Carrier-Bound Methotrexate. I. Water-Soluble Polyaspartamide–Methotrexate Conjugates with Ester Links in the Polymer–Drug Spacer

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ABSTRACT: The antifolate-type anticancer drug methotrexate (MTX) has for many years, in numerous laboratories, been a "workhorse" drug for conjugation with natural and synthetic macromolecular carriers for the purpose of enhancing bioavailability and lowering toxic side effects. In the project here described the polymer–drug conjugation strategy is utilized for the preparation of water-soluble polyaspartamide–methotrexate conjugates in which the drug is carrier-anchored through short spacers containing ester groups as biofissionable links. To this end, polyaspartamide carriers **1,** poly-α,β-D,L-*N*-(2-hydroxyethyl)aspartamide, and **2,** poly-α,β-D,L-*N*-[2-(2-hydroxyethoxy)ethyl]aspartamide, are treated with MTX in DMF solution in the presence of a carbodiimide coupling agent and 4-(dimethylamino)pyridine catalyst. The molar MTX/OH feed ratios, 0.28 and lower, are chosen in these coupling reactions so as to provide conjugates featuring drug-loading levels in the approximate range of 3–16 mol % MTX, roughly corresponding to 6–28% by mass. The water-soluble product polymers are purified by aqueous dialysis, collected in the solid state by freeze-drying, and structurally characterized by ^{1}H–NMR spectroscopy. © 2001 John Wiley & Sons, Inc. J Appl Polym Sci 82: 1844–1849, 2001

Key words: methotrexate; polymer–drug conjugation; bioavailability; macromolecular carrier; antifolate agent

INTRODUCTION

The classical anticancer drug methotrexate (MTX, amethopterin), in clinical use now for some four decades, is a potent antifolate agent, showing activity against a number of neoplasias as a result of inhibition of dihydrofolate reductase, a key enzyme in the folate cycle. However, in parallel with other antineoplastic agents, the drug exerts severe toxic side effects. In addition, it shows a notorious propensity for inducing acquired resistance in the target tissue caused by deficiencies in the active carrier-mediated transport mechanism normally available to folate derivatives including MTX. The topic has been amply reviewed, most recently by Piper[1] and others.[2,3]

Realization of these pharmacological shortcomings prompted early efforts, well extended into the present, to bring about structural modifications aimed at widening the activity spectrum (notably against solid tumors), ameliorating the systemic toxicity, enhancing cell specificity, and reducing the resistance problem. Although increased knowledge of the mechanisms of cytotoxic action, membrane transport, and intracellular

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drug retention has in more recent years led to the development of numerous promising, monomeric structural MTX analogs,[4–6] the most visible progress has been in the development of polymeric MTX derivatives, notably conjugates of the drug with water-soluble macromolecular carriers. In fact, ever since Ringsdorf's groundbreaking contributions to the polymer–drug conjugation technology,[7] MTX has served as a "workhorse" drug for polymer anchoring.

Initial conjugation studies with several synthetic and proteinaceous polymer carriers in the laboratories of Ringsdorf,[8] Shen and Ryser,[9] Chu with coworkers,[10] and Blair and Ghose with collaborators,[11] demonstrated synthetic feasibility of polymer–drug conjugation, conjugate retention in the serum and endocytotic cell entry, elevated intracellular drug levels, and ultimate cytotoxic activity. Shen and Ryser's early pioneering work in particular, using poly-L-lysine and poly-D-lysine carriers,[12,13] demonstrated the ability of their conjugates, once inside cytosolic space, to undergo intracellular drug release, either smoothly, where the biodegradable poly-L-lysine carrier was used, or conditionally, where the conjugates were based on the poorly degradable poly-D-lysine carrier type. Significantly, these investigations also showed that poly-L-lysine–anchored MTX can undergo uptake by cell systems that are inherently resistant as a result of drug influx-inhibiting transport deficiencies. These early findings paved the way for extensive polymer–drug anchoring studies pursued worldwide, including *inter alia,* the outstanding investigations by Kato,[14] Garnett,[15] and Tsou[16] with collaborators.

Polymer–drug conjugation work in our laboratory has been based exclusively on man-made polymers as carriers, specifically on water-soluble aliphatic polyamides, the rationale being that design-guided synthesis would allow for the tailoring of physical and chemical properties in accordance with pharmacological demands. It has been an overriding goal in that work to provide water-soluble polymer–drug conjugates capable of being isolated in the solid state for proper handling, analysis, storage (if necessary, in matrix-embedded form), and ultimate redissolution for use. The present communication deals with our efforts to achieve bioreversible MTX anchoring to synthetic carrier polymers. In this study, the anchoring tether contains an ester group as the biocleavable site.

EXPERIMENTAL

Solid-state IR spectra (KBr pellets) were recorded over the region of 4000–200 cm^{-1}. ^1H–NMR spectra (400 MHz) were taken on D_2O solutions; chemical shifts δ are given in ppm relative to internal sodium 3-(trimethylsilyl)-2,2,3,3-d_4-propionate (integration error limits ± 12%). Immediately prior to recording, the pH of the solutions was adjusted to 10 (KOH) to eliminate potential protonation effects.

Cannon–Fenske tubes were used for the determination of inherent viscosities η_{inh} in deionized H_2O at 30.0 ± 0.5°C; the concentration was c = 0.2 g/100 mL; the findings are given in units of mL g^{-1}. Spectra/Por 4 membrane tubing (Spectrum Industries, Los Angeles, CA), with molecular mass cutoff limits of 12,000–14,000, was used routinely for dialysis of carriers and conjugates. The carrier polymers were additionally dialyzed in Spectra/Por 6 wet tubing with cutoff limit 25,000. The outer phase in all dialysis operations was magnetically stirred deionized water, with pH adjusted where necessary and indicated. Freeze-drying operations were carried out with the aid of a Virtis Bench Top 3 freeze-drier at −30°C, 13 Pa. Carrier polymers were routinely postdried in a Sartorius Thermo Control Infrared Drying System (heating program, twice for 8 min at 65°C); alternatively, an Abderhalden Drying Tube was used (2 days at 60°C, 1.3–2.7 kPa). The Abderhalden equipment was also employed for a postdrying operation (2 days at 60–65°C, 1.3 kPa) of sample material prepared for microanalysis; more forcing conditions were avoided so as to maintain product integrity. This generally left some 2% of moisture in the hygroscopic polymers, reflected in the slightly low carbon values obtained. The microanalytical work was performed by W. Dindorf, Wiesbaden, Germany.

Solvents, Reagents, and Reactants

The reaction solvent, *N,N*-dimethylformamide (DMF), was predried over 4-Å molecular sieves and redistilled under reduced pressure in a faint stream of N_2. Deionized H_2O was used for all preparative work. All other solvents were laboratory grade, received from commercial sources. The monomeric reactants, ethanolamine and 2-(2-aminoethoxy)ethanol, both *purum* (min. 98%), were used as received (Fluka Chemie, Buchs, Switzerland), and so were the esterification cata-

lyst, 4-(dimethylamino)pyridine (DMAP) and the coupling agent, *N,N′*-dicyclohexylcarbodiimide (DCC). L-(+)-Amethopterin (methotrexate, MTX) was a gift from Lederle Laboratories, American Cyanamid Co. The compound was dried for 2 days at 45–50°C in an Abderhalden tube under reduced pressure prior to use to remove any solvating H_2O. Poly-D,L-succinimide was obtained by high-temperature solution polymerization of D,L-aspartic acid as described by Neri and Antoni.[17] The polymer used in this project was taken from a master batch having a mass-average molecular mass of 41,500, determined viscometrically.[18]

Carrier Polymers

The polyaspartamide carrier **1,** poly-α,β-D,L-*N*-(2-hydroxyethyl)aspartamide, was prepared essentially by the described procedure.[17] However, the workup steps were modified as follows. Upon completed reaction, the solution was poured with rapid stirring into excess (approximately double volume) of a primary/secondary BuOH mixture, and the precipitated polymer, washed with EtOH and redissolved in H_2O, was dialyzed for 2 days in Spectra/Por 4 tubing and for another 2 days in Spectra/Por 6 tubing against several batches of stirred deionized H_2O. The carrier was isolated by freeze-drying as a water-soluble solid in a yield of 75%; η_{inh}, 28 mL g^{-1}.

^{1}H–NMR (D_2O; δ/ppm; expected proton counts in parentheses): 4.7–4.5, 0.9 H (1H; peptidic CH); 3.7–3.55, 2 H (2H; CH_2OH); 3.4–3.25, 2.2 H (2H; CONHCH_2); 2.8–2.5, 1.8 H (2H; CH_2 CONH).

The IR spectrum showed the characteristic O—H stretching and C—O, O—H bending absorptions in the 3400 and 1100 cm^{-1} regions in addition to the strong amide-I and -II bands near 1650 and 1500 cm^{-1}. The carrier **2,** poly-α,β-D,L-*N*-[2-(2-hydroxyethoxy)ethyl]aspartamide, was synthesized by an analogous procedure, with ethanolamine replaced by the same equivalent of 2-(2-aminoethoxy)ethanol and the reaction time increased to 14 h. The yield of the water-soluble solid compound was 80%; η_{inh}, 29 dL g^{-1}.

^{1}H–NMR (D_2O; δ/ppm): 4.75–4.5, 1.2 H (1H; peptidic CH); 3.8–3.5, 6.4 H (6H; CH_2OCH_2CH_2OH); 3.4, 2 H (2H; CONHCH_2); 2.9–2.6, 2 H (2H; CH_2CONH). The IR spectrum resembled that of **1,** except for enhanced intensity of the C—O, O—H bending modes.

Amounts of carriers are given as base moles and, hence, correspond to structures **1** and **2** normalized to $x + y = 1$.

MTX Conjugates

The amounts of polymeric conjugates are given as base moles and, thus, correspond to the simplest repeat units. These are represented by the structures **1-MTX** and **2-MTX,** each normalized to $y = 1$.

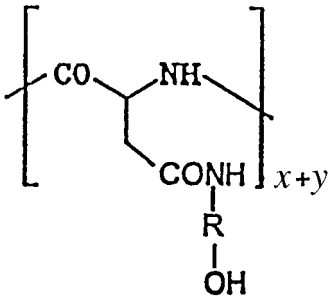
For the **1-MTX** series, the procedure described in the following for the preparation of **1-MTX** (10.8) is representative.

To the stirred solution of **1,** 79 mg (0.5 mmol), in 1.5 mL of DMF was added MTX, 34 mg (0.075 mmol), dissolved in 0.5 mL of the same solvent. DCC, 18 mg (0.085 mmol), and DMAP, 9 mg (0.07 mmol), dissolved together in 0.5 mL of DMF, were added, and the resulting, initially clear solution was stirred in the dark for 3 days at ambient temperature and for a further 4 h at 45–50°C in an incubator. During this reaction period, the solution tended to turn turbid because of some precipitation of the dicyclohexylurea by-product, but reclarified at the elevated temperature. The polymeric product was precipitated with excess Et_2O–hexane (1 : 1), washed with precipitant, and redissolved in 10 mL of H_2O, with the pH adjusted to 9 (Na_2CO_3). The solution was dialyzed in Spectra/Por 4 tubing for 0.5 h against H_2O at the same pH and for another 30 h against several batches of plain H_2O. Freeze-drying of the retentate afforded 87 mg (89.1%) of yellowish, water-soluble solid.

^{1}H–NMR (D_2O; δ/ppm): 8.5, 7.7, 6.8, total 5 H (5H; aromatic H of MTX); 3.7–3.55, 23.6 H (23.6H; CH_2CH_2O); 3.4–3.25, 21 H (23.6H; CONHCH_2).

The IR spectra of **1-MTX** (10.8) and other conjugates were virtually identical with the respective carrier spectra, with MTX bands buried in the polymer absorption features.

The procedure described in the following for conjugate **2-MTX** (6.5) is representative of the conjugation reactions using carrier **2.** A quantity of 202 mg (1.0 mmol) of **2** was dissolved in 3 mL of DMF. MTX, 91 mg (0.2 mmol), dissolved in 2 mL of DMF, was added dropwise to the stirred polymer solution. Upon the addition of DCC, 41 mg (0.2 mmol), and DMAP, 24 mg (0.2 mmol), the mixture was stirred for 3 days at room temperature and another 4 h at 45–50°C in the dark,



1: R = —$(CH_2)_2$—

2: R = —$(CH_2)_2O(CH_2)$—

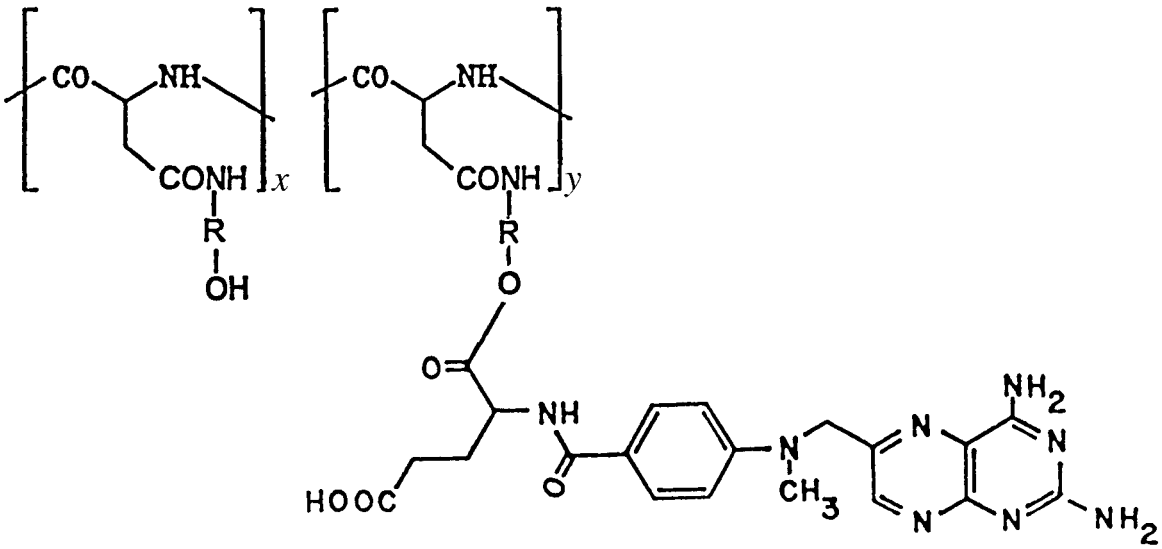
Figure 1

forming a fine suspension as small amounts of urea by-product precipitated from the solution. The conjugate was precipitated with excess Et_2O–hexane (1 : 1), washed, and largely dissolved in 20 mL of H_2O. Centrifugation removed undissolved urea, and the supernatant was dialyzed as described before. The conjugate was isolated by freeze-drying as a yellowish, water-soluble solid in a yield of 192 mg (73.7%).

^{1}H–NMR (D_2O; δ/ppm): 8.5, 7.7, 6.8, total 5 H (5H; aromatic H of MTX); 3.8–3.55, 45 H (45H; $CH_2OCH_2CH_2O$); 3,4, 14.4 H (15H; $CONHCH_2$).

RESULTS AND DISCUSSION

The polyaspartamides serving as the carriers in these ester-forming coupling reactions were water-soluble homopolymers of types **1** and **2** (Figure 1; α- and β-peptide units randomly distributed along the main chain). The compounds were obtained from polysuccinimide by nucleophilic ring opening[19] mediated by ethanolamine (giving **1**) and 2-(2-aminoethoxy)ethanol (giving **2;** only the α-forms are shown for these and other polyaspar-



1—MTX (32.6):	R = —(CH2)$_2$—	x/y = 32.6
1—MTX (27.7):	R = —$(CH_2)_2$—	x/y = 27.7
1—MTX (22):	R = —$(CH_2)_2$—	x/y = 22
1—MTX (10.8):	R = —$(CH_2)_2$—	x/y = 10.8
1—MTX (6.5):	R = —$(CH_2)_2$—	x/y = 6.5
1—MTX (5.3):	R = —$(CH_2)_2$—	x/y = 5.3
2—MTX (34.6):	R = —$(CH_2)_2O(CH_2)_2$—	x/y = 34.6
2—MTX (28.7):	R = —$(CH_2)_2O(CH_2)_2$—	x/y = 28.7
2—MTX (8.9):	R = —$(CH_2)_2O(CH_2)_2$—	x/y = 8.9
2—MTX (6.5):	R = —$(CH_2)_2O(CH_2)_2$—	x/y = 6.5

Figure 2

Table I Carrier Binding of MTX Through Ester Linking

Carrier Designation	Molar Feed Ratio[a]				Conjugate	
	Carrier	MTX	DCC	DMAP	Designation[b]	Yield (%)
1	1	0.07	0.075	0.06	**1-MTX** (32.6)	31
	1	0.08	0.1	0.08	**1-MTX** (27.7)	55
	1	0.1	0.1	0.08	**1-MTX** (22)	66
	1	0.15	0.17	0.14	**1-MTX** (10.8)	89
	1	0.25	0.28	0.25	**1-MTX** (6.5)	84
	1	0.28	0.28	0.25	**1-MTX** (5.3)	81
2	1	0.08	0.09	0.072	**2-MTX** (34.6)	70
	1	0.1	0.1	0.08	**2-MTX** (28.7)	33
	1	0.15	0.17	0.14	**2-MTX** (8.9)	73
	1	0.2	0.2	0.2	**2-MTX** (6.5)	74

[a] MTX = methotrexate; DCC = *N,N′*-dicyclohexylcarbodiimide; DMAP = 4-(dimethylamino)pyridine.
[b] Parenthetic label indicates *x*/*y* ratio in conjugate structure; see Table II.

tamide structures). Fractionation by dialysis in tubing with 25,000 molecular mass cutoff removed all material with molecular masses substantially below that limit.

MTX anchoring to carriers **1** and **2** was accomplished through esterification of a carboxyl function of the drug with carrier-attached hydroxyl groups, mediated by *N,N′*-dicyclohexylcarbodiimide (DCC) and catalyzed by 4-(dimethylamino)pyridine (DMAP). The coupling reactions were performed in *N,N*-dimethylformamide (DMF) solution over 2–3 days at ambient temperature and a brief heating period to complete the process. Aqueous dialysis served to remove unreacted MTX and other low-molecular constituents, and freeze-drying of the retentate provided the conjugates **1-MTX** and **2-MTX** (Figure 2; subunits randomly distributed along the main chain) in yields of 30–90% as water-soluble solids. MTX contents were determined by ^{1}H–NMR spec-

Table II Spectroscopic and Compositional Data for Conjugates 1-MTX and 2-MTX

Conjugate Designation	^{1}H–NMR[a] (ppm)			*x*/*y*[b]	[M][c]	MTX Content	
	8.6–6.7	3.7–3.6	3.4–3.3			Mole Percentage (mol %)[d]	Percentage by Mass
1-MTX (32.6)	5 (5)	67.1 (67.1)	65.7 (67.1)	32.6 : 1	5751	3.0	7.9
1-MTX (27.7)	5 (5)	57.3 (57.3)	56.4 (57.3)	27.7 : 1[e]	4976	3.5	9.1
1-MTX (22)	5 (5)	46 (46)	44.1 (46)	22 : 1[f]	4075	4.4	11.2
1-MTX (10.8)	5 (5)	23.6 (23.6)	21 (23.6)	10.8 : 1[g]	2303	8.5	19.8
1-MTX (6.5)	5 (5)	15 (15)	14.9 (15)	6.5 : 1	1623	13.3	28.0
1-MTX (5.3)	5 (5)	12.6 (12.6)	12.9 (12.6)	5.3 : 1	1275	15.9	35.7
2-MTX (34.6)	5 (5)	213.9 (213.9)	72.2 (71.3)	34.6 : 1	7636	2.8	6.0
2-MTX (28.7)	5 (5)	178 (178)	60 (59.3)	28.7 : 1	6443	3.4	7.1
2-MTX (8.9)	5 (5)	59.4 (59.4)	18.8 (19.8)	8.9 : 1	2439	10.1	18.7
2-MTX (6.5)	5 (5)	45 (45)	14.4 (15)	6.5 : 1[h]	1954	13.3	23.3

[a] Proton counts of selected band groups per base unit; see text for assignments. Numbers in parentheses are proton counts calculated for *x*/*y* ratios in subsequent column.
[b] Ratios derived from proton counts in 3.7–3.6 ppm region.
[c] Base molecular mass, that is, molecular mass of simplest recurring unit (structures **1-MTX** and **2-MTX** normalized to $y = 1$).
[d] Mol % of drug-bearing subunit.
[e] In repeat experiment: 24 : 1.
[f] In repeat experiment: 21 : 1.
[g] In repeat experiment: 9 : 1.
[h] In repeat experiment: 7 : 1.

troscopy, utilizing the aromatic proton signals in the 8.6–6.7 ppm region of the spectra in relation to other bands. (See Table II.) In the structures drawn the α-carboxyl group was assigned, somewhat arbitrarily, as the anchoring site in MTX because of reportedly[20] higher chemical reactivity of this group relative to that of the γ-carboxyl function.

Given that it was intended to load the carriers only fractionally, up to an arbitrary level not exceeding 15 mol % to maintain aqueous solubility, the molar MTX/OH feed ratios were restricted to 0.07–0.28. The mole fractions of DCC in the feed were kept equal to, or slightly larger than, the MTX fractions, and the DMAP fractions chosen were the same as, or insignificantly smaller than, those of MTX. Under these conditions, the extent of drug incorporated ranged from about 3 to 16 mol %, corresponding roughly to 6–28% of MTX by mass. The *x*/*y* ratios derived from the drug-loading data were in the approximate range of 5–35, and these ratios are indicated in the product designations as parenthetic numbers. Pertinent experimental and product compositional data are summarized in Tables I and II.

CONCLUSIONS

The data reveal the expected trend of increasing drug incorporation with growing mole fraction of MTX in the feed, and the trends are similar for the conjugates of both the **1-MTX** and the **2-MTX** series. With very roughly one-half of the drug equivalents in the feed effectively utilized for carrier binding, the esterification process is clearly lacking efficaciousness in both series. Experiments conducted with considerably larger molar excess of coupling agent over drug gave slightly higher (typically by about 10%) drug incorporation levels. In a greater number of repeat experiments, however, this caused partial crosslinking, presumably through involvement of both carboxyl groups of the drug. For this reason, such larger mole fractions of DCC in the feed were not routinely used in this work.

Selected conjugates of this study will be submitted for *in vitro* screening against the LNCaP human metastatic prostate carcinoma cell line.

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