Antineoplastic Activity of Lipid-Soluble Dialkyl Esters of Methotrexate

The folate antagonist methotrexate (4-amino-4-deoxy-N^{10}-methylpteroylglutamate; MTX), a potent inhibitor of the enzyme dihydrofolate reductase, is widely used in the treatment of acute lymphocytic leukemia and other forms of neoplastic disease[1]. We have recently prepared a series of dialkyl esters of this compound for use as model substrates in drug metabolism structure-activity studies in vitro[2]. Since the physicochemical properties of the MTX esters were found to differ markedly from those of the lipid-insoluble parent drug, we felt it to be of interest to assess the pharmacologic activity of these compounds as antitumor agents in vivo, and also as inhibitors of the target enzyme for MTX, dihydrofolate reductase. The results of these studies are reported below.

Materials and methods. The carboxylic acid groups of the glutamyl moiety of MTX were esterified by a synthetic method described elsewhere[2]. Di-*n*-alkyl esters of chain-lengths from 1 to 8 carbons were prepared. Dihydrofolate reductase from mouse L1210/A (XL) leukemia cells was partially purified by ammonium sulfate fractionation and gel filtration on Sephadex G-200; enzyme activity was assayed spectrophotometrically as previously described[3]. Specific activity of the enzyme preparation used was 34 μmoles/min/mg. Compounds were tested for in vivo antineoplastic activity in male CDF_1 mice bearing L1210 leukemia cells; the size of the inoculum was 2×10^5 cells for animals receiving the tumor intracerebrally and 4×10^5 cells for animals receiving the tumor intraperitoneally. Drug treatment was started 24 h later by the intraperitoneal route and was continued once daily for 10 days. Because of limited water solubility of the dialkyl esters, a non-ionic surfactant (Emulphor EL 620) was used in preparing drug solutions; the solvent system used was ethanol: EL 620:water::5:5:90 v/v. The sensitivity of the tumor to administered drugs was determined from prolongation of survival time of drug-treated animals over that of solvent-treated control mice. Serum levels of MTX and MTX esters were determined by the dihydrofolate reductase inhibition titration method of BERTINO and FISCHER[4].

Results. The dialkyl esters were effective inhibitors of dihydrofolate reductase from mouse L1210 leukemia cells; their inhibitory activity, however, was less than that of the non-esterified parent compound, MTX (Table I). Within the ester series, enzyme inhibitory activity increased with ester chain-length up to a maximum with the 4- and 5-carbon esters; further increases in chain length were accompanied by a decrease in inhibitory activity.

In contrast to the results obtained in the dihydrofolate reductase inhibition assay, the antitumor activity of the esters in vivo, in terms of extension of life-span, was equal to that of MTX when tested against both the intracerebrally and intraperitoneally implanted tumor (Table II). The maximum effective dose of the esters was also the same as that of the parent compound (1.5 mg/kg/day i.p.) except for the diethyl ester, which was maximally effective at a dose of 4 mg/kg/day.

Since the in vivo activity of the MTX esters was approximately equal to that of MTX despite the marked differences in their activities in the in vitro test system, it was felt to be likely that hydrolysis of the esters was occurring in vivo, with release of the parent drug. To test this hypothesis, male CDF_1 mice were given the esters i.p. at a dose-level of 5 mg/kg, and serum levels of esterified and free MTX determined at 15, 30 and 60 min by partitioning deproteinized serum between *n*-butanol and water and determining activity in both phases by the dihydrofolate reductase inhibition titration assay. As

Table I. Inhibition of dihydrofolate reductase from mouse L1210 leukemia cells by MTX and MTX di-*n*-alkyl esters

Inhibitor	Drug concentration (*M*) required to reduce rate of reduction of dihydrofolate to tetrahydrofolate to 50% of control value[a]
MTX	1.0×10^{-9}
MTX, Dimethyl ester	3.8×10^{-7}
MTX, Diethyl ester	1.3×10^{-7}
MTX, Di-*n*-butyl ester	5.4×10^{-9}
MTX, Di-*n*-amyl ester	6.0×10^{-9}
MTX, Di-*n*-hexyl ester	1.0×10^{-8}
MTX, Di-*n*-octyl ester	1.3×10^{-8}

[a] Control rate: 0.024 absorbance units/min at 340 nm.

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Table II. Effect of i.p. administered MTX and MTX di-*n*-alkyl esters on survival time of male CDF_1 mice inoculated with L1210 leukemia cells

Compound	Route of tumor cell implantation	Optimal daily dose (mg/kg)	Average survival (days ± SE)
None	Intraperitoneal (IP)		8.6 ± 0.1 (30)[a]
MTX	IP	1.5	17.1 ± 0.5 (28)
MTX, Diethyl ester	IP	4.0	16.1 ± 0.7 (10)
MTX, Di-*n*-amyl ester	IP	1.5	16.8 ± 0.4 (10)
MTX, Di-*n*-octyl ester	IP	1.5	18.3 ± 0.6 (10)
None	Intracerebral (IC)		8.5 ± 0.1 (30)
MTX	IC	1.5	12.6 ± 0.2 (29)
MTX, Diethyl ester	IC	4.0	11.8 ± 0.2 (10)
MTX, Di-*n*-amyl ester	IC	1.5	13.2 ± 0.3 (10)
MTX, Di-*n*-octyl ester	IC	1.5	12.5 ± 0.4 (10)

[a] Values in parentheses indicate number of animals in each treatment group

early as 15 min after intraperitoneal injection, more than 90% of the activity in the serum was found to be present as free MTX except in the case of the diethyl ester, for which hydrolysis was 81% complete at this time. At the later times, more than 99% of the serum MTX activity was present as the non-esterified parent compound.

Discussion. These studies indicate that while di-*n*-alkyl esters of MTX exhibit significant antitumor activity in the mouse, this activity is due to rapid hydrolysis to the parent drug. A similar conclusion was reached by EISENFELD et al.[5] in studies with the dimethyl ester of MTX. Extension of these studies to other mammalian species would however be of interest: comparative studies have indicated that plasma esterase levels in the mouse are significantly higher than in larger species such as monkey, dog, and man[6], and the possibility therefore exists that in the latter species the esters would persist for a sufficient length of time to reach tumor sites inaccessible to the lipid-insoluble parent drug. Replacement of the ester groups of the present series with chemical structures having equivalent lipid solubility, but resistant to biological hydrolysis, would also appear to offer promise[7].

Résumé. L'action biologique de 6 esters liposolubles di-*n*-alkyle de la méthotrexate (MTX) a été comparée à celle du composé non estérifié. En tant qu'inhibiteurs de la réductase dihydrofolate de cellules leucémiques L1210 de la souris, les esters ont été moins efficaces que la MTX. In vivo, les esters dialkyles ont eu une action égale à celle de la MTX, en prolongeant la survie des souris inoculées par l'injection de cellules leucémiques L1210. Puisque les esters sont rapidement hydrolysés in vivo on conclut que l'action antitumeur chez la souris est due à la libération de la MTX.

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The Effect of Quinidine *bis*-(7-Theophylline Acetate) on the Isolated Papillary Muscle of the Rabbit Heart

In the previous papers, a combination of diphenhydramine and theophylline (dimenhydrinate) was found to have a significant antiarrhythmic action[1,2] with none of the undesirable effects of diphenhydramine alone[3]. These observation caused the authors of the present comminication to investigate the properties of a chemical combination of theophylline and quinidine[4,5].

Methods. Quinidine *bis*-(7-theophylline acetate) (QTA) has been obtained by refluxing quinidine (0.01 *M*) and theophylline-7-acetic acid (0.02 *M*) in ethanol solution. After recrystallisation from ethanol, it melted at 201–203 °C, lit. m.p. being 203–207 °C[6].

The experiments were performed on the papillary muscles isolated from the right ventricle of the rabbit heart, perfused with oxygenated warm (35 °C) Tyrode's solution. The preparations were driven with rectangular, double-threshold pulses at a rate of 1 Hz. The electrical activity was recorded by means of intracellular microelectrodes, and the contractions of the preparations with a transducer RCA 5734. The recordings were taken before and 10–15 min after administration of QTA in a concentration of 5 mg $\times 1^{-1}$.

Results and discussion. The results are shown in the Table and the Figure. As results from Table and Figure, QTA practically does not affect either the amplitude of the resting and action potential or the rapid upstroke in the ventricular fibres. Thus, it cannot cause any significant change in the velocity of the impulses propagation. On the contrary, quinidine alone markedly slows down both the rapid upstroke and the velocity of propagation[7–9]. Prolongation of the refractory period after

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Investigated parameters in control conditions and after QTA administration

	Resting potential	Action potential			Effective	Mechanograms	
	(mV)	Amplitude (mV)	Duration of phase 0 (msec)	Duration of AP (msec)	refractory period period (msec)	Amplitude (%)	Duration (msec)
Control	87 ± 6.3	123 ± 8.5	1.9 ± 0.08	220 ± 16.7	170 ± 12.5	100	300 ± 10.6
QTA (5 mg/l	90 ± 4.1	121 ± 5.5	1.9 ± 0.1	310 ± 21.2	280 ± 17.2	98 ± 11.8	380 ± 15.5