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METHOTREXATE INTERACTION WITH A LIPID MEMBRANE MODEL OF DPPC

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Abstract

Dipalmitoylphosphatidylcholine (DPPC) liposomes were employed as membrane models for the investigation of the interaction occurring between methotrexate (MTX) and bilayer lipid matrix. Liposomes were obtained by hydrating a lipid film with 50 mM Tris buffer (*pH* 7.4). The differential scanning calorimetry (DSC) evaluation of the thermotropic parameters associated with the phase transitions of DPPC liposomes gave useful information about the kind of drug-membrane interaction. The results showed an electrostatic interaction taking place with the negatively charged molecules of MTX and the phosphorylcholine head groups, constituting the outer part of DPPC bilayers. No interaction with the hydrophobic phospholipid bilayer domains was detected, revealing a poor capability of MTX to cross through lipid membranes to reach the interior compartment of a lipid bounded structure. These findings correlate well with *in vitro* biological experiments on MTX cell susceptibility.

Keywords: DSC, interaction, lipid model membrane, methotrexate

Introduction

The 4-aminopteridine analogues of folic acid have been of use in cancer chemotherapy since 1948 [1–6]. The methotrexate (MTX) biological action is due to the inhibition of cell growth by interfering with dihydrofolate reductase. This endocytoplasmatic enzyme is responsible for the reduction of 7,8-dihydrofolic

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acid to 5,6,7,8-tetrahydrofolic acid [7]. The latter is an essential cofactor in DNA synthesis.

Many solid tumours remain refractory to MTX treatment. Different mechanisms have been advanced to explain the resistance of tumour cells to MTX: (i) an elevated intracellular level of dihydropholate reductase [8–10], (ii) a structural mutation in dihydropholate reductase decreasing its affinity for MTX [11–12], (iii) inefficient delivery of MTX into the cytoplasmatic environment of the cell or a higher rate of drug efflux from the cell [13–14]. Therefore, the possibility to ensure different drug entrance pathways into tumoral cells is of great importance for a more suitable antitumor chemotherapy.

The drug delivery device that fulfils this purpose is the liposomal system [17–19], which is able not only to target MTX against tumour cells by means of antibody coated surfaces [18–19], but also to principally prevent the MTX resistance of some tumoral cellular strains [20]. For these reasons MTX should be considered a liposome-dependent drug [21].

In order to design an efficient drug delivery device, it is necessary to understand the interactions occurring between host and guest [22–24]. To this end differential scanning calorimetry (DSC) was used. In fact, the presence of drugs in the vesicular structure of liposomes, depending on their hydrophilic or hydrophobic nature, causes variations in the thermotropic parameters of the phase transition from gel to liquid crystal [26–27]. The target lipid model membrane was dipalmitoylphosphatidylcholine, the most abundant phospholipid in the biological membranes and the one that is the most suitable for drug-membrane interaction studies [28–31]. These studies may also be predictive of the possible passive interactions (no involvement of active or facilitated cellular transport systems) among MTX and tumoral cells, partially elucidating the mechanism of drug resistance and, then, shedding light on the possible solutions to adopt for rendering the drug more active.

Experimental section

Materials

Synthetic 1,2-dipalmitoyl-sn-glycero-3-phosphocholine monohydrate (DPPC) was purchased from Fluka Chemical Co. (Buchs, Switzerland). The phospholipid purity, greater than 99%, was assessed by bidimensional thin layer chromatography on silica gel plates (E. Merck, Darmstadt, Germany) loaded with a solution of the lipid in chloroform-methanol (3:1 v/v) and developed first with a solvent system consisting of chloroform-methanol-5 N ammonium hydroxide (60:3:5 v/v/v) and then with chloroform-methanol-acetic acid-water (12:60:8:2.5 v/v/v/v) and stained with Dragendorff's reagent [32]. Phospholipid phosphorous content was assayed as inorganic phosphate as described

by Bartlett [33]. Methotrexate (MTX) was kindly provided by Cyanamid (Catania, Italy). The purity was greater than 99.5% by HPLC [22]. Tritiated deoxyuridine (15–30 Ci/mmol) was from New England Nuclear (Boston, MA, USA). For the biological assay, solutions of MTX and tritiated deoxyuridine were prepared with Dulbecco's phosphate-buffer saline lacking Ca^{2+} and Mg^{2+} (PBS). Double distilled pyrogen-free water was used. All other materials and solvents were of high analytical grade.

Liposome preparation

DPPC was dissolved in chloroform in a round-bottomed flask containing 20 g of glass beads (2–3 mm mean size) (Carlo Erba, Italy). The aim of the glass bead addition was to increase the surface area of the dried lipid film, thus enhancing the phospholipid contact with the buffer solution containing the drug. In this way the lipid hydration and dispersion was easier. The final volume of lipid solution was adjusted to 20 ml with chloroform. The organic solution was removed at 35°C on a rotary evaporator until the lipid was dried and distributed as a thin film on the wall of the vessel and on the surface of the glass beads. Any possible trace of organic solvent was eliminated by overnight high vacuum storage, 150 torr pressure (Edwards high vacuum pump mod. Serial E 2M8 42810).

MTX dispersions in DPPC liposomes were carried out by adding the component to the DPPC film in the desired amount to obtain a different molar fraction of MTX with respect to DPPC.

Liposome dispersions were prepared by adding 50 mM Tris buffer at *pH* 7.4 to the film. The vessel was alternatively vortexed and incubated in a water bath at 55°C for a total of 10 min; this procedure was repeated four times. During this process the samples were maintained at a higher temperature than that of DPPC gel-liquid crystal phase transition to allow for the full hydration of the phospholipid. Finally, the liposome suspension was left at room temperature for 1 h to anneal the bilayer structures. All the procedures were carried out under N_2 to prevent oxidation of the lipid. Of each sample, 40 µl containing 5 mg of DPPC were sealed in an aluminium pan and submitted to DSC analysis.

The kinetic experiments were carried out by putting the exact amount of MTX to obtain a 0.24 molar fraction on the bottom of the pan and then adding 40 µl of DPPC colloidal suspension prepared as described above.

Differential scanning calorimetry analysis

The calorimetric experiments were performed with a Mettler DSC12E differential scanning calorimeter, equipped with a Haake D8-G. An Indium standard sample was employed to calibrate the temperature scale and the enthalpy changes (ΔH). The detection system was a Mettler Pt100 sensor.

The sensor presented a thermometric sensitivity of 56 μV/°C, a calorimetric sensitivity of about 3 μV/mW and a noise lower than 60 nV (<20 μW) peak to peak. The baseline reproducibility was <3 μV (<1 mW). Each DSC scan presented an accuracy of ±0.4°C, a reproducibility and a resolution of 0.1°C. An aluminium pan loaded with 40 μl of 50 mM *pH* 7.4 Tris buffer was employed as reference. Samples were submitted to several heating and cooling cycles in the temperature range of 30–50°C at scanning rate of 1 deg·min^{-1}. Enthalpy changes were calculated from the peak areas by means of Mettler TA89E and FP89 system software Version 2.0 running on a IBM PS/2 mod. 57SX, equipped with 8 MB Ram.

After the DSC scans the content of the pan was submitted to phospholipid phosphorous analysis [33].

Cell lines and culture

The biological experiments utilised a methotrexate-resistant cell line CEM/MTX, which was kindly provided by Prof. Mazzarino, Institute of Pathology, University of Catania. These lines were derived from a MTX-sensitive human leukemic lymphoblast *T* cell line designated CEM/O. CEM/ MTX cells possess levels of dihydropholate reductase identical to those present in the parent cells. However, the CEM/MTX cells revealed an 80% reduction in both the initial rate of MTX uptake and the steady-state intracellular drug concentration, and a concomitant 120-fold increased resistant to MTX.

The cell lines were cultured in flasks with 100 ml of RPMI 1640 medium with the addition of fetal calf serum (10%), penicillin (100 μg/ml), streptomycin (100 μg/ml), and gentamycin (10 μg/ml). The initial inoculum was of $2 \cdot 10^5$ cells per ml. After 72 h of cell growth at 37°C in 5% CO_2, a 5- to 10-fold increase in cell density was reached. The cell concentration was again reduced to the initial value by addition of an appropriate inoculum to flasks containing fresh medium. Cells were harvested by centrifugation (4°C for 10 min, at 750×g), washed twice with PBS, and then resuspended in the culture medium with 10% fetal calf serum (no presence of antibiotics) at a final concentration of $2.2 \cdot 10^5$ viable cells per ml; viability was determined by trypan blue exclusion.

Deoxyuridine incorporation

The cell proliferation assay was carried out in 96-well tissue culture plates (Coster No. 3596) in triplicate. Each well initially contained $4 \cdot 10^4$ washed cells in 180 μl of medium. The addition of MTX was made in 20 μl of saline. After 4 h incubation, 10 μl (1 μCi) of tritiated deoxyuridine was added to the culture, and the incubation was further prolonged for 18 h. Then, the cells were collected onto glass fiber paper by means of an automatic harvester. Deoxyuridine

incorporation was linear up to 24 h. The paper discs were washed with water, dried with ethanol, and counted in Instagel (Packard Instruments Co. Inc., IL 20515, USA) with a Packard scintillation counter. Results are expressed as a percentage of control incorporation, which was calculated from the arithmetic means of the radioactivity taken up by the cells.

Results and discussion

DSC analysis represents a simple, powerful and precise experimental method, which is useful not only to provide information concerning the interaction occurring between pharmaceutically active substance-loaded liposomes and the lipid matrix [30, 34], but also to study *in vitro*, by means of lipid membrane model, the possible *in vivo* interactions among drugs and biological substrates [28–29]. Phospholipid vesicles when submitted to DSC analysis show a particular endothermic peak that is attributed to the transition from the gel state to the liquid crystal phase ($L_\beta \rightarrow L_\alpha$ transition), which is characterised by a derangement of the lipidic core of the liposome lamellae, leading to a less packed and more fluid state. It is worth noting that the thermotropic characteristics, i.e. enthalpy change (ΔH), entropy change (ΔS), and transition temperature (T_m) of the gel to liquid crystal phase transition are approximately due to the following energetic contributions: (i) hydrogen bond energy occurring among polar heads, (ii) Van der Waals reticular energy of the hydrophobic chains, (these two parameters increase the T_m and ΔH values) (iii) electrostatic and steric repulsion energy, which lowers the transition ΔH values.

DPPC liposomes, despite the main transition peak, also present a less intense pretransition peak due to the mesophasic transition from the gel state to the ripple phase. Definitively, DPPC shows three phases: gel state (L_β), ripple phase (P_β),and liquid crystal phase (L_α). Then, the presence of a molecule in the environment of DPPC liposomes could be capable, depending on its own chemico-physical behaviour, of interacting and influencing the thermotropic behaviour of the vesicle, e.g. the presence of cholesterol first depress $L_\beta \rightarrow P_\beta$ transition and then the main transition peak [35].

The presence of MTX did not influence the shape of DPPC thermograms (Fig. 1) and no depression effect on the $L_\beta \rightarrow P_\beta$ transition was observed. In fact, as shown in Fig. 1 by the black arrow, the $L_\beta \rightarrow P_\beta$ transition was maintained by the DPPC system in spite of the increasing molar fraction of MTX. The presence of MTX did not shift even the $L_\beta \rightarrow P_\beta$ and $P_\beta \rightarrow L_\alpha$ transition peak temperatures (Fig. 1 and Table 1). The absence of modification either in the pretransition or in the transition peak temperatures of the phospholipid normally stands for a quite negligible interaction between MTX and liposomes, and particularly with their lipid lamellar core. It is likely that the drug is placed entirely within the aqueous environment of the phospholipid vesicle [36], and then the interactions, which can take place between host and guest, are electrostatic influences.

Evidence of the absence of interaction between MTX and the lipidic core of the DPPC liposome bilayers arose from the analysis of the variation of the $P_\beta \rightarrow L_\alpha$ transition peak width half-height ($\Delta T_{1/2}$) as a function of MTX molar fraction. This parameter is related to the co-operation behaviour of the liposomal system. The smaller the $\Delta T_{1/2}$ value is, the higher is the co-operativity of the system and the number of phospholipid molecules that change phase simultaneously, giving origin to a very large molecular aggregate called 'co-operative unit' [23]. This parameter takes into account the degree of drug penetration into the lipidic bilayer zone and then of the packing order of the phospholipid chains [23]. In the case of MTX, as shown in Fig. 2, the $\Delta T_{1/2}$ values remain almost constant while the drug molar fraction increases, confirming the total absence of interaction between MTX and the hydrophobic alkyl phospolipid chains.

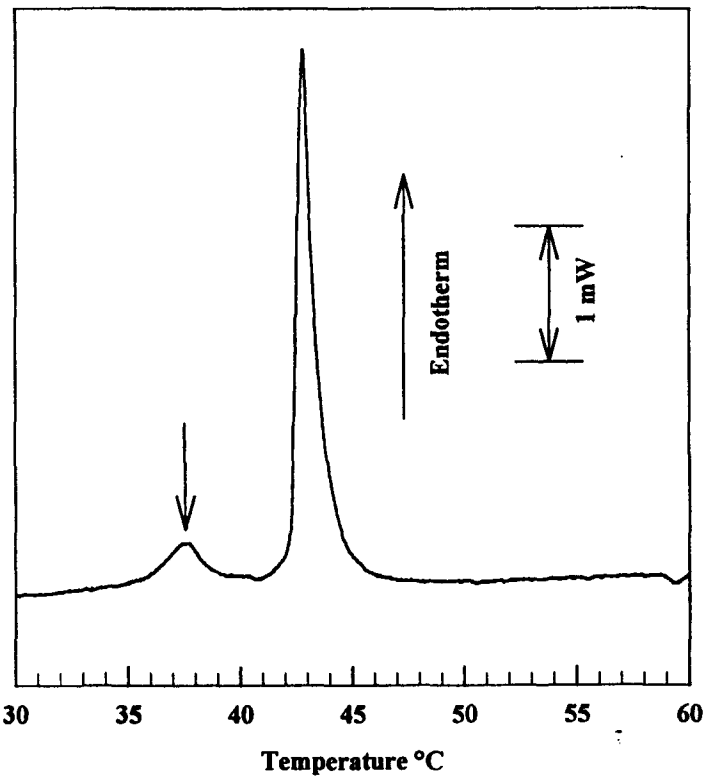

Fig. 1 DSC traces in heating mode of DPPC multilamellar vesicle colloidal dispersions in 50 mM *pH* 7.4 Tris buffer containing MTX at a mole fraction of 0.24. The black arrow indicates the transition peak from gel to ripple phase ($L_\beta \rightarrow P_\beta$). Similar thermograms were obtained in the presence of different molar fractions (0.06; 0.12; 0.18; 0.36) of MTX

Table 1 Mesophase transition temperatures from gel (L_β) to ripple phase (P_β) and from ripple phase to liquid crystalline phase (L_α) of multilamellar vesicle dispersions made up of DPPC at different molar fractions of MTX*

Molar fraction of *MTX*	Transition temperature /°C	
	$L_\beta \rightarrow P_\beta$	$P_\beta \rightarrow L_\alpha$
0	36.7	42.2
0.03	36.2	42.0
0.06	35.9	42.0
0.12	35.8	42.0
0.18	35.8	42.0
0.24	35.6	42.0
0.36	35.7	42.1

*Each experiment was carried out 1 h after the preparation of the liposome suspension. The data, herein reported, are the average of nine measurements obtained from DSC heating curves. The percentage of standard deviation was lower than 0.5

The thermotropic parameters influenced by the increasing MTX molar fraction were ΔH and ΔS values linked to the $P_\beta \rightarrow L_\alpha$ transition peaks. This result showed the capability of MTX to interact with the outer part of the phospholipid polar heads. In particular, as shown in Fig. 3, the enhancement of the MTX molar fraction caused a linear increase in ΔH values. This trend was probably due to an electrostatic interaction between the negatively charged molecule of MTX and the polar choline head groups of DPPC, in this way MTX behaved like a choline group counterion. Therefore, the presence of MTX lowers the

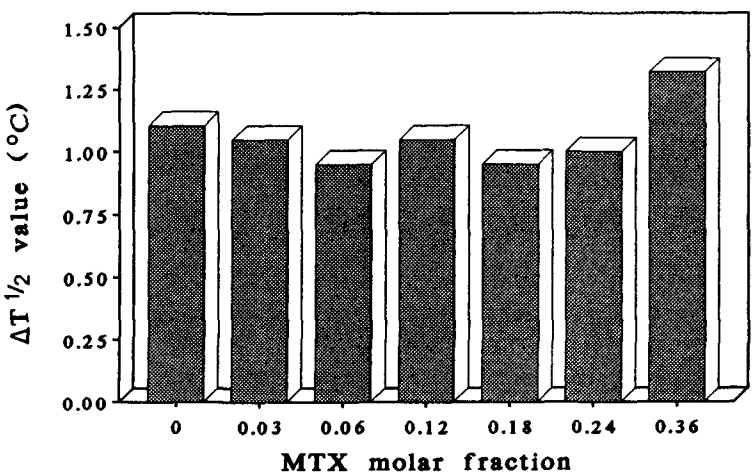

Fig. 2 Variation of the main transition peak width half-height ($\Delta T_{1/2}$/°C) values for DPPC liposomal dispersions in 50 mM *pH* 7.4 Tris buffer as a function of MTX molarfraction

electrostatic repulsion forces among choline groups ensuring a tight packing of the phospholipid alkyl chains, which enhances the Van der Waals reticular energy of hydrophobic domain, and leads to the enhancement of ΔH values.

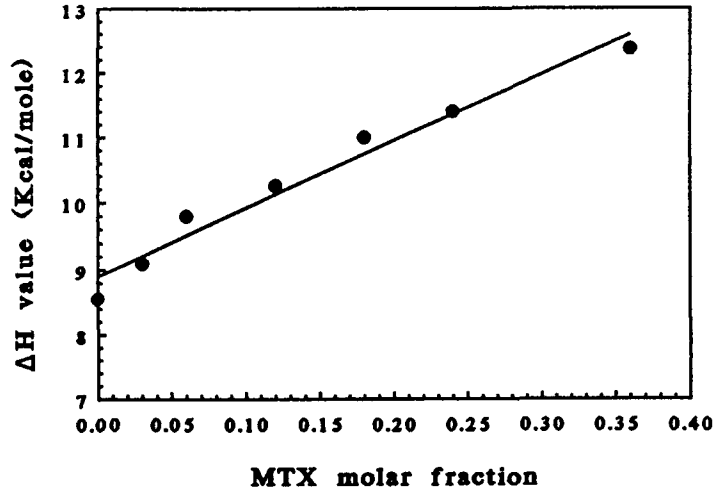

Fig. 3 Enthalpy changes (ΔH values) associated to the $P_\beta \rightarrow L_\alpha$ transition peak of DPPC liposome dispersions in 50 mM *pH* 7.4 Tris buffer as a function of MTX molar fraction. The experiments were carried out 1 h after sample preparation

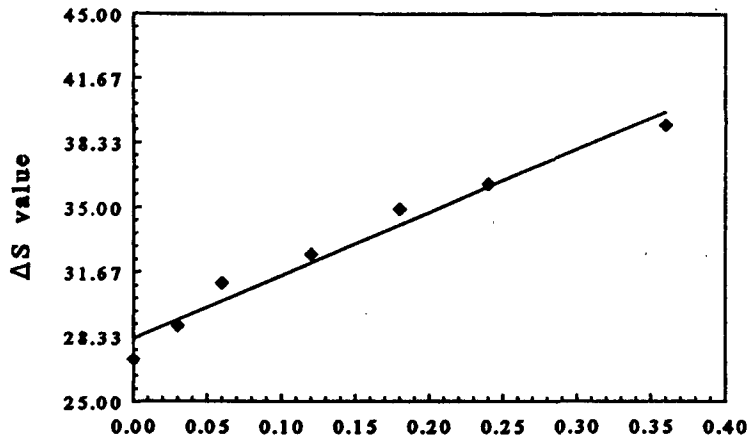

Fig. 4 Entropy changes (ΔS values) associated to the $P_\beta \rightarrow L_\alpha$ transition peak of DPPC liposome dispersions in 50 mM *pH* 7.4 Tris buffer as a function of MTX molar fraction. The experiments were carried out 1 h after sample preparation. The ΔS values were calculated according to the following equation: $\Delta S = \delta Q_{rev} \cdot T^{-1}$
where δQ_{rev} is the heat quantity exchanged during the reversible process $P_\beta \rightarrow L_\alpha$.
T is the transition peak temperature in Kelvin degrees

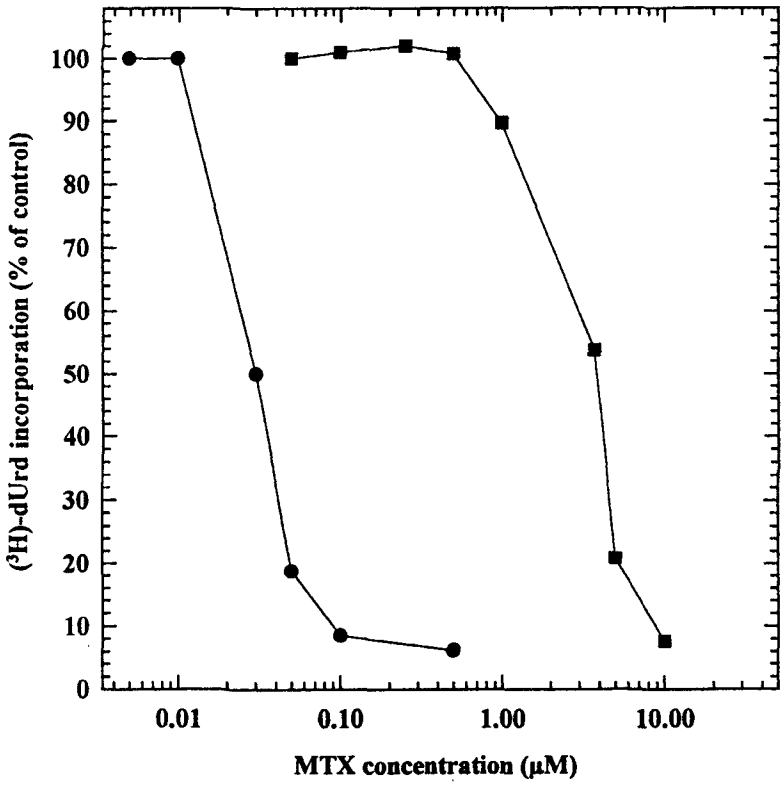

Fig. 5 Inhibition of CEM/O (•) and CEM/MTX (■) cell proliferation by MTX. This value was calculated by determining the percentage of the tritiated deoxyuridine ([^3H]d*Urd*) incorporated within cells with respect to control cells (absence of MTX). Control incorporation was 127397±10853 cpm for CEM/O and 119276±7265 cpm CEM/MTX cells

The most efficient packing of the hydrophobic phospholipid chains due to the presence of MTX is also proved by the linear increase of ΔS as a function of the drug concentration (Fig. 4). In fact, a better bilayer chain packing provided a more ordered gel state and, as a consequence, a reduction of the DPPC system entropy. During the $P_\beta \rightarrow L_\alpha$ transition the variation of entropy [$S_{gel(stabilised\ phase)} \rightarrow S_{liquid}$] ($\Delta S$) is higher when the gel state is more packed and co-operative.

These data showed that MTX was able to interact with the highly hydrophilic outer surface of a model membrane by means of electrostatic bonds; but is was not capable of influencing the hydrophobic hydrocarbon region of the membrane bilayers, owing to its strongly polar molecule. In fact, MTX showed an experimental partition coefficient value of −1.7, expressed as the decimal logarithm of the ratio between the drug concentration in the octanol phase and that in the aqueous buffer phase.

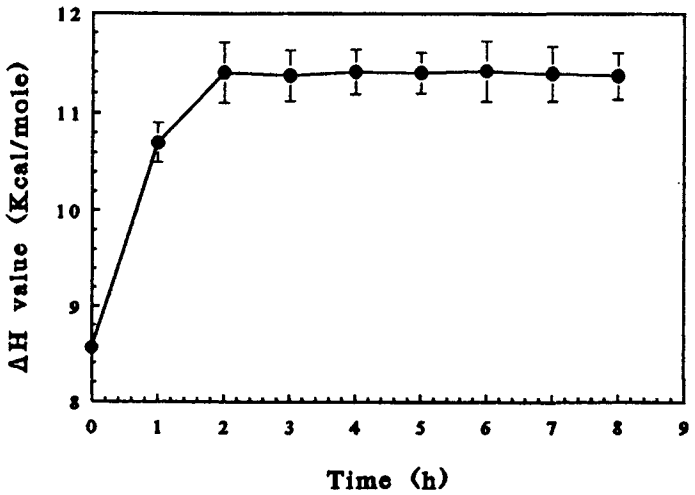

Fig. 6 Kinetic experiment of the interaction between MTX and model membranes. Enthalpy changes of $P_\beta \rightarrow L_\alpha$ transition peak of DPPC liposome dispersion in 50 mM *pH* 7.4 Tris buffer at 0.24 MTX molar fraction as a function of time

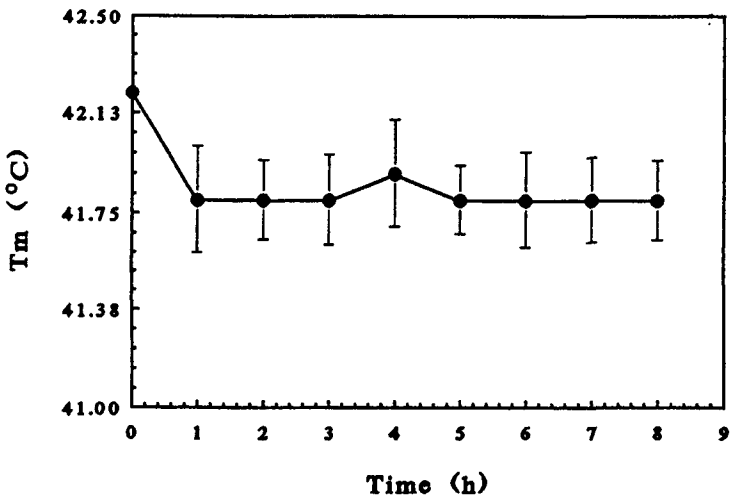

Fig. 7 Kinetic experiment of the interaction between MTX and model membranes. Temperatures changes of $P_\beta \rightarrow L_\alpha$ transition peak of DPPC liposome dispersion in 50 mM *pH* 7.4 Tris buffer at 0.24 MTX molar fraction as a function of time

These results are in agreement with the biological evidence about the interaction between MTX and mammalian cells. MTX is not able to penetrate living cells by simple passive diffusion through the biological membrane, but needs a folate transport system [20], being a transport-dependent drug. For this reason, mutation permeability of the cells and/or change in the affinity of folate trans-

port protein for MTX result in a MTX resistant tumour cell. In fact, as shown in Fig. 5, MTX presented an effective chemotherapeutic activity against CEM/O tumoral cells, which have an active folate transport system, able to ensure the MTX entrance into the cytoplasmatic compartment where the target enzyme is located. A decrease of the tumoricidal activity of MTX was revealed *vs.* tumoral cell strains (CEM/MTX) that did not ensure an effective MTX transport. Particularly, Fig. 5 shows the effect of MTX on incorporation of deoxyuridine into tumoral DNA by both CEM/O and CEM/MTX cells. The curves showed that incorporation would be inhibited 50% by 0.03 μM and 3.7 μM MTX in the case of CEM/O and CEM/MTX cells, respectively; confirming that the transport defective cells are at least 100-fold less sensitive to the drug [37]. Therefore, in the case of CEM/MTX cells the biological activity might be ensured by a different penetration pathway. Previous papers [38–40] dealt with the penetration of MTX-prodrugs, which achieved no difference in the cancer effectiveness against MTX sensitive cells, but an improvement in the biological activity against MTX resistant cells. These findings may be explained in terms of a different penetration pathway.

The difficulty of MTX to overcome the biological membrane barrier, also lead to the failure of MTX to penetrate through the stratum corneum and reach an effective concentration in the viable epidermis, representing the major problem in MTX topical pharmacological therapy [41]. For these reasons, encapsulation of MTX in liposomes is of great interest in topical and systemic treatment [42]. The liposomal device could ensure a suitable biological delivery of MTX in terms of enhanced penetration into the tumoral cell through the bilayer structures of the biological membranes.

The fact that the limiting step in MTX therapy was due to the difficult penetration into the cells via a passive membrane diffusion and to the obligation to reach the cellular cytoplasm through a MTX transport system was shown by the diffusion kinetic studies. As shown in Figs 6 and 7, MTX is able to rapidly diffuse in the aqueous medium, as well as in the biological aqueous environment. The ΔH value increase reached a plateau phase after 2 h of incubation. MTX, after having reached the liposome structure, interacted with the outer hydrophilic layer without passing through, as revealed by the T_m and ΔH values as a function of time. This hypothesis was also confirmed by the absence of interaction between the drug and the hydrophobic bilayer core, causing no alteration in the co-operation energy of the phospholipid hydrocarbon chains constituting the vesicular system (no variation in $\Delta T_{1/2}$ values, data not reported).

In conclusion, our findings show that it is necessary to take advantage of different cellular pathway of MTX entrance by means of various drug delivery devices, in order to obtain a suitable MTX antitumoral chemotherapy, particularly against MTX-resistant cells because of a lack of folate transport systems. Therefore, this study is of interest not only to evaluate *in vitro* drug-biological membrane interaction (the DSC experiments correlate well with *in vitro* biological

experiments on the MTX-resistant cell lines, CEM/MTX), but also to investigate and to predict the possible therapeutic effectiveness of MTX-prodrug and MTX-loaded delivery systems (work in progress). Furthermore, a rationale for the realization of MTX liposome formulation could be attained. The findings, herein reported, show that MTX is totally placed within the aqueous core, and then a suitable liposome preparation process, capable of high water-phase capture, is very useful in the realization of a drug delivery system with high drug carrier capacity [22].

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Zusammenfassung — Dipalmitoylphosphatidylcholin (DPPC) Liposome wurden als Membranmodelle zur Untersuchung der Wechselwirkung zwischen Methotrexat (MTX) und doppelschichtigen Lipidmatrizen angewendet. Die Liposome erhielt man durch Hydratation eines Lipidfilmes mit 50 mM Tris-Puffer (*pH* 7.4). Die DSC-Auswertung der mit der Phasenumwandlung der DPPC-Liposome zusammenhängenden thermotropischen Parameter lieferte nützliche Informationen über die Art der Wechselwirkung zwischen Droge und Membran. Die Ergebnisse zeigten eine elektrostatische Wechselwirkung zwischen negativ geladenen Molekülen von MTX und den Phosphorylcholin-Endgruppen, die den äußeren Teil der DPPC-Doppelschichten bilden. Eine Wechselwirkung mit den hydrophoben Phospholipid-Doppelschichtregionen konnte nicht beobachtet werden, was auf eine geringe Fähigkeit von MTX zur Durchdringung der Lipidmembranen hinweist. Diese Resultate stehe in guter Übereinstimmung mit biologischen *in vitro* Versuchen zur MTX-Zellsuszeptibilität.