Biochemical Pharmacology, Vol. 40, No. 4, pp. 851–857, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

INFLUENCE OF LIPOPHILICITY AND CARBOXYL GROUP CONTENT ON THE RATE OF HYDROXYLATION OF METHOTREXATE DERIVATIVES BY ALDEHYDE OXIDASE

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(*Received* 22 *September* 1989; *accepted* 18 *January* 1990)

Abstract—The influence of lipophilicity and carboxyl group content on the ability of methotrexate (MTX) derivatives to undergo 7-hydroxylation *in vitro* by partly purified rabbit hepatic aldehyde oxidase was examined. Addition of two to four γ-glutamyl residues to the MTX molecule caused a progressive decrease in the rate of hydroxylation associated mainly with a decrease in V_{max} rather than an increase in K_m. These results suggest that the number of carboxyl groups in the side chain has a relatively small effect on affinity for the enzyme active site, but hinders the formation of product. The catalytic efficiency of hydroxylation of MTX tetraglutamate, estimated from V_{max}/K_m ratios, was 36-fold lower than that of the monoglutamate. In contrast, when the number of carboxyl groups was decreased to one, as in 4-amino-4-deoxy-N^{10}-methylpteroic acid, N^{α}-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-lysine, and γ-*t*-butyl-3′-chloromethotexate, enhanced catalytic efficiency was observed, involving both a decrease in K_m and an increase in V_{max}. The catalytic efficiency of hydroxylation of these three substrates was 88-, 360- and 2100-fold higher than that of MTX. γ-*t*-Butyl-3′-chloromethotrexate was a better substrate than γ-*t*-butyl-MTX, demonstrating the strong contribution of a lipophilic Cl atom on the phenyl ring. N^{α}-(4-Amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine, with two carboxyl groups, showed substrate activity similar to that of MTX. The γ-*t*-butyl esters of MTX, 3′-chloromethotrexate, and 3′,5′-dichloromethotrexate were compared with the parent acids as inhibitors of the growth of cultured human leukemic lymphoblasts (CEM cells) and an MTX-resistant subline (CEM/MTX) defective in MTX transport and polyglutamylation. Although the esters were less effective than the acids against CEM cells except at high concentrations, they were more effective against CEM/MTX cells. This "collateral sensitivity" of CEM/MTX cells to lipophilic MTX esters is consistent with a decreased ability to take up and utilize reduced folates from the culture medium.

Hepatic aldehyde oxidase (aldehyde:O_2 oxidoreductase, EC 1.2.3.1) is a metalloflavin enzyme that contains both iron and molybdenum and accepts a variety of heterocyclic ring systems as substrates [1]. While no obvious physiological role has been identified for this enzyme, it resembles xanthine oxidase in its ability to accept as substrates a variety of N-heterocyclic ring systems with unsubstituted carbon adjacent to nitrogen [2]. The excellent substrate activities of the classical folate antagonists methotrexate (MTX§), aminopterin, and 3′,5′-dichloromethotrexate have long been known [3]. Their hydroxylation by aldehyde oxidase constitutes a detoxification route, since the resulting 7-hydroxy derivatives are less effective than the parent compounds as inhibitors of dihydrofolate reductase [4]. On the other hand, 7-hydroxy-MTX is converted *in vitro* [5–7] and in cultured cells [8] to polyglutamate derivatives that cannot be overlooked as possible contributors to the overall biochemical action of MTX [9–13].

Despite its importance as a detoxification mechanism for MTX, relatively little is known about the role of 7-hydroxylation in the pharmacology of other antifolates. Attempts to correlate molecular structure with hepatic aldehyde oxidase substrate activity among classical antifolates have focused previously on the favourable influence of lipophilicity. Thus, 3′,5′-dichloromethotrexate, which is more lipophilic than MTX because of the two Cl atoms on the phenyl ring, is also a better substrate than MTX [3]. Moreover, the very lipophilic diesters of MTX and 3,5-dichloromethotrextate are better substrates than the parent acids [14]. On the other hand, aminopterin, which is somewhat less lipophilic than MTX because it lacks a methyl group at N^{10}, is a poorer substrate for the rabbit enzyme [3, 15] but a better substrate for the guinea pig enzyme [3]. In the only other published studies on MTX analogues, the γ-*t*-butyl ester of MTX [16] and the lysine analogue of MTX [17] were both better substrates than MTX. From the data accumulated to date, therefore, it appears that 7-hydroxylation is sensitive not only to

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§ Abbreviations: MTX or MTX-G_1, 4-amino-4-deoxy-N^{10}-methylpteroyl-L-glutamate; MTX-G_2, *N*-[*N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-γ-glutamyl]-L-glutamate; MTX-G_3, *N*-[*N*-[*N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-γ-glutamyl]-L-γ-glutamyl]-L-glutamate; MTX-G_4, *N*-[*N*-[*N*-[*N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-γ-glutamyl]-L-γ-glutamyl]-γ-L-glutamyl]-L-glutamate; FPGS, folypolyglutamate synthetase; mAPA, 4-amino-4-deoxy-N^{10}-methylpteroic acid; mAPA-Lys, N^{α}-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-lysine; APA-Hemiphth-Orn, N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine; DME, Dulbecco's modified essential medium; and FBS, fetal bovine serum.

the overall lipophilicity of the antifolate but also to the functionality in the amino acid side chain.

Polyglutamates of 7-hydroxy-MTX can, in principle, arise in cells either by polyglutamylation of 7-hydroxy-MTX or by 7-hydroxylation of MTX polyglutamates. The present study was undertaken with the aim of comparing the abilities of MTX polyglutamates to undergo hydroxylation *in vitro* by rabbit liver aldehyde oxidase. Our results indicate that successive introduction of one to three γ-glutamyl residues in MTX causes a progressive decrease in the hydroxylation rate, and that this is due mainly to an effect on V_{max} rather than K_m in the enzyme-catalyzed reaction. This suggests that increasing the number of carboxyl groups in the side-chain, and hence the negative charge, has relatively little influence on binding to the active site but substantially hinders progress through the transition state to give product.

We also report the synthesis of γ-*t*-butyl-3′-chloromethotrexate, a previously undescribed MTX analogue in which lipophilicity and decreased negative charge are combined. This compound, like the 3′,5′-dichloro analogue we described earlier [16] and for which an improved synthesis is presented here, was more active *in vitro* than γ-*t*-butyl-MTX against MTX-resistant human lymphoblasts (CEM/MTX cells) with a transport defect. It was also a superb substrate for hepatic aldehyde oxidase, and thus would be expected to undergo detoxification more rapidly than MTX or γ-*t*-butyl-MTX *in vivo*. The efficiency of oxidation of γ-*t*-butyl-3′-chloromethotrexate, estimated from K_m/V_{max} ratios, was three orders of magnitude greater than that of MTX.

MATERIALS AND METHODS

Materials. MTX was obtained as the disodium salt from the National Cancer Institute, and its purity was determined to be greater than 98% by HPLC. γ-*t*-Butyl-MTX [16], 4-amino-4-deoxy-N^{10}-methylpteroic acid (mAPA) [18], N^{α}-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-lysine (mAPA-Lys) [19], and N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine (APA-Hemiphth-Orn) [20] were available in our laboratory from previous work. 3′-Chloromethotrexate and 3′,5′-dichloromethotrexate were prepared as previously reported [21]. *t*-Butyl hypochlorite for the synthesis of γ-*t*-butyl-3′-chloromethotrexate and γ-*t*-butyl-3′,5′-dichloromethotrexate from γ-*t*-butyl MTX was freshly prepared by the method of Mintz and Walling [22], and used without distillation. Sodium iron (III) ethylenediaminetetraacetate was obtained from the Sigma Chemical Co. (St. Louis, MO). TLC was performed on Whatman MK6F silica gel plates using the following chloroform–methanol–acetic acid mixtures as eluting solvents: 10:5:1 (system 1), 20:5:1 (system 2); and 40:9:1 (system 3). MTX polyglutamates containing two to four glutamate groups (MTX-G_2, MTX-G_3, and MTX-G_4) were obtained from Dr. M. G. Nair (University of South Alabama, Mobile, AL) and were purified by small-scale preparative HPLC prior to use. This purification step was essential, because non-purified polyglutamates appeared to contain an unknown impurity (or impurities) that either blocked hydroxylation entirely, or caused the kinetics of hydroxylation to be highly variable. The method of HPLC purification was similar to the one used earlier to separate MTX polyglutamates in CEM cell lysates [16]. Briefly, each compound was chromatographed on a Waters Novapak C_{18} radial compression cartridge (0.5 cm i.d. × 10 cm long, 5 μm particle size), by elution with 2% acetonitrile in 0.1 M ammonium acetate, pH 6.0, at a rate of 0.7 mL/min for 10 min followed by 2.0 mL/min for 30 min. The elution times were 40 min (MTX-G_2), 35 min (MTX-G_3), and 30 min (MTX-G_4). The elution time for each 7-hydroxy-MTX polyglutamate was slightly shorter than for the corresponding MTX polyglutamate. Non-polyglutamylated MTX (i.e. MTX-G_1) and 7-hydroxy-MTX required a larger amount of acetonitrile (4%) in order to be eluted at 35–40 min.

Enzyme kinetics. Hepatic aldehyde oxidase was partially purified from 200 g of fresh liver from New Zealand white rabbits. The livers were perfused with ice-cold 0.9% NaCl, and frozen at −80° until used. A procedure based on that of Johns and Loo [23] was used to obtain 12-fold purified enzyme with an overall recovery of 74%. The preparation yielded 1.8 units of enzyme with a specific activity of 6.6 nmol/mg/min, one unit being defined as the amount to catalyze the hydroxylation of 50 μM MTX at a rate of 1 mol/min. Protein concentration was determined by the Bradford assay [24]. The enzyme was stored at −70°, and its specific activity was checked prior to every series of assays. Enzyme-catalyzed hydroxylation reactions were carried out at 25° in a covered quartz cuvette but without rigorous exclusion of air. Reactions were performed in 0.1 M sodium phosphate buffer, pH 7.8, containing 0.1 mM ammonium chloride and 0.2 mM sodium iron (III) ethylenediaminetetraacetate. Substrates were dissolved in 950–990 μL of the reaction buffer at various concentrations, and the reaction was initiated by adding 10–50 μL of the enzyme solution (8.3 mg protein/mL), giving a total volume of 1 mL. The reaction was allowed to proceed until no further change in absorbance at 340 nm occurred (generally 1 hr or less), and the change over the linear part of the progress curve was plotted as a function of time. Product analysis by HPLC indicated >97% substrate disappearance except for MTX-G_2 to MTX-G_4, which showed decreasing yields of hydroxylated product at the plateau of the progress curve. Absence of conjugase activity in the enzyme solution was established from the fact that treatment of authentic MTX-G_2 or MTX-G_3 with the enzyme gave no HPLC-detectable MTX. Reaction velocities were determined from the linear part of the curve, and the kinetic parameters K_m and V_{max} were determined from standard Lineweaver–Burk plots and expressed as the mean ± standard deviation for replicate experiments performed on different days (see Table 2).

Synthesis of γ-t-butyl-3′-chloromethotrexate. t-Butyl hypochlorite (0.05 mL, 0.045 g, 0.41 mmol) was added dropwise to a stirred solution of γ-*t*-butyl-MTX (0.18 g, 0.36 mmol) in glacial acetic acid (2.5 mL) and methylene chloride (10 mL), and the reaction mixture left to stir at room temperature

overnight. A precipitate formed, which was filtered and dried *in vacuo* for 2 hr to obtain a light yellow powder (0.16 g, 84%). HPLC analysis showed this material to contain 0.2% of unchanged starting material, 2% of γ-*t*-butyl-3′-5′-dichloromethotrexate, and no detectable amount of either MTX, 3′,5′-dichloromethotrexate, or 3′-chloromethotrexate. For microanalysis, a 25-mg portion of the product was reprecipitated from 1 mL of 0.05 M ammonium hydroxide with 50 μL of 20% (v/v) glacial acetic acid, then filtered, washed with 5 mL of distilled water, and dried overnight in a lyophilizer; m.p. 175–181°. TLC: R_f 0.70 (system 1), 0.47 (system 2), and 0.28 (system 3) versus 0.65, 0.42 and 0.23, respectively, for γ-*t*-butyl MTX. HPLC: elution time 8 min versus 5 min for γ-*t*-butyl MTX (25% acetonitrile in 0.1 M ammonium acetate, pH 6). UV: λ_{max} (0.1 N NaOH) 224 nm (ε 19,800), 260 (27,300), 360 (6,700). Anal. calcd. for $C_{24}H_{29}ClN_8O \cdot H_2O$: C, 51.19; H, 5.56; N, 19.90. Found: C, 51,25; H, 5.43; N, 20.29.

Synthesis of γ-t-butyl-3′,5′-dichloromethotrexate. *t*-Butyl hypochlorite (0.18 mL, 1.5 mmol) was added to a solution of γ-*t*-butyl-MTX (0.27 g, 0.5 mmol) in 10 mL of a 1:1 methanol–glacial acetic acid at 0–4°, and the stirred mixture was allowed to come to room temperature. After 1 hr, it was evaporated nearly to dryness on a rotary evaporator. After addition of 4:1 ether–hexane (25 mL), a precipitate formed. Stirring was continued overnight, and the solid was collected, washed with 4:1 ether–hexane (10 mL), and dried *in vacuo* at 50° for 3 days to obtain a light-yellow powder (0.27 g, 87% yield). For microanalysis, a sample of the product was recrystallized from aqueous ethanol and dried *in vacuo* at 50° overnight; dec >185°. TLC: R_f 0.82 (system 1), 0.56 (system 2), and 0.38 (system 3). HPLC: elution time 14 min (25% acetonitrile in 0.1 M ammonium acetate, pH 6). UV: λ_{max} (0.1 N NaOH) 224 nm (ε 14,800), 259 (26,500), 360 (7,400). Anal. calcd. for $C_{24}H_{28}N_8Cl_2O_5 \cdot H_2O$: C, 48.25; H, 5.06; Cl, 11.87; N, 18.76. Found: C, 47.94; H, 4.77; Cl, 10.32; N, 18.95.

Cell growth inhibition assays. Human leukemic lymphoblasts (CEM and CEM/MTX cells) were grown in Dulbecco's modified Eagle's (DME) medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin. An initial inoculum of 5×10^5 cells/mL was used, and the Trypan blue excluding cells were counted in a hemocytometer after incubation at 37° in an 8% CO_2 humidified atmosphere for 72 hr (2–3 doublings) in the presence of 10^{-2} to 10^{-8} M drug. Survival was calculated as a percent of control (no drug). Replicate experiments were performed, and the mean percent survival at each drug concentration was determined. Drug stability in the growth medium was confirmed by HPLC.

RESULTS

Kinetics of 7-hydroxylation of MTX polyglutamates and other substrates. As indicated in Table 1, MTX was hydroxylated at pH 7.5 by rabbit liver aldehyde oxidase with a K_m of 189 ± 58 μM and a V_{max} of 30 ± 9 nmol/min/mg protein, whereas for MTX-G_2, these values were 230 ± 63 μM and

Table 1. Kinetics of hydroxylation of MTX polyglutamates and MTX analogues by rabbit hepatic aldehyde oxidase

Substrate*	Concn range (μM)	Molar absorbance change† (au/M/cm)	N‡	K_m§ (μM)	V_{max}§ (nmol/min/mg protein)	V_{max}/K_m‖ (relative)
MTX (MTX-G_1)	25–100	8,800	5	189 ± 58	30 ± 9	1.0
MTX-G_2	25–100	8,800	3	230 ± 63	10 ± 4	0.27
MTX-G_3	20–100	8,800	3	120 ± 49	2.7 ± 0.9	0.14
MTX-G_4	25–60	8,800	3	150 ± 70	0.67 ± 0.22	0.028
mAPA	5–25	8,900	2	37 ± 0.9	527 ± 23	88
γ-*t*-Butyl-MTX	2.5–50	9,700	5	24 ± 0.4	240 ± 11	63
γ-*t*-Butyl-3′-Cl-MTX	1–5	10,000	3	2.4 ± 0.3	821 ± 25	2100
mAPA-Lys	5–25	9,000	1	8.5	489	360
APA-Hemiphth-Orn	20–100	8,000	3	183 ± 54	19 ± 4.6	0.63

* The n in MTX-G_n is the total of glutamyl residues; mAPA: 4-amino-4-deoxy-N^{10}-methylpteroic acid; mAPA-Lys: N^{α}-(4-amino-4-deoxy-N^{10}-methylpteroyl-L-lysine; APA-Hemiphth-Orn: N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine.
† Values were determined spectrophotometrically.
‡ N is the number of separate experiments.
§ Values are expressed as means ± standard deviations for N individual determinations when N is greater than 2, and as the mean ± range when N = 2.
‖ V_{max}/K_m ratios are normalized relative to that for MTX, which is assigned arbitrarily a value of 1.0. Data for γ-*t*-butyl MTX are from Ref. 16.

10 ± 4 nmol/min/mg protein, respectively. Introduction of a second glutamate residue did not alter K_m appreciably, but decreased V_{max}. The K_m values for MTX-G_3 and MTX-G_4 were marginally lower than those of MTX and MTX-G_2, but the V_{max} values were decreased to 2.7 ± 0.9 and 0.67 ± 0.22 nmol/min/mg protein respectively. Introduction of each additional glutamate residue led to a further decrease in V_{max}. 4-Amino-4-deoxy-N^{10}-methylpteroic acid (mAPA) gave K_m and V_{max} values of 37 ± 0.9 μM and 527 ± 23 nmol/min/mg protein respectively. Replacement of the glutamate side chain by a carboxyl group, in contrast to the addition of extra glutamate residues, caused a decrease in K_m as well as an increased in V_{max}. Esterification of the γ-carboxyl of MTX had a similar effect; thus, γ-*t*-butyl-MTX had a K_m of 24 ± 0.4 μM, which was lower than that of MTX, and a V_{max} of 240 ± 11 nmol/min/mg protein which was higher than that of MTX.

The K_m and V_{max} values for mAPA-Lys, which contains one positively charged and one negatively charged group in the side chain at pH 7.5, were 8.5 μM and 489 nmol/min/mg protein respectively. This corresponded to a very substantial decrease in K_m relative to MTX, along with an increase in V_{max}.

N^{δ}- (4 -Amino- 4 -deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine gave a K_m of 183 ± 54 μM and a V_{max} of 19 ± 4.6 nmol/min/mg protein, and was essentially identical to MTX in its ability to undergo hydroxylation despite the greater distance between the two carboxyl groups in the molecule and the additional lipophilicity contributed by the $NHCOC_6H_4$ moiety.

Finally, γ-*t*-butyl-3′-chloromethotrexate, with one carboxyl group in the side chain and a lipophilic Cl atom on the phenyl ring, had a K_m of 2.4 ± 0.3 μM and a V_{max} of 821 ± 25 nmol/min/mg protein. The K_m for this compound was lower than the values for γ-*t*-butyl MTX and MTX, whereas the V_{max} was greater. Efforts to determine the kinetics of the reaction with γ-*t*-butyl-3′,5′-dichloromethotrexate were unsuccessful because of its low solubility.

Cytotoxicity of γ-t-*butyl-3′-chloromethotrexate.* As shown in Table 2, the growth of MTX-sensitive CEM cells was inhibited to an approximately equal extent by MTX and its 3′-chloro and 3′,5′-dichloro derivatives, which all had IC_{50} values in the range 10^{-7}–10^{-8} M. Almost complete cross-resistance among these compounds was observed in the MTX-resistant CEM/MTX subline. Exposure of the CEM/MTX cells to 10^{-6} M MTX for 72 hr led to 99% survival relative to controls, whereas on the same treatment with the 3′-chloro and 3′,5′-dichloro compounds the survival was 89 and 90% respectively.

γ-*t*-Butyl-MTX was less effective than MTX against CEM cells until the drug concentration reached 10^{-4} M, at which point the ester and acid became comparable in their effect on growth. The survival of CEM cells treated with 10^{-6} M γ-*t*-butyl-MTX was 65% relative to controls, whereas the same treatment with the γ-*t*-butyl esters of 3′-chloromethotrexate and 3′,5′-dichloromethotrexate afforded only 40 and 35% survival respectively. Treatment of CEM/MTX cells with 10^{-6} M γ-*t*-butyl-MTX led to 92% survival, compared with 65% in the parental line; however, when the same concentration of the mono- and dichloro ester was used, the survival of CEM/MTX cells was only 44 and 29% respectively. At a concentration of the mono- and dichloro esters of 10^{-5} M, survival of the CEM/MTX cells relative to controls was 10 and 5.2% respectively, whereas for the parental lines these values were 32 and 21%. Similar "collateral sensitivity" was observed at higher concentrations, with 10^{-3} M γ-*t*-butyl-3′-chloromethotrexate giving the greatest difference in survival, namely 29% with CEM cells and 1.4% with CEM/MTX cells. When CEM/MTX cells were exposed to γ-*t*-butyl-3′-chloromethotrexate for 72 hr at a concentration of 10^{-2} M, the surviving fraction was only 0.85%.

DISCUSSION

The results shown in Table 1 for the kinetics of MTX hydroxylation by rabbit hepatic aldehyde oxidase confirm the previous results of Johns *et al.* [14], who reported a K_m of 190 μM, and are also in agreement with the K_m of 164 μM reported by McGuire *et al.* [17] using partly purified enzyme obtained in the same manner. Good agreement also exists between our results with mAPA-Lys and the K_m of 9 μM which these authors reported [17]. Our K_m for mAPA, on the other hand, is about 6-fold lower than the value reported by Valerino and associates [25]. The substantial decrease in K_m which we observed for both mAPA and mAPA-Lys is consistent with the idea that a relationship exists between the ionic charge on a molecule and its ability to bind to the active site of aldehyde oxidase. The side chain in MTX contains two ionized carboxyl groups, giving a net charge of −2 at pH 7.5, whereas for mAPA and mAPA-Lys the net charges at this pH are −1 and 0, respectively. The relative catalytic efficiencies of mAPA and mAPA-Lys, as estimated from V_{max}/K_m ratios, were 88- and 360-fold higher than that of MTX. Thus, a decrease in negative charge from −2 to −1, with all other parts of the structure held constant, produced an increase in hydroxylation efficiency at the 7-position of the pteridine ring of almost two orders of magnitude, whereas a further change in the charge from −1 to 0 produced an additional increase in catalytic efficiency which was smaller but not insignificant. It is of interest to note that Johns *et al.* [14] found 3′,5′-dichloromethotrexate to have a K_m of 36 μM and a V_{max} of 160 nmol/min/mg protein, giving a V_{max}/K_m ratio 65-fold greater than that of MTX. Since 3′,5′-dichloromethotrexate has two carboxyl groups, the increased catalytic efficiency of this compound relative to MTX must reflect an important contribution by the lipophilic chloro substituents on the phenyl ring. However, our data with mAPA, mAPA-Lys, and γ-*t*-butyl-MTX, none of which contain aromatic Cl substituents, indicate that the elimination of one carboxyl group is at least as important as the addition of two Cl atoms where rabbit hepatic aldehyde oxidase is concerned. More detailed studies to compare, for example, the ease of 7-hydroxylation of γ-esters versus α-esters, and the ability of human versus rabbit enzyme to hydroxylate a given substrate, would be of interest.

Table 2. Inhibition of CEM and CEM/MTX cell growth by γ-*t*-butyl-3′-chloromethotrexate and other MTX analogues

Compound	Cell line	Cell survival* (% of control) Drug concn (M) 10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
MTX	CEM	82 ± 26	40 ± 18	30 ± 15	30 ± 9	30 ± 13
	CEM/MTX	96 ± 0.1	106 ± 16	99 ± 11	42 ± 22	32 ± 21
3′-Cl-MTX	CEM	79 ± 22	43 ± 14	46 ± 7	36 ± 5	31 ± 2
	CEM/MTX	102 ± 2	96 ± 23	89 ± 7	30 ± 12	20 ± 12
3′,5′-Cl_2-MTX	CEM	89 ± 54	47 ± 11	32 ± 8	33 ± 8	33 ± 6
	CEM/MTX	102 ± 20	104 ± 28	90 ± 39	28 ± 2	24 ± 9
γ-*t*-Butyl-MTX	CEM	102 ± 7	110 ± 4	65 ± 14	23 ± 7	18 ± 4†
	CEM/MTX	95 ± 10	93 ± 21	92 ± 16	22 ± 0.14	6.6 ± 0.01†
γ-*t*-Butyl-3′-Cl-MTX	CEM	97 ± 0	86 ± 19	40 ± 18	32 ± 17	22 ± 12‡
	CEM/MTX	86 ± 7	83 ± 19	44 ± 47	10 ± 11	3 ± 4‡
γ-*t*-Butyl-3′,5′-Cl_2-MTX	CEM	106 ± 0	92 ± 9	35 ± 2	21 ± 3	19 ± 3§
	CEM/MTX	80 ± 11	86 ± 2	29 ± 5	5.2 ± 0.8	3.2 ± 0.9§

* Assay conditions are described in Materials and Methods. Untreated CEM controls grew from 0.58 ± 0.04 × 10^6/mL to 1.1 ± 0.2 × 10^6/mL and untreated CEM/MTX controls from 0.55 ± 0.09 × 10^6/mL to 5.4 ± 3.0 × 10^6/mL over 72 hr, in four experiments on different days. Survival is expressed as the mean of control survival percentage ± range for two experiments on different days.

† At a concentration of 10^{-3} M the surviving fraction relative to controls was 21% for CEM cells and 4.6% for CEM/MTX cells (single determination).

‡ At a concentration of 10^{-3} M the surviving fraction relative to controls was 29 ± 11% for CEM cells and 1.4 ± 0.8% for CEM/MTX cells; at a concentration of 10^{-2} M the corresponding values were 12 ± 12 and 0.85 ± 0.92%.

§ Poor solubility prevented γ-*t*-butyl-3′,5′-Cl_2-MTX from being tested at a concentration greater than 10^{-4} M.

The results presented in Table 1 also reveal an apparent relationship between hydroxylation efficiency and carboxyl groups among the MTX polyglutamates. Thus, the V_{max}/K_m values for MTX, MTX-G_2, MTX-G_3, and MTX-G_4 were progressively *lower* as the total number of carboxyl groups in the side chain increased from two to five. The increase in catalytic efficiency was 4-fold in going from MTX to MTX-G_2, 2-fold in going from MTX-G_2 to MTX-G_3, and 5-fold in going from MTX-G_3 to MTX-G_4, and was inversely proportional to the number of carboxyl groups per molecule. MTX-G_4 was 36-fold less efficient than MTX in undergoing 7-hydroxylation. It is interesting to note that an inverse relationship has also been found between the number of glutamyl residues in MTX polyglutamates and the relative V_{max}/K_m for further polyglutamylation by beef liver [6] and human [26] FPGS. From these results it would appear that the chain length of 7-hydroxy-MTX polyglutamates in liver cells is controlled by differences in catalytic efficiency not only during the polyglutamylation of MTX and 7-hydroxy-MTX by FPGS, but also during the hydroxylation of MTX and MTX polyglutamates by hepatic aldehyde oxidase.

Our Michaelis constant for the 7-hydroxylation of MTX (K_m = 189 μM) is considerably different from that of Fabre *et al.* [15], who found MTX to have a K_m of 34.5 μM. These authors also reported a K_m value of 9.6 μM for 3′,5′-dichloromethotrexate. The K_m values obtained earlier by Johns *et al.* [14] for MTX and 3′,5′-dichloromethotrexate were 190 and 36 μM respectively. It should be noted, however, that Fabre and coworkers used an enzyme that had been subjected to chromatography on hydroxylapatite followed by Sephacryl S-200. This resulted in a 120-fold purification relative to the activity in crude supernatant fraction, whereas Johns and coworkers had used enzyme purified 73-fold and our experiments were performed with enzyme purified only 12-fold. It is possible that the more rigorous purification scheme employed by Fabre and coworkers activated their enzyme, or else removed an endogenous inhibitor.

In addition to an apparent increase in catalytic activity toward MTX and 3′,5′-dichloromethotrexate, the enzyme used by Fabre *et al.* [15] differed from ours in its interaction with MTX polyglutamates. Thus, while they observed a progressive increase in the K_m from 49.3 to 1627 μM in going from the monoglutamate to the tetraglutamate suggesting decreased enzyme binding with increasing chain length, our K_m values for these substrates varied only slightly. Moreover, while these authors found that the V_{max} for the reaction was much lower for MTX-G_2 and MTX-G_3 than for MTX, and that MTX-G_4 and MTX had the same V_{max}, we observed a strikingly regular 3- to 4-fold decline in V_{max} for every added glutamate residue (each of which contributes one extra negative charge and two lipophilic CH_2 groups). The reason for the quantitative difference between our results and those of Fabre and coworkers is not clear. However, our data suggest that the catalytic efficiency of 7-hydroxylation of MTX polyglutamates by rabbit hepatic aldehyde oxidase depends less on the binding affinity, as deduced from the relatively constant K_m values of the homologues, than on the rate of passage through the transition state.

The growth inhibitory activity of γ-*t*-butyl-3′-chloromethotrexate and γ-*t*-butyl-3′,5′-dichloromethotrexate against the MTX-resistant CEM/MTX cells (Table 2) is consistent with our earlier results with other esters including γ-*t*-butyl-MTX [16], and supports the idea that transport-based MTX resistance

can be overcome with lipophilic MTX derivatives [27]. The greater activity of these monochloro and dichloro esters in comparison with γ-*t*-butyl-MTX presumably reflects the contribution of the lipophilic Cl atom. Since 3′-chloromethotrexate and 3′,5′-dichloromethotrextate themselves were only slightly better than MTX as inhibitors of the CEM/MTX cells, we conclude that γ-esterification is more effective than halogenation in enhancing uptake and overcoming transport-based resistance.

CEM-MTX cells have been shown previously to have normal dihydrofolate reductase levels, as measured by [^{3}H]MTX binding in cell lysates, but a profound defect in the ability to take up and polyglutamylate MTX [16, 27]. Since reduced folates and MTX share a common transport pathway, CEM/MTX cells presumably are less efficient than the parent line in taking up reduced folates from the growth medium. The "collateral sensitivity" of these cells to γ-*t*-butyl-MTX, γ-*t*-butyl-3′-chloromethotrexate and γ-*t*-butyl-3′,5′-dichloromethotrexate, which has been noted previously with the lipophilic di-*n*-butyl and γ-*n*-butyl esters of MTX [27], may be due to impaired ability to utilize reduced folates in comparison with wild-type CEM cells.

The inability of MTX, 3′-chloromethotrexate, and 3′,5′-dichloromethotrexate to produce >70% inhibition of growth of the parental CEM cells, even after 72 hr of exposure to 10^{-4} M drug has been noted for MTX by earlier investigators [28]. The ability of a fraction of these cells to survive in the presence of such high MTX concentrations appears not to reflect generalized drug resistance, cellular heterogeneity, or the presence of debris, since (a) this phenomenon was not observed with Adriamycin®, vincristine, mitomycin C, or nor-nitrogen mustard, and (b) several subclones of the original CEM cell line were found to have the same property (data not shown). The most likely explanation for these results is that the cells were grown in DME medium, which contains 2.2 μM folic acid, and that the medium was supplemented with FBS which was not dialyzed and therefore contained 5-methyltetrahydrofolate as well as salvageable purines and pyrimidines.

Efficient 7-hydroxylation of 3′,5′-dichloromethotrexate in comparison with MTX is generally considered to be responsible for the fact that a much higher dose of this compound has to be given to obtain a therapeutic effect in mice [29] and humans [for a review of recent clinical literature on 3′,5′-dichloromethotrexate, see Ref. 30]. Since γ-*t*-butyl-3′-chloromethotrexate is a much better hydroxylation substrate than 3′,5′-dichloromethotrexate, one would expect it to have very low toxicity *in vivo*. Whether this will result in an improved therapeutic index, however, remains to be determined. A more promising analogue for overcoming resistance may be N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine, which is also superior to MTX against CEM/MTX cells [20], but, in contrast to the *t*-butyl esters, is less efficiently detoxified than MTX by hydroxylation. *In vivo* studies comparing γ-*t*-butyl-3′-chloromethotrexate and N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine are now in progress.

Acknowledgements—This work was supported in part by Grants PO1-CA19589 and RO1-CA25394 from the National Cancer Institute (DHHS). The authors are grateful to Ms. Dorothy Trites and Ms. Carol A. Cucchi for their expert technical assistance.

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