# Carrier-bound Methotrexate. IV. Antiproliferative Activity of Polyaspartamide-MTX Conjugates against Leukemic Lymphoblast Cell Lines

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Received 17 May 2005; revised and accepted 7 November 2006.

ABSTRACT

**Polymeric conjugates of methotrexate (MTX) with macromolecular carriers, obtained from amine-functionalized polyaspartamides by coupling with one of the drug's carboxyl groups, are used in this preliminary screening project for *in vitro* cytotoxicity assessment. The water-soluble conjugates, crudely fractionated by aqueous dialysis, possess mass-average molecular masses in the range of 20000–30000. Screens are performed by standard procedures against cultured CEM/S human leukemic lymphoblast cells, a drug-sensitive line, and against CEM/E, its drug-resistant subline, for comparison also against unconjugated MTX. All compounds tested, including the unconjugated drug, display decreasing activity on going from CEM/S to CEM/E, resistance factors ($IC_{50}$ [CEM/E]/$IC_{50}$[CEM/S]) being in the vicinity of 15–20 for MTX as well as for the majority of conjugates. On the other hand, comparisons of $IC_{50}$ values for conjugates *versus* free drug, both against CEM/S and CEM/E, show vastly superior antiproliferative performance of the drug in the carrier-anchored state over the free form, with activity factors ($IC_{50}$[free MTX]/$IC_{50}$ [conjugate]) typically in the 10–50 range and higher. On the basis of these promising *in vitro* findings, the polyaspartamide-MTX conjugates are considered to be excellent candidates for further cell culture and extended *in vivo* tests.**

KEYWORDS

**Methotrexate conjugates, polyaspartamide, CEM leukemic lymphoblasts.**

## 1. Introduction

For several decades now, the antifolate drug methotrexate (+ amethopterine, MTX), a classical, highly potent anticancer agent, has been in clinical use both *per se* and in combination with other drugs.[2–4] The compound acts as an inhibitor of dihydrofolate reductase, thus preventing the reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate. This leads to inhibition of DNA synthesis and eventual cell death.[3]

To the detriment of patient compliance, however, the drug suffers from severe pharmacological shortcomings, notably high systemic toxicity and a propensity for eliciting drug resistance in the affected cells.[4,5] In efforts to enhance the compound's therapeutic effectiveness, numerous laboratories worldwide have embarked on projects aiming at the bioreversible attachment (conjugation) of the drug to biocompatible carrier polymers that would serve as vehicles capable of enhancing bioavailability while circumventing the toxicity and resistance problems. Here we cite only the pioneering work by Ringsdorf[6] and, later, by Shen and Ryser.[7]

In our laboratory, synthetic polymers have been given preference over natural macromolecules as carriers for drug anchoring. This choice is based on the lower immunogenicity generally shown by suitably constructed synthetics, paired with the overriding advantage that a synthetic polymer may be tailor-made to accord with biomedical specifications and unique compositional requirements. Thus, 'blueprints' for the structural build-up of both the hydrosolubilizing and the drug-binding subunits, as well as their relative frequencies along the backbone, can be predetermined and synthetically executed. Such structural versatility is strikingly displayed by peptidic polymers of the α,β-DL-polyaspartamide type, and we have used these extensively for drug conjugation.[8] The inclusion of β-peptidic and D-configurated units in the main chain prevents rapid exopeptidase-mediated degradation through 'unzipping', thus ensuring sufficient stability of the conjugate while in central circulation. In an earlier project in this laboratory[9] polyaspartamides were conjugated with MTX through tethers containing an ester group as the biofissionable site, and in a recent study[1] we prepared a series of related conjugates with biocleavable carboxamide links in the connecting spacer (Scheme 1; for convenience, only the α-form is depicted here). Representative conjugates of both classes have now entered preliminary *in vitro* screening studies, in which their antiproliferative properties are assayed against a series of human cancer cell lines. In the present communication we present the screening results obtained with selected conjugates containing amide-tethered MTX against human CEM/S leukemic lymphoblasts and the derived multidrug-resistant CEM/E subline. It has been our special objective in this study to compare these results with the cytotoxic behaviour of free, i.e. unconjugated MTX tested in the same screens.

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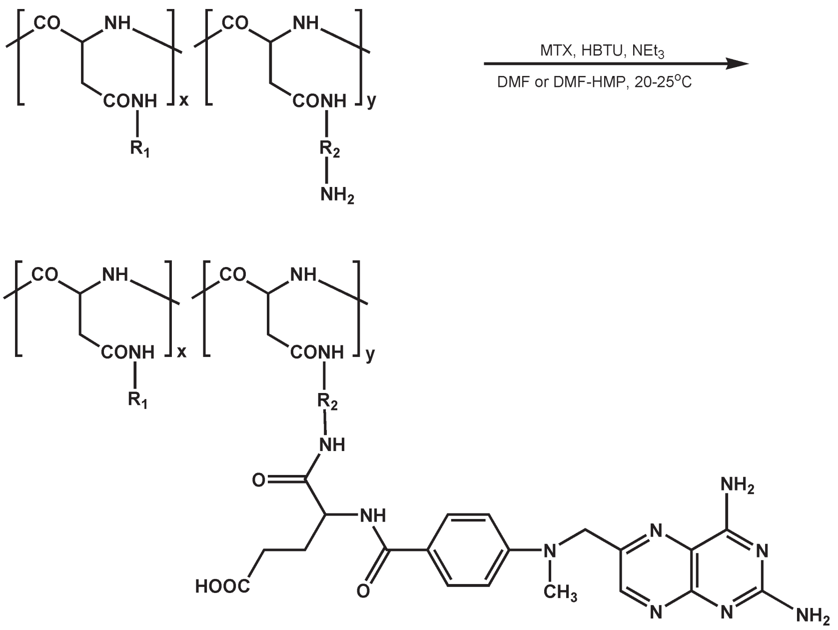
## 2. Results and Discussion

It was demonstrated in the preceding investigation[1] that MTX can be conjugated in aprotic medium with polyaspartamides bearing short, amine-terminated side chains, as depicted in Scheme 1. The reactions are mediated by coupling agents, such as HBTU, (2-(1 H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) or EEDQ, 1-ethoxycarbonyl-2-ethoxy-




**Scheme 1**

1,2-dihydroquinoline. This coupling technique was employed with minor modifications for the preparation of most of the conjugates used in the present project, while a few more conjugates were taken from that earlier work.[1] The drug is carrier-bound in these reactions through one of its carboxyl groups, the α-COOH functionality being the preferred one in view of its higher reactivity.[10] We used a 20–30% molar excess of MTX and coupling agent in order to enforce acceptable drug incorporation, but even under these facilitating conditions complete acylation of available $NH_2$ groups was not always achieved; in the majority of reactions, product polymers tended to be a few per cent short of complete acylation. In exceptional cases, where drug incorporation remained below 90%, the products were retreated with MTX and coupling agent, which brought the extent of N-acylation up to an acceptable level of 95% or higher.

The collection of conjugates made available for testing is reproduced in Fig. 1, and compositional data are collected in Table 1. Whereas in compounds **1** to **8** the hydrosolubilizing functionality $R_1$ is represented by a tertiary amine side chain terminal acting as a potentially cationic species on protonation under physiological conditions, conjugate **9** features an indifferent methoxy side group terminal, and in **10** to **14** the group $R_1$ is represented by a hydroxyl-terminated alkyl chain.

The conjugates were tested *in vitro* by standard procedure[11] against the drug-sensitive CEM/S cell line and, in parallel, against the multidrug-resistant CEM/E subline. Free MTX was tested under the same conditions for comparison. The cytotoxic activities determined for the individual samples are listed in Table 2, expressed in terms of $IC_{50}$ values (drug concentration, in $\mu$g MTX $mL^{-1}$, required to retain 50% cell viability relative to drug-free control). The table also contains entries for the resistance factor, RF, defined here as the ratio of $IC_{50}$[CEM/E] over $IC_{50}$[CEM/S].

A cursory comparison of the results tabulated in the two CEM columns immediately reveals the expected trend of lowered activity on going from CEM/S to CEM/E, with resistance factors generally in the vicinity of 15–20, and the same trend obtains for free MTX (RF = 20.5). Against the two CEM lines, then, the carrier-bound drug, on balance, exhibits no selective ability to circumvent resistance. For conjugates **3** and **8** resistance factors below 5 are apparent from the tabulation. These are exceptional, however, and a larger number of repetitively synthesized conjugates will be required to confirm and rationalize this deviating behaviour.

Comparing now performance data of individual conjugate structures, we detect only minor differences on going from type to type. Significantly, against CEM/S, the overall $IC_{50}$ range (~0.01–0.2) for polymers with tertiary amine side functionalities (**1** to **8**) does not substantially differ from that (~0.01–0.3) determined for the conjugates featuring hydroxyl-terminated side

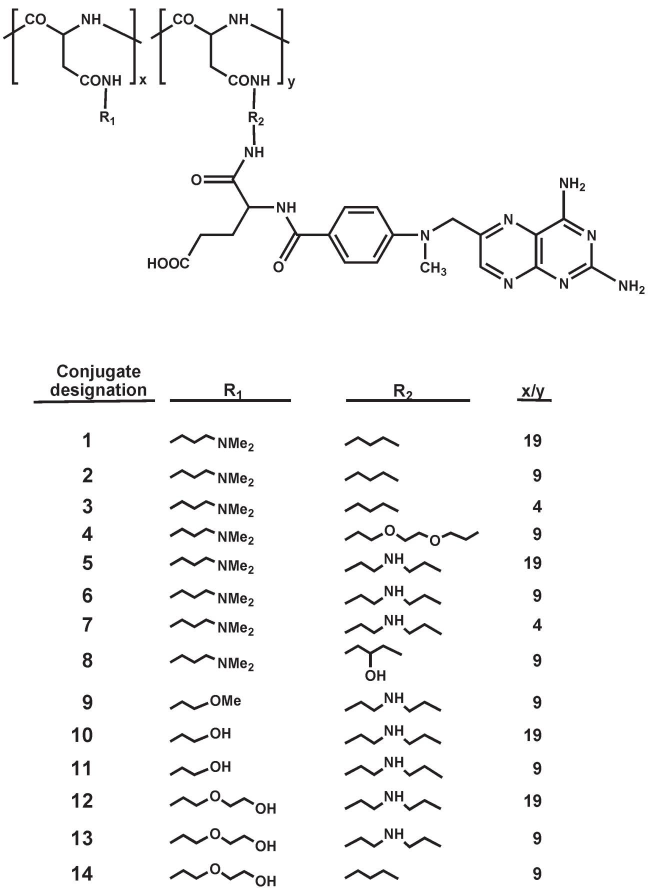



**Fig. 1**

**Table 1** Structural data for conjugates **1** to **14.**

| Conjugate designation | Base molecular mass [a] | $\eta_{inh}$/mL g$^{-1}$ | Extent of acylation [b] | % MTX by mass |
|---|---|---|---|---|
| **1** | 4393.4 | | 101 | 10.4 |
| **2** | 2387.8 | 16.2 | 97 | 18.5 |
| **3** | 1382.8 | 20.0 | 95 | 31.2 |
| **4** | 2466.3 | 15.4 | 98 | 18.1 |
| **5** | 4400.6 | | 95 | 9.8 |
| **6** | 2429.9 | 16.8 | 101 | 18.7 |
| **7** | 1411.9 | 20.2 | 95 | 30.6 |
| **8** | 2408.2 | | 98 | 18.5 |
| **9** | 2186.3 | 13.9 | 100 | 20.8 |
| **10** | 3615.52 | 18.6 | 94 | 11.8 |
| **11** | 2047.0 | | 97 | 21.5 |
| **12** | 4474.3 | | 99 | 10.0 |
| **13** | 2456.6 | | 100 | 18.5 |
| **14** | 2405.7 | | 95 | 17.9 |

[a] Molecular mass of recurring unit.
[b] Percentage substitution by MTX of available $NH_2$.

groups (**10** to **14**). The same argument holds for the $IC_{50}$ data determined against CEM/E. (The only outstanding case is conjugate **9**, which shows poor performance relative to all other samples; this may be an artifact, however, which will be reinvestigated). Evidently, realistic structure-performance relationships can only be derived on the basis of an increased sample number for each structural type, and future investigations will focus on this topic.

The most striking aspect of the here described series of tests emerges as we compare for each CEM column the data derived for the carrier-drug conjugates with those pertaining to unconjugated MTX. With just one exception (conjugate **9**), the conjugate-derived $IC_{50}$ values are considerably lower than the respective values for the free drug. This indicates the cytotoxic activities of the polymer-bound drug to exceed monomeric MTX activity by a large factor (40- to 50-fold in about one-third of all tested samples). The tabulated figures in the activity factor column (AF = $IC_{50}$[MTX]/$IC_{50}$ [conjugate]) provide the details.

In summary: methotrexate, both conjugated and non-conjugated, shows essentially the same trend of decreasing antiproliferative activity on going from the sensitive to the resistant CEM lines. Conjugation thus provides no panacea for circumvention of drug resistance in tests against CEM. On the other hand, vastly superior activities, up to 50-fold and higher, against both CEM/S and CEM/E are observed for the carrier-anchored MTX derivatives in relation to the unbound drug. In view of the common experience that realistic pharmacological benefits, as they arise from drug binding to a carrier polymer, will manifest themselves predominantly, if not solely, in the living organism,[12–14] these findings are highly significant and warrant ongoing studies involving further synthetic work and extensive *in vitro*/*in vivo* screens.

## 3. Experimental

### 3.1. General Procedures

Inherent viscosities, $\eta_{inh}$, were determined with the aid of Cannon-Fenske viscometers in $H_2O$ at 30.0 ± 0.5°C; the concentration was c = 0.2 g100 mL$^{-1}$. Data are reported in units of dL g$^{-1}$. $^1$H NMR spectra (400 MHz; integration error limits, ± 12%) were

**TABLE 2** Antiproliferative activity of conjugates **1** to **14** against CEM/S and CEM/E.

| Comp. | CEM/S | | | CEM/E | | | RF [b] |
|---|---|---|---|---|---|---|---|
| | $IC_{50}$ | | AF [a] | $IC_{50}$ | | AF [a] | |
| | μg MTX mL$^{-1}$ | M [c] | | μg MTX mL$^{-1}$ | M [c] | | |
| **1** | 0.023 | $5.06 \times 10^{-8}$ | 36.3 | 0.548 | $1.21 \times 10^{-6}$ | 31.1 | 23.8 |
| **2** | 0.107 | $2.35 \times 10^{-7}$ | 7.8 | 1.556 | $3.42 \times 10^{-6}$ | 11.0 | 14.5 |
| **3** | 0.137 | $3.01 \times 10^{-7}$ | 6.1 | 0.542 | $1.19 \times 10^{-6}$ | 31.5 | 4.0 |
| **4** | 0.163 | $3.59 \times 10^{-7}$ | 5.1 | 2.120 | $4.67 \times 10^{-6}$ | 8.1 | 13.0 |
| **5** | 0.016 | $3.52 \times 10^{-8}$ | 52.1 | 0.331 | $7.28 \times 10^{-7}$ | 51.6 | 20.7 |
| **6** | 0.121 | $2.66 \times 10^{-7}$ | 6.9 | 1.773 | $3.90 \times 10^{-6}$ | 9.6 | 14.7 |
| **7** | 0.02 | $4.40 \times 10^{-8}$ | 41.7 | 0.467 | $1.03 \times 10^{-6}$ | 36.5 | 23.4 |
| **8** | 0.145 | $3.19 \times 10^{-7}$ | 5.8 | 0.633 | $1.39 \times 10^{-6}$ | 27.0 | 4.4 |
| **9** | >20 | $>4.4 \times 10^{-5}$ | – | >20 | $>4.4 \times 10^{-5}$ | – | – |
| **10** | 0.031 | $6.82 \times 10^{-8}$ | 26.9 | 0.577 | $1.27 \times 10^{-6}$ | 29.6 | 18.6 |
| **11** | 0.273 | $6.01 \times 10^{-7}$ | 3.1 | 6.433 | $1.42 \times 10^{-5}$ | 2.7 | 23.6 |
| **12** | 0.019 | $4.18 \times 10^{-8}$ | 43.9 | 0.377 | $8.30 \times 10^{-7}$ | 45.3 | 19.8 |
| **13** | 0.013 | $2.86 \times 10^{-8}$ | 64.1 | 0.281 | $6.18 \times 10^{-7}$ | 60.7 | 21.6 |
| **14** | 0.011 | $2.42 \times 10^{-8}$ | 75.8 | 0.228 | $5.02 \times 10^{-7}$ | 74.9 | 20.7 |
| MTX | 0.834 | $1.84 \times 10^{-6}$ | – | 17.07 | $3.76 \times 10^{-5}$ | – | 20.5 |

[a] Activity factor: $IC_{50}$[free MTX]/$IC_{50}$[conjugate].
[b] Resistance factor: $IC_{50}$[CEM/E]/$IC_{50}$[CEM/S].
[c] Moles MTX/L.

taken on $D_2O$ solutions. Chemical shifts, δ, are given in ppm relative to sodium 3-(trimethylsilyl)-2,2,3,3-$d_4$-propionate; unless stated otherwise, $D_2O$ solutions of polymeric materials were routinely adjusted to pD 10 just prior to scanning to eliminate spurious protonation. A VIRTIS Bench Top 3 freeze-drier operating at –30°C, 0.1 torr, was used for the lyophilization of aqueous polymer solutions. Dialysis was performed in Spectra/Por 4 membrane tubing (12 000–14 000 molecular mass cut-off) and in Spectra/Por 6 wet tubing (25 000 molecular mass cut-off), for separation of second polymer fractions also in Spectra/Por 3 (6000 molecular mass cut-off). The operations were conducted against frequently changed batches of magnetically stirred $H_2O$ at specified pH. Size exclusion chromatography was performed on Sephadex G-25. Polymer samples were dried in a SARTORIUS Thermo Control Infrared Drying System (heating program: 2 × 5 min at 65°C) or in an Abderhalden tube (2 d at 50°C) under reduced pressure.

Cell culture tests were performed over a 72-h period against the CCRF CEM/S leukemic lymphoblasts cell line and its resistant CCRF CEM/E sublime. SEM limits did not exceed 10% from the mean in all tests. The protocol used has previously been described.[11]

### 3.2. Solvents, Reagents, Monomeric Reactants

Deionized water was used for preparative, chromatographic, and dialysis operations. N,N-Dimethylformamide (DMF) and N-methylpyrrolidone (NMP), both predried over Molecular Sieves 4A, were redistilled under reduced pressure in a gentle stream of $N_2$; the first 5% of distillate were discarded. All other solvents, laboratory grade, were used as received, and so were the coupling agents, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), as well as monomeric amines and other reactants (Fluka Chemie AG). Methotrexate (+ amethopterin, MTX) was a gift from Dr J. Kreisher, Theraplex Corporation; additional quantities were purchased from Fluka Chemie.

### 3.3. Poly-DL-succinimide

This educt polymer was prepared as a master batch by the procedure of Neri and Antoni,[15] the mass-average molecular mass derived from viscosity data[16] was 34100. The polymer, finely pulverized, was dried in an Abderhalden tube (1 d, 70–75°C) under reduced pressure.

### 3.4. MTX Conjugates

Amounts of conjugates and precursor polymers are given as base moles and thus refer to the simplest recurring units defined by structures **1** to **14** normalized to $y = 1$.

*Conjugate* **1**. The preparation of **1** exemplifies MTX conjugation with the aid of EEDQ coupling agent. The carrier serving as the educt polymer for this conjugate, poly-α,β-DL-[N-(3-(dimethylamino)propyl)aspartamide(95)-*co*-N-(3-aminopropyl)aspartamide(5)], was prepared by the established general procedure for aminolytic ring opening in polysuccinimide. To the stirred solution of polysuccinimide, 1.94 g (20 mmol) in 15 mL of DMF, was added 3-(dimethylamino)propylamine, 1.941 g (19 mmol), dissolved in 1 mL of DMF. The solution, saturated with $N_2$, was stirred in the stoppered flask for 8 h at ambient temperature, followed by dropwise addition to 1,3-diaminopropane, 222 mg (3 mmol), predissolved in 15 mL of DMF, stirred and cooled in an ice bath. Resaturated with $N_2$, the resulting solution was stirred for 20 h in an ice bath and another 3 h at room temperature. Up to this point, moisture access was strictly precluded to prevent inadvertent hydrolytic imide ring opening that would generate carboxylic acid side groups. Partial solvent removal under reduced pressure (bath temperature <50°C) and precipitation with excess $Et_2O$-hexane (2:1) afforded a resinous product, which was washed thoroughly with hot toluene and $Me_2CO$ for removal of monomeric amine. Redissolved in 25 mL of $H_2O$, with pH adjusted to 7–8, the product was dialysed for 2 d in Spectra/Por 4 and for another 2 d in Spectra/Por 6 tubing against frequently changed, magnetically stirred batches of $H_2O$. For the last 6 h of the second dialysis step, the retentate pH was raised to 9 (aq. ammonia) for elimination of spurious N-protonation. The tube contents were freeze-dried and post-dried, giving 2.05 g (51.8%) of light cream-coloured carrier polymer as a water-soluble solid.

$^1H$ NMR, δ/ppm (expected proton counts in brackets): 3.2, 39H (40H; CONHC$H_2$); 2.8–2.3, 78H (80H; NHCOC$H_2$, C$H_2$N(CH$_3$)$_2$, C$H_2$NH$_2$); 2.2 110H (114H; CH$_3$); 1.8, 40H (40H; CH$_2$C$H_2$CH$_2$).

For drug conjugation, the carrier, 396 mg (0.1 mmol), was dissolved in 8 mL of DMF. MTX, 56 mg (0.12 mmol), was added in small portions with stirring, followed by the dropwise addition by syringe of EEDQ, 30 mg (0.12 mmol), dissolved in 2 mL of NMP, and triethylamine, 24 mg (0.24 mmol). The solution, saturated with $N_2$, was stirred for 24 h at room temperature and for another 0.5 h at 50°C. The conjugate was precipitated from the cooled (5°C) solution with excess $Et_2O$-hexane (2:1) and redissolved in 20 mL of $H_2O$. The solution, with pH adjusted to 10 ($Na_2CO_3$) in order to dissociate and remove unreacted MTX, was passed through a column (1.5 × 15 cm) charged with Sephadex G-25 and equilibrated with $H_2O$ at the same pH. The eluate (exclusion volume) containing the light yellow product band was dialysed for 2 d in Spectra/Por 6 tubing against $H_2O$ at pH 6.8. For the last 6 h of this operation, the retentate pH was reduced to 4 (0.1 M HCl) and, after several minutes, raised again to 6 (aq. ammonia) in order to liberate the pendent carboxyl group of the attached drug from its Na salt. The retentate was freeze-dried to afford 270 mg (61.5%) of yellow, water-soluble **1**.

$^1H$ NMR, δ/ppm: 8.6–6.6, with individual signals at 8.6, 7.7, and 6.8, 5.1H (1H + 2H + 2H = 5H; aromatic and heteroaromatic CH of MTX); 1.7, 40H (40H; CH$_2$C$H_2$CH$_2$). The data indicate 101% MTX incorporation of available $NH_2$ sites, corresponding to 10.4% by mass.

The combined outer phases collected in this dialysis operation were redialysed for 2 d in Spectra/Por 3 tubing, and from the retentate another portion, 92 mg (21%), of lower-molecular conjugate was isolated as a yellow, water-soluble solid, NMR data indicating an MTX content of 8.5% by mass.

*Conjugate* **2**. In this coupling experiment, HBTU was used as the coupling agent. The precursor polymer, poly-α,β-DL-[N-(3-(dimethylamino)propyl)aspartamide(90)-*co*-N-(3-aminopropyl)aspartamide(10)], was synthesized from polysuccinimide, 3-(dimethylamino)-propylamine, and 1,3-diaminopropane by a previously described procedure.[18] It was isolated as a water-soluble solid in 51% yield.

$^1H$ NMR, δ/ppm: 3.25, 19H (20H; CONHC$H_2$); 2.8–2.3, 39H (40H; NHCOC$H_2$, C$H_2$N(CH$_3$)$_2$, C$H_2$NH$_2$); 2.2, 56H (54H; CH$_3$); 1.75, 20H (20H; CH$_2$C$H_2$CH$_2$).

For MTX conjugation, 196 mg (0.1 mmol) of the carrier was dissolved in 4 mL of DMF together with MTX, 56 mg (0.12 mmol). A solution of HBTU, 42 mg (0.11 mmol), in 0.5 mL of DMF was added dropwise with stirring, followed by triethylamine, 26 mg (0.26 mmol), and stirring of the $N_2$-saturated solution was continued for 2 h at room temperature. The conjugate was precipitated and further worked up as described for conjugate **1**, to give 135 mg (55%) of yellow, water-soluble **2**.

$^1$H NMR, δ/ppm: 8.6–6.5 combined, 4.85H (5H; aromatic and heteroaromatic CH of MTX); 1.7–1.6, 20H (20H; $CH_2CH_2CH_2$). Thus, 97% of available $NH_2$ is substituted by MTX, corresponding to a drug content of 17.0% by mass.

In a repeat drug coupling experiment, only 87% of available $NH_2$ sites were substituted. The polymer was re-treated with 0.4, 0.3, and 0.8 equivalents of MTX, HBTU, and $NEt_3$, respectively, for 2.5 h at ambient temperature. Work-up as above gave conjugate for which NMR data indicated an MTX content of 18.6% by mass.

*Conjugate* **3**. The carrier polymer, poly-α,β-DL-[N-(3-(dimethyl amino)propyl)aspartamide-(80)-*co*-N-(3-aminopropyl)asparta mide(20)], was synthesized as described for the precursor to **1**, except with these reagent amounts: polysuccinimide, 20 mmol; 3-(dimethylamino)-propylamine, 16 mmol; 1,3-diamino propane, 12 mmol; in a total of 35 mL of DMF. Yield, 47%.

$^1$H NMR, δ/ppm: 3.3–3.2, 10.5H (10H; $CONHCH_2$); 2.8–2.28, 18H (20H; $NHCOCH_2$, $CH_2N(CH_3)_2$, $CH_2NH_2$); 2.2, 26H (24H; $CH_3$); 1.7, 10H (10H; $CH_2CH_2CH_2$).

The carrier so obtained was conjugated with MTX by the procedure described for **2**, except with the following mole ratio of reactants and reagents in the feed:carrier, 0.3 mmol; MTX, 0.39 mmol; HBTU, 0.33 mmol; $NEt_3$, 0.78 mmol. The yellow, water-soluble conjugate **3** was obtained in a yield of 48%.

$^1$H NMR, δ/ppm: 8.6–6.6 combined, 4.75H (5H; aromatic and heteroaromatic CH of MTX); 1.7, 10H (10H; $CH_2CH_2CH_2$). An MTX incorporation of 95%, corresponding to 31.2% by mass, is indicated by these data.

In repeat experiments (and similar coupling experiments conducted at these reactant ratios in the feed) a faint precipitation of solids was observed in the early stages of the reaction. The addition of a few mL of hexamethylphosphoramide resulted in clear solutions.

*Conjugate* **4**. This conjugate, taken from the preceding investigation,[1] was redialysed (20 h in Spectra/Por 4) and isolated upon freeze-drying as a water-soluble polymer. $^1$H NMR data indicated 98% MTX incorporation, corresponding to 18.1% by mass.

In two repeat experiments, drug incorporation was 79 and 83%, requiring retreatment as described for conjugate **2**. This raised the figures to 93 and 95%, respectively, corresponding to 17.3 and 17.6% by mass.

*Conjugate* **5**. The required carrier, poly-α,β-DL-[N-(3-(dimethylamino)propyl)aspartamide-(95)-*co*-N-(3,6-diazahexyl)aspartamide(5)], was prepared by the procedure leading to the carrier required for **1**, except that 1,3-diaminopropane was replaced by the same amount (3 mmol) of diethylenetriamine. The water-soluble polymer was isolated in 59% yield.

$^1$H NMR, δ/ppm: 3.1, 42H (40H; $CONHCH_2$); 2.8–2.2, 61H (64H; $NHCOCH_2$, $CH_2N(CH_3)_2$, $CH_2NH(CH_2)_2NH_2$); 2.15, 117H (114H; $CH_3$); 1.7, 38H (38H; $CH_2CH_2CH_2$).

MTX conjugation with the carrier so obtained, 399 mg (0.1 mmol), was carried out as described for conjugate **1**. This gave 230 mg of light yellow, water-soluble **5**.

$^1$H NMR, δ/ppm: 8.6–6.8 combined 4.4H (5H; aromatic and heteroaromatic CH of MTX); 1.8, 38H (38H; $CH_2CH_2CH_2$). An MTX incorporation of 88% of available $NH_2$ is indicated by these data.

The conjugate was retreated as described for **2** to give polymer with 95% MTX incorporation, corresponding to 9.8% by mass. Yield, 175 mg (39.8%).

*Conjugates* **6** *and* **7**. Derived from the respective carriers, poly-α,β-DL-[N-(3-(dimethylamino)-propyl)aspartamide(90)-*co*-N-(3,6-diazahexyl)aspartamide(10)] and its (80)-(20) analog, these conjugates had previously been described and were taken from that investigation.[1] Redialysis for 1 d in Spectra/Por 4 and freeze-drying provided the conjugates with 101% and 95% MTX incorporation, corresponding to 18.7 and 30.6% MTX by mass.

*Conjugate* **8**. The required carrier, poly-α,β-DL-[N-(3- (dimethyl amino)propyl)aspartamide-(90)-*co*-N-(3-amino-2-hydroxy propyl)aspartamide(10)], was prepared in 54% yield from polysuccinimide, 970 mg (10 mmol), dissolved in 8 mL of DMF, 3-(dimethylamino)-propylamine, 920 mg (9 mmol), in 3 mL of DMF, and 1.3-diaminopropan-2-ol, 270 mg (3 mmol), in 8 mL of DMF by the general procedure described for the preparation of the precursor polymer of conjugate **1**. Yield, 1.05 g (53.5%).

$^1$H NMR, δ/ppm: 3.7, 1H (1H; $CH_2CH(OH)CH_2$); 3.2, 21H (20H; $CONHCH_2$); 2.9–2.1, 95H (94H; $NHCOCH_2$, $CH_2N(CH_3)_2$, $CH_2NH_2$); 1.7, 18H (18H; $CH_2CH_2CH_2$).

For MTX conjugation, the carrier, 198 mg (0.1 mmol), was dissolved in 2 mL of DMF together with MTX, 57 mg (0.125 mmol). HBTU, 47 mg (0.125 mmol), in 0.5 mL of DMF was added dropwise to the stirred solution. Upon the further addition of triethylamine 25 mg (0.25 mmol), the solution was saturated with $N_2$ and stirred for 2 h at room temperature. Work-up as described for conjugate **1** gave **8** as a yellowish, water-soluble solid in a yield of 110 mg (45.7%).

$^1$H NMR, δ/ppm: 8.6–6.6 combined, 4.3H (5H; aromatic and heteroaromatic CH of MTX); 1.8, 18H (18H; $CH_2CH_2CH_2$). MTX incorporation: 98%, corresponding to 18.5% by mass.

A repeat experiment provided **8** with 73% substitution of available $NH_2$

*Conjugate* **9**. This compound was taken from the preceding investigation.[1] It was redialysed for 1 d in Spectra/Por 4 tubing and, after freeze-drying, was collected as a yellow, water-soluble solid.

$^1$H NMR, δ/ppm: 8.6–6.5 combined, 5H (5H; aromatic and heteroaromatic CH of MTX); 3.6–3.2, 65H (65H; $CH_3OCH_2$, $CONHCH_2$). An MTX incorporation of 100% is inferred from these data, corresponding to 20.8% by mass.

*Conjugate* **10**. The precursor polymer, poly-α,β-DL-[N-(2-hydroxyethyl)aspartamide(95)-*co*-N-(3,6-diazahexyl) aspartamide(5)], was prepared in 43% yield as described for the synthesis of the educt polymer for conjugate **1**, except that ethanolamine replaced 3-(dimethylamino)propylamine, and diethylenetriamine was used in place of the diaminopropane, both in the respective amounts.

$^1$H NMR, δ/ppm: 3.7–3.6, 35H (38H; $CH_2OH$); 3.4-3.3, 41H (40H; $CONHCH_2$); 3.0–2.5, 43H (46H; remaining $CH_2$).

For drug conjugation, the carrier, 321 mg (0.1 mmol), was dissolved in 4 mL of DMF. To the stirred solution was slowly added MTX, 59 mg (0.13 mmol), a solution of HBTU, 49 mg (0.13 mmol) in 2 mL of NMP, and $NEt_3$, 27 mg (0.27 mmol), in that order. The stirred solution, saturated with $N_2$, was stirred for 2.5 h at ambient temperature. Precipitated and worked up as before, the conjugate was obtained as a yellowish, water-soluble solid in a yield of 240 mg (65%).

$^1$H NMR, δ/ppm: 8.7–6.9 combined, 4.7H (5H; aromatic and heteroaromatic CH of MTX); 3.7–3.6, 38H (38H; $CH_2OH$). An MTX incorporation of 94% is inferred from these data, corresponding to a drug content of 11.6% by mass.

*Conjugate* **11**. This conjugate was taken from the preceding investigation.[1] Redialysis for 1 d in Spectra/Por 4 tubing and conventional work-up provided **11** as a yellow water-soluble solid.

$^1$H NMR, δ/ppm: 8.5–6.4 combined, 4.85H (5H; aromatic and heteroaromatic CH of MTX); 3.6, 18H (18H; C*H*$_2$OH). These data indicate 97% MTX incorporation, corresponding to an MTX content of 21.5% by mass.

*Conjugate* **12**. The precursor polymer, poly-α,β-DL-[N-(3,6-dioxahexyl)aspartamide(95)-*co*-N-(3,6-diazahexyl)aspartamide(5)] was prepared in 53% yield by the method used for the synthesis of the educt polymer for conjugate **1**, except that 2-(2-aminoethoxy)ethanol and diethylenetriamine replaced 3-(dimethylamino)propylamine and 1,3-diaminopropane, respectively. The water-soluble carrier was routinely isolated in 53% yield.

$^1$H NMR, δ/ppm: 3.8–3.6, 114H (114H; O(CH$_2$)$_2$OCH$_2$); 3.5-3.2, 44H (40H; CONHC*H*$_2$); 3.0–2.5, 42H (46H; remaining CH$_2$).

MTX conjugation was brought about by dissolving the carrier, 404 mg (0.1 mmol), in 4 mL of DMF, and adding MTX, 64 mg (0.14 mmol), to the stirred solution. This was followed by the addition of HBTU, 49 mg (0.13 mmol) in 2 mL of NMP, and NEt$_3$, 28 mg (0.28 mmol). The resulting, N$_2$-saturated solution was stirred for 2.5 h at ambient temperature. Work-up by the conventional procedure gave light-yellow, water-soluble conjugate **12** in a yield of 264 mg (59%).

$^1$H NMR, δ/ppm: 8.6–6.8 combined, 4.95H (5H; aromatic and heteroaromatic CH of MTX); 3.8–3.6, 114H (114H; O(CH$_2$)$_2$ OCH$_2$). The data indicate 99% MTX incorporation, corresponding to a drug content of 10.1%.

*Conjugate* **13**. The precursor polymer, poly-α,β-DL-[N-(3.6-dioxahexyl)aspartamide(90)-*co*-N-(3.6-diazahexyl)aspartamide(10)], was taken from an earlier investigation,[17] there designated carrier **9**. It was redialysed for 20 h in Spectra/Por 4 tubing and, upon conventional work-up, was obtained as a beige-coloured, water-soluble solid.

$^1$H NMR, δ/ppm: 3.75–3.6, 54H (54H; O(CH$_2$)$_2$OCH$_2$); 3.5–3.1, 21H (20H; CONHC*H*$_2$); 3.0–2.5, 26H (26H; remaining CH$_2$).

Initial drug-coupling experiments employing MTX/NH$_2$ molar ratios in the feed of 1.2–1.3 afforded conjugates with deficient drug incorporation. This required a modification of the procedure. Briefly, the carrier, 202 mg (0.1 mmol), dissolved in 3 mL of DMF, was treated with MTX, 68 mg (0.15 mmol), a solution of HBTU, 53 mg (0.14 mmol), in 2 mL of NMP, and NEt$_3$, 30.5 mg (0.3 mmol), in that order. The N$_2$-saturated solution was stirred for 2 h at room temperature and worked up by the conventional procedure. This gave yellow, water-soluble conjugate **13** in a yield of 169 mg (69%).

$^1$H NMR, δ/ppm: 8.6–6.5 combined, 5H (5H; aromatic and heteroaromatic CH of MTX); 3.8–3.5, 54H (54H; O(CH$_2$)$_2$OCH$_2$). The data indicate 100% MTX incorporation, corresponding to 18.5% by mass.

*Conjugate* **14**. The precursor carrier, poly-α,β-DL-[N-(3.6-dioxahexyl)aspartamide(90)-*co*-N-(3-aminopropyl)aspartamide(10)], taken from an earlier project[18] (there designated **11**) was redialysed for 1 d in Spectra/Por 6 tubing and isolated as a water-soluble solid by the established procedure.

$^1$H NMR, δ/ppm: 3.8–3.55, 53.5H (54H; O(CH$_2$)$_2$OCH$_2$); 3.4–3.1, 20.5H (20H; CONHC*H*$_2$); 2.9–2.4, 21H (22H; NHCOC*H*$_2$, C*H*$_2$NH$_2$); 1.8–1.7, 2H (2H; CH$_2$C*H*$_2$CH$_2$).

The carrier, 199 mg (0.1 mmol), was conjugated with MTX, 59 mg (0.13 mmol), HBTU, 46 mg (0.12 mmol), and NEt$_3$, 27 mg (0.26 mmol), in 4 mL of DMF + 1 mL of NMP over a period of 2 h at room temperature and conventional work-up. There was obtained 149 mg (62%) of yellow, water-soluble **14**.

$^1$H NMR, δ/ppm: 8.6–6.5 combined, 4.75H (5H; aromatic and heteroaromatic CH of MTX); 3.8–3.5, 54H (54H; O(CH$_2$)$_2$OCH$_2$). The data show 95% of MTX to be incorporated, corresponding to a drug content of 18.0% by mass.

## Acknowledgements

Support of this investigation is gratefully acknowledged to the H.E. Griffin Cancer Trust, the Anglo American Chairman's Fund, and to the Cancer Association of South Africa in conjunction with the THRIP Project. Dr J. Kreisher, Theraplex Corporation, is thanked for a generous gift of methotrexate. D.D. N'Da is indebted to the Mellon Foundation for a study grant.

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