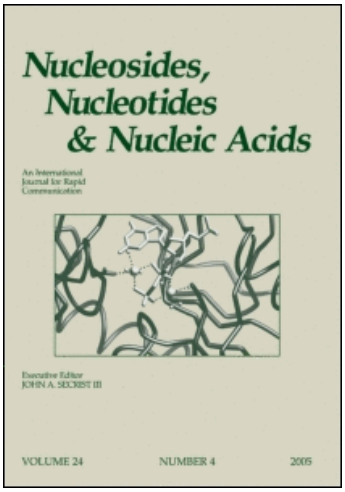

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6-Substituted 5-Fluorouracil Derivatives as Transition State Analogue Inhibitors of Thymidine Phosphorylase

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6-SUBSTITUTED 5-FLUOROURACIL DERIVATIVES AS TRANSITION STATE ANALOGUE INHIBITORS OF THYMIDINE PHOSPHORYLASE

Thomas I. Kalman and Li Lai ▫ *Department of Chemistry, University at Buffalo, Amherst, New York, USA*

▫ *A combination of mechanism-based and structure-based design strategies led to the synthesis of a series of 5- and 6-substituted uracil derivatives as potential inhibitors of thymidine phosphorlase/platelet derived endothelial cell growth factor (TP/PD-ECGF). Among those tested, 6-imidazolylmethyl-5-fluorouracil was found to be the most potent inhibitor with a K_i-value of 51 nM, representing a new class of 5-fluoropyrimidines with a novel mechanism of action.*

INTRODUCTION

Thymidine phosphorylase (TP), the enzyme responsible for the reversible phosphorolysis of thymidine to thymine and 2-deoxy-D-ribose-1-phosphate (dR-1-P) was first described by Friedkin and Roberts in 1954.[1] TP is overexpressed in a variety of tumors and was recently recognized to be identical to an angiogenic protein known as platelet-derived endothelial growth factor (PD-ECGF). The angiogenic activity of TP/PD-ECGF is a consequence of the conversion of one of the products of the TP reaction, dR-1-P to 2-deoxy-D-ribose, which stimulates migration of endothelial cells and the release of a variety of angiogenic factors,[2] including VEGF, integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$, IL-8, and MMP-1 (Figure 1), and is believed to promote tumor metastasis. 2-Deoxy-L-ribose can antagonize these effects of 2-deoxy-D-ribose downstream from the action of TP.[12] Since the enzymatic activity is essential for the angiogenic activity of TP/PD-ECGF, TP-selective enzyme inhibitors can block the angiogenic activity of this protein. Due to the dependence of the growth of solid tumors on vascularization,[13] antiangiogenic agents found therapeutic utility in the management of cancer,[14] and TP/PD-ECGF is considered a potentially important molecular target for non-cytotoxic anticancer drug development. The structures of a few representative examples of the variety of TP inhibitors reported in the literature are shown in Figure 2. Compounds **1–6** are

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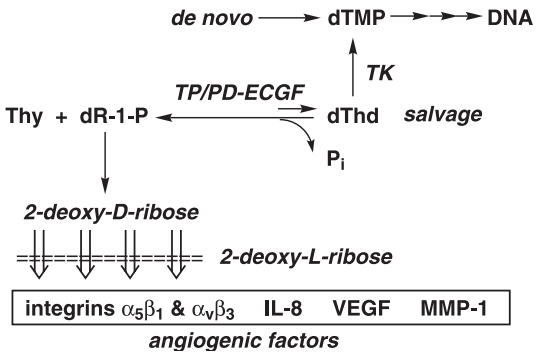


FIGURE 1 Outline of the role of TP/PD-ECGF in thymidine metabolism and angiogenesis.

pyrimidine derivatives, whereas **7–9** are purine analogues, **1**, **2**, **7**, and **8** (a multisubstrate analogue) are derivatives of 6-aminouracil. Compounds **1**, **2**, and **7** are neutral molecules, **4** is positively charged, **8** is negatively charged, and **3**, **5**, **6**, and **9** are protonated at neutral pH. 6-Aminothymine and 5-bromo-6-aminouracil (**1**), first reported by Langen et al. in 1967,[3] are weak inhibitors of the enzyme, and so are **2**, **4**, **7–9**, whereas **3**, **5**, and **6** are potent inhibitors with submicromolar IC_{50}- or K_i-values.

This article describes our efforts to rationally design 6-substituted uracil derivatives as potent transition state analogue inhibitors of TP/PD-ECGF and the effects of fluorine substitution at the 5-position of the pyrimidine ring.

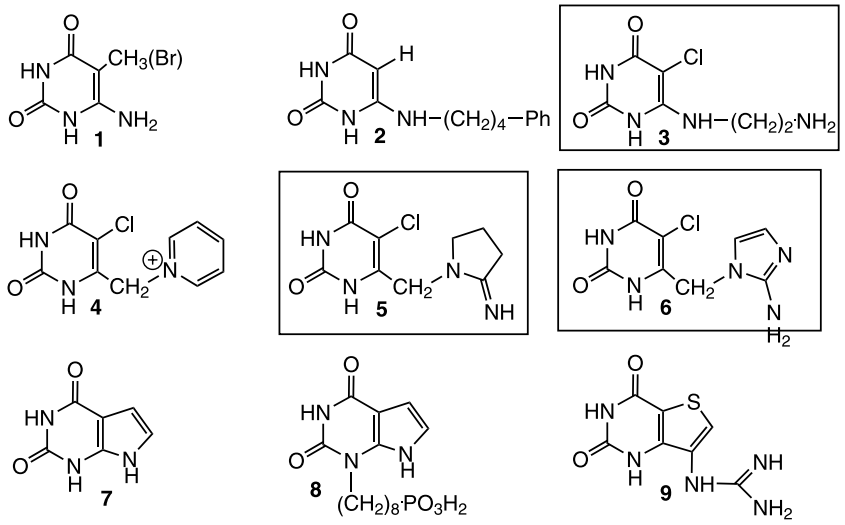


FIGURE 2 Examples of TP inhibitors. References for the compounds shown: **1**[3]; **2**[4]; **3**[5]; **4**[6]; **5**[7]; **6**[8]; **7**[9]; **8**[10]; **9**.[11] Structures of submicromolar inhibitors are framed.

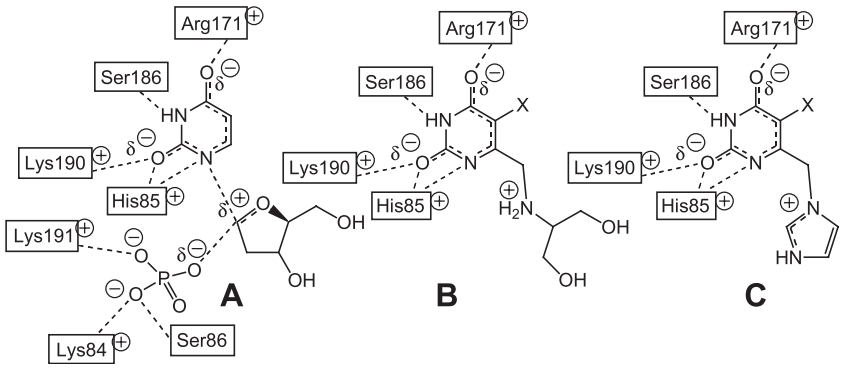
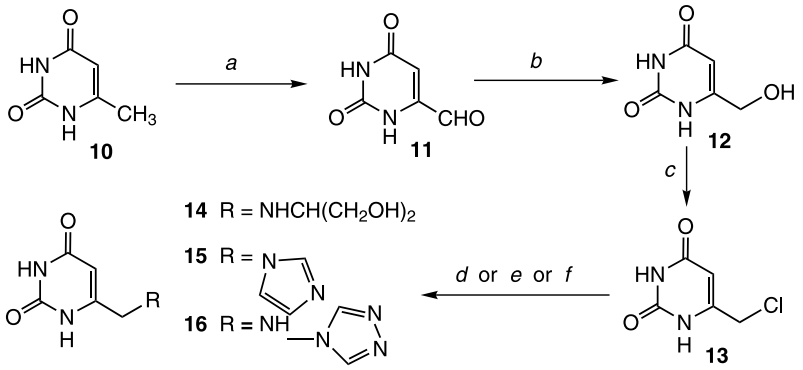


FIGURE 3 Rationale for transition state analogue design. **A**, oxacarbenium ion-like transition state of TP (*E. coli*); **B**, prototype acyclic base-substituted analogue; **C**, prototype cyclic base-substituted analogue. X = H or F.

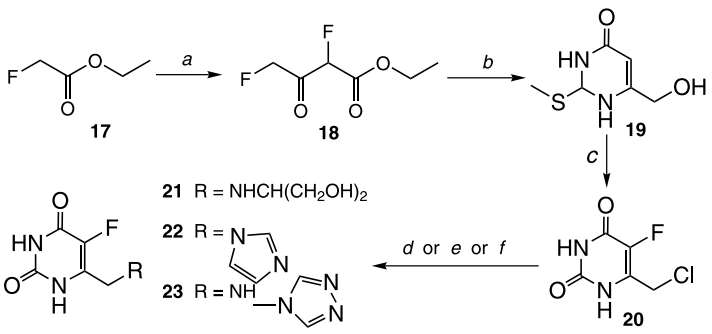
RESULTS AND DISCUSSION

Design Rationale

The availability of the crystal structures of *E. coli* TP,[15] which is highly homologous with the human enzyme, provided the structural framework for our inhibitor design. An oxacarbenium ion-like transition state common in reactions catalyzed by phosphorylases[16] was considered for TP. Figure 3(A) is a schematic representation of such a hypothetical transition state, incorporating relevant structural information about the active site.[15] Figure 3(B) and (C) show the two types of analogue design considered, both having a basic side-chain at the 6-position to mimic the oxacarbenium ion transition state, and retaining the active site contacts of the pyrimidine ring. The inhibitor structures in (B) represent analogues **14–15**, which retain the two OH-groups of dThd. The inhibitor structures in (C) represent



SCHEME 1 Outline of the synthesis of 6-substituted uracil derivatives **14**, **15**, and **16**. *a*, SeO_2, AcOH, reflux, 88%; *b*, $NaBH_4$, MeOH, 88%; *c*, $SOCl_2$, CH_2Cl_2, reflux, 93%; *d*, serinol, $CaCO_3$, DMA, 30%; *e*, imidazole, KI, DMF, RT, 28%; *f*, 4-amino-1,2,4-triazole, 2-propanol, reflux, 31%.



SCHEME 2 Outline of the synthesis of 6-substituted 5-fluorouracil derivatives **21**, **22**, and **23**. *a*, NaH, Et_2O, 40°C, 68%; *b*, 2-methyl-2-thiopseudourea sulfate, $NaOCH_3$, MeOH, reflux, 31%; *c*, 6N HCl, reflux, 70%; *d*, serinol, $CaCO_3$, DMA, 56%; *e*, imidazole, KI, DMF, RT, 40%; *f*, 4-amino-1,2,4-triazole, 2-propanol, reflux, 43%.

analogues **21–22**, in which the 5-membered ring of the side-chain mimics the sugar moiety of dThd.

Chemistry

The synthesis of the 6-substituted uracil analogues are outlined in Scheme 1. Oxidation of 6-methyluracil (**10**) with SeO_2 led to the 6-aldehyde **11**, which was reduced to the hydroxymethyl derivative **12**. Conversion to the chloromethyl derivative **13** was followed by alkylation of serinol, imidazole and 4-amino-1,2,4-triazole to give target compounds **14**, **15**, and **16**, respectively.

The synthesis of the 6-substituted 5-fluorouracil derivatives (see Scheme 2) involved cyclization of 2-methyl-2-thiopseudourea (**19**), derived from ethylfluoroacetate (**17**), to the hydroxymethylpyrimidine **19**, which was converted to 5-fluoro-6-chloromethyluracil (**20**). The latter was used to alkylate serinol, imidazole and 4-amino-1,2,4-triazole to give target compounds **21**, **22**, and **23**, respectively.

Enzyme Inhibitory Activity

Table 1 shows the results of the evaluation of compounds **14–16** and their 5-fluoro-substituted derivatives **21–23** as inhibitors of *E. coli* TP. All 5-fluorouracil

TABLE 1 Comparison of the Potencies of the Uracil Derivatives **14–17** and the Corresponding 5-Fluorouracil Derivatives **21–23**

Compound	5-Substituent	K_i (μM)	K_m/K_i
14	H	8.8 ± 2.2	150
15	H	4.7 ± 1.4	280
16	H	5.4 ± 1.6	240
21	F	1.0 ± 0.24	1,300
22	F	0.051 ± 0.012	25,500
23	F	0.37 ± 0.12	3,500

Conversion of dThd to thymine in arsenate buffer (pH 6.5) at 37°C was determined by HPLC. Kinetics of inhibition were competitive with respect to dThd; K_m (dThd) = 1.3 ± 0.13 mM.

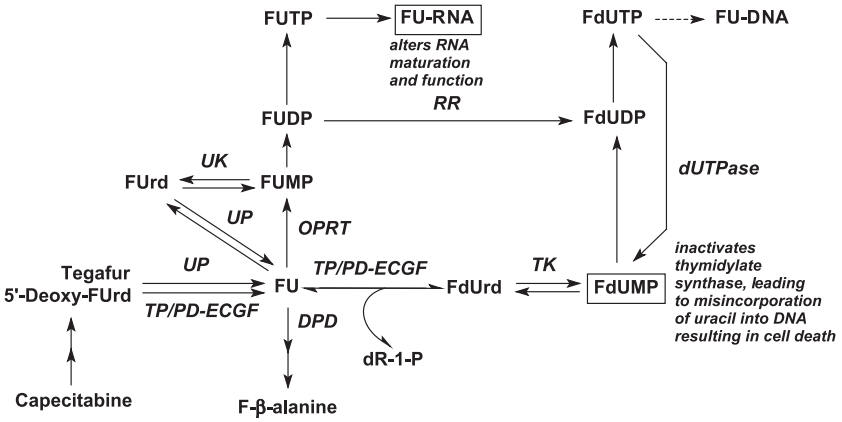


FIGURE 4 Outline of the role of thymidine phosphorylase (TP/PD-ECGF) in 5-fluorouracil (FU) and 5-fluoro-2′-deoxyuridine (FdUrd) metabolism and the activation of FU prodrugs capecitabine, 5-fluoro-5′-deoxyuridine (5′-deoxy-FUrd), and tegafur. *DPD*, dihydropyrimidine dehydrogenase; *OPRT*, orotidylate phosphoribosyl transferase; *RR*, ribonuclease reductase; *TK*, thymidine kinase; *UK*, uridine kinase; *UP*, uridine phosphorylase.

derivatives were more potent than the corresponding uracil analogues. The most potent compound was 6-imidazolylmethyl-5-fluorouracil (**22**) with a K_i-value of 0.051 μM, 2 orders of magnitude lower than the corresponding uracil derivative **15** (K_i = 4.7 μM). It is possible that *E. coli* TP is mechanistically identical to the human enzyme, and involves S_N2- instead of an S_N1-like transition state.[17] There is no oxacarbenium ion formation in the S_N2 transition state; therefore, the positively charged side-chains of the TP inhibitors most likely bind to the enzyme-bound phosphate ion.[17] This is all the more likely, because phosphate is known to bind before dThd,[18] and the pyrimidine binding site is dominated by several positively charged residues[15] unaccommodating to the protonated 6-substituents. Thus, **22** may bind to the active site of TP in its zwitter-ionic form, which is its preferred ionization state at pH 6.5 of the assay mixture. Consistent with this hypothesis is the 2 orders of magnitude lower K_i of the 5-fluoro derivative **22** (with the lower pK_a) than its unsubstituted counterpart **15**, indicating that the pyrimidine ring binds in its negatively charged form. Recently, the crystal structure of the human TP complexed with **5** was solved,[19] confirming the assumed binding interactions of the pyrimidine ring. However, the reported structure had no phosphate ion present, therefore the postulated interaction of the protonated side-chains of the potent TP inhibitors **3**, **5**, **6**, and **22** remains to be confirmed.

Therapeutic Implications

Since the enzymatic activity of TP/PG-ECGF is essential for its angiogenic activity, **22** may serve as a lead for the development of anti-angiogenic drugs with potential utility in the treatment angioproliferative disorders, such as cancer, diabetic retinopathy, macular degeneration, rheumatoid arthritis, psoriasis, and atherosclerosis. The TP activity of TP/PD-ECGF is also involved in the metabolism

of the 5-fluoropyrimidines, an important class of anticancer drugs (Figure 4). It is responsible for the activation of 5-fluorouracil (FU) and its prodrug derivatives, capecitabin, 5′-fluoro-2′-deoxyuridine and tegafur, as well as the moderate selectivity of these prodrugs. In contrast, it curtails the utility of the more potent and DNA-selective 5-fluoro-2′-deoxyuridine (FdUrd), by rapidly converting it to FU. Thus, TP inhibitors cannot be combined with FU and its prodrugs. However, their combination with FdUrd may have potential therapeutic benefit by preventing the metabolic degradation of this drug. It should be noted that due to the presence of their 6-substituent, the fluoropyrimidines **21–23** described in this paper are not subject to the metabolism outlined in Figure 4. Thus, these TP inhibitors represent a new class of 5-fluoropyrimidines with a novel mechanism of action.

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