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METABOLISM OF METHOTREXATE AND γ-*tert*-BUTYL METHOTREXATE BY HUMAN LEUKEMIC CELLS IN CULTURE AND BY HEPATIC ALDEHYDE OXIDASE *IN VITRO*

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Abstract—The cellular uptake and metabolism of methotrexate (MTX) and γ-*tert*-butyl methotrexate (TBM) were compared in CEM human leukemic lymphoblasts and a highly MTX-resistant subline (CEM/MTX) in which MTX uptake is defective. The CEM/MTX cells were found previously to be as sensitive as the parent line to TBM. While MTX was polyglutamylated extensively in the CEM cells, giving abundant levels of non-effluxing conjugates, polyglutamylation in CEM/MTX cells was reduced severely, even after exposure to a high MTX concentration (100 μM) in the medium. This treatment provided free intracellular MTX in >100-fold excess over the dihydrofolate reductase level. In contrast to MTX, the ester TBM was unmetabolized in either cell line. Uptake levels after incubation of CEM and CEM/MTX cells with 2 μM TBM for 24 hr were 17 and 15 pmol/mg protein respectively. Thus, TBM accumulated equally in both cells and was well retained despite the lack of polyglutamylation. These results, together with the previously observed affinity of the drug for dihydrofolate reductase, provide a plausible rationale for the comparable sensitivity of CEM and CEM/MTX cells to TBM. Experiments were also performed to determine the susceptibility of TBM to metabolic detoxification by hepatic aldehyde oxidase. K_m values were 8-fold lower for TBM than for MTX in assays using an enzyme preparation from rabbit liver, and V_{max} values were 8-fold higher. Neither MTX nor TBM was oxidized to its 7-hydroxy derivative in intact CEM or CEM/MTX cells. Because TBM is capable of overcoming at least one of the modalities of MTX resistance, defective polyglutamylation, and may be more efficiently detoxified than MTX by the action of hepatic aldehyde oxidase, it has the potential to be a useful agent for the treatment of MTX-resistant tumors.

Almost four decades after its discovery [1], MTX‖ is still the most widely used antimetabolite in the armamentarium of cancer chemotherapy [2]. Over the years, a complex pattern of transport, metabolism, and receptor binding has been uncovered in order to explain its mechanism of action. Elucidation of the biochemical and genetic pathways leading to antifolate resistance [3] has led to the development of improved dose regimens [4] and more effective combinations of MTX with other drugs [5]. At the same time, there has been an ongoing search for new agents that are less toxic to the host and more active against MTX-resistant cancer cells [6]. Systematic molecular modifications of MTX have uncovered new and useful structure–activity relationships [7]. Consideration of these findings has prompted us to design a series of γ-substituted derivatives of MTX [8, 9]. Because of their increased lipophilicity and decreased free carboxyl content, such compounds should be taken up more readily into the tumor cell to give a higher concentration of tight-binding dihydrofolate reductase inhibitor [10]. These same properties may enhance hepatic detoxification [11].

One of the compounds, γ-*tert*-butyl methotrexate (TBM), is of particular interest. It was prepared via a regiospecific route to keep the α-carboxyl group free, since this is required for high-affinity binding to dihydrofolate reductase [9]. The choice of a *tert*-butyl ester group was based, in part, upon its hydrolytic stability under ordinary physiological conditions, in contrast with primary and secondary esters [12]. Additionally, it seemed reasonable that resistance developed through loss of polyglutamylating activity should not affect a hydrolytically stable γ-blocked derivative.

The ability of TBM to overcome MTX resistance was tested in an appropriate cell culture model. MTX-sensitive controls in this system consisted of CEM human T-cell leukemic lymphoblasts [13]. A 210-fold MTX-resistant subline, CEM/MTX, was fully sensitive to TBM when compared with the parental control cells in a 48-hr cytotoxicity assay [14].

In earlier reports, we characterized the uptake and

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‖ Abbreviations: MTX, *N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-glutamic acid (methotrexate); MTX-G_n (n = 1 to 5), MTX polyglutamates having n additional glutamyl residues; TBM, γ-*tert*-butyl methotrexate; PIC, paired ion chromatography; LSC, liquid scintillation counting; DME, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; and au, absorbance units.

receptor binding of MTX and TBM in CEM and CEM/MTX cells [15]. As those studies indicated, dihydrofolate reductase overproduction is not an important mode of MTX resistance in CEM/MTX cells, since levels of the enzyme do not differ significantly from those of the parent cells. On the other hand, MTX uptake plateau levels are much lower in the resistant cells, due to more rapid efflux of the drug [15]. Thus, transport is an important determinant of MTX resistance in this model.

In the present studies, the polyglutamylation of MTX [16, 17] in intact CEM and CEM/MTX cells was examined, as well as the possibility of intracellular hydrolysis of TBM to MTX. In addition, the 7-hydroxylation of MTX and TBM was compared, both in intact CEM and CEM/MTX cells and in a cell-free assay using rabbit hepatic aldehyde oxidase.

MATERIALS AND METHODS

Materials

7-Hydroxymethotrexate was obtained from the urine of an adult male cancer patient, following a high-dose MTX treatment and was purified by HPLC. It was characterized by TLC, i.r. and u.v. and found to be identical with a sample prepared by enzymatic oxidation of MTX using rabbit liver aldehyde oxidase.

γ-tert-Butyl 3′,5′-dichloromethotrexate was prepared from 1 mmol of TBM dissolved in 5 ml of glacial acetic acid. The solution was stirred at room temperature, and 4 mmol of *tert*-butyl hypochlorite [18] dissolved in 5 ml of glacial acetic acid was added dropwise over 0.5 hr. After one additional hour, the solution was evaporated under reduced pressure at 55–65°. The residue was purified by column chromatography on 80 g of Baker 60–200 mesh activated silica gel eluted with chloroform–methanol–acetic acid (40:10:1). TLC homogeneous product fractions were pooled and evaporated, and the residue was treated with 150 ml of anhydrous ethyl ether to obtain a yellow precipitate. The solid was washed with hexane and dried under vacuum. Yield: 55%. TLC: R_f 0.69 (Whatman MK6F silica gel, 15:5:1 $CHCl_3$–MeOH–AcOH. Infrared (KBr): ν cm^{-1} 3590–3000, 2990, 1710, 1640, 1600, 1570, 1460, 1420, 1390, 1370, 1000. NMR (3:1 d_6-DMSO–$CDCl_3$) δ ppm 1.4 (s, 9H, Me_3C), 2.3 (m, 6H, $(CH_2)_3$), 2.8 (s, 3H, NMe), 4.5 (s, 2H, NCH_2), 7.9 (s, 2H, aromatic protons), 8.8 (s, 1H, C_7–H). Anal. Calcd for $C_{24}H_{28}N_8Cl_2O_5 \cdot 0.5H_2O$: C, 48.99; H, 4.97; N, 19.04; Cl, 12.05. Found: C, 48.88; H, 4.98; N, 19.08; Cl, 11.88.

[^{3}H]MTX and [^{3}H]TBM were obtained by tritiation of 160 mg of the appropriate 3′,5′-dichloro derivatives of MTX or TBM. The Pt-catalyzed exchange reaction with 25 Ci of tritium gas, removal of excess catalyst and exchangeable tritium, and reoxidation of reduced antifolate were performed by the Tritium Labeling Service, Amersham Corp, Arlington Heights, IL. The crude product was then returned to our laboratory and purified to homogeneity by preparative HPLC. Specific activities were 17–20 Ci/mmol for both compounds. Purified yields: [^{3}H]MTX, 0.5 to 5.0 Ci; [^{3}H]TBM, 0.2 Ci. Analytical HPLC monitoring of radiochemical decomposition and repurification by preparative HPLC at appropriate intervals were undertaken to maintain the purity of both labeled probes.

Cell culture and treatment

Growth cultures were maintained as previously described [14]. Prior to incubation with radiolabeled probes, 6×10^6 cells from either line were pelleted and resuspended in 30 ml of fresh DME medium containing penicillin, 100 units/ml; streptomycin, 0.1 mg/ml; and L-glutamine, 0.3 mg/ml, with 10% fetal bovine serum (FBS). They were incubated for 72 hr (2.5 doublings), pelleted, and resuspended in DME with 10% *dialyzed* FBS, 10 μM thymidine and 100 μM deoxyinosine. Cells were treated with 2 μM [^{3}H]MTX or [^{3}H]TBM for 24 hr or with 100 μM [^{3}H]MTX for 17 hr. They were pelleted and resuspended in 2 ml of ice-cold distilled water in a 30-ml Corex centrifuge tube. The cells were then sonicated on ice with three 10-sec bursts at maximum microprobe power. Microscopic observation of sonicates showed that disruption was complete. Three 50-μl aliquots of the cell suspension were dissolved in 950 μl of 1 M NaOH for the assay of protein by the method of Lowry *et al.* [19].

Polyglutamylation assay

Cytosols were diluted with 6 ml of absolute methanol and kept for 2 hr at −5°. Aliquots of the cytosols (5×5 μl) were taken for LSC to determine total drug uptake. Precipitated proteins were removed by centrifugation at 20,000 *g* for 30 min, and the supernatant fraction was concentrated under a stream of N_2 at 35° on a Pierce Reactivap. The residue was redissolved in 150 μl of HPLC eluent, and a 100-μl portion was injected onto the HPLC column along with a mixture of unlabeled standards (200 nmol each) of MTX, 7-hydroxymethotrexate, MTX polyglutamates G_1 to G_5 and, whenever appropriate, TBM. The column was eluted with a linear gradient of 0–5% CH_3CN in 0.1 M ammonium acetate, pH 6.0, over 70 min at a rate of 1.0 ml/min. In the analysis for TBM, following the 70-min initial run the CH_3CN concentration was increased in a single step to 18% and then elution was continued for another 30 min. This procedure gave excellent separation of TBM, MTX, 7-hydroxymethotrexate, and MTX polyglutamates up to G_5. Since no PIC reagent was used, the conjugates eluted in reverse order of chain length (Fig. 1). Fractions of 0.5 ml each were collected in LSC minivials and counted for 10 min.

TLC separations

Parallel TLC analyses were run on the unused portion of the residue from the HPLC assay. Using 5×20 cm glass plates, lanes were inscribed with a razor blade at 0.5-cm intervals. Twenty nanomoles each of TBM, 7-hydroxymethotrexate, MTX, and MTX polyglutamates G_1 through G_5 were spotted onto the first eight lanes. Onto lane 9 a 2-μl volume of deproteinated cytosol and 20 nmol of each unlabeled standard were spotted. Plates were eluted with $CHCl_3$–CH_3OH–CH_3CO_2H (2:2:1) containing 20 mg/ml of cetyl trimethylammonium bromide. Following development, the plates were placed under

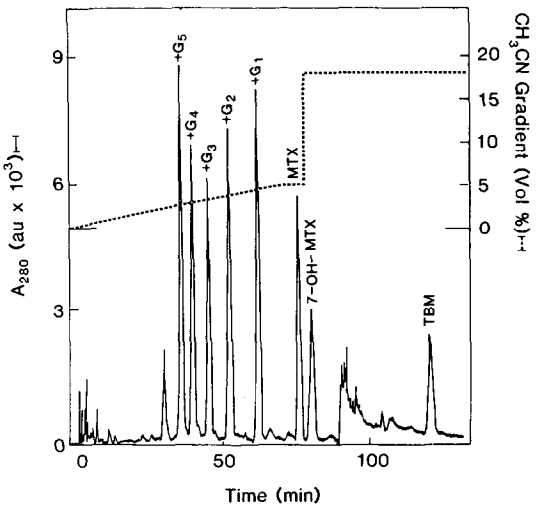

Fig. 1. HPLC separation of TBM, 7-hydroxymethotrexate, MTX and its polyglutamates G_1–G_5.

ordinary fluorescent light for 2 days, rendering the standards intensely fluorescent for visualization under long-wave ultraviolet light. The spots were marked, scraped individually into LSC vials, and counted. Non-radioactive standard spots were removed and counted in the same way to obtain background values. Separation of TBM, 7-hydroxymethotrexate, MTX, and MTX polyglutamates G_1 and G_2 was excellent, whereas the G_3–G_5 conjugates were less clearly resolved.

Dihydrofolate reductase content

[^{3}H]MTX radioligand binding and Lowry protein assays were performed for quantitation of intracellular DHFR as reported previously [19, 20].

Enzyme kinetics

Hepatic aldehyde oxidase was partially purified from 200 g of fresh saline-perfused rabbit essentially as described [21], yielding 1.8 units of enzyme at a specific activity of 6.7 nmol/mg-min. One unit will catalyze the hydroxylation of 50 μM MTX at a rate of 1 μmol/min [22]. The enzyme preparation was diluted to give a final protein concentration of 8.3 mg/ml. Enzyme activity was tested by direct measurement of MTX oxidation rates at 340 nm and by coupling this oxidation to 2,6-dichloroindophenol reduction measured at 600 nm. Preparations stored at 5°, −5°, and −70° showed no loss of activity after 3 months. Inhibition of MTX oxidation by menadione and Triton X-100 was also observed [23, 24].

The concentration dependence of MTX and TBM 7-hydroxylation rates at pH 7.5 was measured directly at 340 nm over a range of 25–100 μM MTX and 2.5–50 μM TBM. Substrates were converted to their sodium salts and stock solutions were prepared in distilled water, rather than 1% ethanol, which was used in the published procedure [11]. Rate calculations were based upon differences in extinction coefficients between substrates and their products obtained from appropriate Beer–Lambert curves over a 0–70 μM concentration range for both substrates. These differences were 8800 and 9700 au/M-cm, respectively, for conversions of MTX and TBM to their 7-hydroxy derivatives, which were complete after 1 hr at 25°. Product analyses by HPLC showed complete disappearance of substrate and formation of a single product eluting 3–4 ml after the corresponding substrate peaks. Kinetic parameters (K_m, V_{max}) were calculated as the mean ± standard deviation of four determinations on different days, all analyzed by the unweighted Lineweaver–Burk method, and did not differ appreciably from values obtained using concentration or velocity squared weightings.

Other procedures

Infrared spectra were obtained on a Perkin–Elmer model 781 spectrophotometer. Ultraviolet and visible spectra were obtained on a Cary model 210 instrument. HPLC separations were performed on a Waters model 400 instrument equipped with a model 660 solvent programmer, model 440 ultraviolet absorbance detector set at 280 nm, and an RCM100 radial compression module with a 0.5 cm i.d. × 10 cm long 5 μm C_{18} column. Cells were sonicated with a Fischer model 300 Dismembrator equipped with a miniprobe. Centrifugation was performed with a Sorvall RC2B instrument using either an SS-34 or a GS-3 rotor, depending upon sample volume.

RESULTS

In CEM cells incubated with 2 μM MTX for 24 hr or with 100 μM MTX for 17 hr, polyglutamylation was extensive, giving conjugates with 3, 4, and 5 total glutamyl residues (MTX-$G_{2,3,4}$) as the predominant intracellular species. These conjugates (MTX-G_1 to MTX-G_5) constituted 93 mol% of total drug at the 2 μM dose and 87 mol% at the 100 μM dose. Total intracellular drug levels were 14 and 190 pmol/mg protein respectively. In CEM/MTX cells, the corresponding proportions of MTX polyglutamates were only 3 and 38 mol%, respectively, based upon total drug uptakes of 6.8 and 84 pmol/mg protein. Uptake of [^{3}H]MTX was always in appreciable excess of the dihydrofolate reductase level, which was 0.45 ± 0.07 pmol/mg protein in the CEM cells and 0.77 ± 0.13 pmol/mg protein in the CEM/MTX cells. In the MTX-resistant cells, therefore, conjugation was reduced considerably and the predominant species was unchanged MTX. No 7-hydroxymethotrexate was found. These results are summarized in Table 1.

CEM and CEM/MTX cells were incubated with 2 μM [^{3}H]TBM for 24 hr, and the total incorporation of radioactivity was measured. TBM levels were 17 and 15 pmol/mg protein in the CEM and CEM/MTX cells, respectively, indicating that the cells do not differ appreciably in their ability to accumulate and retain this drug. Analysis of the cytosols for TBM, MTX, 7-hydroxymethotrexate and MTX polyglutamates revealed that only TBM was present. Thus, TBM is taken up and retained in unaltered form, in amounts slightly greater than the level of

Table 1. Cellular metabolism of MTX

Compound	Intracellular concentration (pmol/mg protein)			
	CEM cells		CEM/MTX cells	
	A*	B	A	B
MTX	0.9 (7)†	24.1 (13)	6.6 (97)	51.8 (62)
MTX-G_1	1.4 (10)	18.5 (10)	0.1 (1)	4.4 (5)
MTX-G_2	4.6 (34)	48.6 (26)	0.07 (1)	11.1 (13)
MTX-G_3	3.7 (27)	46.2 (24)	0.0	8.1 (10)
MTX-G_4	2.8 (21)	46.2 (24)	0.0	6.5 (8)
MTX-G_5	0.2 (1)	5.3 (3)	0.0	1.9 (2)
7-OH-MTX	0.0	0.0	0.0	0.0

* A: 2 μM MTX, 24 hr; B: 100 μM MTX, 17 hr.
† Mol% of total MTX + polyglutamates.

polyglutamylated MTX found in CEM cells under equivalent conditions of drug concentration and incubation time.

Qualitative confirmation of the MTX and TBM metabolism assay data was obtained using a TLC method developed for resolution of MTX and its polyglutamates, 7-hydroxymethotrexate and TBM. Some deviation from the HPLC values occurred with the less thoroughly resolved MTX polyglutamates of greater chain length, especially in the 100 μM incubations, where error limits were as high as 30% in the worst cases (Fig. 2).

In vitro oxidation of MTX by partially purified hepatic aldehyde oxidase followed Michaelis–Menten kinetics (Fig. 3) as previously described [22], with $K_m = 189 \pm 58$ μM and $V_{max} = 30 \pm 9$ nmol/mg protein-min. Corresponding parameters for TBM were $K_m = 23.8 \pm 1.4$ μM and $V_{max} = 240 \pm 11$ nmol/mg protein-min. Thus, the efficiency of oxidation of TBM ($V_{max}/K_m = 10$) was about 62-fold greater than that of MTX ($V_{max}/K_m = 0.16$). Substrate inhibition was observed with MTX above 125 μM, as others have reported [25], but not with TBM (data not shown).

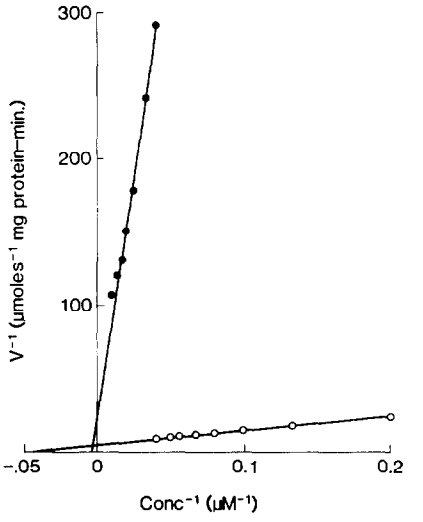

Fig. 3. Lineweaver–Burk plots of MTX and TBM 7-hydroxylation kinetics. Key: (●) MTX; and (○) TBM.

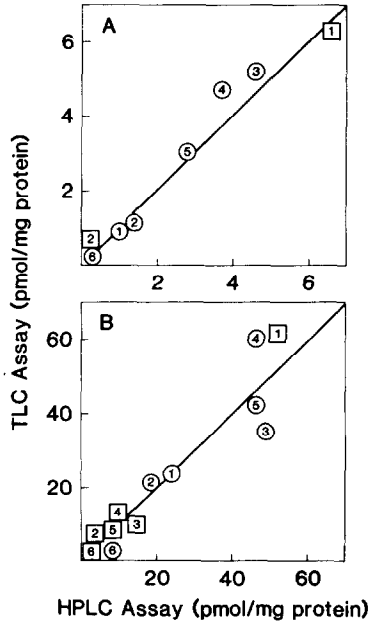

Fig. 2. Scattergram comparison of TLC assay results with those from the standard HPLC assay. (A) 24-hr incubation with 2 μM MTX. (B) 17 hr, 100 μM. Key: (○) CEM cells; and (□) CEM/MTX cells. Integers inside the symbols: total glutamyl residues.

DISCUSSION

In this study we have compared some of the properties of MTX and TBM in a model system consisting of MTX-sensitive and MTX-resistant human leukemic lymphoblasts in culture. These cells were used in earlier studies in which resistance was shown to be associated with increased MTX efflux, resulting in a lower steady-state plateau. We have now further characterized the model by showing that even at an external MTX concentration of 100 μM, which is sufficient to give cellular levels in >100-fold excess of the intracellular dihydrofolate reductase, polyglutamylation in CEM/MTX cells lags far behind that in CEM cells. Since only a fraction of this abundant pool of free MTX was polyglutamylated, we must conclude that the polyglutamylation mechanism is deficient or, alternatively, that folylpolyglutamate hydrolase activity is enhanced markedly in the resistant cells. Because MTX polyglutamylation is required for intracellular retention [26], its absence in the resistant cells contributes an additional component to their defective MTX uptake. Recent clinical observations of wide variations in polyglutamylation after high-dose MTX treatment with leucovorin rescue for osteosarcoma [17] add to the relevance of this resistance modality.

Having determined that a defect of conjugative metabolism contributes to the resistance of CEM/MTX cells, we sought to determine the role of TBM

in circumventing this problem. In studies with γ-*n*-butyl MTX in the Rhesus monkey, gradual cleavage to MTX was observed [27]. Since subsequent polyglutamylation of the MTX is probable, primary ester prodrugs of MTX are likely to be as susceptible as the parent drug to the resistance mechanism described above. Under the alkaline conditions typically used for *in vitro* chemical hydrolysis of primary and secondary esters, tertiary esters show great kinetic stability [12]. Since we have shown that this generalization applies to TBM in cell culture, we may now understand the ability of the drug to overcome resistance based upon defective uptake and polyglutamylation. Since TBM is not a prodrug of MTX in our cell culture system, it is not subject to γ-glutamylation. Rather, it seems to accumulate to high concentrations by virtue of some inherent physical property such as lipophilicity. According to this rationale, the equipotency of TBM against the MTX-sensitive CEM cells and the MTX-resistant CEM/MTX mutants [14] can be explained by the ability of the ester to attain comparable levels in the two cells after 24 hr of incubation. Although a comparison of the IC_{50} values for the two drugs in CEM cells indicates that MTX is about 20-fold more potent than TBM [14], this is also reasonable, in view of the 2-fold decrease in binding affinity for dihydrofolate reductase reported earlier [8]. We intend to explore this phenomenon in greater detail in future studies.

While HPLC remains the method of choice for the rigorous assay of MTX and TBM metabolism, we have investigated the possible usefulness of TLC as an alternative. Although lacking the resolving power and quantitative precision of the HPLC method, TLC was, nonetheless, found to possess several distinct advantages for the estimation of TBM, MTX, 7-hydroxymethotrexate and MTX polyglutamates. TLC assays needed only one-tenth the amount of cold standard required for HPLC, and simultaneous multiple determinations of up to twenty-seven samples could be done on a single 20 × 20 cm plate. Estimates of metabolite levels were quite suitable for assessment of the relative amounts of effluxing versus non-effluxing γ-glutamyl conjugates of MTX.

Reports of intracellular 7-hydroxylation of MTX by CEM cells from our laboratory [28] and by another group [29] have now been found to be erroneous. The discrepancy in our earlier study was caused by a misidentified radioactive compound which happened to coelute with the cold standard for 7-hydroxymethotrexate. Preparative HPLC repurification of [^{3}H]MTX just prior to assay and improvements in peak separation due to a change in eluent composition have reproducibly shown that no 7-hydroxymethotrexate forms in CEM or CEM/MTX cells after 24 hr with 2 μM, or 17 hr with 100 μM, [^{3}H]MTX. Nor was any evidence of intracellular 7-hydroxylation of TBM found after 24 hr at the 2 μM level.

These findings suggest an additional rationale for the use of TBM, since its specificity for malignant cells may depend in part upon the lack of hepatic aldehyde oxidase activity in target tissues. The differential activity of this enzyme is believed to contribute to the specificity of cyclophosphamide, whose metabolite aldophosphamide is detoxified in normal, but not in malignant, tissues by being converted to carboxyphosphamide by aldehyde oxidase [30]. Conventional MTX therapy can benefit only minimally from variations in this aldehyde oxidase effect because of the relatively low affinity of MTX for the enzyme, as shown by other workers [31] and confirmed herein. Esters of MTX, however, have proven to be much better substrates [11], and we have found that TBM is no exception. Due to its 8-fold lower K_m and 8-fold higher V_{max}. TBM may be subject to differential detoxification analogous to that of cyclophosphamide. Toxicology tests in appropriate *in vivo* systems would be necessary to establish whether this concept, arising as it does from *in vitro* and cell culture data, can be translated into meaningful improvements in therapeutic index.

REFERENCES

1. D. R. Seeger, D. B. Cosulich, J. M. Smith, Jr. and M. E. Hultquist, *J. Am. chem. Soc.* **71**, 1753 (1949).
2. B. Chabner, *Pharmacologic Principles of Cancer Treatment*, pp. 229–55. W. B. Saunders, Philadelphia (1982).
3. R. T. Schimke, *Cancer Res.* **44**, 1735 (1984).
4. N. Jaffe, E. Frei, III, D. Traggis and Y. Bishop, *New Engl. J. Med.* **291**, 994 (1974).
5. E. Cadman, in *Folate Antagonists as Therapeutic Agents* (Eds. F. M. Sirotnak, J. J. Burchall, W. D. Ensminger and J. A. Montgomery), Vol. 2, pp. 23–42. Academic Press, New York (1984).
6. E. Mini, B. A. Moroson, C. T. Franco and J. R. Bertino, *Cancer Res.* **45**, 325 (1985).
7. J. A. Montgomery and J. R. Piper, in *Folate Antagonists as Therapeutic Agents* (Eds. F. M. Sirotnak, J. J. Burchall, W. D. Ensminger and J. A. Montgomery), Vol. 2, pp. 219–60. Academic Press, New York (1984).
8. A. Rosowsky, R. A. Forsch, J. Uren and M. Wick, *J. med. Chem.* **24**, 1450 (1981).
9. A. Rosowsky, R. Forsch, C-S. Yu, H. Lazarus and G. P. Beardsley, *J. med. Chem.* **27**, 605 (1984).
10. L. M. Werbel, in *Folate Antagonists as Therapeutic Agents* (Eds. F. M. Sirotnak, J. J. Burchall, W. D. Ensminger and J. A. Montgomery), Vol. 1, pp. 261–84. Academic Press, New York (1984).
11. D. G. Johns, D. Farquhar, M. K. Wolpert, B. A. Chabner and T. L. Loo, *Drug Metab. Dispos.* **1**, 580 (1973).
12. J. March, *Advanced Organic Chemistry*, pp. 309–13. McGraw-Hill, New York (1968).
13. G. E. Foley, H. Lazarus, S. Farber, B. G. Uzman, B. A. Boone and R. E. McCarthy, *Cancer, N.Y.* **18**, 522 (1965).
14. A. Rosowsky, J. H. Freisheim, H. Bader, R. Forsch, S. S. Susten, C. A. Cucchi and E. Frei, III, *J. med. Chem.* **28**, 660 (1985).
15. A. Rosowsky, J. E. Wright, C. A. Cucchi and E. Frei, III, *Proc. Am. Ass. Cancer Res.* **27**, 255 (1986).
16. I. Fabre, G. Fabre and I. D. Goldman, *Cancer Res.* **44**, 3190 (1984).
17. K. H. Cowan and J. Jolivet, *J. biol. Chem.* **259**, 10793 (1984).
18. M. J. Mintz and C. Walling, *Org. Syn. Col.* **5**, 184 (1973).
19. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
20. B. A. Kamen, P. L. Takach, R. Vatev and J. D. Caston, *Analyt. Biochem.* **70**, 54 (1976).
21. D. G. Johns and T. L. Loo, *J. pharm. Sci.* **56**, 356 (1967).
22. D. G. Johns, A. T. Iannotti, A. C. Sartorelli, B. A. Booth and J. R. Bertino, *Life Sci.* **3**, 1383 (1964).

23. D. G. Johns, A. T. Iannotti, A. C. Sartorelli, B. A. Booth and J. R. Bertino, *Biochim. biophys. Acta* **105**, 380 (1965).
24. D. G. Johns, *J. clin. Invest.* **46**, 1492 (1967).
25. G. Fabre, R. Seither and I. D. Goldman, *Biochem. Pharmac.* **35**, 1325 (1986).
26. J. Jolivet, R. L. Schilsky, B. D. Bailey, J. C. Drake and B. A. Chabner, *J. clin. Invest.* **70**, 351 (1982).
27. A. Rosowsky, H. T. Abelson, G. P. Beardsley, W. D. Ensminger, D. W. Kufe, G. Steele and E. J. Modest, *Cancer Chemother. Pharmac.* **10**, 62 (1982).
28. J. E. Wright, A. Rosowsky, C. A. Cucchi and E. Frei, III, *Proc. Am. Ass. Cancer Res.* **27**, 255 (1986).
29. P. A. Newton and R. L. Blakley, *Biochem. biophys. Res. Commun.* **122**, 1212 (1984).
30. D. L. Hill, W. R. Laster and R. F. Struck, *Cancer Res.* **32**, 658 (1972).
31. S. A. Jacobs, R. G. Stoller, B. A. Chabner and D. G. Johns, *Cancer Treat. Rep.* **61**, 651 (1977).