Bioorganic & Medicinal Chemistry Letters 20 (2010) 1648–1651

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

The role of phosphate in the action of thymidine phosphorylase inhibitors: Implications for the catalytic mechanism

Harsh V. Jain [a], Roshni Rasheed [a], Thomas I. Kalman [a,b,*]

[a] Department of Chemistry, University at Buffalo, Buffalo, NY 14260, United States
[b] Department of Pharmacology & Toxicology, University at Buffalo, Buffalo, NY 14260, United States

ARTICLE INFO

Article history:
Received 31 December 2009
Accepted 11 January 2010
Available online 21 January 2010

Keywords:
Thymidine phosphorylase
Transition state analog
Enzyme mechanism
Inhibition kinetics

ABSTRACT

The design and synthesis of 5-fluoro-6-[(2-aminoimidazol-1-yl)methyl]uracil (AIFU), a potent inhibitor of thymidine phosphorylase (TP) with K_i-values of 11 nM (ecTP) and 17 nM (hTP), are described. Kinetic studies established that the type of inhibition of TP by AIFU is uncompetitive with respect to inorganic phosphate (or arsenate). The results obtained suggest that AIFU and other zwitterionic thymine analog inhibitors of TP act as transition state analogs, mimicking the anionic thymine leaving group, consistent with an S_N2-type catalytic mechanism, and anchored by their protonated side chains to the enzyme-bound phosphate by electrostatic and H-bonding interactions.

Thymidine phosphorylase (TP) is a multifunctional protein also known as platelet-derived endothelial cell growth factor (PD-ECGF) that stimulates angiogenesis, and as gliostatin, a factor controlling glial cell proliferation.[1,2] TP is frequently overexpressed in many types of cancer and has prognostic value in fluoropyrimidine therapy, since it is mainly responsible for the metabolic activation of 5-fluorouracil prodrugs, such as capecitabine, 5′-deoxy-5-fluorouridine and tegafur.

Figure 1 outlines the TP-catalyzed reaction of the reversible phosphorolysis of thymidine to thymine and 2-deoxy-D-ribose-1-phosphate (dR-1-P). Subsequent dephosphorylation of the product dR-1-P to 2-deoxy-D-ribose in vivo triggers the pro-angiogenic response and promotes cancer metastasis. Thus, TP inhibitors may have utility in the treatment of cancer. Indeed, a potent inhibitor of TP, 5-chloro-6-[(2-iminopyrrolidin-1-yl)methyl]uracil (TPI) (see Fig. 2) entered clinical trials in combination with 5-trifluoromethyl-2′-deoxyuridine as an orally available anticancer treatment under the name of TAS-102.[3,4]

Many laboratories including our own have been involved in the design and synthesis of a variety of TP inhibitors (see Fig. 2). The most potent inhibitors were 5-halogenated uracil derivatives with a basic side chain at the 6-position, such as TPI. The potent inhibitory activity of these zwitterionic analogs bearing a positively charged side chain appeared to confirm a hypothetical S_N1-type mechanism of catalysis involving an oxacarbenium ion transition state (see Fig. 3), in analogy to other glycosyl bond-cleaving enzymatic reactions.[5,6] Based on the same working hypothesis, 5-fluoro-6-imidazol-1-ylmethyl-uracil (**1** IFU, $K_i = 51$ nM; ecTP)[†] was designed in our laboratory as a transition state analog inhibitor of TP (see Fig. 2) as an oxacarbenium ion mimic.[7] Our results indicated that IFU was bound to the enzyme in its zwitterionic form, the fluorine contributing two orders of magnitude increase in affinity, presumably due to a decrease in the pK_a of the uracil ring by two pH units.

In spite of the success of the oxacarbenium ion-like transition state hypothesis as a basis for the design of many potent TP inhibitors, experimental support for an S_N1-type mechanism was lacking. Contrary to the prevalence of the S_N1 mechanisms in nucleoside phosphorylases, Birck and Schramm provided strong experimental evidence for the involvement of a nearly symmetrical S_N2-like transition state in the TP-catalyzed reaction.[8] In addition, they put forward the hypothesis that the positively charged side chain of TPI acts as a phosphate 'chelator' and that the ion-pair complex may show resemblance to the S_N2 transition state.[8,9] In contrast, the fact that the structure of the human TP–TPI binary complex was obtained in the absence of phosphate,[10] was taken as an indication in a recent review that 'phosphate is not necessary for enzyme inhibition' by TPI.[1]

The observed high potency of the zwitterionic inhibitors is consistent with either types of nucleophilic substitution mechanisms. In both cases, the negatively charged 5-halogenated uracil rings may resemble the anionic thymine leaving group, an integral part of both types of transition states. However, the protonated side chains of these inhibitors may play two fundamentally different

* Corresponding author. Tel.: +1 716 645 4184; fax: +1 716 645 6963.
E-mail address: tkalman@buffalo.edu (T.I. Kalman).

[†] ecTP, *Escherichia coli* TP; hTP, human TP.

0960-894X/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2010.01.076

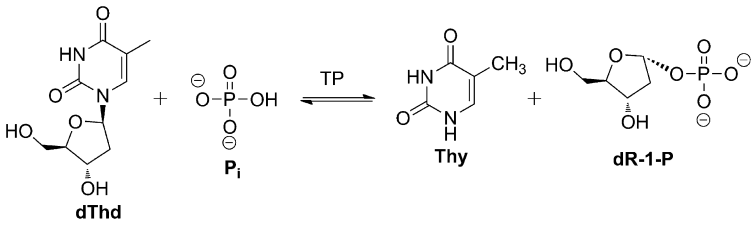


Figure 1. Outline of the reaction catalyzed by TP.

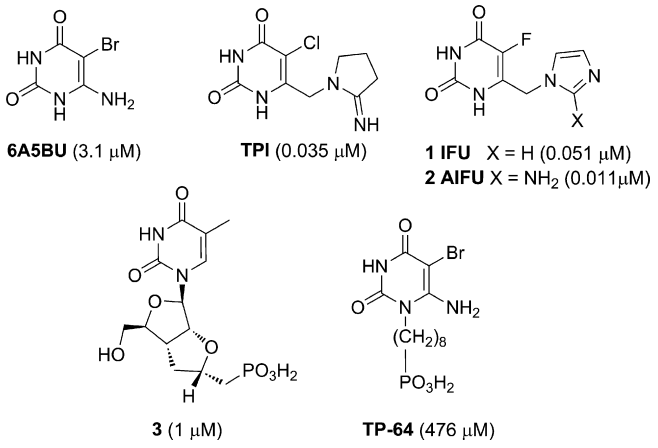


Figure 2. Examples of TP inhibitors (K_i -values indicated).

roles: they can (1) either mimic the oxacarbenium ion of an S_N1 transition state, or (2) interact with the phosphate nucleophile in an S_N2-type mechanism (see Fig. 3). Since AIFU may be classified as a mechanism-based inhibitor, we sought to distinguish between these two alternatives.

In order to shed further light on the catalytic mechanism, we focused our attention on the postulated interaction of the basic side chain with phosphate. We decided to modify IFU to maximize the interaction with P_i by replacing the imidazole (pK_a = 7.18)[‡] with the more basic 2-aminoimidazole (pK_a = 8.56)[‡] yielding 5-fluoro-6-[(2-aminoimidazol-1-yl)methyl]uracil (**2** AIFU) (see Fig. 2). This modification increased the potency ca. fivefold giving K_i-values of 11 nM and 17 nM for ecTP and hTP, respectively, presumably due to the presence of the NH_2-group with additional H-bonding potential. The potency of AIFU is similar to that of the corresponding 5-chloro and 5-bromo analogs previously reported by Reigan et al.[11] While it is difficult to reconcile the dominance of positively charged residues at the active site of TP with an oxacarbenium-like transition state, as we previously pointed out,[7] it is conceivable that they play a role in the stabilization of the negative charge developing on the thymine leaving group in the transition state, since thymine has a relatively high pK_a of 9.86 at NH-1.[12] This is in line with the observed increase in affinity of the more acidic 5-halogenated uracil-containing TP inhibitors with an unsubstituted N-1 position (pK_a = 7.7–7.9 for 5-F-, 5-Cl-, and 5-Br-uracil). This effect is more pronounced for the zwitterionic analogs bearing a basic side chain, which further lowers the pK_a-values by 1.9–2.0 pH units, for example, the pK_a of both TPI and AIFU is 6.05.[‡] This also explains the weaker binding of thymidine analogs, since substitution at N-1 prevents the all-important ionization of the pyrimidine ring at that position.

The synthesis of AIFU is outlined in Scheme 1. The preparation of **4** has been previously described.[7,13] When 2-aminoimidazole

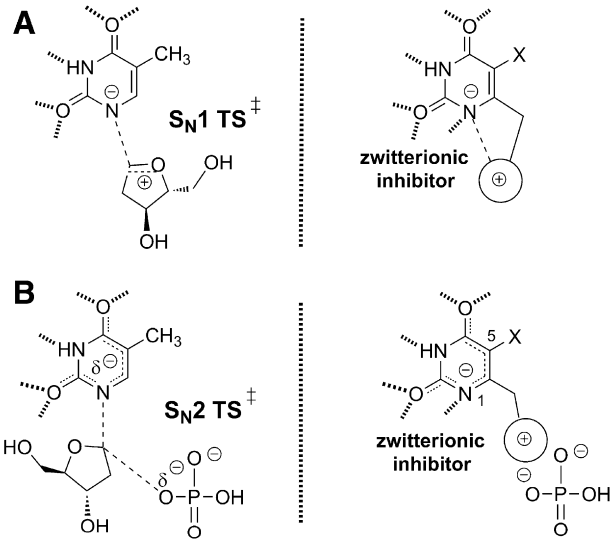


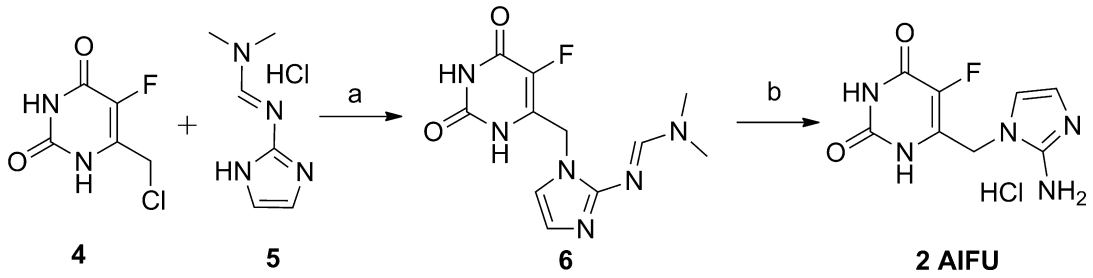
Figure 3. Hypothetical S_N1 (A) and S_N2 (B) transition states for the TP-catalyzed reaction are depicted together with their zwitterionic analogs.

was used without a protecting group, the yield of AIFU was less than 10%. Protection of the amino group of 2-aminoimidazole with *N,N*-dimethylformamide dimethylacetal yielded the known formamidine **5**,[14] which was alkylated by **4** in DMF to give the desired **6**[15] in 65% yield. This was followed by deprotection in concentrated hydrochloric acid at 100 °C to afford target compound **2**[16] in 90% yield. This is a considerable improvement of the 9–13% yields reported by Reigan et al. for the direct coupling of 2-aminoimidazole with 5-bromo- or 5-chloro-6-[(2-chloro-1-yl)methyl]uracil.[11]

A variety of phosphonate derivatives were reported as multisubstrate inhibitors of TP[17,18] and were found to inhibit the enzyme competitively with respect to phosphate. The rigid bicyclic phosphonate **3** (see Fig. 2) was shown to be 30-fold more active than its *exo*-isomer,[17] clearly showing the importance of the orientation of the phosphonate, which may occupy the phosphate binding site. To identify the likely location of the phosphate at the active site, **3** was docked into the hTP structure in the closed conformation, using the coordinates of the previously reported crystal structure of the human TP-TPI binary complex,[10] keeping the respective pyrimidine rings in the same orientation (see Fig. 4A). Similarly, AIFU was docked in place of TPI to reveal the orientation of the 2-aminoimidazole side chain. Figure 4B illustrates that in the energy minimized conformation, the protonated side chain points toward a cavity occupied by several water molecules, which presumably fill up the empty phosphate binding site. At the same time, it is positioned in close proximity to the putative location of the phosphate identified by the phosphonate group of **3**, conducive to charge-charge and H-bonding interactions.

Experiments were performed using ecTP to determine the kinetics of inhibition of the enzyme by AIFU. Enzyme activity was monitored spectrophotometrically at 350 nm, as described,[19]

[‡] pKa values were calculated by ACD/Labs pKa DB program.



Scheme 1. Outline of the synthesis of AIFU. Reagents and conditions: (a) DMF, DIEA (3 equiv), 60 °C, 12 h, argon; (b) concd HCl/EtOAc (1:1), 100 °C, 2 h.

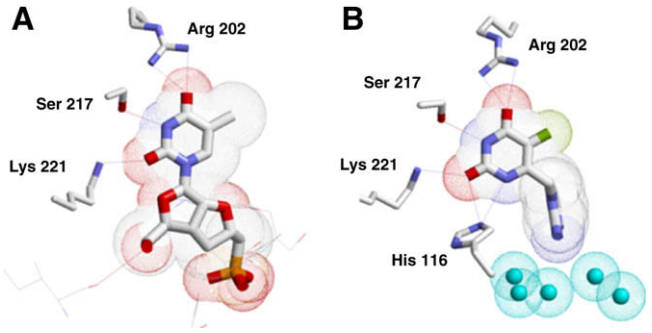


Figure 4. Ligand binding at the active site of hTP (pdb code 1UOU). Van der Waals surfaces of docked molecules of **3** (A), and **2** (B) are shown; water molecules are depicted as cyan-colored balls. Docking was performed using Sybyl 7.3; illustration by RasTop 2.1.

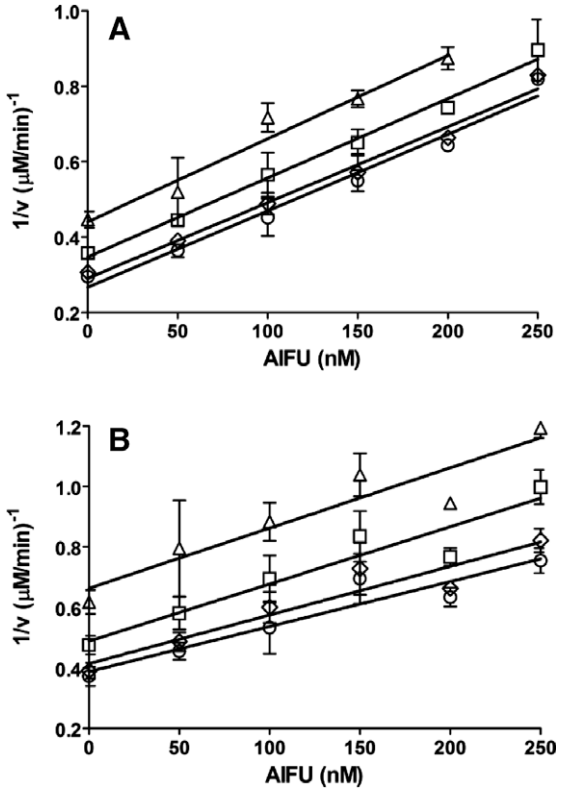


Figure 5. 1/*v* versus [I] plot of the inhibition of ecTP by AIFU. The concentrations of arsenate (plot **A**) and phosphate (plot **B**) were: (△)1 mM; (□) 2 mM; (◇) 5 mM; (○) 10 mM; 5-NO_2–dUrd (0.13 mM) was used as substrate.

using a fixed concentration of 5-NO_2–dUrd (0.13 mM), as chromogenic substrate. Rates were measured at varying concentrations of AIFU in the presence of 1, 2, 5, and 10 mM of either arsenate (Fig. 5A) or phosphate (Fig. 5B). The nearly identical slopes of the Dixon plots of Figure 5 clearly indicate uncompetitive type of inhibition by AIFU with respect to arsenate or phosphate, as described by the following equation:

$$\frac{1}{v_i} = \frac{1}{V_{max}K_i}[I] + \frac{1}{V_{max}}\left(1 + \frac{K_m}{[S]}\right)$$

Accordingly, arsenate or phosphate must bind first to the enzyme before AIFU can bind, to form an inactive ternary complex.

The validity of the interpretation of data obtained by using arsenate as a second substrate has been questioned before.[20] The similar patterns of inhibition kinetics obtained in this work justify the use of arsenate for the study of TP inhibitors. Arsenate is generally used only to avoid interference by the product dR-1-P formed in the presence of orthophosphate, and to obtain more reliable kinetics, since the analogous arsenate product is unstable and rapidly degraded by hydrolysis.[21]

The results of our studies described in this communication showing the obligatory order of binding of phosphate before AIFU suggest that the positively charged side chain of all structurally related zwitterionic inhibitors of TP may interact with the enzyme-bound phosphate, rather than act as a mimic of an oxacarbenium ion-like transition state and strongly support Birck and Schramm's hypothesis.[8] Nevertheless, the importance of the involvement of an oxacarbenium ion-like transition state as a working hypothesis in guiding the rational design of potent TP inhibitors in a number of laboratories, including our own, must be recognized.

With respect to future inhibitor design, it is important to consider that the uncompetitive relationship of the zwitterionic TP inhibitors with phosphate, that is, the requirement for prior phosphate binding, is an advantage in their predicted in vivo activity, since the intracellular concentrations of orthophosphate are usually maintained around 1 mM,[22] exceeding its K_m-value for TP. In contrast, phosphonate derivatives, like TP-64 (and most likely **3**) inhibit TP competitively with respect to orthophosphate,[18] which is to their disadvantage. The bound phosphate may permit inhibitors with protonated side chains to have high affinity to the active site occupied by several repulsive positively charged amino acid side chains (Arg202, Lys221, His116, and Lys115). These residues may serve to stabilize the negatively charged pyrimidine ring resembling the transition state structure, in addition to attracting phosphate (or arsenate) to the active site.

In summary, the results of our enzyme kinetic studies are consistent with the hypothesis that the protonated side chains of zwitterionic inhibitors of TP, such as AIFU, interact with the enzyme-bound phosphate (or arsenate). Validation of this hypothesis, however, will require the determination of the 3D-structure of an enzyme-phosphate-inhibitor ternary complex.

Acknowledgments

Helpful comments by Drs. John Richard and Andrew Murkin are gratefully acknowledged.

References and notes

1. Perez-Perez, M. J.; Priego, E. M.; Hernandez, A. I.; Camarasa, M. J.; Balzarini, J.; Liekens, S. *Mini-Rev. Med. Chem.* **2005**, *5*, 1113.
2. Liekens, S.; Bronckaers, A.; Pérez-Pérez, M.-J.; Balzarini, J. *Biochem. Pharmacol.* **2007**, *74*, 1555.
3. Yano, S.; Kazuno, H.; Sato, T.; Suzuki, N.; Emura, T.; Wierzba, K.; Yamashita, J.; Tada, Y.; Yamada, Y.; Fukushima, M.; Asao, T. *Bioorg. Med. Chem. Lett.* **2004**, *12*, 3443.
4. Emura, T.; Suzuki, N.; Fujioka, A.; Ohshimo, H.; Fukushima, M. *Int. J. Oncol.* **2005**, *27*, 449.
5. Ganem, B. *Acc. Chem. Res.* **1996**, *29*, 340.
6. Schramm, V. L. *Nucleic Acids Symp. Ser.* **2003**, *3*, 107.
7. Kalman, T. I.; Lai, L. *Nucleosides Nucleotides Nucleic Acids* **2005**, *24*, 367.
8. Birck, M. R.; Schramm, V. L. *J. Am. Chem. Soc.* **2004**, *126*, 2447.
9. Birck, M. R.; Clinch, K.; Gainsford, G. J.; Schramm, V. L.; Tyler, P. C. *Helv. Chim. Acta* **2009**, *92*, 823.
10. Norman, R. A.; Barry, S. T.; Bate, M.; Breed, J.; Colls, J. G.; Ernill, R. J.; Luke, R. W.; Minshull, C. A.; McAlister, M. S.; McCall, E. J.; McMiken, H. H.; Paterson, D. S.; Timms, D.; Tucker, J. A.; Pauptit, R. A. *Structure* **2004**, *12*, 75.
11. Reigan, P.; Edwards, P. N.; Gbaj, A.; Cole, C.; Barry, S. T.; Page, K. M.; Ashton, S. E.; Luke, R. W.; Douglas, K. T.; Stratford, I. J.; Jaffar, M.; Bryce, R. A.; Freeman, S. *J. Med. Chem.* **2005**, *48*, 392.
12. Ganguly, S.; Kundu, K. *Can. J. Chem.* **1994**, *72*, 1120.
13. Duschinsky, R.; Pleven, E.; Heidelberger, C. *J. Am. Chem. Soc.* **1957**, *79*, 4559.
14. Lahue, B. R.; Wan, Z.-K.; Snyder, J. K. *J. Org. Chem.* **2003**, *68*, 4345.
15. *For compound* **6**: ^{1}H NMR (500 MHz, D_2O) δ 8.02 (s, 1H), 6.96 (d, J = 2.5 Hz, 1H), 6.86 (d, J = 2.5 Hz, 1H), 4.98 (d, J = 2.1 Hz, 2H), 3.19 (d, J = 6.1 Hz, 1H), 3.06 (s, 3H), 2.94 (s, 3H). ^{13}C NMR (75 MHz, D_2O) δ 159.52 (d, JC_4F = 25.6 Hz), 158.21 (s), 150.60 (s), 149.40 (s), 138.69 (d, JC_5F = 233.0 Hz), 133.97 (d, JC_6F = 23.6 Hz), 117.83 (s), 114.26 (s), 41.11 (s), 40.41 (s), 34.69 (s). ^{19}F NMR (376 MHz, DMSO-d_6) δ −170.41 (s). MS $(M+H)^+$ = 281.2.
16. *For compound* **2**: ^{1}H NMR (500 MHz, D_2O) δ 6.76 (d, J = 15.1 Hz, 2H), 4.95 (s, 2H). ^{13}C NMR (126 MHz, D_2O) δ 159.41 (d, J = 25.7 Hz), 150.65 (s), 146.70 (s), 139.03 (d, JC_5F = 233.5 Hz), 132.33 (d, J = 23.6 Hz), 115.70 (d, J = 5.1 Hz), 113.49 (d, J = 2.7 Hz).^{19}F NMR (376 MHz, D_2O) δ -168.79 (t, J = 2.2 Hz). MS $(M+H)^+$ = 226.2.
17. Allan, A. L.; Gladstone, P. L.; Price, M. L.; Hopkins, S. A.; Juarez, J. C.; Donate, F.; Ternansky, R. J.; Shaw, D. E.; Ganem, B.; Li, Y.; Wang, W.; Ealick, S. *J. Med. Chem.* **2006**, *49*, 7807.
18. Balzarini, J.; Degrève, B.; Esteban-Gamboa, A.; Esnouf, R.; De Clercq, E.; Engelborghs, Y.; Camarasa, M.-J.; Pérez-Pérez, M.-J. *FEBS Lett.* **2000**, *483*, 181.
19. Wataya, Y.; Santi, D. W. *Anal. Biochem.* **1981**, *112*, 96.
20. Edwards, P. N. *J. Enzyme Inhib. Med. Chem.* **2006**, *21*, 483.
21. Parks, R. E., Jr.; Agarwal, R. P., 3rd ed.. In *The Enzymes*; Boyer, P. D., Ed.; Academic Press: New York, 1972; Vol. 7, pp 483–514.
22. Katz, L. A.; Swain, J. A.; Portman, M. A.; Balaban, R. S. *Am. J. Physiol. Heart Circ. Physiol.* **1988**, *255*, H189.