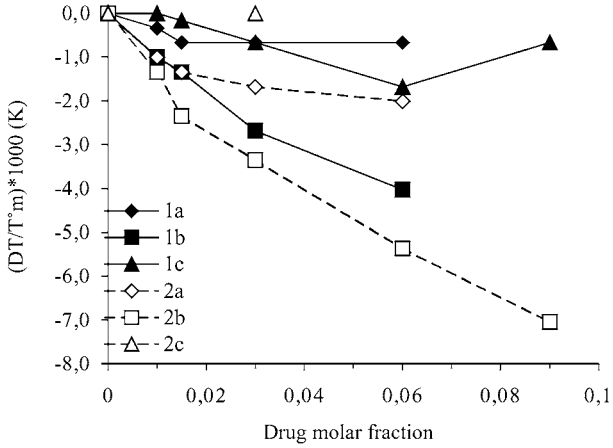


Fig. 8. Comparison of the thermotropic effect exerted on the T_m value of DMPC multilamellar liposomes by increasing molar fractions of GL-MTX conjugates.

glucose residue, on the interaction with the biomembrane model.

It is also noteworthy that the pair of conjugates with the same tetradecyl chain (**1b** and **2b**) that are able to interact deeper with vesicle bilayers (Fig. 8), are also the ones that showed the highest biological activity (Table 2) [9]. These data further confirm the good correlation existing between

Table 1
Experimental DSC thermotropic data of DMPC liposomes containing different molar fractions (X_{DRUG}) of conjugates **1a–c** and **2a–c**

X_{DRUG}	T_m (°C)	ΔT	$\Delta T/T^0$ ($\times 10^3$)	ΔH (J/g)	$T_{10\%} - T_{95\%}$ (°C)[a]
DMPC	24.9 (T^0)	–	–	34.0	24.4–25.7
Compound **1a**					
0.01	24.8	−0.1	−4.02	29.6	24.5–26.7
0.03	24.7	−0.2	−8.03	20.0	24.5–26.7
0.06	24.7	−0.2	−8.03	19.5	24.5–27.0
Compound **1b**					
0.01	24.6	−0.3	−12.04	26.7	24.0–27.4
0.015	24.5	−0.4	−16.06	27.4	23.7–27.3
0.03	24.1	−0.8	−32.12	24.5	23.2–26.5
0.06	23.7	−1.2	−48.19	14.0	22.6–26.0
Compound **1c**					
0.01	24.9	0	0	26.6	24.4–27.0
0.03	24.7	−0.2	−8.03	23.0	23.6–27.3
0.06	24.4	−0.5	−20.08	20.0	22.8–27.3
0.09	24.7	−0.2	−8.03	18.0	22.9–27.2
Compound **2a**					
0.015	24.5	−0.4	−16.06	21.1	24.1–25.2
0.03	24.4	−0.5	−20.08	28.5	23.8–25.8
0.06	24.3	−0.6	−24.09	26.4	23.1–26.3
Compound **2b**					
0.01	24.5	−0.4	−16.06	21.0	24.1–25.1
0.03	23.9	−1	−40.16	26.2	22.6–26.8
0.06	23.3	−1.6	−64.25	25.7	21.5–26.2
0.09	22.8	−2.1	−84.33	25.6	20.9–26.3
Compound **2c**					
0.03	24.9	0	0	24.7	24.6–26.8

[a] Transition temperature interval.

DSC findings, relative to the interaction of drugs with a phospholipid biomembrane model, and their biological activity observed at least in vitro.

The conjugates with the longest aliphatic chain (octadecyl derivatives **1c** and **2c**) displayed both a low inhibitory activity against tumor cell growth (Table 2) and a minimal ability to interact with the liposome bilayers (Fig. 8), very probably due to their poor solubility in the experimental aqueous media used.

By comparing each pair of conjugates, with an acetylated or free glucose residue, containing the same LAA moiety (compounds of **a**, **b**, or **c** series), it was seen that the conjugates with a free glucose group exerted a more marked effect

Table 2
In vitro growth inhibitory activity (EC_{50}, μM) of conjugates **1** and **2** against sensitive and resistant CCRF-CEM cells (incubation time: 72 h)

Compound	CCRF-CEM	CEM/MTX
1a	34.5 ± 2.2	n.t.[a]
1b	23.9 ± 4.3	24.5 ± 2.2
1c	>50	n.t.
2a	15.4 ± 0.4	23.5 ± 3.9
2b	10.9 ± 0.7	8.3 ± 0.2
2c	>50	n.t.
MTX	0.09 ± 0.8	10.1 ± 0.3

[a] Not tested.

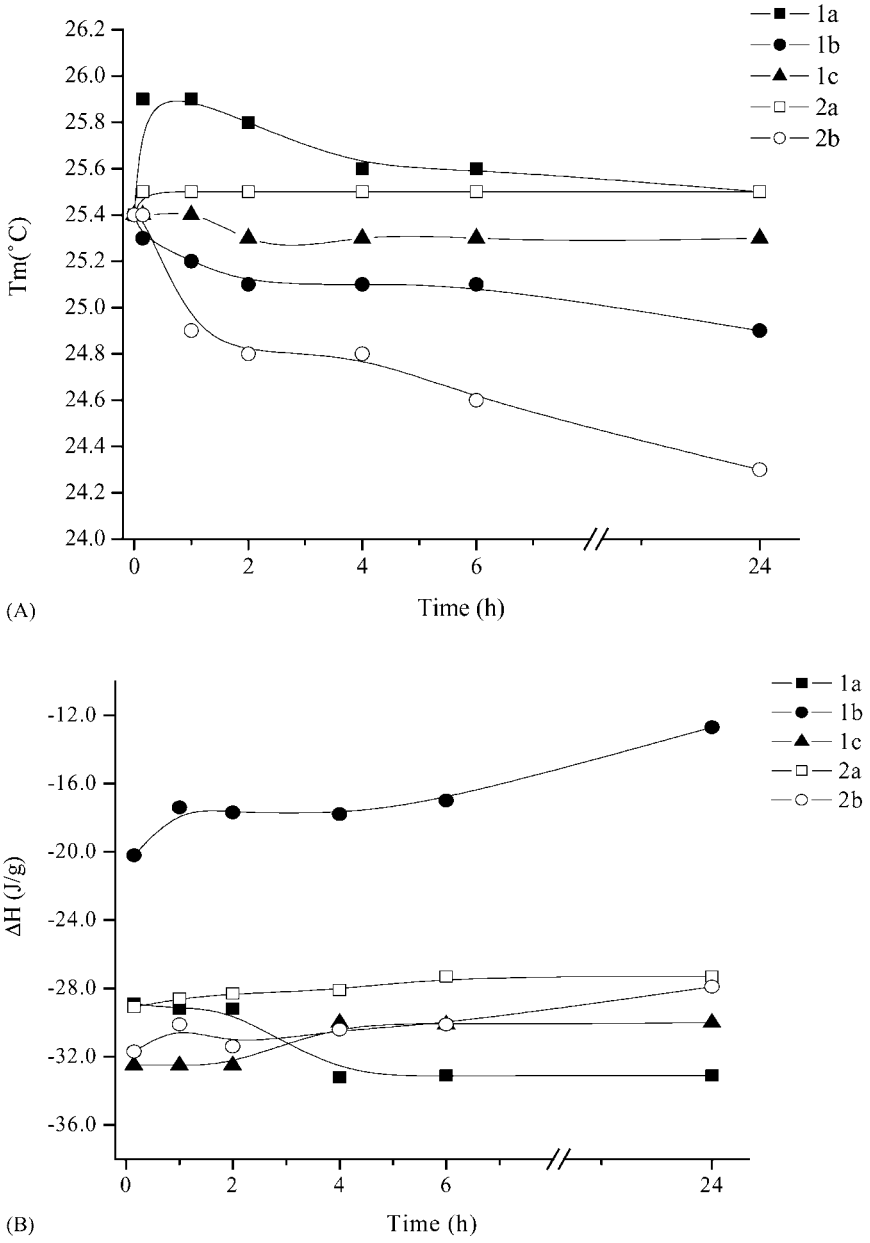


Fig. 9. Differential scanning calorimetry effects (T_m changes, panel A, and enthalpy variations, panel B) observed upon incubation of conjugates **1** and **2** with empty DMPC liposomes.

upon the transition curves of the liposome bilayers (cf., for instance, Figs. 1 and 2). This could be explained by the presence of the free glucose hydroxyl groups, which allow the formation of hydrogen bonds with the phospholipid polar heads, with a consequent change of their gel-to-liquid crystal transition energy. In the corresponding peri-acetylated conjugates **1a**–**c** such polar interaction was reduced or absent and the main effect was exerted on the DMPC acyl chains, mainly affecting the ΔH value of the phospholipid phase transition.

A series of kinetic calorimetric experiments were also performed to further analyze the interaction of these compounds with the biomembrane model. Basing on the preliminary DSC results, a 0.06 molar fraction of each conjugate was chosen for incubation with empty DMPC MLVs. Samples were submitted to heating and cooling cycles between 5 and 37 °C. From the DSC scans at predetermined time intervals it was then possible to gather the rate and entity of diffusion of each conjugate into liposomes. Fig. 9 resumes the effects on DMPC T_m and ΔH values registered as a function of time in the presence of the different conjugates. Moreover, for a better comparison between a pair of conjugates belonging to the acetylated or free glucose residue series, and containing the same 2-amino-tetradecanoic acid moiety, Figs. 10 and 11 report the DSC curves registered during the incubation for compounds **1b** and **2b**, respectively.

The analysis of Fig. 9 allows interesting comments. Among the series **1** containing peri-acetylated glucose residues, the lowest term **1a** increased the T_m only in the first phase of the incubation, thereafter assuming a location within the vesicle bilayers that did not disturb their package; similarly, the effects on ΔH changes were visible only in the first 2 h of incubation (Fig. 9B). The intermediate term **1b**, which contains tetradecyl moieties, progressively depressed the DMPC T_m value the along incubation, in the meantime giving the strongest reduction of the associated enthalpy changes since the beginning of the incubation. The more lipophilic conjugate **1c** had only limited effects on both DMPC T_m and ΔH, visible only after a longer time of incubation with the liposomes (2–4 h). Furthermore, with the lower conjugates of the series (**1a** and **1b**) the phospholipid pretransition endothermic peak was still partially visible around 15–16 °C (see Fig. 10 for compound **1b**), indicating a weak interaction of these drugs with the vesicle bilayers.

Such parabolic profile, i.e., the conjugates with the shortest or longest alkyl chain being less able to interact with the biomembrane model, had been already described for the corresponding series of MTX-LAA conjugates and was associated with a similar profile of in vitro inhibitory activity against tumor cell growth [12].

As concerns the conjugates of the glucosyl-LAA series, the lower homologue **2a** only showed an initial weak interaction with liposomes (after about 1 h of incubation), followed by a substantial stability of the system. This was confirmed by the almost unchanged T_m and ΔH values registered in the presence of such compound (Fig. 9). The conjugate **2b**, conversely, exerted a progressive, strong depression of T_m

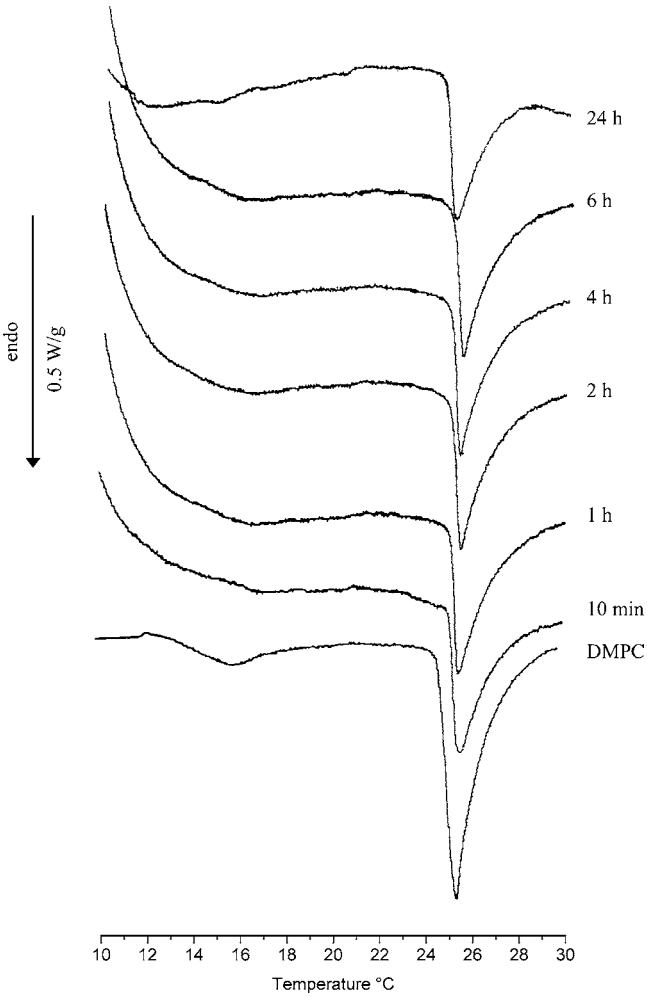


Fig. 10. Transfer kinetics of conjugate **1b** (molar fraction: 0.06) to void DMPC MLVs.

values (Fig. 9A) and caused a rapid phase segregation within the phospholipid bilayers, giving a broad endothermic signal deriving from the overlapping of several thermotropic components (Fig. 11). This effect was clearly due to the ability of this conjugate, in contrast with the acetylated compounds, to penetrate the DMPC bilayers, at least the external ones, perturbing their packing order and causing a change in the thermal transition profile. Because of the presence of the two sugar residues and/or to steric hindrance, such an interaction did not continued in the inner bilayers of the multilamellar liposomes and the formation of a homogeneous drug/DMPC system was not reached, even after 24 h of incubation.

The presence of the polar glucose residues in conjugates **2** allowed them to interact at more levels with DMPC bilayers than the acetylated counterparts: as Fig. 9A shows, in fact, the former conjugates (open symbols) always showed a greater depression of T_m values than the corresponding acetyl-glucosyl conjugates.

A further consideration about the different affinity of the two series of derivatives for the DMPC bilayers was linked

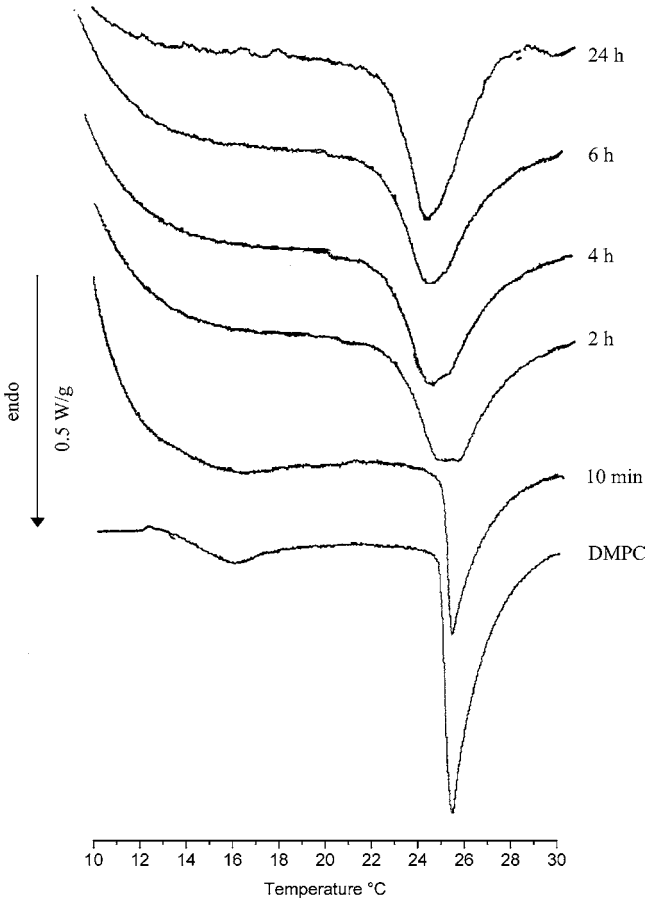


Fig. 11. Transfer kinetics of conjugate **2b** (molar fraction: 0.06) to void DMPC MLVs.

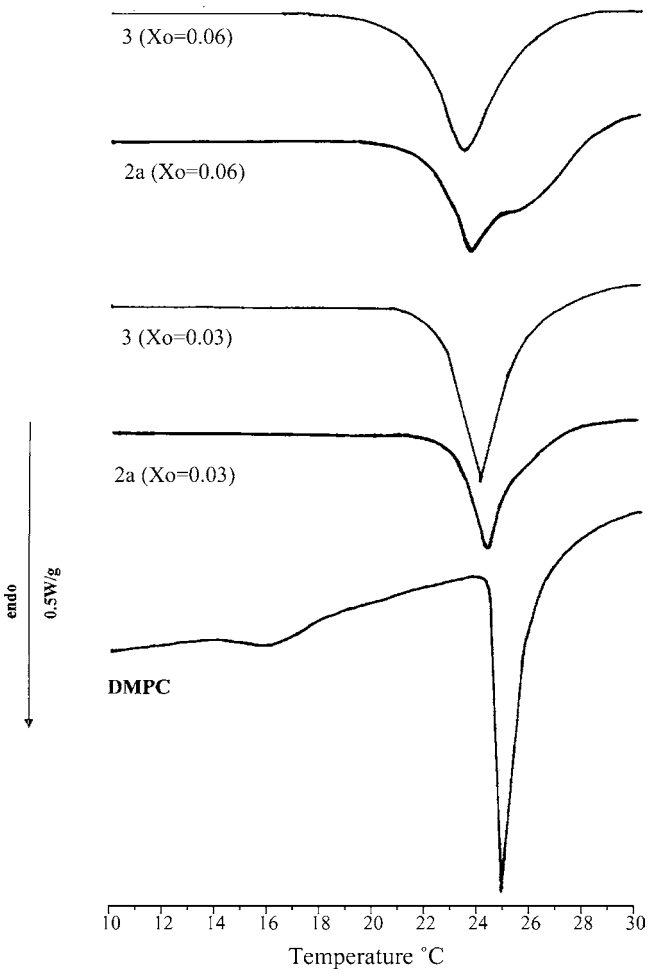


Fig. 12. Comparative DSC runs of different molar fractions of conjugate **1b** and the corresponding MTX-α,γ-bis(tetradecyl)-LAA conjugate described in [1].

to the analysis of ΔH changes measured at the end of the incubation period (Fig. 9B) in respect to the ΔH values given by a 0.06 molar fraction of each compound, when dispersed with DMPC during the formation of liposomes (Figs. 2–7). For instance, after 24 h of incubation with the acetyl-glucosyl derivative **1a**, DMPC vesicles gave a ΔH value of −33.3 J/g, whereas the same compound gave an enthalpy change of −19.5 J/g when mixed with the phospholipid. On the contrary, compounds **2a** and **2b** gave ΔH values of −26.4 and −25.6, and −27.3 and −27.9 J/g, respectively, after 24 h of incubation with empty liposomes or after their dispersion in the DMPC film. Thus, only the conjugates containing free glucose residues are able to penetrate slowly the phospholipid acyl chains, reaching a somewhat complete interaction with the bilayers.

Finally, we compared the thermotropic behavior of two conjugates containing the same tetradecyl LAA residues but with (compound **2a**) or without the glucose moiety linked (compound **3** in Ref. [1]) (Fig. 12). The latter, more lipophilic compound caused more marked effects on the DSC profile of DMPC vesicles, at both the tested molar fractions (0.03 and 0.06), with a clear tendency to cause a phase segregation within the phospholipid bilayers and to split the phase transition peak into two or more components. Such behavior suggests a reduced solubility of this conjugate within DMPC bilayers; its higher lipophilicity and/or the lack of contribution of the glucose residues to electrostatic interactions with DMPC head groups, observed for the glucosyl-LAA derivative, can be addressed to explain the different behavior of the two related conjugates.

4. Conclusions

The thermotropic effects of two series of lipophilic α,γ-bis(conjugates) of the anticancer drug methotrexate with glucosyl-lipoamino acids were investigated. Changes in the alkyl length of the lipoamino acid moieties and in the free or acetylated state of the glucose portion allow to modulate the overall lipophilic/hydrophilic balance of these conjugates, as well as their solubility in aqueous media, thus conditioning their biological activity profile.

In particular, the calorimetric analysis of their interaction with a biomembrane model, consisting of DMPC multilamellar vesicles, could help to understand why these conjugates showed a reduced inhibitory activity against tumor cell cultures in vitro, as compared to the corresponding MTX-LAA conjugates previously described [1].

In fact, when the GL-MTX conjugates were encapsulated in the liposomes during the preparation of the latter (i.e., in the organic phase), we observed that they were able to affect even at low molar fractions the packing order of DMPC bilayers in a concentration-dependent way. Conversely, by incubating the same conjugates at 37 °C with preformed empty vesicles, they failed in penetrating the phospholipid bilayers, probably because of the presence of the two sugar residues.

Translated into a biological aspect, this behavior would suggest that these compounds are likely not able to penetrate into living cell membranes and inhibit their intracellular target enzyme DHFR, although we had observed, in an in vitro cell-free assay, a relevant inhibitory activity against this enzyme [9].

In conclusion, the further modification of the structure of the previously described lipophilic MTX-LAA conjugates [1] did not improve their anticancer activity profile, at least in vitro. Of course, in vivo studies would more information about their potential activity, since their more suitable hydrolipophilic balance could favor their absorption and circulation in the blood, as well as their bioconversion into the active parent drug.

References

[1] R. Pignatello, G. Jansen, I. Kathmann, G. Puglisi, I. Toth, J. Pharm. Sci. 87 (1998) 367–371.
[2] R. Pignatello, G. Spampinato, V. Sorrenti, L. Vicari, C. Di Giacomo, J.J. McGuire, C.A. Russell, G. Puglisi, I. Toth, Eur. J. Pharm. Sci. 10 (2000) 237–245.
[3] G.B. Henderson, in: R.L. Blakley, V.M. Whitehead (Eds.), Folates and Pterins, vol. 3, Wiley, New York, 1986, pp. 207–250.
[4] A. Rosowsky, H. Lazarus, G.C. Yuan, W.R. Beltz, L. Mangini, H.T. Abelson, E.J. Modest, E. Frei III, Biochem. Pharmacol. 29 (1980) 648–652.
[5] I. Toth, J. Drug Targeting 2 (1994) 217–239.
[6] R. Pignatello, S. Guccione, S. Forte, C. Di Giacomo, V. Sorrenti, L. Vicari, G. Uccello Barretta, F. Balzano, G. Puglisi, Bioorg. Med. Chem. 12 (2004) 2951–2964.
[7] A. Wong, I. Toth, Curr. Med. Chem. 8 (2001) 1123–1136.
[8] I. Toth, J.P. Malkinson, N.S. Flinn, B. Drouillat, A. Horvath, J. Erchegyi, M. Idei, A. Venetianer, P. Artursson, L. Lazorova, B. Szende, G. Keri, J. Med. Chem. 42 (1999) 4010–4013.
[9] R. Pignatello, L. Vicari, V. Sorrenti, C. Di Giacomo, G. Spampinato, G. Puglisi, I. Toth, Drug Develop. Res. 52 (2001) 454–464.
[10] F. Castelli, G. Giammona, G. Puglisi, B. Carlisi, S. Gurrieri, Int. J. Pharm. 59 (1990) 19–25.
[11] G. Puglisi, M. Fresta, R. Pignatello, Drug Develop. Res. 44 (1998) 162–169.
[12] E. Theodoropoulou, D. Marsh, Biochim. Biophys. Acta 1461 (1999) 135–146.
[13] M. Fresta, M. Ricci, C. Rossi, P.M. Furneri, G. Puglisi, J. Colloid Interf. Sci. 226 (2000) 222–230.
[14] R. Pignatello, I. Toth, G. Puglisi, Thermochim. Acta 380 (2001) 255–264.