Pergamon

Bioorganic & Medicinal Chemistry Letters 10 (2000) 391–394

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Mechanism-Based Inactivation of Thymidylate Synthase by 5-(3-Fluoropropyn-1-yl)-2′-deoxyuridine 5′-Phosphate

Thomas I. Kalman,* Zhe Nie and Ashwini Kamat

Department of Medicinal Chemistry, State University of New York at Buffalo, Buffalo, New York 14260, USA

Received 27 October 1999; accepted 17 December 1999

Abstract—5-Fluoropropynyl-2′-deoxyuridine 5′-phosphate (**3**) was designed as a mechanism-based inactivator of thymidylate synthase (TS). The inhibitor was synthesized from 5-iodo-2′-deoxyuridine and propargyl alcohol by palladium-catalyzed coupling, followed by fluorination and selective phosphorylation. Incubation of TS with **3**, in the presence or *absence* of the CH_2H_4folate cofactor, caused rapid, irreversible inactivation of the enzyme.

Thymidylate (dTMP) synthase (EC 2.1.1.45) catalyzes the conversion of deoxyuridylate (dUMP) and 5,10-methylenetetrahydrofolate (CH_2H_4folate) to dTMP and 7,8-dihydrofolate. This reaction is the sole de novo biosynthetic source of thymine in DNA. As a consequence, inhibition of dTMP synthase, in the absence of preformed thymidine, blocks DNA synthesis, and causes "thymineless death" of the cell.[1] Due to its essential role in cellular proliferation, this enzyme is recognized as a major target in cancer chemotherapy.[2–4]

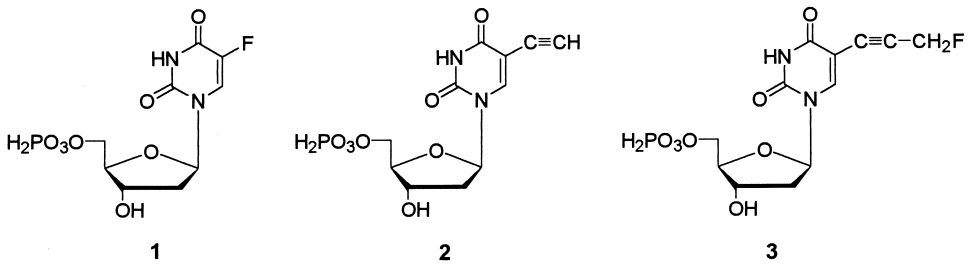
The most important dTMP synthase inhibitors of clinical utility are the 5-fluoropyrimidines: 5-fluorouracil (5-FU), 5-fluoro-2′-deoxyuridine (FdUrd), and their prodrug derivatives.[5] The active form of these drugs is the 5′-phosphate of FdUrd, FdUMP (**1**), which is a mechanism-based inhibitor of this enzyme. Its potent inhibitory activity is due to the reversible formation of an inactive, covalently bound ternary complex composed of **1**, the CH_2H_4folate cofactor, and the enzyme.[2,6] The stability of this complex in vivo depends on the intracellular levels of the polyglutamylated forms of CH_2H_4folate, which are often inadequate for effective inhibition of dTMP synthase. This prompted the search for novel analogues that do not require the cofactor for potent inhibitory activity. 5-Ethynyl-2′-deoxyuridine 5′-phosphate (**2**) was considered a promising candidate, because it has the potential for irreversible interactions via enzyme-mediated conversion of the acetylenic side-chain to a chemically reactive allene.[7] However, inactivation of dTMP synthase by **2** was found also to require the presence of CH_2H_4folate; in addition, the activity of the enzyme recovered very rapidly.[8–11] 5-Fluoropropynyl-dUMP (**3**), an improved derivative of **2**, was designed, based on a mechanistic rationale, as a prototype of a new generation of dTMP synthase inhibitors,[12,13] operating by a novel strategy of enzyme inactivation. In this communication, we describe the synthesis of **3**, and the initial results of our enzyme inhibition studies. These results demonstrate that **3** can cause rapid, irreversible inactivation of dTMP synthase, in both the presence and the *absence* of CH_2H_4folate.

Results and Discussion

Title compound **3** was synthesized as outlined in Scheme 1. The sugar hydroxyl groups of 5-iodo-2′-deoxyuridine (**4**) were protected by acetylation to yield **5**, which was reacted with propargyl alcohol by Pd(0)-catalyzed coupling[14] to yield **6**. Several methods of fluorination were tried for the conversion of **6** to **7**. Triflation of the alcohol followed by S_N2 displacement with fluoride failed to yield the desired product. Fluorination with Selectfluo (F-TEDA-BF_4), in the presence of Me_2S, was also unsuccessful. Direct fluorination of compound **6** could be carried out successfully with an excess of DAST at −78 °C, in anhydrous methylene chloride.[15] At the end of the reaction, the excess reagent was quenched with MeOH. After flash column chromatography, **7** was obtained in 30–40% yield.[16] Standard deprotection conditions could not be used, due to the reactivity of the fluoropropynyl side-chain. Dilute methanolic ammonia converted **7** to the corresponding amine, whereas the use of aqueous potassium carbonate

*Corresponding author.

0960-894X/00/$ - see front matter
PII: S0960-894X(00)00012-3



1 2 3

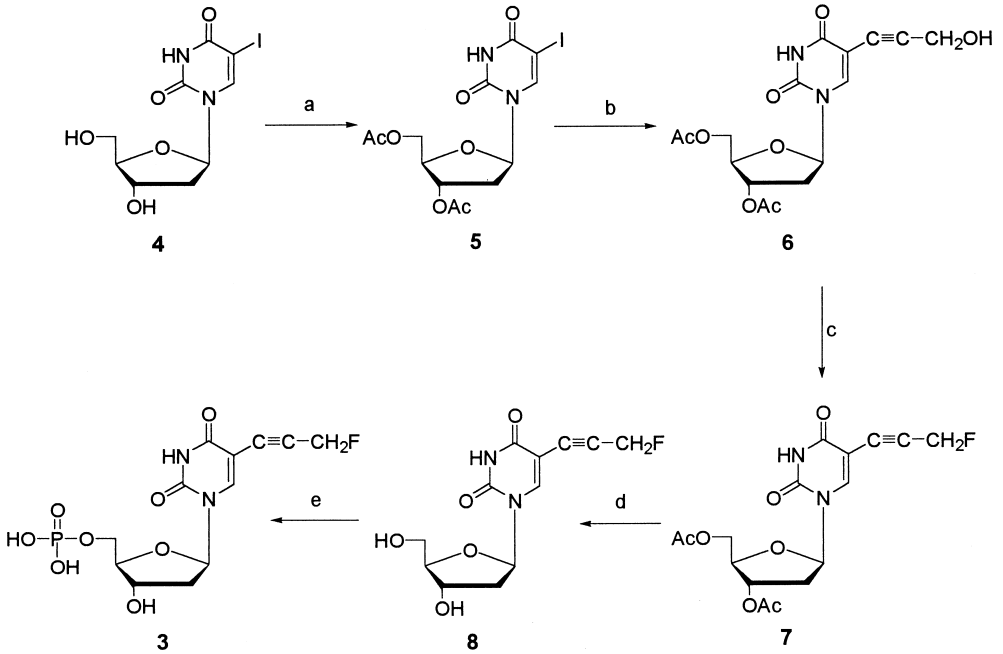
resulted in hydrolysis of the fluoromethyl group. Successful removal of the acetate protective groups of **7** could be carried out at 50 °C in MeOH, in the presence of a catalytic amount of HCl to yield **8**.[16] The 5′-OH group of **8** was selectively phosphorylated to yield **3**,[16] using $POCl_3$ in $PO(OEt)_3$, as described.[17]

Under standard conditions,[18] using the spectrophotometric assay, **3** was found to be a competitive inhibitor of *L. casei* dTMP synthase[19] with a K_i of 14.8 ± 3.0 nM. Preincubation of the enzyme with **3**, either in the presence, or in the absence of CH_2H_4folate, resulted in a rapid loss of enzyme activity. Figure 1 shows the time dependence of enzyme inactivation by **3** in the absence of CH_2H_4folate. It is shown that 5 μM **3** led to the inactivation of dTMP synthase with a $t_{1/2}$ of <1 min. The enzyme was protected by a saturating concentration of the substrate, dUMP, from inactivation by **3** (Fig. 1), indicating that the interaction occurs at the active site.

It was of interest to examine the reversibility of the inactivation of the enzyme by **3**, in light of the fact that most mechanism-based inhibitors of dTMP synthase, such as **1** and **2**, cause transient inactivation that can be reversed by dialysis.[6,8–11] When dTMP synthase, inactivated in the absence of CH_2H_4folate with **3**, was dialyzed exhaustively, no enzyme activity could be recovered over a period of 24 h. A typical dialysis experiment is shown in Figure 2.

These results strongly suggest that the inactivation process is irreversible, and that a stable, covalent binary complex is formed between dTMP synthase and **3**. Therefore, the mechanism of inhibition of the enzyme by **3** must be fundamentally different from that by either **1** or **2**.

It is postulated that the inactivation of dTMP synthase by **3** involves initial nucleophilic attack by the active site thiol at the 6-position of the pyrimidine ring, forming a covalent enolate intermediate, analogous to the normal enzyme catalyzed reaction.[6] This is followed by rapid expulsion of the fluoride ion, forming a covalently attached cumulene derivative (Fig. 3), in accordance with our design strategy.[12,13] This working hypothesis is consistent with the observed lack of requirement for the folate cofactor by the inactivation process.



4 5 6 7 8 3

Scheme 1. (a) Ac_2O, DMAP (cat.), rt, 12 h; (b) $CH{\equiv}CCH_2OH$, $Pd(PPh_3)_4$, CuI, Et_3N, DMF, rt, overnight; (c) DAST, CH_2Cl_2, −78 °C→rt, 2 h; (d) HCl (cat.), MeOH, 50 °C, 20 h; (e) 1. $POCl_3$, $PO(OEt)_3$, pyridine (cat.), −10 °C, 5 h; 2. H_2O.

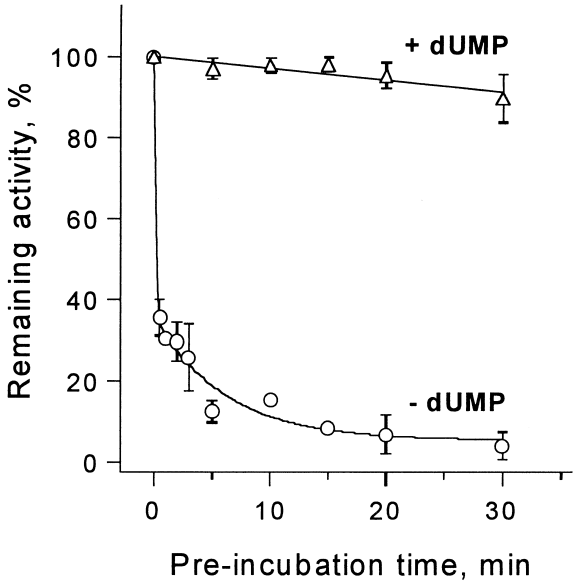


Figure 1. Time-dependent inactivation of *L. casei* dTMP synthase by 5 μM 5-fluoropropynyl-dUMP (**3**), in the presence (△) or absence (○) of 20 mM dUMP, as indicated.

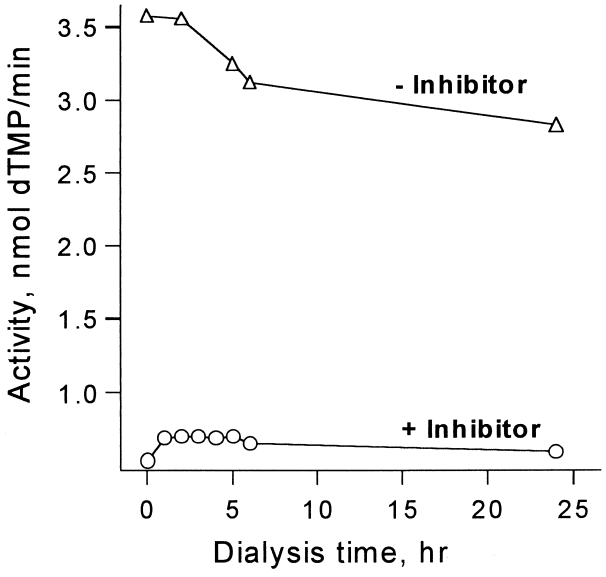


Figure 2. Time course of dTMP synthase activity during dialysis. Enzyme inactivated by **3** in the absence of CH_2H_4folate in 100 μL assay mixture was dialyzed against 350 mL phosphate buffer (changed every 2 h). Remaining activity was assayed at the indicated time periods; (○) inactivated enzyme, (△) free enzyme control.

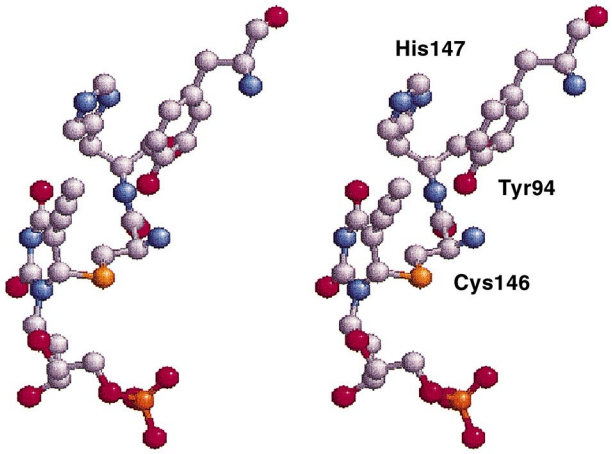


Figure 4. A stereoview model (Sybyl) of the active site of the inactivated dTMP synthase, showing the cumulene derivative of **3** covalently attached to Cys146. (X-ray coordinates of covalent complexes of *E. coli* dTMP synthase with known nucleotide analogues were provided by D. Matthews.)

The validity of this hypothesis remains to be determined, since the enzyme-mediated formation of the postulated cumulene derivative is without precedent in the literature. Figure 4 shows a model of the covalent enzyme-inhibitor complex. The model reveals that the cumulene side-chain is accommodated at the active site, without steric interference by the adjacent His147 and Tyr94, the closest active site residues around. However, it is conceivable that further reactions of the electrophilic cumulene may take place at the active site that may involve the imidazole of His147, or the phenolic OH-group of Tyr 94. Studies are in progress to elucidate the exact mechanism that leads to inactivation of dTMP synthase by **3**.

References and Notes

1. Cohen, S. S. *Ann. N.Y. Acad. Sci.* **1971**, *186*, 292.
2. Danenberg, P. V. *Biochim. Biophys. Acta* **1977**, *473*, 73.
3. Douglas, K. T. *Med. Res. Rev.* **1987**, *7*, 441.
4. Harrap, K. R.; Jackman, A. L.; Newell, P. R.; Taylor, G. A.; Hughes, L. R.; Calvert, H. A. *Adv. Enzyme. Reg.* **1989**, *29*, 161.
5. Grem, J. L. In *Cancer Chemotherapy: Principles and Practice,* Chabner, B. A.; Collins, J. M., Eds.; Lippincott, New York, 1990.

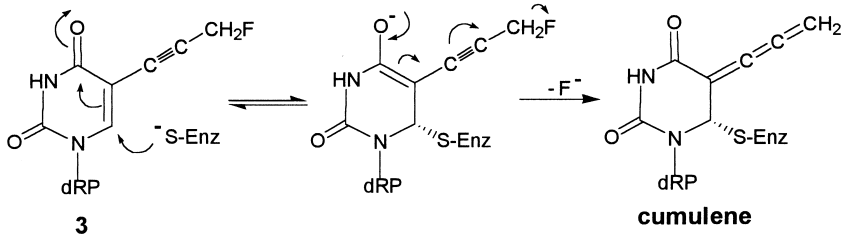


Figure 3. Hypothetical mechanism of the interaction of **3** with dTMP synthase.

6. Carreras, W. C.; Santi, D. V. *Annu. Rev. Biochem.* **1995**, *64*, 721.
7. Rando, R. R. *Methods Enzymol.* **1977**, *46*, 158.
8. Barr, P. J.; Nolan, P. A.; Santi, D. V. *J. Med. Chem.* **1981**, *24*, 1385.
9. Danenberg, P. V.; Bhatt, R. S.; Kundu, N. G.; Danenberg, K.; Heidelberger, C. *J. Med. Chem.* **1981**, *24*, 1537.
10. Barr, P. J.; Robins, M. J.; Santi, D. V. *Biochemistry* **1983**, *22*, 1696.
11. Kalman, T. I.; Yalowich, Y. C. In *Drug Action and Design: Mechanism-Based Enzyme Inhibitors,* Kalman, T. I., Ed., Elsevier/North Holland, New York, 1979; pp 75–91.
12. Nie, Z.; Kalman, T. I.; Kamat, A. *Abstracts,* 218th National Meeting, Amer. Chem. Soc., 1999, MEDI 221.
13. Kalman. T. I.; Nie, Z. *Nucleosides Nucleotides* **2000**, in press.
14. Hobbs, Jr., F. W. *J. Org. Chem.* **1989**, *54*, 3420.
15. Card, P. J. *J. Org. Chem.* **1983**, *48*, 393.
16. **3**: ^{1}H NMR (D_2O, Inova-400) δ 2.15–2.26 (m, 2H, 2′ + 2″-H), 3.89–3.93 (m, 2H, 5′ + 5″-H), 4.01 (m, 1H, 4′- H), 4.36 (m, 1H, 3′-H), 5.04 (d, J = 47.6 Hz, 2H, CH_2F), 6.08 (t, J = 6.4 Hz, 1H, 1′-H), 8.04 (s, 1H, 6-H). **7**: ^{1}H NMR ($CDCl_3$, Inova-500) δ 2.12–2.25 (m + 2s, 7H, 2′-H + $COCH_3$), 2.56 (m, 1H, 2″-H), 4.31–4.41 (m, 3H, 4′,5′ + 5″-H), 5.14 (d, J = 47.5 Hz, 2H, CH_2F), 5.24 (m, 1H, 3′-H), 6.30 (t, J = 6.75 Hz, 1H, 1′-H), 7.94 (s, 1H, 6-H). **8**: ^{1}H NMR (DMSO-d_6, Inova-500) δ 2.11 (m, 2H, 2′ + 2″-H), 3.54–3.58 (m, 2H, 5′ + 5″-H), 3.77 (m, 1H, 4′-H), 4.20 (m, 1H, 3′-H), 5.12 (t, J = 4.8 Hz, 1H, 5′-OH), 5.23 (d, J = 4.4 Hz, 1H, 3′-OH), 5.25 (d, J = 47.2 Hz, 2H, CH_2F), 6.07 (t, J = 6.4 Hz, 1H, 1′-H), 8.32 (s, 1H, 6-H), 11.68 (s, 1H, NH).
17. Sowa, T.; Ouchi, S. *Bull. Chem. Soc. Jpn.* **1975**, *48*, 2084.
18. Kalman, T. I.; Goldman, D. *Biochem. Biophys. Res. Commun.* **1981**, *102*, 682.
19. Leary, R. P.; Kisliuk, R. L. *Prep. Biochemistry* **1971**, *1*, 47.