Biochemical Pharmacology, Vol. 37, No. 3, pp. 449–451, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

INHIBITION OF CHICKEN LIVER 5-AMINOIMIDAZOLE-4-CARBOXAMIDE RIBONUCLEOTIDE TRANSFORMYLASE BY 5,8-DIDEAZA ANALOGUES OF FOLIC ACID

W. THOMAS MUELLER,* GARY K. SMITH,* STEPHEN J. BENKOVIC* and JOHN B. HYNES†‡

*Department of Chemistry, The Pennsylvania State University, University Park, PA 16802; and †Department of Pharmaceutical Sciences, Medical University of South Carolina, Charleston, SC 29425, U.S.A.

(*Received* 1 *May* 1987; *accepted* 30 *June* 1987)

Abstract—A series of fourteen 5,8-dideaza analogues of folic and pteroic acids was evaluated for inhibition of 5-aminoimidazole-4-carboxamide ribonucleotide transformylase (AICAR TFase) from chicken liver. Of the 5,8-dideaza folate derivatives studied, 10-oxa-5,8-dideazafolic acid was the most potent inhibitor. The addition of one L-glutamate moiety to the γ-carboxyl group caused a 6- to 7-fold reduction in K_i in three instances. Two compounds devoid of an L-glutamate were 4- to 6-fold less inhibitory than their parent counterparts possessing one L-glutamate residue.

5-Aminoimidazole-4-carboxamide ribonucleotide transformylase (AICAR TFase§) catalyzes the conversion of AICAR to inosine monophosphate and as such is the last enzyme in the *de novo* biosynthetic pathway to the purine ring in both procaryotic and eucaryotic organisms. The coenzyme for this transformation as well as the glycinamide ribonucleotide transformylase (GAR TFase) reaction, the other folate dependent reaction in purine *de novo* biosynthesis, is 10-formyltetrahydrofolate [1, 2]. Recent studies have provided evidence which suggests that one of the modes of action of the classical folate antagonist methotrexate (MTX) in MCF-7 cells in culture is the inhibition of AICAR TFase [3, 4]. Within 5 hr of MTX treatment, purine *de novo* biosynthesis in these cells decreases to 15% of control, but 10-formyltetrahydrofolate levels in these cells do not decrease by more than 50% over 24 hr [4]. Further, 10-formyldihydrofolate, which is also a good coenzyme for AICAR TFase [5], accumulates in cells during this period and, thus, may offset the decrease in the natural coenzyme. Therefore, the purine deficiency which results from MTX treatment does not appear to derive from the lack of a coenzyme for GAR or AICAR TFase. However, MTX pentaglutamate, which accumulates in the cells upon treatment with MTX, is a competitive inhibitor of AICAR TFase with a K_i of 57 nM against 10-formyltetrahydrofolate and 5.9 μM against 10-formyltetrahydropteroylpentaglutamate [3]. Similarly, dihydropteroylpentaglutamate, which also accumulates upon MTX treatment due to inhibition of dihydrofolate reductase, is also a competitive inhibitor of this reaction with a K_i of 43 nM against 10-formyltetrahydrofolate and 2.7 μM against 10-formyltetrahydropteroylpentaglutamate [3]. However, dihydrofolate and MTX are weak competitive inhibitors of AICAR TFase, regardless of the degree of glutamylation of the folate cofactor [3]. The rate of increase in concentration of these polyglutamates parallels the decrease in the rate of purine *de novo* biosynthesis in MCF-7 cells [4, 6]. Therefore, rather than depletion of the folate coenzyme, a more likely explanation for the decrease in purine *de novo* biosynthesis following MTX treatment is the direct inhibition of AICAR TFase by polyglutamates of MTX and dihydrofolate [3–5]. If this antipurine mechanism of action of MTX proves to be correct, then AICAR TFase constitutes a logical target for new cancer chemotherapeutic agents.

A variety of classical quinazoline analogues of folic acid (5,8-dideazafolates) have been shown to be effective inhibitors of mammalian thymidylate synthase [7–9]. In addition, 10-formyl-5,8-dideazafolic acid, **2**, was found to be an excellent substrate for GAR TFase from chicken liver [2]. Since certain of these dideazafolates have the ability to bind to enzymes which require reduced folates as cofactors despite the fact that they are fully aromatic, it appeared that certain of these compounds might also be effective inhibitors of other reduced folate requiring enzymes. This study concerns the inhibition of chicken liver AICAR TFase by fourteen 5,8-dideaza analogues of folic and pteroic acids.

MATERIALS AND METHODS

Enzyme assay. AICAR TFase was purified to apparent homogeneity from fresh chicken liver as previously described and was assayed under anaerobic conditions [10]. The continuous spectrophotometric assay, which follows the conversion of 10-formyltetrahydrofolate to tetrahydrofolate at 298 nm, was used for all inhibition studies [11]. Assay mixtures maintained at 25° con-

‡ Address correspondence to: Dr. John B. Hynes, Department of Pharmaceutical Sciences, Medical University of South Carolina, 171 Ashley Ave., Charleston, SC 29425.

§ Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; AICAR TFase, 5-aminoimidazole-4-carboxamide ribonucleotide transformylase; GAR TFase, glycinamide ribonucleotide transformylase; and MTX, methotrexate.

tained 32.5 mM Tris–HCl, pH 7.4, 5 mM 2-mercaptoethanol, 25 mM KCl, 50 μM AICAR (Ba^{2+}), 10 nM enzyme, and various concentrations of (±)-10-formyltetrahydrofolate. Inhibition constants were calculated using the enzyme kinetics program of Cleland [12]. For several of the less potent inhibitors, the inhibition of AICAR TFase was measured at three different inhibitor concentrations using 50 μM AICAR and 100 μM (±)-10-formyltetrahydrofolate. To determine K_i values, it was assumed that the inhibition was competitive and that the apparent K_m for (−)-10-formyltetrahydrofolate was 68 μM [1]. Complete kinetic analyses were performed for compounds **3**, **7**, **9**, and **11**, and double-reciprocal plots of the data showed competitive inhibition in each case.

The preparation of most of the compounds employed in this study has been reported earlier [13–19]. Methods for preparing **10**, **11** and **14** will be described elsewhere.

RESULTS

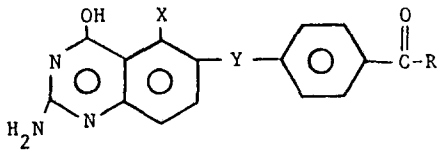
The K_i values obtained for compounds **1–14** against AICAR TFase are summarized in Table 1. It can be seen that 5,8-dideazafolic acid, **1**, was a modestly effective inhibitor having a K_i of 24 μM. The addition of a formyl group at position 10 yielding **2** had little effect, while a methyl group in this position, **3**, reduced the K_i 2-fold. However, the inclusion of a methyl group at position 5, resulting in **4**, caused a dramatic decrease in inhibitory potency. Reversal of the 9,10 bridge yielding 5,8-dideazaisofolic acid, **8**, resulted in a 4-fold increase in K_i compared to **1**. However, the introduction of a formyl group at position 9, resulting in compound **9**, caused an 8-fold reduction in K_i. Compound **9** is, therefore, 2.5-fold more inhibitory than its normal bridge isomer **2**.

Structural alterations at position 10 produced some unexpected effects upon affinity for AICAR TFase. For example, while 10-thia-5,8-dideazafolic acid, **5**, had a K_i similar to that of **1**, the 10-deaza modification, **6**, was 3-fold and the 10-oxa derivative, **7**, 8-fold more inhibitory than the parent compound **1**. The addition of one γ-L-glutamate moiety to compounds **1**, **3**, and **8** (compounds **10–12** respectively) resulted in a 6- to 7-fold reduction in K_i. Conversely, compounds **13** and **14**, which may be considered as 5,8-dideaza analogues of pteroic acid, were 4- to 5-fold less inhibitory than their counterparts containing a single L-glutamyl moiety, **1** and **2**.

DISCUSSION

The data presented in Table 1 show that a number of 5,8-dideazafolates are effective inhibitors of AICAR TFase. The 10-oxa derivative, **7**, had the lowest K_i of compounds possessing a single L-glutamyl moiety and, therefore, has approximately 29-fold higher affinity for this enzyme that the natural substrate, (−)-10-formyltetrahydrofolate [1]. A terminal L-glutamyl moiety appears necessary for good affinity, since the pteroic acid analogues, **13** and **14** were significantly less potent than their folate counterparts, **1** and **2**. It is noteworthy that the enhancement in inhibitory activity resulting from the addition of one γ-L-glutamyl residue was 6- to 7-fold for compounds **10–12**, whereas a 26-fold increase has been observed for MTX diglutamate toward AICAR TFase from MCF-7 cells [3]. It is surprising that 10-formyl-5,8-dideazafolic acid, **2**, was 2.5-fold less inhibitory than its reversed bridge isomer, **9**, particularly in view of the earlier observation that **2**

Table 1. Inhibition of 5-aminoimidazole-4-carboxamide ribonucleotide transformylase by 5,8-dideazafolates and 5,8-dideazapteroates



Compound No.	X	Y	R	K_i (μM)
1	H	CH_2NH	L-Glu	24
2	H	$CH_2N(CHO)$	L-Glu	29
3	H	$CH_2N(CH_3)$	L-Glu	11.6
4	CH_3	CH_2NH	L-Glu	>200
5	H	CH_2S	L-Glu	27
6	H	CH_2CH_2	L-Glu	8
7	H	CH_2O	L-Glu	2.9
8	H	$NHCH_2$	L-Glu	94
9	H	$N(CHO)CH_2$	L-Glu	11.8
10	H	CH_2NH	L-Glu-γ-L-Glu	3.5
11	H	$CH_2N(CH_3)$	L-Glu-γ-L-Glu	1.8
12	H	$NHCH_2$	L-Glu-γ-L-Glu	13
13	H	CH_2NH	OH	100
14	H	$CH_2N(CHO)$	OH	160

possesses weak substrate activity for AICAR TFase [1]. It would be of interest to determine whether **9** can also serve as a substrate for this enzyme.

Recent studies have shown that a wide variety of 5,8-dideaza analogues of folic acid, isofolic acid, and aminopterin are substrates for mammalian folylpoly-γ-glutamate synthetase [20]. Several of these have greater substrate affinity for this enzyme than displayed by aminopterin which, in turn, is a superior substrate to MTX [20]. It is apparent, therefore, that 5,8-dideazafolates such as **7** may inhibit *de novo* purine biosynthesis after being converted to poly-γ-glutamyl metabolites intracellularly as has been shown in the case of MTX in MCF-7 cells [3]. However, it should be noted that several of the monoglutamates shown in Table 1 are effective inhibitors of mammalian thymidylate synthase [7–9] and that the presence of additional γ-L-glutamyl residues should enhance this activity. The results suggest that other 5,8-dideazafolates, which are not potent inhibitors of dihydrofolate reductase or thymidylate synthase, should be evaluated as inhibitors of AICAR TFase in an effort to develop antitumor agents having this unique mechanism of action.

Acknowledgements—This investigation was supported by Grants GM 24129 (to S. J. B.) and CA 25014 (to J. B. H.).

REFERENCES

1. G. K. Smith, W. T. Mueller, P. A. Benkovic and S. J. Benkovic, *Biochemistry* **20**, 1241 (1981).
2. G. K. Smith, P. A. Benkovic and S. J. Benkovic, *Biochemistry* **20**, 4034 (1981).
3. C. J. Allegra, J. C. Drake, J. Jolivet and B. A. Chabner, *Proc. natn. Acad. Sci. U.S.A.* **82**, 4881 (1985).
4. C. J. Allegra, R. L. Fine, J. C. Drake and B. A. Chabner, *J. biol. Chem.* **261**, 6478 (1986).
5. J. E. Baggott, W. H. Vaughn and B. B. Hudson, *Biochem. J.* **236**, 193 (1986).
6. J. Jolivet, R. L. Schilsky, B. D. Bailey, J. C. Drake and B. A. Chabner, *J. clin. Invest.* **70**, 351 (1982).
7. K. J. Scanlon, B. A. Moroson, J. R. Bertino and J. B. Hynes, *Molec. Pharmac.* **16**, 261 (1979).
8. T. R. Jones, A. H. Calvert, A. L. Jackman, M. A. Eakin, M. J. Smithers, R. F. Betteridge, D. R. Newell, A. J. Hayter, A. Stocker, S. J. Harland, L. C. Davies and K. R. Harrap, *J. med. Chem.* **28**, 1468 (1985).
9. T. R. Jones, M. J. Smithers, R. F. Betteridge, M. A. Taylor, A. L. Jackman, A. H. Calvert, L. C. Davies and K. R. Harrap, *J. med. Chem.* **29**, 1114 (1986).
10. W. T. Mueller and S. J. Benkovic, *Biochemistry* **20**, 337 (1981).
11. S. L. Black, M. J. Black and J. H. Mangum, *Analyt. Biochem.* **90**, 397 (1978).
12. W. W. Cleland, *Adv. Enzymol.* **29**, 1 (1967).
13. S. P. Acharya and J. B. Hynes, *J. heterocyclic Chem.* **12**, 1283 (1975).
14. J. B. Hynes, D. E. Eason, C. M. Garrett, P. L. Colvin, Jr., K. E. Shores and J. H. Freisheim, *J. med. Chem.* **20**, 588 (1977).
15. J. E. Oatis, Jr. and J. B. Hynes, *J. med. Chem.* **20**, 1392 (1977).
16. J. B. Hynes, Y. C. S. Yang, G. H. McCue and M. B. Benjamin, *Folyl and Antifolyl Polyglutamates* (Eds. I. D. Goldman, B. A. Chabner and J. R. Bertino), p. 101. Plenum Publishing, New York (1983).
17. T. R. Jones, *Eur. J. Cancer* **16**, 707 (1980).
18. J. B. Hynes, Y. C. S. Yang, J. E. McGill, S. J. Harmon and W. L. Washtein, *J. med. Chem.* **27**, 232 (1984).
19. J. B. Hynes, J. M. Buck, L. D'Souza and J. H. Freisheim, *J. med. Chem.* **18**, 1191 (1975).
20. J. B. Hynes, D. J. Cichowicz and B. Shane, *Chemistry and Biology of Pteridines 1986* (Eds. B. A. Cooper and V. M. Whitehead), p. 997. Walter de Gruyter & Co., Berlin (1986).