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Thermochimica Acta 380 (2001) 255–264

thermochimica acta

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Structural effects of lipophilic methotrexate conjugates on model phospholipid biomembranes

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Abstract

Lipophilic conjugates of the antitumor drug methotrexate (MTX) with lipoamino acids (LAAs) have been previously described as a tool to enhance MTX passive entrance into cells, overcoming a form of transport resistance which makes tumour cells insensitive to the antimetabolite. A knowledge of the mechanisms of interaction of such lipophilic derivatives with cell membranes could be useful for planning further lipophilic MTX derivatives with an optimal antitumour activity. To this aim, a calorimetric study was undertaken using a biomembrane model made from synthetic 1,2-dipalmitoyl-glycero-3-phosphocholine (DPPC) multilamellar liposomes. The effects of MTX and conjugates on the phase transition of liposomes were investigated using differential scanning calorimetry.

The interaction of pure MTX with the liposomes was limited to the outer part of the phospholipid bilayers, due to the polar nature of the drug. Conversely, its lipophilic conjugates showed a hydrophobic kind of interaction, perturbing the packing order of DPPC bilayers. In particular, a reduction of the enthalpy of transition from the gel to the liquid crystal phase of DPPC membranes was observed. Such an effect was related to the structure and mole fraction of the conjugates in the liposomes.

The antitumour activity of MTX conjugates was evaluated against cultures of a CCRF–CEM human leukemic T-cell line and a related MTX resistant sub-line. The in vitro cell growth inhibitory activity was higher for bis(tetradecyl) conjugates than for both the other shorter- and longer-chain derivatives. The biological effectiveness of the various MTX derivatives correlated very well with the thermotropic effects observed on the phase transition of DPPC biomembranes.

Keywords: Methotrexate; Lipoamino acids; Differential scanning calorimetry; Membrane model; CCRF–CEM cells; CEM/MTX cells

1. Introduction

Dihydrofolate reductase (DHFR) inhibitors play an important role in the clinical treatment of many tumour forms. Methotrexate (MTX) is a DHFR inhibitor widely used in many human malignancies [1], as well as for the treatment of rheumatoid arthritis and psoriasis [2,3]. However, clinical MTX effectiveness is often hampered by the development of acquired forms of cell resistance [4]. In particular, in human and murine tumours an impaired cellular uptake of the drug occurs [5,6], as a consequence of a defective active carrier system (reduced folate carrier (RFC)) which regulates the cell entrance of folate analogues [7]. On the other hand, the passive diffusion of MTX through cell membranes is limited, due to its hydrophilic nature.

Modifications in the MTX molecule to increase its lipophilicity without affecting the binding to DHFR represents a valid route to overcome such transport resistance. For instance, functionalisation of the α- and

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0040-6031/01/$ – see front matter
PII: S0040-6031(01)00679-7

γ-carboxyl groups in the glutamate MTX residue allows for active lipophilic MTX derivatives and conjugates to be obtained [8].

We have previously described some MTX conjugates with aliphatic amides as well as lipoamino acids (LAAs) [9–12]. The latter are a family of lipid-modified amino acids whose linkage to drug molecules can confer to them a ''membrane-like'' character, allowing greater stability in serum and an easier uptake by target cells [13]. MTX conjugates were prepared using different LAA residues, in order to modulate the overall lipophilicity of the resulting compounds [10,12]. Some conjugates showed an inhibitory activity against in vitro growth of lymphoblastoid CCRF–CEM cells. Despite they are in general less potent than the parent drug, however, they showed to maintain the same level of activity also against an MTX resistant sub-line (CEM/MTX), which displays an unpaired cellular uptake for reduced folates and MTX, and against which MTX itself shows a 200-fold reduced potency. As previously considered [10,12], these compounds do not represent optimal terms from a therapeutic point of view, since they do not improve MTX activity against sensitive cells and display problems of water solubility that, for example, can compromise the formulation of injections. Nevertheless, the approach of increasing lipophilicity of MTX through an intervention at the level of its glutamate end confirmed the possibility of by-passing cell resistance. In further works in progress at present, we are trying to balance the excessive lipophilicity of the above MTX–LAA conjugates by linking a sugar moiety to the conjugates or by using shorter-chain LAA derivatives.

However, the comprehension of the mode of interaction of lipophilic MTX conjugates with cell membranes can help in planning drugs with the best physico-chemical properties and biological activity. To this aim, we undertook a calorimetric study by evaluating the effects of selected MTX–LAA conjugates (Fig. 1) on the thermotropic properties of a model membrane, consisting of dipalmitoylphosphatidylcholine (DPPC) multilamellar liposomes. Differential scanning calorimetry (DSC) was used as a tool to assay the behaviour of DPPC biomembranes as a function of the drug and of its mole fraction [14–16]. A correlation between these results with in vitro antitumour activity on lymphoblastoid human cell lines was also suggested.

2. Materials and methods

2.1. Chemicals

Synthesis of MTX conjugates with LAAs (Fig. 1) was described in two previous notes [10,12].

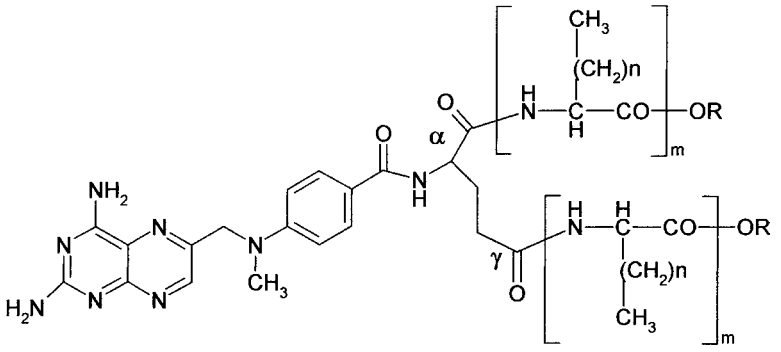
Synthetic DPPC monohydrate was a Genzyme Ltd. Product, purchased from ChemProgress S.r.l. (Sesto U., Milan, Italy). It was used within 3 months without further purification. DPPC purity was assessed by two-dimensional TLC using Merck (Darmstadt, Germany) silica gel aluminium foils. TLC plates were loaded with DPPC solutions in chloroform–methanol (9:1, v/v) and eluted firstly with a chloroform–methanol–5-*N*-ammonium hydroxide mixture (60:3:5, v/v) and for the second perpendicular run with a chloroform–methanol–glacial acetic acid–water mixture (12:60:8:3, v/v). Spots were evidenced by Dragendorff's reagent [17]. Double-distilled water was used; pre-weighed tablets (Sigma) were used to prepare weekly a pH 7.4 phosphate buffered saline (PBS). All other reagents were analytical grade commercial products.

2.2. Liposome preparation

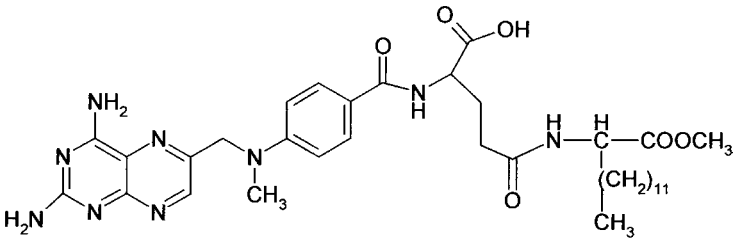
DPPC, either pure or in the presence of the required mole fraction of drug ($X_{\mathrm{DRUG}} = 0.01$–0.12), was dissolved in a glass tube by a 9:1 (v/v) chloroform–methanol mixture, to gain a final lipid concentration of 5 mg/ml. Solvents were evaporated under a nitrogen flow at 30°C under slow rotation to obtain a thin lipid film along the internal tube walls. The phospholipid films were left overnight under vacuum at about 35°C in a Büchi TO-51 oven, to remove all volatiles. The lipid film was hydrated with 60 μl PBS/mg DPPC. The tube was alternatively warmed in a water bath at 50–55°C for 2 min and vortexed for the same time; the process was repeated three to four times. The vesicle suspension was left at 50°C for 1 h and then at room temperature for another hour before use, to anneal the bilayers structure. DSC runs were carried out within the same day of liposomes preparation.

2.3. DSC experiments

Calorimetric experiments were performed on a Mettler DSC12E instrument. Samples (40 μl of liposomal suspension) were sealed in an aluminium pan



Compound	*n*	*m*	R
ROP 101	7	1	H
ROP 102	7	1	Me
ROP 105	11	1	H
ROP 106	11	1	Me
ROP 107	11	2	H
ROP 108	11	2	Me
ROP 109	13	1	H
ROP 110	13	1	Me



ROP 106-γ

Fig. 1. MTX–LAA conjugates chosen for DSC experiments.

and scanned using PBS (40 μl) in the reference pan. An indium standard was used to calibrate the instrument. The detection system was a Mettler Pt100 sensor, with a thermometric sensitivity of 56 μV/°C, a calorimetric sensitivity of about 3 μV/mW, and a noise level lower than 60 nV (<20 μW) from peak-to-peak.

The tested samples were submitted to four complete heating and cooling cycles between 25 and 60°C, at a scanning speed of 2°C/min. The data from the first scan were always discarded to avoid false results coming to the possible lipid–drug mixing in the sample under heating. For a better quantitative comparison of results between different samples, a control

experiment was carried out each time using pure DPPC liposomes prepared under the same conditions as above. The endothermic peak of the second heating scan of such a control sample was taken as a reference value for DSC studies. Molar enthalpies of transition (ΔH) were calculated from peak areas by means of a Mettler TA89E and FP89 system software (version 2.0). Results are reported in Table 1.

2.4. Partitioning studies of conjugates between water and phospholipid bilayers

In order to evaluate the solubility of some MTX conjugates in liposomes, partition experiments were carried out as follows. For each compound three DPPC liposomes samples were prepared containing three different drug molar fractions (0.015, 0.06, and 0.12), obtained by adding to a known liposome amount the required weight of conjugate. DPPC concentration in the dispersion (pH 7.4 phosphate buffer) was kept at 10 mg/ml. Drug–liposomes suspension was incubated at 50°C until the equilibrium between two phases was attained, as verified by DSC tests. About 500 ml were then withdrawn and centrifuged for 30 min at 4°C and 12,300 rpm. The resulting pellet and supernatant were separated and freeze-dried. Residues were dissolved in the suitable volume of a chloroform–methanol (9:1, v/v) mixture, and analysed spectrophotometrically at $\lambda = 303$ nm. Samples prepared with known amount of each conjugate in the same solvent mixture were used for absorbance reference.

From the resulting drug concentration, known the initial amounts of drug and DPPC, the percent of conjugate in the aqueous and lipid bilayer phases were calculated; results are reported in Table 2.

2.5. Partition coefficient evaluation

To increase the significance of the partitioning experiments of conjugates in DPPC vesicles, evaluation of the octanol–water partition coefficient was performed by the classical shake-flask method. However, due to the very low solubility of MTX conjugates in water, significant results were only obtained for the carboxylic derivatives (Fig. 1) by using a pH 7.4 phosphate buffer as the aqueous phase (log $D_{7.4}$, or apparent log P).

Different amounts of drugs were dissolved at room temperature in *n*-octanol (previously saturated with the aqueous buffer and centrifuged) and the UV absorbance at 303 nm was measured. A two- or five-fold volume of phosphate buffer (previously saturated with the pure organic phase) was then added and the mixture was magnetically stirred for 8–12 h. After phase separation, drug absorbance in the octanol was measured again and the distribution coefficient calculated by the classical Hansch equation. Values reported in Table 2 are the average of the different experiments.

A dedicated software was also used for collecting the calculated log P values (ACD/log P 4.5, within the ACD/I-Lab software, Toronto, Canada). Results are also reported in Table 2.

2.6. In vitro cell growth inhibition assay

The biological activity of some of the conjugates studied in the present work has been reported previously [10,12] (Table 3). For the other terms, tests were carried out as described below.

The human T-lymphoblastic leukemia cell line CCRF–CEM and an MTX resistant (transport-defective) cell line (CEM/MTX) [18] were grown at 37°C, in a 5% CO_2 atmosphere, in RPMI 1640 (HyClone) supplemented with 10% fetal calf serum, glutamine (2 mM) and antibiotics. Cells were plated in plastic 24-well plates at a concentration of 1×10^5 cells/ml per well and test compounds were concomitantly added in a concentration range of 0.01–100 μM. Dilutions were made using the culture medium from 1 mM stock solutions of the drugs in anhydrous DMSO. Controls were treated with an equivalent amount of solvent, diluted as above, to assess the absence of toxicity due to the solvent.

After 72 h of incubation, viable cells were counted with a haemocytometer by the trypan blue exclusion assay. EC_{50} (drug concentration reducing viability to 50% that of untreated control) were deter-mined from plots of relative growth (percent of untreated control) versus the logarithm of drug concentration, by using the Origin 6.0 software (MicroCal Software Inc., USA) and are reported in Table 3. Each experiment was repeated three times in duplicate.

Table 1
Thermotropic results (heating mode) of the gel-to-liquid crystal phase transition of DPPC multilamellar vesicles loaded with different amounts of the MTX–LAA conjugates

Compound	Drug mole fraction	Actual mole fraction[a]	$L_\beta \rightarrow P_\beta$[b] (°C)	T_m[c] (°C)	Transition range[d]		$\Delta T^{1/2}$[e] (°C) (±S.E.)	ΔH (kJ/mol) (±S.E.)
					$T_{10\%}$ (°C)	$T_{95\%}$ (°C)		
DPPC	0.00	–	36.2	42.1	41.6	43.4	0.9 (0.02)	61.3 (0.3)
ROP 101	0.01	0.00765	34.7	42.2	41.8	45.7	1.1 (0.02)	59.0 (1.1)
	0.03	0.022	–	42.2	41.6	45.3	1.4 (0.1)	58.6 (1.2)
	0.06	0.046	–	42.1	40.2	45.8	2.0 (0.0)	56.1 (2.2)
	0.12	0.092	–	42.2	40.1	46.1	1.7 (0.2)	52.4 (2.0)
ROP 102	0.01	0.0084	34.2	42.1	41.5	45.2	0.4 (0.0)	64.5 (1.4)
	0.03	0.025	34.4	42.0	41.3	45.0	0.4 (0.015)	64.7 (0.9)
	0.06	0.05	–	42.0	39.9	44.9	0.4 (0.01)	66.1 (3.1)
ROP 105	0.01	0.0081	–	41.9	41.3	45.8	1.0 (0.0)	44.9 (2.2)
	0.03	0.025	–	41.5	40.8	45.0	1.8 (0.02)	43.0 (3.2)
	0.06	0.049	–	40.9	38.7	45.8	2.1 (0.04)	41.7 (1.1)
	0.12	0.098	–	41.0[f]	38.1	45.2	1.7 (0.12)	37.6 (2.6)
ROP 106	0.01	0.0087	35.0	41.6	39.4	45.6	1.4 (0.13)	64.0 (5.5)
	0.03	0.026	–	41.4	39.2	45.5	1.6 (0.14)	57.1 (7.1)
	0.06	0.052	–	41.6	39.8	45.8	1.6 (0.2)	46.1 (2.3)
	0.12	0.104	–	41.5	39.8	45.2	1.4 (0.14)	30.3 (0.3)
ROP 106-γ	0.01	0.0058	35.9	42.1	41.6	45.9	1.0 (0.0)	62.1 (4.1)
	0.03	0.017	34.7	41.9	41.8	44.6	0.8 (0.02)	40.0 (4.0)
	0.06	0.035	34.5	41.9	41.3	45.1	1.1 (0.12)	53.6 (3.2)
	0.12	0.07	–	42.0	41.0	44.9	0.9 (0.04)	56.2 (8.1)
ROP 107	0.01	0.0089	37.2	42.1	41.6	45.2	1.2 (0.8)	41.1 (0.8)
	0.03	0.027	–	41.8	40.8	44.8	1.9 (0.03)	37.4 (1.2)
	0.06	0.053	–	41.8	39.9	45.0	2.4 (0.09)	32.4 (1.2)
	0.12	0.107	–	42.1[f]	40.0	45.0	2.0 (0.1)	36.8 (1.7)
ROP 108	0.01	0.0093	34.9	42.1	39.9	45.2	1.4 (0.11)	47.5 (2.9)
	0.03	0.028	36.0	42.0	38.2	45.0	1.2 (0.11)	49.5 (5.4)
	0.06	0.056	–	41.8	38.0	44.7	1.3 (0.11)	45.1 (5.1)
	0.12	0.112	–	41.8	37.9	45.0	1.2 (0.18)	47.7 (2.3)
ROP 109	0.01	n.c.	–	42.0	41.9	45.0	0.9 (0.08)	33.2 (2.1)
	0.03	n.c.	–	41.8	41.0	45.9	1.1 (0.07)	34.1 (1.2)
	0.06	n.c.	–	41.4	40.4	44.9	1.1 (0.0)	25.0 (0.9)
	0.12	n.c.	–	41.0[f]	38.7	43.9	1.0 (0.1)	26.7 (1.6)
ROP 110	0.01	n.c.	–	42.2	42.5	43.8	0.8 (0.04)	30.7 (1.6)
	0.03	n.c.	–	41.9	41.2	45.2	0.8 (0.09)	29.0 (0.9)
	0.06	n.c.	–	41.8[f]	39.8	44.8	1.6 (0.1)	25.3 (2.5)
	0.12	n.c.	–	41.4[f]	37.9	44.3	0.9 (0.1)	18.7 (3.2)

[a] Corrected drug molar fraction on the basis of partitionin studies (see Table 2).
[b] Pre-transition peak temperature.
[c] Main transition peak temperature.
[d] Temperature range from 10 to 95% of the main transition peak.
[e] Width at half-height of the main transition peak of DPPC vesicles.
[f] Signs of phase segregation observed.

Table 2
Calculated and experimental log P values, and partition data between aqueous phase (pH 7.4 phosphate buffer) and DPPC bilayers of some MTX–LAA conjugates (mean of three determinations at three different drug mole fractions)

Compound	Drug in the aqueous phase (%)	Drug in the bilayers (%)	log $D_{7.4}$[a]	C log P[b]
ROP 101	23.5	76.5	−3.08	3.94
ROP 102	17.0	83.0	–	6.26
ROP 105	18.8	81.2	−2.19	8.02
ROP 106	13.1	86.9	1.65	10.33
ROP 106-γ	42.2	57.8	−3.58	5.91
ROP 107	11.2	88.8	–	22.18
ROP 108	6.6	93.4	–	22.86

[a] See Section 2.
[b] ACD/log P 4.5 software.

Table 3
In vitro cell growth inhibitory activity (IC_{50} (μm)) of MTX and its LAA conjugates against sensitive (CEM/S) and resistant (CEM/MTX) CCRF–CEM cell lines [10,12][a]

Compound	CEM/S	CEM/MTX
ROP 101	>50	>50
ROP 102	22.3 ± 8.3	19.7 ± 1.1
ROP 105	16.4 ± 1.8	13.5 ± 1.9
ROP 106	3.7 ± 0.8	2.2 ± 1.0
ROP 106-γ	0.085 ± 0.7	>5
ROP 107	>10	>10
ROP 108	24.9 ± 2.6	23.0 ± 4.7
MTX	0.013 ± 0.003	1.7 ± 0.2

[a] Incubation time was 72 h.

3. Results and discussion

The investigation of the thermotropic changes in liposome behaviour in the presence of foreign (drug) molecules, provides useful data on the nature and depth of their interactions and localisation within the membrane model.

Pure phospholipid vesicles display a typical gel-to-liquid crystal phase transition temperature (T_m), associated with a constant enthalpy value related to the acyl chain melting [19,20] (Table 1). The perturbing action of a drug distributed inside the phospholipid bilayers can induce two kind of effects: firstly, if a significant downfield shift of the T_m value is observed, this indicates that the drug is mainly interacting with the polar heads of the phospholipid molecules. Secondly, a strong interaction of the drug with the phospholipid acyl chains in the ordered vesicle bilayers results in the lowering of the ΔH associated with the main phase transition [21–24]. In the meantime, a more or less marked broadening of the transition peak shape can be observed, as shown by an increase of the width at half-height and the expansion of temperature range between $T_{10\%}$ and $T_{95\%}$. Insertion of a foreign molecule between liposome bilayers reduces the phase transition co-operativity and leads to a depression of the main phase transition thermotropic parameters [15,19–21].

Among the MTX–LAA conjugates previously described [10], we selected some representative compounds (Fig. 1) in which the length and number of aliphatic side-chain changed to cover a suitable range of lipophilicity and solubility. One γ-mono-LAA derivative (compound **ROP 106-γ**) was also examined, with the glutamate α-carboxyl group left unsubstituted. The presence of a free carboxyl group of course affects physico-chemical properties of the derivative, but is also known to influence the pharmacological profile of the drug [12].

In DSC experiments, incorporation of increasing amount of the MTX–LAA lipophilic conjugates caused a progressive perturbation of DPPC thermotropic behaviour (Table 1). The effect was strictly related to the actual drug concentration in liposomes, the higher the conjugate mole fraction, the greater the depression of gel-to-liquid crystal phase transition ($P_\beta \rightarrow L_\beta$ transition). In particular, even at low drug mole fractions the disappearance of the $L_\beta \rightarrow P_\beta$ pretransition peak of pure DPPC (associated to the transition from the gel state to the ''ripple'' phase) was observed in most cases, along with a broadening of the T_m and a reduction of the molar enthalpy of transition. The former phenomenon is known to be unspecific, indicating that an interaction occurred between drug and phospholipid, whose structural conformation becomes altered [25].

The feature of a pair of corresponding carboxyl- and ester-type MTX–LAA conjugates was compared in Figs. 2 and 3, where the DSC runs of void DPPC vesicles or loaded with compounds **ROP 105** and **ROP 106** at different mole fractions is, respectively, reported. The latter conjugate displayed the best biological activity among the described compounds

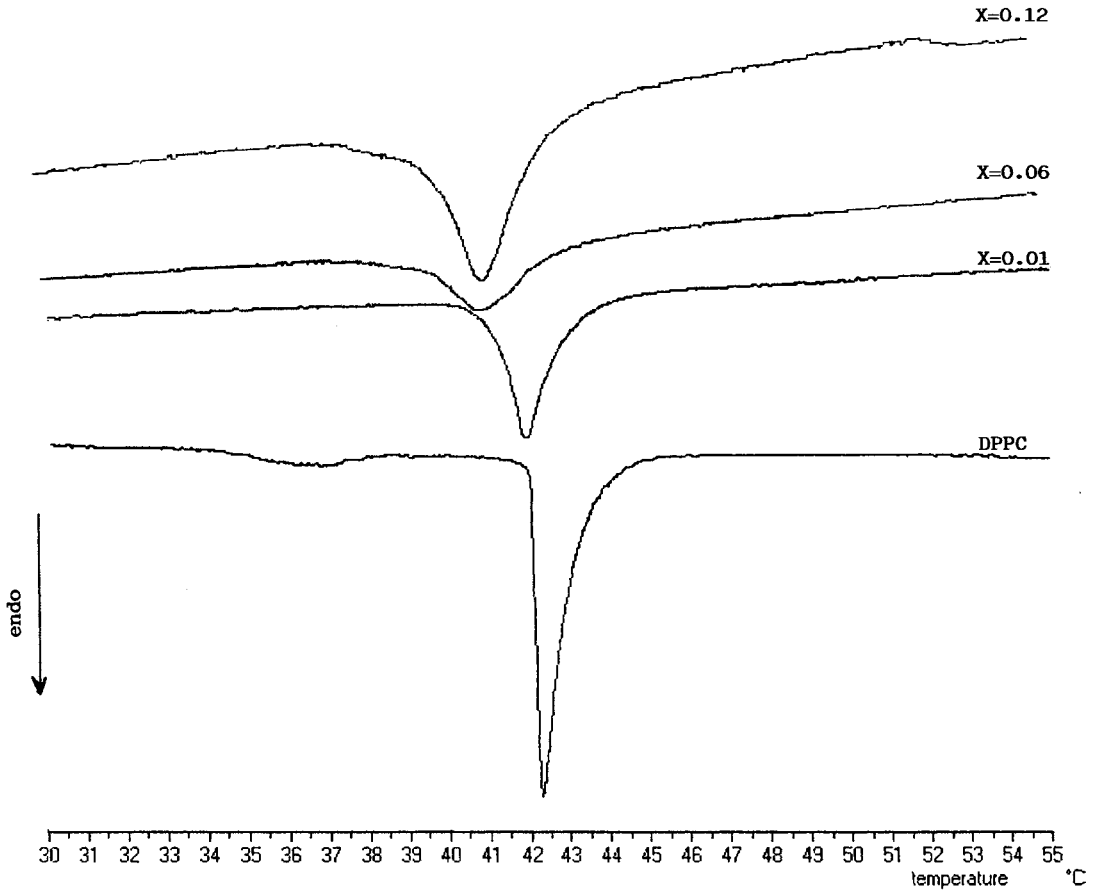


Fig. 2. DSC runs of conjugate **ROP 105**, at different mole ratios with respect to DPPC.

against two lines of MTX sensitive and resistant tumour cells [10].

For the ester-type derivative **ROP 106**, the enthalpy change associated to the phospholipid phase transition progressively dropped off with increasing drug mole fraction in the vesicles, while T_m value remained almost constant, at a temperature 0.5–0.7°C lower than the T_m of pure DPPC vesicles (Fig. 3). Such behaviour is consistent with a perturbing effect on DPPC bilayers mainly at the level of the acyl chains, but without a significant involvement of the polar choline head groups [21]. The high partition coefficient of compound **ROP 106** (Table 2) confirms its affinity for liposome bilayers. Conversely, when the corresponding conjugate with two free carboxyl groups was tested (compound **ROP 105**), the thermotropic effects were more complex (Fig. 2). Increasing the drug mole fraction from 0.01 to 0.06, lowered the T_m from 41.9 to 40.9°C, with a concomitant strong reduction of the associated enthalpy change and an initial sign of phase segregation, as evidenced by the shoulder preceding the main transition peak. By mathematical deconvolution, such a transition peak was split in two partially overlapped peaks, one centred at 38.3°C and the other at 41.2°C. This effect was even more visible at the highest tested mole fraction (0.12) (Fig. 2). The above findings indicate that compound **ROP 105** is able to strongly interact with DPPC bilayers at both aqueous compartment and lipid domain level. However, above a certain drug–phospholipid mole ratio it is prone to form separate drug-poor and drug-rich phospholipid domains within the vesicle bilayers, as often already observed in the literature [15,26]. In fact, increasing the drug mole

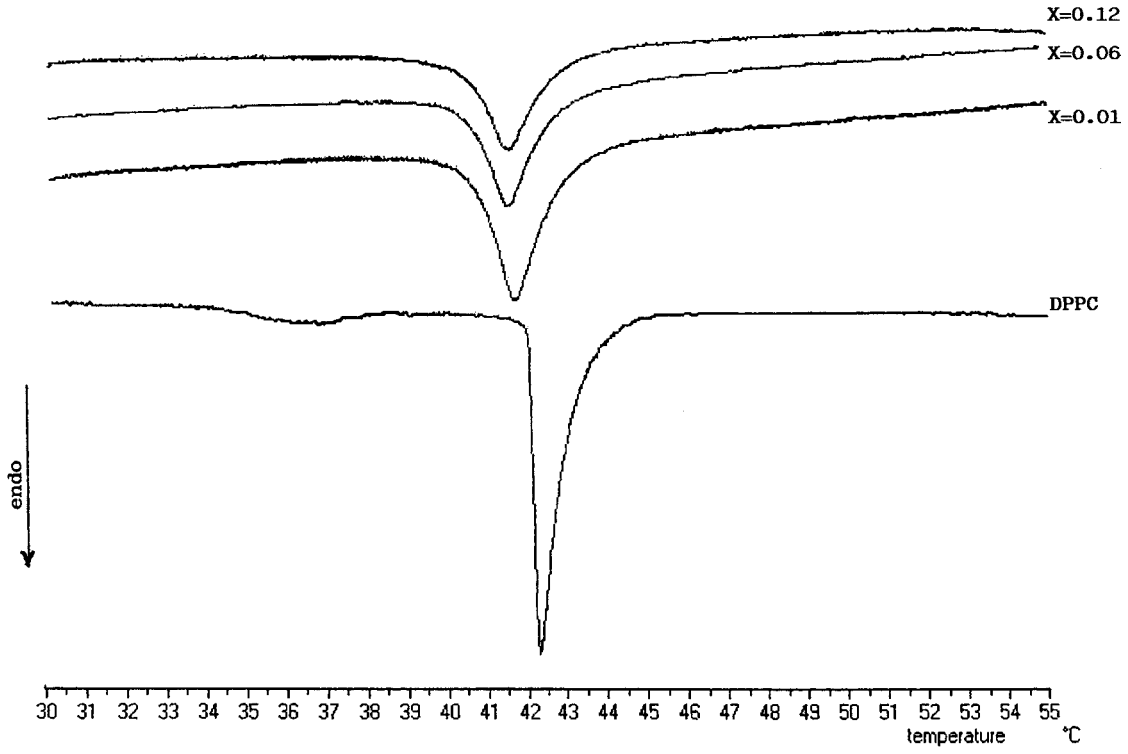


Fig. 3. DSC runs of conjugate **ROP 106**, at different mole ratios with respect to DPPC.

fraction from 0.06 to 0.12 did not led to a further reduction of T_m value, but enhanced the tendency to phase segregation. This behaviour suggests that conjugate molecules in excess with respect to their solubility in DPPC bilayers, formed separate aggregates, which had only a limited perturbing effect on the vesicle thermotropic profile.

Since this conjugate displayed a high partition coefficient in DPPC bilayers (Table 2), is must be presumed that its tendency to form separate aggregates occurred only under liposome formation conditions, and is only in part to be ascribed to its great lipophilicity.

In the case of the more lipophilic pairs of conjugates, **ROP 107** versus **ROP 108**, and **ROP 109** versus **ROP 110**, the effects on DPPC bilayers were less marked. Phase transition temperatures remained almost constant and close to that of pure DPPC (Table 1), while transition enthalpy changes were much lower and transition peaks generally appeared broader, as confirmed by their $\Delta T^{1/2}$ values (Table 1).

From the very high partition coefficient values of the latter conjugates in DPPC bilayers (Table 2), a deep effect on vesicle thermotropic pattern could have been expected. On the contrary, to explain their limited effects on DPPC transition process, the very low solubility of these compounds in the hydration buffer must be taken into account. During liposome formation, they probably tend to form insoluble aggregates outside DPPC vesicles, thus giving an actual drug mole fraction much lower than the theoretical one. The amount of conjugate which remained associated to liposomes exerted a great disrupting effect upon DPPC acyl chain order, by virtue of their higher lipophilicity. The very low water solubility of these conjugates also explain their reduced activity usually observed in the biological tests (see below).

The tendency of the more lipophilic compounds to form molecular aggregates within DPPC bilayers was confirmed by a separate experiment, in which liposomes containing compound **ROP 110** at 0.01 mole fraction (Table 1), was allowed to stand at room

temperature for 2 days and then submitted to DSC. Although the T_m did not changed (42.1°C), ΔH lowered from 47.1 to 34.6 J/mol and some additional broad endothermic signals appeared around 34–35.5, 39.5, and 46–47°C. This set of peaks indicate a progressive penetration of the drug into DPPC bilayer, causing a greater perturbation of DSC profile. Moreover, the presence of a peak at a higher temperature than DPPC T_m confirmed that the lipophilic derivative formed some aggregates which 'stabilise', instead of perturbing the phospholipid structures.

By evaluating the relatively less lipophilic pair of conjugates **ROP 101** and **ROP 102** (with shorter 10 carbon long side chains) different data, as expected, were experimented. Neither of them influenced the pre-transition and main transition parameters (Table 1), however, both induced a greater reduction of T_m peak width at half-height values and a slight increase of associated enthalpy changes, compared to pure DPPC liposomes.

These findings attested that the presence of the two free carboxyl groups in LAA residue allowed a relevant electrostatic interaction with DPPC choline head groups, whereas the corresponding methyl ester derivatives showed their main effects on the phospholipid acyl chains.

By comparing the whole set of tested derivatives, a parabolic-type behaviour was observed, as already reported for a series of lipophilic MTX bis(amide) derivatives [16]: the tetradecyl derivatives (compounds **ROP 105** and **ROP 106**) showed the more marked effects on vesicle DSC behaviour. Lowering or increasing lipophilicity resulted in a reduced capacity of perturbing the phospholipid phase transition phenomena.

Analysis of the mono-substituted derivative **ROP 106-γ** showed only minor effects on DPPC bilayers, up to the higher examined mole fraction. DSC runs displayed both the pre-transition peak at close values to void DPPC vesicles and no shift of the main transition peak (Table 1). As above discussed, such a behaviour accounts for a poor interaction at the level of DPPC choline head-groups. On the other hand, and in contrast with the results of the corresponding di-substituted conjugates **ROP 105** and **ROP 106**, also the enthalpy changes and $\Delta T^{1/2}$ values were not significantly different from those of pure DPPC liposomes. This behaviour indicates that compound **ROP 106-γ** had a limited influence on phospholipid bilayers, also at the level of acyl chains. A similar feature better resembles that one shown by the parent MTX molecule. The effects of the latter on DPPC thermotropic parameters were in fact found to be limited to the enthalpy values associated with phospholipid phase transition. The negatively charged molecules of MTX are only able to interact with the outer portions of DPPC bilayers, which contain positively charged choline groups [16].

The present DSC experiments then confirmed the suitability of the conjugation strategy adopted for these MTX derivatives [10,12,13], to obtain drugs with the required level of lipophilicity, solubility and biological activity on the target enzymes.

The correlation between calorimetric features and in vitro cell-growth inhibitory activity of conjugates against CCRF–CEM cells was also attempted for some terms. Biological data are not available for all the tested compounds, mainly because of the very low solubility of the more lipophilic ones in the cell culture media. However, comparison of the available cytotoxicity data (Table 3) with the calorimetric results, agreed in indicating that the two tetradecyl derivatives, **ROP 105** and mainly **ROP 106** had both the more pronounced effects on DPPC vesicle bilayers and the greater growth inhibitory activity against CEM cells. The corresponding conjugates with shorter alkyl chain (**ROP 102**) or two linked tetradecyl chains (**ROP 108**) were less active as cell growth inhibitors. Interestingly, compounds with free carboxylic functions (**ROP 101** and **ROP 107**, respectively), were almost inactive against CEM cell cultures. Since in DSC experiments they showed some ability in interacting with the phospholipid choline head groups, it could be hypothesised that, once reaching tumour cell membranes, a portion of the drug remain associated with the membrane, instead of crossing it and reaching cell cytoplasm, where the antimetabolic activity has to be exerted.

When tested against a cell sub-line resistant to MTX by virtue of an impaired folate transport system (CEM/MTX cells), most of the described conjugates kept the same order of cell outgrowth inhibitory activity, whereas MTX was about 200 times less potent (Table 3) [10,12]. The different behaviour of the parent drug and LAA-conjugates upon DPPC biomembranes, as shown by the DSC studies, further

support the starting thesis that the latter are able to bypass the cell transport resistance by means of an enhanced positive (passive) penetration through cell membranes.

In conclusion, evaluation of the mode and depth of interaction of lipophilic MTX–LAA conjugates with a lipid model membrane gave precious information about the possibility of carrying these drugs in liposomes. A similar approach is important in planning the incorporation of a new drug molecule in such a colloidal delivery system. In particular, the differential behaviour on DPPC biomembrane model of acid- and ester-type LAA ends in MTX conjugates suggested that limiting the formation of hydrogen bonds between drug molecule and membrane components, enhanced the ability of the conjugates to reach the cell cytoplasm and the target enzyme.

The overall lipophilic character resulting from the substitution of MTX with LAAs affected the calorimetric profile of DPPC vesicle bilayer phase transition. Moreover, the substitution of only one carboxyl group on MTX molecule gave a compound able to perturb DPPC bilayers similarly to the parent drug. Thus, the strategy of using MTX–LAA conjugates for over-crossing an altered MTX uptake in transport-resistant cells seems to be better satisfied by substituting both the α- and γ-carboxyl groups in the MTX glutamate moiety.

Of course, the qualitative evaluation of the interaction between drugs and DPPC vesicles, here reported, gave only a partial prevision of the in vivo behaviour of these conjugates. A compound able to penetrate into and interact with these biomembrane model could in fact not to be able to be effectively released into cell interior, but can remain entangled within lipid bilayers.

The above DSC experiments also showed that the drug concentration in the lipid mixture during liposome preparation must be accurately chosen, to avoid a separation of the excess (unincorporated) drug outside the phospholipid bilayers. Work is in progress to evaluate the biological activity of MTX and its lipophilic conjugates with LAAs after incorporation in liposomes with different composition and surface charges.

The usefulness of the DSC technique has also been further confirmed by the present study to explore the importance of physico-chemical and steric parameters in conditioning the interaction of drugs with cell membranes.

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