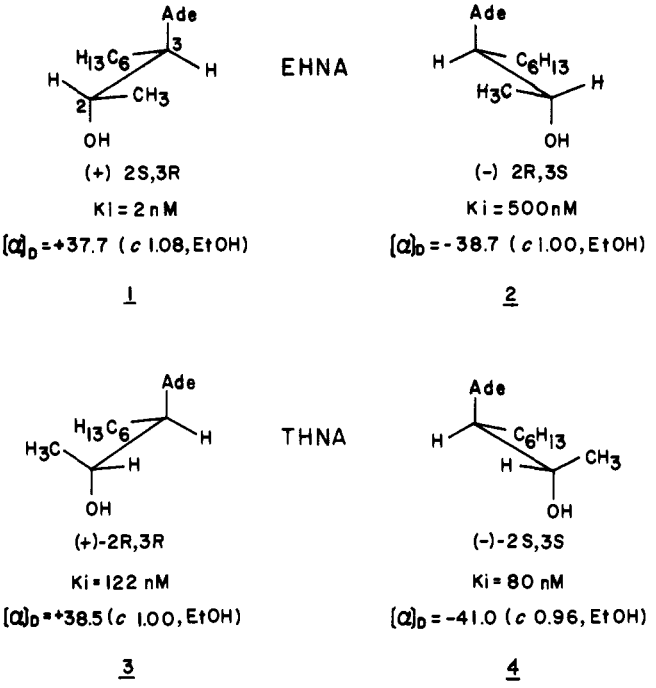
Chart I



pyranoside[15] 18 followed by catalytic reduction, and formation of the 6-deoxy derivative (**20**) according to known methods (Scheme II).[16]

Inhibition of human erythrocytic ADA was performed on all four isomers (**1–4**), and the K_i values obtained are listed in Chart I. The most active compound was found to be (+)-*erythro*-9-[2(*S*)-hydroxy-3(*R*)-nonyl]adenine (**1**).[17] Its enantiomer was the least active, while the two threo isomers, where only one chiral center is inverted, had reduced but comparable activities. Their effect on purine metabolism as well as their chemotherapeutic activity are currently under investigation.

It is worth noting that the synthetic methods described herein are versatile, utilize inexpensive starting materials, and offer the opportunity to incorporate the amino alcohols **5** into other heterocycles. Of special interest will be future attempts to attach **5d** to the aglycon of coformycin.[3] Furthermore, the Wittig reaction can be modified to introduce functional groups on the lipophilic hydrocarbon portion of **5**. Such groups could play an important role either as ligands, which might aid in the purification of ADA, or as probes of the hydrophobic center of the enzyme, resulting in a more potent inhibitor.

Acknowledgment. This work was supported by PHS Grants CA 24885 and CA 07340. It is a collaborative effort of the Roger Williams Cancer Center, CA 20892 and CA 13943. We thank Richard Ware of Pfizer, Inc., for the high-resolution mass spectra and Dr. G. Menichi for the synthesis of compound **17**.

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(18) Chargé de Recherches, INSERM, France.

Gerard Bastian,[18] Michel Bessodes[18]
Raymond P. Panzica,* Elie Abushanab*
Department of Medicinal Chemistry
University of Rhode Island
Kingston, Rhode Island 02881

Shih-Fong Chen, Johanna D. Stoeckler
Robert E. Parks, Jr.
Section of Biochemical Pharmacology, Brown University
Providence, Rhode Island 02912
Received July 16, 1981

Inhibition of Thymidylate Synthetase by 5-Alkynyl-2′-deoxyuridylates[1]

Sir:

Thymidylate (dTMP) synthetase (EC 2.1.1.45) catalyzes the conversion of 2′-deoxyuridylate (dUMP) and 5,10-methylenetetrahydrofolate (CH_2-H_4folate) to 2′-deoxythymidylate (dTMP) and 7,8-dihydrofolate. This enzyme represents the sole de novo pathway for dTMP synthesis and has received much attention as a target for inhibitors with potential chemotherapeutic value. The catalytic mechanism and inhibition of this enzyme have recently been reviewed.[2,3] One class of potent inhibitors of this enzyme is 5-substituted dUMP's, which act as mechanism-based inhibitors. An early event in the normal enzymatic reaction involves nucleophilic attack of a cysteine thiol of the enzyme at the 6 position of dUMP to form 5,6-dihydropyrimidine intermediates which are covalently bound to the enzyme during the remaining catalytic sequence. The 5-substituted dUMP's, which are mechanism-based inhibitors of this enzyme, undergo similar nucleophilic attack at the 6 position; subsequently, the analogue either remains attached to the enzyme or a moiety at the 5 position of the inhibitor is activated so that it may covalently interact with the enzyme.

β,γ-Acetylenic carbonyl compounds have received much attention as suicide inactivators of enzymes.[4,5] The acetylenic functional group is normally inert toward nucleophiles, but enzyme-catalyzed generation of a carbanion at the α carbon can result in isomerization to a conjugated allene; the latter is a powerful Michael acceptor, and if a nucleophile of the enzyme is juxtaposed to the reactive β carbon, covalent bond formation can occur. As suggested by other workers,[4,6] since the initial covalent bond formation between dTMP synthetase and 5-substituted dUMP's generates a transient carbanion at the 5 position, 5-alkynyl-dUMP's are potential suicide inactivators of this enzyme. 5-Ethynyl-dUrd (EdUrd) has recently been

(1) Abbreviations used: E, 5-ethynyl; HOP, 5-(3-hydroxypropynyl); HOB, 5-(4-hydroxybutynyl); H, hexynyl; PhE, 2-phenylethynyl. All other abbreviations are those recommended by IUPAC.
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0022-2623/81/1824-1385$01.25/0

prepared and shown to be a potent growth inhibitor of cells grown in tissue culture;[7,8] in addition, a preparation of 5-ethynyl-dUMP (EdUMP) was found to inhibit dTMP synthetase.[7] Unfortunately, the tedious synthesis of these compounds precluded a thorough evaluation of EdUMP, and its mechanism of action was unresolved. Recently, a facile, general synthesis of 5-alkynyl-dUrd's has been reported.[9] Here, we describe preliminary observations of the inhibitory properties of a number of 5-alkynyl-dUMP's toward dTMP synthetase, as well as the growth inhibitory properties of the corresponding nucleosides toward S-49 mouse lymphoma cells.

dTMP synthetase was obtained from a methotrexate-resistant strain of *Lactobacillus casei* and purified as previously described.[10] Enzyme[11,12] and growth inhibition[13] assays were performed using reported procedures. HPLC was performed using a Lichrosorb C_{18} column (4.6 × 250 mm) at 30 °C with a flow rate of 2 mL/min. System A was 5 mM $(n\text{-Bu})_4N^+HSO_4^-$ and 5 mM KH_2PO_4 (pH 7.1) in specified amounts of MeOH/H_2O; solvent system B was 10% MeOH in H_2O. The 5-alkynyl-2′-deoxyuridines were those previously reported.[9] (+)L-CH_2-[6-^{3}H]H_4folate (34.5 mCi/mmol) was a gift from T. W. Bruice. EdUMP was prepared by phosphorylation of EdUrd using *Escherichia coli* dThd kinase and partially purified by DEAE-cellulose chromatography using methods previously described.[10] To remove residual AMP, the product was dissolved in 1 mL of 1 M $Et_3NH^+\cdot HCO_3^-$ (pH 8.5) and applied to an Affigel 601 boronate column (1.5 × 8 cm) which was previously equilibrated in the same buffer. After washing with 20 mL of the above buffer, the combined eluate was lyophilized to dryness and then coevaporated with H_2O (3 × 10 mL). The product was obtained in 85% overall yield and shown to be pure by HPLC using system A/8% MeOH. [2-^{14}C]EdUMP (59 mCi/mmol) was prepared from [2-^{14}C]-EdUrd in a similar manner, except after phosphorylation the mixture was deproteinized with 2 volumes of cold MeOH and the nucleotide was purified by HPLC (system A/8% MeOH): yield 78%. HOPdUMP and HOBdUMP were prepared in 23 and 30% yields, respectively, using carrot phosphotransferase as previously described.[14] After DEAE-cellulose chromatography, HPLC of the preparations (system A/16% MeOH) showed HOPdUMP (k' = 7.2) containing 20% of HOPdUrd-3′-P (k' = 9.8) and HOBdUMP (k' = 8.3) containing 18% of HOBdUrd-3′-P (k' = 12.2). PhEdUMP and HdUMP were prepared by chemical phosphorylation using $POCl_3$/triethyl phosphate.[15] After purification by DEAE-cellulose chromatography, PhEdUMP and HdUMP were obtained in 25 and 26% yields, respectively. PhEdUMP (k' = 4.7) and HdUMP (k' = 4.6) were shown to be pure by HPLC using system B. Nitrocellulose filter assays of the EdUMP–CH_2-H_4folate–dTMP synthetase complex were performed as previously described for the FdUMP complex.[16] A solution containing 1.0 μM enzyme, 10 μM [2-^{14}C]EdUMP (17.7 mCi/mmol), and 50 μM CH_2-[6-^{3}H]H_4folate (34.5 mCi/mmol) in the standard assay buffer[11] was incubated at 25 °C for 15 min. Aliquots (100 μL) were applied to

Table I. Inhibition of dTMP Synthetase by 5-Alkynyl-dUMP's

compd	5-substituent	K_i, μM
EdUMP	C≡CH	0.1
HOPdUMP	C≡CCH_2OH	3.0
HOBdUMP	C≡CCH_2CH_2OH	1.9
HdUMP	C≡C$CH_2CH_2CH_2CH_3$	2.6
PhEdUMP	C≡CC_6H_5	2.0

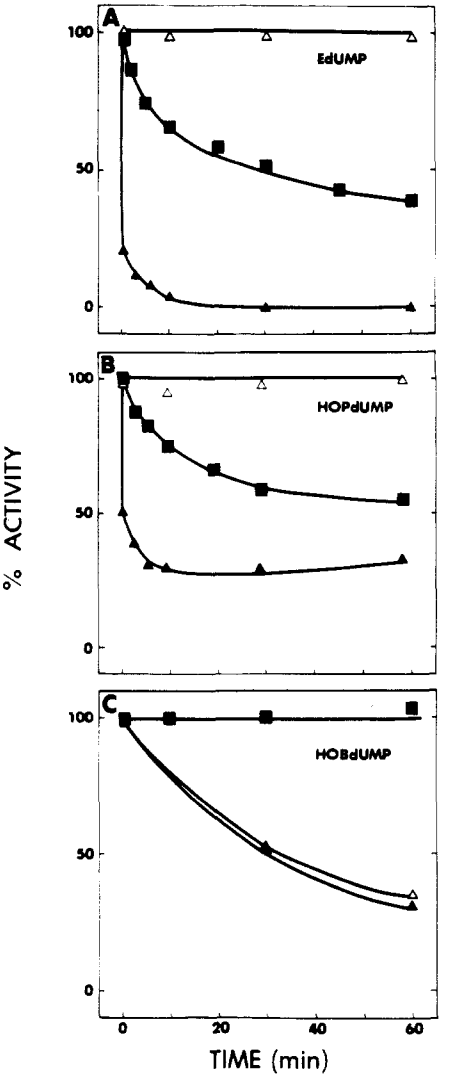

Figure 1. Time-dependent inactivation of 3 × 10^{-7} M dTMP synthetase by 10^{-5} M 5-alkynyl-dUMP's. A: (△) EdUMP; (▲) EdUMP and 10^{-4} M CH_2-H_4folate; (■) EdUMP, 10^{-4} M CH_2-H_4folate, and 5 × 10^{-4} M CldUMP. B: (△) HOPdUMP; (▲) HOPdUMP and 10^{-4} M CH_2-H_4folate; (■) HOPdUMP, 10^{-4} M CH_2-H_4folate, and 10^{-5} M ClduMP. C: (△) HOBdUMP; (▲) HOBdUMP and 10^{-4} M CH_2-H_4folate; (■) HOBdUMP and 10^{-3} M dUMP. At indicated times, 100-μL aliquouts of the incubation mixture were added to 900 μL of a solution containing excess dUMP (0.84 mM) and CH_2-H_4folate (0.1 mM) in the standard buffer, and the initial velocity was recorded.[11,12]

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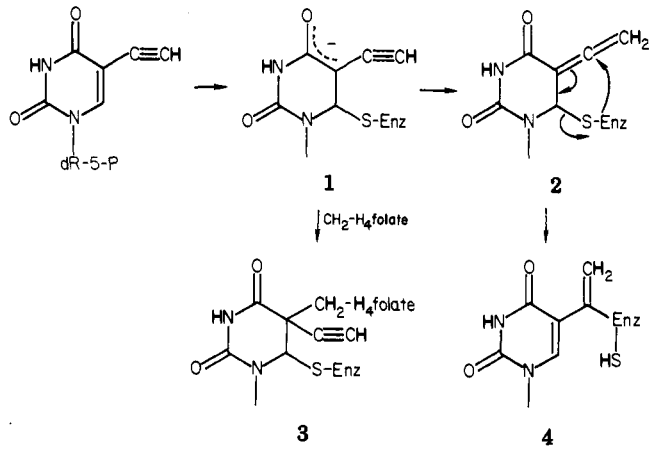
nitrocellulose filters, and unbound ligands were removed as previously described;[16] filters were dissolved in Aqueous Counting Scintillant (ACS; Amersham Corp.), and the radioactivity was determined by liquid scintillation counting.

The 5-alkynyl-dUMP's examined were good inhibitors of the initial velocity of dTMP formation as catalyzed by dTMP synthetase (Table I); all have approximate K_i values below the K_m of dUMP (5 μM), with EdUMP being the most potent (K_i = 0.1 μM). As previously discussed, K_i values of time-dependent inactivators which are determined by inhibition of initial velocities can be subject to error,[12] and the values reported for E-, HOP-, and HOBdUMP should be considered as approximations (see below).

To test whether the 5-alkynyl-dUMP's were mechanism-based inhibitors of dTMP synthetase, they were examined for their ability to cause time-dependent inactivation of the enzyme. At 50 μM concentration, PhE- and HdUMP caused no loss of activity after as long as 2-h incubation with the enzyme and 0.1 mM CH_2-H_4folate. As shown in Figure 1, incubation of the enzyme with EdUMP (10 μM) and CH_2-H_4folate caused a very rapid loss of activity (ca. 90% in 3 min) which could be protected against by the competitive inhibitor, CldUMP (K_i = 0.2 μM).[12] When cofactor was omitted from the incubation mixture, there was no inactivation after as long as 2 h. Similarly, HOPdUMP caused a rapid loss of activity in the presence but not in the absence of cofactor, which could be protected against by including CldUMP in the incubation mixture. The inactivation by HOPdUMP was somewhat slower than that observed with EdUMP and, for reasons not currently understood, did not proceed to completion. We have recently found that EdUMP and HOPdUMP undergo enzyme-catalyzed conversions to noninhibitory products which are competitive with enzyme inactivation. Such reactions may account for the unusual inactivation kinetics observed here and are currently being investigated. In contrast to EdUMP and HOPdUMP, HOBdUMP caused a time-dependent inactivation of the enzyme even in the *absence* of CH_2-H_4folate, which could be protected against by including 1 mM dUMP in the incubation mixture. None of the corresponding 5-alkynyl-dUrd's (0.1 mM) or the 3′-monophosphates of HOBdUrd and HOPdUrd (10 μM) caused any decrease in enzyme activity in the presence of cofactor (0.1 mM) over a 2-h period of incubation. It was also demonstrated that when the enzyme was incubated with an excess of [2-^{14}C]EdUMP and CH_2-[6-^{3}H]H_4folate for 15 min, a ternary complex could be trapped on nitrocellulose membranes which possess the nucleotide and cofactor in a ratio of 1.0:1.07. Taken together, the above data indicate that EdUMP, HOPdUMP and HOBdUMP inhibit dTMP synthetase by an active-site-directed process. First, although the kinetics of inactivation require clarification, they clearly show a time-dependent inactivation and protection against inactivation by nucleotides which bind to the active site (i.e., CldUMP and dUMP). Second, the inability of the corresponding 5-alkynyl-dUrd's to inactivate the enzyme is in accord with the known requirement of the 5′-phosphate of dUMP analogues for reversible binding to the enzyme and the fact that acetylenic moieties are not in themselves intrinsically reactive. Third, as has been demonstrated for a number of other inactivators of this enzyme,[17] time-dependent inhibition by EdUMP and HOPdUMP requires the presence of CH_2-H_4folate. The involvement of the cofactor in forming inhibitory complexes is further illustrated by the isolation of a complex which contains EdUMP and CH_2-H_4folate tightly bound to the enzyme in equivalent amounts.

Scheme I



1 2 3 4

Although the data described here do not permit assignment of the exact mechanism for the inactivation of dTMP synthetase by 5-alkynyl-dUMP's, it is of interest to consider a number of the more reasonable possibilities. Of course, the inactivation could result from slow formation of very tight E–I complexes which do not involve covalent bond changes; this could occur if a slow conformational change of the enzyme was required for formation of thermodynamically favorable noncovalent interactions. More interesting possibilities arise if the data are interpreted with regard to the combined knowledge of the catalytic mechanism of dTMP synthetase,[2] the behavior of this enzyme toward a number of mechanism-based inhibitors,[2,3,11] and the chemistry of acetylenic suicide substrates.[4,5] As shown for EdUMP in Scheme I, after formation of reversible E–I complexes, the catalytic nucleophile of the enzyme could attack the 6 position of the heterocycle to form the carbanion intermediate **1**; depending on the compound and conditions used, the latter could then react in one of a number of ways. For example, isomerization of **1** would form the conjugated allene **3**, which could further react with a nucleophile of the enzyme to form covalent E–I complexes. This could account for the time-dependent inactivation observed with HOBdUMP in the absence of CH_2-H_4folate. In this mechanism, the cofactor requirement for enzyme inactivation by EdUMP and HOPdUMP could be explained by conformational effects it imposes on the protein and by the formation of ternary complexes in which the cofactor is tightly but not covalently bound. Alternatively, inactivation of the enzyme might be due to the reaction of **1** with the cofactor to form a covalent ternary complex such as **2**; the latter is directly analogous to the covalent ternary complex formed with FdUMP and is in accord with the cofactor requirement for inactivation by EdUMP and HOPdUMP and the isolation of a stable EdUMP–CH_2-H_4folate–enzyme complex. Other mechanisms involving covalent bond changes induced by formation of **1** can also be envisioned but are not presented here. Work is in progress which is aimed toward elucidation of the mechanism of inhibition of dTMP synthetase by 5-alkynyl-dUMP's.

The inhibitory properties of 5-alkynyl-dUMP's toward dTMP synthetase prompted a study of the cytotoxicity of the corresponding nucleosides. EdUrd was a potent growth inhibitor of wild-type S49 cells (EC_{50} = 0.12 μM) but had no effect on a mutant line lacking dThd kinase

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(S49/TK$^-$) at 0.1 mM. Together with the enzyme studies reported here, these results provide strong support for previous proposals that the cytotoxicity of EdUrd in tissue culture cells primarily results from inhibition of dTMP synthetase.[6,8] The fact that HOP-, PhE-, and HdUrd were not cytotoxic toward S49 cells (wild type and TK$^-$) at 0.1 mM is probably due to their inability to serve as substrates for dThd kinase, since the corresponding nucleotides are all reasonably good inhibitors of dTMP synthetase, with HOPdUMP causing time-dependent inactivation. Further, dUrd analogues with large 5-substituents have generally proven to be poor substrates for dThd kinase.[18] In this regard, HOBdUrd appears to be an exception, since it is cytotoxic to wild-type S49 cells (EC_{50} = 70 μM) but has no effect on S49/TK$^-$ cells at 0.1 mM. In view of this curious finding, it would be of interest to investigate the structural specificity of dThd kinase in more detail.

Acknowledgment. This work was supported by USPHS grant CA 14394 from the National Cancer Institute.

Philip J. Barr, Patricia A. Nolan, Daniel V. Santi*
Department of Biochemistry and Biophysics and Department of Pharmaceutical Chemistry University of California San Francisco, California 94143

Morris J. Robins
Department of Chemistry, University of Alberta Edmonton, Alberta, Canada T6G 2G2

Received July 2, 1981

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Articles

Cell-Specific Ligands for Selective Drug Delivery to Tissues and Organs

Mitree M. Ponpipom,* Robert L. Bugianesi, James C. Robbins, T. W. Doebber, and T. Y. Shen

Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey 07065. *Received May 7, 1981*

Various numbers of D-mannose residues have been attached via spacer arms to lysine, dilysine, and oligolysine backbones. These D-mannosyl peptide analogues were found to be potent competitive inhibitors of the uptake of ^{125}I-labeled D-mannose–bovine serum albumin conjugate by rat alveolar macrophages. The inhibitory potency of these synthetic ligands increased with increasing number of carbohydrate moieties. The chirality of the peptide backbone did not appear to play a major role in binding, whereas variations of the length and linkage of the spacer arm notably affected the inhibitory activities. The saccharide specificity of the macrophage receptor was demonstrated by the inactivity of the corresponding D-galactosyl peptide analogues. The L-fucosyl peptide derivative was only weakly active. The trimannosyldilysine ligand (K_I = 3.9 μM) and its analogues are potentially useful in selective delivery of therapeutic agents to macrophages.

The use of drugs for therapeutic purposes would be greatly improved by the possibility of introducing them selectively into those cells where the pharmacological action is required. Many selective drug delivery systems have been described,[1] including binding a drug or radioactive atom to tumor-specific antibodies that seek only one type of malignant tissue. While this has worked well in some laboratory tests, treatment efforts have been much less successful. The targeting has usually been too imprecise and the payload too small. Deoxyribonucleic acid (DNA) has also been used as a carrier for antitumor drugs such as daunorubicin, adriamycin, and ethidium bromide.[1] Possible drawbacks of DNA as a drug carrier are its immunogenicity and its possible genetic effects. Human albumin microspheres covalently coupled to succinoyl-Ala-Ala-Pro-Val-CH_2Cl, an active-site-directed inhibitor of human leukocyte elastase, were successfully used to direct the inhibitor to the lungs of rats.[2] Another attractive drug-delivery system is the use of liposomes as carriers.[3,4] Liposomes are well tolerated by experimental animals and have been used with some success to treat respiratory distress syndrome in premature infants.[5] Substantial improvement in efficacy was also shown with liposome preparations of antimony potassium tartrate[6] and 8-aminoquinolines[7] against the parasite *Leishmania donovani* infection in mice. Liposomes containing antiinflammatory drugs were found to be selectively localized in the phagocytic synovial lining cells.[8,9] Beneficial responses were demonstrated in the treatment of joint inflammation of rabbits with experimental arthritis[10] and human patients with rheumatoid arthritis.[11] Glycolipid-containing liposomes with a terminal D-glucose or D-

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