Enzymatic Assays for 2,4-Diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)methyl]quinazoline, a Promising New "Nonclassical" Antifolate

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Received November 19, 1979, from the **Vermont Regional Cancer Center and Pharmacology Department, College of Medicine, University of Vermont, Burlington, VT 05405,* and the ‡*Laboratory of Medicinal Chemistry and Biology, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, MD 20205.* Accepted for publication January 7, 1981. (Inquiries should be directed to the University of Vermont.)

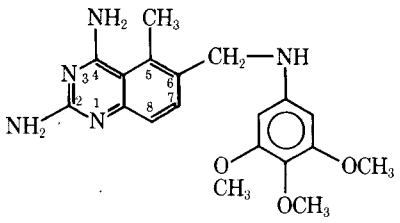
Abstract □ 2,4-Diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)-methyl]quinazoline (I) is a promising new "nonclassical" antifolate. Inhibition of dihydrofolate reductase from bacterial (*Lactobacillus casei*) and mammalian (beef liver) sources was employed to develop useful enzymatic assays for this compound. A linear relationship was obtained by plotting the I concentration *versus* $1/V$. The resultant standard curves maintained linearity particularly well between the 30 and 70% control range, with a correlation coefficient of 0.99 for both enzyme systems. The two enzyme systems are characterized by differences in sensitivity, stability, and day-to-day variation. The ID_{50} for the beef liver reductase system was 1.6×10^{-9} *M* (±0.3); for the *L. casei* system, it was 1.35×10^{-8} *M* (±0.2). The apparent advantage for the beef liver enzyme was offset somewhat by its relative instability and its higher day-to-day variability. Studies in mice demonstrated that these assays are suitable for pharmacokinetic studies *in vivo*. Such studies indicated that I has a serum $t_{1/2}$ of 45 min in mice; a similar serum $t_{1/2}$ (50 min) was estimated in studies with ^{14}C-labeled I in position 6.

Keyphrases □ 2,4-Diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)-methyl]quinazoline—two enzyme assays using beef liver reductase and *Lactobacillus casei* □ Enzymatic analyses—2,4-diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)methyl]quinazoline using beef liver reductase and *Lactobacillus casei* □ Antifolates—2,4-diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)methyl]quinazoline enzymatic analyses using beef liver reductase and *Lactobacillus casei*

2, 4-Diamino-5-methyl-6-[(3, 4, 5-trimethoxyanilino)-methyl]quinazoline (I) is a potent inhibitor of dihydrofolate reductase [5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase (EC 1.5.1.3)]. Compound I was originally synthesized by Elslager and colleagues as part of an effort to develop quinazoline antifolates with useful antiprotozoal activity (1). Interest in possible antineoplastic applications of I was stimulated when the agent was reported (2) to be a potent inhibitor of the enzyme from several sources, including the L-1210 murine leukemia line.

In comparison to methotrexate, I offers several qualities that may prove advantageous clinically. Since it is a much more lipophilic derivative than is methotrexate, it may penetrate the central nervous system (CNS) more readily and thus be more effective than methotrexate against CNS neoplasms.

Bertino (3) commented upon the increased uptake of I into neoplastic cells compared to methotrexate, and he and his colleagues (4) reported also that I is effective against a wider range of transplantable murine tumors than is methotrexate. Methotrexate, a weak dicarboxylic acid (pKa 4.7), can be metabolized to a 7-hydroxy metabolite, which may contribute to its nephrotoxicity (5). High-dose methotrexate therapy is associated with significant clinical nephrotoxicity in patients, despite vigorous hydration and alkalinization of the urine (6). It is possible that I may offer an advantage as a substitute for methotrexate in high-dose antifolate regimens.



I

Studies are underway to determine the pharmacokinetic and pharmacodynamic properties of I relative to those of methotrexate. This article reports two enzymatic assays for this "nonclassical" antifolate.

EXPERIMENTAL

Beef liver dihydrofolate reductase is available commercially[1] in 5-unit vials. The enzyme was prepared just prior to use by diluting 3 μl of the commercial suspension in 2 ml of chilled 0.1 *M* phosphate buffer at pH 7.0. *Lactobacillus casei* dihydrofolate reductase was obtained[2] in 20-unit vials. This stock enzyme solution was prepared by dissolving 1 mg of powder in 4 ml of 0.1 *M* phosphate buffer at pH 7.0.

The assay system employed was a slight modification of that described by Burchall and Hitchings (7), involving measurement of the decrease in absorbance at 340 nm, catalyzed by dihydrofolate in a 1-ml system. The system was composed of NADPH[3] (1×10^{-4} *M*), 2-mercaptoethanol (1×10^{-2} *M*), and dihydrofolate [6.6×10^{-5} *M*, prepared according to Futterman (8)] in 0.1 *M* phosphate buffer at pH 7.0. The quantity of enzyme solution used for each assay was sufficient to produce an absorbance decrease of 0.17 unit for the beef liver and of 0.2 unit for the *L. casei* systems over a 10.5-min assay period. The enzyme assays were performed[4] at a chamber temperature of 37°. A reference blank containing buffer, enzyme, and 2-mercaptoethanol was employed during each run.

Compound I was dissolved in dimethyl sulfoxide at 1.1×10^{-3} *M* and then serially diluted in 0.1 *M* phosphate buffer at pH 7.0 to give a suitable range of concentrations. After a 5-min preincubation at 37°, first NADPH and then dihydrofolate (as a 1:3 dilution in 0.1 *M* phosphate buffer at pH 7.0) were added to the cells to initiate the enzymatic reaction. Controls without inhibitor were run with each assay. A NADPH control (reaction mixture minus dihydrofolate) was run daily to assess absorbance changes due to NADPH oxidation. This value was subtracted from each value determined to give a more accurate control and inhibitor value. Absorbance changes were measured every 90 sec for 10.5 min, and ID_{50} values were obtained by a least-squares regression analysis of the lines determined by plotting the enzyme activity *versus* the inhibitor concentration.

Compound I was formulated for intravenous administration to mice by solubilizing 10 mg in 0.4 ml of propylene glycol with stirring. After solubilization, the addition of 0.6 ml of normal saline produced a solution suitable for injection. Injections were made into the tail vein of female

[1] Sigma Chemical Co., St. Louis, MO 63178.
[2] New England Enzyme Center (Tufts University, School of Medicine), Boston, MA 02153.
[3] P. L. Biochemicals, Milwaukee, WI 53205.
[4] Beckman 25 kinetic spectrophotometric system, Beckman Instruments, Irvine, CA 92634.

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Table I—Levels of I in Mouse Serum after Intravenous Administration

Minutes	Concentration, $M \times 10^{-6}$	
	Enzymatic Assay[a]	Isotopic Assay[b]
15	20.7 (±0.3)	2.3 (±0.1)
30	16.5 (±0.5)	1.8 (±0.1)
60	9.5 (±1.3)	1.25 (±0.1)
90	6.1 (±1.3)	0.73 (±0.06)
120	4.3 (±1.1)	0.59 (±0.07)
180	1.7 (±0.2)	—

[a] *L. casei* enzyme; I was administered at 10 mg/kg. Estimated serum elimination $t_{1/2}$ was 45 min. Data are from six separate experiments (one mouse for each time point). Figures in parentheses are the standard errors of the mean. [b] Data from four experiments; I was administered at 1 mg/kg. Estimated serum elimination $t_{1/2}$ was 50 min.

mice (Swiss-Webster, 20–22 g), and blood samples were collected by retro-orbital puncture. Compound I labeled in position 6 with carbon 14 (specific activity of 21 μCi/mg) was used[5]. For the isotopic studies, 10 μl of serum was placed in 10 ml of scintillation fluid[6] and radioactivity was measured by liquid scintillation spectrometry[7].

RESULTS AND DISCUSSION

In anticipation of pharmacokinetic studies of I, two assay procedures were developed based on the inhibitory activity of I on dihydrofolate reductase from bacterial and mammalian sources. A linear relationship was obtained by plotting the I concentration *versus* $1/V$[8]. This linearity held well for I concentrations producing 30–70% inhibition of dihydrofolate reductase. The correlation coefficient for such lines was 0.99 for both enzyme systems. The standard curves obtained by linear or semilog plots of the I concentration *versus* the percent control are suitable for pharmacokinetic studies.

By using this enzymatic approach to drug assay, studies in mice indicated a serum elimination half-time of ~45 min (Table I). Sigel and colleagues[9], using a different assay, found a similar serum elimination half-time for I in the dog. A small amount of ^{14}C-labeled I was obtained for exploratory pharmacokinetic studies in our laboratories. As shown in Table I, the elimination profile determined by the isotopic method was similar to that observed with the enzymatic method. The I levels were lower in the isotopic studies because a lower dose of I was employed (1 mg/kg).

Both enzyme assays are advantageous in that they are highly sensitive with a lower limit of sensitivity of 5×10^{-10} *M* for the beef system and of 5×10^{-9} *M* for the *L. casei* system (Table II). The sensitivity of both assays can be increased by lowering the amount of dihydrofolate present and/or reducing the amount of enzyme used per assay.

The two enzyme systems differ in sensitivity, stability, and accuracy. The ID_{50} was 1.6×10^{-9} *M* (±0.3) for the beef system and 1.35×10^{-8} *M* (±0.2) for the *L. casei* system. Under the experimental conditions employed, the beef liver enzyme was about one order of magnitude more sensitive to I than was the bacterial enzyme; however, the instability of the beef preparation was reported previously (9). On the other hand, the *L. casei* enzyme was considerably more stable and easier to work with. For example, the beef enzyme began to lose activity after several hours in solution, while the bacterial enzyme solution was essentially stable throughout the day. In addition, the latter solution may be refrozen with a large measure of its activity retained upon subsequent thawing.

A disadvantage of both enzyme assays is relatively high day-to-day variability: 20% for the beef liver assay and 15% for the *L. casei* assay. Other investigators (10, 11) automated the methotrexate enzymatic assay to improve these figures. In our studies, a standard curve was generated daily. Another disadvantage of both enzyme assays is the relatively narrow range of linearity of the concentration–inhibition curve (30–70% control values have the highest reliability). However, reasonable estimates of required dilutions can be made, which will reduce assay time.

Table II—Relationship between Inhibition of Dihydrofolate Reductase and I Concentration

Concentration, $M \times 10^{-9}$	Percent Control[a]	
	L. casei	Beef Liver
44	28 (±1.0)	—
33	33.5 (±1.0)	—
22	41.3 (±1.7)	—
16.5	46.7 (±2.3)	—
11	53.7 (±2.3)	—
8.8	58.3 (±3.7)	—
6.6	63 (±5.0)	—
5.5	67.3 (±4.3)	27.7 (±1.7)
4.4	70.7 (±5.3)	32 (±2.0)
3.3	75.5 (±5.5)	37 (±3.0)
2.2	83.3 (±6.7)	44.3 (±3.3)
1.1	—	57 (±5.0)
0.88	—	61.3 (±5.7)
0.66	—	66.7 (±6.0)
0.55	—	70 (±7.0)

[a] Each value is the mean of three separate determinations of the standard curve. Figures in parentheses are the standard errors of the mean.

The described methods for determining I levels are sensitive, relatively specific, and accurate. These enzyme assays employ common laboratory equipment and readily available reagents and should prove applicable in various preclinical and clinical research situations. Measuring levels of I in biological material will be of added importance in preclinical and clinical studies in view of observations by Duch *et al.* (12) that I and related compounds can produce substantial concentration-dependent inhibition of histamine metabolism and consequent elevation of histamine levels in experimental animals.

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[5] Obtained from Dr. Robert Engle, Pharmaceutical Resources Branch, National Cancer Institute, Silver Spring, Md.

[6] Filtron-X, National Diagnostics, Somerville, NJ 08876.

[7] Beckman LS 7000 liquid scintillation system, Beckman Instruments, Irvine, CA 92713.

[8] Where $1/V = V$ control/V inhibitor = (absorbance change control/absorbance change inhibited).

[9] Dr. Carl Sigel, Wellcome Research Laboratories, Research Triangle Park, NC 27709, personal communication.