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Synthesis and characterization of methotrexate-dimyristoylphosphatidylethanolamine derivatives and the glycerophosphorylethanolamine analogs

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Research in this laboratory is currently focused on the biochemical and pharmacological properties of liposomes in which an otherwise water-soluble drug is anchored to the lipid bilayers via an appropriate non-polar residue. To this end, we have synthesized three (I–III) methotrexate (MTX) derivatives of dimyristoylphosphatidylethanolamine (DMPE) by conjugation of the α and/or γ glutamyl carboxyl groups of the drug with the amino function of the phospholipid. These derivatives have been characterized analytically and chromatographically as MTX-γ-DMPE (I), MTX-α-DMPE (II), and MTX-α,γ-diDMPE (III). The corresponding glycerophosphorylethanolamine analogs have also been prepared and identified. The biological activity of these compounds (as inhibitors of in vitro cell proliferation and dihydrofolate reductase) is described in the following paper.

Introduction

The use of monoclonal antibodies to target a drug(s) to specific cells has been extensively investigated in recent years. For this purpose, the drug may be either covalently coupled directly to the antibody or be associated with a vehicle, such as liposomes, to which the antibody has been attached. Furthermore, in the case of liposome-mediated delivery, the drug can either be trapped within the aqueous compartments or incorporated in the lipid bilayers depending on its solubility properties.

Although each of these modes has unique advantages and disadvantages, we need briefly mention here only those that prompted the present studies. In the case of an antibody-drug conjugate, the antibody may tolerate substitution with only a limited number of drug molecules before immunological activity is destroyed. Additionally, the synthesis of a conjugate in which an antibody molecule carries two or more different drugs may prove quite difficult. In contrast, combined therapy would appear feasible with liposomes bearing encapsulated drugs, particularly in light of the development of methods for preparing unilamellar liposomes with a very large capture volume. However, in vivo leakage of drugs from the aqueous compartments of such liposomes remains a serious problem in spite of efforts to control this by modification of liposome composition. Leakage (i.e., covalent bond rupture) of an active drug from its antibody carrier has not been documented yet.

Leakage also may not be a significant problem

Abbreviations: MTX, methotrexate; DMPE, dimyristoylphosphatidylethanolamine; glyceroPE, glycerophosphorylethanolamine.

with liposomes in which the drug is incorporated within the lipid bilayers as a minor integral component. Moreover, the liposomal bilayers could accommodate several drugs simultaneously, although probably much lower amounts could be inserted than the quantity of drugs that can be encapsulated within the aqueous compartments. To date, however, studies of the properties of these liposomes have been confined to those prepared with single drugs (e.g., amphotericin B, actinomycin D) that are sufficiently soluble in a non-polar environment. By employing appropriate amphipathic derivatives of water-soluble drugs, the potential therapeutic applications of such liposomes should, accordingly, be extended.

These considerations led us to prepare the three possible derivatives of methotrexate (MTX) in which its carboxyl groups are conjugated to the amino function of dimyristoylphosphatidylethanolamine (DMPE). Methotrexate was chosen for this purpose because a basis for comparison is provided by the numerous in vitro investigations utilizing liposomes that contain this potent cytotoxic drug trapped in the aqueous regions (see, for example, Refs. 1–6). In the present paper, we describe the synthesis, isolation, and characterization of the MTX-DMPE derivatives and their glycerophosphorylethanolamine (glyceroPE) analogs. The following paper [7] details the ability of liposomes prepared with these derivatives, and the capacity of the analogs, to inhibit in vitro cell proliferation and dihydrofolate reductase (EC 1.5.1.3). A subsequent report (in preparation) concerns the immunogenicity of liposomes sensitized with these derivatives insofar as the induction of anti-methotrexate antibodies may influence in vivo liposomal therapy.

Materials and Methods

Chemicals. These were obtained commercially as follows: dimyristoylphosphatidylethanolamine (Avanti Polar Lipids, Birmingham, AL); chloroform, dioxane, ethylacetate, methanol, and triethylamine (Fisher Scientific Co., St. Louis, MO); *N*,*N*′-dicyclohexylcarbodiimide, *N*-hydroxysuccinimide, and methotrexate (Sigma Chemical Co., St. Louis, MO). The above were used without further purification except that *N*-hydroxysuccinimide was recrystallized from ethylacetate, and methanol and triethylamine were redistilled (the latter over ninhydrin to remove primary amine contaminants).

Synthesis and purification of MTX-DMPE derivatives. Methotrexate (40 μmol) was dissolved in 0.8 ml of a 1 : 1 volume mixture of chloroform and methanol (hereafter abbreviated C/M) containing triethylamine (240 μmol). The following were then added sequentially to this solution while stirring; DMPE (120 μmol) dissolved in 5.6 ml of C/M; *N*-hydroxysuccinimide (200 μmol) dissolved in 0.8 ml of C/M; *N*,*N*′-dicyclohexylcarbodiimide (200 μmol) dissolved in 0.8 ml of C/M. After incubation for 3 h at room temperature, the reaction mixture was taken to dryness by rotary evaporation under reduced pressure at 40°C, and the residue was redissolved in 2 ml of C/M.

Chromatographic separation of the MTX-DMPE derivatives was accomplished by streaking 250 μl of this fraction on each of 8 analytical thin-layer plates (Silica gel 60 F-254, 0.25 mm, Brinkmann Instruments, Inc., Westbury, NY). The plates were developed in a solvent system of chloroform/methanol/water (65 : 30 : 5, by vol.). After development, four yellow bands (I–IV) were visible that also gave a positive test for phosphate when sprayed with an acid molybdate reagent (Applied Science, Deerfield, IL). These bands had approximate R_F values of 0.18 (I), 0.28 (II), 0.39 (III), and 0.49 (IV), whereas the R_F for the unreacted methotrexate band, which did not stain for phosphate, was 0.06. Bands I–IV were individually scraped from the plates and suspended in 5 ml of methanol. After centrifugation (750 × *g* for 10 min at 4°C), the yellow supernatant was recovered, and the pelleted silica gel particles were reextracted with another 5 ml of methanol. Ten ml of chloroform was added to the combined supernatants, and this solution was layered over a 50 mm high bed of Unisil (Clarkson Chemical Co., Williamsport, PA) at the bottom of a 1 × 20 cm column. The Unisil previously had been washed extensively with chloroform, followed by C/M. The yellow compounds (designated MTX-DMPE derivatives I, II, III, and IV) were subsequently eluted by passage of 20 ml of C/M. Each eluate was taken to dryness, and the residue was redissolved in 5 ml of C/M and stored at −20°C.

Synthesis and purification of MTX-glyceroPE analogs. The analogs were prepared from the corresponding MTX-DMPE derivatives by mild alkaline hydrolysis. This was accomplished using a minor modification of the procedure employed by Dawson [8] for phospholipid analysis. Briefly, each derivative (2 μmol) was dissolved in methanol (3.2 ml), followed by the addition of ethanol (30 ml), water (2.6 ml) and 1 M NaOH (1 ml). After incubation at 37°C for 20 min, the reaction mixture was chilled and passed over a column (1.6 × 7.5 cm) of Bio-Rex-70 (Bio-Rad, Richmond, CA). The resin had been previously converted to the H^+ form with 1 M HCl (30 ml), followed by successive washes with water (100 ml) and 80% ethanol (30 ml). The initial eluate and the fraction collected with 80% cold ethanol (40 ml) were combined and taken to dryness. The residue was resuspended in water and fatty acid was removed by partition with a mixture of chloroform-isobutyl alcohol, and extraction with *n*-hexane, essentially as described elsewhere for the isolation of dinitrophenylated-glyceroPE [9]. The material in the final aqueous fraction was recovered by lyophilization.

Miscellaneous. The phosphate content of the MTX-DMPE derivatives and MTX-glyceroPE analogs was assayed by a minor modification of the method of Gerlach and Deuticke [10]. The methotrexate content of these compounds was determined from their peak b absorbance between 298 and 304 nm. The precise location of this peak, as well as the corresponding molar extinction coefficient for methotrexate, was determined in the appropriate solvent system specified in the figure legends.

Results and Discussion

Characterization of MTX-DMPE derivatives

Methotrexate contains two carboxyl groups in the glutamyl portion that are capable of forming amide bonds with the amino group of DMPE. Accordingly, three MTX-DMPE derivatives might be expected as products under the synthetic conditions employed. One of these derivatives should contain DMPE residues linked to both the α and γ carboxyl groups. The other two derivatives should contain only a single DMPE residue attached to either the α or γ carboxyl group.

However, as mentioned above (Materials and Methods), four phosphate-containing bands (I–IV) were consistently detected after thin-layer chromatographic resolution of the reaction mixture. The yellow materials eluted from each of these bands gave spectra, which had absorbance maxima (peaks a, b, and c), that very closely resembled the spectrum of methotrexate (Fig. 1). The calculated molar extinction coefficients for methotrexate and the derivatives were nearly identical as indicated by the values for peak b (Fig. 2).

MTX-DMPE IV possessed unusual properties (Fig. 2), which lead us to suspect that it may be either a side product or an intermediate in the formation of derivatives I, II, and III. This derivative had a phosphate : methotrexate ratio of 1, indicating that only one of the carboxyl groups had been substituted with DMPE. However,

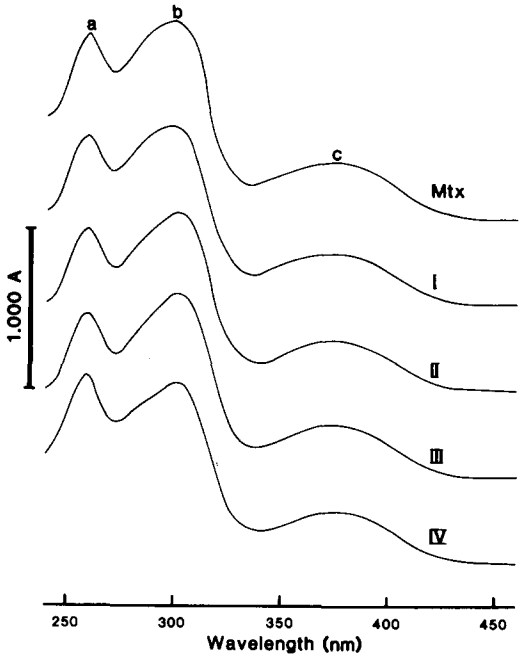
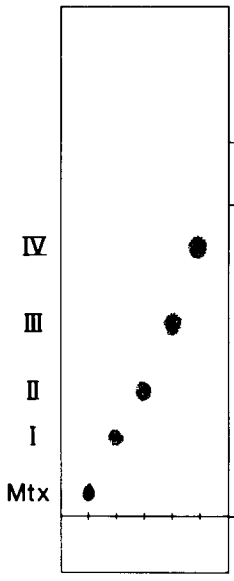


Fig. 1. Spectra of methotrexate and MTX-DMPE derivatives I, II, III, and IV. Solutions were made on the basis of the phosphate content of each derivative, and contained 40 μM phosphate (in the case of I, II, and IV) and 80 μM phosphate (in the case of III). The spectrum of methotrexate (Mtx) was determined on a 40 μM solution prepared on a weight basis. The solvent was chloroform/methanol/water (1 : 2 : 0.8, by vol.).



	Rf	Yield (%)	ε (peak b)	P:Mtx ratio
IV	0.53	5.9	28,310	1.03
III	0.38	9.4	28,560	1.98
II	0.24	7.5	27,660	0.97
I	0.15	17.5	27,690	1.01
Mtx	0.04	–	30,220	0

Fig. 2. Properties of methotrexate and MTX-DMPE derivatives. The R_F values were determined by thin-layer chromatography in chloroform/methanol/water (70:30:5, by vol.). Approx. 40 nmols of methotrexate and each derivative were spotted. Methotrexate was visualized by its yellow color and a positive reaction with iodine vapor; these tests were also used to detect the derivatives along with a positive reaction for phosphate (acid molybdate spray) and a negative reaction for amino groups(ninhydrin spray). The yields, calculated relative to the starting amount of methotrexate (Mtx), was the average obtained from three preparations. The molar extinction coefficients for peak b were determined from the spectra presented in Fig. 1. These calculations take into consideration the fact that the solution of III had twice the phosphate (P) concentration of the solutions of I, II, and IV.

MTX-DMPE IV was considerably more non-polar than either derivative I, II, or III as evidenced by a higher R_F value in the solvent system employed. Additionally, in the course of developing the purification procedure, we found that MTX-DMPE IV could be eluted from silica gel by 70% chloroform in methanol. On the other hand, MTX-DMPE I and MTX-DMPE II, which also had a phosphate: methotrexate ratio of 1, required 100% methanol for quantitative elution. These observations suggest that the second carboxyl in MTX-DMPE IV is neither ionizable nor substituted by a charged (DMPE) group. This carboxyl could, for example, be present in ester linkage with *N*-hydroxysuccinimide or as an acylurea adduct with dicyclohexylcarbodiimide [11]. Further characterization of IV was not attempted, particularly because the compound slowly decomposed during isolation and/or storage with the trace appearance of materials that behaved chromatographically like derivatives I and II.

In contrast to MTX-DMPE IV, the other derivatives were stable to storage at −20°C for at least 6 weeks. MTX-DMPE III had a phosphate: methotrexate ratio of 2, indicating that both carboxyl groups were linked to DMPE. This conclusion is also supported by the fact that MTX-DMPE III possesses a higher R_F value than either derivative I or II (Fig. 2). As already noted, MTX-DMPE I and II each contained equivalent amounts of phosphate and methotrexate, which is consistent with a structure in which only one of the carboxyl groups is linked to DMPE. Proof of this relationship between the derivatives is also provided by the demonstration that MTX-DMPE I and MTX-DMPE II can each be converted to MTX-DMPE III (as well as the side product,

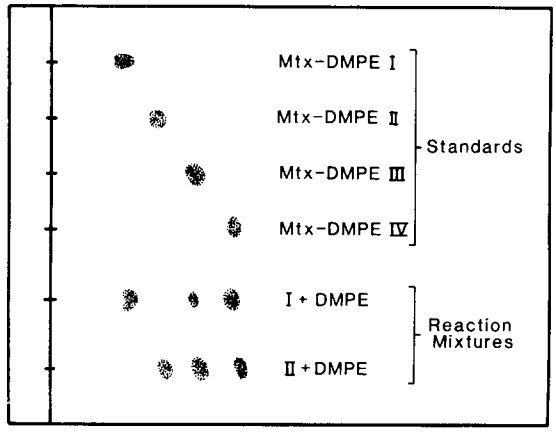


Fig. 3. Conversion of MTX-DMPE I and MTX-DMPE II to MTX-DMPE III and MTX-DMPE IV. Tubes initially contained 0.5 μmol of either MTX-DMPE I or MTX-DMPE II, 1.5 μmol of DMPE, and 3 μmol of triethylamine in 300 μl of chloroform/methanol (1:1, v/v) (C/M). *N*-Hydroxysuccinimide (2.5 μmol in 100 μl of C/M) and *N*,*N*′-dicyclohexylcarbodiimide (also 2.5 μmol in 100 μl of C/M) were then added. After incubation for 20 h at room temperature, aliquots of each reaction mixture (50 μl) and appropriate amounts of each reference derivative (30 nmol) were analyzed by thin-layer chromatography in chloroform/methanol/water (70:30:5, by vol.). The compounds were detected as described in the legend to Fig. 2.

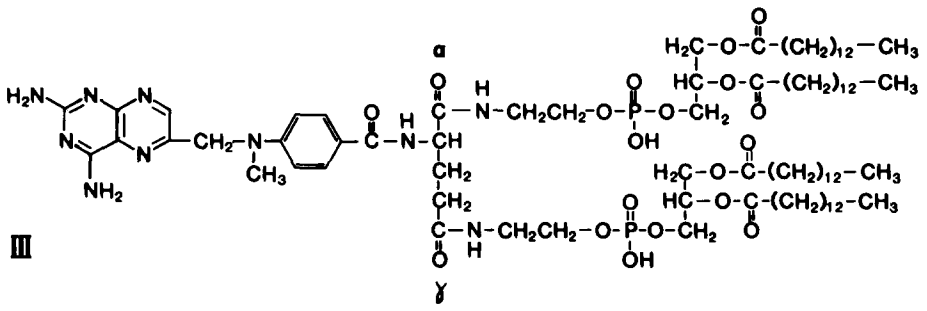


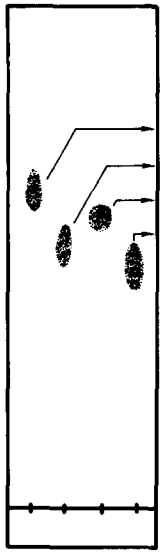
Fig. 4. Proposed structure of MTX-DMPE derivative III. Both the α and γ glutamyl carboxyl groups of methotrexate are attached via amide bonds to the amino group of DMPE. The available evidence indicates that derivative I contains a single DMPE residue conjugated to the γ carboxyl, whereas derivative II has a single DMPE residue attached to the α carboxyl. See text for additional details.

MTX-DMPE IV) by further incubation with DMPE in the presence of *N*-hydroxysuccinimide and *N,N'*-dicyclohexylcarbodiimide (Fig. 3).

On the basis of the preceding results, the structure proposed for MTX-DMPE III is MTX-α,γ-di-DMPE (Fig. 4). Regarding the location of the DMPE residues in derivatives I and II, it is significant that MTX-DMPE I is more polar than MTX-DMPE II as revealed by a lower R_F value (Fig. 2). Because the glutamyl α carboxyl of methotrexate is a stronger acid than the γ carboxyl group by 1.4 pK_a units [12], this finding suggests that the DMPE residue is linked to the γ carboxyl in MTX-DMPE I, and the α carboxyl in MTX-DMPE II. This suggestion finds precedence in the work of Rosowsky and Yu [11]. They characterized the three major products formed between methotrexate and diethyl-L-glutamate in the presence of various coupling reagents. One of the products, methotrexate-γ-L-glutamate diethyl ester (akin to MTX-DMPE I) displayed a slower mobility when subjected to thin-layer chromatography than methotrexate-α-L-glutamate diethyl ester (akin to MTX-DMPE II). It is also significant that the third product, methotrexate-α,γ-bis(L-glutamate tetraethyl ester), had solubility properties comparable to those of MTX-DMPE III.

Characterization of MTX-glyceroPE analogs

Treatment of the MTX-DMPE derivatives with mild alkali converts them into substances that remain at the origin when analyzed by thin-layer chromatography in the chloroform/methanol/water (70 : 30 : 5, by vol.) solvent system used to separate the parent materials. This behavior is consistent with the hydrolytic removal of the fatty acid (myristic) residues. The glyceroPE analogs can, however, be reproducibly distinguished in a solvent system of ethanol/water/acetic acid (70 : 30 : 5, by vol.). A diagram of a representative plate is shown in Fig. 5, along with the essential



	Rf	Yield (%)	ε (peak b)	P:Mtx ratio
Mtx	0.63	—	25980	0
Mtx-GPE I	0.52	69	23595	1.10
Mtx-GPE II	0.58	65	24180	1.07
Mtx-GPE III	0.48	68	24040	2.16

Fig. 5. Properties of methotrexate and MTX-glyceroPE analogs. The R_F values were determined by thin-layer chromatography in ethanol/water/acetic acid (70 : 30 : 5, by vol.). Thirty nmols of methotrexate (Mtx) and each analog were spotted; after development, each compound was located by its yellow color which was coincident with an iodine positive reaction. The yields were based on the starting amounts of each MTX-DMPE derivative. The molar extinction coefficients for peak b were calculated from absorption spectra recorded in 0.1 M NaOH.

properties of these compounds. Each glyceroPE analog gave an absorption spectrum (not shown) that was identical to that of methotrexate, as demonstrated by the calculated molar extinction coefficients for peak b. The phosphate : methotrexate ratio of each glyceroPE analog was the same as the ratio of the original DMPE derivative indicating that the dilute alkali did not cleave any amide or phosphoryl bonds (cf. Fig. 4).

Thus, MTX-glyceroPE III corresponds to MTX-α,γ-diglyceroPE. In the following paper, enzymatic evidence will be presented which is consistent with the conclusion that MTX-glyceroPE I and MTX-glyceroPE II can be equated with MTX-γ-glyceroPE and MTX-α-glyceroPE, respectively. Briefly, over a wide concentration range, methotrexate and MTX-glyceroPE I were found to be equal in their ability to inhibit dihydrofolate reductase isolated from L1210 cells, whereas MTX-glyceroPE II was significantly less potent. These results are compatible with the extensive experiments of Piper et al. [13] showing that various γ-substituted amide and peptide analogs of methotrexate (but not the corresponding α-substituted compounds) are as effective enzyme inhibitors as methotrexate.

In our opinion, therefore, the MTX-DMPE derivatives and the glyceroPE analogs, which are described herein, have been sufficiently characterized for the objectives mentioned in the Introduction.

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