When the cofactor concentration was varied, the substrate concentration was maintained at saturating levels, 100 μM.

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5-Cyano-2′-deoxyuridine 5′-Phosphate: A Potent Competitive Inhibitor of Thymidylate Synthetase

Charles T.-C. Chang, Michael W. Edwards, Paul F. Torrence, and Mathias P. Mertes*[1]

Department of Medicinal Chemistry, School of Pharmacy, University of Kansas, Lawrence, Kansas 66045, and the Laboratory of Chemistry, National Institute of Arthritis, Metabolism and Digestive Diseases, U.S. National Institutes of Health, Bethesda, Maryland 20014. *Received March 1, 1979*

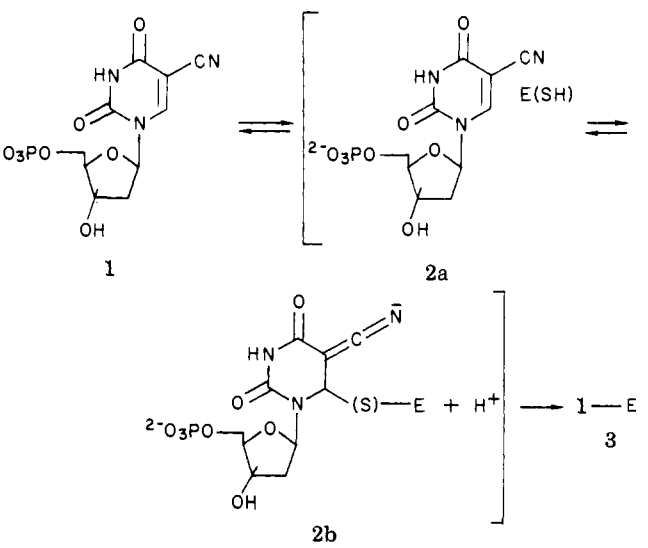
The 5′-phosphate (1) of the antiviral nucleoside 5-cyano-2′-deoxyuridine was synthesized and evaluated for inhibition of thymidylate synthetase purified from methotrexate-resistant *Lactobacillus casei*. Compound 1 was a potent competitive inhibitor with a K_I of 0.55 μM. Irreversible enzyme inhibition by this compound could not be detected.

Thymidylate synthetase (EC 2.1.1.45) catalyzes a two-step reductive alkylation of 2′-deoxyuridine 5′-phosphate (dUMP) to give thymidine 5′-phosphate (dTMP). Inhibition of this enzyme by 5-substituted derivatives of the substrate has been clinically useful in the control of cancer and viral infections.[2a,b] Two of the most effective compounds in this regard, the 5-fluoro and 5-(trifluoromethyl) derivatives, are notably electron withdrawing. A quantitative assessment of the electronic and polar effects on inhibition by various 5-substituted 2′-deoxyuridine 5′-phosphates has been reported by Hansch and co-workers[3] who concluded that the electronic effect (electron withdrawal) is the major factor contributing to enzyme affinity. Since the inductive and resonance effects of the cyano group are great, the introduction of this group at the 5 position of 2′-deoxyuridine 5′-phosphate should result in potent inhibition.

5′-Cyano-2′-deoxyuridine is able to arrest the multiplication of vaccinia virus in cell cultures,[4] albeit not as effectively as the halogenated analogues 5-bromo-2′-deoxyuridine and 5-iodo-2′-deoxyuridine. Evidence has been presented elsewhere that suggests that 5-cyano-2′-deoxyuridine must first be phosphorylated to the corresponding 5′-monophosphate[5] and that this may then block thymidylate synthetase.[6] This latter hypothesis was based largely on the observation that 5-cyano-2′-deoxyuridine inhibited labeled 2′-deoxyuridine incorporation into DNA but had no effect on labeled deoxythymidine incorporation. For this reason and because of the wealth of literature relating to the biological activity of the 5-halo-2′-deoxyuridines to inhibition of thymidylate synthetase, it was of considerable interest to examine the effect of the 5-cyano derivative 1 on the enzyme.

A further aspect of this study was the potential irreversible k_{cat} type inhibition that would be expected if an

Scheme I



enzyme nucleophile (cysteine SH) at the active site added to carbon-6 of the pyrimidine ring. Under similar experimental conditions, enzyme inactivation has been noted[2] for the 5-fluoro-, 5-(trifluoromethyl)-, 5-carboxyaldehyde-, and 5-nitro-2′-deoxyuridine 5′-phosphate.[7,8] The enzyme–inhibitor complex which requires cofactor for inactivation has been characterized[9,10] only for the 5-fluoro derivative. Santi and Sakai,[11] on the basis of model studies, formulated a two-step mechanism for the inactivation of thymidylate synthetase by 5-(trifluoromethyl)-2′-deoxyuridine 5′-phosphate.

As seen in Scheme I, if the enzyme nucleophile adds to carbon-6 of 5-cyano-2′-deoxyuridine 5′-phosphate (1) the resultant complex (2) would be a ketenimine,[12] a strong

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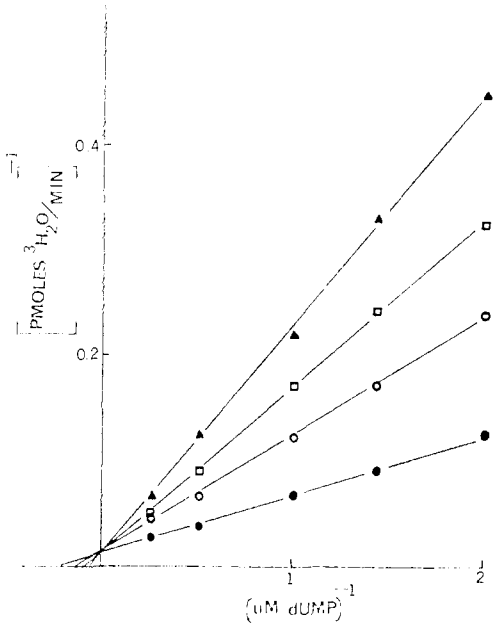

Figure 1. Reciprocal plot of the velocity of thymidylate synthetase catalysis vs. concentration of 2′-deoxyuridine 5′-phosphate in the absence and presence of the inhibitor 5-cyano-2′-deoxyuridine 5′-phosphate: I = 0 (●); 0.5 μM (○); 1.0 μM (□); 2.0 μM (▲).

electrophile that could interct with a nucleophilic group of the enzyme, resulting in enzyme inactivation. Such a mechanism, enzymatic interaction leading to formation of a chemically reactive group as an intermediate in the catalysis, is termed a k_{cat} or suicide inhibitor.[13] Thus, compound 1 was prepared as a potential suicide inhibitor of thymidylate synthetase based on its potential for acting as a substrate for the first step in the enzyme-catalyzed reaction.

Results

5-Cyano-2′-deoxyuridine 5′-phosphate was evaluated as an inhibitor of *Lactobacillus casei* thymidylate synthetase. In this assay, a measure of ^3H_2O released, the enzyme–substrate complex has a K_m of 4.4 μM which compares favorably with the range of 0.7–5.2 μM using the spectrophotometric assay.[14] A reciprocal plot of the velocity vs. substrate (dUMP) concentration (Figure 1) shows competitive inhibition and a K_I of 0.55 μM for 1 calculated from the line slopes.

Acrylonitrile, widely recognized as a strong electrophile in cyanoethylation reactions,[15] is a classic example of a Michael acceptor. The electronic effect of the cyano group in compound 1 would be expected to enhance nucleophilic or Michael addition to carbon-6 of the pyrimidine ring. Coupled with the fact that the enzyme catalysis is thought to occur by addition of a cysteine SH group to carbon-6 of the substrate,[9,10] compound 1 clearly should enhance this addition reaction. If this occurs, 1, acting as a suicide inhibitor, could interact with the enzyme as a mechanism-based affinity-labeling reagent[7] as depicted in Scheme I.

Irreversible inhibition of this enzyme can be established by the usual techniques of enzyme preincubation with the inhibitior in the absence of cofactor for varying times, followed by assaying at high substrate concentrations to measure remaining activity. By this method, two affinity labeling reagents, the 5-nitro[7] and 5-(α-bromoacetyl)[16] derivatives of 2′-deoxyuridine 5′-phosphate, were observed to irreversibly inactivate thymidylate synthetase. At concentrations up to 4 μM, approximately 10 times the K_I value and 100 times the enzyme concentration, compound 1 did not show any significant loss of enzyme activity in the absence of cofactor over a 12-min incubation period. Thus, the second step, conversion of the reversible EI complex 2 to an irreversible complex 3, could not be established for 1.

Discussion

5-Halo-substituted 2′-deoxyuridine 5′-phosphates show K_I values for binding to this enzyme ranging from 0.014 (5-F) to 1.6 μM (5-I).[3] Considering the bulk of the cyano group as a cylinder diameter of 3.6 Å, this compares to the sphere bulk of a 5-chloro (K_I = 0.19 μM) estimated to have a diameter of 3.6 Å or 5-bromo (K_I = 1.4 μM) with a diameter of 3.9 Å.[17] The K_I of compound 1 at 0.55 μM is intermediate in this range and, further, approximates the steric bulk of the chloro or bromo derivative. Hansch and co-workers[3] concluded from their regression analysis that the major effect contributing to enzyme binding was electronic in nature. The inductive and resonance σ values for cyano exceed the values for halo groups and, considering the importance of the electronic effects, 1 should have significantly greater enzyme affinity than the 5-chloro derivative. Using σ_p substituent constants[18] the cyano group (σ_p = 0.63), being intermediate between the trifluoromethyl group (σ_p = 0.55) and the nitro group (σ_p = 0.78), should have enzyme affinity in the same range. This is not the case, since both the 5-(trifluoromethyl)[3] (K_I = 0.039 μM) and the 5-nitro[7,8] (K_I = 0.029) derivatives have ten times greater affinity for this enzyme than does the cyano derivative 1. Another exception to the electronic effect of the 5-substituent as the major contribution to enzyme affinity is the 5-mercapto derivative.[19] The sulfhydryl group in 5-mercapto-2′-deoxyuridine 5′-phosphate is essentially ionized at pH 7, yet this compound has high affinity for thymidylate synthetase (K_i = 0.04 μM).

Although verified only for the 5-fluoro derivative, mechanism-based irreversible inactivation of this enzyme by the 5-fluoro, 5-(trifluoromethyl), 5-(nitro), 5-(α-bromoacetyl), and 5-carboxaldehyde derivatives of the substrate has been postulated wherein the cysteine SH adds to carbon-6 to give the covalent complex. With this background, the absence of any detectable inactivation of the enzyme by compound 1 was completely unexpected. It would appear that bimolecular model studies could aid in understanding the reaction sequence for the 5-carboxaldehyde and 5-nitro derivatives and perhaps explain why 5-cyano-2′-deoxyuridine 5′-phosphate (1) does not inactivate the enzyme. Another condition that could be important for inactivation is the presence of cofactor during the incubation period. This is recognized as a requirement for inactivation by the fluoro and carboxyaldehyde derivative of the substrate.

Experimental Section

Thymidylate synthetase purified from methotrexate-resistant *Lactobacillus casei* was purchased from the New England Enzyme Center, Tufts University, at a specific activity of 1.03 μmol of TMP formed min^{-1} (mg of protein)$^{-1}$ using the radioisotope assay. The enzyme was activated by dialysis for 4 days at 4 °C against 0.1 M potassium phosphate (pH 6.8) containing 50 mM mercaptoethanol. The substrate 2′-deoxy[5-^{3}H]uridine 5′-phosphate at a specific activity above 15 Ci/mmol was purchased from Moravek Biochemicals, Industry, Calif., and diluted with cold substrate purchased from Sigma Chemical Co., St. Louis, to give a specific activity of 500 μCi/μmol. The cofactor, *dl*-tetrahydrofolic acid, was also purchased from Sigma Chemical Co.

Preparation of 5-Cyano-2′-deoxyuridine 5′-Monophosphate (1). A modification of the procedure of Yoshikawa et al.[20] was followed to phosphorylate 5-cyano-2′-deoxyuridine. 5-Cyano-2′-deoxyuridine (0.2 mmol), prepared as described elsewhere,[4] was

dissolved in 0.6 mL of a trimethyl phosphate solution containing 0.061 mL (0.02 mmol) of phosphorous oxychloride and 5 μL of water. The mixture was maintained at 0 °C for 4 h; then diethyl ether (8 mL) and water (0.5 mL) were added consecutively to the reaction mixture. After shaking in a separatory funnel, the separated aqueous layer was applied to Whatmann 3MM paper. Development of the chromatogram was accomplished with isopropyl alcohol–ammonia–water (7:1:2) as solvent. After elution from the paper and lyophilization of the aqueous solution to dryness, a hygroscopic colorless solid (6 mg, 10% yield) was obtained. The product had R_f values of 0.89 and 1.36 (relative to 2′-deoxyuridine 5′-monophosphate) in ethanol–1 M ammonium acetate (pH 7.5) (7:3, v/v) and isopropyl alcohol–ammonia–water (7:1:2), respectively. The UV λ_{max} of the product was the same as the unphosphorylated nucleoside [λ_{max} (H_2O) 278 nm]. Digestion of a portion of the product with bacterial alkaline phosphatase gave 5-cyano-2′-deoxyuridine as the sole product, as determined by thin-layer chromatography (silica gel, $CHCl_3$–CH_3OH).

Enzyme Assay. The enzyme was assayed by modification of the radioisotope assays described by Roberts[21] and Lomax and Greenberg.[22] The solution, 0.1 mL, contained 25 mM mercaptoethanol; 0.22 mM *dl*-tetrahydrofolic acid; 6.75 mM formaldehyde; 5 mM sodium bicarbonate; 3 mM magnesium chloride; 0.12 mM EDTA; 6 mM Tris–acetate buffer, pH 6.8; 5 μL of the diluted enzyme solution; substrate; and, when indicated, inhibitor. Control reactions lacked the cofactor, tetrahydrofolic acid. The substrate 2′-deoxy[5-^{3}H]uridine 5′-phosphate was used at a specific activity of 500 μCi/μmol. The assays were started by the addition of the enzyme to the complete mixture and then incubated at 30 °C. Incubation was stopped at 30 s by the addition of 50 mL of 20% trichloroacetic acid. A 20% aqueous suspension of charcoal (0.25 mL) was added to the solution, and then the solution was vortexed and allowed to stand for 15 min. The suspension was filtered through a glass-wool-plugged Pasteur pipet, and 0.1 mL of the filtrate was counted in a scintillation fluid containing 0.5% 2,5-diphenyloxazole and 10% Beckman BBS-3 solubilizer in toluene. Counting efficiency was 33%; control samples lacking the cofactor were found to have less than 5% of the respective sample counts. On the basis of specific activity, velocity is reported in the adjusted value of picomoles of ^3H_3O formed per minute in the assay.

Preincubation Studies. The enzyme (5×10^{-8} M) was preincubated at 30 °C in 50 μL of solution containing 5 mM 2-mercaptoethanol; 6 mM magnesium chloride; 0.24 mM EDTA; 12 mM Tris–acetate buffer, pH 6.8; and varying concentrations of inhibitor. After incubation for the indicated time period, the assay for the remaining active enzyme was started by the addition of 50 μL of a solution containing buffer and other components of the assay to give the same concentrations as noted in the enzyme assay. A high substrate concentration (40 μM) was used in these assays to afford a reasonably high velocity and to competitively reduce the enzyme inactivation by the inhibitor during the assay. The assay was run for 30 s and treated as described in the enzyme assay section. Inactivation of the enzyme was measured by comparing the velocity at time zero to that at the indicated incubation times. Under the conditions of the assay, the uninhibited enzyme retained 95% of the initial activity after 20 min of incubation.

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