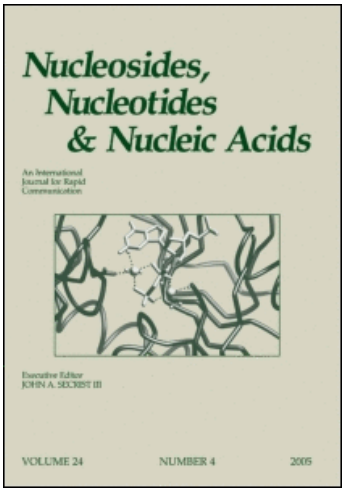

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RATIONAL DESIGN OF SELECTIVE ANTIMETABOLITES OF DNA-THYMINE BIOSYNTHESIS: 5-PROPYNYLPYRIMIDINE NUCLEOSIDE DERIVATIVES

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Dedicated to the memory of Gertrude B. Elion

ABSTRACT: A novel series of 5-propynylpyrimidine nucleosides are proposed as potential antimetabolites of DNA-thymine biosynthesis. This proposal is based on the results of detailed mechanistic analyses of the molecular interactions between dTMP synthase and its inhibitors. It is proposed that a propynyl side-chain at the 5-position of dUMP, bearing an appropriate leaving group, would cause irreversible inactivation of dTMP synthase, which would not require the presence of the cofactor, CH_2H_4folate.

INTRODUCTION

Half a century ago, Elion and Hitchings pioneered the design of structural analogues of the nucleic acid bases, as antimetabolites of RNA and DNA biosynthesis.[1-3] These purine and pyrimidine analogues proved useful in the treatment of cancer and other disease conditions, and opened the modern era of rational drug design and development, well before detailed knowledge about the targets of drugs became available. The success of the base analogues led to the development of a growing family of nucleoside derivatives,[4] which continue to play a major role in contemporary medicine. Recent advances in biochemistry, and molecular and structural biology, have produced a wealth of detailed structural and functional information about therapeutic targets and their interactions with drugs at the molecular level. Elion and Hitchings' legacy compels us to

This paper is dedicated to the memory of the late Trudy Elion, whose pioneering contributions, and self-sacrificing dedication to science and humanity, will remain an inspiration to all of us.

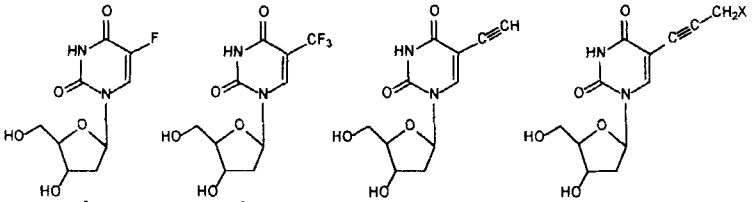
fully exploit this information for the rational design of new therapeutic agents. In this communication, the mechanism-based design of a family of novel inhibitors of thymidylate (dTMP) synthase is described. The design strategy attempted to overcome

RESULTS AND DISCUSSION

5-Fluoropyrimidines, Thymidylate Synthase, and the Design of New Mechanism-Based Inhibitors. Following the footsteps of Elion and Hitchings, Heidelberger designed 5-fluorouracil (5-FU), as an analogue of uracil, using the isosteric replacement of H with F at the 5-position, based on the preferential utilization of uracil by tumor cells, as a rationale.[5] 5-FU, a prototype of the fluoropyrimidines,[5] is currently the drug of choice, alone or in combinations, for the treatment of colorectal cancer, and a major weapon against solid tumors of head and neck, breast, and ovarian cancer.[6] The active form of the fluoropyrimidines is 5-fluoro-2'-deoxyuridine-5'-phosphate (FdUMP) that is a potent inhibitor of thymidylate (dTMP) synthase, the enzyme responsible for *de novo* synthesis of DNA-thymine. Thus, 5-FU should be considered an antimetabolite of thymine, in addition to that of uracil. While the uracil antagonism of 5-FU is simply structure-based, the thymine antagonism is mechanism-based. Furthermore, it is the thymine antagonism of 5-FU that is primarily responsible for the anticancer activity of the fluoropyrimidines, which could not be predicted by Heidelberger. It should be noted that long before the discovery of 5-fluorouracil, 5-bromouracil (5-BU) was recognized as a thymine antagonist, having the isosteric replacement of CH_3 with Br at the 5-position. Due to its close structural resemblance to thymine, 5-BU is readily incorporated into DNA in place of thymine. As a consequence, 5-BU is strongly mutagenic which precludes its use as a drug.

There are major shortcomings of the fluoropyrimidines in their therapeutic applications. The three most important are: (1) incorporation of 5-FU into RNA in place of uracil, causing a lack of target selectivity, which may be responsible for some of the toxicities to normal tissues; (2) the requirement for adequate intracellular levels of the cofactor, 5,10-methylenetetrahydrofolate (CH_2H_4folate), for full inhibition of dTMP synthase *in vivo*; (3) reversibility of the formation of the inactive, covalent ternary complex. The last two are interrelated and believed to be associated with both intrinsic and acquired resistance of tumor cells against the fluoropyrimidines.

The deoxyribonucleoside derivative of 5-FU, 5-fluoro-2'-deoxyuridine (FdUrd, **1**), was shown to be a much more potent cytotoxic agent, and a very selective inhibitor of DNA synthesis *in vitro*.[5] However, rapid degradation of **1** to 5-FU by thymidine (dThd) phosphorylase *in vivo* abolished these differences. The 5-trifluoromethyl-dUrd (**2**), a 'second generation' fluoropyrimidine, a structural analogue of dThd, was found to be a very potent inhibitor of dTMP synthase.[5] Due to its larger 5-substituent, it could not enter uracil metabolism, and was not incorporated into RNA. However, its chemical reactivity and significant incorporation into DNA, restricted its use to the topical treatment of herpes viral infections of the eye.



Major advances in the elucidation of the molecular mechanism of dTMP synthase,[7] and the structural details of its interaction with FdUMP, facilitated the rational design of novel inhibitors that could overcome the shortcomings of the fluoropyrimidines. Rando predicted that an ethynyl group at the 5-position would lead to irreversible inactivation of dTMP synthase, due to enzyme-mediated formation of a reactive allene group that may alkylate an active site amino acid residue.[8] Indeed, it was shown that 5-ethynyl-2'-deoxyuridine (**3**) is cytotoxic,[9] and that its 5'-phosphate is a potent inhibitor of dTMP synthase, capable of time-dependent inactivation of the enzyme.[10] However, the formation of the inhibitory complex required the presence of the cofactor, CH_2H_4folate, and the enzyme inactivation was found to be even more rapidly reversible than that of the corresponding FdUMP complex.[11]

Since complex formation and stabilization depend on the intracellular levels of the cofactor, CH_2H_4folate, the therapeutic effectiveness of the fluoropyrimidines may vary from tissue to tissue, and patient to patient. Inadequate levels of intracellular CH_2H_4folate may render cancer cells resistant to the fluoropyrimidines. This resistance can be intrinsic, or can be acquired by selection of folylpolyglutamate synthetase mutants deficient in their ability to maintain long chain polyglutamates of folate cofactors. These and other difficulties prompted the development of therapeutic combinations of the

fluoropyrimidines with leucovorin.[12] These combinations significantly improved the response rate, particularly in the case of colorectal cancer patients. However, the success was only partial and the search continued for novel inhibitors of dTMP synthase. This led to the discovery of a number of cofactor analogue inhibitors of the enzyme that entered clinical trials, some of which received regulatory approval recently.[13]

In order to overcome the deficiencies of the substrate analogues, a detailed analysis of the mechanisms of action of these dTMP synthase inhibitors was undertaken. The covalent ternary complex of dTMP synthase-FdUMP-CH_2H_4folate is a 'dead-end' intermediate, since its further conversion along the reaction coordinate toward product formation is blocked by the inability of the enzyme to cleave the carbon-fluorine bond (see **FIG. 1**).

The formation of the complex is reversible, because it is 'enzyme-catalyzed' and governed by the microscopic reversibility principle, just like the formation of the corresponding intermediate in the normal reaction.[11] Denaturation of the protein permanently stabilizes the linkages between the three components, because cleavage of the two covalent bonds requires the native structure of the catalytic machinery. The rate-determining step of the reactivation of the enzyme is the cleavage of the C-C bond between FdUMP and the cofactor. This step is very slow, with an activation energy of 26.5 kcal/mol,[11] whereas the decomposition of the binary complex is much faster. This fully accounts for the cofactor requirement of stable complex formation by FdUMP.

It was observed that the activity of dTMP synthase, after inactivation by **3** in murine leukemia L1210 cells, recovered much faster ($t_{1/2}$ = 24 min) than that by **1** ($t_{1/2}$ = 120 min), albeit the concentration of **3** used was 10-fold higher than **1**.[11] This suggested that the regeneration of enzyme activity may involve different mechanisms, depending on the structure of the inhibitor. Time-dependent inactivation of purified dTMP synthase by 5-ethynyl-dUMP could be observed only in the presence of the cofactor, CH_2H_4folate,[10] as is the case with FdUMP. However, in the absence of the cofactor, 5-ethynyl-dUMP served as a substrate for the enzyme and reacted with a nucleophile, such as 2-mercaptoethanol, in the medium.[14] Thus, the rapid recovery of dTMP synthase activity in cells[11] treated with **3** is the consequence of the consumption of the inhibitor at the active site of the enzyme. Substrate activity of 5-ethynyl-dUMP was not detected in the

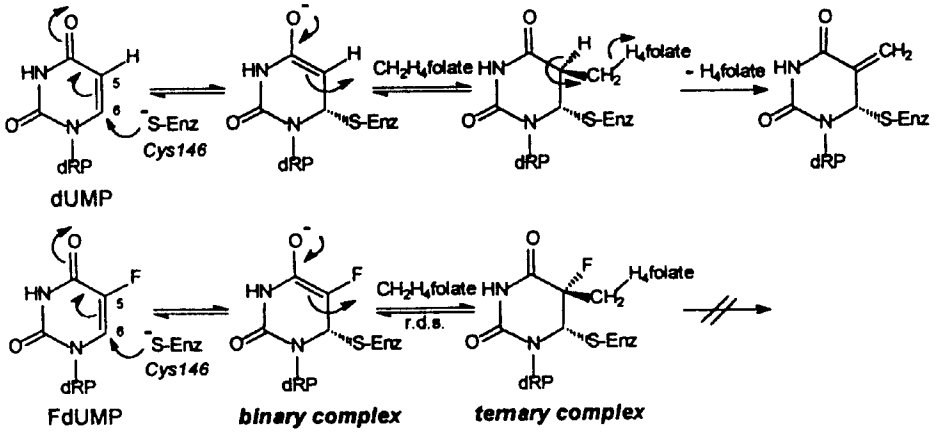


FIGURE 1. Outline of the initial steps of the dTMP synthase catalyzed reaction and the formation of the covalent ternary complex between the enzyme, FdUMP, and the cofactor, CH_2H_4folate.

presence of the cofactor, presumably, because the active site in the ternary complex is closed by a C-terminal flap,[7] and thus is not accessible to the medium. Alternatively, 5-ethynyl-dUMP may form a covalent ternary complex, analogous to that of FdUMP, much faster than the allene can form. A plausible mechanism for the interaction between the 5-ethynyl-dUMP and the enzyme is outlined in **FIG. 2.**

According to the mechanism outlined in **FIG. 2,** the binary covalent complex may partition between the reversible formation of the ternary complex, and the irreversible formation of the allene. This latter requires the protonation of the β-carbon of the acetylenic side-chain, which may happen only in the absence of the cofactor, when the active site is open. Every time the allene intermediate is captured by a nucleophile, one molecule of the inhibitor is destroyed, and simultaneously the enzyme is reactivated by elimination of the SH-group of Cys146 (*E. coli* numbering), at the active site. In cells treated with **3**, the activity of the enzyme should return, as the pool of the inhibitor is exhausted.

It is suggested that the failure of 5-ethynyl-dUMP to fulfill Rando's prediction[8] and inactivate dTMP synthase irreversibly, is due to the absence of a properly oriented

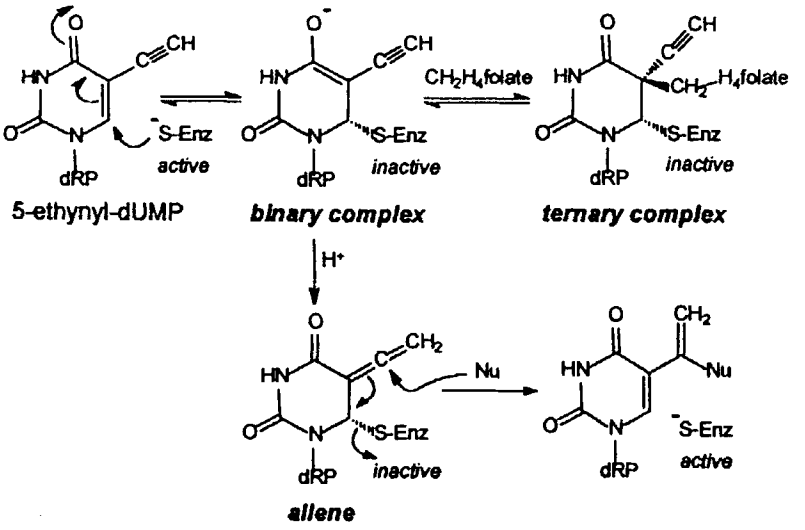


FIGURE 2. Hypothetical mechanism of the interactions of the 5-ethynyl-dUMP with dTMP synthase.

nucleophilic amino acid side-chain that would be able to compete effectively with the nucleophiles in the medium and react with the allene.

This analysis suggested that appropriate modification of the ethynyl substituent at the 5-position of the pyrimidine ring may promote irreversible inactivation of dTMP synthase. Based on mechanistic considerations, a family of 5-propynylpyrimidines (**4**), characterized by a leaving group (X) at the γ-position, was chosen, as potential mechanism-based inactivators of dTMP synthase. **FIG. 3** outlines the proposed mechanism of interactions of the 5'-phosphate of **4** with dTMP synthase.

It is postulated that the initial nucleophilic addition of the active site SH-group of the enzyme to the pyrimidine ring is followed by the expulsion of the leaving group (X), leading to the formation of a cumulene side-chain (see **FIG. 3**). Leaving group departure should be spontaneous, in contrast to the conversion of the acetylene side-chain of 5-ethynyl-dUMP to the allene, which depends on the availability of a proton, which may or may not be provided by an active site residue. The reaction of the α-carbon of the

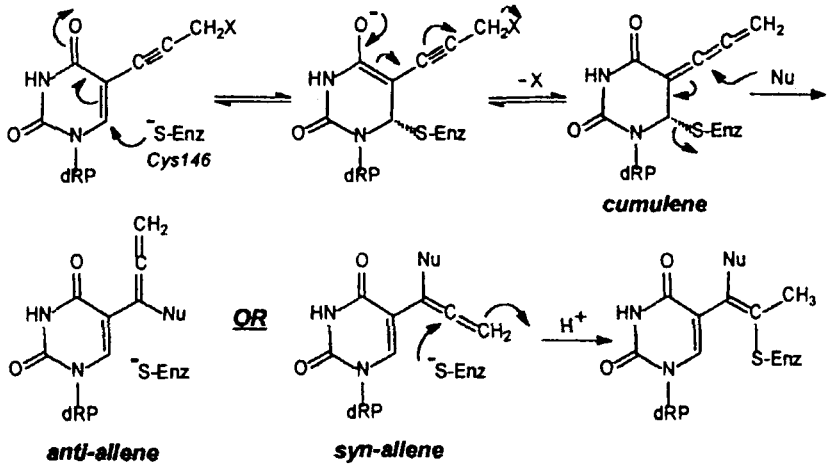


FIGURE 3. Hypothetical mechanism of the interactions of the 5'-phosphate of 4 with dTMP synthase.

cumulene with a nucleophile from the medium would produce an allene, accompanied by the elimination of the SH-group of the enzyme as a thiolate ion (see **FIG. 3**).

If the resulting allene is the *syn*-isomer, its proximity to the side-chain of Cys146 may allow recapturing of the active site SH-group, leading to an alkylated, inactive enzyme (see **FIG. 3**). This reaction is predicted to be irreversible, because this alkylation step is *not* on the reaction coordinate of the normal, enzyme-catalyzed reaction. This is in contrast to the initial 1,4-addition reaction. Depending on the relative orientation of the reactants, it may happen that the allene derived from the cumulene is the *anti*-isomer (**FIG. 3**).

While the reactive β-carbon of the *syn*-allene is within 2.3 Å from the SH-sulfur of Cys146, as revealed by molecular modeling (see **FIG. 4A**), the orientation of the *anti*-allene side-chain appears unfavorable for a reaction to occur. However, two other active site residues, Tyr94 and His147, are located in the vicinity, so that a covalent linkage may form between one of these residues of the enzyme and the inhibitor. The phenolic oxygen of Tyr94, and the imidazole-N^{τ} of His147, is within 3 Å, and 4.9 Å, respectively, from the reactive central C-atom of the *anti*-allene side chain (see **FIG 4B**), making alkylation

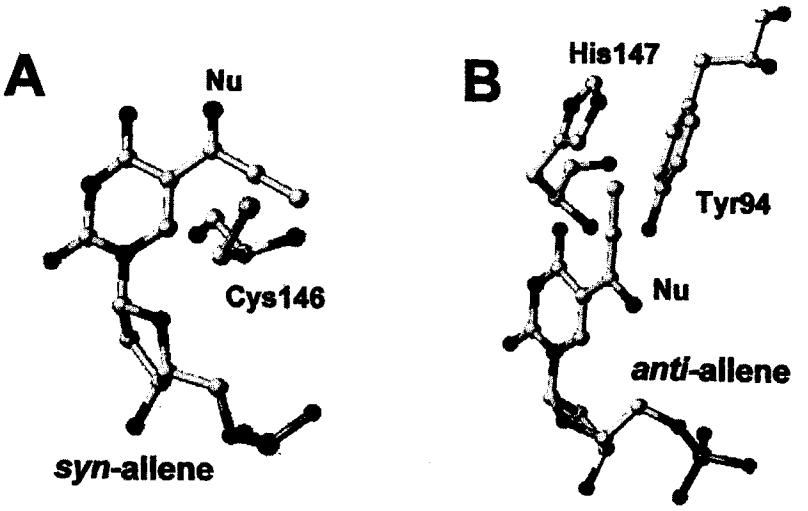
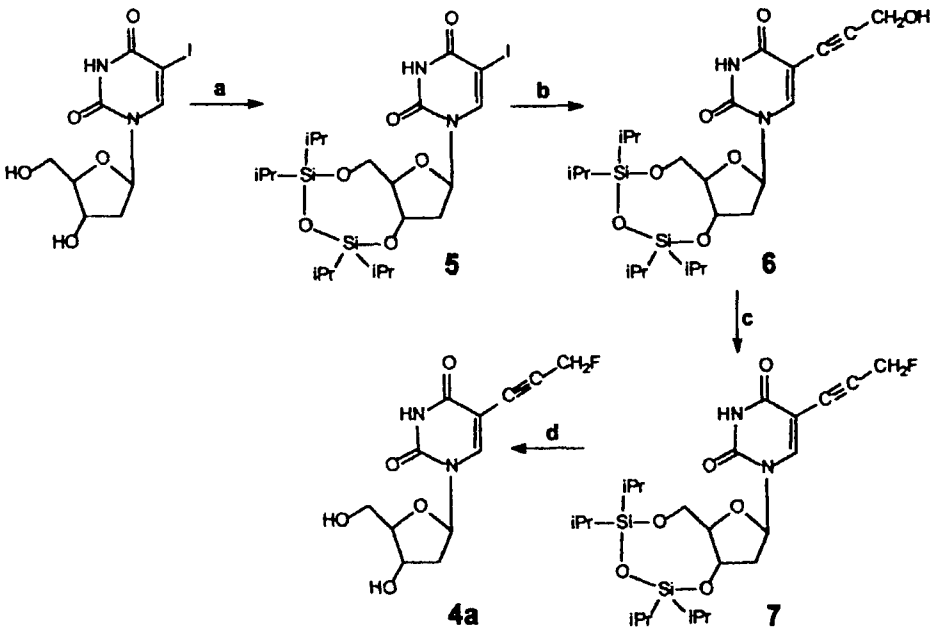


FIGURE 4. Models of *syn*-allene (**A**) and *anti*-allene (**B**) at the active site of *E. coli* dTMP synthase. **Nu**, external nucleophile, after reaction with the cumulene intermediate.

likely to occur. Thus, it is conceivable that irreversible inactivation of dTMP synthase may be achieved by the propyne ⇒ cumulene ⇒ allene ⇒ alkylated enzyme sequence, involving one of three possible active site residues of the enzyme.

5-Fluoropropynyl-2'-deoxyuridylate, a Prototype Mechanism-Based Inhibitor of dTMP Synthase. The nature of the X-substituent in **4** must be optimized between good leaving group ability and susceptibility to S_N2 displacement that would jeopardize stability and selectivity. The smallest X-substituent that can serve as a leaving group is a fluorine atom, as in **4a**, thus it was selected as a prototype. The synthesis of **4a** is outlined in **SCHEME 1**.

Reaction of 5-iodo-2'-deoxyuridine with 1,3-dichloro-1,1,3,3-tetraisopropyl-disiloxane[15], in the presence of imidazole as base, in anhydrous DMF, gave **5** in quantitative yield.[16] This protecting reagent was chosen, because trimethylsilyl protection is incompatible with the fluorination conditions employed in step (c). Pd(0)-catalyzed coupling[17] of **5** with propargylalcohol led to **6**, which was purified by flash column



SCHEME 1. (a) imidazole, 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane, DMF, r.t., 3 h; (b) $CH{\equiv}CCH_2OH$, $Pd(PPh_3)_4$, CuI, Et_3N, r.t., 2-10 h; (c) DAST, CH_2Cl_2, 0 °C, 10 min; (d) KF, DMF, r.t., 3 h.

chromatography, after extraction with 5% EDTA. Fluorination of **6** was carried out at 0 °C by stirring for 10 min in anhydrous CH_2Cl_2, using (diethylamino)sulfur trifluoride (DAST)[18,19] in slight excess. After quenching the excess reagent with MeOH, the resulting **7** was purified by column chromatography and deprotected, using KF, to yield **4a**. The 5'-monophosphate, 5-fluoropropynyl-dUMP, was synthesized by selective phosphorylation of **4a**, using $POCl_3$ in $PO(OEt)_3$, as described.[20]

Preliminary results demonstrated that 5-fluoropropynyl-dUMP can rapidly inactivate dTMP synthase *in the absence* of the cofactor, CH_2H_4folate.[21] A typical experiment is illustrated in **FIG. 5**, which shows the loss of dTMP synthase activity as a function of the time of the preincubation of the enzyme with the inhibitor, in the presence or absence of a saturating concentration of the substrate, dUMP, with no cofactor present.

The protection of the enzyme by the substrate indicates that the inactivation is due to interactions at the active site. It is clear that the presence of the cofactor is not required

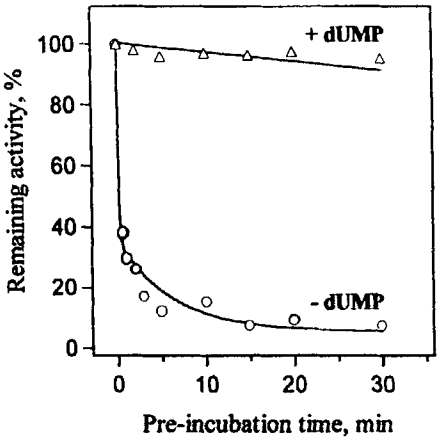


FIGURE 5. Time-dependent loss of activity of *L. casei* dTMP synthase during preincubation with 5 μM 5-fluoropropynyl-dUMP, in the presence or absence of 20 mM dUMP, as indicated.

for inactivation of dTMP synthase by 5-fluoropropynyl-dUMP, in contrast to that by closely related 5-ethynyl-dUMP,[10,14] a finding that supports the validity of the proposed hypothesis. It will be interesting to explore further the structural determinants of cofactor dependence of enzyme inhibitory activity.

Further studies are in progress to demonstrate the irreversibility of the enzyme inactivation process, and to elucidate the underlying molecular mechanism. It will be important to determine, how the nature of the X-substituent of the 5-propynylpyrimidine nucleosides affects not only the enzyme inhibitory activity of these analogues, but also their cellular uptake and phosphorylation — two prerequisites for antimetabolite activity and therapeutic potential.

EXPERIMENTAL

3',5'-*O*-(Tetraisopropyldisiloxane-1,3-diyl)-5-iodo-2'-deoxyuridine (5). To a solution of 5-iodo-2'-deoxyuridine (3.54 g, 10 mmol) in anhydrous DMF (10 mL), imidazole (2.72 g, 40 mmol) was added, followed by 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (3.4 g, 10.7 mmol). The reaction mixture was stirred at room temperature under argon for 3 h. After the solvent was evaporated, the residue was dissolved in CH_2Cl_2, washed by ice-cold water. The organic layer was dried over sodium

sulfate, and evaporated to a foam-like solid, which was crystallized from ethanol to give **5** as a colorless solid. Mp. 180-182 °C; ^{1}H NMR ($CDCl_3$, Inova-400) δ 0.93-1.11 (m, 28H, iPr_4), 2.28 (m, 1H, 2'-H), 2.48 (m, 1H, 2"-H), 3.77 (m, 1H, 4'-H), 4.02 (m, 1H, 5'-H), 4.13 (m, 1H, 5"-H), 4.45 (m, 1H, 3'-H), 5.98 (dd, J = 1.6 Hz & 7.2 Hz, 1H, 1'-H), 8.01 (s, 1H, 6-H), 8.24 (br s, 1H, NH). Anal. calcd. for $C_{21}H_{37}IN_2O_6Si_2$: C, 42.28; H, 6.25; N, 4.70. Found: C, 42.10; H, 6.25; N, 4.70.

3',5'-*O*-(Tetraisopropyldisiloxane-1,3-diyl)-5-(3-hydroxypropyn-1-yl)-2'-deoxyuridine (6). To 596 mg of **5** (1 mmol) in 5 mL of freshly distilled triethylamine, 58 mg (0.05 mmol) of $Pd(PPh_3)_4$ and 19 mg (0.10 mmol) of CuI were added, followed by slow addition of 145 μL (2.5 mmol) of propargyl alcohol. The reaction mixture was stirred at room temperature for 2-10 hours. After the solvent was evaporated *in vacuo*, the residue was dissolved in CH_2Cl_2, washed by 5% EDTA solution and water, and chromatographed on silica gel (ethyl acetate/hexane = 1:1) to give 360 mg (69%) of **6** as a white solid. Mp. 96-98 °C. ^{1}H NMR ($CDCl_3$, Inova-500) δ 0.90-1.13 (m, 28H, iPr_4), 2.30 (m, 1H, 2'-H), 2.53 (m, 1H, 2"-H), 3.31 (br s, 1H, OH), 3.79 (m, 1H, 4'-H), 4.03 (m, 1H, 5'-H), 4.17(m, 1H, 5''-H), 4.43-4.48 (s & m, 3H, 3'-H + CH_2OH), 6.04 (d, J = 6 Hz, 1H, 1'-H), 7.95 (s, 1H, 6-H), 9.83 (br s, 1H, NH). Anal. calcd. for $C_{24}H_{40}N_2O_7Si_2$: C, 54.93; H7.68; N, 5.34. Found: C, 54.93; H, 7.73; N, 5.17.

3',5'-*O*-(Tetraisopropyldisiloxane-1,3-diyl)-5-(3-fluoropropyn-1-yl)-2'-deoxyuridine (7). To 524 mg (1 mmol) of **6** in 20 mL of anhydrous CH_2Cl_2 at 0 °C, was added DAST (165 μL, 1.25 mmol). The reaction mixture was stirred for 10 min at 0 °C, then MeOH was added at 0 °C to quench the excess DAST. Flash column chromatography on silica gel (CH_2Cl_2:MeOH = 100:1) afforded 116 mg (22%) of **7**, which was used directly for next step. ^{1}H NMR ($CDCl_3$, Inova-500) δ 0.89-1.08 (m, 28H, iPr_4), 2.25 (m, 1H, 2'-H), 2.50 (m, 1H, 2"-H), 3.75 (m, 1H, 4'-H), 3.99 (m, 1H, 5'-H), 4.14 (m, 1H, 5'-H), 4.42 (m, 1H, 3'-H), 5.10 (d, J = 47.5 Hz, 2H, CH_2F), 5.98 (d, J = 7 Hz, 1H, 1'-H), 8.00 (s, 1H, 6-H), 9.32 (br s, 1H, NH).

5-(3-Fluoropropyn-1-yl)-2'-deoxyuridine (4a). The above obtained 116 mg of **7** in 5 mL of DMF was treated with 100 mg (2 mmol) of KF for 3 h. Chromatography on a silica gel column (CH_2Cl_2:MeOH = 20:1) gave 45 mg (66%) of **4a.** ^{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). ^{13}C NMR (DMSO-d_6, Inova-500) δ 40.2, 60.8, 70.0, 71.9, 82.5, 84.9, 86.4, 87.7, 96.8, 145.2, 149.4, 161.4. UV λ_{max} 287 nm (ε 10,400, pH = 2; ε 10,800, pH = 7). Anal. calcd. for $C_{12}H_{13}FN_2O_5 \cdot 0.6H_2O$: C, 48.85; H, 4.85; N, 9.49. Found: C, 48.86; H, 4.51; N, 9.45.

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