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IRREVERSIBLE INHIBITION OF THYMIDYLATE SYNTHASE BY PYRIDOXINE (B6) ANALOGUES†

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Three vitamin B_6 analogues have been synthesized and tested as inhibitors of thymidylate synthase. The compounds are: 4′,5′-dichloro-, 4′,5′-dibromo- and 4′, 5′-diiodo-pyridoxine. All three analogues inhibited the enzyme irreversibly. The kinetic data for the chloro- and bromo-analogues showed that a limiting rate of inhibition is approached as the inhibitor concentration is increased, which indicates that a reversible enzyme: inhibitor affinity complex is formed prior to the irreversible reaction. 4′,5′-Dibromo-pyridoxine exhibited a greater binding affinity (lower K_i) for thymidylate synthase than 4′,5′-dichloro-pyridoxine, and it also reacted faster to irreversibly inhibit the enzyme. The presence of the substrate dUMP (10 μM) completely protected thymidylate synthase from inhibition. These data suggest that the halogenated vitamin B_6 analogues are active site-directed inhitors of thymidylate synthase, which first bind reversibly to the catalytic site and then react irreversibly with the enzyme.

KEY WORDS: Thymidylate synthase, inhibition, vitamin B_6 analogues, pyridoxine analogues.

INTRODUCTION

Thymidylate synthase (5,10-methylene tetrahydrofolate: deoxyuridine-5′-monophosphate C-methyl transferase, EC 2.1.1.45) catalyzes the following reaction:

$$dUMP + CH_2\text{-}H_4folate \rightarrow H_2folate + dTMP.$$

Since thymidylate synthase provides the sole *de novo* source of thymidylate, it is crucial for DNA biosynthesis and cell growth. As a result, this enzyme is an important target of cancer chemotherapeutic agents.[1] The previous kinetic studies by Chen *et al.*[2] showed that pyridoxal phosphate (PLP) was a reversible inhibitor of thymidylate synthase and that the inhibition was competitive when dUMP was the variable substrate and noncompetitive when methylenetetrahydrofolate was the variable substrate. Surprisingly, PLP was an impressive inhibitor with an inhibition constant of approximately 1 μM. The change in the absorption spectrum of PLP produced by thymidylate synthase suggested that the inhibition resulted from the reaction of the aldehyde group of PLP with the catalytically essential sulfhydryl group to form a thiohemiacetal, rather than by forming a Schiff base with lysine residues. Direct spectral evidence that PLP acts at the active site was obtained by showing that increasing concentrations of dUMP counteracted the spectral changes of PLP produced by thymidylate synthase.[3] Similar results were obtained with increasing concentrations of the active site-directed inhibitor 5-fluorodeoxyuridylate. The spectrum

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Abbreviations: CH_2-H_4folate, 5,10-methylene-5,6,7,8-tetrahydrofolate; DBP, 4′,5′-dibromopyridoxine: DCP, 4′,5′-dichloropyridoxine; DIP, 4′,5′-diiodopyridoxine; dTMP, thymidylate; dUMP, deoxyuridylate; PIPES, piperazine-N,N′-bis(2-ethanesulfonic acid); PLP, pyridoxal 5′-phosphate.

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of PLP was not changed by the addition of thymidylate synthase that had been treated with either iodoacetamide or methylmethane thiosulfonate to block sulfhydryl groups.[3] It was also shown that dUMP and dTMP interfered substantially with PLP binding.[4] In this study, the 4 and 5 positions of pyridoxine (vitamin B_6) were replaced with a methylhalide ($-CH_2X$; X = Cl, Br, I) and the kinetics of the irreversible reaction between these analogues and thymidylate synthase was investigated.

MATERIALS AND METHODS

Chemicals and Supplies

Thionyl chloride, hydrobromic acid and hydroiodic acid were purchased from Aldrich Chemical Company, Inc., Milwaukee, WI; Hydroxyapatite (Bio-Gel HT) was from Bio-Rad Laboratories, Richmond, CA. Carboxymethyl Sephadex C-50, and PD-10 Sephadex G 25 M columns were from Pharmacia Fine Chemicals, Piscataway, NJ. 2′-Deoxyuridine-5′-monophosphoric acid (disodium salt), folic acid, and 2-mercapto-ethanol, were from Sigma Chemical Co., St Louis, MO. All other chemicals were obtained commercially and were of reagent grade or better.

Thymidylate synthase was purified from amethopterin-resistant *Lactobacillus casei* by the method of Lyon *et al.*,[5] and the final preparations had specific activities of approximately 2.5 units/mg. The concentration of thymidylate sythase was calculated from the absorbance at 280 nm using either 105,000 $M^{-1} cm^{-1}$ or 1.55 $mg^{-1} cm^{-1}$ as the extinction coefficient.[5] Before use, thymidylate synthase was activated by dialysis for 24 h against 20 mM potassium phosphate buffer, pH 6.8 containing 25 mM 2-mercaptoethanol. Activated enzyme was dethiolated[6] immediately before use to prevent interference by mercaptoethanol.

Epimeric (6-*ambo*)-H_4folate was prepared by the catalytic hydrogenation of folic acid in glacial acetic acid with platinum oxide catalyst according to the procedure of Hatefi *et al.*[7] The lyophilized, white product was divided into 6 mg portions, packaged under argon in sealed serum bottles,[8] and stored at −50°C until used.

Three analogues of vitamin B_6, 4′,5′-dichloro-, 4′,5′-dibromo-, and 4′,5′-diiodo-pyridoxine, were prepared by treating pyridoxine with thionyl chloride, hydrobromic acid and hydroiodic acid, respectively, as described previously.[9] The compounds were packaged in small bottles with aluminium foil covers and stored 0–5°C until used. They were identified by their melting points, infrared (IR), proton nuclear magnetic resonance (^1H NMR) and mass spectra. 4′,5′-Dichloro-pyridoxine (DCP): mp 179–197°C (decompose); IR ν_{max} (KBr) 3190 (–OH), 3080 (C–H, aromatic), 2850 (N · HCl), 1625 (aromatic), 1225, 1183, 940, 855, 718 cm^{-1}; ^1H NMR (D_2O): 2.63 (s, 3H, C-2-CH_3), 4.97 (s, 2H, C-5′-CH_2Cl), 5.00 (s, 2H, C-4′-CH_2Cl), 8.42 (s, 1H, C-6-H); MS (rel. intensity) 206, 208 and 210 (isotopic cluster for 2Cl; M^+, $M^+ + 2$ and $M^+ + 4$; 100%, 68% and 11%), 191 (M-CH_3; 3%), 170 and 172 (M-HCl; 19% and 8%). 4′,5′-Dibromo-pyridoxine (DBP): mp 216–228°C (decompose); IR ν_{max} (KBr) 3190 (−OH), 3080 (C–H, aromatic), 2845 (N · HBr), 1625 (aromatic), 1220, 1165, 932, 845, 758, 610 cm^{-1}; ^1H NMR (D_2O): 2.60 (s, 3H, C-2-CH_3), 4.80 (s, 2H, C-5′-CH_2Br), 4.92 (s, 2H, C-4′-CH_2Br), 8.24 (s, 1H, C-6-H); MS (rel. intensity) 294, 296 and 298 (isotropic cluster for 2Br; 51%, 100% and 49%), 214 and 212 (M-HBr; 9% and 11%). 4′,5′-Diiodo-pyridoxine (DIP): mp 120–153°C (decompose); IR ν_{max} (KBr) 3190 (–OH), 3090 (C–H, aromatic), 2845 (N · HI), 1625 (aromatic), 1355, 1225, 1145, 1030, 925, 850, 751 cm^{-1}; ^1H NMR (D_2O); 2.60 (s, 3H, C-2-CH_3), 4.83 (s, 2H,

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C-5′-CH_2I), 4.86 (s, 2H, C-4′-CH_2I), 8.31 (s, 1H, C-6-H); MS (rel. intensity) 390 (M^+), 308 (20%), 264 (7%), 156 (100%).

Enzyme Assay

Thymidylate synthase activity was measured at 25°C as previously described[10] with a Gilford Model 250 spectrophotometer (Gilford Instruments Co., Oberlin, OH).

Inhibition of Thymidylate Synthase by Vitamin B_6 Analogues

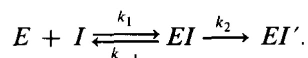
Stock solutions of the inhibitors in distilled water ranging in concentration from 1–10 mM were prepared the same day that they were used. Inhibition reactions were carried out at 25°C and begun by adding 30 μl of inhibitor solution to 270 μl of activated, dethiolated thymidylate synthase solution in 20 mM PIPES buffer, pH 6.8. The enzyme concentration in the inhibition reaction mixtures was approximately 6 μM. Residual enzyme activities were determined by periodically withdrawing 20 μl aliquots of the inhibition reaction mixtures and adding them to 0.98 ml portions of assay mixture contained in spectrophotometer cuvettes. Initial velocities (ΔA_{340}/min) were obtained at 25°C from the linear portions of recorder tracings using a Gilford Model 250 spectrophotometer. In each experiment, it was found that essentially no activity was lost in a control that contained the same amount of enzyme but no inhibitor.

The effect of dUMP on the rate of inhibition of thymidylate synthase was determined by adding various amounts of dUMP to the enzyme solutions prior to adding the inhibitors, followed by incubation and determination of residual activities as described above.

To show that the inhibition was irreversible, enzyme solutions that were nearly completely inactivated were dialyzed at 5°C in phosphate buffer (pH 6.8) containing 25 mM 2-mercaptoethanol for 12 h, and then assayed for recovered enzyme activity.

Determination of Inhibition Constants (K_i) and Rate Constants (k_2)

The following kinetic scheme describes the reaction of an enzyme (E) with an inhibitor (I) to reversibly form a Michaelis complex (EI), followed by the irreversible formation of a stable complex (EI') between enzyme and inhibitor (reference 11 and references therein):



$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} EI'.$$

The following equation can be derived[11,12] that allows the determination of the Michaelis-type constant (K_i) and the first order rate constant (k_2) for the conversion of the EI complex to the stable EI' complex.

$$\frac{[I]}{k'} = \frac{[I]}{k_2} + \frac{K_i}{k_2} \quad \text{where } k' = \frac{k_2}{1 + K_i/[\mathrm{I}]}; \quad K_i = \frac{k_{-1}}{k_1}.$$

The pseudo first-order rate constant (k') for a given concentration of inhibitor can be obtained from logarithm % residual enzyme activity *vs* time plots either from the slopes of the lines which are equal to $-k'/2.3$, or from the time required for the loss of 50% of the initial enzyme activity $t_{1/2}$, and is numerically equal to (ln 2)/$t_{1/2}$, where

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ln 2 is the naperian logarithm of 2. Secondary plots of $[I]/k'$ against $[I]$ gives a line with a slope of $1/k_2$ and an intercept on the abscissa equal to $-K_i$.

RESULTS

Inhibition of Thymidylate Synthase

The results of the inhibition of thymidylate synthase by DCP (4′,5′-dichloropyridoxine), DBP (4′,5′-dibromopyridoxine) and DIP (4′,5′-diiodopyridoxine) at various inhibitor concentrations and at various incubation times are shown in Figure 1. All three vitamin B_6 analogues inhibited thymidylate synthase though the relative rates of inhibition for these compounds differed significantly. The inhibition reactions were conducted in the presence of a large molar excess of inhibitor relative to enzyme, thus the concentration of inhibitor remained constant during the inhibition reaction. The semilogarithmic plots of residual activity *vs* time (Figure 1, left panels) were linear for the loss of at least 50% of the enzyme activity for all inhibitors and at all concentrations tested, which indicates that the inhibition of thymidylate synthase by these compounds is a pseudo first-order process with respect to enzyme. The pseudo first-order rate constants (k') for each inhibitor concentration were obtained from the time required for the loss of 50% of the activity ($t_{1/2}$): $k' = (\ln 2)/t_{1/2}$. For the three inhibitors tested, plots of k' *vs* inhibitor concentration were not linear (Figure 1, right panels) which means that the average order of the reactions are not first order with respect to inhibitor. A limiting rate of inhibition was approached as the concentration of DCP or DBP was increased (Figure 1, panels A and B) which suggests that a reversible enzyme : inhibitor affinity complex is formed between thymidylate synthase and DCP and DBP prior to the irreversible reaction. Values for K_i and k_2 for DCP and DBP were determined by replotting $[I]/k'$ *vs* $[I]$ (see Figure 2 and Methods section). As shown in Table I, DBP exhibited a greater binding affinity (lower K_i) for thymidylate synthase than DCP, and it also reacted faster (larger k_2) to irreversibly inhibit the enzyme. The inhibition data for DIP showed that the average order of this reaction was second order with respect to inhibitor, and since there was no indication that a limiting rate of inhibition was approached (Figure 1, panel C) the formation of an affinity complex was not evident, although it cannot be ruled out.

The presence of the substrate dUMP decreased the rate of inhibition of thymidylate synthase by DCP, DBP and DIP. At a concentration of $10\,\mu M$, dUMP completely protected thymidylate synthase from inhibition (Figure 3). These data suggest that the halogenated vitamin B_6 analogues are active-site-directed inhibitors of thymidylate synthase, which bind to the dUMP binding site.

Dialysis of thymidylate synthase that had been completely inactivated by treatment with these inhibitors did not result in significant recovery of activity, demonstrating that the inhibition of thymidylate synthase by DCP, DBP and DIP is irreversible.

DISCUSSION

The mechanism of inhibition of thymidylate synthase by the chlorinated(DCP) and brominated(DBP) vitamin B_6 analogues probably involves displacement of the halide (X^-) by nucleophilic attack of an enzyme sulfhydryl group at the C-4′ of the inhibitor to form a stable carbon-sulfur bond as shown below. This mechanism is similar to the

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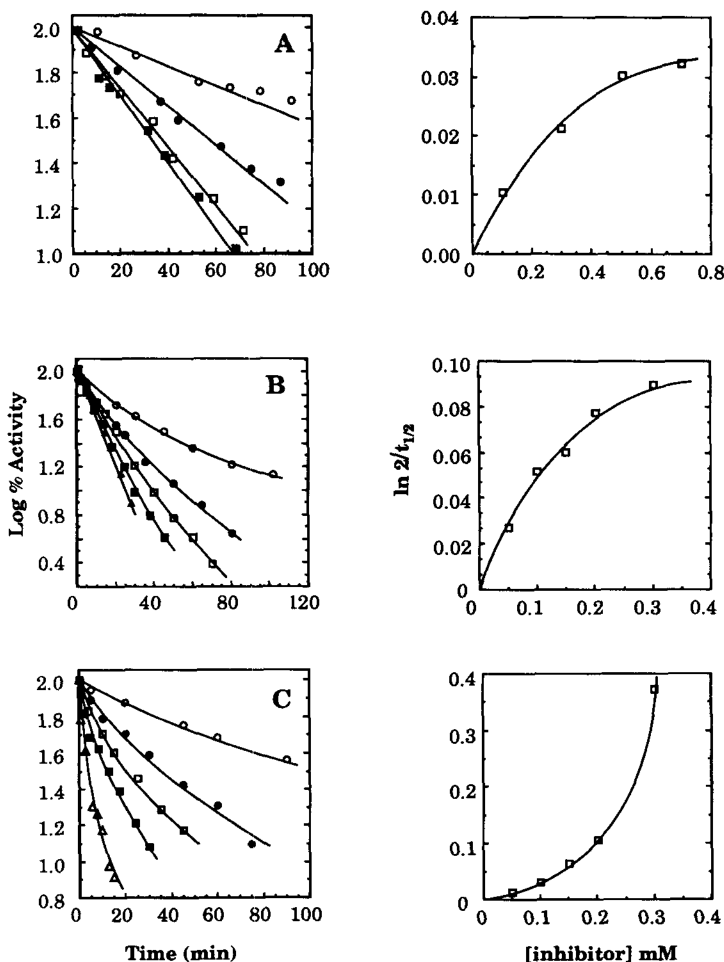

FIGURE 1 Inhibition of thymidylate synthase by DCP (panel A), DBP (panel B) and DIP (panel C): *Left panels*: Semi-log plots of % remaining enzyme activity *vs* time of preincubation with inhibitor. The concentrations of DCP (panel A) were 0.1 mM (○), 0.3 mM (●), 0.5 mM (□), and 0.7 mM (■); the concentrations of DBP (panel B) were 0.05 mM (○), 0.10 mM (●), 0.15 mM (□), 0.2 mM (■), and 0.3 mM (△); and, the concentrations of DIP (panel C) were 0.05 mM (○), 0.10 mM (●), 0.15 mM (□), 0.2 mM (■), and 0.3 mM (△). Inhibition reaction mixtures contained thymidylate synthase (6 μM) and inhibitors in 20 mM PIPES buffer, pH 6.8 at 25°C. Aliquots (20 μl) were withdrawn at the times shown and assayed for enzyme activity. Each point represents a single determination. Log % activities are log residual activities expressed as a percentage of the enzymatic activity in reaction mixtues lacking inhibitor, but otherwise identical. *Right panels*: Replots of (ln 2)/$t_{1/2}$ *vs* [DCP] (panel A), [DBP] (panel B), and [DIP] (panel C). Values for $t_{1/2}$ (time required for a 50% loss of activity were obtained from the corresponding semi-log plots (left panels).

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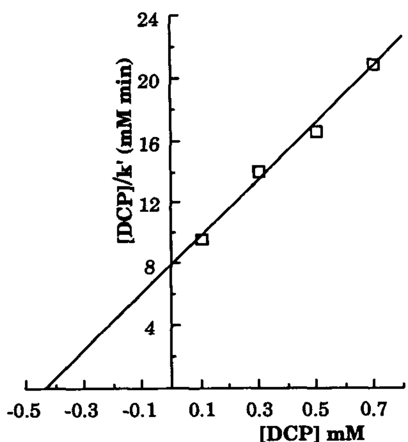

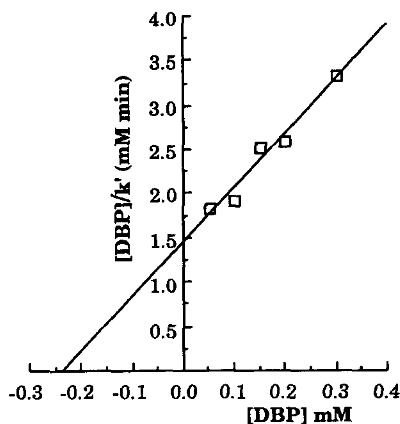

FIGURE 2 Replots of [DCP]/k' *vs* [DCP] (*upper panel*), and [DBP]/k' *vs* [DBP] (*lower panel*). The slope of the line equals $1/k_2$ and the intercept on the abscissa equals $-K_i$. Each of the k' values were obtained from one of the lines in Figure 1, where each line was obtained from 6 or 7 activity determinations at different inhibition times.

TABLE I
Inhibition constant (K_i) and rate constant (k_2) for DCP and DBP[a]

Compound	K_i(mM)	k_2(min^{-1})
DCP	0.44	0.055
DBP	0.24	0.163

[a] Values for K_i and k_2 were calculated from Figure 2 as described in the Methods section.

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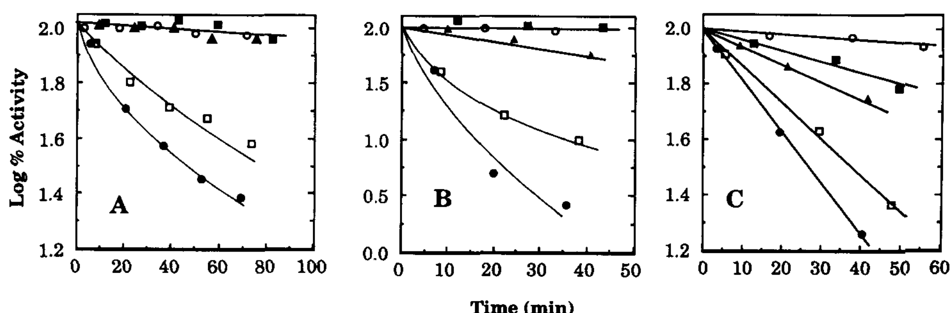
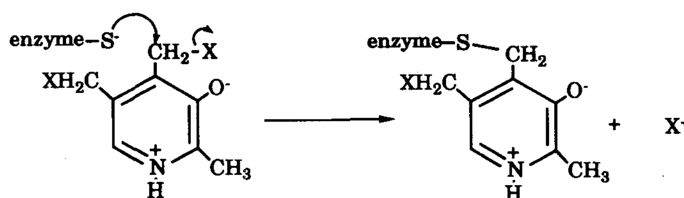


FIGURE 3 Protection of thymidylate synthase by dUMP against inhibition by 0.5 mM DCP (panel A), 0.1 mM DBP (panel B), and 0.1 mM DIP (panel C). dUMP was added to the enzyme solution prior to adding inhibitor. Aliquots (20 μl) were withdrawn at the times shown and assayed for enzyme activity. One reaction mixture contained no inhibitor (○). The dUMP concentrations were (○) 0, (●) 0, (□) 1 μM, (▲) 5 μM, and (■) 10 μM.



mechanism suggested by Chen *et al.*[2] for the reversible inhibition of thymidylate synthase by pyridoxal phospate (PLP), which involved nucleophilic attack of an enzyme sulfhydryl on the C-4′carbonyl of PLP to form a thiohemiacetal. We suggest displacement of the halogen at the C-4′ rather than the C-5′ because our kinetic[2] and binding[4,13] studies indicate that the C-4′ of PLP is properly positioned to react with an enzyme sulfhydryl group when PLP is bound to the active site; and the C-4′ is more electrophilic than the C-5′. Bromide is known to be a better leaving group than chloride which provides a reasonable explanation of why DBP inhibited thymidylate synthase more rapidly than DCP. The more complex kinetcs exhibited by the iodinated compound (DIP) suggests a different mechanism of inhibition, perhaps involving heterolytic cleavage of the C–I bond to produce I^- and a carbocation which is subsequently attacked by the enzyme sulfhydryl. Alternatively, the C–I bond could break homolytically to produce free radicals which could react with catalytically essential amino acids.

Previous studies have shown that the phosphate group on the 5′-position of pyridoxal phospate is important for the inhibition of thymidylate synthase since PLP was a better inhibitor of thymidylate synthase than pyridoxal,[14] and it formed a tight affinity complex with the enzyme with a K_i of about 1 μM.[2] It has been suggested that the phosphate group of PLP binds to the enzyme site that normally binds the phosphate group of the substrate(dUMP),[2] which would account for the general observation that phosphorylated compounds bind better to thymidylate synthase than the corresponding non-phosphorylated ones.[14] The bromine at the 5′-position of DBP, which is larger and more polarizable than the chlorine in DCP, may bind to the positively charged arginine residues in the phosphate binding site of the enzyme,

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providing a possible explanation for the tighter binding (lower K_i) of DBP. This rationalization suggests that 4′monohalogenated derivatives of pyridoxine 5′-phosphate should be very good inhibitors of thymidylate synthase because they would be superior to the non-phosphorylated compounds in forming an enzyme: inhibitor affinity complex.

Acknowledgement

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