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Carrier-bound Methotrexate. III.‡ Antiproliferative Activity of Macromolecular MTX Conjugates Against the Human HeLa and Colo Carcinoma Cell Lines

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ABSTRACT

In continuation of studies in these laboratories aiming at the bioevaluation of macromolecular anticancer drug models, *in vitro* cytotoxicity screens are performed on several series of water-soluble polymer-methotrexate conjugates. The methotrexate drug in these conjugates is bound through amide or ester linkages to water-soluble polyamide- or polyamidoamine-type carriers by previously developed anchoring techniques. Tests were conducted against the HeLa human cervical carcinoma cell line generally considered to be drug-sensitive, and against two variants of the rather refractory Colo 320 DM, a human colon adenocarcinoma line.

KEYWORDS

Drug conjugation, Colo cell line, HeLa cell line, methotrexate, polyaspartamide, polyamidoamine.

1. Introduction

The concept of binding (conjugating) a medicinal agent to a macromolecular carrier for the purpose of enhancing the drug's overall clinical effectiveness dates back to the mid-seventies, and Helmut Ringsdorf must be credited with having laid the groundwork for this highly successful technology[2]. It was in Ringsdorf's laboratory where the prototype carrier-drug conjugate originated and the venerable anticancer agent, methotrexate, served in that work as the drug constituent.[3] Subsequently, Ringsdorf's strategy was creatively utilized in other laboratories, notably those of Ghose[4] Chu and Howell[5], Shen and Ryser[6], Garnett and Baldwin[7], Kanellos[8] and Deguchi[9], with co-workers, who pioneered the development of methotrexate conjugates with antibodies and other natural, proteinaceous polymers. Successful activities in this field have later been reported also by Pouton and Marriott[10], Stehle *et al.*[11], Boratyński *et al.*[12], Ghosh *et al.*[13], and most notably, by the prolific group of Umemoto, Kato and Hara[14]. The subject has more recently been reviewed by the last-named authors[15]. In our laboratory, man-made, i.e. fully synthetic, macromolecules as drug carriers have been given preference over proteinaceous and other natural polymers for a number of incisive reasons elaborated previously[1]. Most importantly, such macromolecules can be constructed precisely by design in terms of structural types, biodegradability, and frequency in the chain of the various subunits with solubilizing, drug-binding, and other facilitating functions. Polyaspartamides lend themselves particularly well to this application. Accordingly, as part of our ongoing programme in carrier-bound drug development we recently synthesized MTX conjugates in which the drug is anchored to polyaspartamide carriers through short connecting segments containing ester or carboxamide groups as the biofissionable links. A preliminary *in vitro* evaluation of selected polyaspartamide-MTX conjugates in screens against CEM human leukemic lymphoblasts showed outstanding cytotoxic behaviour[1], prompting further exploratory work with this conjugate system in tests against other cancer cell lines. In the present communication we present the results of *in vitro* tests performed against the Colo 320 DM human colon adenocarcinoma cell line. Colorectal malignancies are notoriously unresponsive to chemotherapy, and the Colo line may therefore serve as a useful tool to assess an anticancer agent's ability to circumvent drug resistance. The conjugates tested were predominantly of the polyaspartamide-MTX type, but several conjugates with the drug bound to poly(amidoamine) carriers were included in this project.

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2. Experimental

2.1. General Procedures

^{1}H NMR spectra (400 MHz; integration error limits, ±12%) were 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. Inherent viscosities, η_{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 g 100 mL^{-1}. Data are reported in units of dL g^{-1}. 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 programme: 2 × 5 min at 65°C) or in an Abderhalden tube (2 d at 50°C) under reduced pressure.

Conjugates were tested *in vitro* against the HeLa human cervical epitheloid carcinoma and against two variants of the Colo DM 320 human colon adenocarcinoma cell line. Tests were performed over a 72 h period; the protocol has previously been described[16].

2.2. Solvents, Reagents, 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 was 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 hexafluorophos-phate (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 A. Bogdanov; additional quantities were purchased from Fluka Chemie. The compound was dried for 2 d at 40°C in an Abderhalden tube under reduced pressure to remove water of hydration.

Poly-DL-succinimide, the educt polymer for the polyaspartamide carriers, was prepared as a master batch by the procedure of Neri and Antoni[17]; the mass-average molecular mass derived from viscosity data[18] was 31 600. The polymer, finely pulverized, was dried in an Abderhalden tube (1 d, 70–75°C) under reduced pressure.

2.3. N-Protection of Diamines

The diamine monomers required for the synthesis of conjugates **19**–**24**, 1,3-diaminopropane, diethylenetriamine, and 1,2-bis(2-aminoethoxy)ethane, were mono-N-protected with the BOC (*tert*-butoxycarbonyl) group. The procedure described below for the preparation of N-(*tert*-butoxycarbonyl)-1,3-diaminoethane, is representative.

The solution of the BOC protection agent, di-*tert*-butyl dicarbonate, 21.8 g (0.1 mol) in 140 mL of dry dioxan, was added dropwise to a solution of 1,3-diaminopropane, 59.3 g (0.8 mol) in 60 mL of dioxan. After a stirring period of 1 d at ambient temperature, the solvent, together with excess diamine, was removed by rotatory evaporation at 50°C bath temperature. Water, 100 mL, was added to the residue, and a small portion of insoluble N,N-bis-BOC-protected diamine was removed by filtration. The filtrate was exhaustively extracted with several 50-mL portions of methylene chloride; this left any residual unreacted diamine as the most hydrophilic constituent in the aqueous phase. From the combined extracts, dried over anhydrous $MgSO_4$, the solvent was removed on a rotavapor, leaving N-BOC-1,3-diaminopropane as an oily liquid in a yield of 12.5 g (72%). The crude compound, giving a very clean ^{1}H NMR spectrum, was used as collected.

^{1}H NMR, δ/ppm: 3.1 (t), 2H ($CONHCH_2$); 2.66 (t), 2H (CH_2NH_2); 1.6 (m), 2H ($CH_2CH_2CH_2$); 1.46 (s), 9H (CH_3).

For the preparation of N-(*tert*-butoxycarbonyl)-1,4,7-triazaheptane, diethylenetriamine, 31 g (300.7 mmol) dissolved in 50 mL of dioxan, was treated with the BOC reagent, 9.2 g (42 mmol), predissolved in 60 mL of the same solvent. Work-up as in the preceding experiment afforded 7.1 g (83%) of oily liquid.

^{1}H NMR, δ/ppm: 3.2 (t), 2H ($CONHCH_2$); 2.65 (m), 6H (remaining CH_2); 1.46 (s), 9H (CH_3).

The third N-protected diamine, N-(*tert*-butoxycabonyl)-4,7-dioxa-1,10-diazadecane, was prepared in an analogous fashion from 1,2-bis(2-aminoethoxy)ethane, 69.7 g (470 mmol), and BOC-reagent, 14.8 g (68 mmol). The crude, oily product was obtained in a yield of 14.3 g (85%).

^{1}H NMR, δ/ppm: 3.7 (s), 4H (OCH_2CH_2O); 3.6 (m), 4H ($NHCH_2CH_2O$); 3.2 (t), 2H ($CONHCH_2$); 2.7 (t), 2H (CH_2NH_2); 1.45 (s), 9H (CH_3).

2.4. 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 **24** normalized to y = 1 (x = 1 in **25**).

Conjugate 1. 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 from polysuccinimide by stepwise aminolytic ring opening as previously described[1]. Briefly, 3-(dimethylamino)propylamine, 971 mg (9.5 mmol), dissolved in 1 mL of DMF, was added to the stirred solution of polysuccinimide, 970 mg (10 mmol) in 6 mL of DMF. The N_2-saturated solution was stirred for 8 h at room temperature and subsequently added dropwise to 1,3-diaminopropane, 111 mg (1.5 mmol), predissolved in 8 mL of DMF and stirred in an ice bath. Upon resaturation with N_2, stirring was continued for 20 h in the ice bath and for another 1 h at ambient temperature. Moisture access was strictly precluded up to this point to prevent hydrolytic imide ring opening in the educt polymer. 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 10 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) to eliminate N protonation. Freeze-drying and post-drying in the SARTORIUS unit gave water-soluble carrier polymer in a yield of 960 mg (48.5%).

^{1}H NMR, δ/ppm (expected proton counts in parentheses): 3.2, 38H (40H; $CONHCH_2$); 2.8–2.3, 83H (80H; $NHCOCH_2$, $CH_2N(CH_3)_2$, CH_2NH_2); 2.2 112H (114H; CH_3); 1.75, 40H (40H; $CH_2CH_2CH_2$).

A 0.1 mmol sample of the carrier, 396 mg, dissolved in 8 mL of DMF, was treated with MTX, 56 mg (0.12 mmol), added in small portions with stirring. EEDQ, 30 mg (0.12 mmol), dissolved in 2 mL of NMP, was added dropwise, and so was triethylamine, 24 mg (0.24 mmol). Upon saturation with N_2, the resulting solution was stirred for 24 h at ambient temperature and for another 0.5 h at 50°C. Precipitation of the polymeric conjugate was brought about by addition of excess Et_2O-hexane (2:1) to the precooled solution, and the resinous polymer was redissolved in 20 mL of H_2O. For dissociation and removal of unreacted MTX the pH was adjusted to 10 (Na_2CO_3), and the solution was passed through a column (1.5 × 15 cm) charged with Sephadex G-25 and equilibrated with H_2O at that 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 5 h of this treatment the retentate pH was lowered to 4 (0.1 M HCl) and after several minutes raised again to 6 (aq. ammonia); this served to liberate the pendent carboxyl group of MTX from its Na salt. Freeze-drying of the retentate afforded conjugate **1** as a yellow, water-soluble solid in a yield of 247 mg (56.3%).

^{1}H NMR, δ/ppm: 8.6–6.6, with individual signals at 8.6, 7.7, and

6.7, 4.8H (1H + 2H + 2H = 5H; aromatic and heteroaromatic CH of MTX); 1.7, 40H (40H; $CH_2CH_2CH_2$). The data indicate 96% MTX incorporation of available NH_2 sites, corresponding to 9.9% by mass.

The combined outer phases collected in the dialysis operation were redialysed for 2 d in Spectra/Por 3 tubing, and from the retentate another portion, 105 mg (24%), of lower-molecular conjugate was isolated as a yellowish, water-soluble solid with an MTX content of 8.5% by mass (NMR).

Conjugate 2. The precursor polymer, poly-α,β-DL-[N-(3-(dimethylamino)propyl)-aspartamide(90)-*co*-N-(3-aminopropyl)aspartamide(10)], was synthesized from polysuccinimide (10 mmol), 3-(dimethylamino)propylamine (9 mmol), and 1,3-diaminopropane (3 mmol) in a total of 15 mL of DMF by the general procedure described for the carrier of 1. The water-soluble carrier was isolated in 55% yield.

^{1}H NMR, δ/ppm: 3.2, 20H (20H; CONHCH_2); 2.8-–2.3, 37H (40H; NHCOCH_2, CH_2N$(CH_3)_2$, CH_2NH_2); 2.2, 55H (54H; CH_3); 1.7, 20H (20H; $CH_2CH_2CH_2$).

A 0.1 mmol sample of the carrier, 196 mg, 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 with stirring, and so was triethylamine, 26 mg (0.26 mmol). N_2 saturation was followed by further stirring for 2 h at ambient temperature. A slight turbidity appearing during the stirring period was eliminated by the addition of 0.5 mL of NMP. Precipitation and further work-up as described for **1** gave 125 mg (51%) of **2** as a yellow, water-soluble solid.

^{1}H NMR, δ/ppm: 8.6–6.5 combined, 4.7H (5H; aromatic and heteroaromatic CH of MTX); 1.7, 20H (20H; $CH_2CH_2CH_2$). The data indicate 94% NH_2 substitution by MTX, corresponding to an MTX content of 16.5% by mass.

Conjugate 3. The carrier polymer, poly-α,β-DL-[N-(3-(dimethylamino)propyl)aspartamide-(80)-*co*-N-(3-aminopropyl)aspartamide(20)], was synthesized as described for the precursor to **1**, except with these reagent amounts: polysuccinimide, 10 mmol; 3-(dimethylamino)- propylamine, 8 mmol; 1,3-diaminopropane, 6 mmol; in a total of 16 mL of DMF. The yield of water-soluble carrier was 54%.

^{1}H NMR, δ/ppm: 3.3–3.2, 10.5H (10H; CONHCH_2); 2.8–2.3, 17H (20H; NHCOCH_2, CH_2N$(CH_3)_2$ CH_2NH_2); 2.2, 25H (24H; CH_3); 1.7-1.6, 10H (10H; $CH_2CH_2CH_2$).

MTX conjugation to this carrier was achieved by the procedure used for the preparation of **2**, the amounts of reactants and reagents in the feed being: carrier, 0.2 mmol; MTX, 0.26 mmol; HBTU, 0.22 mmol; NEt_3, 0.52 mmol. Yield, 53%.

^{1}H NMR, δ/ppm: 8.6–6.6 combined, 4.7H (5H; aromatic and heteroaromatic CH of MTX); 1.7, 10H (10H; $CH_2CH_2CH_2$). These data indicate an MTX incorporation of 94%, corresponding to 30.9% by mass.

Conjugate 4. The carrier, poly-α,β-DL-[N-(3-(dimethylamino)propyl)aspartamide(75)-*co*-N- (3-aminopropyl)aspartamide(25)], was prepared as previously described[19] (there designated **11** (75)).

^{1}H NMR, δ/ppm: 3.3–3.1, 8H (8H; CONHCH_2); 1.8, 8H (8H; $CH_2CH_2CH_2$).

The drug was conjugated to the carrier as in the preceding experiments. The yellow, water-soluble conjugate was collected in 39% yield.

^{1}H NMR, δ/ppm: 8.6–6.5 combined, 4.1H (5H; aromatic and heteroaromatic CH of MTX); 1.8-1.7, 8H (8H; $CH_2CH_2CH_2$). A coupling extent of 82% was indicated by these data.

The conjugate was retreated with 0.4, 0.3, and 0.8 equivalents of MTX, HBTU, and NEt_3, respectively, for 3 h at room temperature and was conventionally worked up. Recovery yield, 59%.

^{1}H NMR, δ/ppm: 8.6–6.5, 4.8H (5H), indicating 96% MTX incorporation (36.2% MTX by mass).

Conjugates 5-11 These were taken from the preceding investigation[1], there designated **5**, **6**, **7**, **4**, **8**, **10** and **11**, respectively.

Conjugate 12. The required carrier, poly-α,β-DL-[N-(2-hydroxyethyl)aspartamide(90)-*co*-N-(3-amino-2-hydroxypropyl)aspartamide(10)], was synthesized in 57% yield by the general procedure described for the carrier of **1**, with these reactants and amounts: polysuccinimide, 970 mg (10 mmol), ethanolamine, 550 mg (9 mmol), and 1,3-diaminopropan-2-ol, 270 mg (3 mmol), in a total of 19 mL of DMF.

^{1}H NMR, δ/ppm: 3.7, 19H (19H; CH_2OH, C*H*OH); 3.3–3.2, 19.8H (20H; CONHCH_2).

Drug coupling was achieved by treatment of the carrier, 400 mg (0.248 mmol), dissolved in 7 mL of DMF, with a solution of MTX, 135 mg (0.298 mmol), in 2 mL of the same solvent, followed by the dropwise addition of HBTU, 101 mg (0.273 mmol), dissolved in 1 mL of DMF, and NEt_3, 50 mg (0.5 mmol). The N_2-saturated solution was stirred for 2 h at room temperature, and the product was worked up by the conventional procedure. There was obtained 380 mg (74.2%) of yellow, water-soluble **12**.

^{1}H NMR, δ/ppm: 8.5–6.5 combined, 4.75H (5H; aromatic and heteroaromatic CH of MTX); 3.7, 19H (19H; CH_2OH, C*H*OH). The data indicate 95% NH_2 substitution by MTX, corresponding to an MTX content of 21.2%.

Conjugates 13-15. These were taken from the previous study[1], there numbered **12**, **13** and **14**, respectively.

Conjugate 16. The carrier, poly-α,β-DL-[N-(3,6-dioxahexyl)aspartamide(90)-*co*-N-(3-amino-2-hydroxypropyl) aspartamide(10)], was synthesized as described for the carrier in the foregoing experiment, except that ethanolamine was replaced by an equal amount of 2-(2-amino-ethoxy)ethanol. The water-soluble carrier was collected in 61% yield.

^{1}H NMR, δ/ppm: 3.8–3.7, 55H (55H; O$(CH_2)_2$OCH_2, C*H*OH); 3.4–3.2, 19H (20H; CONHCH_2).

MTX conjugation as described for **12** afforded conjugate **16** as a yellow, water-soluble solid in 69% yield.

^{1}H NMR, δ/ppm: 8.6–6.5 combined, 5H (5H; aromatic and heteroaromatic CH, MTX); 3.8–3.5, 55H (55H; O$(CH_2)_2$OCH_2, C*H*OH). The data indicate 100% NH_2 substitution by MTX, corresponding to an MTX content of 18.5%.

Conjugate 17, 18. These conjugates with ester-bound MTX were taken from an earlier project[20], there designated **1-MTX**(10.8) and **2-MTX**(8.9), with MTX contents of 19.8 and 18.7%, respectively.

Conjugate 19. For the preparation of the carrier polymer, ethylenebisacrylamide, 2.48 g (16 mmol) was dissolved with warming in 20 mL of H_2O. After cooling to ambient temperature, N-(*tert*-butoxycarbonyl)-1,3-diaminopropane, 558 mg (3.2 mmol), was added and dissolved. The resulting solution, saturated with N_2, was stirred for 1 d at room temperature and for another 20 h at 50°C. Upon the addition of 3-(dimethylamino) propylamine, 1.31 g (12.8 mmol), the solution was resaturated with N_2. Stirring was continued for 40 h at 55–60°C and, after the addition of ethanolamine, 49 mg (0.8 mmol), for another 1 h at that temperature. The last-named step served to eliminate any terminal vinyl groups in the product as a potential cause of subsequent crosslinking. Complete solvent removal under reduced pressure (50°C bath temperature) was followed by stirring (1 h) of the residue with 5 mL of trifluoroacetic acid for N-deprotection. Excess acid was removed by rotatory evaporation (30°C bath temperature). The product polymer was precipitated with excess Et_2O-EtOH-hexane (2:1:2), thoroughly washed with hot

toluene, and redissolved in 20 mL of H_2O. The solution, with pH adjusted to 7, was dialysed for 2 d in Spectra/Por 4 tubing and for another 2 d in Spectra/Por 6 tubing. Freeze-drying and post-drying on the SARTORIUS unit gave 559 mg (14%) of carrier polymer as a water-soluble solid.

^{1}H NMR, δ/ppm: 4.54, 10H (10H; NHCH_2NH); 2.75, 20.5H (20H; NHCOCH_2); 2.7–2.25, 44H (40H; $CH_2N(CH_2)(CH_2)$, CH_2NH$_2$, CH_2N$(CH_3)_2$); 2.21, 24.5H (24H; CH_3); 1.6, 10.2H (10H; $CH_2CH_2CH_2$).

The carrier so obtained was conjugated with MTX by a method similar to that used for the preparation of conjugates **1**–**18**. A 200-mg portion of the carrier (0.16 mmol) was dissolved in 5 mL of DMF. MTX, 87 mg (0.192 mmol) was added and dissolved, followed by the dropwise addition of HBTU 66 mg (0.178 mmol), predissolved in 0.5 mL of DMF, and then, triethylamine, 32 mg (0.319 mmol). After saturation with N_2, the solution was stirred for 2h at ambient temperature. From the cooled (~5°C) solution, the conjugate was precipitated with excess Et_2O-Me_2CO (2:1), redissolved in 5 mL of H_2O, and further treated as described for conjugate **1**. This gave yellow, water-soluble conjugate **19** in a yield of 136 mg (49.8%).

^{1}H NMR, δ/ppm: 8.5–6.5 combined, 4.9H (5H; aromatic and heteroaromatic CH of MTX); 4.55 10H (10H; NHCH_2NH); 1.75, 9.5H (10H; $CH_2CH_2CH_2$). The data indicate 98% substitution of available NH_2 groups by MTX, corresponding to an MTX content of 26.4% by mass.

Conjugate 20. The water-soluble carrier was synthesized in 17% yield as described in the foregoing, except that N-BOC-1,3-diaminopropane was replaced by an equal amount of N(*tert*-butoxycarbonyl)-1,4,7-triazaheptane.

^{1}H NMR, δ/ppm: 4.55, 10H (10H; NHCH_2NH); 2.8, 22H (20H; NHCOCH_2); 2.76–2.5, 48H (44H; $CH_2N(CH_2)(CH_2)$, CH_2NHCH_2CH_2NH$_2$, CH_2N$(CH_3)_2$); 2.21, 22H (24H; CH_3); 1.6, 7H (8H; $CH_2CH_2CH_2$).

Conventional conjugation with MTX as described for **19** gave yellow, water-soluble conjugate **20** in 53% yield.

^{1}H NMR, δ/ppm: 8.5–6.5 combined, 4.95H (5H; aromatic and heteroaromatic CH of MTX); 1.6, 8H (8H; $CH_2CH_2CH_2$). The extent of MTX incorporation was thus 99%, corresponding to an MTX content of 26.0% by mass.

Conjugate 21. The water-soluble carrier was prepared in 18.6% yield by the general procedure used for the carrier of **19**; however, N-BOC-1,3-diaminopropane was replaced by an equal amount of N-(*tert*-butoxycarbonyl)-4,7-dioxa-1,10-diazadecane.

^{1}H NMR, δ/ppm: 4.5, 10H (10H; NHCH_2NH); 3.65–3.5, 9H (8H; CH_2OCH_2); 2.8–2.23, 64H (60H; NHCOCH_2, $CH_2N(CH_2)(CH_2)$, CH_2NH$_2$, CH_2N$(CH_3)_2$); 2.2, 23H (24H; CH_3); 1.6, 8.2H (8H; $CH_2CH_2CH_2$).

Conjugation of this carrier with MTX by the procedure described for **19** gave yellowish, water-soluble conjugate in a yield of 47%. ^{1}H NMR data indicated the extent of NH_2 substitution to be 86%. The polymer was, therefore, retreated with 0.3, 0.3, and 0.6 equivalents of MTX, HBTU, and triethylamine, respectively. This gave **21** in a recovery yield of 42%.

^{1}H NMR, δ/ppm: 8.5–6.6 combined, 5H (5H; aromatic and heteroaromatic CH of MTX); 4.55, 10H (10H; NHCH_2NH). The data indicate 100% of NH_2 groups to be substituted, corresponding to an MTX content of 25.8% by mass.

Conjugate 22. The carrier was obtained in 12% yield by the general procedure described for the carrier of **19**; however, 2-(dimethylamino)ethylamine was used in place of 3-(dimethylamino)propylamine. The water-soluble carrier was collected in 21% yield.

^{1}H NMR, δ/ppm: 4.55, 10H (10H; NHCH_2NH); 2.8–2.7, 27H (20H; NHCOCH_2); 2.7–2.25, 39H (40H; $CH_2N(CH_2)(CH_2)$, CH_2NH$_2$, CH_2N$(CH_3)_2$; 2.2, 24.5H (24H; CH_3); 1.6, 2.2H (2H; $CH_2CH_2CH_2$).

The carrier was conjugated with MTX by the general procedure described for **19**. There was obtained yellow, water-soluble conjugate **22** in a yield of 36%.

^{1}H NMR, δ/ppm: 8.5–6.6 combined, 5H (5H; aromatic and heteroaromatic CH of MTX); 4.51, 10H (10H; NHCH_2NH). The extent of MTX incorporation was thus 100%, corresponding to an MTX content of 27.8% by mass.

Conjugate 23. The water-soluble carrier required for this conjugate was synthesized in 15% yield by the general procedure described for the preparation of the carrier of **19** with these modifications: 3-(dimethylamino)propylamine was replaced by 2-(dimethylamino)ethylamine, and N-(*tert*-butoxycarbonyl)-4,7-dioxa-1,10-diazadecane was used in lieu of the N-BOC-1,3-diaminopropane.

^{1}H NMR, δ/ppm: 4,5, 10H (10H; NHCH_2NH); 3.7–3.6, 8.6H (8H; CH_2OCH_2); 2.8–2.3, 62H (60H; NHCOCH_2, $CH_2N(CH_2(CH_2))$, CH_2NH$_2$, CH_2N$(CH_3)_2$).

The carrier was conjugated with MTX by the procedure described for **19**. The yellow water-soluble conjugate was collected in 44% yield.

^{1}H NMR, δ/ppm: 8.5–6.6 combined, 4.25H (5H; aromatic and heteroaromatic CH of MTX); 4.5, 10H (10H; NHCH_2NH). An extent of MTX incorporation of 85% is calculated from these data. A retreatment as described for conjugate **4** gave polymer with 93% MTX incorporation, corresponding to an MTX content of 25.2% by mass.

^{1}H NMR, δ/ppm: 8.5–6.6, 4.65H (5H); 4.5, 10H (10H).

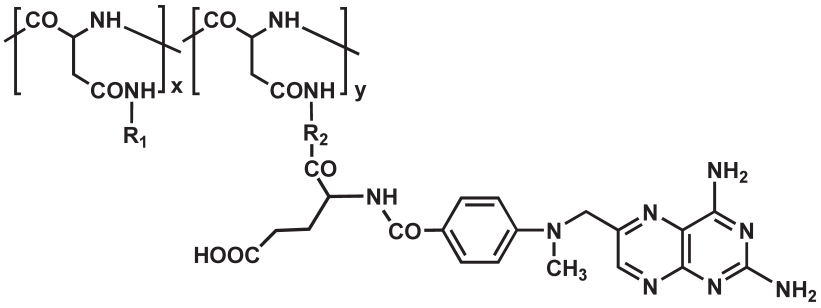
Conjugate 24. The carrier was obtained in 12.5% yield as a water-soluble polymer by the general procedure described for the carrier of **19**, yet with an equal amount of 2-(2-aminoethoxy) ethanol used in place of 3-(dimethylamino)propylamine.

^{1}H NMR, δ/ppm: 4.52, 10H (10H; NHCH_2NH); 3.75–3.55, 23H (24H; CH_2OCH_2CH_2OH); 1.6, 1.8H (2H; $CH_2CH_2CH_2$).

The procedure described for **19** was used for MTX conjugation with the carrier, giving yellow, water-soluble conjugate in 48% yield. ^{1}H NMR data indicated 90% MTX incorporation. The polymer was re-treated with MTX and coupling agent as described for conjugate **4** (recovery, 64%).

^{1}H NMR, δ/ppm: 8.5–6.6 combined, 4.7H (5H; aromatic and heteroaromatic CH of MTX); 4.51, 10H (10H; NHCH_2NH). The data indicate 94% NH_2 substitution, corresponding to an MTX content of 25.5% by mass.

Conjugate 25. To the solution of methylenebisacrylamide, 1.24 g (8 mmol) in 4 mL of isopropanol-H_2O (4:1) (gentle heating required), was added N-(*tert*-butoxycarbonyl)-1,3-diaminopropane, 698 mg (4 mmol), dissolved in 1 mL of isopropanol. The solution, saturated with N_2, was stirred for 3 d at ambient temperature. The solvent was removed by rotatory evaporation, and the residual intermediary monomer was redissolved in 50 mL of the same solvent blend, thus providing the high dilution ([MBA] ≤ 0.2 M) required to prevent disubstitution on N in the second reaction step. O,O′-Bis(3-aminopropyl)poly (ethylene glycol) 1500, 6 g (4 mmol), was dissolved in the cooled (~5°C) solution, followed by triethylamine, 405 mg (4 mmol). Upon resaturation with N_2, the solution was stirred overnight in an ice bath, 1 d at ambient temperature, and for another 36 h at 60°C. The solvent was removed once more on the rotavapor, 5 mL of trifluoroacetic acid was added to the residue, and stirring was continued for 1 h at room temperature. The acid was distilled off on the rotavapor (40°C bath temperature) and the residual material was treated with Et_2O-hexane (1:1) and repeatedly



Conjugate designation	R_1	R_2	x/y	MTX content % by mass (found)
1	NMe_2	NH	19	9.9
2	NMe_2	NH	9	16.5
3	NMe_2	NH	4	30.9
4	NMe_2	NH	3	36.2
5	NMe_2	NH–NH	19	9.8
6	NMe_2	NH–NH	9	18.7
7	NMe_2	NH–NH	4	30.6
8	NMe_2	O–O–NH	9	18.1
9	NMe_2	NH (OH)	9	18.5
10	OH	NH–NH	19	11.6
11	OH	NH–NH	9	21.5
12	OH	NH (OH)	9	21.2
13	O–OH	NH–NH	19	10.1
14	O–OH	NH–NH	9	18.5
15	O–OH	NH	9	18.0
16	O–OH	NH (OH)	9	18.5
17	OH	O	10.8	19.8
18	O–OH	O–O	8.9	18.7

Figure 1

washed with boiling toluene for removal of all traces of unreacted poly(ethylene glycol). The crude polymer was purified by dialysis and isolated as described for **19**. The yield of the water- and methanol-soluble carrier was 2.1 g (27.8%).

^{1}H NMR, δ/ppm: 4.54, 4H (4H; NHC*H*$_2$NH); 2.8, 8.2H (8H; NHCOC*H*$_2$); 2.7–2.3, 14.8H (16H; $CH_2N(CH_2)(CH_2)$, C*H*$_2$NH$_2$, C*H*$_2$NHC*H*$_2$); 1.8–1.55, 4.8H (6H; $CH_2CH_2CH_2$).

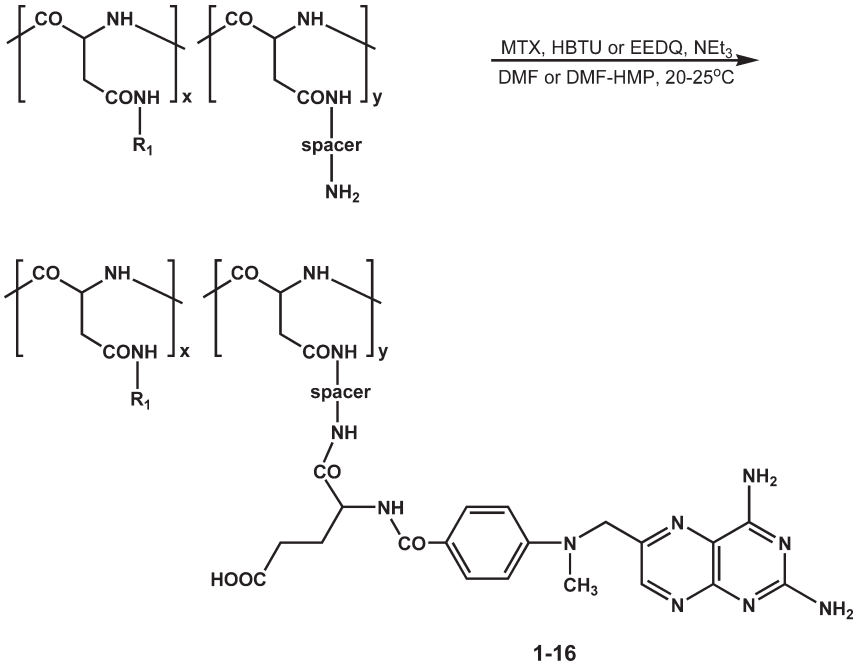
The carrier, 200 mg (0.106 mmol), together with MTX, 69 mg (0.149 mmol), was dissolved in 5 mL of DMF. HBTU, 49 mg 0.13 mmol), dissolved in 0.5 mL of the same solvent, was added dropwise, and so was triethylamine, 22 mg (0.216 mmol). The N_2-saturated solution was stirred for 2 h at room temperature, followed by cooling in an ice bath. The conjugate was precipitated with excess Et_2O-Me_2CO and separated from the supernatant by centrifugation. It was redissolved in 5 mL of H_2O, and the solution, with pH adjusted to 10, was passed through Sephadex G-25, dialysed in Spectra/Por 6, and freeze-dried as described for **1**. The yellowish, water-soluble conjugate was isolated in a yield of 130 mg (55%).

^{1}H NMR, δ/ppm: 8.5–6.6 combined, 4H (5H; aromatic and heteroaromatic CH of MTX; 4.5, 4H (4H; NHC*H*$_2$NH); These data indicate an 80% MTX incorporation, according to an MTX content of 15.7% by mass. Attempted retreatment with MTX failed to raise this percentage.

3. Results and Discussion

3.1. Conjugate Compositions

The polyaspartamide-MTX conjugates assayed in this study, most of them obtained in the preceding investigations or newly synthesized by the procedures there described[1,20,21], conform to the structures **1** to **18** depicted in Fig. 1. In **1** to **16** the biofissionable groups in the connecting spacer are represented by the carboxamide link, and the compounds were synthesized from amine-functionalized polyaspartamides by a carboxyl-amine coupling process mediated by the HBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) or EEDQ (1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline) coupling agents (Scheme 1; spacer = aliphatic segment). In conjugates **17** and **18** the biofissionable groups are represented by an ester functionality, and the synthesis, starting out from hydroxyl-functionalized polyaspartamides, proceeds by a carboxyl-hydroxyl coupling step mediated by the DCC (dicyclohexyl carbodiimide) coupling agent and catalysed by DMAP (4-(dimethylamino)pyridine) (Scheme 2; spacer = aliphatic segment). A unifying feature of the conjugates listed in Fig. 1 is their copolymeric construction comprising subunits with hydrosolubilizing side group terminals of the *tert*-amine (**1** to **9**) or hydroxyl (**10** to **18**) types in addition to other

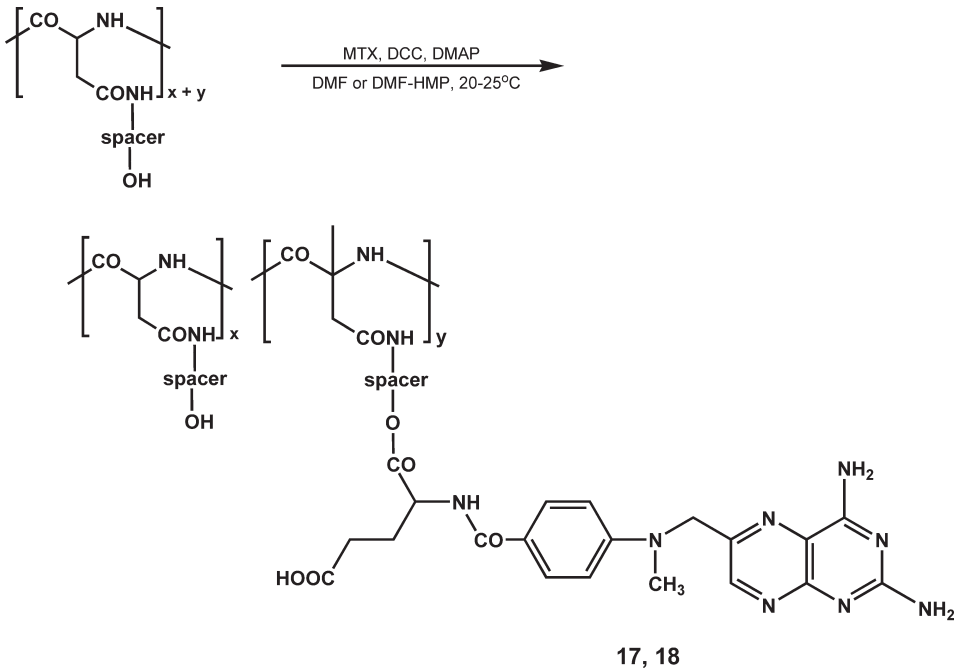


Scheme 1

subunits containing the amide- or ester-coupled MTX molecule, with x > y.

In view of the considerable run-to-run differences observed in many instances, most of the conjugates were prepared in duplicate or triplicate by *de novo* carrier synthesis and drug coupling. Individual repeat runs are identified in Table 1 as Expt. 1, 2, and 3, as applicable.

The second class of MTX conjugates, newly synthesized and investigated in this project, is based on poly(amidoamine) carrier structures formed by a Michael polyaddition process from bisacrylamides and amines. This type of Michael addition reaction, pioneered by Ferruti's group[22] and amply utilized in our own laboratory[23–25], proceeds in aqueous phase. As the propagation step is militated against by hydrolysis under the common reaction conditions (3-4 d at ambient temperature or shorter periods at 50–60°C), overall yields of polymer in the required molecular mass region of about 20000–40000 are generally low. In a preceding project[25], the chosen bisacrylamide monomer, methylenebisacrylamide (MBA), was copolymerized with pairs of amine comonomers, one of these a *tert*-amine- or hydroxyl-functionalized primary amine introduced there as a solubilizing entity, and the other, a diamine mono-N-protected by the BOC (*tert*-butoxycarbonyl) group. N-deprotection with trifluoroacetic acid and rigorous fractionation and purification treatment then provided the desired primary amine-functionalized poly(amidoamine) carriers. Seven carrier structures selected from that preceding investigation were used in the present project. The carriers were coupled to MTX by the standard procedures[1] (Scheme 3). The individual structures **19** to **24** so obtained are listed in Fig. 2. Lastly, the described



Scheme 2

Table 1 Cell culture test data for Polyaspartamide-MTX conjugates.

Conjugate		HeLa			Colo (1)[a]			Colo (2)[a]		
		IC_{50}		AF[b]	IC_{50}		AF[b]	IC_{50}		AF[b]
		μg MTX /mL	M[c]		μg MTX /mL	M[c]		μg MTX /mL	M[c]	
1	Expt. 1[d]	0.067	1.48×10^{-7}	2.1	0.027	5.95×10^{-8}	42.8			
	Expt. 2	0.02	4.40×10^{-8}	7.0	0.027	5.95×10^{-8}	42.8			
2	Expt. 1	0.075	1.65×10^{-7}	1.9						
	Expt. 2	0.013	2.86×10^{-8}	10.8	0.043	9.47×10^{-8}	26.9	0.196	4.32×10^{-7}	14.8
3	Expt. 1	0.119	2.62×10^{-7}	1.2	0.050	1.10×10^{-7}	23.1			
	Expt. 2	0.031	6.83×10^{-8}	4.5	0.011	2.42×10^{-8}	105.0			
4	Expt. 1	0.037	8.15×10^{-8}	3.8	0.030	6.61×10^{-8}	38.5			
5	Expt. 1	0.012	2.64×10^{-8}	11.7	0.0079	1.74×10^{-8}	146.2			
6	Expt. 1	0.018	3.96×10^{-8}	7.8	0.0053	1.17×10^{-8}	217.9			
	Expt. 2	0.015	3.30×10^{-8}	9.3	0.0046	1.01×10^{-8}	251.1			
	Expt. 3	0.003	6.61×10^{-9}	46.7				0.149	3.28×10^{-7}	19.5
7	Expt. 1	0.019	4.19×10^{-8}	7.4	0.013	2.86×10^{-8}	88.8			
	Expt. 2	0.012	2.64×10^{-8}	11.7	0.004	8.81×10^{-9}	288.8			
8	Expt. 1	0.027	5.95×10^{-8}	5.2	0.075	1.65×10^{-7}	15.4			
	Expt. 2	0.048	1.06×10^{-7}	2.9				0.264	5.81×10^{-7}	11.0
9	Expt. 1	0.01	2.20×10^{-8}	14.0	0.031	6.83×10^{-8}	37.3			
	Expt. 2	0.025	5.51×10^{-8}	5.6				0.49	1.08×10^{-6}	5.9
10	Expt. 1[d]	0.024	5.29×10^{-8}	5.83	0.017	3.74×10^{-8}	67.9			
11	Expt. 1	0.689	1.52×10^{-6}	0.20				1.084	2.39×10^{-6}	2.7
	Expt. 2	0.449	9.89×10^{-7}	0.31	0.116	2.56×10^{-7}	10.0			
	Expt. 3	0.379	8.35×10^{-7}	0.4				1.214	2.67×10^{-6}	2.4
12	Expt. 1	4.918	1.08×10^{-5}	0.03				12.96	2.85×10^{-5}	0.2
13	Expt. 1	0.0039	0.59×10^{-9}	35.9	0.0093	2.05×10^{-8}	124.2			
14	Expt. 1	0.594	1.31×10^{-6}	0.23				1.967	4.33×10^{-6}	1.5
	Expt. 2	0.261	5.75×10^{-7}	0.54	0.154	3.39×10^{-7}	7.5			
	Expt. 3	0.536	1.18×10^{-6}	0.3				1.712	3.77×10^{-6}	1.7
15	Expt. 1	0.0093	2.04×10^{-8}	15.1	0.0034	7.49×10^{-9}	339.7			
	Expt. 2	0.027	5.95×10^{-8}	5.2				0.507	1.12×10^{-6}	5.8
16	Expt. 1	4.228	9.31×10^{-6}	0.03				16.37	3.61×10^{-5}	0.2
17	Expt. 1	0.325	7.16×10^{-7}	0.4	0.107	2.36×10^{-7}	10.8			
	Expt. 2	0.159	3.50×10^{-7}	0.9	0.064	1.41×10^{-7}	18.0			
18	Expt. 1	0.066	1.45×10^{-7}	2.1	0.028	6.17×10^{-8}	41.3			
	Expt. 2	0.195	4.30×10^{-7}	0.7	0.080	1.76×10^{-7}	14.4			
	Expt. 3	0.473	1.04×10^{-6}	0.3	1.449	3.19×10^{-6}	0.8			
MTX		0.140	3.08×10^{-7}		1.155	2.54×10^{-6}		2.902	6.39×10^{-6}	

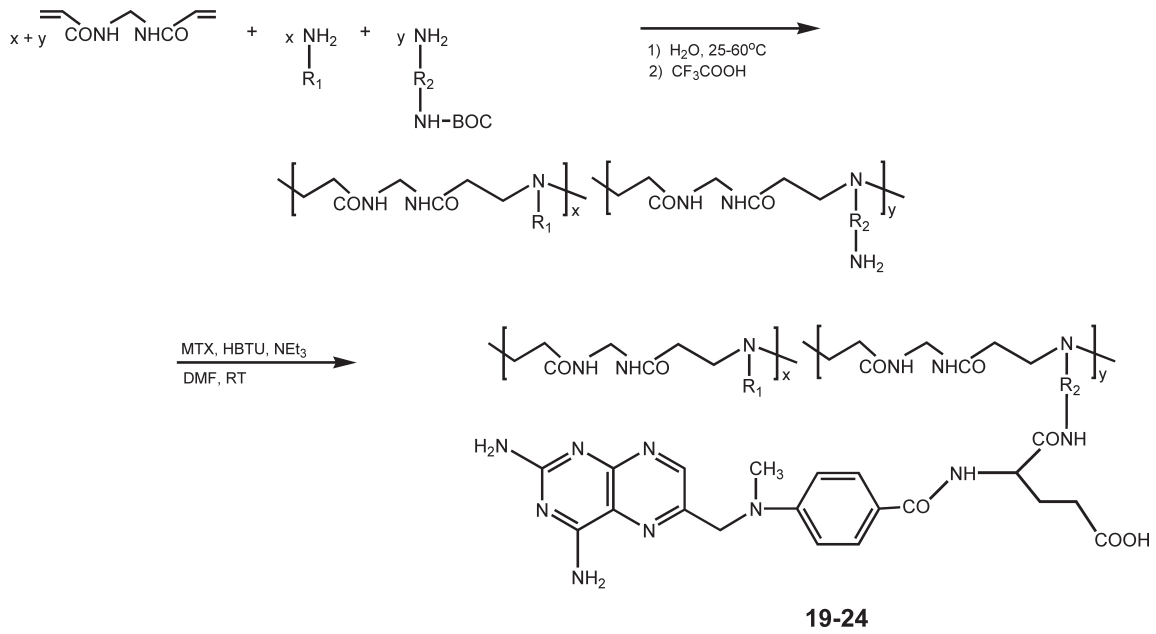
[a] Variants of Colo 320 DM: moderately resistant (1) and strongly resistant (2) lines.
[b] Activity factor, here defined as: IC_{50}(MTX): IC_{50}(Conjugate), rounded off to first or second decimal.
[c] Moles MTX/L.
[d] Expt.: refers to separately synthesized conjugates.

technology was utilized for a related poly(amidoamine)-MTX conjugate **25** (Scheme 4; structure idealized, MTX incorporation 80%)[25].

3.2. Cell Culture Testing

The conjugates of both classes were evaluated in cell culture tests for antiproliferative activity. Members of the first class (conjugates **1** to **18**) were tested against a moderately drug-resistant cell line derived from the Colo 320 DM human colorectal carcinoma, hereinafter briefly designated Colo (1). Selected conjugates were also tested against a more refractory, i.e. less drug-responsive Colo 320 DM cell line variant, hereafter named Colo (2). The latter line was also, exclusively, employed in the tests of the members of the second class (**19** to **25**). For comparison, the samples of both classes were separately screened for activity against the HeLa human cervical epitheloid adenocarcinoma line generally found to be drug-sensitive and frequently used as a standard. Activities were determined in triplicate by the earlier described procedure[16], and the findings, expressed as IC_{50} values (drug concentration required to achieve 50% cell kill relative to drug-free control) were averaged for each sample. In order to obtain a measure of drug activity of the conjugates relative to unconjugated drug, we have also included free MTX in these test series, and the results derived from five determinations against each cell line, averaged for each line, have been used for this comparison.

It is convenient to present and discuss the test results separately for the two classes of conjugates.



Scheme 3

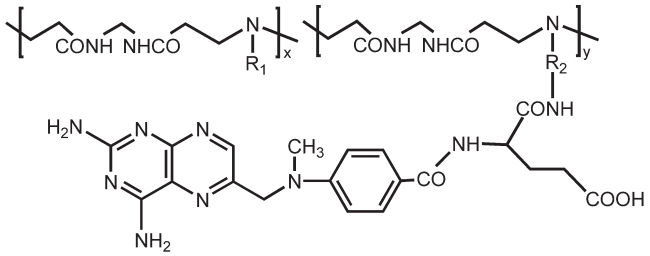
3.2.1. Polyaspartamide-MTX Conjugates

For the polyaspartamide-based conjugates **1** to **18**, including repeat runs, the IC_{50} values, in terms of μg MTX mL^{-1} and μM MTX, have been listed in Table 1 for the tests against HeLa and the two Colo 320 DM variants. The table also contains the averaged results shown by free MTX for each cell line. Furthermore, we have included activity factors, AF, here defined as the ratio of conjugate activities over MTX activity and expressed in terms of IC_{50} (MTX):IC_{50} (conjugate).

It is instructive to consider first the data in the HeLa column. A superficial examination reveals that IC_{50} values for the first one-half of the listed conjugates are lower than that for MTX. For these first 17 samples (conjugates **1** to **9**) an averaged activity factor of 4.3 can be calculated from the averaged IC_{50} values (0.0324 μg MTX mL^{-1}). These conjugates as a group, hence, are some four times more active than the free drug. We recall that these 17 samples are characterized by *tert*-amine side chain terminals as hydrosolubilizing groups in R_1.

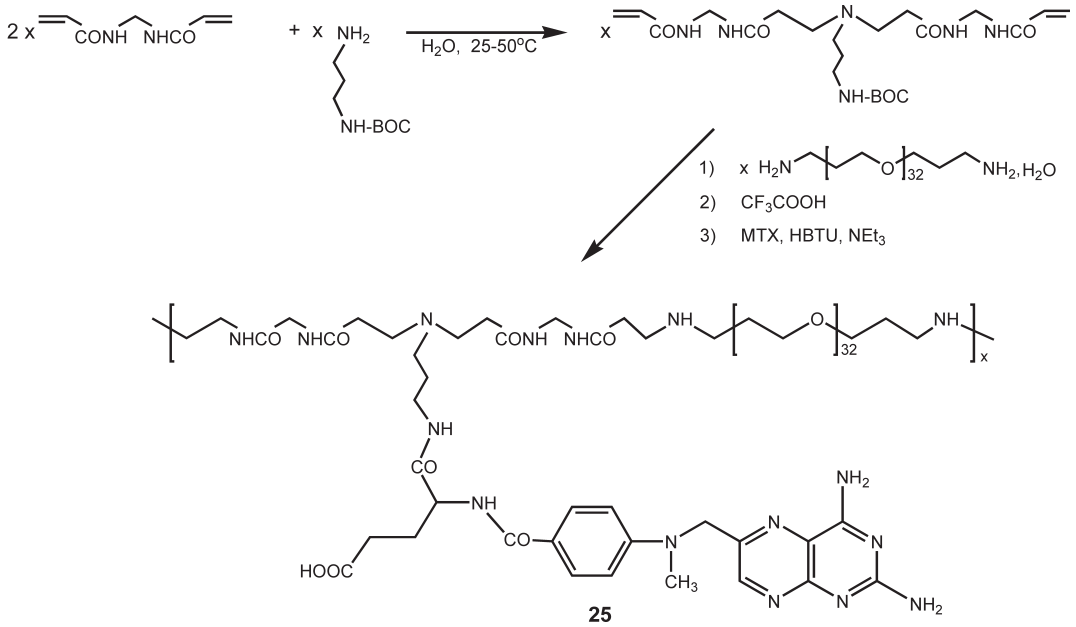
The situation is different for the second one-half of the tabulated samples (conjugates **10** to **18**) identified by hydroxyl side chain terminals in R_1. For these 17 samples the averaged IC_{50} value, 0.889 μg MTX mL^{-1}, suggests an average activity factor of 0.16, thus reflecting a reverse behaviour, with free MTX now some six times more active than the samples as a group. It is clear that, against the HeLa line, the *tert*-amine functionality in conjugates **1** to **9** exerts a positive influence on the *in vitro* cytotoxic data. In the biological environment, this functionality is partially protonated, which renders the polymer moderately basic and may thus facilitate pinocytic cell entry with resultant activity enhancement[26]. Larger sample numbers will be required, however, to corroborate this explanation.

An even more favorable constellation emerges as one inspects the IC_{50} values in the two Colo columns. Let us first consider the Colo (1) data. Against this moderately refractory cell line, free MTX, as expected, encounters some eight times higher resistance (IC_{50}[Colo (1)]:IC_{50}[HeLa] = 8.25). In stark contrast, the conjugates as a group experience practically no resistance relative to HeLa. In fact, 18 of the total of 23 samples tested show IC_{50} values lower than observed in the tests against HeLa, indicating higher activity against the ostensibly resistant line than against



Conjugate designation	R_1	R_2	x/y	MTX content/ % by mass (found)
19	NMe_2		4	26.4
20	NMe_2	NH	4	26.0
21	NMe_2	O O	4	25.8
22	NMe_2		4	27.8
23	NMe_2	O O	4	25.2
24	O OH		4	25.5

Figure 2



Scheme 4

the sensitive one. Compared with IC_{50} of free MTX in this column (1.155 μg MTX mL^{-1}), all samples except one (conjugate **18**, Expt. 3) are distinctly more active than the free drug. From the averaged IC_{50}, 0.1025 μg MTX/mL, an activity factor of 11.3 is calculated, indicating the 23 samples as a group to exceed the free drug more than 10-fold in activity.

More specifically, considering the first 13 samples of conjugates **1** to **9**, we determine average values of IC_{50} and activity factors of 0.0253 μg MTX mL^{-1} and 45.7, respectively, for these conjugates *in toto.* Individually within this group of *tert*-amine-functionalized conjugates, five samples are over 100-fold more active than MTX. Even the 10 hydroxyl-functionalized samples of conjugates **10** to **18** *in toto* give an average activity factor of 5.69 derived from the averaged IC_{50} data (0.2028 μg MTX mL^{-1}).

Let us proceed now to the Colo (2) column. To the unconjugated drug, the Colo (2) line proves to be some 20 times more refractory than HeLa, which clearly identifies that Colo variant as a strongly drug-resistant line. Here, for the first four samples listed in this column (conjugates **2**, **6**, **8**, and **9**) the averaged IC_{50} value (0.275 μg MTX mL^{-1}) leads to an average activity factor of 10.55, showing these *tert*-amine-functionalized polymers as a group to be over 10 times more active than free MTX. The following seven samples in the category of hydroxyl-functionalized conjugates (**11**, **12**, **14**, and **16**) are distinctly less active as a group against this strongly resistant line. With an activity ratio of 0.57, derived from the averaged IC_{50} values (5.1162 μg MTX mL^{-1}), they are even less active than the free drug. This average AF value is misleading, however, being strongly affected by the two outsiders (possibly artifacts), the conjugates **12** and **16**. If these are omitted from the calculations, an activity factor of 2.24 results from the averaged IC_{50} value (1.2968 μg MTX mL^{-1}). This indicates that even these hydroxyl-functionalized conjugates for the most part are twice as active as the unconjugated drug.

3.2.2. Poly(amidoamine)-MTX Conjugates

This class comprises a small number of conjugates (**19** to **25**; one sample per conjugate) made available for a very preliminary evaluation of activities against the HeLa and Colo (2) lines. The results in terms of IC_{50} and activity factor are presented in Table 2, which includes data obtained in this test series for unconjugated MTX.

We shall first inspect the data in the HeLa column. For the first five conjugates **19** to **23**, characterized by the presence of *tert*-amine terminals on short side chains functioning as hydrosolubilizing moieties, the IC_{50} values approximate that of

Table 2 Cell culture test data for poly(amidoamine)-MTX conjugates.

Conjugate	HeLa			Colo (2)[a]		
	IC_{50}		AF[b]	IC_{50}		AF[b]
	μg MTX /mL	M[c]		μg MTX /mL	M[c]	
19	0.134	2.95×10^{-7}	0.6	1.348	2.97×10^{-6}	3.1
20	0.014	3.08×10^{-8}	5.7	0.089	1.96×10^{-7}	12.9
21	0.058	1.28×10^{-7}	1.4	0.884	1.86×10^{-6}	4.9
22	0.086	1.89×10^{-7}	0.9	0.675	1.49×10^{-6}	6.1
23	0.025	5.51×10^{-8}	3.2	1.719	3.79×10^{-6}	2.4
24	2.659	5.86×10^{-6}	0.03	15.62	3.44×10^{-5}	0.27
25	1.324	2.92×10^{-6}	0.06	9.58	2.11×10^{-5}	0.43
MTX	0.08	1.76×10^{-7}		4.15	9.14×10^{-6}	

[a] Strongly resistant variant of Colo 320 DM. See text.
[b] Activity factor. For definition, see footnote b, Table 1.

free MTX. The average activity factor, 1.26, derived from the averaged IC_{50} data (0.0634 μg MTX mL^{-1}), identifies this group of conjugates as slightly more active than MTX proper. Conjugate **24**, in contrast, containing a hydroxyl side chain terminal for solubilization, gives an activity factor of 0.03, and **25**, with a solubilizing intrachain oligo(ethylene oxide) segment, provides an AF of 0.06. The activities of these two conjugates are thus considerably lower than that of MTX.

The same basic trend is apparent for all conjugates from the Colo data. While conjugates **19** to **23** as a group are five times more active than free MTX, with averaged IC_{50} = 0.783 μg MTX mL^{-1} and resultant AF = 5.3, the remaining two conjugates turn out to be less active than free MTX, with activity factors of 0.27 and 0.43, respectively. In compound **24**, the presence of the hydroxyl (rather than *tert*-amine) side group most likely accounts for the inferior performance against both cell lines in accordance with the trend described above for the polyaspartamide-MTX conjugate series. The oligo(ethylene oxide) segment in **25** possesses protein-repellant characteristics, and this may lead to inhibition of intracellular enzymatic cleavage of the drug-binding carboxamide links and resultant lower bioavailability.

In conclusion, cell culture tests performed against the drug-sensitive HeLa and the two moderately or strongly resistant Colo lines reveal a vastly superior cell killing potential for polymer-bound MTX as a class in relation to the unconjugated drug. Best performers belong to the group of polyaspartamide-based conjugates featuring *tert*-amine side chain terminals as hydrosolubilizing entities, with selected compounds some 10 to 100 times more active than free MTX. The superiority of this group of conjugates is particularly apparent in tests against the resistant Colo (1): whereas free MTX displays an activity decreased over 10-fold relative to HeLa, the conjugates as a group are even more active against this resistant line than against the sensitive HeLa standard. While in tests against Colo (2) all compounds show lowered cytotoxic performance, this loss of activity is much less severe with the polymeric than with the monomeric drug. Polyaspartamide-bound MTX derivatives, notably those containing *tert*-amine side chain terminals, thus stand out as eminently promising candidates for further bioevaluation work in tests against both sensitive and multidrug-resistant cancer cells.

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