Carrier-Bound Methotrexate. II. Water-Soluble Polyaspartamide Methotrexate Conjugates with Amide Links in Polymer–Drug Spacer

Gregg Caldwell, Maria G. Meirim, David D. N'Da, Eberhard W. Neuse

School of Chemistry, University of the Witwatersrand, WITS 2050, Witwatersrand, South Africa

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ABSTRACT: In Part I of this series, conjugation of methotrexate (MTX) to water-soluble polyaspartamide carriers was accomplished through ester link formation between an MTX carboxyl group and a carrier-attached hydroxyl function. Contrasting with that type of anchoring link, this project utilizes amide formation as the means of drug conjugation. This is achieved through condensation of one of the drug's carboxyl groups with a carrier-attached primary amine function. Derived from polysuccinimide by a time-proven nucleophilic ring-opening process in the presence of aliphatic diamines, polyaspartamide-type carriers **1–12** comprise subunits equipped with *tert*-amine or hydroxyl side group terminals for hydrosolubilization and other subunits equipped with primary amine terminals as drug-binding sites. MTX conjugation with these carriers is effected in aprotic solvent, the reaction being mediated by 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate. The water-soluble conjugates are fractionated and purified by size exclusion chromatography and dialysis; they are isolated by freeze-drying in typical yields of 40–65%. In the molar MTX/NH_2 feed ratios that are chosen (generally 1.2–1.3) and with the mole fractions of drug-binding subunits restricted to 10 and 20%, drug loading in the resultant conjugates approximates 20–30% by mass. In follow-on study, conjugates **1-MTX–12-MTX** thus obtained will be screened in cell culture tests for antiproliferative activity against a number of human cancer lines. J Appl Polym Sci 100: 3415–3424, 2006

Key words: methotrexate; antifolate agent; polymer conjugation; amide links; polyaspartamide carriers

INTRODUCTION

Methotrexate (MTX), a deoxyfolic acid derivative, has found clinical application for many years as a highly potent anticancer agent. (For leading reviews, see Refs. 1–3). Folate-dependent enzymes play a vital role in the provision of nucleotides required for the build-up of DNA by both normal and transformed (i.e., cancerous) cells, but predominantly so in the latter type because of their excessive rates of metabolism. MTX acts as a tight-binding inhibitor of dihydrofolate reductase, the key enzyme in intracellular folate metabolism. As a result of this process, folate cofactors accumulate in their inactive dihydrofolic acid form, which in turn inhibits purine and thymidylate synthesis, leading to cell death. This process is somewhat selective, antifolate action being stronger in transformed tissues than in normal ones. Intracellular polyglutamylation of MTX contributes beneficially to the drug's accumulation in the tumor cell. MTX is active against choriocarcinoma and acute lymphocytic leukemia and in combination with other drugs, it is active against osteosarcoma and carcinomas of the lung, cervix, ovaries, bladder, and other organs. Multiagent chemotherapy including MTX has recently also been used successfully against AIDS-related lymphoma, with complete remission observed in some 50% of test cases.[4] As a typical cytotoxic agent, MTX causes toxic side effects and development of resistance; its uptake by the tumor cell proceeds by an active transport mechanism, which appears to be inhibited as resistance is building up.[5,6]

The bioreversible tying (anchoring) of a medicinal agent to a macromolecular carrier possessing water solubility entails a number of significant, clinically demanded advantages (recently reviewed by Duncan and Spreafico,[7] Putnam and Kopeček,[8] Maeda,[9] and others[10,11]), such as a reduction in systemic toxicity, enhanced accumulation in tumor tissue, and facilitated cell entry, thereby compensating for any inhibition of the active transport mechanism or accelerated P-glycoprotein-mediated drug efflux from the cell. With the aforementioned pharmacological deficiencies of MTX taken into account, this drug should thus serve as an excellent candidate for polymer anchoring.

Correspondence to: E. W. Neuse (dept@aurum.wits.ac.za).
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The literature cites numerous reports of polymer–MTX conjugation. In most of these, natural proteinaceous polymers have served as the carriers; among the more outstanding publications are those from the laboratories of Uemto et al.,[12] Ghose et al.,[13] Fitz-Patrick and Garnett,[14] Stehle et al.,[15] and, most recently, Boratyńtiski et al.[16] Less frequently used carrier types include dextrans 18–20 or human-made polymers exemplified by poly(ethylene oxide)-containing blocopolymers,[20] divinyl ether–maleic anhydride copolymers,[21,22] and polypeptides.[23,24] Poly(L-lysine) has proved to be a particularly valuable substrate for MTX anchoring in the early pioneering studies of Przybylski et al.,[25] Ryser and Shen,[26] Chu et al.,[17,27] and Arnold et al.[28] Both in vitro and in vivo tests conducted with selected conjugates have provided highly encouraging results with cytotoxic activities equal to or better than those shown by the free drug.

As part of the ongoing program in our laboratory to develop carrier-conjugated anticancer agents, MTX binding to hydroxyl-functionalized polyaspartamides via ester links was reported in Part I of this series,[29] the ester groups in the spacer segments providing the site of biocleavage for ultimate in vivo release of the free drug. In drug-anchoring studies researchers generally favor the amide over the ester link for conjugation because of its superior hydrolytic stability, permitting extended circulation half-lives of the conjugates prior to the endocytotic step of entry into the target cell. For example, MTX bound by amide to a dextran carrier reportedly had a half-life in PBS medium of some 20 days, whereas that of the ester-bound counterpart polymer was a mere 3 days.[19] Similar relative stability observations were made in other laboratories.[12,20]

Against that background, in an effort to study relative stability and drug release behavior, we have embarked on a project to synthesize polyaspartamide–MTX conjugates with amide links in the spacer, complementing the series of ester-bound conjugates dealt with in the foregoing article.[29] This work is described in the present communication.

EXPERIMENTAL

General procedures

Proton NMR (^{1}H-NMR) spectra were recorded at 400 MHz on D_2O solutions, and the chemical shifts (δ) are given in parts per million relative to internal sodium 3-(trimethylsilyl)-2,2,3,3-d_4-propionate (integration error limits = ±12%). Immediately prior to recording, the pD was adjusted to 10 (NaOH) in order to eliminate potential protonation effects. The spectra of conjugates were scanned in duplicate and the derived intensity data averaged for proton count determination. The proton counts given in parentheses in the ^{1}H-NMR descriptions refer to those calculated for the respective carrier (**1–12**) or conjugate (**1-MTX–12-MTX**) structures.

The inherent viscosities (η_{inh}) were determined at 30.0 ± 0.5°C in Cannon–Fenske tubes on aqueous solutions, with a concentration of 0.2 g/100 mL. Results are expressed in milliliters per gram. Polymeric compounds dissolved in H_2O were routinely dialyzed in Spectra/Por 4 membrane tubing (molecular mass cutoff limit = 12,000–14,000) and/or Spectra/Por 6 wet tubing (cutoff limit = 25,000). The aqueous outer phases were magnetically stirred and periodically replaced by fresh batches. A Virtis Bench Top 3 freeze-drier operating at −30°C and 0.1 torr was used for freeze-drying operations. Carrier polymers were routinely postdried in a Sartorius Thermo Control Infrared Drying System (2 × 8 min at 65°C heating program). Sample material prepared for microanalysis was further dried for 2 days at 60–65°C and 10 torr in an Abderhalden tube. This relatively mild drying procedure, which was used to avoid structural changes, generally left 1–3% moisture in the hygroscopic polymers, reflected in the slightly low carbon values that were determined. Microanalyses were conducted by W. Dindorf (Wiesbaden, Germany).

Solvents, reagents, and reactants

Deionized water was used for preparative and dialysis operations. *N*,*N*-Dimethylformamide (DMF) and *N*-methylpyrrolidone (NMP), both predried over 4-Å molecular sieves, were redistilled under reduced pressure in a faint stream of N_2. Hexamethylphosphoramide (HMP) was predried over 4-Å molecular sieves. All other solvents were laboratory grade and used as received. All monomers and monomeric reactants (reagent-grade commercial products, Fluka Chemie AG) were used as received, as was the coupling agent 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU).

Poly-D,L-succinimide was prepared as a master batch by the published procedure;[30] the mass-average molecular mass, derived from viscosity data[31], was 41,500. MTX (amethopterin hydrate) was a gift from Wyeth–Ayerst Research and the Massachusetts General Hospital; additional compound was purchased from Sigma–Aldrich.

Polyaspartamide carriers

The amounts of polymeric educts and products are given as base moles, thus corresponding to structures **1–12** and **1-MTX–12-MTX**, each normalized to $y = 1$.

Polyaspartamide 1

This carrier, poly-α,β-DL-[*N*-(3-(dimethylamino)propyl)aspartamide(90)-*co*-*N*-(3-aminopropyl)aspart-

amide(10)], was synthesized essentially by the procedure elaborated elsewhere,[32] with slight modifications. 3-(Dimethylamino)propylamine (920 mg, 9 mmol) dissolved in 3 mL of DMF was added dropwise to the stirred 970 mg (10 mmol) solution of predried polysuccinimide in 8 mL of DMF. The solution, which was saturated with N_2, was stirred in a stoppered flask for 10 h at room temperature and then added dropwise to 222 mg (3 mmol) of 1,3-diaminopropane dissolved in 7 mL of DMF and precooled in an ice bath. Upon resaturation with N_2, stirring of the solution was continued for 24 h at ice bath temperature and another 6 h at ambient temperature. Up to this point, all operations were performed in the absence of moisture to prevent hydrolytic imide ring opening in intermediary stages with concomitant generation of free carboxyl groups. The solvent was partially removed under reduced pressure, and the product polymer was precipitated with 2 : 1 Et_2O–hexane (25 mL). The sticky precipitate, which was washed with 1 : 1 Me_2CO–hexane and hot toluene for removal of any unreacted amines, was redissolved in 20 mL of H_2O. After pH adjustment to 7.5 (HCl), the solution was dialyzed against H_2O for 2 days in Spectra/Por 4 tubing and for another 2 days in Spectra/Por 6 tubing. For the last 6 h of dialysis, the pH of the inside solution was readjusted to 9 with aqueous ammonia to reverse nitrogen protonation of the *tert*-amine side group terminals. In this last dialysis step, rapid agitation and frequent exchange of the outer phase was essential for complete removal of NH_4CI from the product polymers. (In the lysosomal compartment, this ammonium salt would raise the pH and thus deactivate lysosomal enzymes[33] with potentially retarding effects on drug detachment from the carrier.) Freeze-drying of the retentate afforded the polymer as a cream-colored, fluffy solid that was completely soluble in H_2O. Yield = 1.06 g (54.1%); η_{inh} = 11.6 mL g^{-1}. ^{1}H-NMR (δ): 3.2, 22H (20H, CONHCH_2); 2.8–2.2, 38H [40H, CH_2CONH, CH_2N(CH_3)$_2$, CH_2NH$_2$]; 2.2–2.1, 54H (54H; CH_3); 1.8–1.7, 20H (20H; CH$_2$CH_2CH$_2$).

Polyaspartamide 2

By the general procedure described in the foregoing description, carrier **2**, poly-α,β-DL-[*N*-(3-(dimethylamino)propyl)aspartamide(80)-*co*-*N*-(3-aminopropyl)-aspartamide(20)], was prepared from polysuccinimide (10 mmol), 3-(dimethylamino)propylamine (8 mmol), and 1,3-diaminopropane (6 mmol) in a total of 20 mL of DMF. Work-up as in the preceding experiment afforded **2** as a water-soluble solid in 44% yield; η_{inh} = 14.5 mL g^{-1}. ^{1}H-NMR (δ): 3.2, 10H (10H, CONHCH_2); 2.8–2.25, 19H [20H, CH_2CONH, CH_2N(CH_3)$_2$, CH_2NH$_2$]; 2.2, 25H (24H; CH_3); 1.8, 10H (10H, CH$_2$CH_2CH$_2$).

Polyaspartamide 3

The basic procedure described for polyaspartamide **1**, except that 1,3-diaminopropane was replaced by diethylenetriamine in the same feed ratio, gave **3**, poly-α,β-DL-[*N*-(3-(dimethylamino)propyl)aspartamide(90)-*co*-*N*-(3,6-diazahexyl)aspartamide(10)], in 43% yield as a water-soluble solid; η_{inh} = 10.3 mL g^{-1}. ^{1}H-NMR (δ): 4.7–4.5, 9H (10H, COC*H*NH); 3.2, 19H (20H, CONHCH_2); 2.8–2.1, 97H [98H, CH_2CONH, CH$_2$N(CH$_3$)$_2$, CH_2NH(CH_2)$_2$NH$_2$]; 1.7–1.6, 18H (18H, CH$_2$CH_2CH$_2$).

Polyaspartamide 4

By the general procedure described for **1**, the water-soluble carrier **4**, poly(α,β-DL-[*N*-(3-(dimethylamino) propyl)aspartamide(80)-*co*-*N*-(3,6-diazahexyl) aspartamide(20)], was obtained from polysuccinimide (10 mmol), 3-(dimethylamino)propylamine (8 mmol), and diethylenetriamine (6 mmol) in a total of 22 mL of DMF. Yield = 60.4%; η_{inh} = 17.0 mL g^{-1}. ^{1}H-NMR (δ): 3.2–3.1, 9.5H (10H, CONHCH_2); 2.8–2.2, 21.5H [24H, CH_2CONH, CH_2N(CH_3)$_2$, CH_2NH (CH_2)$_2$NH$_2$]; 2.2–2.1, 24H (24H, CH_3); 1.9–1.8, 8H (8H, CH$_2$CH_2CH$_2$).

Polyaspartamide 5

This carrier, poly-α,β-DL-[*N*-(3-(dimethylamino) propyl)aspartamide(90)-*co*-*N*-(9-aza-3,6-dioxanonyl) aspartamide(10)], was taken from a previous investigation.[34] It was redialyzed for 30 h in Spectra/Por 6 tubing and collected by freeze-drying as a water-soluble solid; η_{inh} = 10.6 mL g^{-1}.

Polyaspartamide 6

The compound poly-α,β-DL-[*N*-(3-(dimethylamino) propyl)aspartamide (80)-*co*-*N*-(9-aza-3,6-dioxanonyl) aspartamide(20)] was prepared by the general procedure described for **1** from polysuccinimide (10 mmol), 3-(dimethylamino)propylamine (8 mmol), and ethylenedioxy-O,O′-bis(2-ethylamine) (6 mmol). However, the room-temperature stirring period in the second step was increased to 24 h. The polymer was obtained in 66% yield as a water-soluble solid; η_{inh} = 14.1 mL g^{-1}. ^{1}H-NMR (δ): 4.7–4.5, 4.5H (5H, COC*H*NH); 3.6, 7.5H (8H, CH$_2$O); 3.4–3.2, 9.6H (10H, CONHCH_2); 2.8–2.2, 21H [20H, CH_2CONH, CH_2N(CH_3)$_2$, CH_2NH$_2$]; 2.2–2.1, 24.2H (24H; CH_3); 1.7, 8H (8H, CH$_2$CH_2CH$_2$).

Polyaspartamide 7

The method for the synthesis of **7**, poly-α,β-DL-[*N*-(2-hydroxyethyl)-aspartamide(90)-*co*-*N*-(3,6-diazahexy1)-aspartamide(10)], was similar to that described for **1**.

The solution of ethanolamine (550 mg, 9 mmol) in 3 mL of DMF was added dropwise to 970 mg (10 mmol) of polysuccinimide dissolved in 8 mL of DMF and saturated with N_2. After resaturation with N_2, the solution was stirred for 6 h at ambient temperature and subsequently added dropwise to the stirred and precooled (ice bath) solution of 311 mg of diethylenetriamine (3 mmol) in 10 mL of DMF. Stirring was continued for 20 h in the ice bath and another 24 h at room temperature. The polymeric product was precipitated, washed, and dialyzed as described for **1**, except that the pH was readjusted to 8 for the final 6-h dialysis period in Spectra/Por 6 tubing. The water-soluble polymer was isolated by freeze-drying in a yield of 658 mg (40.5%); η_{inh} = 16.2 mL g^{-1}. ^{1}H-NMR (δ): 4.75–4.55, 9.6H (10H, COC*H*NH); 3.7–3.6, 18H (18H, CH_2OH); 3.4–3.2, 20H (20H, CONHCH_2); 3.0–2.5, 26H (26H, remaining CH_2).

Anal. Calcd for $(C_{62}\ H_{106}N_{22}O_{29})_n$ $(1623.6)_n$ (**7**): C, 45.87%; H, 6.58%; N, 18.98%. Found: C, 43.55%; H, 6.71%; N, 17.99%.

Polyaspartamide 8

This carrier, poly-α,β-DL-[*N*-2-hydroxyethyl)aspartamide(80)-*co*-*N*-(3,6-diazahexyl)aspartamide(20)], was synthesized as described in the foregoing experiment from polysuccinimide (20 mmol), ethanolamine (16 mmol), and diethylenetriamine (12 mmol) in a total of 42 mL of DMF. The water-soluble **8** was collected in 55% yield; η_{inh} = 12 mL g^{-1}. ^{1}H-NMR (δ): 3.7–3.6, 8H (8H; CH_2OH); 3.4–3.1, 10.2H (10H; CONHCH_2); 2.9–2.5, 15H (16H, remaining CH_2).

Polyaspartamide 9

Carrier poly-α,β-DL-[*N*-(3,6-dioxahexyl)aspartamide-(90)-*co*-*N*-(3,6-diazahexyl)aspartamide(10)] was obtained by a procedure analogous to that described for the synthesis of **7**, with ethanolamine replaced by 2-(2-aminoethoxy)ethanol in the same feed ratio. Furthermore, the stirring period in the first reaction step was extended to 12 h to accommodate the somewhat lower reactivity of the aminoethoxyethanol as compared to ethanolamine. The water-soluble solid was isolated by freeze-drying in a yield of 58.3%; η_{inh} = 12.1 mL g^{-1}. ^{1}H-NMR (δ): 4.7–4.5, 8H (10H, CO-C*H*NH); 3.75–3.6, 54H [54H, CH_2O(CH_2)$_2$OH]; 3.4–3.2, 20H (20H, CONHCH_2); 2.9–2.5, 28H (26H, remaining CH_2).

Anal. Calcd for $(C_{80}H_{142}N_{22}O_{38})_n$ $(2020.2)_n$ (**9**): C, 47.56%; H, 7.09%; N, 15.26%; O, 30.10%. Found: C, 45.10%; H, 7.15%; N, 14.09%.

Polyaspartamide 10

As in the preceding experiment, yet in the appropriately changed feed ratio, polysuccinimide (20 mmol) was treated with 2-(2-aminoethoxy)ethanol (16 mmol) and diethylenetriamine (12 mmol), to give carrier **10** in 49% yield; η_{inh} = 11.1 mL g^{-1}. ^{1}H-NMR (δ): 3.7–3.6, 24H [24H, CH_2O(CH_2)$_2$OH]; 3.4–3.2, 9H (10H, CONHCH_2); 2.9–2.5, 15H (16H, remaining CH_2).

Polyaspartamide 11

For the synthesis of this carrier, the same procedure was used as for the preparation of **1** except that 3-(dimethylamino)propylamine and 1,3-diaminopropane were replaced by 2-methoxyethylamine and diethylenetriamine, respectively, in the same molar ratio of 10:9:3. In addition, for the final 6-h period of dialysis in Spectra/Por tubing the pH was adjusted to 8. Carrier **11** was obtained as a water-soluble solid in a yield of 48%; η_{inh} = 8.9 mL g^{-1}. ^{1}H-NMR (δ): 3.6, 18H (18H; CH_3OCH_2); 3.4–3.2, 46.8H (47H; CH_3OCH$_2$, CONHCH_2); 2.9–2.6, 25.2H (26H; CH_2CONH, CH_2NHCH_2CH_2).

Polyaspartamide 12

This carrier was prepared as in the preceding experiment except that the amounts of reactants in the feed were 10 mmol polysuccinimide, 8 mmol 2-methoxyethylamine, and 6 mmol diethylenetriamine. Water-soluble compound **12** was collected in 59% yield; η_{inh} = 12.8 mL g^{-1}. ^{1}H-NMR (δ): 3.6, 8H (8H; CH_3OCH_2); 3.5–3.2, 21.7H (22H; CH_3OCH$_2$, CONHCH_2); 2.9–2.5, 17H (16H; CH_2CONH, CH_2NHCH_2CH_2).

Polyaspartamide–MTX conjugates

Conjugate 1-MTX

A solution was prepared from 200 mg (0.102 mmol) of carrier **1** in 5 mL of DMF. Then, 56 mg (0.122 mmol) of MTX was added and dissolved. HBTU (46 mg, 0.12 mmol) dissolved in 2 mL of DMF was added dropwise to the rapidly stirred, N_2-saturated solution over a period of 20 min. This was followed by the addition of 21 mg of triethylamine (28 μL, 0.204 mmol). The solution was stirred in the stoppered flask for 2 h at ambient temperature, followed by chilling in an ice bath. The polymeric product was precipitated with 15 mL of Et_2O–hexane (2 : 1), thoroughly washed with warm Me_2CO, and dissolved in 6 mL of H_2O. A pH adjustment to 10 (Na_2CO_3) was followed by size exclusion chromatography on a Sephadex G25, and the aqueous eluate (exclusion volume) was dialyzed in Spectra/Por 6 tubing for 2 days. The pH, now ~7, was adjusted to 4 (1*M* HCL) to regenerate the unconjugated carboxyl group of MTX from its sodium salt. After pH readjustment to 6 (NH_4OH), dialysis in the same tubing was continued for another 8 h with numerous changes of the aqueous outer phase for com-

plete removal of inorganic salts. The retentate was freeze-dried to give 125 mg (51.5%) of yellow, water-soluble conjugate **1-MTX**. ^{1}H-NMR (δ): 8.6–6.6 (individual signals 8.5, 7.6, and 6.6), 4.9H (1H + 2H + 2H = 5H; aromatic and heteroaromatic CH of MTX); 1.6, 20H (20H, $CH_2CH_2CH_2$). These data indicate MTX incorporation to be 98% of available NH_2 anchoring groups.

Conjugate 2-MTX

For the preparation of this conjugate the basic procedure described for the foregoing experiment was used with appropriate modifications. A rapidly stirred solution of 200 mg (0.207 mmol) of carrier **2** in 5 mL of DMF was prepared, and 113 mg (0.249 mmol) of MTX was added in small portions and dissolved. A solution of 86 mg (0.228 mmol) of HBTU in 2 mL of DMF was added dropwise over a 0.5-h period with continuous stirring, and this was followed by the addition of 42 mg (0.414 mmol) of triethylamine. The voluminous precipitate formed was redissolved by the addition of 2 mL of HMP. The yellow solution saturated with N_2 was stirred for 2 h at room temperature and cooled in an ice bath for 0.5 h. The conjugate formed was precipitated, washed, further purified, and isolated as described for **1-MTX**, yielding 155 mg (53.3%) of yellow, water-soluble solid. ^{1}H-NMR (δ): 8.5–6.5, 4.8H (5H; aromatic and heteroaromatic CH of MTX); 1.6, 10H (10H; $CH_2CH_2CH_2$). A 96% MTX incorporation is indicated by these data.

Conjugate 3-MTX

The general procedure leading to **1-MTX** was used for the preparation of **3-MTX** from 199 mg (0.1 mmol) of carrier **3**, 55 mg (0.12 mmol) of MTX, 46 mg (0.12 mmol) of HBTU, and 24 mg (0.24 mmol) of triethylamine, in a total of 7 mL of DMF. Work-up by the conventional procedure afforded 125 mg (51.5%) of water-soluble conjugate. ^{1}H-NMR (δ): 8.5–6.7, 5.05H (5H; aromatic and heteroaromatic CH of MTX); 1.6, 18H (18H; $CH_2CH_2CH_2$). MTX incorporation was 101%.

In a separate experiment carried out essentially as described above, the incorporation of MTX was only 85%. The polymer (50 mg, ~0.025 mmol) was retreated with 9 mg (0.02 mmol) of MTX, 5 mg (0.013 mmol) of HBTU, and 5 mg (0.05 mmol) of triethylamine and the reaction period was extended to 4 h. After routine work-up, the conjugate was isolated in a recovery yield of 35 mg (70%). ^{1}H-NMR data showed the compound to contain the drug at the 100% level.

Conjugate 4-MTX

By the procedure leading to **2-MTX**, yet with an MTX/NH_2 feed ratio of 1.3, 1O0 mg (0.1 mmol) of carrier **4** was treated with 59 mg (0.13 mmol) of MTX, 46 mg (0.12 mmol) of HBTU, and 26 mg (0.26 mmol) of triethylamine, in 4 mL of DMF plus 1 mL of HMP. We obtained 85 mg (59.3%) of water-soluble conjugate. ^{1}H-NMR (δ): 8.6–6.7, 4.75H (5H; aromatic protons, MTX); 1.65, 8H (8H; $CH_2CH_2CH_2$). MTX incorporation was 95%.

In a parallel experiment conducted with a molar MTX/NH_2 feed ratio of 1.2, the extent of MTX incorporation was unsatisfactory (82%). In order to achieve essentially quantitative MTX incorporation, the conjugate was retreated as follows. A 25-mg sample (0.025 mmol) of the polymer was dissolved in 2 mL of DMF together with 2.0 mg (0.02 mmol) of triethylamine and 4.6 mg (0.01 mmol) of MTX, the latter added predissolved in 0.5 mL of HMP. Upon saturation with N_2 and the dropwise addition of 3.8 mg (0.01 mmol) of HBTU (predissolved in 0.5 mL of NMP), the solution was stirred from 3 h at ambient temperature and worked up as described above. Twelve milligrams of yellow, water-soluble conjugate **MTX** were obtained. ^{1}H-NMR (δ): 8.6–6.7, 4.7H (5H; aromatic and heteroaromatic CH of MTX); 1.6, 8H (8H; $CH_2CH_2CH_2$). MTX incorporation was 94%.

Conjugate 5-MTX

Under the conditions of the experiment affording **1-MTX**, except that an MTX/NH_2 molar feed ratio of 1.3 was used, 1O2 mg (0.05 mmol) of carrier **5** was treated with 30 mg (0.065 mmol) of MTX, 23 mg (0.06 mmol) of HBTU, and 13 mg (0.13 mmol) of triethylamine, in a total of 4 mL of DMF. After conventional work-up, a yield of 55 mg (44.5%) of water-soluble **5-MTX** was collected. ^{1}H-NMR (δ): 8.6–6.7, 4.9H (5H; aromatic and heteroaromatic CH of MTX); 1.65, 18H (18H; $CH_2CH_2CH_2$). MTX incorporation was 98%.

In a parallel experiment performed as described above, only 70% of available NH_2 groups were acylated. A retreatment with MTX (0.7 eq), HBTU (0.5 eq), and triethylamine (1.9 eq), conducted over a 4-h period at room temperature, raised the MTX incorporation level to 97.5%.

Conjugate 6-MTX

This water-soluble polymer was obtained by the general procedure described for **2-MTX** from 104 mg (0.1 mmol) of carrier **6**, 59 mg (0.13 mmol) of MTX, 46 mg (0.12 mmol) of HBTU, and 26 mg (0.26 mmol) of triethylamine, in a total of 3 mL of DMF–HMP. The reaction period was extended to 4 h. Yield = 65 mg (44%). ^{1}H-NMR (δ): 8.6–6.6, 4.85H (5H; aromatic and heteroaromatic CH of MTX); 8H (8H; $CH_2CH_2CH_2$), corresponding to 97% NH_2 acylation.

Conjugate 7-MTX

Because of a lack of complete solubility during the coupling step that was occasionally experienced, the preferred solvent for this conjugation experiment was a 3 : 2 blend of DMF and HMP. In addition, the relative amounts of MTX and HBTU in the feed were increased to 1.5 and 1.3, respectively. A representative procedure follows. Carrier **7** (200 mg, 0.123 mmol), dissolved in 5 mL of DMF–HMP, was treated with 84 mg (0.185 mmol) of MTX, 37 mg (0.37 mmol) of triethylamine, and 61 mg (0.16 mmol) of HBTU, predissolved in 0.5 mL of DMF and added dropwise over a period of 30 min. After saturation with N_2, the solution was stirred for 2 h at room temperature and cooled to ~5°C before the polymeric product was precipitated and further treated as described for **1-MTX**. This gave 140 mg (55.3%) of yellowish, water-soluble conjugate. ^{1}H-NMR (δ): 8.6–6.4, 4.85H (5H; aromatic and heteroaromatic CH of MTX); 3.7, 18H (18H; CH_2OH). The data indicate 97% acylation.

A separate experiment conducted as above but with a molar MTX/HBTU/NH_2 feed ratio of 1.2 : 1.1 : 1 gave a polymer product with only 66% acylation.

Conjugate 8-MTX

An experiment was conducted as described for the preparation of **2-MTX**, with the following compounds in the feed: 200 mg (0.24 mmol) of carrier **8**, 132 mg (0.29 mmol) of MTX, 1O0 mg (0.27 mmol) of HBTU, and 59 mg (0.58 mmol) of triethylamine. The water-soluble conjugate (159 mg, 52%) was incompletely acylated. ^{1}H-NMR (δ): 8.5–6.4, 3.9H (5H; MTX); 3.7–3.5, 8H (8H; CH_2OH). NH_2 acylation = 78%. The polymer (42 mg, 0.05 mmol) was retreated with 18 mg (0.04 mmol) of MTX, 10 mg (0.025 mmol) of HBTU, and 10 mg (0.1 mmol) of triethylamine over a period of 4 h at ambient temperature. The routinely isolated conjugate was recorded in a yield of 27 mg (54%). ^{1}H-NMR (δ): 8.6–6.4, 5H (5H; aromatic and heteroaromatic CH of MTX); 3.7–3.5, 8H (8H; CH_2OH). NH_2 acylation was 100%.

Conjugate 9-MTX

For the preparation of this conjugate the procedure employed for the synthesis of **7-MTX** was used, except that carrier **7** was replaced by **9** and the reaction period extended to 3 h. Conventional work-up provided **9-MTX** as a water-soluble polymer in a yield of 172 mg (56.9%). ^{1}H-NMR (δ): 8.6–6.5, 4.8H (5H; aromatic and heteroaromatic CH of MTX); 3.8–3.5, 54H (54H; OCH_2). The data indicate 96% acylation.

Conjugate 10-MTX

An experiment performed as described for **2-MTX**, with the reactants carrier **10**, MTX, HBTU, and triethylamine employed in the same molar feed ratio of 1 : 1.2 : 1.1 : 2, gave the polymer product in 51% yield, the MTX incorporation being 88%. The polymer (50 mg, 0.05 mmol) was retreated in a total of 4 mL of DMF–HMP (2 : 1) with 18 mg (0.04 mmol) of MTX, 10 mg (0.025 mmol) of HBTU, and 1O mg (0.1 mmol) of triethylamine over a 4-h reaction period. This gave 35 mg (70%) of water-soluble **10-MTX**. ^{1}H-NMR (δ): 8.5–6.5, 5H (5H; aromatic and heteroaromatic CH of MTX); 3.7–3.4, 24H (24H; OCH_2). This corresponded to 100% acylation.

Conjugate 11-MTX

By the general procedure leading to **1-MTX**, 200 mg (0.114 mmol) of carrier **11** was treated with 62 mg (0.137 mmol) of MTX, 48 mg (0.126 mmol) of HBTU, and 23 mg (0.229 mmol) of triethylamine, in a total of 6 mL of DMF. We obtained 170 mg (68.2%) of water-soluble conjugate. ^{1}H-NMR (δ): 8.5–6.5, 5.1H (5H; aromatic and heteroaromatic CH of MTX); 3.6–3.2, 65H (65H; CH_3OCH_2, CONHCH_2). Effectively 100% acylation is indicated by these data.

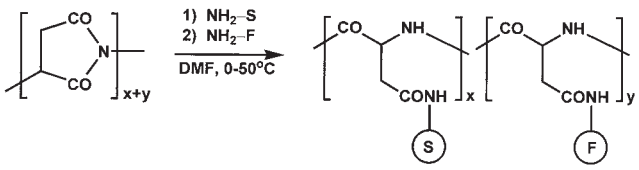
Conjugate 12-MTX

By the basic procedure leading to **2-MTX**, yet with slightly increased equivalents of MTX and HBTU and the reaction period extended to 3 h, **12-MTX** was prepared from 100 mg (0.12 mmol) of carrier **12**, 71 mg (0.156 mmol) of MTX, 55 mg (0.144 mmol) of HBTU, and 32 mg (0.317 mmol) of triethylamine over a reaction period of 3 h. The water-soluble polymer was conventionally isolated in a yield of 79 mg (49.7%). ^{1}H-NMR (δ): 8.5–6.5, 4.9H (5H; aromatic and heteroaromatic CH of MTX); 3.6–3.2, 30H (30H; CH_3OCH_2, CONHCH_2). The data correspond to 98% acylation.

RESULTS AND DISCUSSION

The carriers utilized in this project for MTX anchoring were α,β-DL-polyaspartamides obtained from polysuccinimide by stepwise aminolytic imide ring opening, a reaction type pioneered by Neri and Antoni[30] and others.[31] In our laboratory, numerous water-soluble polyaspartamide derivatives have been synthesized in more recent years in accordance with Scheme 1 (random sequence of subunits; only the α-peptide forms are shown). In the structure depicted, S generally represents a side chain attached solubilizing group and F stands for a segment containing a functional group introduced here as the drug-binding site.

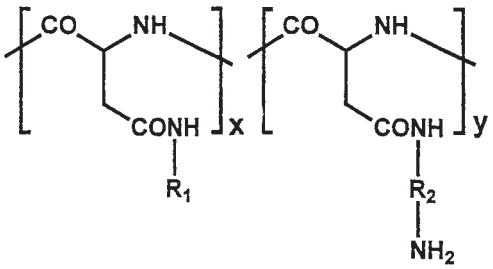
Chains possessing the natural α-L-peptidic linking system undergo rapid enzyme-mediated degradation in the aqueous vascular system and thus would prove dysfunctional as drug carriers destined to experience



Scheme 1 The method for synthesizing water-soluble polyaspartamide derivatives.

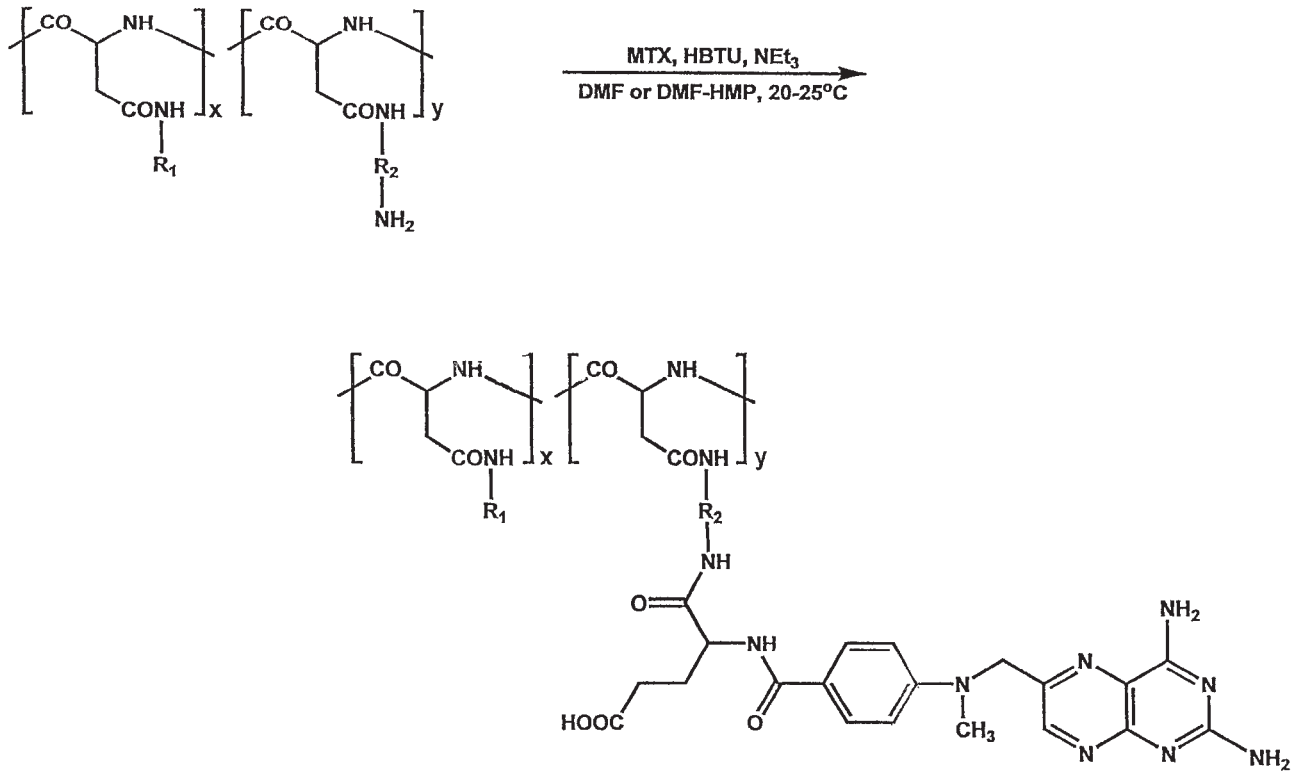
cell entry without prior backbone fission. However, the α,β-DL forms arising in the process of Scheme 1 feature an optimal combination of backbone links with sufficient resistance to α-peptidase-mediated "unzipping" and other enzymatic fission mechanisms; yet, they are still prone to ultimate breakdown and elimination in the "spent" (i.e., drug depleted) state.

In the presently described project, the chosen carriers of the type shown in Scheme 1 featured primary amino groups introduced as terminals in component F, serving as the MTX-binding functionality. The subunits incorporating this binding site comprised 10 and 20 mol % of the chains, leaving 90 and 80 mol % of S-labeled subunits of the *tert*-amine or hydroxyl types for hydrosolubilization, respectively. Carrier structures **1–12** (Fig. 1) exemplify these compositional specifications, the choice of which was based on the necessity to restrict drug loading to levels commensurate with the retention of water solubility of the resulting conjugates. Experiments with carriers containing larger mole percentages of F-Iabeled subunits previously demonstrated the need for utilization of a disproportionately large excess of MTX in the feed for complete drug incorporation. In addition, the cou-



Carrier	R_1	R_2	x/y
1	$-(CH_2)_3N(CH_3)_2$	$-(CH_2)_3-$	9
2	$-(CH_2)_3N(CH_3)_2$	$-(CH_2)_3-$	4
3	$-(CH_2)_3N(CH_3)_2$	$-(CH_2)_2NH(CH_2)_2-$	9
4	$-(CH_2)_3N(CH_3)_2$	$-(CH_2)_2NH(CH_2)_2-$	4
5	$-(CH_2)_3N(CH_3)_2$	$-(CH_2)_2O(CH_2)_2O(CH_2)_2-$	9
6	$-(CH_2)_3N(CH_3)_2$	$-(CH_2)_2O(CH_2)_2O(CH_2)_2-$	4
7	$-(CH_2)_2OH$	$-(CH_2)_2NH(CH_2)_2-$	9
8	$-(CH_2)_2OH$	$-(CH_2)_2NH(CH_2)_2-$	4
9	$-(CH_2)_2O(CH_2)_2OH$	$-(CH_2)_2NH(CH_2)_2-$	9
10	$-(CH_2)_2O(CH_2)_2OH$	$-(CH_2)_2NH(CH_2)_2-$	4
11	$-(CH_2)_2OCH_3$	$-(CH_2)_2NH(CH_2)_2-$	9
12	$-(CH_2)_2OCH_3$	$-(CH_2)_2NH(CH_2)_2-$	4

Figure 1 Composition of carriers **1–12**.



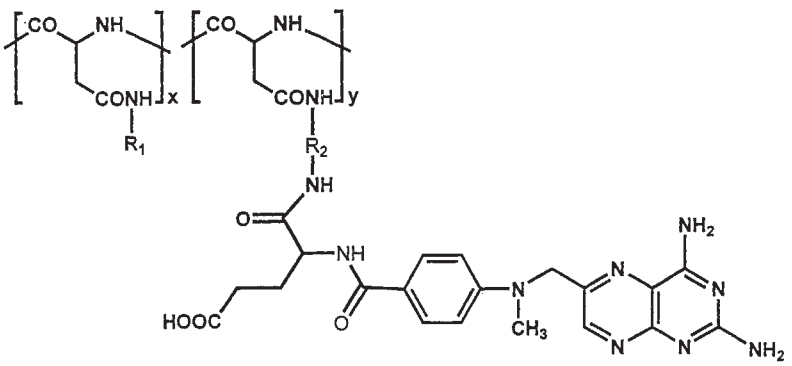
Scheme 2 The coupling reactions performed by treatment of the carrier with MTX, organic base, and HBTU for 2 h at ambient temperature in DMF solution.

pling products thus obtained displayed a propensity for branching and crosslinking with concomitantly decreasing solubility once they had been isolated in the solid state. All carriers were fractionated by dialysis to remove constituents with molecular masses substantially below 25,000.

The literature provides numerous examples of MTX anchoring that involves the covalent, reversible attachment to the carrier through amide bond formation. Coupling with the aid of such water-soluble carbodiimides as 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride has been the preferred technique of anchoring in these studies, notably where proteinaceous carriers have been used and the reactions conducted in the aqueous phase.

Coupling in anhydrous media such as DMF has generally been brought about through the intermediacy of in situ presynthesized, active *N*-hydroxysuccinimide esters. In our work the presynthesized, in situ used active ester, especially if applied in higher mole ratios, tended to cause gradual crosslinking of the ultimate conjugates, presumably because of the presence of bifunctionally activated drug molecules. The preferred method adopted in our laboratory therefore involved the direct acid–amine coupling mediated by the HBTU coupling agent. The coupling reactions were performed by treatment of the carrier with MTX, organic base, and HBTU for 2 h at ambient temperature in DMF solution (Scheme 2). The typically employed MTX/NH_2 molar reactant ratios were 1.2 or 1.3. Under these conditions, MTX binding in most experiments approached the 100% level. However, in polymer-homologous reactions, such as carrier–drug conjugation, one commonly observes greater variability than in analogous nonpolymeric processes. Accordingly, a small number of coupling experiments in our work failed to achieve an extent of conjugation larger than 70–90%, with corresponding proportions of the F-modified subunits left intact in the conjugates. In those cases, a retreatment with drug, base, and coupling agent generally resulted in essentially complete anchoring.

Target conjugates **1-MTX–12-MTX** (Fig. 2) after precipitation from solution by added nonsolvent were subjected to size exclusion chromatography and staged aqueous dialysis under carefully controlled pH conditions for removal of unreacted drug and byproducts. They were isolated as water-soluble solids by freeze-drying. Ultimate product yields typically ranged from 40 to 65%. Pertinent viscosity data, reaction variables, and conjugate yields are summarized in Table I, which also lists the MTX contents in mass percents. These were determined by ^{1}H-NMR spectroscopy, assessing the relative intensities of the aromatic resonances in the 6.5–8.5 ppm region against other prominent bands in the spectra. For several con-



Conjugate	R_1	R_2	x/y
1-MTX	$-(CH_2)_3N(CH_3)_2$	$-(CH_2)_3-$	9
2-MTX	$-(CH_2)_3N(CH_3)_2$	$-(CH_2)_3-$	4
3-MTX	$-(CH_2)_3N(CH_3)_2$	$-(CH_2)_2NH(CH_2)_2-$	9
4-MTX	$-(CH_2)_3N(CH_3)_2$	$-(CH_2)_2NH(CH_2)_2-$	4
5-MTX	$-(CH_2)_3N(CH_3)_2$	$-(CH_2)_2O(CH_2)_2O(CH_2)_2-$	9
6-MTX	$-(CH_2)_3N(CH_3)_2$	$-(CH_2)_2O(CH_2)_2O(CH_2)_2-$	4
7-MTX	$-(CH_2)_2OH$	$-(CH_2)_2NH(CH_2)_2-$	9
8-MTX	$-(CH_2)_2OH$	$-(CH_2)_2NH(CH_2)_2-$	4
9-MTX	$-(CH_2)_2O(CH_2)_2OH$	$-(CH_2)_2NH(CH_2)_2-$	9
10-MTX	$-(CH_2)_2O(CH_2)_2OH$	$-(CH_2)_2NH(CH_2)_2-$	4
11-MTX	$-(CH_2)_2OCH_3$	$-(CH_2)_2NH(CH_2)_2-$	9
12-MTX	$-(CH_2)_2OCH_3$	$-(CH_2)_2NH(CH_2)_2-$	4

Figure 2 Composition of conjugates **1-MTX–12-MTX**.

jugates, the MTX contents were independently determined from UV spectra (257 nm, 0.1*M* NaOH), giving similar results. From these findings we deduce that in aqueous solution under the conditions of our NMR experiments the conjugates of this study do not undergo molecular association; such self-assembly would cause MTX signal attenuation in the NMR spectra as observed, for example, with MTX conjugates based on poly(ethylene oxide)-terminated block copolymers.[20]

The actual binding site on the glutamyl constituent of the drug in these reactions is unknown, and most researchers in the field leave this question open. In the conjugate structures shown in Figure 2 we have ascribed the α-carboxyl group to this function, our arbitrary choice being corroborated by a literature report giving preference to that group as the more reactive one in coupling reactions with polylysine.[28] It may be recalled, parenthetically, that MTX–dihydrofolate reductase binding likewise involves the drug's α-carboxyl group,[35] although steric factors in the complex may contribute to this regiospecific binding.

Cell culture tests probing the antiproliferative activity against selected human cancer cell lines will be performed on the described conjugates in forthcoming investigations.

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TABLE I
Polyaspartamide–MTX Conjugates

Carrier			Mol ratio in feed	Conjugate						
						M				
Designation	*M*	η_{inh} (mL g^{-1})	MTX/NH_2	Yield (%)	Designation	Actual	Calcd	NH_2 acylation (%)	MTX content[a] (%)	η_{inh} (mL g^{-1})
1	1964.5	11.6	1.2	51	1-MTX	2392.2	2400.9	98	18.6	16.1
2	968.2	14.5	1.2	53	2-MTX	1387.2	1404.6	96	31.45	19.9
3	1993.5	10.3	1.2	52	3-MTX	2429.9	2429.9	101[b]	18.7	16.7
4	997.3	17.0	1.3	59	4-MTX	1411.8	1433.6	95[b]	30.6	20.2
5	2038.5	10.6	1.3	45	5-MTX	2466.3	2474.9	98[b]	18.1	15.4
6	1042.3	14.1	1.3	44	6-MTX	1465.6	1478.7	97	30.1	22.9
7	1623.7	16.2	1.5	55	7-MTX	2047.0	2060.1	97[b]	21.5	18.7
8	832.9	12.0	1.2	52	8-MTX	1269.3	1269.3	100	35.8	21.8
9	2020.2	12.1	1.5	57	9-MTX	2439.1	2456.6	96	17.9	—
10	1009.1	11.1	1.2	70	10-MTX	1445.5	1445.5	100	31.4	21.2
11	1750.0	8.9	1.2	68	11-MTX	2186.4	2186.4	100	20.8	13.9
12	889.0	12.8	1.3	50	12-MTX	1316.7	1325.4	98	33.8	22.0

M, base molecular mass, actual or actual and calculated (for 100% acylation).
[a] By mass.
[b] In parallel experiments conducted under identical or modified conditions, the percentage of acylation ranged from 66 to 85%, requiring retreatment (see Experimental section).

sachusetts General Hospital, and to Wyeth–Ayerst Research, Pearl River, NY, for donations of MTX. Merck SA (Pty) Ltd. is thanked for a generous solvent donation.

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