International Journal of Pharmaceutics, 85 (1992) 189–197

IJP 02860

Synthesis of methotrexate-dimyristoylphosphatidylethanolamine analogs and characterization of methotrexate release in vitro

Anwen S. Williams, W.G. Love and B.D. Williams

Rheumatology Research Laboratories, University of Wales College of Medicine, Heath Park, Cardiff (UK)

(Received 3 February 1992)
(Modified version received 2 March 1992)
(Accepted 16 March 1992)

Key words: Methotrexate; Conjugate; Hydrolysis; HPLC; Esterase; Arthritis; Phospholipid

Summary

Lipophilic amide derivatives of methotrexate (MTX) were synthesized by covalent linkage to dimyristoylphosphatidylethanolamine (DMPE). These derivatives were characterized by infrared spectroscopy, thin-layer chromatography and colorimetrically as MTX-γ-DMPE (B), MTX-α-DMPE (C) and MTX-α,γ-diDMPE (D). The in vitro release of free drug from each of the conjugates was determined by HPLC after incubation in phosphate buffer pH 7.4 at 37°C. MTX-diDMPE (D) was most stable whilst the mono-substituted derivatives (B and C) released free drug more readily ($t_{10\%}$ = 10, 2.1 and 1.3 days, respectively). The susceptibility of MTX-gamma-DMPE to hydrolysis under more physiological conditions was also investigated. In fresh human plasma and in the presence of high esterase concentrations (10 U/ml), the rate of hydrolysis was increased ($t_{10\%}$ 19 and 1.7 h). Furthermore, MTX was liberated from its MTX-γ-DMPE derivative more rapidly at alkaline pH values than under acidic conditions (pH 8.7, $t_{10\%}$ = 1.4 days and pH 2.3, $t_{10\%}$ = 11 days).

Introduction

Methotrexate (MTX) is used in the treatment of rheumatoid arthritis (RA) (Kremer and Lee, 1986; Rose et al., 1990; Shiroky et al., 1991). The exact mechanism and site of action of low dose MTX in RA are unclear, although the beneficial effects of MTX may be related to its ability to inhibit synovial cell turnover and impair responses to histamine and other vasoactive substances (Brooks et al., 1990). Controlled clinical studies with MTX in patients with RA have not only demonstrated its efficacy, but have also high-lighted the frequency and severity of the side effects which are related in part to its non-specific action (Furst and Kremer, 1988).

MTX is given in weekly oral doses of 5–15 mg in RA (Furst and Kremer, 1988; Sinnett et al., 1989; Fries et al., 1991; Sany et al., 1990). The drug has also been administered intra-articularly (i.a) to control the synovitis in arthritic joints, but the results have generally been disappointing, possibly because adequate concentrations of drug could not be maintained in the joint (Sany et al., 1990). Improved uptake of liposomal [^{3}H]MTX by the inflamed synovium and reduced clearance from the joint compared with free [^{3}H]MTX were

Correspondence to: A.S. Williams, Rheumatology Research Laboratories, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, U.K.

noted following i.a injection in rabbits (Foong and Green, 1988). Thus, liposomal conjugation should improve the efficacy and reduce the side effects of MTX when delivered directly into the joint cavity. Following intravenous administration, liposomes accumulate within the inflamed paw tissue of rats with adjuvant-induced arthritis and within the actively inflamed joints of patients with RA (Williams et al., 1986, 1987; O'Sullivan et al., 1988). Thus when liposomal characteristics are defined for optimal localization, a drug such as MTX may be targeted to the site of inflammation following entrapment.

The in vitro cytotoxic effect of liposomes prepared with three MTX-dimyristoylphosphatidylethanolamine (DMPE) derivatives has previously been demonstrated (Hashimoto et al., 1985a; Noe et al., 1988). Those authors showed that these liposomes containing either the MTX-γ-DMPE, MTX-α-DMPE or MTX-diDMPE blocked DNA synthesis to different extents and concluded that liposomes prepared with the MTX-γ-DMPE conjugate were most active. In contrast, 10-fold lower concentrations of free MTX were required to give the same degree of inhibition (Hashimoto et al., 1985a). The greater potency of MTX was a reflection of the fact that free drug enters cells by a different, more efficient, pathway than that of the liposomes. Furthermore MTX-γ-DMPE was not degraded extracellularly (Hashimoto et al., 1985a) but had to undergo intracellular metabolism to exert optimal inhibition of L1210 cell proliferation (Kinsky et al., 1987). Thus liposomes containing MTX-DMPE are non-toxic until they are endocytosed by the cell, consequently such a system might be employed to reduce the systemic side-effects reported with low dose MTX therapy in the treatment of RA.

In this paper we describe the synthesis, isolation and characterization of three MTX derivatives of DMPE. The release of MTX from the γ-derivative was quantified by a high-pressure liquid chromatography (HPLC) method and characterized in buffer pH 7.4, fresh human plasma and in the presence of esterase, at 37°C. In addition, the effect of changing pH upon drug release from the conjugate is described at the same temperature.

Materials and Methods

Chemicals

Conjugation reagents were of Analar grade and obtained from Sigma Chemical Co., U.K. These included DMPE, triethylamine, *N,N'*-dicyclohexylcarbodiimide, *N*-hydroxysuccinimide and methotrexate. Solvents were HPLC grade, and had been dried by storage over molecular sieve (sodium alumino-silicate beads 1/8 inch diameter).

Synthesis and purification of MTX-DMPE derivatives

The synthesis of three MTX-DMPE derivatives was achieved by following methods previously described by Hashimoto et al. (1985b). All reagents and solvents were of the highest purity and dried before use to facilitate the conjugation reaction. The resulting conjugates, MTX-γ-DMPE, MTX-α-DMPE and MTX-α,γ-diDMPE were separated on 10 analytical thin-layer plates (Silica gel, 60 A, 20 × 20 cm, 0.2 mm thickness with fluorescent indicator 254 nm; Sigma Chemical Co.) eluted with a solvent system containing chloroform : methanol : water (70 : 30 : 5). These spots had R_f values of 0.49, 0.58 and 0.65, respectively. Each spot gave a positive test for phospholipid using the Stewart assay (Trudinger, 1970; Stewart, 1980). Unconjugated methotrexate had an R_f value of 0.21 and gave a negative result when tested for phospholipid. The resulting bands were individually scraped from the plates, suspended in 5.0 ml chloroform : methanol (1 : 1) and passed through a sintered glass filter under vacuum. The filtrates were reserved and stored at −20°C.

Linkage of MTX to the phospholipid was confirmed by infrared spectroscopy. Spectra were run on a Perkin Elmer 681 infrared spectrophotometer using a sodium chloride cell (2.5 mm × 4.0 mm).

Effect of pH on the hydrolysis of MTX-γ-DMPE

Phosphate citrate (McIlvaine) buffers were prepared at pH values ranging from 2.3 to 8.7. 400 μl of each conjugate solution (in chloroform : methanol 1 : 1) was diluted to 5.0 ml with

the respective buffer solutions and incubated at 37°C for 24 h. At 1-h intervals, 200-μl aliquots were removed and analysed for free methotrexate by the HPLC method described. Percentage methotrexate release was determined following complete hydrolysis of the conjugate upon addition of sodium hydroxide (1.0 M).

Release rates for both MTX-DMPE and MTX-diDMPE conjugates in buffer pH 7.4 at 37°C were quantified. In addition, the effect of pH upon drug release from the MTX-γ-DMPE derivative at 37°C was investigated.

Hydrolysis of MTX-γ-DMPE in the presence of esterase

Since a series of hydrolytic enzymes are found in a physiological environment, the effect of one such enzyme upon MTX cleavage from the conjugate was investigated in buffer pH 7.4 at 37°C.

Test solutions were prepared as above with the addition of 10 U/ml esterase (Sigma Chemical Co., EC 3.1.1.1, from porcine liver; 1 unit will hydrolyse 1.0 μmol of ethyl butyrate to butyric acid and ethanol per min at pH 8.0 at 25°C). This enzyme is present in most tissues but has highest activity in the liver. Free methotrexate released at various time points was determined as above.

Hydrolysis of MTX-γ-DMPE in fresh, pooled human plasma

30 ml fresh blood from four healthy volunteers was collected into EDTA-coated vials. These were centrifuged at 2500 rpm for 15 min. The plasma was pooled and 4.0 ml aliquots taken for the hydrolysis experiment. 600 μl of the γ-derivative was measured into a glass tube and evaporated to dryness before addition of the fresh plasma. The sample was mixed and incubated at 37°C in a water-bath for 24 h. 200 μl volumes were removed at 15-min intervals, diluted with 200 μl of methanol and frozen immediately in liquid nitrogen before being stored at −70°C. Samples were thawed prior to analysis by an HPLC method.

HPLC assay for methotrexate in buffer solution

An LKB Bromma HPLC system with 2152 LC controller, 2150 pump, 2151 variable wavelength detector and 200 μl loop was used.

200-μl samples were injected directly onto an HPLC column (Hichrom S50DS2, 250 mm × 4.6 mm) which was eluted with 0.067 M phosphate buffer, pH 6.8, containing 30% methanol. Flow rate was set at 1 ml/min. The column effluent was monitored at 305 nm with peak areas quantified using a computing integrator (Jones Chromatography). MTX concentrations were determined after comparison with a standard curve, which was linear over the concentration range of 10–0.01 μg/ml, prepared on each day of assay. MTX was eluted after 5 min, whilst MTX-α-DMPE, MTX-diDMPE and MTX-γ-DMPE had retention times of 18, 24 and 28 min, respectively.

HPLC assay for MTX in human plasma

Hydrolysis samples were thawed before dilution with 100 μl phosphate buffer, pH 6.8. Each sample was mixed thoroughly before centrifugation at 13000 rpm for 10 min. 450 μl of super-

TABLE 1

Characterisation of MTX and MTX-DMPE derivatives

Sample	R_f	% yield ± S.D.	% yield [a]	DMPE : MTX ratio	Release in buffer, pH 7.4 [b]
A	0.21	12.00 ± 4.0	60.00	–	–
B	0.49	42.00 ± 9.0	17.50	0.98	2.1 days
C	0.58	15.00 ± 5.0	7.50	1.20	1.3 days
D	0.65	23.00 ± 8.0	9.40	2.06	10 days

[a] Data from Hashimoto et al. (1985b).

A, MTX; B, MTX-γ-DMPE; C, MTX-α-DMPE; D, MTX-α,γ-diDMPE. % yield is expressed as mean ± S.D. for separate conjugate batches ($n = 3$).

[b] $t_{10\%}$, time required to release 10% of total MTX from each conjugate at 37°C.

natant was then transferred to ultrafiltration units (EMIT-system 1, Syva) which were centrifuged at 3100 rpm for 30 min. This final ultrafiltration step was included to prolong column life by avoiding the possibility of on-column plasma protein precipitation. The coefficient of variation for the extraction procedure ($n = 5$) was 6.1% and a sample recovery of 92% was attained. 100 μl of filtrate was diluted with 100 μl buffer, pH 6.8, and loaded directly onto the HPLC column as described above.

Results

Conjugate synthesis and characterization

Following the reaction of MTX with DMPE in the presence of *N,N'*-dicyclohexylcarbodiimide and *N*-hydroxysuccinimide, free drug and three MTX-DMPE derivatives were consistently separated by TLC. They had retention factors of 0.21 (A), 0.49 (B), 0.58 (C) and 0.65 (D), respectively. Each band contained varying percentages of MTX (determined by HPLC method and UV spectrometry), namely 12% (A), 42% (B), 15% (C) and 23% (D) (Table 1), but only bands B–D gave a positive test for phospholipid, by Stewart assay (Trudinger, 1970; Stewart, 1980). Bands B and C had a phospholipid/MTX ratio of 1:1 and were characterized as MTX-γ-DMPE and MTX-α-DMPE, respectively. D contained two DMPE residues per MTX molecule and was identified as the MTX-α,γ-diDMPE derivative.

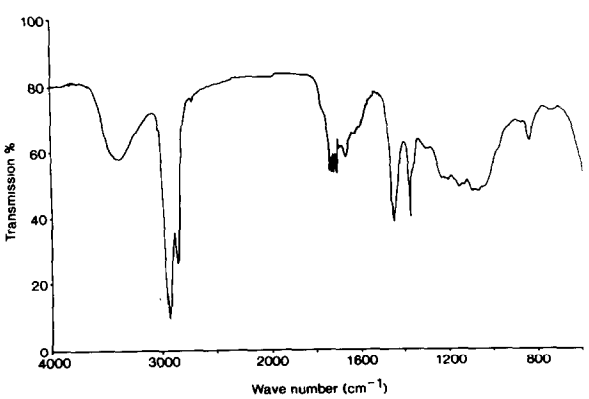

Fig. 1. Infrared spectrum for MTX-DMPE derivatives.

TABLE 2

Interpretation of infrared spectrum for MTX-DMPE conjugates(derived after comparison with standard methotrexate and DMPE samples)

Wavenumber (cm^{-1})	Spectral assignment of band
3600–3200	broad band associated with primary and secondary amide stretch.
2960, 2920 and 2850	strong sharp bands characteristic of C-H stretch of the carbon skeleton of the phospholipid.
1670 and 1630	amide I and amide II carboxyl stretch vibrations and N-H deformation.
1450 and 1360	two bands associated with C-H deformation of alkane, CH_3-CH_2-CH_2 assigned to phospholipid.
840	very characteristic C-H out of plane deformation of *para*-substituted benzene–assigned to aromatic portion of MTX molecule.

In each conjugate the amine group of the phospholipid is attached to MTX via an amide linkage. This was confirmed by infrared spectroscopy, which was carried out on each derivative (Fig. 1). Characteristic amide stretch bands were observed (3600–3200 cm^{-1}) whilst peaks at 1670 and 1630 cm^{-1} were assigned to amide I and amide II carboxyl stretch and N-H deformation. Strong bands at 2960, 2920 and 2850 cm^{-1} were assigned to C-H stretch of the carbon skele-

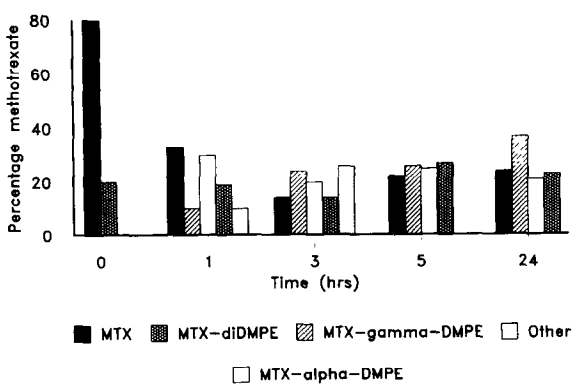

Fig. 2. Characterization of MTX-DMPE conjugate formation over a 24 h period.

ton of DMPE (Table 2). The infrared spectra for conjugates B–D were identical.

The conjugation reaction was monitored over a 24 h period. At specific time intervals 40 μl of reaction mixture was removed, separated by TLC, individual bands isolated and percentage conjugation at each time point determined, the results being presented in Fig. 2. Upon addition of MTX to the phospholipid solution, the MTX-diDMPE derivative formed immediately, accounting for 20% of the total MTX in the reaction mixture. However, at this same instance, approx. 80% of MTX was still present in its free form, which was reduced to 14% after 3 h. After 24 h constant stirring at 40°C, there was little difference with 19% unbound MTX remaining. Therefore, the optimal reaction time is 3 h which is in agreement with that adopted by Hashimoto et al., (1985b) for conjugate synthesis, although at this time point these researchers found 60% of the MTX was present in its free form. In our improved procedure all reagents were kept as dry as possible and HPLC grade solvents were used: this factor may have contributed to the better conjugation efficiency observed. In one batch where Analar grade reagents were utilized, 80% of MTX remained unbound after 3 h, 14% was present as the γ-derivative, 4% as the α-derivative and 2% as the diDMPE substituent.

Hydrolysis of MTX-DMPE in buffer and plasma

The stability of both mono- and di-substituted MTX-DMPE was studied in McIlvaine buffer, pH 7.4, at 37°C. The three phospholipid conjugates were stable to hydrolysis, MTX-diDMPE (D) being most stable with 10% of the total MTX released ($t_{10\%}$) after 10 days. The mono-substituted derivatives (B and C) had substantially shorter $t_{10\%}$ values of 2.1 and 1.3 days, respectively (Fig. 3).

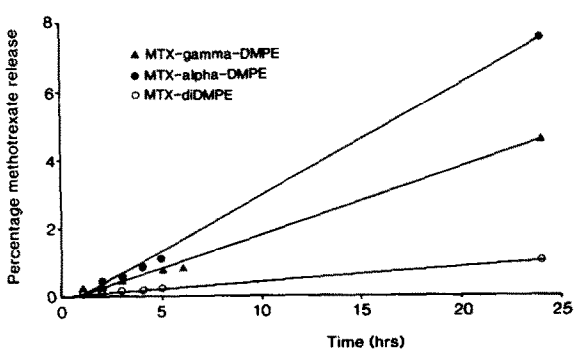

Fig. 3. Release of MTX from its mono-DMPE and diDMPE derivatives in buffer pH 7.4 at 37°C.

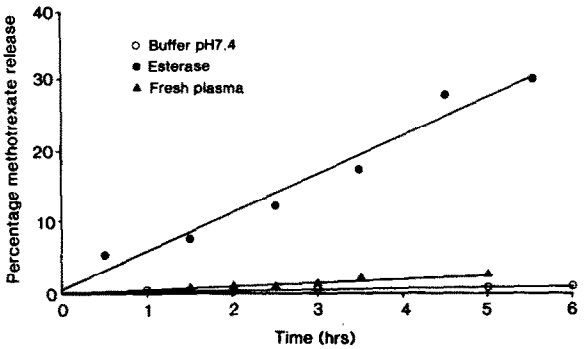

Fig. 4. Hydrolysis of MTX-γ-DMPE in the presence of buffer, carboxylic ester hydrolase and fresh human plasma at 37°C.

The susceptibility of MTX-γ-DMPE to hydrolysis under more physiological conditions was also investigated. In fresh human plasma the rate of MTX release from its phospholipid conjugate was raised ($t_{10\%}$ = 19 h), whilst in the presence of high esterase concentrations (10 U/ml) the hydrolysis rate was increased further ($t_{10\%}$ = 1.7 h, Fig. 4).

Finally, the effect of changing pH upon MTX release from MTX-γ-DMPE was investigated. When examined over a 6 h period, MTX was liberated more rapidly at pH 8.7 than at pH 2.3 ($t_{10\%}$ = 1.4 and 11 days). At pH 7.0, 6.0 and 4.0 the times for 10% MTX release were 3.6, 6.3 and 8.7 days, respectively (Fig. 5).

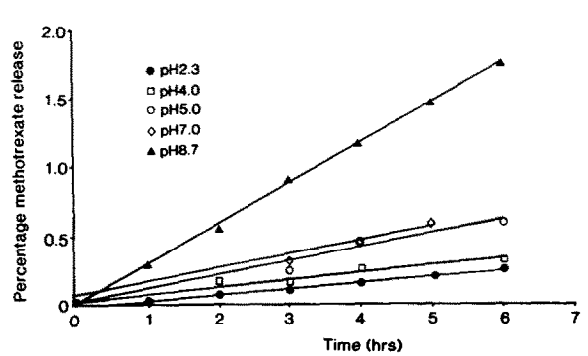

Fig. 5. Effect of pH on the hydrolysis of MTX-γ-DMPE at 37°C.

Discussion

Methotrexate contains two carboxylic acid groups in the glutamyl portion of the molecule. Thus, under the synthetic conditions employed, three MTX-DMPE conjugates might be expected, viz., two mono-substituted derivatives where DMPE was attached either to the α- or the γ-carboxyl group and MTX-diDMPE where one DMPE residue is attached to each of the acid functionalities (Fig. 6).

When examined by TLC, MTX and its three MTX-DMPE derivatives migrated as discrete fluorescent entities having retention factors of 0.21 (A), 0.49 (B), 0.58 (C) and 0.65 (D), respectively. Following complete hydrolysis in aqueous alkali, the amount of free MTX released from each band was quantified by the HPLC method described above. The γ-derivative was formed preferentially, containing 42% of total MTX, whilst A, C and D contained 12, 15 and 23% MTX, respectively. Only bands B–D gave a positive test for phospholipid (Stewart assay). Bands B and C had a phospholipid ratio of 1:1 whilst D contained two DMPE residues per MTX molecule.

The α-carboxyl group of MTX is a much stronger acid ($pK_a = 3.36$) than the γ-carboxyl group ($pK_a = 4.70$) (Rosowsky and Forsch, 1982). Thus, in MTX-γ-DMPE the more polar α-carboxylic acid group is free and would retard the chromatographic movement of the derivative in a hydrophobic environment. Consequently bands

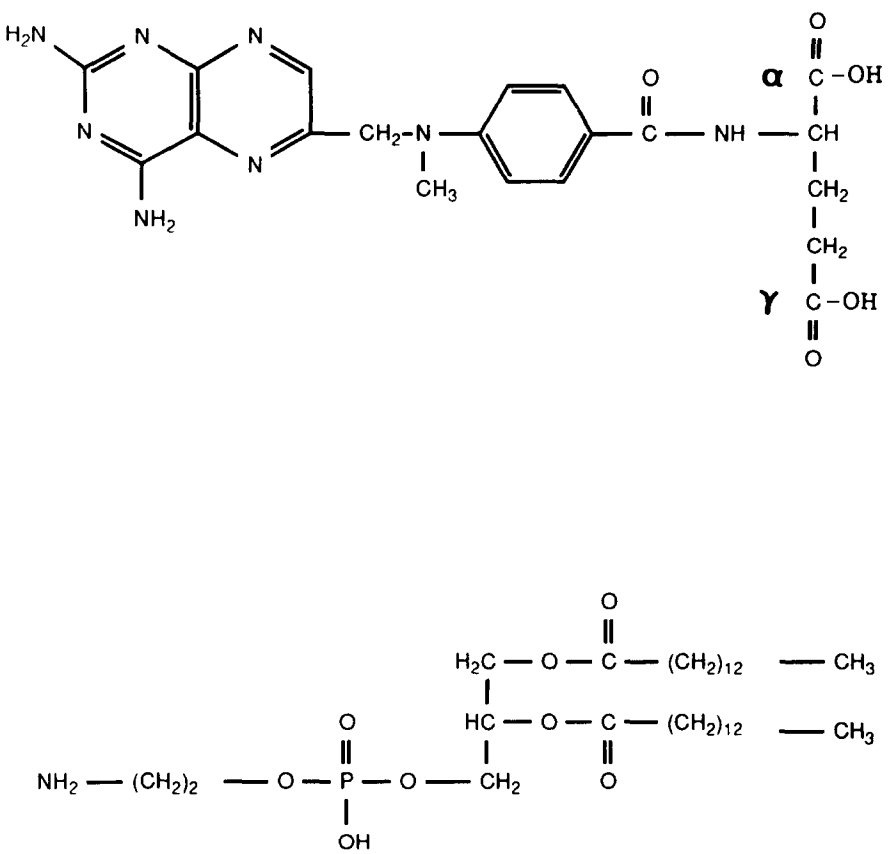

Fig. 6. Structures of (A) methotrexate and (B) dimyristoylphosphatidylethanolamine. Either the α-, γ- or both glutamyl carboxyl groups are attached via amide bonds to the amino group on DMPE.

A–D can be assigned to MTX, MTX-γ-DMPE, MTX-α-DMPE and MTX-α,γ-diDMPE, respectively.

These findings are in agreement with those previously reported by Hashimoto et al. (1985b). However, we obtained better conjugate yields, 42% of the MTX-γ-DMPE compared to 17.5% being present after 3 h reaction. Similarly, the reaction of MTX with diethyl L-glutamate in the presence of peptide bond-forming agents such as *N,N'*-dicyclohexylcarbodiimide was investigated (Rosowsky and Yu, 1978). This gave three ester derivatives, namely, the MTX γ-L-glutamate diethyl ester, α-L-glutamate diethyl ester and α,γ-bis(L-glutamate tetraethyl ester). The tetraethyl ester was separated by solvent extraction in chloroform whilst the more polar diesters were separated by TLC. The faster moving component of the diester mixture was the least acidic α-derivative with the free γ-COOH group.

In each MTX-DMPE conjugate synthesized, the amine group of the phospholipid is attached to MTX via an amide linkage. This was confirmed by infrared spectroscopy; the spectra obtained for each conjugate (B, C and D) were identical.

The conjugation reaction was monitored over a 24 h period, and the optimal reaction time for MTX-γ-DMPE synthesis was 3 h. This incubation time is consistent with that adopted by Hashimoto et al. (1985b), although, by modifying the latter method slightly MTX-γ-DMPE yield was doubled. In this improved procedure all reagents were kept as dry as possible and HPLC grade solvents were used; these factors may have contributed to the better conjugation efficiency observed.

Similar observations were made by Fan et al. (1991) who studied the interaction of MTX with dihydrofolate reductase. This involved the synthesis of a fluorescent derivative of MTX by coupling the α- or γ-carboxyl of the glutamate moiety via a diaminopentane spacer, to fluorescein isothiocyanate (Gapski et al., 1975; Fan et al., 1991). The original procedure describing the synthesis of such a fluorescent derivatives resulted in low yields (15%) of an impure mixture of isomers (Gapski et al., 1975). Fan et al. (1991) found that the overall yield of the two isomers depended upon the synthetic conditions employed. Anhydrous reaction conditions and dry reagents coupled with a 1.5 molar excess of carbodiimide/*N*-hydroxysuccinimide relative to MTX gave yields of 7 and 44% for the α- and γ-derivatives, respectively.

Though the synthesis and characterization of MTX-DMPE (B–D) have been described previously (Hashimoto et al., 1985b), little attention has been paid to the in vitro release behaviour of MTX from such derivatives. The three phospholipid conjugates were stable to hydrolysis in McIlvaine buffer, pH 7.4, at 37°C. MTX-diDMPE (D) was more stable than the mono-substituted derivatives (B and C) ($t_{10\%}$ = 10, 2.1 and 1.3 days).

At pH 7.4, MTX is 99% ionised and exists in a dianionic form. Thus, the uptake of folates is an electrostatically unfavourable interaction between the ionized glutamate carboxylic acid functionalities and the negatively charged cell membrane, such that the species actually penetrating the cell membrane may be undissociated (non-charged), this represents only a minute fraction of the total extracellular drug concentration (Rosowsky and Forsch, 1982).

γ-Monoamides of MTX have been synthesized as potential prodrugs of MTX or as active agents in their own right. Modification of the γ-carboxyl in MTX does not greatly affect binding to the target enzyme dihydrofolate reductase (DHFR). Thus, even if formation of free MTX did not occur from the monoamide inside the cell, these derivatives might still inhibit tumour cell growth. An important consequence of the γ-substitution in MTX is that the replacement of the negative charge in the glutamate side chain by a hydrophobic group may favour uptake into the cell via a different mechanism (Rosowsky et al., 1986). In the case of classical antifolates with a glutamate side chain, the efficiency with which they are converted to non-effluxing polyglutamate metabolites is a major determinant of their ability to be transported across the cell membrane and to accumulate in cells (McGuire and Coward, 1984; Rosowsky et al., 1991).

The release of MTX following hydrolysis of MTX-γ-DMPE in fresh human plasma and in

esterase (10 U/ml) was more rapid ($t_{10\%} = 19$ and 1.7 h) than in buffer pH 7.4 ($t_{10\%} = 48$ h) at the same temperature. Previously, the in vivo hydrolysis, in mouse plasma, of the γ-amide bond in a benzylamide derivative of MTX was investigated and was insignificant over a 2.5 h period (Rosowsky et al., 1986). A variety of enzymes are present in human plasma or serum. Carboxylic acid ester hydrolases (EC 3.1.1.) are a group of enzymes which attack molecules such as simple esters, fats and phospholipids. Choline esterase and lipase are enzymes of this class predominantly found in human serum, and have enzyme activities of approx. 1.9–3.8 and 0.14 U/ml, respectively, at 25°C (Willis, 1985). Thus, the esterase concentration used to study the stability of MTX-γ-DMPE is in excess of those normally found in human plasma. The experiment demonstrates the susceptibility of the conjugate to enzymatic cleavage resulting in the liberation of free drug.

Finally, the effect of changing pH upon MTX release from MTX-γ-DMPE was investigated. MTX was liberated more rapidly at pH 8.7 than at pH 2.3 ($t_{10\%} = 1.4$ and 11 days).

In this paper we describe an improved method for the synthesis of MTX-γ-DMPE with a view to liposomally incorporating the conjugate and investigating its effect in vitro upon inflammatory cell populations and in vivo against experimental models of rheumatoid arthritis. The γ-derivative is relatively stable to hydrolysis in fresh human plasma but release of MTX from the conjugate is dependent upon hydrolytic enzyme concentrations. At physiological pH the conjugate releases free MTX ($t_{10\%} = 2.1$ days) more rapidly than at the more acidic pH values found within the lysosome (pH 4.0, $t_{10\%} = 8.7$ days). Thus, degradation of the conjugate within the lysosome is likely to be dependent upon lysosomal enzyme activity and not pH.

Acknowledgement

A.S.W. would like to thank the ARC for their financial support during the course of this study.

References

Brooks, P.J., Spruill, W.J., Parish, R.C. and Birchmore, D.A., Pharmacokinetics of methotrexate administered by intramuscular and subcutaneous injections to patients with rheumatoid arthritis. *Arthritis Rheum.*, 33 (1990) 91–94.

Fan, J., Pope, L.E., Vitols, K.S. and Huennekens, F.M., Affinity labelling of folate transport proteins with the N′-hydroxysuccinimide ester of the γ-isomer of fluorescein-methotrexate. *Biochemistry*, 30 (1991) 4573–4580.

Foong, W.C. and Green, K.L., Retention and distribution of liposome entrapped triturated methotrexate injected into normal and arthritic joints. *J. Pharm. Pharmacol.*, 40 (1988) 464–468.

Fries, J.F., Singh, G., Lenert, L. and Furst, D.E., Aspirin, hydroxychloroquine and hepatic enzyme abnormalities with methotrexate in rheumatoid arthritis. *Arthritis Rheum.*, 33 (1990) 1611–1619.

Furst, D.E. and Kremer, J.M., Methotrexate in rheumatoid arthritis. *Arthritis Rheum.*, 31 (1988) 305–313.

Gapski, G.R., Whiteley, J.M., Rader, J.I., Cramer, P.L., Henderson, G.B., Neef, V. and Huennekens, F.M., Synthesis of a fluorescent derivative of amethopterin. *J. Med. Chem.*, 18 (1975) 526–528.

Hashimoto, K., Loader, J.E. and Kinsky, S.C., Synthesis and characterization of methotrexate-DMPE derivatives and the glycerophosphorylethanolamine analogs, *Biochim. Biophys. Acta*, 816 (1985b) 163–168.

Hashimoto, K., Loader, J.E., Knight, M.S. and Kinsky, S.C., Inhibition of cell proliferation and dihydrofolate reductase by liposomes containing methotrexate-DMPE derivatives and by the glycerophosphorylethanolamine analogs. *Biochim. Biophys. Acta*, 816 (1985a) 169–178.

Kinsky, S.C., Loader, J.E. and Hashimoto, K., Inhibition of cell proliferation by putative and non-degradable analogs of methotrexate-DMPE. *Biochim. Biophys. Acta*, 917 (1987) 211–218.

Kremer, J.M. and Lee, J.K., The safety and efficacy of the use of methotrexate in long term therapy for rheumatoid arthritis. *Arthritis Rheum.*, 29 (1986) 822–831.

McGuire, J.J. and Coward, J.K., *Folates and Pterins*. In Blakeley, R.L. and Benkovic, S.J. (Eds), Wiley, New York, 1984, pp. 135–190.

Noe, C., Hernandez-Borrell, J., Kinsky, S.C., Matsura, E. and Leserman, L., Inhibition of cell proliferation with antibody-targeted liposomes containing methotrexate-γ-DMPE. *Biochim. Biophys. Acta*, 946 (1988) 253–260.

O'Sullivan, M.M., Powell, N., French, A.P., Williams, K.E., Morgan, J.R. and Williams, B.D., Inflammatory joint disease: a comparison of liposome scanning, bone scanning and radiography. *Ann. Rheum. Dis.*, 47 (1988) 485–491.

Rose, C.D., Singsen, B.H., Eichenfield, A.H., Goldsmith, D.P. and Athreya, B.H., Safety and efficacy of methotrexate therapy for juvenile rheumatoid arthritis. *J. Paediatr.*, 117 (1990) 653–658.

Rosowsky, A., Bader, H., Smith, M.R., Cucchi, C.A. and Wick, M.M., Methotrexate analogues 28. Synthesis and

biological evaluation of new gamma-monoamides of aminopterin and methotrexate. *J. Med. Chem.*, 29 (1986) 1703–1709.

Rosowsky, A. and Forsch, R., Methotrexate analogues 16. Improvement of the side chain amide carbonyl group as a structural determinant of biological activity. *J. Med. Chem.*, 25 (1982) 1454–1459.

Rosowsky, A. and Yu, C.S., Methotrexate analogues 10. Direct coupling of methotrexate and diethyl L-glutamate in the presence of peptide bond forming reagents. *J. Med. Chem.*, 21 (1978) 170–175.

Rosowsky, A., Forsch, R.A., Bader, H. and Freisheim, J.H., Synthesis and in vitro biological activity of new deaza analogues of folic acid, aminopterin and methotrexate with an L-orthinine chain. *J. Med.Chem.* 34 (1991) 1447–1454.

Sany, J., Kaliski, S., Couret, M., Cuchacovich, M. and Daures, J.P., Radiologic progression during intramuscular treatment of rheumatoid arthritis. *J. Rheumatol.*, 17 (1990) 1636–1641.

Shiroky, J.B., Frost, A., Skelton, J.D., Haegert, D.C., Newkirk, M.M. and Neville, C., Complications of immunosuppression associated with weekly low dose methotrexate. *J. Rheumatol.*, 18 (1991) 1172–1175.

Sinnett, M.J., Groff, G.D., Raddatz, D.A., Franck, W.A. and Bertino, J.S., Methotrexate pharmacokinetics in patients with rheumatoid arthritis. *J. Rheumatol.*, 16 (1989) 745–748.

Stewart, J.C.M., Colorimetric determination of phospholipids with ammonium ferro thiocyanate. *Anal. Biochem.*, 104 (1980) 10.

Trudinger, P.A., Determination of lipid phosphorus in the nanomolar range, *Anal. Biochem.*, 36 (1970) 225.

Williams, B.D., O'Sullivan, M.M., Saggu, G.S., Williams, K.E., Williams, L.A. and Morgan, J.R., Imaging in rheumatoid arthritis using liposomes labelled with technetium. *Br. Med. J.*, 293 (1986) 1143–1144.

Williams, B.D., O'Sullivan, M.M., Saggu, G.S., Williams, K.E., Williams, L.A. and Morgan, J.R., Synovial accumulation of technetium labelled liposomes in rheumatoid arthritis. *Ann. Rheum. Dis.*, 46 (1987) 314–318.

Willis, E.D., Role of subcellular organelles: lysosomes, chap. 3. In Wright, J. (Ed.), *Biochemical Basis of Medicine*, Pitman, London, 1985, pp. 33–43.