RESEARCH COMMUNICATION

Differences in methotrexate and 7-hydroxymethotrexate inhibition of folate-dependent enzymes of purine nucleotide biosynthesis

Joseph E. BAGGOTT,* Sarah L. MORGAN and William H. VAUGHN

Department of Nutrition Sciences, University of Alabama at Birmingham, UAB Station, Birmingham, AL 35294, U.S.A.

7-Hydroxymethotrexate (7-OH-MTX) is the major and, frequently the only, pteridine metabolite found in bone-marrow aspirates of patients chronically treated with low-dose oral methotrexate (MTX) [Sonneveld, Schultz, Nooter and Hahlen (1986) *Cancer Chemother. Pharmacol.* **18**, 111–116]. The K_i values for MTX and 7-OH-MTX for avian liver 5-aminoimidazole-4-carboxamide ribonucleotide transformylase differ

by 4.5-fold in favour of 7-OH-MTX as the better inhibitor, while K_i values for avian liver glycylamide ribonucleotide transformylase differ by 1.9-fold favouring MTX as the better inhibitor. Thus 7-OH-MTX possesses a different enzyme-inhibiting repertoire from its parent drug and this information may be useful in explaining the mechanism of action of low-dose MTX therapies used to treat autoimmune disease.

INTRODUCTION

The mechanism of action of low-dose methotrexate (MTX) therapy for autoimmune disease remains unclear. Since MTX at low-dose levels would be devoid of efficacy in the treatment of neoplastic disease, there is reason to believe that the target enzymes are not the same. MTX is readily metabolized to 7-hydroxy-MTX (7-OH-MTX) (Johns and Loo, 1967), and this metabolite is much less potent as an inhibitor of dihydrofolate reductase when comparing K_i or IC_{50} values (Johns and Loo, 1967; Farquhar et al., 1972). Inhibition of other folate-metabolizing enzymes by 7-OH-MTX has not been reported, even though this metabolite is the major circulating form of the drug in patients 24 h after high-dose MTX therapy (Watson et al., 1978; Erttman et al., 1985; Borsi et al., 1990) and 24 h after low-dose oral MTX therapy (Sonneveld et al., 1986). 7-OH-MTX is also the major, and frequently the only, pteridine species of the drug found in bone-marrow aspirates 24 h after low-dose oral MTX therapy (Sonneveld et al., 1986). Although 7-OH-MTX is one or two orders of magnitude less cytotoxic to bone marrow and lymphoma cells in culture (Fabre et al., 1986), its overwhelming *in vivo* prevalence following MTX therapy strongly suggests that at least some pharmacological effects of MTX are mediated by this drug metabolite (Slordal et al., 1988).

In order to investigate its biochemical properties further, 7-OH-MTX was tested as an inhibitor of the two folate-dependent enzymes of purine nucleotide biosynthesis: 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) formyltransferase and glycylamide ribonucleotide (GAR) formyltransferase. This information may be important in explaining the mechanism of action of chronic low-dose MTX therapy where 7-OH-MTX may actually be the 'real' pharmaceutical agent (Slordal et al., 1988).

MATERIALS AND METHODS

Sources or synthesis of AICAR, (α , β) GAR, MTX and avian liver transformylase enzymes are described previously (Baggott et al., 1986; Chan and Baggott, 1982). [6R]-10-HCO-H₄folate

was prepared from [6S]-5-HCO-H₄folate (a gift from Lederle Labs) as described previously (Rabinowitz, 1963). 7-OH-MTX was biosynthesized from MTX using a rabbit liver homogenate and recrystallized as described by Cairnes and Evans (1983). Purified 7-OH-MTX had a u.v. spectrum (λ_{max} , 260, 304; pH 7.4; $A_{304}/A_{260} = 1.76$) nearly identical with that reported previously (λ_{max} , 261, 303; pH 7.6; $A_{303}/A_{261} = 1.78$; λ_{max} , 259, 303; pH 7.4; $A_{303}/A_{259} = 1.66$) and migrated as a single u.v.-absorbing spot in paper chromatography (Johns and Loo, 1967; Farquhar et al., 1972).

The spectrophotometric assay of GAR and AICAR transformylase employed has been described previously (Smith et al., 1981; Baggott et al., 1986). Assay conditions for GAR transformylase (final concentrations) were 50 mM Tris/HCl buffer, pH 7.4, 50 mM KCl, 5 mM mercaptoethanol, 50 μ M (α , β) GAR, 1.6–33 μ M [6R]-10-HCO-H₄folate, 0.25% (v/v) glycerol, 0.1% (v/v) dimethyl sulphoxide (DMSO) and enzyme in a 1-cm path-length cuvette at 25 °C. Assay conditions for AICAR transformylase (final concentrations) were 100 mM Tris/HCl buffer, pH 7.4, 150 mM KCl, 10 mM mercaptoethanol, 200 μ M AICAR, 8–350 μ M [6R]-10-HCO-H₄folate, 1.7% (v/v) glycerol, 0.7% (v/v) DMSO and enzyme in a 0.2-cm path-length cuvette at 25 °C. Concentrations of MTX and 7-OH-MTX are given in Figure 1.

Initial velocities were estimated as described previously (Baggott et al., 1986). Initial velocities (with their S.D.s) and substrate concentrations were fitted to inhibition models using EZ-fit program (E. I. DuPont de Nemour). This program also computed the S.E.M. of the fit. Student's *t* test (two-tailed) was used to detect differences in K_i , K_m and V_m values.

RESULTS AND DISCUSSION

The double-reciprocal plots of the inhibition of GAR and AICAR transformylases by MTX and 7-OH-MTX are shown in Figure 1. With respect to 10-HCO-H₄folate, MTX and 7-OH-MTX were competitive inhibitors of GAR transformylase and non-competitive and competitive inhibitors of AICAR transformylase

Abbreviations used: MTX, methotrexate; 7-OH-MTX, 7-hydroxymethotrexate; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; GAR, glycylamide ribonucleotide; DMSO, dimethyl sulphoxide.

* To whom correspondence should be addressed.

Figure 1 Inhibition of GAR and AICAR transformylase by MTX and 7-OH-MTX

Double-reciprocal plots, $1/V$ versus $1/[10\text{-HCO-H}_4\text{folate}]$ are shown for the inhibition of GAR transformylase by MTX (a) and 7-OH-MTX (b) and for inhibition of AICAR transformylase by MTX (c) and 7-OH-MTX (d). Data at low concentrations of 10-HCO-H₄folate or having low initial rates are not shown.

Table 1 Inhibition of GAR and AICAR transformylases by MTX and 7-OH-MTX

All K_i values are for competitive inhibition (with respect to the folate coenzyme) except for $K_{i,\text{MTX}}$, AICAR transformylase, which is non-competitive. Values given in parentheses are S.E.M.s. K_m values given are for 10-HCO-H₄folate.

Enzyme	K_m (μM)	V_m (arbitrary units)	$K_{i,\text{MTX}}$ (μM)	$K_{i,7\text{-OH-MTX}}$ (μM)
GAR transformylase	4.61 (0.74)	151 (8)	9.27 (1.34)	17.7 (2.8)
	5.52 (0.86)	164 (9)		
AICAR transformylase	143 (14)	313 (14)	596 (75)	133 (20)
	152 (21)	305 (21)		

respectively. An attempt was made to fit the data to competitive, non-competitive and mixed non-competitive inhibition models. Models were excluded on the basis of a relatively high S.E.M. of the fit and/or K_i constants which were not statistically different from zero. Estimated K_m values for 10-HCO-H₄folate and V_m values were not altered by the presence of MTX and 7-OH MTX (Table 1), which also suggests a good fit to the inhibition models selected.

K_i values were different from each other at $P = 0.01$ and $P < 0.001$ levels of confidence for GAR and AICAR transformylases respectively (Table 1). Thus, metabolism of MTX to 7-OH-MTX decreases inhibitory potency toward GAR transformylase in concert with increasing the inhibitory potency toward AICAR

transformylase by 4.5-fold. Also the K_i of 7-OH-MTX for AICAR transformylase has a value similar to that of the K_m of 10-HCO-H₄folate, indicating that this drug metabolite competes effectively with the coenzyme for the active site. The opposite is true when considering inhibition of GAR transformylase by 7-OH-MTX. Since the above results are observed using avian enzymes, a note of caution is in order and results using mammalian enzymes may be different.

With this communication, the following changes in biochemical properties have been reported when MTX is metabolized to 7-OH-MTX. (i) 7-OH-MTX is a much less potent inhibitor of dihydrofolate reductase than MTX. However, the K_i of 7-OH-MTX for dihydrofolate reductase is 6 nM, and polyglutamates

of 7-OH-MTX have increased affinity for this enzyme, therefore this MTX metabolite is certainly not inactive at this metabolic step (Johns and Loo, 1967; Fabre and Goldman, 1985). (ii) 7-OH-MTX is polyglutamylated in tumour cells *in vitro* at a rate 2.7-fold faster than MTX (Fabre et al., 1984). (iii) Direct inhibition of GAR transformylase is relieved, and conversely the direct inhibition of AICAR transformylase is increased, by the metabolic conversion of MTX into 7-OH-MTX.

Finally, adenine is the most effective metabolite at rescuing cultured cells from 7-OH-MTX toxicity when compared with thymidine and folinic acid, and this suggests that this drug metabolite strongly targets purine nucleotide biosynthesis (Fabre et al., 1986). The above observations, taken together, suggest that the effect 7-OH-MTX on folate and purine metabolism is different from that of MTX, its parent compound, and this information may be useful in explaining the mechanism of action of low-dose MTX therapy for diseases such as rheumatoid arthritis.

This work was supported in part by the National Institutes of Health, Department of Research Resources Clinical Research Center grant RR 32-31S1 and United States

Public Health Service grants CA-28103-10, AR-20614 and AR-03555. We thank Ms. Tonya Veitch and Ms. Sharon Matlock for their assistance.

REFERENCES

- Baggott, J. E., Vaughn, W. H. and Hudson, B. B. (1986) *Biochem. J.* **236**, 193–200
- Borsi, J. D., Sagen, E., Romslo, I. and Moe, P. J. (1990) *Med. Pediatr. Oncol.* **18**, 217–224
- Cairnes, D. A. and Evans, W. E. (1983) *Ther. Drug Monit.* **5**, 363–366
- Chan, V. T. and Baggott, J. E. (1982) *Biochim. Biophys. Acta* **702**, 99–104
- Erttman, R., Bielack, S. and Landbeck, G. (1985) *Cancer Chemother. Pharmacol.* **15**, 101–104
- Fabre, G. and Goldman, I. D. (1985) *Cancer Res.* **45**, 80–85
- Fabre, G., Fabre, I., Matherly, L. H., Cano, J. P. and Goldman, I. D. (1984) *J. Biol. Chem.* **259**, 5066–5072
- Fabre, J., Fabre, G. and Cano, J. P. (1986) *Eur. J. Cancer Clin. Oncol.* **22**, 1247–1254
- Farquhar, D., Loo, T. L. and Vadlamundi, S. (1972) *J. Med. Chem.* **15**, 567–569
- Johns, D. G. and Loo, T. L. (1967) *J. Pharm. Sci.* **56**, 356–359
- Rabinowitz, J. C. (1963) *Methods Enzymol.* **6**, 814–815
- Slordal, L., Sager, G. and Aarbakke, J. (1988) *Lancet* **i**, 591–592
- Smith, G. K., Benkovic, P. A. and Benkovic, S. J. (1981) *Biochemistry* **20**, 4034–4036
- Sonneveld, P., Schultz, F. W., Nooter, K. and Hahlen, K. (1986) *Cancer Chemother. Pharmacol.* **18**, 111–116
- Watson, E., Cohen, J. L. and Chan, K. K. (1978) *Cancer Treat. Rep.* **62**, 381–387