Mice were placed backwards on the rotating rod in such a way that they had to turn around to face the movement. Mice were pretrained for a 5-min period 30 min before the beginning of the experiment. The ability of each mouse to stay on the rod for 1 min was then pretested just before the first drug treatment. The occasional mouse not succeeding this pretest was eliminated. Mice were then injected with diazepam (2 mg/kg) followed 10 min later by compound 13 (up to 20 mg/kg) or saline and tested every 10 min during the next hour. Each experiment was repeated on 10 mice.

In Vivo Inhibition of [^{3}H]Flunitrazepam Binding by Compound 13. Tritiated flunitrazepam (200 μCi/kg diluted in saline to a dose volume of 0.1 mL/10 g of weight) was administered through the tail vein of mice (Swiss, male) 20 min before decapitation. The test molecule 13 (10 mg/kg) suspended in Tween 80 was administered 10 min after [^{3}H]flunitrazepam treatment and 10 min before decapitation.[50] Total binding values were obtained from mice treated only with [^{3}H]flunitrazepam. Nonspecific binding values were determined from mice injected with diazepam (10 mg/kg)[51] 60 min before decapitation.

After decapitation, brains were rapidly excised. The cerebellum, cortex, and hippocampus were dissected over ice and weighed and then homogenized with a Polytron in an appropriate volume of ice-cold Tris·HCl (50 mM, pH 7.4): 3 mL for the cortex and cerebellum and 1.5 mL for the hippocampus. Two 600-μL aliquots of these crude homogenates (12 mg of wet tissue) were immediately filtered through Whatman GF/B glass fiber filters and washed twice with 5 mL of ice-cold buffer. Radioactivity retained on the filters was counted in 10 mL of Aquasol scintillation liquid (NEN) by using an LKB Rackbeta 2 counter. Results are given in Table II.

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(50) In initial experiments, compound 13 was, like diazepam, injected 20 min or more before [^{3}H]flunitrazepam. However, no displacement of the radioactive ligand by 13 was observed. In view of the potential metabolic instability of 13, this compound was therefore injected 10 min *after* [^{3}H]flunitrazepam. Control experiments have shown that, when this protocol is used, standard β-carbolines (e.g., β-CCE) effectively displace tritiated benzodiazepines in vivo.

(51) The dose of diazepam that half-maximally inhibits the in vivo binding of [^{3}H]flunitrazepam in mice (ID_{50} value) is ca. 0.5 mg/kg.[49b] A much higher dose of diazepam was used here (10 mg/kg) merely to determine nonspecific binding, that is, the maximum [^{3}H]flunitrazepam binding inhibition that can be expected by the test compound. The dose of compound 13 chosen for the in vivo binding experiments (10 mg/kg) thus represents 20 times the ID_{50} of diazepam, and, despite a fivefold-lower affinity than diazepam, 13 would be expected to displace at least a fraction of the bound [^{3}H]flunitrazepam if it reached the brain.

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N^{10}-Substituted 5,8-Dideazafolate Inhibitors of Glycinamide Ribonucleotide Transformylase

Carol A. Caperelli

Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111. *Received December 30, 1986*

A series of 5,8-dideazafolates bearing ethyl, isopropyl, cyclopropylmethyl, propargyl, 3-cyanopropyl, carboxymethyl, 2-carboxyethyl, phenacyl, 3-fluorobenzyl, and 5-uracilylmethyl substituents at N^{10} were tested as inhibitors of purified L5178Y glycinamide ribonucleotide transformylase (GAR TFase), which requires 10-formyltetrahydrofolate as cofactor. All of these cofactor analogues exhibited competitive inhibition against N^{10}-formyl-5,8-dideazafolate, with K_i's ranging from 2 to 32 μM.

We have recently demonstrated that both 5,8-dideazafolate (1) and the N^{10}-acetyl (7) analogue could inhibit the GAR TFase catalyzed formylation of glycinamide ribonucleotide (GAR) by N^{10}-formyl-5,8-dideazafolate in a competitive manner.[1] This reaction is the first of two folate-dependent formyl group transfers in the de novo purine biosynthetic pathway. Previous studies had indicated that the N^{10}-formyl analogue was utilized very efficiently as a substrate by GAR TFase isolated from both avian[2] and mammalian sources[3,4] and that this analogue was very effective as an affinity ligand for enzyme purification.[3,5]

Recently, a series of N^{10}-substituted 5,8-dideazafolates that displayed preferential inhibition of thymidylate synthase (TS) vs. dihydrofolate reductase (DHFR) were reported.[6] Several of these analogues were also quite cytotoxic toward L1210 cells in culture. However, none of the new analogues were more effective than the N^{10}-propargyl compound (5), which is the most potent antifolate inhibitor of TS (K_i = 4.5 nM) described to date[7] and which has an ID_{50} of 5 μM for L1210 cells in culture.[6] Since other 5,8-dideazafolates have proven to be extremely useful tools for studies on GAR TFase,[3-5] we wished to examine the interaction of these new 5,8-dideazafolate analogues with L5178Y GAR TFase in order to gain insight into the

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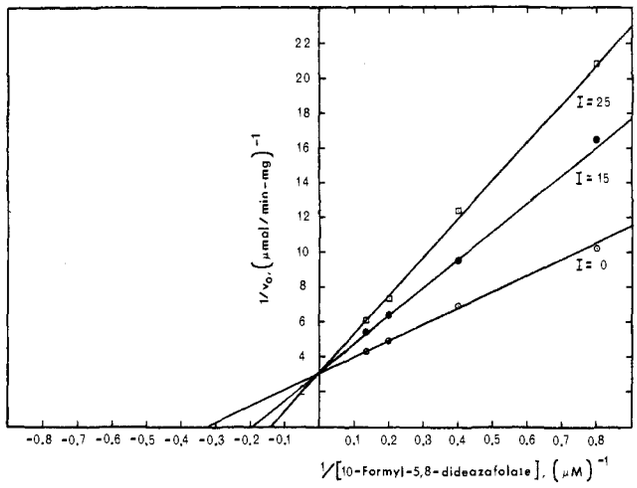


Figure 1. Lineweaver–Burk plot of the initial velocity data for inhibition of GAR TFase by N^{10}-isopropyl-5,8-dideazafolate (**3**) as a function of N^{10}-formyl-5,8-dideazafolate concentration. The concentration of **3** was 0 μM (○), 15 μM (●), and 25 μM (□).

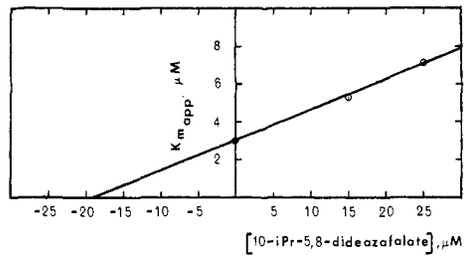


Figure 2. Intercept replot. Plot of apparent K_m for N^{10}-formyl-5,8-dideazafolate as a function of N^{10}-isopropyl-5,8-dideazafolate (**3**) concentration.

cofactor binding site of the enzyme and to determine whether GAR TFase might be an in vivo target for these antifolates.

Biological Evaluation

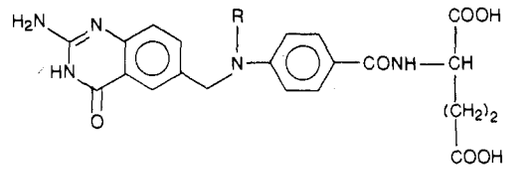
The analogues of **2–6**, **8–12** were tested as inhibitors of purified GAR TFase isolated from the murine lymphoma cell line L5178Y.[3] These assays were performed with saturating levels of the substrate GAR and with N^{10}-formyl-5,8-dideazafolate as formyl group donor.[3] The data were analyzed with Lineweaver–Burk plots in order to assess both the mode of inhibition and the kinetic parameters.

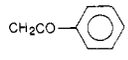
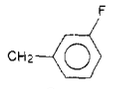
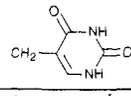
Results and Discussion

All of the N^{10}-substituted 5,8-dideazafolate analogues tested displayed competitive inhibition patterns against N^{10}-formyl-5,8-dideazafolate as illustrated in the Lineweaver–Burk plot (Figure 1) of the data for **3**. The inhibition constants (K_i's) were estimated from the intercept replots, as shown for **3** (Figure 2), and are summarized in Table I, along with the data for **1** and **7** determined previously.[1]

The most striking thing to note is that, despite the large chemical and steric differences among the substituents, all of the analogues are effective inhibitors of L5178Y GAR TFase with K_i's in the μM range. None of the new analogues, however, were more effective than **1** or **7**. Several observations can be made from a comparison of the inhibitory potency to the chemical type of the substituent: (i) Alkyl substituents, viz., ethyl (**2**) and isopropyl (**3**), decreased effectiveness about 10-fold compared to hydrogen (**1**) and acetyl (**7**), but this could be partially counteracted by increased s-bonding character, viz., cyclopropylmethyl (**4**) and propargyl (**5**). (ii) Negatively charged substituents, such as carboxymethyl (**8**) and carboxyethyl (**9**), were also inhibitory, although this homologation resulted in a less effective analogue. (iii) Bulky substituents such as phenacyl (**10**), 3-fluorobenzyl (**11**), and 5-uracilylmethyl (**12**) were also accommodated by the cofactor binding site, but their effectiveness did not parallel their relative polarities.[6]

Table I. Inhibition of GAR TFase by N^{10}-Substituted 5,8-Dideazafolic Acids



no.	R	K_i, μM[a]	no.	R	K_i, μM[a]
1	H[b]	2	10		3
2	CH_2CH_3	16			
3	$CH(CH_3)_2$	19			
4	CH_2-c-C_3H_5	8	11		18
5	$CH_2C{\equiv}CH$	6			
6	$CH_2CH_2CH_2CN$	11			
7	$COCH_3$[b]	1.3	12		7
8	CH_2COOH	5			
9	CH_2CH_2COOH	32			

[a] K_i's were determined as described in the text. [b] From ref 1.

Previous studies with this enzyme indicated that the kinetic mechanism is sequential,[1] suggesting that both substrate and cofactor must reside at the active site simultaneously. The accommodation of bulky N^{10} substituents (**10–12**) in the cofactor binding site is consistent with this suggestion. A reasonable chemical mechanism for the transformylation reaction is a direct formyl group transfer from cofactor to substrate through the intermediacy of the tetrahedral intermediate formed from nucleophilic attack by the terminal amino group of GAR on the formamide carbonyl carbon of the cofactor. The inhibitory efficacy of the carboxymethyl analogue (**8**) may reflect its ability to (partially) mimic the proposed tetrahedral intermediate.

Conclusion

A wide variety of substituents at the N^{10} position of 5,8-dideazafolate are accommodated by the cofactor binding site of GAR TFase, which requires N^{10}-formyltetrahydrofolate as cofactor, but will accept N^{10}-formyl-5,8-dideazafolate with comparable efficiency. These analogues showed competitive inhibition against the N^{10}-formyl cofactor analogue, with K_i's in the μM range, but none were better than the previously described N^{10}-hydrogen (**1**) and -acetyl (**7**) analogues.[1] It is unlikely that GAR TFase is a major in vivo target for these analogues, since the K_i's determined with the purified enzyme are, for the most part, larger than the ID_{50}'s of these analogues for L1210 cells in culture.[6]

Experimental Section

General Procedures. Compounds **2–6**, **8–12** were generously provided by Dr. Terence R. Jones and his colleagues (Drug Development Section, Institute of Cancer Research, Sutton, Surrey, SM2 5PX England, and Imperial Chemical Industries PLC, Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire, SK10 4TG England). Stock solutions of the analogues were prepared in 10 mM potassium phosphate, pH 7.5, and their concentrations were determined from the absorption spectra.[6] UV–vis spectra were recorded with a Varian 2200 spectrophotometer. Enzyme assays were performed with a Gilford 260 spectrophotometer equipped with an automatic cell programmer,

recorder, and a circulating constant-temperature bath. Glycinamide ribonucleotide was prepared according to the literature procedure.[8] 5,8-Dideazafolate (1) and N^{10}-acetyl-5,8-dideazafolate (7) were prepared as described previously.[1] Glycinamide ribonucleotide transformylase was purified from L5178Y as previously described.[3]

Enzyme Assays. Glycinamide ribonucleotide transformylase activity was assayed by monitoring the production of 5,8-dideazafolate at 295 nm ($\Delta\epsilon = 18.9 \times 10^3$ mM^{-1}).[2] The assays were performed at 35 °C and pH 7.5 with the solution containing 100 mM potassium phosphate, pH 7.5, 1 mM (α,β) glycinamide ribonucleotide, and enzyme in 0.5 mL. For the inhibition studies, the concentration of N^{10}-formyl-5,8-dideazafolate was varied from 1.25 to 7.5 μM. The inhibitors were maintained at fixed concentrations, which were varied from 0 μM up to 50 μM for the less effective inhibitors. All components, except cofactor, were incubated at 35 °C for 10 min, and the reaction was initiated by the addition of cofactor in a small volume. The rate of product formation was dependent on enzyme concentration and linear with time during the initial part of the reaction. No turnover of cofactor was observed in the absence of either enzyme or substrate.

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Folate Analogues. 30. Synthesis and Biological Evaluation of N^{10}-Propargyl-5,8-dideaza-5,6,7,8-tetrahydrofolic Acid and Related Compounds[1]

M. G. Nair,*† Rashmi Dhawan,† M. Ghazala,† T. I. Kalman,‡ R. Ferone,§ Y. Gaumont,§ and R. L. Kisliuk‖

Department of Biochemistry, University of South Alabama, Mobile, Alabama 36688, Department of Medicinal Chemistry, State University of New York, Buffalo, New York 14260, Department of Microbiology, The Wellcome Research Laboratories, Burroughs Welcome Co., Research Triangle Park, North Carolina 27709, and Department of Biochemistry and Pharmacology, Tufts University, Health Science Campus, Boston, Massachusetts 02111. *Received October 14, 1986*

The 5,6,7,8-tetrahydro derivative (1) of the powerful thymidylate synthase inhibitor N^{10}-propargyl-5,8-dideazafolic acid (PDDF) has been synthesized and evaluated for its antifolate activity. A convenient method for the preparation of the key intermediate 2-amino-6-(bromomethyl)-4-hydroxy-5,6,7,8-tetrahydroquinazoline (18) is described. Two closely related analogues of 1 were also synthesized and evaluated for their antifolate activity and thymidylate synthase inhibition. N^{10}-Propargyl-5,8-dideaza-5,6,7,8-tetrahydrofolate (1) and N^{10}-methyl and N^{10}-hydrogen analogues **2** and **3** were weaker inhibitors of *Lactobacillus casei* thymidylate synthase compared to PDDF. N^{10}-Methyl-5,8-dideaza-5,6,7,8-tetrahydrofolate (**2**) exhibited the most potent antifolate activity against *L. casei* (IC_{50} = 2.8 nM) and *Streptococcus faecium* (IC_{50} = 0.57 nM). In intact and permeabilized murine leukemia L1210 cells, the replacement of the quinazoline moiety with its tetrahydro derivative resulted in a marked decrease in potency and a loss of the contribution of the propargyl substituent to enzyme inhibition, indicating an altered binding mode to thymidylate synthase.

Coenzyme analogues structurally related to folic acid that are specific inhibitors of thymidylate synthase (TS) are potentially useful antineoplastic agents. Analogues of 5,8-dideazafolic acid that were substituted at N^{10} with various substituents have exhibited varying degrees of inhibition of thymidylate synthase (EC 2.1.1.45).[2,3] The powerful antileukemic agent N^{10}-propargyl-5,8-dideazafolic acid (**4**) (Chart I) has been shown to be a specific inhibitor of L1210,[4] *Lactobacillus casei*,[5] *Streptococcus faecium*,[5] and human[6] thymidylate synthases. N^{10}-Propargyl-5,8-dideazapteroyl tri-, tetra-, and pentaglutamates were equipotent with 5-fluorodeoxyuridylate as inhibitors of thymidylate synthesis in permeabilized L1210 cells.[7] These polyglutamates of N^{10}-propargyl-5,8-dideazafolic acid (PDDF) isolated as metabolites[8] were shown to be the most potent antifolate inhibitors of *L. casei* and L1210 thymidylate synthases yet described.[7,9,10] In spite of its excellent inhibition ($K_i = 1 \times 10^{-9}$ M) of L1210 TS, the therapeutic utility of PDDF has suffered because of its poor solubility and consequent nephrotoxicity.[11] Several analogues (**5–7**) of PDDF were subsequently synthesized from this[7] and other laboratories[12] and evaluated for their biological activity. None of these compounds were found to be superior to PDDF as TS inhibitors or as antitumor agents. N^{10}-Propargylfolic acid (**8**), which is the closest pteridine analogue of PDDF, has been recently synthesized and evaluated for its antifolate activity and TS inhibition.[13] The folate analogue **8** exhibited a considerably lower de-

† University of South Alabama.
‡ State University of New York.
§ Burroughs Welcome Co.
‖ Tufts University.

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