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Liposome-mediated delivery of pteridine antifolates to cells in vitro: potency of methotrexate, and its α and γ substituents

Timothy D. Heath [a,b,c], Ninfa G. Lopez [c], James R. Piper [e], John A. Montgomery [e], Walter H. Stern [c,d] and Demetrios Papahadjopoulos [a,b]

[a] *Cancer Research Institute, and Departments of* [b] *Pharmacology and* [c] *Ophthalmology, University of California Medical Center, San Francisco, CA 94143 (U.S.A.),* [d] *Veterans Administration Medical Centre, San Francisco, CA 94121 (U.S.A.) and* [e] *Southern Research Institute, Birmingham, AL 35255 (U.S.A.)*

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We have examined the growth-inhibitory potency of several pteridines encapsulated in negatively charged liposomes, including methotrexate, methotrexate-γ-methylamide, methothrexate-γ-dimethylamide, methotrexate-α-aspartate, and a lipophilic methotrexate-phosphatidylethanolamine conjugate. The potency of encapsulated methotrexate is greater than the potency of the free drug for CV1-P cells, but not for other cell lines. The potency of methotrexate-γ-methylamide and methotrexate-γ-dimethylamide is only minimally improved by encapsulation. The potency of methotrexate-α-aspartate is increased by encapsulation. In addition, the lipophilic methotrexate derivative has demonstrable potency when incorporated in liposomes. We have also examined the potency of several pteridines under conditions where the cells are exposed to the drug for periods shorter than the entire growth assay. Reduction of the exposure time decreases the potency of both encapsulated and free drugs. However, the difference in potency between the encapsulated and free drug is increased, because the potency of the encapsulated drug is affected less. Consequently, encapsulated methotrexate-γ-aspartate is 300-fold more potent than free drug, if CV1-P cells are exposed to drug for 4 h. Moreover, encapsulated methotrexate is more potent than free methotrexate for growth inhibition of L929 fibroblasts, if the term of exposure is less than 8 h. Potency is least affected by reduction of exposure length for the lipophilic methotrexate derivative.

Introduction

The mechanism by which liposomes deliver drugs is one of the most important considerations for optimal liposome-mediated drug delivery. Liposome dependence may be defined as the ability of a drug to act exclusively on the cell to which it is delivered by the liposome [1]. Liposome dependence is most simply demonstrated in cases where the potency of the drug is increased by encapsulation, because the ambiguity arising from the possible uptake of free drug that leaks from the liposomes is eliminated. We have exemplified liposome dependence with methotrexate-γ-aspartate, a methotrexate derivative whose potency is increased by antibody-directed liposomes [2], or by negatively charged liposomes [1]. Negatively charged liposomes will increase the potency of this drug for a wide variety of cell types. Consequently, the in vitro use of negatively charged liposomes may allow the identification of other

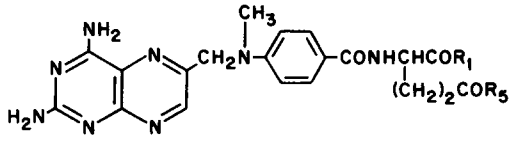
Correspondence address: Dr. T.D. Heath, The University of Wisconsin, School of Pharmacy, 425 North Charter Street, Madison, WI 53706, U.S.A.

liposome-dependent drugs, and may also allow the further study of the mechanism of liposome-dependent drug delivery [1,3].

To further our understanding of liposome-dependent drugs, we have examined the pteridine antifolates in more detail. We have examined whether methotrexate can be liposome-dependent when encapsulated in negatively charged liposomes, particularly in a cell line that is very sensitive to liposome-mediated delivery. In addition, we have examined the liposome-mediated delivery of several other substituents of methotrexate, including methotrexate-γ-dimethylamide, methotrexate-γ-methylamide, methotrexate-α-aspartate, and a lipophilic derivative. We have also examined the effects of changing the length of the exposure time on the relative potency of these drugs.

Materials and Methods

All phospholipids and reagents were obtained or purified as previously described [1]. Large unilamellar liposomes were prepared by the method of Szoka and Papahadjopoulos [4] and extruded by passage through polycarbonate filters [5]. Sonicated liposomes were prepared as previously described [1]. Methotrexate was obtained from Lederle (Pearl River, NY). The derivatives of methotrexate used in this study are listed in Fig. 1, and were synthesized as outlined below, or as previously described [6]. The drugs were dissolved in a buffer solution pH 6.7, 290 mosM · kg^{-1}, containing 50 mM morpholinoethanesulfonic acid,



Compound No.	R_1	R_5	Abbreviation
4a	OH	Asp	MTX-γ-Asp
4b	OH	NHMe	MTX-γ-NHMe
4c	OH	$N(Me)_2$	MTX-γ-$N(Me)_2$
4d	Asp	OH	MTX-α-Asp
4e	Gly	NH_2	MTX-α-Gly-γ-NH_2

Fig. 1. The structure of methotrexate derivatives.

50 mM morpholinopropanesulfonic acid, 1 mM ethylenediaminetetraacetic acid, and NaCl. Drugs were used at an original concentration of 5 mM for liposome preparation. The liposomes were separated from the unencapsulated drug by passage through a 1×10 cm Sephadex G75 column (Pharmacia, New Jersey) equilibrated with the same buffer solution. Sterility was maintained throughout the procedure as previously described [1]. The liposome fractions were analysed for their drug content after extraction [7]. The absorbance of the upper phase was determined at 370 nm, and the drug content was calculated assuming a molar extinction coefficient of 7943 for all compounds. The phospholipid content of the vesicles was measured by phosphorus determination [8].

The growth-inhibitory properties of the encapsulated drugs were measured on five cell lines. The five cell lines, their sources, and the media and conditions for growth, and the growth-inhibition assay have been previously described [1]. For some growth-inhibition studies, the length of cell exposure to drug was less than the total period of growth. In these studies, the medium containing the drug was aspirated at the end of the drug incubation period, the cells were gently washed with phosphate-buffered saline containing 0.8 mM calcium and 0.8 mM magnesium, and fresh medium at 37°C was added. The cells were returned to incubation at 37°C for the rest of the growth period. Cells that were preloaded with methotrexate were similarly washed after a 1 h incubation in medium containing 3 μM methotrexate. In our previous experiments, CV1-P cells were grown for 48 h [1]. In the present study, the growth period for CV1-P cells was 72 h, unless otherwise indicated in the legends, because this cell line grows more slowly than the others.

The route used to prepare MTX-α-Gly-γ-NH_2 (4e) is outlined in Fig. 2. This approach is an adaptation of the general route used for the synthesis of analogous 4a–d [6]. The protected N^2-(4-(methylamino)benzoyl)-L-glutamine derivative 1 was coupled with glycine benzyl ester by the mixed anhydride method using *i*-butyl chloroformate to give the pure protected glutaminylglycine derivative 2 (m.p. 140–142°C, from acetonitrile) [9].

Deprotection by hydrogenolysis with 30% pal-

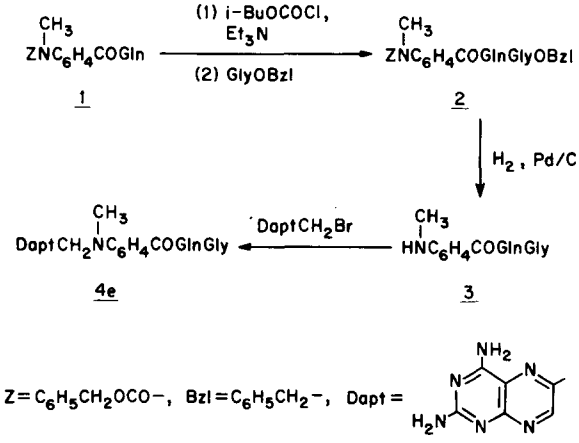


Fig. 2. The synthesis of *N*-[*N*^{2}-[4-[[(2,4-diamino-6-pteridinyl) methyl]methylamino]benzoyl]-L-glutaminyl]glycine (4e).

ladium on carbon in dioxane/methanol (9 : 1) gave *N*-[*N*^{2}-(4-(methylamino)benzoyl)-L-glutaminyl] glycine (3). Alkylation of 3 with 6-(bromomethyl) -2,4-pteridinediamine produced 4e [10]. Elemental analysis of 2, 3 and 4e gave values which agree with calculated values. The mass and ^{1}H-NMR spectra of 3 and 4e were consistent with the assigned structures. The use of HPLC showed 4e to be of greater than 99% purity [11].

50 μmol egg phosphatidylethanolamine (transesterified) was dissolved in 5 ml dry methanol together with 70 μmol 4e and 100 μmol triethylamine. The conjugation was initiated by the addition of 70 μmol of dicyclohexylcarbodiimide, and was allowed to proceed overnight at room temperature. Thin-layer chromatography on silica gel plates developed with acetone/methanol (3 : 1) showed a fast-moving spot ($R_F = 0.8$), which was positive for phosphorus stain, negative to ninhydrin, and visibly yellow. A small amount of a phosphorus-containing component with an R_F of 0.15 was detected. This corresponded to unmodified phosphatidylethanolamine. The material was purified on a silica gel column eluted with acetone/methanol (3 : 1). The fractions containing the derivative were pooled, dried down to remove the solvent, and stored in chloroform/methanol (1 : 1). Purity was checked by thin-layer chromatography, and by analysis for phosphorus and pteridine content. The predicted structure for the product is shown in Fig. 3. This compound will be

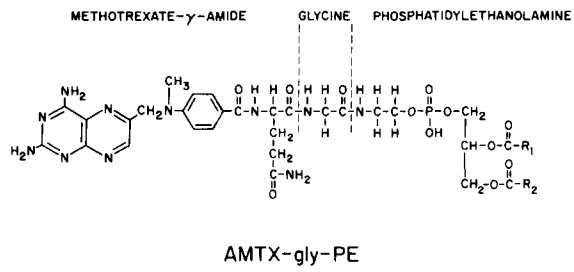


Fig. 3. The structure of AMTX-gly-PE.

referred to as AMTX-gly-PE.

Liposomes were prepared from AMTX-gly-PE by first mixing it with known amounts of phosphatidylserine and cholesterol in chloroform/methanol (1 : 1). The mixture was dried down in a small screw-capped tube. Buffer was added to suspend the lipid, and the liposomes were sonicated for 30 min. The quantitative incorporation of the pteridine into the bilayer was verified with some batches of liposomes by passing them through a 1 × 10 cm column of Sephadex G-50. The pteridine and the liposomes eluted as a single peak in the early fractions from the column. For all preparations, the pteridine content of the vesicles was determined from the absorbance at 373 nm after solubilization of a sample of the vesicles in chloroform/methanol/water (1 : 3 : 1). The vesicles were then diluted and used for growth-inhibition studies as described above.

Results and Discussion

Methotrexate

Methotrexate encapsulated in antibody-conjugated liposomes has been extensively studied by Leserman et al., and was shown to be liposome-dependent [12]. Liposome-dependent delivery of methotrexate has also been demonstrated using a transport-resistant cell line [13]. However, methotrexate in negatively charged liposomes was no more potent than the free drug, and was shown only to act after leakage from the vesicles [14]. This apparent discrepancy seems surprising, as one might expect a liposome-dependent drug to be liposome-dependent in all types of liposomes. Therefore, we wished to establish whether methotrexate can be liposome-dependent when en-

capsulated in negatively charged liposomes, particularly in a cell line that is very sensitive to liposome-mediated delivery.

Table I shows the growth-inhibitory potency of free and encapsulated methotrexate for the five cell lines that we previously used to study methotrexate-γ-aspartate [1]. In most cases, the potency of methotrexate was not increased by encapsulation in liposomes. However, encapsulated methotrexate is 9-times more effective than the free drug for growth inhibition of CV1-P cells. CV1-P cells were also the most sensitive of the five lines that we had tested for delivery of methotrexate-γ-aspartate [1]. This result suggests that liposome-dependent delivery of methotrexate in negatively charged liposomes can be demonstrated if it is efficient enough not to be surpassed by the effects of free drug. It is interesting to note that for CV1-P cells the IC_{50} of methotrexate encapsulated in 0.1 μm phosphatidylserine/cholesterol (67 : 33) liposomes is the same as the IC_{50} of methotrexate-γ-aspartate in the same liposome type [1]. These two compounds will have similar potency if delivered intracytoplasmically. This result suggests that methotrexate and methotrexate-γ-aspartate are comparably delivered to the cells by the liposomes.

Methotrexate-γ-methylamide and methotrexate-γ-dimethylamide

There are several other γ-substituents of methotrexate other than methotrexate-γ-aspartate that exhibit higher values for the influx K_m than methotrexate, while possessing a similar K_i for dihydrofolate reductase [6]. We wished to examine methotrexate-γ-methylamide and methotrexate-γ-dimethylamide to see if their potency could also be increased by encapsulation in liposomes. Table II shows the growth-inhibitory potency of methotrexate-γ-methylamide and methotrexate-γ-dimethylamide. Methotrexate-γ-methylamide is more effective for growth inhibition of CV1-P cells and Cl 18 cells when encapsulated in liposomes. However, encapsulated methotrexate-γ-methylamide is no more effective for growth inhibition of RAW 264 than the free compound, and less effective than the free compound for growth inhibition of L929 and AKR/J SL2 cells. Encapsulated methotrexate-γ-dimethylamide is

TABLE I

GROWTH-INHIBITORY POTENCY OF FREE AND ENCAPSULATED METHOTREXATE

Drug was encapsulated in phosphatidylserine : cholesterol (67 : 33)liposomes prepared by reverse-phase evaporation and extruded to 0.1 μm. The final drug : lipid ratio was 6.5 mmol/mol. Growth inhibition due to lipid was assessed from previous growth-inhibition studies with nonloaded liposomes [1], and is indicated by (−) = no lipid effects, (+) = partial lipid involvement, (++) = growth inhibition totally due to the lipid. CV1-P cells were allowed to grow for 48 h.

Cell type	IC_{50} (μM)	
	free drug	encapsulated
AKR/J SL2	0.015	0.022 (−)
L929	0.011	0.017 (−)
Cl 18	0.017	0.029 (−)
RAW 264	0.017	0.016 (−)
CV1-P	0.04	0.0045 (−)

TABLE II

GROWTH-INHIBITORY POTENCY OF FREE AND ENCAPSULATED METHOTREXATE-γ-METHYLAMIDE, AND METHOTREXATE-γ-DIMETHYLAMIDE

Drug was encapsulated in phosphatidylserine : cholesterol 67 : 33 liposomes prepared by reverse-phase evaporation and extruded to 0.1 μm. The final drug : lipid ratio was 6.4 mmol/mol for methotrexate-γ-methylamide and 6.7 mmol/ml for methotrexate-γ-dimethylamide. Growth inhibition due to lipid was assessed as described in Table I. CV1-P cells were allowed to grow for 48 h.

Cell type	IC_{50} (μM)			
	MTX-γ-methylamide		MTX-γ-dimethylamide	
	free drug	encaps.	free drug	encaps.
AKR/J SL2	0.06	0.13 (−)	0.06	0.14 (−)
L929	0.08	0.12 (−)	0.14	0.20 (−)
Cl 18	0.11	0.03 (−)	0.20	0.16 (−)
RAW 264	0.06	0.06 (−)	0.07	0.14 (−)
CV1-P	0.35	0.04 (−)	0.42	0.11 (−)

less effective than the free compound for growth inhibition of AKR/J SL2, L929 and RAW 264 cells, marginally more effective for CL 18 cells, and only 4-times more effective for CV1-P cells. These results suggest that these compounds are less effectively delivered to cells by liposomes than either methotrexate or methotrexate-γ-aspartate. This can most clearly be seen by examining the potency of the encapsulated drugs for growth inhibition of CV1-P cells. Encapsulated methotrexate-γ-methylamide has an IC_{50} of 0.04 μM, while encapsulated methotrexate-γ-dimethylamide has an IC_{50} of 0.11 μM for CV1-P cells. These values are 9- and 24-times higher than the IC_{50} of either methotrexate or methotrexate-γ-aspartate in liposomes of the same composition and size.

There are a number of possible explanations for this unexpected result. These two compounds may be inactivated through lysosomal processing of the liposomes, or they may be less readily transferred from the lysosomal compartment to the cytoplasm. Alternatively, these compounds may leak more rapidly from the liposomes before delivery can occur. Such leakage may be due to the lower net charge of these monocarboxylic drugs, as compared to methotrexate, which is a dicarboxylate, and methotrexate-γ-aspartate, which is a tricarboxylate. This is consistent with our recent study of fluoroorotate, another monocarboxylate, which is only effectively delivered to cells by liposomes composed of lipids with high phase-transition temperatures [3]. These liposomes are likely to be more resistant to leakage than vesicles composed of low transition temperature lipids.

Methotrexate-α-aspartate

Derivatives of methotrexate with substituents on the α-carboxyl of the glutamyl moiety show high values for the K_m of influx. These derivatives also exhibit K_i values for dihydrofolate reductase that are 10^5-times greater than the K_i of methotrexate [6]. The α-substituents of methotrexate might act as lysosomally cleavable, liposome-dependent pro-drugs, because the α-moiety is linked to methotrexate via a peptide bond that may be susceptible to cleavage by lysosomal peptidases or amidases.

Table III shows the growth-inhibitory potency

TABLE II

THE EFFECT OF NH_4Cl ON THE GROWTH-INHIBITORY POTENCY OF FREE AND ENCAPSULATED METHOTREXATE-α-ASPARTATE

Methotrexate-α-aspartate was encapsulated at 5 mM original concentration in phosphatidylserine:cholesterol 67:33 REV extruded to 0.2 μm, final drug:lipid ratio = 10 mmol/mol. + indicates that the cells were incubated with 5 mM NH_4Cl, which was added 30 min before drug addition. − Indicates no treatment with NH_4Cl. Growth inhibition due to lipid was assessed as described in Table I.

Cell type	IC_{50} (μM)				Growth inhibition by NH_4Cl (% control)
	free drug		encapsulated		
	−	+	−	+	
AKR/J SL2	> 30	2.1	0.47 (++)	0.86 (++)	81
L929	2.8	9.5	0.25 (−)	3.00 (−)	23
Cl 18	2.1	1.7	0.18 (−)	0.21 (−)	17
RAW 264	2.0	2.0	0.12 (−)	0.16 (−)	26
CV1-P	12.1	1.9	0.05 (−)	0.05 (−)	43

of methotrexate-α-aspartate in 0.2 μm phosphatidylserine/cholesterol (67 : 33) liposomes. Encapsulated methotrexate-α-aspartate is in all cases more effective for growth inhibition than the free compound. However, we have assessed the possible contribution of lipid to growth inhibition as previously described [1], and find that the effects of the encapsulated drug on AKR/J SL2 cells are wholly due to the lipid. Lipid does not contribute to growth inhibition for the other cell lines, and the increase in potency ranges from 11-fold for L929 cells to 38-fold for CV1-P cells. The increased potency of methotrexate-α-aspartate will depend on its conversion to methotrexate through the action of lysosomal peptidases. Consequently, the potency of this compound in liposomes supports the previous conclusion that liposome-mediated drug delivery requires lysosomal processing [1,2,12].

The inclusion of 5 mM ammonium chloride in the medium selectively inhibits the potency of encapsulated methotrexate-α-aspartate for L929 cells, which further confirms the involvement of lysosomes in the delivery of the drug. Ammonium

chloride does not affect the potency of the encapsulated drug for growth inhibition of other cell lines. However, we similarly observed the failure of ammonium chloride to block the delivery of encapsulated methotrexate-γ-aspartate to all lines except L929 and CV1-P, and have discussed the difficulties inherent in these experiments [1].

AMTX-gly-PE

The ability of cells to take up and activate methotrexate-α-aspartate prompted us to consider whether lipid conjugates based on this structure might not be used in liposomes. We designed α-glycylmethotrexate-γ-amide specifically as a molecule which could be conjugated to phosphatidylethanolamine through its only carboxyl, and which could be degraded by a peptidase to release an active methotrexate derivative, methotrexate-γ-amide (see Fig. 3). Table IV shows the potency of this compound in liposomes as a function of its concentration in the liposomal membrane. For all liposome preparations, the IC_{50} for CV1-P cells appears to be close to 1 μM. The preparation with the lowest drug : lipid ratio is the most potent, but calculations indicate a likely involvement of lipid in the effects of this preparation.

The above results with AMTX-gly-PE demonstrate that this compound can be delivered and is degraded to a cytotoxic molecule, but are somewhat disappointing when compared to the results obtained with methotrexate-α-aspartate. This suggests that this molecule may not be rapidly degraded, perhaps due to the low susceptibility of the molecule to degradative enzymes. This conclusion is supported by the observations of Hashimoto et al. [15], who have recently synthesized several conjugates of methotrexate with phosphatidylethanolamine. They have found that the conjugate in which the linkage is via the α-carboxyl of methotrexate is much less effective than the conjugate linked via the γ-carboxyl [16]. More effective lipophilic derivatives might result from the inclusion of a peptide spacer that is more readily degraded by lysosomal enzymes [17].

The inhibition of cell growth by methotrexate requires the complete saturation of the dihydrofolate reductase pool [18,19]. This complete saturation, which requires a relatively large amount of methotrexate, is only maintained if the cells contain a small amount of free methotrexate. Loading of the cells with the drug and subsequent removal will result in the inhibition of 98% of the enzyme, but will not in any way impair cell growth. We therefore considered that preloading of cells with methotrexate might selectively enhance the potency of encapsulated drugs, particularly in cases such as AMTX-gly-PE where the release of the active drug may be slow.

We first tested the approach on CV1-P cells, using methotrexate-γ-aspartate encapsulated in sonicated liposomes at two different drug : lipid ratios (Table V). Preloading increased the potency by 2.3-fold for the free drug, and by 2.7- and 3.1-fold for the encapsulated drug. Two points should be noted from the non-preloaded data in this experiment. First, the liposomes containing 10.3 mmol drug per mol lipid are the most potent, and are about half as potent as 0.1 μm vesicles, which is consistent with previous observations [1].

TABLE IV

THE GROWTH-INHIBITORY POTENCY OF AMTX-gly-PE FOR CV1-P CELLS

Liposomes were prepared from phosphatidylserine : cholesterol 67 : 33 by suspension and sonication. The AMTX-gly-PE was added to the lipid mixture in the amounts indicated, substituting derivative for phosphatidylserine. Normal indicates that the CV1-P cells were treated directly with the drug. Pretreated cells were exposed to 3 μM methotrexate for 1 h, were washed with phosphate-buffered saline, and returned to fresh medium prior to drug treatment. The control wells, which were also treated in this way, showed no growth inhibition from the transient methotrexate exposure. The ratio of the IC_{50} obtained on normal and pretreated cells indicates the extent to which the pretreatment has increased the potency of the preparation. Growth inhibition due to lipid was assessed as described in Table I.

Drug : lipid (mmol/mol)	IC_{50} (μM)		Normal/ pretreated
	normal	pretreated	
10	0.94	0.60	1.57
	(+)	(−)	
25	0.81	0.47	1.72
	(−)	(−)	
100	1.05	0.27	3.89
	(−)	(−)	
200	1.05	0.26	4.04
	(−)	(−)	

TABLE V

THE EFFECT OF METHOTREXATE PRETREATMENT OF CV1-P CELLS ON THE POTENCY OF FREE AND ENCAPSULATED METHOTREXATE-γ-ASPARTATE

Liposomes were prepared from phosphatidylserine : cholesterol 67 : 33 by suspension and sonication. Vesicles containing 1.2 mmol drug per mol lipid were prepared in a buffer solution containing 5 mM methotrexate-γ-aspartate and 20 mM carboxyfluorescein, and the drug content was estimated from the carboxyfluorescein capture. Vesicles containing 10.3 mmol drug per mol lipid were prepared in a buffer solution containing 50 mM methotrexate-γ-aspartate, and the drug was estimated directly as described in Methods. Normal and pretreated and the ratio between them are as described in Table IV. Growth inhibition was assessed as described in Table I.

Drug : lipid (mmol/mol)	IC_{50} (μM)		Normal/ pretreated
	normal	pretreated	
Free drug	0.7	0.3	2.33
1.2	0.054	0.017	3.18
	(−)	(−)	
10.3	0.0094	0.0035	2.68
	(−)	(−)	

Second, the vesicles containing 1.2 mmol drug per mol lipid were 5-fold less potent than the vesicles containing 10.3 mmol drug per mol lipid. We previously observed no change in drug potency with changes in drug : lipid ratio using 0.1 μm liposomes. However, we did not go to such low drug : lipid ratios, and we were using larger liposomes, which would require a lesser number of particles to be taken up in order to allow for comparable drug delivery.

For AMTX-gly-PE, preloading of the cells with methotrexate increases the potency of the preparations by as much as 4-fold. This is slightly higher than the enhancement seen for encapsulated methotrexate-γ-aspartate, and twice the enhancement seen for free methotrexate-γ-aspartate. This supports our hypothesis, and suggests that the limited potency of the AMTX-gly-PE may be due to the slow rate of degradation. We noted above that there is likely to be some involvement of lipid in the effects seen for the lowest concentration of the derivative in the liposomes. This probably explains why the effects of this preparation are least enhanced by the preloading of the cells with methotrexate.

Length of cell exposure to drug

Fig. 4 shows the effect of changing the length of exposure on the potency of methotrexate-γ-aspartate for growth inhibition of CV1-P cells. Potency changes with the length of exposure for both free and encapsulated drug, but changes most for the free drug. The difference between the potency of the free and the encapsulated drug is 90-fold when the exposure is continuous, but greater than 300-fold at the earliest exposure time where comparison can be made (4 h). Similar results were obtained with L929 cells (not shown).

Fig. 5 shows the change in potency of free and encapsulated methotrexate with exposure time. The IC_{50} of free and encapsulated drug is the same when the L929 cells are exposed for 24–48 h. The potency of both free and encapsulated drug is reduced by reducing the length of exposure. However, the encapsulated drug can be as much as 10-times more effective than the free drug if the cells are exposed to drug for only 1–4 h. This result confirms that the potency of encapsulated pteridines is much less affected by the length of

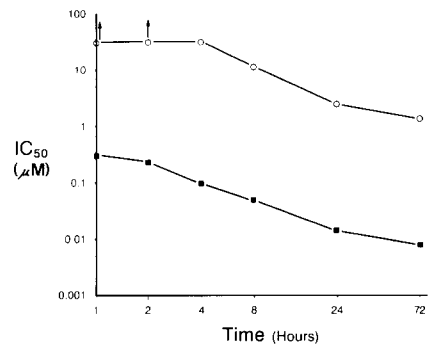


Fig. 4. The effect of the length of exposure of CV1-P cells on the potency of free and encapsulated methotrexate-γ-aspartate. The drug was encapsulated in liposomes prepared from phosphatidylserine : cholesterol 67 : 33 by the method of reverse-phase evaporation. The liposomes were sized to 0.2 μm by extrusion through polycarbonate filters. The original drug concentration was 5 mM and the final drug : lipid ratio was 10 mmol/mol. The cells were exposed to the drug and were washed free of the drug at the times indicated as described in Methods. No liposome effects can be attributed to lipid as the lipid concentration at the IC_{50} does not exceed that which would cause growth inhibition even in continuous exposure. In 1 and 2 h of exposure, the IC_{50} of the free drug is greater than the highest drug concentration used (30 μM), and is indicated by ↑ next to these values.

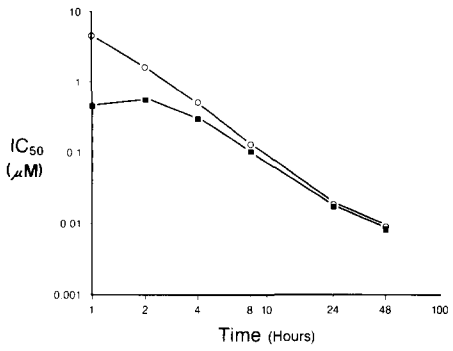


Fig. 5. The effect of the length of exposure of L929 cells on the potency of free and encapsulated methotrexate. The drug was encapsulated in liposomes prepared from phosphatidylserine:cholesterol 67:33 by reverse-phase evaporation. The liposomes were not sized by extrusion. The original drug concentration was 5 mM and the final drug:lipid ratio was 24 mmol/mol. The liposome effects cannot be attributed to lipid.

the exposure than the potency of the free compound. This result also confirms that methotrexate is a liposome-dependent drug.

In designing AMTX-gly-PE, we considered that its primary advantage should derive from its inability to leak from the liposomes. Therefore, the potency of AMTX-gly-PE might best be demonstrated in a limited-exposure study similar to the above experiments with methotrexate-γ-aspartate and methotrexate. Fig. 6 shows the potency of this compound in liposomes for growth inhibition of CV1-P cells as a function of the time of cell

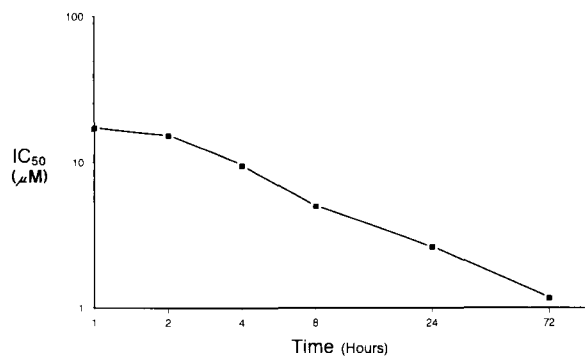


Fig. 6. The effect of the length of exposure of CV1-P cells on the potency of AMTX-gly-PE. Liposomes were prepared from phosphatidylserine:cholesterol:AMTX-gly-PE, 57:33:10, by extensive sonication. No effects can be attributed to lipid other than the AMTX-gly-PE.

exposure. The slope of the curve is clearly shallower than that seen with methotrexate-γ-aspartate. The potency of encapsulated methotrexate-γ-aspartate is increased 43-fold by increasing the exposure time time from 1 to 72 h (Fig. 4), while the potency of AMTX-gly-PE is increased only 15-fold by the same change in exposure time.

The above experiments suggest that the potency of encapsulated pteridines exceeds that of the free drug by the most when the length of exposure is shortest. Previous studies have suggested that endocytosis of the liposomes is the major pathway of drug deliver [1,2,12]. Association studies show that liposomes rapidly associate with the cells and suggest that they are subsequently internalized at a rate which is much slower than the initial binding rate [20,21]. If cells are exposed to the liposomes for only a short period, the liposomes that bind rapidly to the cell surface would act as a reservoir of material to be internalized over a longer period. In optimal circumstances the reservoir of drug would be created rapidly, and would provide sufficient drug to maintain growth inhibition throughout the entire 48 or 72 h. If this occurred, the IC_{50} would be the same regardless of the period of exposure. In fact, the IC_{50} decreases with the increase in the exposure length. This decrease may be due either to leakage of drug from the surface-bound liposomes, or to the inability of sufficient drug to be associated with the cells in this way, or to both phenomena.

In conclusion, much information can be gained on the mechanism of drug delivery from in vitro drug delivery studies. It is also possible by rigorous design of experiments to develop systems that will allow the selection of appropriate drugs for liposome-dependent delivery. In this paper we further explored the selection of pteridines and have demonstrated the use of lipophilic conjugates, of the alpha substituents, and of procedures involving transient drug exposure. We hope in future studies to explore other classes of drugs significant in the therapy of cancer and other diseases for which drug delivery is a relevant approach.

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