Lipoamino Acid Conjugates of Methotrexate with Antitumor Activity

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Abstract □ The synthesis, characterization, and in vitro antitumor activity against a wild and a transport-resistant CCRF-CEM cell line is described for a series of α,γ-bisamide lipoamino acid and oligomer conjugates of methotrexate. The influence of the lipophilicity of the conjugates on the cytotoxicity and the dihydrofolate reductase inhibition was investigated. All compounds were more active than their fatty acid conjugate analogues. Compound **1e** with a 12-carbon atom aliphatic side chain showed the highest in vitro activity.

Introduction

Methotrexate (4-amino-4-deoxy-N^{10}-methylpteroyl-L-glutamic acid, MTX, **1a**), a classical inhibitor of dihydrofolate reductase (DHFR), is widely used alone or as a basic component of combination regimes for treatment of human neoplastic diseases, like choriocarcinoma, lymphocytic leukemia, and diffuse lymphomas and sarcomas, and for the therapy of severe cases of rheumatoid arthritis and psoriasis.[1] However, as well as its marrow-related toxicity, the clinical efficacy of MTX is often limited by inherent or acquired cell resistance, due to different mechanisms. In particular, impairment of the active transport system, associated with both reduced folates and the majority of MTX uptake, has been recognized as the basis of a "transport resistance" in some cases.[2,3]

It was reasoned that an overall reduction in the polarity of the MTX molecule may enhance passive cellular uptake into tumor cells and therefore evade the transport-resistance barrier. Modification of the carboxyl groups present in the glutamic acid moiety of MTX, defined as a region of "bulk tolerance", appeared an ideal target, since the high-affinity binding of MTX to target enzymes derives from the diamino pyrimidine portion of the pteridine ring.[4] Unsurprisingly, this has been the focus of many studies, mainly exploiting mono- and diesters of MTX[5−7] and aromatic or short chain aliphatic mono- and bis-amides.[8−13]

In our previous work,[14] a series of linear aliphatic MTX α,γ-bis(amides) were prepared and tested in vitro to investigate the effects of different substitutions of the free carboxyl groups present in MTX on the affinity toward DHFR and its growth inhibitory activity on different tumor cell lines. Preliminary results demonstrated that bis-(tetradecyl)amide had the highest in vitro antitumor potency. Interestingly, despite these derivatives being less active than MTX against wild CCRF-CEM cells, they retained almost the same activity against an MTX-resistant CEM line, which lacks the physiological transport system for the folates.[15]

To investigate this area further, it was reasoned that the bifunctional nature of various lipoamino acids (LAAs)[16,17] could be exploited to allow the incorporation of different levels of lipidic substitution onto the MTX molecule, thereby increasing the propensity for passive cellular uptake of the chemotherapeutic conjugate. We would therefore like to report the synthesis and biological evaluation of a series of novel MTX α,γ-bis LAA conjugates **1b**−**r**.

Materials and Methods

MTX (purity >98%, HPLC) was kindly gifted by Cyanamid (Catania, Italy). Lipoamino acids and their esters were racemic and prepared as described by Gibbons *et al.*[17] ^{1}H NMR spectra were recorded in DMSO-d_6, $CDCl_3$, or a 2:1 $CDCl_3$−MeOD-d_4 mixture, with either a Bruker AM250 or AM500 instrument operating at 250 or 500 MHz, respectively; chemical shifts are reported in ppm downfield from internal TMS. Fast-atom bombardment (FAB) mass spectra were run on a VG analytical ZAB-SE instrument, using a 20 kV Cs^+ bombardment and 2 μL matrix (*m*-nitrobenzoic acid or thioglycerol−glycerol−TFA); a NaI or KI methanol solution was added to produce salted species when no protonated molecular ions were observed. Electron-impact (EI) mass spectra were carried out on a VG analytical ZAB-SE instrument, using electrons at 70 eV. Elemental analysis was performed on a Carlo Erba 1106 analyzer; experimental values are within ±0.3% of theoretical ones (Table 1). TLC was carried out on Merck $F_{254+366}$ silica gel aluminum-backed plates. Purification was achieved by gravity column chromatography on either Merck Kieselgel G60 or *dry* G60 silica gel (230−400 mesh), with the eluent systems indicated. Fractions identified by MS mass matching were further checked for purity using RP-HPLC. Since we used racemic lipoamino acids for conjugation, the MTX lipidic conjugates were diastereomeric mixtures (melting points not reported).

Method A—*Methotrexate α,γ-bis(methyl 2-aminodecanoate) (**1b**)*—Methotrexate **1a** (90 mg, 0.2 mmol), triethylamine (50 mg, 0.5 mmol), 1-hydroxybenzotriazole hydrate (65 mg, 0.42 mmol), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC; 100 mg, 0.52 mmol) were dissolved in 4 mL of dry dimethylformamide at 0 °C in an ice bath; after 30 min, a solution of methyl α-aminodecanoate hydrochloride[16] (100 mg, 0.42 mmol) in 2 mL of dry dichloromethane was added and the mixture was stirred at 0 °C for 2 h and then at room temperature for 24 h. Solvents were removed under high vacuum, and the residue was dissolved in 30 mL of dichloromethane and washed with water (30 mL), 5% acetic acid solution (20 mL), 10% sodium bicarbonate solution (30 mL) and brine (2 × 30 mL). The organic layer was then

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Table 1—Analytical Data of MTX Lipoamino Acid Conjugates

compd	method	% yield appearance	TLC R_f	HPLC t_R (min)	formula	elemental analysis
1b	A	75 yellow	0.51, 0.52[a]	6.63	$C_{42}H_{64}N_{10}O_7$ (821.044)	calcd C 60.12, H 7.93, N 16.69 (·H_2O) found C 60.15, H 8.00, N 16.82
1c	A	66 yellow	0.53, 0.57[a]	9.15	$C_{62}H_{102}N_{12}O_9$ (1159.59)	calcd C 62.76, H 8.92, N 14.16 (·1.5H_2O) found C 62.83, H 8.80, N 14.14
1d	A	65 yellow	0.54[a]	7.57	$C_{46}H_{72}N_{10}O_7$ (877.15)	calcd C 61.11, H 8.36, N 15.49 (·1.5H_2O) found C 61.01, H 8.49, N 15.69
1e	A	68 yellow	0.51, 0.53[a]	9.75	$C_{50}H_{80}N_{10}O_7$ (933.26)	calcd C 63.13, H 8.69, N 14.72 (·H_2O) found C 62.93, H 8.78, N 14.52
1f	A	71 yellow	0.53[a]	11.88	$C_{78}H_{134}N_{12}O_9$ (1384.02)	calcd C 67.79, H 9.63, N 12.16 found C 67.59, H 9.60, N 11.93
1g	A	80 yellow	0.52, 0.54[a]	10.77	$C_{54}H_{88}N_{10}O_7$ (989.42)	calcd C 62.70, H 9.06, N 13.54 (·2.5H_2O) found C 62.78, H 9.24, N 13.71
1h	A	71 yellow	0.56, 0.59[a]	11.77	$C_{86}H_{150}N_{12}O_9$ (1496.23)	calcd C 69.04, H 10.10, N 11.23 found C 69.30, H 10.27, N 11.09
1i	A	80 yellow	0.52, 0.53[a]	11.63	$C_{58}H_{96}N_{10}O_7$ (1045.53)	calcd C 64.95, H 9.30, N 13.06 (·2H_2O) found C 64.75, H 9.40, N 13.14
1j	B	87 orange-yellow	0.18[b]	6.08	$C_{40}H_{60}N_{10}O_7$ (792.99)	calcd C 57.33, H 7.82, N 16.71 (·2.5H_2O) found C 57.48, H 7.80, N 16.55
1k	B	68 dark yellow	0.18[b]	8.77	$C_{60}H_{98}N_{12}O_9$ (1131.53)	calcd C 62.69, H 8.77, N 14.62 (·H_2O) found C 62.66, H 8.80, N 14.59
1l	B	82 dark yellow	0.21[b]	7.02	$C_{44}H_{68}N_{10}O_7$ (849.10)	calcd C 60.95, H 8.14, N 16.15 (·H_2O) found C 60.87, H 8.26, N 16.08
1m	B	74 dark yellow	0.26[b]	8.65	$C_{48}H_{76}N_{10}O_7$ (905.25)	calcd C 63.69, H 8.46, N 15.47 found C 63.88, H 8.65, N 15.34
1n	B	77 dark yellow	0.27[b]	11.08	$C_{76}H_{130}N_{12}O_9$ (1355.94)	calcd C 63.92, H 9.74, N 11.77 (·4H_2O) found C 63.76, H 9.94, N 11.77
1o	B	90 dark yellow	0.28[b]	9.57	$C_{52}H_{84}N_{10}O_7$ (961.36)	calcd C 59.40, H 9.01, N 13.32 (·5H_2O) found C 59.58, H 8.86, N 13.52
1p	B	75 dark yellow	0.28[b]	11.25	$C_{84}H_{146}N_{12}O_9$ (1468.22)	calcd C 68.72, H 10.02, N 11.45 found C 68.90, H 10.23, N 11.12
1r	B	88 orange-yellow	0.29[b]	10.74	$C_{56}H_{92}N_{10}O_7$ (1017.47)	calcd C 63.84, H 9.18, N 13.29 (·2H_2O) found C 64.03, H 9.22, N 13.06

TLC systems: [a] CH_2Cl_2-MeOH, 8:2. [b] CH_2Cl_2–MeOH–AcOH 8.5:1:0.5.

dried ($MgSO_4$) and the solvent removed in vacuo. The crude product was purified by column chromatography on silica gel [eluent system, CH_2Cl_2 (400 mL), CH_2Cl_2-MeOH, 95:5 (1000 mL)]: yield 123 mg (75%) yellow solid; ^{1}H NMR ($CDCl_3$) δ 8.56 (1H, s, H-7), 8.10 (1H, d, CONH-Glu), 7.63, 6.68 (4H, 2d, arom), 6.55 (1H, d, CONH), 4.68 (2H, s, 9-CH_2), 4.44 (2H, bs, 2 α-CH), 3.69−3.59 (6H, m, 2 $COOCH_3$), 3.11 (3H, s, NCH_3), 2.50−2.05 (2H, m, CH_2), 1.16, 1.15 (28H, 2s, 14 CH_2), 0.79 (6H, t, 2 CH_3); FAB-MS (*m*/*z*, %) 844, 843 $[M + Na]^+$ (22, 55), 822 $[M + 1]^+$ (30), 821 $[M]^+$ (50), 669 (10), 659 (15), 621,620 (10, 21), 461 (14), 446 (13), 439 (12), 308 (100); EI-MS (*m*/*z*, %) 662 (29), 648, 647 (37, 93), 530 (38), 515 (32), 441 (15), 316 (19), 220 (25), 191 (25), 136 (34), 109 (100).

Methotrexate α,γ-Bis[methyl 2-(2-aminodecanoyl)-aminodecanoate] (***1c***)—^{1}H NMR ($CDCl_3$) δ 8.60 (1H, bs, H-7), 7.65, 6.75 (4H, 2 d, arom), 6.55 (1H, s, CONH-Glu), 4.50 (4H, m, 4 α-CH), 3.70 (6H, bs, 2 $COOCH_3$), 3.18 (3H, s, NCH_3), 2.40−1.60 (4H, m, $^{\beta}CH_2{}^{\gamma}CH_2CO$), 1.24 (56H, s, 28 CH_2), 0.85 (12H, t, 4 CH_3); FAB-MS (*m*/*z*, %) 1183, 1182 $[M + Na]^+$ (30, 42), 1160 $[M + 1]^+$ (15), 1006 (11), 789 (12), 445 (16), 437 (18), 279 (19).

Methotrexate α,γ-Bis(methyl 2-aminododecanoate) (***1d***)—^{1}H NMR ($CDCl_3$) δ 8.65 (1H, d, H-7), 7.71, 6.75 (4H, 2 m, arom), 5.26 (1H, bs, CH), 4.76 (2H, s, 9-CH_2), 4.60, 4.50 (2H, 2m, 2 α-CH), 3.73 (6H, m, 2 $COOCH_3$), 3.18 (3H, d, NCH_3), 2.45−2.00 (4H, br m, $^{\beta}CH_2{}^{\gamma}CH_2CO$), 1.27−1.21 (36H, 4s, 18 CH_2), 0.88 (6H, t, 2 CH_3); FAB-MS (*m*/*z*, %) 900 $[M^+ + Na]^+$ (100), 878 $[M + 1]^+$ (82), 725 (20), 716 (24), 648 (27), 583 (76), 473 (36), 409 (30), 402 (39).

Methotrexate α,γ-Bis(methyl 2-aminotetradecanoate) (***1e***)—^{1}H NMR ($CDCl_3$) δ 8.55 (1H, bs, H-7), 8.00 (1H, br, NH), 7.70, 6.65 (4H, br, arom), 7.40 (2H, br, NH_2), 4.75 (2H, s, 9-CH_2), 4.60, 4.50 (2H, m, 2 α-CH), 3.48 (6H, m, $COOCH_3$), 3.11 (3H, m, NCH_3), 2.60−1.70 (4H, m, $^{\beta}CH_2{}^{\gamma}CH_2$-CO), 1.23−1.18 (44H, 7s, 22 CH_2), 0.85 (6H, t, 3 CH_3); FAB-MS (*m*/*z*, %) 957, 956 $[M^+ + Na]^+$ (10, 22), 934 $[M + 1]^+$ (47), 676 (10), 338 (12), 330 (9), 308 (100), 290 (18), 280 (15), 258 (15).

Methotrexate α,γ-Bis[methyl 2-(2-aminotetradecanoyl)-aminotetradecanoate] (***1f***)—^{1}H NMR ($CDCl_3$) δ 8.65 (1H, br, H-7), 8.05 (1H, br, NH_2), 7.70, 6.77 (4H, br, arom), 4.75 (2H, s, 9-CH_2), 4.40 (4H, br m, 4 α-CH), 3.71 (6H, m, 2 $COOCH_3$), 1.27−1.20 (88H, 6s, 44 CH_2), 0.87 (12H, t, 4 CH_3); FAB-MS (*m*/*z*, %) 1407 $[M + Na]^+$ (64), 1384 $[M + 1]^+$ (21), 1108 (12), 1022 (37), 912 (72), 795 (33), 753 (42), 694 (48), 662 (73), 647 (87), 530 (100), 512 (38).

Methotrexate α,γ-Bis(methyl 2-aminohexadecanoate) (***1g***)—^{1}H NMR ($CDCl_3$) δ= 7.70, 6.70 (4H, br, arom), 4.75 (2H, bs, 9-CH_2), 4.45 (2H, m, 2 α-CH), 3.72 (6H,d, 2 $COOCH_3$), 3.15 (3H, s, NCH_3), 1.25 (52H, s, 26 CH_2), 0.87 (6H, t, CH_3); FAB-MS (*m*/*z*, %) 1012 $[M^+ + Na]^+$ (63), 991 $[M + 1]^+$ (100), 838 (13), 684 (17), 410 (87), 331 (55).

Methotrexate α,γ-Bis[methyl 2-(2-aminohexadecanoyl)-aminohexadecanoate] (***1h***)—^{1}H NMR ($CDCl_3$) δ 8.63 (1H, bs, H-7), 7.70, 6.75 (4H, m, arom), 7.30 (2H, bs, NH_2), 4.76 (2H, bs, 9-CH_2), 4.60, 4.40 (4H, m, 4 α-CH), 3.70 (6H, m, 2 $COOCH_3$), 3.20 (3H, m, NCH_3), 1.24 (104H, s, 52 CH_2), 0.87 (12H, t, 4 CH_3); FAB-MS (*m*/*z*, %) 1519 $[M^+ + Na]^+$ (17), 1497 $[M + 1]^+$ (27), 694 (23), 665 (42), 639 (48), 612 (28), 539 (100), 519 (85), 460 (99), 435 (57), 369 (43), 350 (49).

Methotrexate α,γ-Bis(methyl 2-aminooctadecanoate) (***1i***)—^{1}H NMR ($CDCl_3$) δ 8.65 (1H, s, H-7), 7.70, 6.75 (4H, m, arom), 5.29 (2H, br, CH), 4.75 (2H, s, 9-CH_2), 4.50 (2H, m, 2 α-CH), 3.70 (6H, m, 2 $COOCH_3$), 3.18 (3H, s, NCH_3), 1.27−1.21 (60H, 3s, 30 CH_2), 0.87 (6H, t, 2 CH_3); FAB-MS (*m*/*z*, %) 1068 $[M + Na]^+$ (93), 1046 $[M + 1]^+$ (77), 884 (26), 732 (100), 558 (50), 488 (29), 459 (35), 426 (58).

Method B—*Methotrexate α,γ-bis(2-aminodecanoic acid) (**1j**)*—Compound **3a** (75 mg) was dissolved in MeOH (10 mL), a 1 M MeOH/water (4:1) solution of NaOH (10 mL) was added, and the mixture was stirred at 40 °C. The progress of the reaction was followed by TLC. Upon completion (usually, 10−20 min), the organic solvent was removed in vacuo and the mixture was diluted with water and acidified with a saturated citric acid solution to pH 6. The product was extracted with dichloromethane (2 × 40 mL) and the organic layer was dried ($MgSO_4$) and evaporated off in vacuo, to give an yellow-orange product (63 mg, 87% yield): ^{1}H NMR (DMSO-d_6) δ 8.55 (1H, s, H-7), 7.99 (1H, br s, CONH-Glu), 7.72−7.70, 6.82−6.78 (4H, 2d, arom), 6.57 (1H, s, CO−NH), 4.77 (2H, s, 9-CH_2), 4.40, 4.10 (2H, br, 2 α-CH), 3.19 (3H, s, NCH_3), 2.00−1.40 (4H, br m, $^{\beta}CH_2{}^{\gamma}CH_2CO$), 1.35 (4H, s, 2 β-CH_2), 1.17 (24H, 3s, 12 CH_2), 0.82 (6H, t, 2 CH_3); FAB-MS (*m/z*, %) 837, 815 [M + 2Na, M + Na]$^+$ (10, 35), 794 [M + 1]$^+$ (54), 641 (18), 606 (12), 482 (68), 460 (100), 443 (29), 430 (72), 410 (56); EI-MS (*m/z*, %) 662 (20), 648, 647 (26, 62), 631 (3), 530 (63), 515 (49), 441 (83), 337 (29), 320 (27), 276 (22), 219 (48), 136 (58), 121 (67), 109 (100).

*Methotrexate α,γ-bis[2-(2-aminodecanoyl)-aminodecanoic acid] (**1k**)*—^{1}H NMR ($CDCl_3$−MeOD) δ 7.80, 6.85 (4H, br, arom), 4.89 (2H, s, 9-CH_2), 4.50 (4H, m, 4 α-CH), 3.31 (3H, s, NCH_3), 2.00−1.75 (4H, br m, $^{\beta}CH_2{}^{\gamma}CH_2CO$), 1.37 (56H, s, 28 CH_2), 0.97 (12H, t, 4 CH_3); FAB-MS (*m/z*, %) 1197, 1176, 1154 [M + 3Na, + 2Na, + Na]$^+$ (12, 33, 100), 1132 [M + 1]$^+$ (37), 984 (16), 978 (12), 951 (11), 663 (20), 646 (15), 530 (12).

*Methotrexate α,γ-bis(2-aminododecanoic acid) (**1l**)*—^{1}H NMR ($CDCl_3$−MeOD) δ 8.60 (1H, s, H-7), 8.05 (1H, br, CONH-Glu), 7.77−6.80 (4H, 2d, arom), 6.65 (2H, br, NH_2), 4.83 (2H, s, 9-CH_2), 4.15 (2H, br, 2 α-CH), 3.40 (3H, s, NCH_3), 2.10−1.50 (4H, m, $^{\beta}CH_2{}^{\gamma}CH_2CO$), 1.25 (36H, 2s, 18 CH_2), 0.90 (6H, t, 2 CH_3); FAB-MS (*m/z*, %) 916, 894, 872 [M + 3Na, +2Na, +Na]$^+$ (3, 11, 33), 849 [M + 1]$^+$ (18), 613 (11), 483, 482 (29, 65), 460 (100), 443 (25), 413 (27).

*Methotrexate α,γ-bis(2-aminotetradecanoic acid) (**1m**)*—^{1}H NMR (DMSO-d_6) δ 8.55 (1H, s, H-7), 8.00 (1H, m, CO−NH-Glu), 7.69, 6.79 (4H, 2d, arom), 7.40 (2H, bs, NH_2), 6.53 (1H, s, CO−NH), 4.75 (2H, s, 9-CH_2), 4.35, 4.11 (2H, m, 2 α-CH), 3.17 (3H, s, NCH_3), 2.2−1.5 (4H, m, $^{\beta}CH_2{}^{\gamma}CH_2CO$), 1.18 (44H, s, 22 CH_2), 0.81 (6H, t, 2 CH_3); FAB-MS (*m/z*, %) 971, 949, 928 [M + 3Na, +2Na, +Na]$^+$ (13, 37, 100), 906 [M + 1]$^+$ (46), 881 (13), 753 (31), 663 (59), 649 (52), 590 (43), 530 (50), 491 (47), 469 (70), 460 (66), 441 (85), 421 (62), 412 (52).

*Methotrexate α,γ-bis[2-(2-aminotetradecanoyl)-aminotetradecanoic acid] (**1n**)*—^{1}H NMR ($CDCl_3$−MeOD) δ 8.55 (1H, s, H-7), 8.20 (1H, br, CO−NH-Glu), 7.75, 6.75 (4H, 2d, arom), 4.72 (2H, s, 9-CH_2), 4.40 (4H, m, 4 α-CH), 3.25 (3H, s, NCH_3), 1.75−1.60 (4H, br m, $^{\beta}CH_2{}^{\gamma}CH_2CO$), 1.24 (88H, m, 44 CH_2), 0.86 (12H, t, 4 CH_3); FAB-MS (*m/z*, %) 1379 [M + Na]$^+$ (43), 1356 [M + 1]$^+$ (24), 1204 (21), 1004 (21), 928 (15), 663 (23), 635 (43), 618 (87), 591 (100), 505 (44).

*Methotrexate α,γ-bis(2-aminohexadecanoic acid) (**1o**)*—^{1}H NMR ($CDCl_3$−MeOD) δ 8.55 (1H, s, H-7), 8.15 (1H, m, CO−NH-Glu), 7.70, 6.75 (4H, 2d, arom), 7.50 (2H, bs, NH_2), 6.55 (1H, bs, CO−NH), 4.67 (2H, s, 9-CH_2), 4.25 (2H, m, 2 α-CH), 3.31 (3H, s, NCH_3), 1.8−1.6 (4H, m, $^{\beta}CH_2{}^{\gamma}CH_2CO$), 1.21−1.13 (52H, 4s, 26 CH_2), 0.84 (6H, t, CH_3); FAB-MS (*m/z*, %) 1050, 1028, 1006, 983 [M + 4Na, M + 3Na, +2Na, +Na]$^+$ (13, 100, 42, 7), 960 [M − 1]$^+$ (24), 851 (23), 754 (32), 685 (77), 647 (47), 553 (59), 530 (64), 496 (70), 441 (86), 418 (83), 414 (74).

*Methotrexate α,γ-bis[2-(2-aminohexadecanoyl)-aminohexadecanoic acid (**1p**)*—^{1}H NMR ($CDCl_3$−MeOD) δ 8.45 (1H, bs, H-7), 8.05 (1H, br, CO−NH-Glu), 7.70, 6.87 (4H, br, arom), 7.52 (2H, bs, NH_2), 4.46 (2H, bs, 9-CH_2), 4.30−3.99 (4H, m, 4 α-CH), 3.50 (3H, s, NCH_3), 1.90−1.50 (4H, br m, $^{\beta}CH_2{}^{\gamma}CH_2CO$), 1.24 (104H, s, 52 CH_2), 0.86 (12H, t, 4 CH_3); FAB-MS (*m/z*, %) 1491, 1490 [M + Na]$^+$ (41, 75), 1468 [M]$^+$ (100), 1446 (16), 1365 (19), 1234 (22), 1197 (36), 1147 (33), 1090 (31), 1037 (41), 1002 (34).

*Methotrexate α,γ-bis(2-aminooctadecanoic acid) (**1r**)*—^{1}H NMR (DMSO-d_6): δ = 8.55 (1H, s, H-7), 8.01 (1H, br, CO−NH-Glu), 7.69, 6.79 (4H, 2d, arom), 6.55 (1H, bs, CO−NH), 4.75 (2H, s, 9-CH_2), 4.40, 4.10 (2H, m 2 α-CH), 3.18 (3H, s, NCH_3), 1.9−1.6 (4H, m, $^{\beta}CH_2$-$^{\gamma}CH_2$−CO), 1.20 (60H, s, 30 CH_2), 0.82 (6H, t, 2 CH_3); FAB-MS (*m/z*, %) 1084, 1062, 1040 [M + 3Na, +2Na, +Na]$^+$ (68, 79, 75), 1017, 1016 [M, M − 1]$^+$ (20, 22), 994 (23), 907 (35), 886 (38), 780 (63), 757 (64), 654 (62), 629 (70), 605 (100).

HPLC Analysis—The purity of compounds was assessed by reversed phase HPLC, using a Waters instrument equipped with a 717 plus automatic sampler, a 616 pump, a 600S flow controller, and a 486 UV detector, using a Vydac C_4 column connected to a Beckman Ultrasphere C_8 precolumn, at a constant 1.2 mL/min flow rate. Wavelength and sensitivity were set at 310 nm and 1.0 AUFS, respectively. The mobile phase consisted of 0.1% TFA (v/v) (A) and 90% v/v acetonitrile−0.1% TFA (B); solvents were filtered through a 23 μM membrane filter and degassed by helium flow before use. The solvent gradient ranged from 50% to 100% solvent B in 5 min and then was kept at 100% B for 5 min and finally decreased to 50% acetonitrile in 3 min. Samples were dissolved in 30% (v/v) aqueous acetic acid and filtered through on a 0.8−0.22 μM polyamide filter (Acrodisc PF, Gelman Sciences, UK) before injection. Retention times of conjugates usually ranged between 8.5 and 11 min; the peaks of artificial mixtures of conjugates and MTX always displayed a good separation, with baseline resolution of each peak, under the above conditions (MTX t_R = 7.36).

In Vitro Cytotoxicity Assay—Lymphoblastoid CCRF-CEM (CEM/S and CEM/R) cells were grown at 37 °C, in a 5% CO_2 atmosphere, in RPMI 1640, supplemented with 10% fetal calf serum containing 2 mM glutamine and antibiotics (penicillin and streptomycin). Cells concentration at initial inoculum was 1 × 10^5 cell/mL. One milliliter of cells was put in the individual well of a 24-well plate containing 10 μL of drug solution in anhydrous DMSO, at the required drug concentration. Control cultures were included containing 10 μL of DMSO without drugs. The final DMSO dilution (1:100) appeared to be nontoxic to cell growth. Six representative conjugates from the synthesized pool were chosen for the cellular experiments. Compounds **1j**, **1m**, **1r**, and **1n** represented acid conjugates with increasing lipophilicity, and compounds **1e** and **1f** represented methyl ester conjugates with increasing lipophilicity (a medium length monomer and dimer lipoamino acid were chosen). Stock solutions of 5 mM **1e**, **1j**, and **1m** and 2.5 mM **1f** and **1r** were made in DMSO, and compound **1n** was examined in 10 μM solution. After 72 h incubation with the compounds, cell counts and viability were determined with a hemocytometer by tripan blue exclusion assay.

DHFR Inhibition Assay—DHFR activity was measured by following NADPH oxidation at 340 nm as described previously.[14]

Results and Discussion

Chemistry—Diastereomeric methotrexate conjugates **1b**−**i** were obtained by coupling the corresponding racemic methyl α-aminoalkanoates to **1a**. The reactions were carried out in the presence of 1-hydroxybenzotriazole,[18] using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride assisted coupling method, for 24 h at room

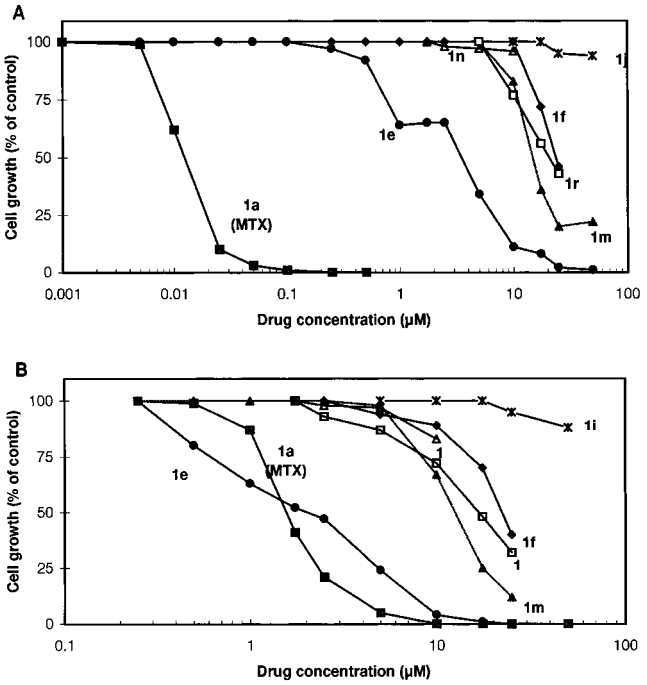
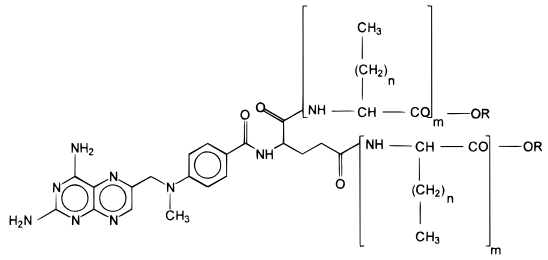


Figure 1—Growth-inhibitory activity of lipoamino acid conjugates of MTX **1e**, **1f**, **1j**, **1m**, **1n**, and **1r** against CEM/s (A) and CEM/R cells (B).

temperature. Although the resulting yields were always higher than 65%, a small amount of starting compound **1a** and some monosubstituted side products were always found in the reaction mixture, but they were easily removed by chromatography.



l	**a**	**b**	**c**	**d**	**e**	**f**	**g**	**h**	**i**	**j**	**k**	**l**	**m**	**n**	**o**	**p**	**r**
m	0	1	2	1	1	2	1	2	1	1	2	1	1	2	1	2	1
n		7	7	9	11	11	13	13	15	7	7	9	11	11	13	13	15
R	H	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3	H	H	H	H	H	H	H	H

The lipid-modified compounds were always insufficently soluble in water. To increase the water solubility and to introduce a functional group for further potential derivatization, the methyl esters were hydrolyzed by alkaline treatment, resulting in compounds **1j**–**r**.

In Vitro Cytotoxicity—To assess the influence of the conjugation and increased lipophilicity, representatives of MTX conjugates (**1e**, **1f**, **1j**, **1m**, **1n**, and **1r**) were assayed for their in vitro growth inhibitory activity against two lines of lymphoblastoid CCRF-CEM cells, using both the wild (sensitive) line (CEM/S) and a MTX-resistant mutant (CEM/R). The activites were directly compared with that of MTX **1a**.

All the tested compounds possessed a reduced level of activity against CEM/S cells compared with **1a** (Figure 1A, Table 1). Among the MTX conjugates, compound **1e** (tetradecanoyl derivative) showed the highest activity, with an IC_{50} value of 3.7 μM. The other derivatives of MTX had IC_{50} values between 15 and 25 μM, except compound **1j** (with a seven-carbon alkyl chain), which appeared to be inactive. It should be noted that the maximum achievable concentration for compound **1n** was 10 μM, due to its poor solubility in the culture medium.

Table 2—IC_{50} Values (μM) of MTX Conjugates Against CEM Cells

compd	CEM/S line	CEM/R line	selectivity ratio[a]
1a (MTX)	0.013 ± 0.003	1.7 ± 0.2	–99.23
1e	3.7 ± 0.8	2.2 ± 1.0	68.2
1f	24.9 ± 2.6	23.0 ± 4.7	8.3
1j	>50	>50	
1m	16.4 ± 1.8	13.5 ± 1.9	21.5
1n	>10	>10	
1r	22.4 ± 3.6	17.7 ± 4.1	26.6

[a] Expressed as $[(IC_{50}CEM/S)/(IC_{50}CEM/R) - 1] \times 100$.

Table 3—Inhibitory Activity of MTX and Its Lipoamino Acid Conjugates against Bovine Liver DHFR

	% enzyme inhibition				
compd	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M
1a (MTX)	100	100	100	92	89
1e	100	94	75	51	29
1f	100	88	40	31	12
1j	95	59	32	23	11
1m	100	64	54	39	32
1n	90	70	62	43	40
1r	87	70	54	37	21

[a] Results indicate percentage of enzyme inhibition against control.

The above results correlate well with the reported observations that substitution on the MTX α-carboxyl group could hinder the binding of the compound to DHFR.[8,11] It is however noteworthy than many α-substituted MTX derivatives showed interesting features as possible prodrugs in enzyme-assisted therapeutic schemes (e.g., ADEPT approach).[19,20]

The activity of compounds **1e**, **1f**, **1j**, **1m**, **1n**, **1r**, and **1a** were also tested against a CEM cell subline (CEM/R), which is defective in the physiological "reduced folate" carrier system (RFC). MTX (**1a**) is 100 times less potent against CEM/R than against the parental CEM cells. The growth inhibitory activity of conjugates **1e**, **1f**, **1j**, **1m**, **1n**, and **1r** was maintained and in some cases even increased (Figure 1B, Table 2). Compound **1e** was the most active against CEM/R cells, with a very similar IC_{50} value to that of MTX. Compounds with the 12-carbon long alkyl chains (**1e, 1m**) showed the highest activity. The more lipophilic methyl ester **1e** displayed higher activity than the corresponding compound with free acids, **1m**. Interestingly, compound **1j**, with octyl side chains, was practically inactive, and compounds with higher lipophilicity, **1n** (4 × 12-carbon atom chains) and **1r** (2 × 16-carbon atom chains) also showed reduced activity.

It is worth mentioning that incubations of MTX bis-conjugates at 37 °C under different pH conditions [buffers at pH 4.5 (acetate), 7.4 (PBS), and 8.0 (phosphate)] did not show any decomposition or hydrolysis to MTX, up to 48 h incubation.

In conclusion, the lipidic conjugates **1e**, **1f**, **1j**, **1m**, **1n**, and **1r** were able to penetrate tumor cells passively because of their increased lipophilic character and possessed an in vitro cytotoxic effect on MTX-transport-resistant CCRF-CEM cells.

In Vitro DHFR Inhibitory Activity—The effects of MTX on cell proliferation is due to its inhibitory activity on DHFR. Therefore, compounds **1a**, **1e**, **1f**, **1j**, **1m**, **1n**, and **1r** were examined for their ability to inhibit purified bovine liver DHFR. The "inhibition index", defined as [IC_{50}

MTX/IC_{50} bis(amide)] × 100, was employed to compare the activities (Table 3). Interestingly, all compounds still retained DHFR inhibitory activity even at 10^{-9} M concentration, although these measured activities were still lower than that of the parent MTX (**1a**). It is worth noting that conjugation of MTX with lipoamino acids resulted in compounds posessing higher DHFR inhibitory activity than the analogous bis(amides) synthesized via condensation of MTX with alkylamines.[14]

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