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Lipophilic methotrexate conjugates with antitumor activity

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Abstract

Lipophilic methotrexate (MTX)–lipoamino acid conjugates coupled with amide or ester linkages (**1a**–**1r**) were synthesised. The inhibitory activity of the conjugates was evaluated on bovine liver DHFR. The in vitro growth inhibitory effect against MTX-sensitive human lymphoblastoid CCRF-CEM cells and an MTX-resistant sub-line (CEM/MTX), which displays defective intracellular transport of MTX, was determined under short-term and continuous (120-h incubation) exposure conditions. The α, γ, or α,γ amide conjugates showed different activity in inhibiting the growth of parent cells. CEM/MTX cells were much less susceptible than CCRF-CEM cells to inhibition by α or α,γ-substituted lipoamino acid conjugates, whereas both cell lines were almost equally sensitive to the MTX-γ conjugates. Although less potent than MTX, they could partially circumvent the impaired transport system. These findings confirm that lipophilic MTX conjugates may be good lead compounds on the drug development for the treatment of some MTX-resistant tumors. Ester-type conjugates displayed an interesting activity against parent CCRF-CEM cells, although they were less potent against the transport-resistant sub-line. Stability studies on these molecules indicated that they are not degraded into MTX in the culture medium, thus suggesting that they are not able to over-cross cell resistance despite of their lipophilicity.

Keywords: Methotrexate conjugates; Lipoamino acids; Antitumor agents; Lipophilicity; CCRF-CEM cells; CEM/MTX cells

1. Introduction

We have previously reported the synthesis and biological evaluation of methotrexate (MTX) lipophilic amide derivatives. Thus, MTX has been derivatised with aminoalkanes (Pignatello et al., 1996, 1999) and lipoamino acids (LAAs) (Pignatello et al., 1998), in order to achieve compounds able to penetrate passively into tumor cells. With such drugs, structurally related to the 'non-classical' antifolates, the possibility arises of over-crossing one of the forms of resistance commonly shown by many tumor cell lines toward MTX. Such a 'transport resistance' is due to a defective efficiency of the low-affinity, high-capacity active carrier (RFC) used by the cells for the uptake of physiologically circulating reduced folate cofactors and of the antifolate drugs (Henderson, 1986).

As many literature reports suggest, it is reasonable that an overall reduction in the polarity of MTX molecule gives a key for enhancing passive internalization of the cytotoxic agent into transport defective cell clones (Kinsky and Loader, 1987; Rahman and Cchabra, 1988; Westerhof, 1995). The modification of one or both the carboxyl groups, present in the glutamate moiety of the MTX molecule (defined as a region of 'bulk tolerance'), appeared as an ideal target, since the binding of MTX to its target enzymes [dihydrofolate reductase (DHFR) and thymidilate synthetase (TS)], is mainly linked to the diaminopyrimidine moiety of the pteridine ring (Sirotnak et al., 1979). For instance, Rosowsky et al. (1984) found that increasing chain length in a series of alkyl monoesters of MTX had a divergent effect upon their inhibitory activity against DHFR (which is reduced) and CEM cells,

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against which the more lipophilic derivatives displayed the maximum activity.

Among the previously described lipophilic MTX α,γ-bis-conjugates (Pignatello et al., 1996, 1998, 1999), some derivatives showed interesting in vitro growth inhibitory activity, compared to free MTX, against a human leukemic T cell lymphoblastoid CCRF-CEM line. Furthermore, most derivatives showed an inhibitory activity against bovine liver DHFR, despite the absence of a free carboxylic group in the α-position of glutamate moiety, whose importance in the binding of folates to DHFR is known (Sirotnak et al., 1979).

However, when an MTX transport-resistant cell sub-line (CEM/MTX cells) was used, which shows an impaired RFC system activity and a reduced MTX uptake (Rosowsky et al., 1980), lipophilic disubstituted derivatives maintained a good cell growth inhibitory activity with respect to the parent drug. Such findings confirm the possibility of bypassing the cell defective RFC transport system (Pignatello et al., 1996, 1998, 1999).

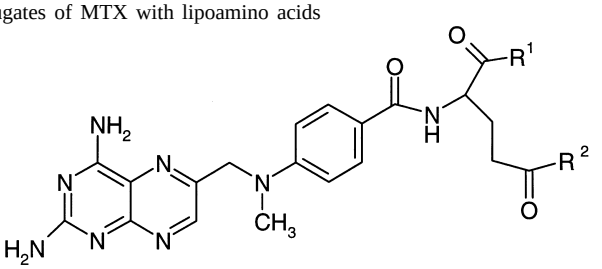
We describe here the synthesis and biological evaluation of further α- and γ-mono-substituted and α,γ-disubstituted lipoamino acid conjugates of MTX coupled with an ester, amide or ester-amide linkage (Table 1). The growth inhibitory activity of the conjugates against sensitive and resistant CCRF-CEM lymphoblastoid cell sublines was evaluated in vitro, along with their inhibitory activity against bovine liver DHFR.

2. Experimental procedures

2.1. Apparatus

IR spectra (not reported) were recorded in KBr disks on a Perkin–Elmer 1600 series FTIR spectrophotometer; the data were in agreement with the structure of the compounds. ^{1}H-NMR were obtained in $CDCl_3$ or in 1:1 (v/v) $CDCl_3$/MeOD with a Brüker AM500 instrument at 500 MHz; chemical shifts are reported in ppm downfield from TMS as the internal reference. Mass spectra were recorded with a VG Analytical ZAB-SE instrument, using fast-atom bombardment (FAB) ionization. A 20-kV Cs^+ ion bombardment was used, with 2 μl of appropriate matrix (either 3-nitrobenzyl alcohol or thioglycerol/glycerol/TFA); a NaI methanol solution was added to produce nitrated species when no protonated molecular ions were observed. TLC was performed on Merck $F_{254+366}$ silica gel aluminum backed plates; spots were detected by UV light, exposure to iodine vapors or pouring into a 10% sulfuric acid ethanol solution. Column chromatography was carried out on Merck dry silica gel (230–400 mesh), with the

Table 1
Structure of mono- and disubstituted conjugates of MTX with lipoamino acids



Compound	$\mathbf{R}^1$	$\mathbf{R}^2$
1a	OH	$NHCH[CONH_2](CH_2)_{13}CH_3$
1b	$NHCH[CONH_2](CH_2)_{13}CH_3$	OH
1c	$NHCH[COOCH_3](CH_2)_7CH_3$	$NHCH[COOCH_3](CH_2)_7CH_3$
1d	$NHCH[COOCH_3](CH_2)_{11}CH_3$	$NHCH[COOCH_3](CH_2)_{11}CH_3$
1e	$NHCH[COOCH_3](CH_2)_7CH_3$	OCH_3
1f	$NHCH[COOCH_3](CH_2)_{11}CH_3$	OCH_3
1g	$NHCH[COOCH_3](CH_2)_7CH_3$	OH
1h	$NHCH[COOCH_3](CH_2)_{11}CH_3$	OH
1i	OH	$NHCH[COOCH_3](CH_2)_7CH_3$
1j	OH	$NHCH[COOCH_3](CH_2)_{11}CH_3$
1k	$OCH_2CONHCH[COOC_2H_5](CH_2)_9CH_3$	$OCH_2CONHCH[COOC_2H_5](CH_2)_9CH_3$
1l	$OCH_2CONHCH[COOC_2H_5](CH_2)_{13}CH_3$	$OCH_2CONHCH[COOC_2H_5](CH_2)_{13}CH_3$
1m	$OCH_2CONHCH[COOC_2H_5](CH_2)_9CH_3$	OH
1n	$OCH_2CONHCH[COOC_2H_5](CH_2)_{13}CH_3$	OH
1o	OH	$OCH_2CONHCH[COOC_2H_5](CH_2)_9CH_3$
1p	OH	$OCH_2CONHCH[COOC_2H_5](CH_2)_{13}CH_3$
1r	$OCH[COOC_2H_5](CH_2)_{13}CH_3$	$OCH[COOC_2H_5](CH_2)_{13}CH_3$

eluent systems reported below. Elemental analysis was carried out on a Carlo Erba mod. 1106 analyzer; samples were kept in vacuo for 24 h over P_2O_5 before analysis; values found are within ±0.4% of theoretical ones.

2.2. Chemicals

MTX (free acid; purity >98%, HPLC) was gifted by Cyanamid (Catania, Italy). MTX-γ-ethyl ester (Piper et al., 1982) was a generous gift of Dr. J. R. Piper (Southern Research Institute, Birmingham, AL, USA). 2-Aminoalkanoic acids (LAAs) were synthesised from their corresponding 1-bromoalkanes and diethylacetamido malonate (Gibbons et al., 1990). Bovine liver DHFR (specific activity 7.8 U/mg protein), dihydrofolic acid and aminopteroic acid emihydrochloride (APA) were from Aldrich–Sigma Chimica, (Milan, Italy). Solid phase synthesis procedures were carried out using Novabiochem (Nottingham, UK) products. *N*,*N*-dimethylformamide (DMF) was distilled and dried over molecular sieves before use; all other solvents and reactants were reagent grade or better and did not need further purification.

2.3. Synthesis of products

2.3.1. ***1a*** *and* ***1b***

A NovaSyn TGR resin (Rink amide linker) (substitution ratio: 0.2 mmol/g) (200 mg) was swollen at room temperature in dry DMF for 60 min and placed in a sintered glass peptide synthesis reaction vessel of a NovaSyn Gem apparatus (Novabiochem). 2-[*N*-1-(4,4,-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl]-aminohexadecanoic acid (Kellam et al., 1998) (48.8 mg, 0.12 mmol) in 2 ml of DMF was coupled to the resin through the assistance of 2-(1 H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (45.5 mg, 0.12 mmol) and in the presence of *N*-hydroxybenzotriazole (HOBt) (10 mg, 0.06 mmol) and diisopropylethylamine (DIEA) (42 μl, 0.24 mmol). The reaction mixture was recycled until a 98% substitution was achieved (about 20 min, ninhydrin test). The resin was then washed with dry DMF for 5 min and the protecting group was removed by 3-min treatment with a 2% hydrazine hydrate solution in DMF; the deprotection was monitored at 288 nm. After a 5-min wash with DMF, the *N*-α-Fmoc-L-glutamic acid α or γ-*t*-butyl ester (51 mg, 0.12 mmol) was injected into the synthesizer along with 10 mg of HOBt, 45.5 mg of HBTU, and 42 μl of DIEA in 2 ml of dry DMF. The conjugation was allowed to proceed and the continuous flow reaction was monitored at 288 nm until a plateau was achieved, determining the end of the acylation phase (about 30 min). After the Fmoc group was removed by treatment with 20% piperidine in DMF, the resin was washed for 5 min with DMF. One equivalent of APA (41 mg) was dissolved in 2 ml of DMF and added with 10 mg of HOBt, 45.5 mg of HBTU, and 42 μl of DIEA. The activation of APA was completed after 5 min. The activated APA was then injected into the system and allowed to recirculate for 2 h (with continuous absorbance check at 288 nm) until the acylation cycle was completed. The resin was finally washed with dry DMF (2×20 ml) and then dry dichloromethane (2×20 ml). The conjugate was then cleaved from the resin by a 90-min treatment with 95% aqueous trifluoroacetic acid (TFA). The crude product was precipitated with ice-cooled diethyl ether and purified by semi-preparative HPLC (see Section 2.4).

2.3.2. Reaction of MTX mono-ester with lipoamino acids

The coupling reaction between MTX γ-methyl ester (Piper et al., 1982) and methyl α-aminodecanoate hydrochloride (Gibbons et al., 1990) was carried out as described previously (Pignatello et al., 1998). Treatment of the reaction crude product by column chromatography on dry silica gel with increasing polarity eluent mixtures (from pure dichloromethane to 7:3, v/v, dichloromethane–methanol), gave the following compounds (FAB-MS peaks and yield given): **1c** (Pignatello et al., 1998) (*m*/*z* 822; 35%); MTX γ-methoxy-α-(methyl 2-aminodecanoate) **1e** (*m*/*z* 653, 652; 24%); a mixture of MTX α-(methyl 2-aminodecanoate) and γ-(methyl 2-aminodecanoate) (**1g, 1i**) (*m*/*z* 624). The latter mixture was subjected to a further chromatographic separation with 7:3 (v/v) dichloromethane–methanol, allowing the collection of the two isomers. According to IR data and literature reports (Rosowsky et al., 1978), the faster eluting compound was identified as the monoconjugate having a free γ-COOH group (i.e., the α-substituted conjugate **1g**). However, the overall yield of these two products was very low (below 10%).

The same reaction was carried out with the methyl α-aminotetradecanoate hydrochloride (Gibbons et al., 1990), giving the corresponding derivatives **1d**, **1f**, **1h** and **1j** with the same order of elution (*m*/*z* 920, 709, and 680, respectively; yields: 23%, 20%, <5%, <5%, respectively).

2.3.3. *α-Bromoethanoyl-ω-methoxy[imino-1(decyl-2-oxo-1,2-ethanediyl)]*

The compound was obtained by a modified procedure of that described by Wood et al. (1992). Bromoacetic acid (140 mg, 0.1 mmol), methyl 2-aminododecanoate hydrochloride (325 mg, 0.1 mmol) (Gibbons et al., 1990), triethylamine (100 mg, 1 mmol), HOBt (155 mg, 1 mmol), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) (200 mg, 1.1 mmol) were dissolved in 5 ml dichloromethane and allowed to react at 0°C for 2 h and then overnight at room temperature. The mixture was diluted with 10 ml dichloromethane and extracted as described above for compound **1c**. Crystallization of the residue from acetonitrile gave the title compound with a 70% yield. Analysis for $C_{15}H_{28}BrNO_3$ (350.30): % calc.

(found)=C 51.43 (51.71), H 8.06 (8.29), N 4.00 (4.21). FAB-MS (*m*/*z*, %): 374, 372 $[M\text{-}H+Na]^+$ (8), 352, 350 $[M]^+$ (19, 22), 330, 328 (12, 32), 308, 306 (34, 100), 248, 246 (23, 77).

The corresponding higher homologous α-bromo-ethanoyl-ω-methoxy[imino-1(tetradecyl-2-oxo-1,2-ethanediyl)] was obtained using the procedure described above, by the reaction of bromoacetic acid with methyl α-aminohexadecanoate hydrochloride (Gibbons et al., 1990). Analysis for $C_{19}H_{36}BrNO_3$ (406.41): % calc. (found)=C 56.15 (56.26), H 8.93 (9.07), N 3.45 (3.26). Yield: 52%. FAB-MS (*m*/*z*, %): 430, 428 $[M\text{-}H+Na]^+$ (28), 408, 406 $[M]^+$ (57, 64), 364, 362 (14, 34), 348, 346 (58, 64), 226, 224 (100, 27), 154 (67), 137 (97), 109, 107 (52, 63). ^{1}H-NMR ($CDCl_3$): δ=7.05, 6.90 (1 H, 2d, NH; J=9 Hz), 4.58 (1 H, m, α-CH), 4.07, 3.89 (2 H, 2 s, CH_2Br), 3.76 (3 H, s, $COOCH_3$), 1.90–1.85, 1.75–1.70 (2 H, 2 m, β-CH_2), 1.28–1.25 (24 H, s, 12 CH_2), 0.88 (3 H, t, CH_3; J=13 Hz).

2.3.4. Compound **1k**

MTX was suspended in methanol and the required amount of a methanol–water (1:1, v/v) KOH solution was added. After 10 min, the resulting yellow solution was filtered, evaporated in vacuo and the residue freeze-dried to give the MTX dipotassium salt. The latter (15 mg, 0.028 mmol) was dissolved in 5 ml methanol and 18-crown-6 ether (25 mg, 0.095 mmol) was added. After stirring at room temperature for 30 min, the solvent was evaporated, the residue treated twice with 10 ml benzene and dried in vacuo. The salt (15 mg, 0.028 mmol) was then dissolved in 2 ml anhydrous DMSO and methyl 2-bromoacetamido-dodecanoate (23 mg, 0.057 mmol) was added. After stirring for 24 h at room temperature, the solvent was removed under high vacuum and the residue treated with a small volume of water. The crude compound was purified by column chromatography using 10:1 (v/v) dichloromethane/methanol mixture.

Yield: 48%; FAB-MS (*m*/*z*, %): 1032 (9) $[M+K]^+$, 993 (100) $[M+1]^+$, 724 (63), 494 (23); ^{1}H-NMR ($CDCl_3$): δ=8.63 (1 H, s, H-7), 7.70, 6.75 (4 H, 2s, aromatic H), 7.37 (2 H, m, NH_2), 5.40 (2 H, bs, CH), 4.75 (2 H, s, 9-CH_2), 4.62 (2 H, m, 2 α-CH), 3.71–3.64 (6 H, m, 2 OCH_3), 3.19 (3 H, s, NCH_3), 1.84–1.80 (4 H, bm, $^{\beta}CH_2^{\gamma}CH_2CO$), 1.26–1.22 (36 H, s, 18 CH_2), 0.88 (6 H, t, 2 CH_3; J=6.6. Hz).

Compound **1o** was obtained as a minor product (yield: 12%). MS *m*/*z* (%)=746 (3) $[M+Na]^+$, 724 (10) $[M^+]^+$, 338 (66), 303 (100), 287 (94); ^{1}H-NMR ($CDCl_3$): δ=8.57 (1 H, s, H-7), 7.66, 6.83 (4 H, 2d, aromatic H; J=8.5 Hz and 8.7 Hz), 4.78 (2 H, s, 9-CH_2), 4.47 (2 H, m, 2 α-CH), 3.81–3.53 (3 H, m, OCH_3), 3.20 (3 H, s, NCH_3), 1.75-1.60 (4 H, bm, $^{\beta}CH_2^{\gamma}CH_2CO$), 1.20 (18 H, s, 9 CH_2), 0.85 (3 H, t, CH_3; J=6.7 Hz); HPLC, R_T: 6.6 min.

Compound **1l** was synthesized using the same procedure described for compound **1k**, by the reaction of MTX dipotassium salt and methyl 2-bromoacetamido-hexadecanoate: yield: 63%; FAB-MS (*m*/*z*, %): 1150 (10) $[M+2Na]^+$, 1128 (59) $[M+Na]^+$, 1106 (33) $[M+1]^+$, 634 (23), 583 (34), 555 (100), 527 (54); ^{1}H-NMR ($CDCl_3$): δ=8.60 (1 H, bs, H-7), 7.70, 6.75 (4 H, 2d, aromatic H; J=6.2 Hz), 7.40 (2 H, d, NH_2; J=6 Hz), 5.29 (2 H, s, CH), 4.76 (2 H, s, 9-CH_2), 4.62 (2 H, m, 2 α-CH), 3.80–3.65 (6 H, m, 2 OCH_3), 3.49–3.40 (4 H, m, $COOCH_2CO$), 3.19 (3 H, s, NCH_3), 1.85–1.65 (4 H, m, $^{\beta}CH_2^{\gamma}CH_2CO$), 1.43 (4 H, s, 2 CH_2), 1.24 (48 H, s, 24 CH_2), 0.89 (6 H, t, 2 CH_3; J=10 Hz); HPLC: R_T 10.48 min.

Minor products (yield<10%) were **1n** and **1p**; FAB-MS: *m*/*z* 780 $[M+1]^+$, 803 $[M+Na]^+$); HPLC: R_T=7.9 min.

2.3.5. Ethyl 2-bromohexadecanoate

2-Bromohexadecanoic acid (Fluka, Sigma–Aldrich Srl, Milan, Italy) was dissolved in a saturated solution of hydrochloric acid in absolute ethanol, under stirring for 1 h at room temperature. The crude product was purified by flash column chromatography (dichloromethane/methanol, 10:1, v/v) and the ester was obtained as oil in a 74% yield (FAB-MS: 366, 364 $[M^+ +1]$).

2.3.6. Compound **1r**

MTX dipotassium salt (11 mg, 0.02 mmol) was treated with 15 mg 18-crown-6 ether in methanol. Ethyl 2-bromo-hexanoate (15 mg, 0.04 mmol) dissolved in 1 ml dry DMF was added and the mixture was left under stirring at room temperature for 16 h. After evaporation under high vacuum, the residue was treated with 2 ml of cold water and extracted with diethyl ether (3×10 ml). Removal of the solvent under vacuum gave a crude product which was further purified by flash column chromatography, using hexane (to remove the unreacted starting bromoester) and then dichloromethane–methanol (8:2, v/v) as the eluent. Compound **1r** was collected with an overall 64% yield.

TLC, R_f: 0.52 (dichloromethane–methanol, 10:1, v/v); FAB-MS (*m*/*z*, %): 1042 (24) $[M+Na]^+$, 1020 (100) $[M+1]^+$, 858 (22), 647 (41), 590 (43), 441 (77). ^{1}H-NMR ($CDCl_3$): δ=8.65 (1 H, bs, H-7), 8.15 (1 H, m, CONHGlu), 7.70, 6.70 (4 H, 2 m, aromatic H), 7.36 (2 H, s, NH_2), 4.80 (2 H, s, 9-CH_2), 4.25 (4 H, q, 2 CH_2), 4.23 (2 H, m, 2 α-CH), 3.20 (3 H, s, NCH_3), 1.79–1.75 (4 H, m, $^{\beta}CH_2^{\gamma}CH_2CO$), 1.43 (4 H, s, 2 CH_2), 1.30–1.25 (54 H, 5s, 24 CH_2+2 $COOCH_2\underline{CH_3}$), 0.88 (6 H, t, 2 CH_3; J=13.5 Hz).

2.4. Conjugate analysis and purification

The purity of the prepared compounds was assessed by means of RP-HPLC, using a Waters 616/600S twin pump system equipped with a 717plus automatic sampler and a 486 tunable UV detector monitored by a Millennium

software package. A Vydac C_4 column (4.6×150 mm) linked to a Beckman Ultrasphere C_8 pre-column was used, at a constant flow-rate of 1.2 ml min^{-1}. Wavelength and sensitivity were set at 310 nm and 1.0 AUFS, respectively. Mobile phase consisted of 0.1% TFA (v/v) (A) and 90% v/v acetonitrile/0.09% TFA (B); solvents were filtered through a 0.2-μm polyamide filter and degassed by a helium flow before use. The solvent gradient ranged linearly from 0 to 100% solvent B in 10 min and then decreased to 0% solvent B in 5 min. Test samples were dissolved in 20% acetic acid and filtered through a 0.45-μm polyamide filter before injection. Analysis of mixtures of each compound with MTX always showed a good separation of the two peaks under the above conditions.

Purification of crude reaction mixtures was also afforded by semi-preparative RP-HPLC on a Vydac C_6 column (8×150 mm). Separation was achieved with the same solvents as above, using a linear gradient between 0 and 60% (solvent B) in 180 min, staying at these conditions for 30 min, and then decreasing steadily to 0% solvent B in 30 min, at a constant flow-rate of 6 ml min^{-1}. The gradient was effected by two microprocessor-controlled Gilson 302 single-piston pumps. Eluates were detected by a Holochrome UV–Vis detector at 300 nm and chromatograms were recorded using a LKB 2210 single-channel recorder. The identity of conjugates in the suitable collected fractions was confirmed by FAB-mass spectrometry.

2.5. Stability evaluation of MTX conjugates

The stability of some compounds, selected as prototypes of the series of prepared conjugates, was evaluated both in the same culture medium used for the cell growth inhibitory assays (see below) and in FCS, in order to test their susceptibility toward the hydrolysis into free MTX.

For the first experiment MTX, MTX-diethyl ester (Rosowsky, 1973) or conjugates **1c**, **1k**, and **1o** were dissolved in 100 μl DMSO at 1 μM concentration and the solution added to 2 ml of complete RPMI 1640 culture medium, in separate screw-capped vials. At time 0 and after 12, 24 and 48 h of incubation at 37°C, 250 μl of medium were withdrawn and added with 500 μl of chloroform. Samples were vigorously stirred and then centrifuged at 10°C, for 10 min at 5000 rpm. The organic layer was discarded and samples of the aqueous phase were spotted on TLC RP-18 glass sheets (Merck F_{254}), together with a pure (untreated) MTX specimen. After elution with a pH 8.0 phosphate buffer, the compounds were visualized by UV light at 254 nm.

To assay the hydrolytic stability of conjugates in a biological medium, compounds **1c** and **1k** (1 mg) were dissolved in 200 μl DMSO and the solution added to 1.8 ml of fresh FCS. Samples were vortexed and incubated at 37°C (±1°C) in a water bath. At the decided time intervals, 50 μl of the solution was withdrawn, added with 450 μl of ice-cooled methanol and centrifuged at 5000 rpm. The supernatant was collected and diluted twice with distilled water, and the mixture was injected in the HPLC column and tested by the procedure described above.

2.6. DHFR inhibition assay

The drug inhibitory effect on DHFR activity was measured by following NADPH oxidation at λ=340 nm (Pignatello et al., 1996). The assay mixture contained 1 M sodium acetate buffer (pH 6.0), 0.6 M KCl, 50 μM NADPH, 50 μl DMSO (control) or the same volume of a DMSO solution of test compounds, to a final concentration ranging between 10^{-5} and 10^{-11} M, and 0.02 units of bovine liver DHFR, in a final volume of 3 ml. After enzyme addition, the mixture was incubated at 23°C for 5 min; reaction was started by adding 33 μM dihydrofolic acid. The change in absorbance at λ=340 nm (Δabs_{340}) was followed for ten min, during which the activity was linear with respect to time. Results are reported as percent inhibition of the enzymatic activity vs. control (Fig. 1); each value is the mean±S.D. of six experiments.

2.7. In vitro cell cytotoxicity tests

2.7.1. Cell growth inhibition assay

The human T-lymphoblastic leukemia cell line CCRF-CEM (Foley et al., 1965) and an MTX-resistant (transport-defective) cell line (CEM/MTX) (Rosowsky et al., 1980; Westerhof et al., 1995) were grown at 37°C, in a 5% CO_2

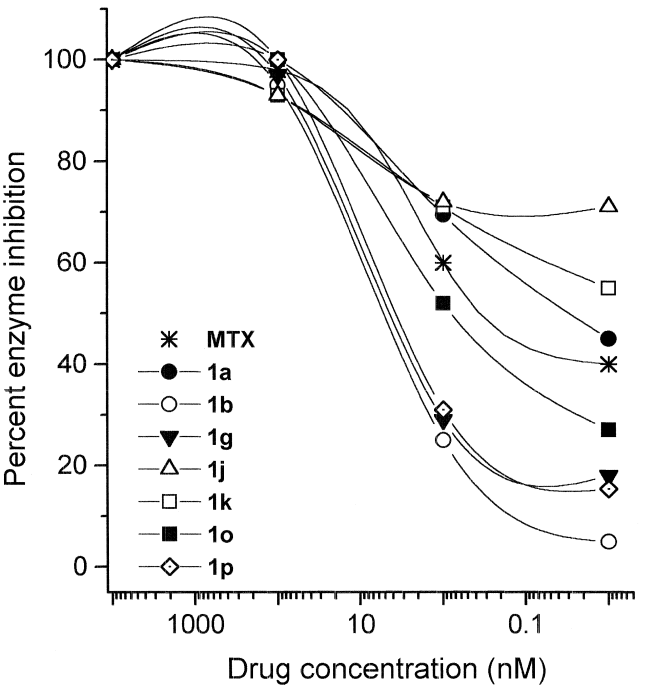


Fig. 1. Bovine liver DHFR inhibition by MTX conjugates.

atmosphere, in RPMI 1640 (HyClone) supplemented with 10% fetal calf serum, glutamine (2 mM) and antibiotics. Cells were plated in plastic 24-well plates at a concentration of 1×10^5 cells per ml per well and test compounds were concomitantly added in a concentration range of 0.01–10 μM. Dilutions were made using the culture medium from 1 mM stock solutions of the drugs in anhydrous DMSO. Controls were treated with an equivalent amount of solvent, diluted as above, to assess the absence of toxicity due to the solvent.

After 72 h of incubation, viable cells were counted with a haemocytometer by trypan blue exclusion assay. EC_{50} (drug concentration reducing viability to 50% that of untreated control) were determined visually from plots of relative growth (percent of untreated control) versus the logarithm of drug concentration and are reported in Table 2. The experiments were repeated three times in duplicate.

2.7.2. Continuous drug exposure test

The CCRF-CEM cell line and its MTX-resistant sub-line CEM/MTX were cultured as described in RPMI 1640 medium (GIBCO) supplemented with 10% horse serum (McCloskey et al., 1991); growth inhibition by continuous drug exposure for 120 h was measured as described (McCloskey et al., 1991). Both cell lines (generation times: CCRF-CEM, 20.4±0.1 h; CEM/MTX, 19.9±0.6 h) grew logarithmically for 120 h if seeded at 10^4 cells per ml at t_{zero}. The 120-h exposure time increases the sensitivity of the growth inhibition assay by allowing the maximum number of logarithmic cell doublings to occur. MTX was dissolved in water; compounds **1a**, **1b** and **1j** were dissolved in DMSO. Solvent controls were included in each experiment and only data generated at DMSO concentrations that were themselves nontoxic (≤0.23% DMSO for both cell lines) were considered valid. Both cell lines were verified to be negative for *Mycoplasma* contamination using the GenProbe test kit. EC_{50} values were determined as reported above (Table 2).

3. Results

3.1. Chemistry

An isomeric pair of γ- and α-substituted monoconjugates of MTX with 2-aminohexadecanoic acid **1a** and **1b** were prepared using standard solid-phase peptide synthesis methods (SPPS) (Merrifield, 1963). An *N*-acetyldimedone (Dde)-protected 2-aminohexadecanoic acid (Kellam et al., 1998) was linked to a TGR (Rink amide linker) resin through HBTU assistance. After removal of the protecting group with 2% hydrazine hydrate, *N*-α-Fmoc-L-glutamic acid α- or γ-*tert.*-butyl ester was added, followed by cleavage with 20% piperidine. The deprotected compound was then reacted with 2,4-diaminopteroic acid hemihydrochloride (APA). Removal of the final compound from resin by treatment with 95% aqueous TFA gave the expected derivatives **1a,b** with a very high purity (>95%, HPLC). The major advantages of this new method, compared with those described in the literature, were (i) the easy Dde deprotection and (ii) the use of non-toxic HBTU as the activating agent.

Monosubstituted conjugates of MTX **1c–j** were also obtained as side products from the reaction between MTX-γ-methyl ester and LAAs. Separation of the of α- and γ-substituted isomer (lower R_f) was accomplished by column chromatography, by taking advantage from the little difference in the pK_a of the two carboxyl functions (Rosowsky et al., 1978; Rahman and Cchabra, 1988). The structure elucidation of the compounds was accomplished by using modern physicochemical methods functions (Rosowsky et al., 1978; Antonjuk et al., 1984a; Rahman and Cchabra, 1988). It is noteworthy that a partial hydrolysis of MTX-γ-methyl ester occurred in the reaction medium, resulting in a free γ-COOH group, which reacted with the LAA residues present in the reaction mixture (Rosowsky et al., 1978).

MTX–LAAs conjugates with an ester linkage **1k–p** were obtained by crown-ether assisted condensation. First

Table 2
In vitro growth inhibitory activity (EC_{50}, nM) of MTX and conjugates against MTX-sensitive CCRF-CEM cells and the transport-resistant CEM/MTX sub-line

Drug	CCFR-CEM (72-h)	CEM/MTX (72-h)	Relative resistance[a] fold	CCRF-CEM (120-h)	CEM/MTX (120-h)	Relative resistance[a] fold
1a	385	390	1.01	310	1800[b]	5.8
1b	900	>5000	n.d.[c]	5800[b]	>10 000	n.d.
1j	85	>5000	n.d.	>1000	>1000	n.d.
1k	35	1900	54.3	–	–	–
1o	60	10 000	167	–	–	–
1p	270	570	2.1	–	–	–
MTX	30	4300	143	14	2200	157[d]

[a] Relative resistance is the ratio of EC_{50} (CEM/MTX) to EC_{50} (CCRF-CEM).
[b] In a second experiment, the EC_{50} was not reached but the trend in the data allowed extrapolation to an approximate EC_{50} which was consistent with the value presented. For **1a** and CEM/MTX, the extrapolated EC_{50} was 1300 nM; for **1b** and CCRF-CEM cells, the extrapolated EC_{50} was 6000 nM.
[c] n.d.=value cannot be determined.
[d] In separate experiments, relative resistance of CEM/MTX sub-line toward MTX ranged between 100- and 150-fold.

dipotassium salt of MTX was reacted with crown-16-ether and then with the methyl ester of the desired LAA (Gibbons et al., 1990) in DMSO or dichloromethane. The purification of **1k–p** was achieved by column chromatography. A similar reaction was used to obtain the long-chain aliphatic MTX α,γ-bis(ester) **1r**.

Qualitative stability studies were carried out on selected terms, i.e., an α,γ-bis(amide) conjugate (**1c**) and two ester derivatives (**1k** and **1o**), by testing the appearance of free MTX, under the same conditions used for the cell outgrowth inhibition assays. Neither compound **1c** or the ester derivatives **1k** and **1o** were converted into MTX after 48 h of incubation in the culture medium, whereas MTX diethyl ester already was partially hydrolyzed into both the monoethyl ester and the free drug after 24 h of incubation. MTX did not display any degradation sign after 48 h of incubation at 37°C.

After incubation in FCS the results are divergent for the amide and ester-type conjugates. Amide conjugate **1c** was almost stable after 5 days of incubation, giving only traces of free MTX (<3%, based on the HPLC peak area). On the contrary, the ester **1k** was rapidly degraded: after 15 min a 20% MTX was detected in the incubation mixture (retention time: 3.85 min), and its concentration remained almost constant up to 8 h incubation. In the meantime, the disappearance of the starting compound (retention time: 8.83 min) was associated by increasing levels of two degradation products (retention times: 6.30 and 6.90 min), corresponding to the isomeric pair of monosubstituted conjugates **1m** and **1o**, as confirmed by the FAB-MS analysis on the collected HPLC fraction. Their combined concentration in the assay medium went from 22% after 15 min of incubation to 55% after 8 h.

3.2. Biochemical screening

Some of the prepared conjugates were tested against DHFR as representative terms of the different series of lipophilic MTX conjugates. All of them showed an inhibitory activity against bovine liver DHFR (Fig. 1). Compounds **1b** and **1g**, substituted on the α-carboxyl function were less potent than MTX. However, the γ mono-amide conjugates **1a** and **1j**, and the α,γ-bis(ester) conjugate **1k** showed a similar or greater inhibitory activity than the parent drug.

3.3. In vitro tumor cell cytotoxicity assay

To perform these assays selected compounds were also chosen, belonging to the three series of lipophilic MTX conjugates. Against the MTX-sensitive (parental) CCRF-CEM cell line, compounds **1a**, **1b**, **1j** and **1p** were less active than MTX, but the activity of ester conjugates **1k** and **1o** was comparable with that one of the parent drug (Table 2). However, data relative to the activity exerted against the transport-deficient resistant cells (i.e., the relative resistance) gave divergent results. In fact, only γ-substituted monoconjugates (**1a** and **1p**) maintained a similar potency against both cell lines, whereas MTX was about 150 times less active against the resistant sub-line than towards the parental line. In Fig. 2, the concentration–activity profile is enlightened for compound **1a** and MTX.

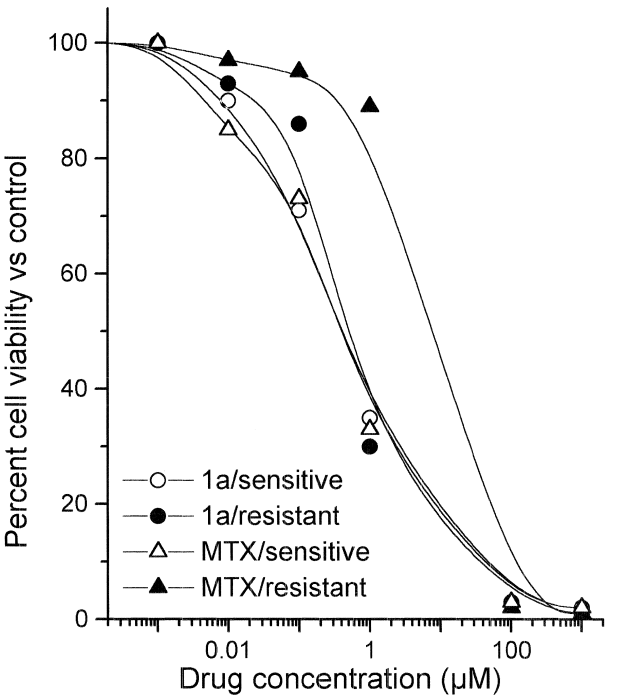


Fig. 2. Growth inhibitory activity of **1a** and MTX against sensitive and resistant CCRF-CEM cells.

Compounds **1a**, **1b** and **1j** were compared to MTX as inhibitors of MTX-sensitive CCRF-CEM human leukemia cells growth in horse serum (instead of FCS, as above) also during continuous exposure in vitro (Table 2). Compounds **1a** and **1b** were 22-fold and 414-fold less potent than MTX as growth inhibitors of CCRF-CEM cells. The well-characterized MTX-transport-defective CEM/MTX sub-line was 157-fold resistant to MTX, but only slightly cross-resistant to **1a** (**1b** did not affect cell growth at 10 μM, the highest concentration testable).

By comparing the results from the two experiments, compound **1a** displayed the same order of cytotoxicity both in the 72-h and 120-h incubation assays (relative resistance of 1.01 and 5.8, respectively). The α-substituted isomer **1b** showed a lower activity against sensitive cells and was almost inactive against the resistant CEM/MTX sub-line.

4. Discussion

In general, the inhibitory activity of the described lipophilic MTX conjugates was lower than the activity of the parent drug against sensitive cells. However, some

conjugates showed the same cytotoxicity to CEM/MTX cells, which were primarily resistant to MTX by virtue of an impaired cellular drug uptake. Similar findings have been reported for other lipophilic antifolates and MTX derivatives (Rosowsky et al., 1980; Piper et al., 1982; Rosowsky et al., 1984; Kinsky and Loader, 1987; Rahman and Cchabra, 1988; Pignatello et al., 1996, 1998, 1999). Since the lack of drug transport represents one important form of resistance to MTX in both human and murine cell lines (Sirotnak, 1980; Westerhof, 1995), the alterations of the transport kinetics have a potential clinical value.

Comparison of results relative to DHFR activity and tumor cell growth inhibition by the conjugates, confirmed that the presence of a free carboxylic group in the α position of MTX glutamate moiety, is important for the binding to the target enzyme (Antonjuk et al., 1984b, 1989); as expected, the γ-mono substituted compounds **1a, 1j** and **1k** inhibited DHFR at the same level than MTX.

The γ- and α-substituted mono-amide conjugates **1a** and **1b** exhibited a very different affinity for DHFR and in vitro inhibition potency against CCRF-CEM cell growth. The γ-substituted isomer **1a** showed a higher inhibitory activity against DHFR than the corresponding α-substituted derivative **1b**, with a higher IC_{50} value (0.034 nM) than MTX itself (0.37 nM) (Fig. 1). Similarly, compound **1a** was 20–50 times more potent than **1b** against sensitive CCRF-CEM cells and resistant (CEM/MTX) sub-line (Table 2).

Conjugate **1a** showed an activity profile close to that one of MTX. However, the lipophilic conjugate retained its activity against the MTX-uptake defective CEM/MTX cell sub-line, showing a relative resistance of 1.02 and 5.8, after 72-h and 120-h drug exposure respectively (Table 2 and Fig. 2.). As expected, MTX was much less potent in both assays. CEM/MTX cells become less sensitive to compound **1a** with exposure time. Its EC_{50} value was in fact 393 nM after 72-h drug exposure and 1800 nM after 120-h exposure. For compound **1b** differences were less marked, while MTX displayed similar EC_{50} values in both the culture conditions. Several interpretations can be given to these results: conjugate **1a** may slowly be degraded into MTX in the long-term (120-h) culture assay, and a time-dependent release of free MTX may occur in the external medium, leading to a progressive reduction of cell growth inhibition. It is noteworthy that we used different culture media (FCS or horse serum) in the two in vitro assays, and this could induce a different hydrolytic activity upon the MTX conjugates.

Alternatively, by considering that these conjugates could not be subjected to intracellular polyglutamylation (McGuire, 1998), a steady-state equilibrium between drug influx and efflux could occur, lowering the effective concentration of the inhibitor inside the tumor cells.

In any case, it must be considered that the 120-h exposure time increases the sensitivity and dynamic range of the growth inhibition assay by allowing the maximum number of logarithmic growth doublings to occur during the assay period. The longer exposure period also allows delayed growth inhibition to be detected, if it occurs. The greater dynamic range allows more closely spaced drug concentrations to be tested (typically spacing of, for example, 10 nM, 20 nM, 50 nM, 100 nM, 200 nM, etc.). This spacing allows a more accurate interpolation of the EC_{50} value than the wider ten- to 100-fold spacing used in the 72-h viability studies (Fig. 2). It should also be noted that the 120-h assay measures growth (based on particle counts), while the shorter assay measures actual cell viability (based on trypan blue dye exclusion). Thus the results of the two assays may not coincide if cells are killed (not viable), but do not lyse (particle intact).

Compound **1j** (a γ-mono-substituted amide conjugate) showed a higher affinity than MTX for DHFR ($IC_{50}<0.01$ nM); it also showed an activity close to MTX against sensitive CCRF-CEM cells, but was inactive against MTX-resistant cells ($EC_{50}>1000$ nM; data not shown). It would then seem that it was not able to take advantage of its lipophilic character to penetrate passively into the transport-resistant cell lines.

An important consideration about the lipophilic conjugates of MTX is that their biological activity can either be the result of a direct effect on cell enzymes, or of being mediated by the intracellular release of free MTX, that will ultimately be responsible for blocking cell outgrowth. For this reason, we prepared further compounds where MTX and the LAA residue were linked through an ester bond (compound **1r**), or an ester–amide bond (compounds **1k–1p**). The hydrolysis rate of an ester in the biological environment is normally faster than the amide bonds (Bundgaard, 1985), so we expected that these compounds would act better as MTX prodrugs.

In our experiments, the biological activity of compounds **1k** and **1o** was very close to that of MTX against both the parent and resistant cell lines (72-h incubation time) (Table 2). By considering that these ester conjugates were not converted into MTX when incubated in the culture medium (see Results), it seems that they are unable to enter resistant cells via passive routes, despite their lipophilic character. However, the gradual hydrolysis into the corresponding monosubstituted esters and then free MTX that we observed after incubation of some ester conjugates in fetal calf serum, made the conjugate **1k** a potential prodrug for the slow systemic release of MTX.

5. Conclusions

We have confirmed that lipophilic antifolates, like MTX–LAA conjugates can be used as lead compounds for the development of drugs for MTX-resistant tumors.

Lipophilic derivatization of MTX glutamate allowed the passive entry of the drug into tumor cells. The activity of the amide conjugates of MTX **1a** and **1p**, which showed an

intrinsic potency against cell growth, was not due to the release of free MTX inside the cells.

The therapeutic association of MTX with one of its lipophilic derivatives may prevent the development of transport resistance to the drug, which has often been observed during clinical use of MTX. Moreover, the described lipophilic MTX conjugates would not be expected to form γ-polyglutamates once they enter a cell, unlike the parent drug. This distinctive property has potential therapeutic implications for the treatment of certain MTX-resistant tumors whose resistance may be associated with a lower than normal capacity to form γ-polyglutamates, in comparison with highly proliferative tissues such as intestinal mucosa or marrow (McCloskey et al., 1991; McGuire, 1998).

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