Brief Articles

Novel Nonsubstrate Inhibitors of Human Thymidine Phosphorylase, a Potential Target for Tumor-Dependent Angiogenesis

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Thymidine phosphorylase/platelet-derived endothelial cell growth factor (TP/PD-ECGF) is an enzyme involved in thymidine metabolism and homeostasis, and its catalytic activity appears to play an important role in angiogenesis. Here we describe the cloning and expression of a His-tagged human TP/PD-ECGF and its assay with uracil and thymine analogues. We present the design, synthesis, and biological evaluation of novel 6-(phenylalkylamino)uracil derivatives which, at micromolar concentrations, inhibit both catabolic and anabolic reactions of human TP in vitro. These base analogues are not converted by the enzyme into the nucleoside form, thus representing pure nonsubstrate inhibitors of the enzyme.

Introduction

Solid tumors are angiogenesis-dependent.[1] Thus, to develop an anticancer therapy based upon the regression or inhibition of vascularization of the tumor mass, it is important to understand how tumors cells elicit the growth of capillaries into the tumor mass and to discover possible ways in which this phenomenon may be prevented. To this purpose it was recently found that thymidine phosphorylase (TP), an enzyme involved in thymidine metabolism and homeostasis, could play an important role in angiogenesis[2,3] because of its endothelial cell chemotactic activity which stimulates endothelial cell migration. This enzyme, which is mainly localized in the platelet cytosol, catalyzes the reversible phosphorolytic degradation of thymidine to thymine and deoxyribose-1-phosphate. TP has a low substrate specificity being able to recognize not only thymidine but also deoxyuridine and other pyrimidine nucleoside derivatives.[4,5] Its levels of expression were elevated up to 10-fold when compared to nonneoplastic regions of the organs in nearly all biopsies examined from carcinomas of the stomach, colon, and ovary,[6] and higher levels of TP also correlate with ovarian malignancy and ovarian tumor blood flow.[7]

Gene sequence comparison has demonstrated that human TP is identical to a novel angiogenic protein, known as platelet-derived endothelial cell growth factor (PD-ECGF).[8−10] PD-ECGF has been purified from human platelets[11] and human placenta[12] and was found to be produced by different cell types in culture, including human foreskin fibroblasts, human vascular smooth muscle cells, and certain cancer cell lines.[12,13] Recently, it was found that TP or PD-ECGF − in this paper the protein will be referred to as TP − is expressed in gastric carcinoma,[14] colon carcinoma,[15] uterine sarcoma and leiomyoma,[16] renal carcinoma,[17] and breast[18] and lung cancer.[19] It was demonstrated that serine residues of TP can be covalently linked to phosphate groups of nucleotides, resulting in a nucleotidylated protein[20] whose functional significance has not been determined. Studies with TP mutants, made by site-directed mutagenesis, indicate that the catalytic activity of the enzyme is essential for its angiogenic effects,[3,21,22] but the mechanism by which it stimulates angiogenesis remains to be elucidated However, it has been suggested that 2-deoxy-D-ribose, the dephosphorylated form of the reaction product of TP, may be responsible for TP-dependent angiogenesis.[22]

Since endothelial cell migration is essential for angiogenesis and the catalytic activity of TP is essential for endothelial cell migration, we hypothesize that selective nonsubstrate inhibitors of TP could be useful anticancer drugs by interfering with the TP-dependent angiogenesis in tumor tissues. Specific inhibitors of human TP could also enhance the efficacy of thymidine analogues such as 5-fluoro-2′-deoxyuridine and 5-iodo-2′-deoxyuridine which would no longer be cleaved and inactivated by TP.[5] A logical starting point for identification of TP inhibitors is thymine and other 5-substituted uracils. Baker reported extensively on the inhibitory activity of numerous uracil and thymine derivatives, including 1- and 6-alkyl-substituted compounds, against *Escherichia coli* TP.[23] Recently, a series of 5-chloro-6-(aminoalkyl)uracils was reported to have potent activity against human TP,[24] and 7-deazaxanthine was shown to both inhibit TP activity and prevent neovascularization in the chicken chorioallantoic membrane.[25] A

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NH_2 terminus of the native human thymidine phosphorylase

Met Ala Ala Leu Met Thr Pro Gly Thr Gly Ala Pro Pro Ala Pro Gly Asp.....
ATG GCA GCC TTG ATG ACC CCG GGA ACC GGG GCC CCA CCC GCG CCT GGT GAC.....

NH_2 terminus of the recombinant human thymidine phosphorylase

Met Gly Gly Ser His His His His His His Gly Met Ala Ser Met Thr Gly
ATG GGG GGT TCT CAT CAT CAT CAT CAT CAT GGT ATG GCT AGC ATG ACT GGT

Gly Gln Gln Met Gly Arg Asp Leu Tyr Asp Asp Asp Asp Lys His Arg Trp
GGA CAG CAA ATG GGT CGG GAT CTG TAC GAC GAT GAC GAT AAG CAT CGA TGG

Ile Arg Pro Arg Pro Pro Ala Pro Gly Asp.....
ATC CGA CCT CGA CCA CCC GCG CCT GGT GAC.....

Figure 1. Amino-terminal sequences of native and recombinant TPs. The bold sequence in the native enzyme has been replaced by the bold sequence in the recombinant form.

preliminary screen of a large number of uracil derivatives and related compounds available in our laboratories allowed us to select the 6-aminouracil moiety as a possible lead structure for the discovery of potent, selective human TP inhibitors. Indeed, 6-aminothymine and 5-bromo-6-aminouracil were inhibitors of human TP.[5] In this paper we present the results of screening, synthesis, and in vitro inhibition testing of 6-(arylalkylamino)uracil analogues and a study of preliminary structure−activity relationships for their inhibition of human TP.

Results

Expression of Recombinant His-Tagged Human TP and Its Comparison with TP Purified from Human Platelets. DNA containing the full-length PD-ECGF/TP cDNA was ligated into a plasmid to give the recombinant expression vector pHisTP containing the sequence for the His-tagged protein. As illustrated in Figure 1, the recombinant TP lacks at its NH_2 terminus the first 11 amino acids present in the native human protein and in their place contains 39 amino acids present in the vector (pTRCHisC, Invitrogen) including the 6 His residues needed for affinity purification on Ni resin. Expression and purification of the protein on a Ni-NTA column gave the recombinant TP, and the SDS−PAGE of the purified recombinant TP is presented in Figure 2A. We demonstrated (Figure 2B) that polyclonal rabbit anti-recombinant TP antibodies recognize both the recombinant TP and the native TP, purified from human platelets as described in the Experimental Section. Similarly to the native enzyme, the recombinant TP is able to perform both catabolic and anabolic reactions using as substrates thymidine and thymine, respectively (data not shown).

6-Substituted Pyrimidine Analogues Inhibit Human TP. Previous studies revealed that numerous hydrophobic uracil and thymine derivatives inhibited *E. coli* TP.[23] Recently, the effect of a 5-chlorouracil derivative as an inhibitor of angiogenesis and apoptosis in tumors has been described.[24] We screened numerous pyrimidines available in our laboratories, and we have identified a class of 6-(phenylalkylamino)uracil derivatives that inhibit human TP.

The general structure of the prototypes and newly synthesized compounds is presented in Scheme 1. All compounds in this study are listed in Table 1, and syntheses and properties of new compounds are presented in the Experimental Section. Compounds **4**−**9** were prepared as described previously[26,27] by reacting the appropriate arylalkylamine with 6-chlorouracil in water or 2-methoxyethanol. The same general procedure was employed for the synthesis of analogous 5-methyl-6-(arylalkylamino)uracil derivatives **10**−**11** and 5-ethyl-6-(arylalkylamino)uracil derivatives **12**−**15**. 5-Chloro-6-(4-phenylbutylamino)uracil (**16**) was obtained by reacting 6-(4-phenylbutylamino)uracil ("PBAU" **7**) with *N*-chlorosuccinimide in acetic acid. N1-Substituted derivatives **18** and **19** were prepared as described previously:[28] the corresponding 6-aminouracils **17** and **26**, respectively, were heated with a mixture of 4-phenylbutylamine and 4-phenylbutylamine hydrochloride at 160 °C in the absence of solvent, followed by demethylation of the methoxy intermediates with trimethylsilyl iodide. N3-Substituted compounds **21** and **22** were prepared from 3-(2-methoxyethyl)-6-chlorouracil (**27**) and 3-(3-methoxypropyl)-6-chlorouracil (**28**), respectively, by heating in 4-phenylbutylamine as solvent.

6-Substituted pyrimidine analogues were assayed in vitro as described in the Experimental Section in order to determine their capacity to inhibit the catabolic reaction of human TP. The reaction of thymidine with sodium arsenate results in thymine and deoxyribose-α-D-1-arsenate. Sodium arsenate is used in place of sodium phosphate (which would give deoxyribose-α-D-1-phosphate as product of reaction) in order to reduce the reversibility of the TP reaction. In fact, deoxyribose-α-D-1-arsenate hydrolyzes rapidly in water − far more rapidly than it is condensed with the released thymine to re-form thymidine − thus allowing the reaction to go to completion. The percent inhibition of the TP reaction by the pyrimidine analogues at a fixed concen-

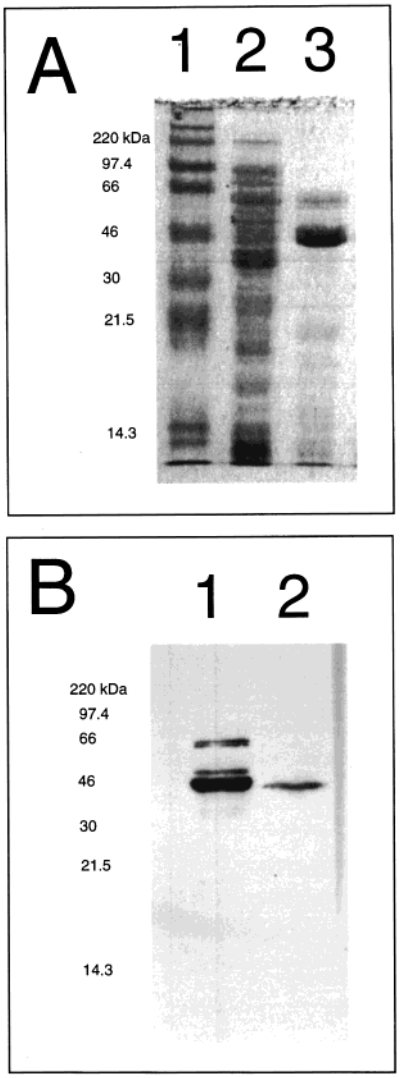
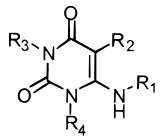


Figure 2. Panel A: SDS−PAGE of the affinity-purified recombinant TP. Lanes: 1, MW markers; 2, IPTG-induced *E. coli* crude extract; 3, 1.6 pg of recombinant TP eluted from a Ni-NTA column in the presence of *E. coli* crude extract. Panel B: Western blot analysis of recombinant TP (lane 1) and native human TP (lane 2). For experimental details, see the Experimental Section.

tration of 200 μM is reported in Table 1. All of the inhibitor assays were performed with thymidine at 100 μM.

Uracil derivatives with 6-phenylalkylamino substituents inhibited the degradation of thymidine with percentages that increased with alkyl group length (Table 1). The inhibition curves determined by assaying TP in the presence of several concentrations of these compounds (Figure 1A, Supporting Information) showed that 6-(4-phenylbutylamino)uracil (**7**, PBAU) was the most potent of this series, inhibiting the catabolic function of TP with an IC_{50} of 30 μM (Table 2). Comparison of the effect of **7** with that of 6-aminothymine (**2**) clearly shows the greater potency of the new compound (Figure 3). On the basis of the reported activity of thymine derivatives, we hypothesized that the double substitution in positions 5 and 6 could lead to more active inhibitors. However, compounds analogous to **4**−**7**, the 5-methyl (**8**−**11**) and 5-ethyl (**12**−**15**) 6-(phenylalkylamino)uracils, were considerably weaker

Table 1. Inhibition of Recombinant TP by 6-Aminouracil Base Analogues at 200 μM



compd	R_1	R_2	R_3	R_4	% inhib[a]
1	H	H	H	H	41
2	H	CH_3	H	H	65
3	H	C_2H_5	H	H	10
4	$CH_2C_6H_5$	H	H	H	0
5	$(CH_2)_2C_6H_5$	H	H	H	50
6	$(CH_2)_3C_6H_5$	H	H	H	70
7	$(CH_2)_4C_6H_5$	H	H	H	86
8	$CH_2C_6H_5$	CH_3	H	H	10
9	$(CH_2)_2C_6H_5$	CH_3	H	H	9
10	$(CH_2)_3C_6H_5$	CH_3	H	H	0
11	$(CH_2)_4C_6H_5$	CH_3	H	H	16
12	$CH_2C_6H_5$	C_2H_5	H	H	14
13	$(CH_2)_2C_6H_5$	C_2H_5	H	H	16
14	$(CH_2)_3C_6H_5$	C_2H_5	H	H	23
15	$(CH_2)_4C_6H_5$	C_2H_5	H	H	31
16	$(CH_2)_4C_6H_5$	Cl	H	H	50
17	H	H	H	CH_3	
18	$(CH_2)_4C_6H_5$	H	H	CH_3	25
19	$(CH_2)_4C_6H_5$	H	H	$(CH_2)_2OCH_3$	30
20	$(CH_2)_4C_6H_5$	H	H	$(CH_2)_2OH$	17
21	$(CH_2)_4C_6H_5$	H	$(CH_2)_2OCH_3$	H	6
22	$(CH_2)_4C_6H_5$	H	$(CH_2)_3OCH_3$	H	6

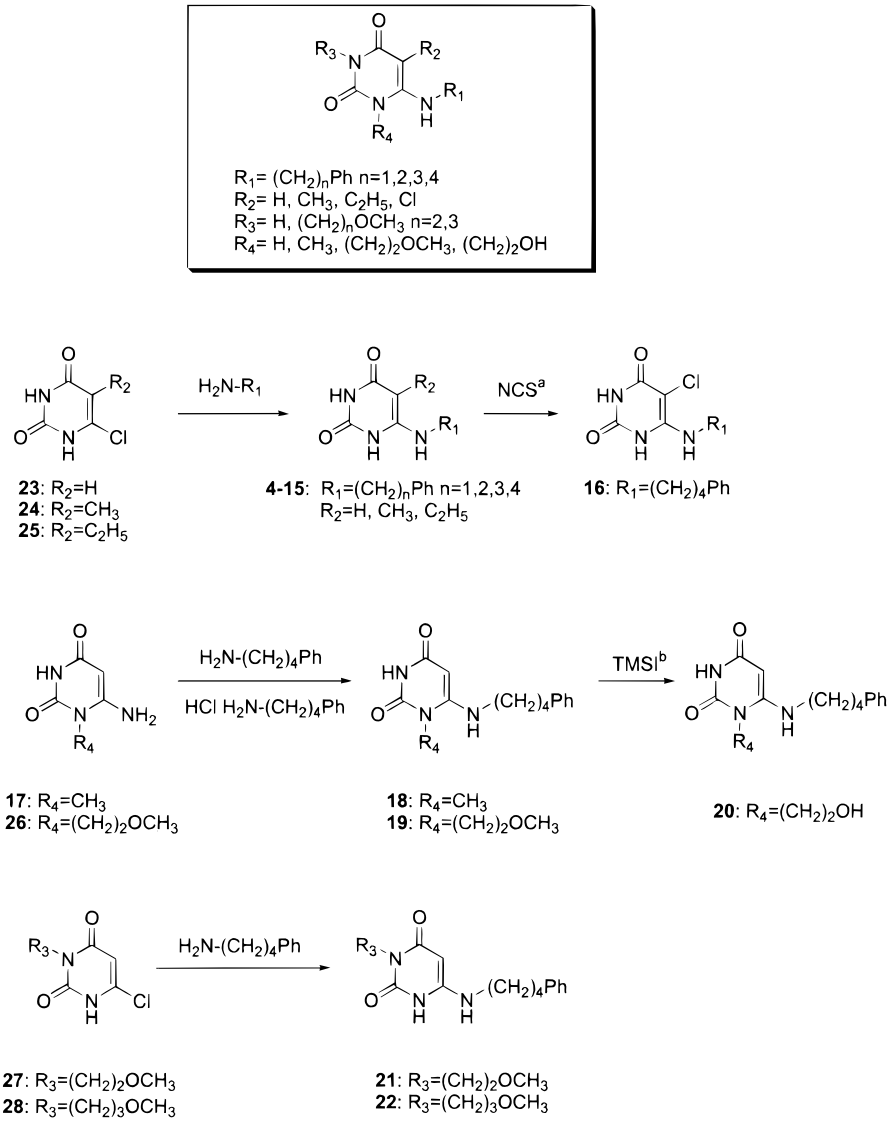
[a] Inhibitor assays employed 100 μM thymine (see Experimental Section for details).

inhibitors (Table 1). These results demonstrate not only the absence of synergistic effects when both substitutions are present but also an interference of the two substitutions on inhibitory activity of the compounds. To evaluate the activity resulting from the introduction of an electron-attracting substituent in the 5-position, 5-chloro-PBAU (**16**) was synthesized. Although this derivative was a weaker inhibitor than **7** (Table 1), the reduction in potency was considerably less than that induced by electron-donating alkyl groups in the 5-position (compare compound **16** to **8**−**15**). Furthermore, substitutions in positions 1 and 3 of the pyrimidine ring do not lead to active compounds, but even reduce the efficacy of the 6-substituted compounds (**18**−**22**).

Apart from 6-aminothymine (**2**), the most active compounds (**5**−**7**) are all 6-(phenylalkylamino)uracils. Additional studies of these compounds and their IC_{50} values for both catabolic (thymidine + P_i → thymine + deoxyribose-α-D-1-phosphate) and anabolic reactions of TP are reported in Table 2. The data demonstrate that these compounds inhibit both forward and reverse reactions of human TP with comparable efficiency. The same results were obtained in assays of the native TP, purified from human platelets following the procedure described by Desgranges et al.[5] Nearly identical concentration:inhibition curves of PBAU (**7**) on both native and recombinant TPs were found (Figure 1B, Supporting Information).

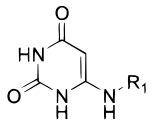
6-Substituted Pyrimidine Analogues Are Not Converted to Their Nucleoside Form by Human TP. We then asked if compounds **2** and **5**−**7**, like thymine, were bona fide substrates of human TP. The experimental approach involved incubating human TP with ribose-1-phosphate and the base analogues under

Scheme 1



[a] NCS: *N*-chlorosuccinimide. [b]TMSI: trimethylsilyl iodide.

Table 2. IC_{50} Values of Inhibitors on Catabolic and Anabolic Activities of Human TP



compd	R_1	IC_{50}, μM Thd → Thy catabolic	IC_{50}, μM Thy → Thd anabolic
4	benzyl	>200[a]	ND
5	phenylethyl	148	200
6	phenylpropyl	82	125
7	phenylbutyl	30	74

[a] Highest concentration tested.

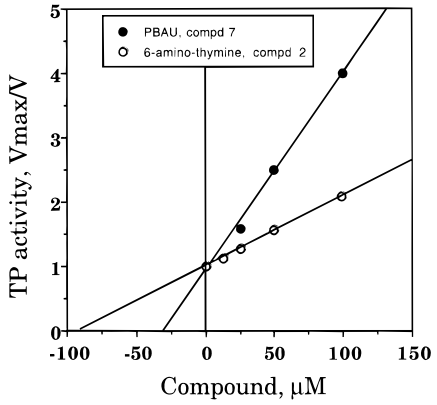


Figure 3. Comparison of the concentration effect of PBAU (**7**) and 6-aminothymine (**2**) on the inhibition of recombinant TP activity. Experimental details are described in the Experimental Section.

conditions promoting the formation of the corresponding nucleosides and monitoring the reaction mixtures by HPLC. Under conditions which allowed the complete transformation of 2.5 μmol of thymine to thymidine, compounds **2** and **5**−**7** were completely resistant to this transformation by human TP − no reduction in compound peak area nor emergence of a peak in the nucleoside region of the HPLC were observed (Figure 2, Supporting Information). These results indicate that these compounds are pure nonsubstrate inhibitors of the enzyme.

Conclusions

This work describes the design, synthesis, and biological evaluation against human TP of several pyrimidine derivatives. Generally, 6-(phenylalkylamino)uracils inhibited the enzyme, and in particular, 6-(2-phenylethylamino)uracil (**5**), 6-(3-phenylpropylamino)uracil (**6**), and 6-(4-phenylbutylamino)uracil (**7**, PBAU) inhibited, at micromolar concentrations, both catabolic and anabolic reactions of human TP in vitro. These base analogues are not converted by the enzyme into the nucleoside form, thus representing pure nonsubstrate inhibitors of the human enzyme. Conversion of the most potent inhibitor PBAU (**7**) to the thymine (5-methyl) or other 5-substituted derivatives resulted in compounds with decreased inhibitory potency. Substituents at N1 or N3 also did not increase potency. These results contrast with the positive effects of substitutions in the pyrimidine ring for inhibition of *E. coli* TP by uracil derivatives.[23]

The role of TP in angiogenesis and the reported ability of a TP inhibitor to inhibit apoptosis and, possibly, angiogenesis in a human tumor xenograft model in mice[24] suggest that specific nonsubstrate inhibitors of TP can have antitumor activity. Such compounds when used in combination with antitumor pyrimidine nucleoside analogues could have a double effect by enhancing the antitumor efficacy of nucleoside analogues[5] and inhibiting TP-dependent angiogenesis in tumor growth regions.[25]

Experimental Section

Biochemistry. Analytical grade reagents were used exclusively. Bacterial media components were from Difco. Ni-NTA Superflow resin was from QIAGEN. Superspher 100 RP18 column (LiChroCART 75-4) was from Merck. Restriction and modification enzymes were from Promega, Sigma or Boehringer. IPTG was from Sigma.

Construction of Recombinant Bacterial Expression Vector. Plasmid pPL5 (kindly provided by Prof. R. Bicknell, University of Oxford), which contains the full length PD-ECGF/TP cDNA (1587 bp containing the 1446-bp coding sequence), was digested with *Apa*I and the 3′-protruding end was removed by the 3′-5′ exonuclease activity of *Pfu* DNA polymerase. The linear pPL5 was then digested with *Eco*RI. *Apa*I restricts 33 bp downstream of the ATG initiation of translation site, whereas *Eco*RI cuts downstream of the stop codon. The resulting DNA was ligated into the multiple cloning site of pTrcHisC (Invitrogen) and restricted with *Ava*I (filled with *E. coli* DNA polymerase I − Klenow fragment) and *Eco*RI, to give the recombinant bacterial expression vector pHisTP containing the His-tagged TP sequence which encodes for a 509-amino acid protein.

Expression and Purification of Cloned PD-ECGF/TP from Bacterial Cells. Expression and purification of the His-tagged PD-ECGF/TP were carried out as described by the manufacturer of the Ni-NTA Superflow resin (QIAGEN). Briefly, a fresh overnight saturated culture of *E. coli* (DH5α strain) transformed with the recombinant pHisTP was diluted 1:50 in 50 mL of 2XTY broth[34] containing ampicillin (100 μg/mL) and incubated at 37 °C with shaking. After 45 min of growth IPTG was added to a final concentration of 1 mM, and the culture was incubated for a further 4 h at 37 °C. The bacterial cell pellet was resuspended in 2 mL of lysis buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole, 1 mM PMSF and 1 mg/mL lysozyme) and incubated on ice for 30 min. Cells were then sonicated on ice, and the lysate was centrifuged at 10000*g* for 30 min at 4 °C. The supernatant was loaded on a Ni-NTA Superflow column (0.5 mL) and eluted at a flow rate of 0.2 mL/min. The column was first washed with lysis buffer and then with 50 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl and 20 mM imidazole. The protein was then step-eluted with 250 mM imidazole in 50 mM sodium phosphate buffer, pH 8.0, and 300 mM NaCl. Fractions were collected for SDS−PAGE and enzymatic activity analysis. The enzyme was then dialyzed against 20 mM potassium phosphate buffer, pH 7.5, containing 10% glycerol, 1 mM DTT and 1 mM EDTA. The enzyme preparation had 250 000 units/mg. (One unit corresponds to conversion of 1 nmol of thymidine to thymine in 1 h.)

Western Blotting. Recombinant TP (80 ng) and platelet extract (100 pg) were electrophoresed in a 10% polyacrylamide gel and then blotted to Biodyne B membrane (PALL) for 45 min at 10 V. The membrane was incubated with 5% powdered milk (Rapilat, MIGROS) in water for 1 h to block nonspecific binding. TP was detected by incubating the membrane with 1 pg/mL of protein-G-purified (Pharmacia) rabbit anti-recombinant TP polyclonal antibodies (K39) for 1 h at room temperature. After incubation with horseradish peroxidase-conjugated anti-rabbit IgG (1:5000, Amersham Life Technologies) for 1 h, immune complexes were detected with an enhanced chemiluminescence ECL detection system (Amersham Life Technologies). The membrane was exposed to autoradiographic film (Hyperfilm MP, Amersham Life Technologies) for 15 s.

Purification of TP from Human Platelets. Human TP was purified from 2.5 g of human platelets prepared as described by Desgranges et al.[5] Briefly, platelets were suspended in 20 mL of Tris-maleate buffer (pH 6.5) containing 30 mM NaCl, 1 mM DTT and 0.5 mM PMSF (buffer A). After 10 min on ice platelets were homogenized with a Dounce homogenizer and then sonicated 3 × 10 s at 20−25 W. The crude extract was centrifuged at 100000*g* for 1 h at 4 °C. The supernatant was loaded on a HiTrapQ column (1 mL; Pharmacia) using the FPLC system. The enzyme was eluted with a linear gradient between 10 and 500 mM NaCl in buffer A. The pooled fractions were diluted with buffer A and then loaded on a MonoQ column (1 mL; Pharmacia), and the enzyme was eluted with a linear gradient between 10 and 500 mM NaCl in buffer A. The enzyme eluted as a single peak at 0.25 M NaCl and was made 20% in glycerol and stored in small aliquots in liquid nitrogen until use.

TP Assays. The TP activity was measured by HPLC analysis of the products of either catabolic or anabolic reaction of the enzyme. For measurement of the catabolic activity, assays in 25-μL reaction mixtures contained 0.6 mM thymidine and 50 mM sodium arsenate, pH 6.2. For measurement of the anabolic activity, assays in 25 μL of reaction mixtures contained 0.1 mM thymine, 10 mM deoxyribose-1 phosphate and 50 mM Tris-maleate, pH 7.25. In both cases an amount of enzyme was added to give linear reaction rates. Mixtures were incubated at 37 °C and then heated for 5 min at 90 °C. The assay tubes were centrifuged at 12 000 rpm for 5 min, and the supernatants (20 μL) were analyzed by HPLC. Thymine and thymidine were resolved on a Superspher 100 RP18 column by elution with the following gradient. Eluents: buffer A (20 mM KH_2PO_4, pH 4.6), buffer B (20 mM KH_2PO_4, pH 4.6, 60% methanol). Gradient conditions: 0−4 min, 0% buffer B; 4−20 min, from 30% to 72% buffer B; 20−40 min, 100% buffer B. Flow rate: 0.5 mL/min. The relative peak areas of thymine and thymidine were used to determine the enzymatic activity of TP.

The above assay conditions were also used to determine the inhibitory effect of the 6-aminouracil base analogues. In these assays 0.1 mM thymidine or thymine was incubated, in its respective mixture, in the presence of different concentrations of the base analogue. Reactions were initiated by addition of an amount of enzyme able to transform approximately 50% of the substrate (thymidine or thymine) present in the assay. The mixtures were incubated for 15 min at 37 °C; 20 μL of the reaction mixtures was then analyzed by HPLC in order to separate thymine from thymidine. IC_{50} values were calculated by analysis of the areas of the peaks eluted from the column.

Chemistry. Melting points were measured using a Meltemp hot-stage apparatus and are uncorrected. ^1H NMR

spectra were recorded in DMSO-d_6 at 300 MHz using a Varian Unity 300 spectrometer. ^{1}H chemical shifts (δ, ppm from internal TMS) and ^{1}H−^{1}H coupling constants (J, Hz) were consistent with the proposed structures in all cases. Elemental analyses were performed on a Carlo Erba 1106 elemental analyzer, and results were within ±0.4% of the theoretical values. All reactions were monitored by TLC on 0.25-mm Merck silica gel (60 F254) visualized by UV light (λ = 264 or 365 nm). Chromatography was performed using silica gel (60−200 μm; Merck). 6-Aminouracil (**1**) and 6-chlorouracil (**23**) were purchased from Aldrich. 6-Aminothymine (**2**),[29] 5-ethyl-6-aminouracil (**3**),[30] 1-methyl-6-aminouracil (**17**),[28] 5-methyl-6-chlorouracil (**24**) and 5-ethyl-6-chlorouracil (**25**),[31] 1-(2-methoxyethyl)-6-aminouracil (**26**),[28] 3-(2-methoxyethyl)-6-chlorouracil (**27**) and 3-(3-methoxypropyl)-6-chlorouracil (**28**)[32] were prepared by the referenced methods. Compounds **4**−**7**[27] and **8** and **12**[33] were prepared as described.

5-Alkyl-6-(phenylalkylamino)uracil Derivatives 9−15. A solution of 5-alkyl-6-chlorouracil (1.0 mmol) and the appropriate amine (2.0 mmol) in 2-methoxyethanol (10 mL) was heated at reflux for 16−48 h. The reaction mixture was allowed to cool to room temperature, and the precipitate resulting from addition of few drops of water was filtered and washed with a mixture of 1:1 EtOH:H_2O. Crystallization of the residue afforded the compounds as colorless crystals.

5-Methyl-6-(2-phenylethylamino)uracil, 9. Yield 46%; mp 260 °C dec (EtOH). ^{1}H NMR (DMSO-d_6): 1.75 (s, 3H, CH_3), 2.89 (m, 2H, CH_2-Ph), 3.07 (m, 2H, NH-CH_2), 6.35 (t, 1H, NH-CH_2), 7.15 (m, 5H, Ph), 10.20 (s, 1H, 3-NH), 10.25 (s, 1H, 1-NH). Anal. ($C_{13}H_{15}N_3O_2$) C, H; N: calcd, 17.13; found, 16.69.

5-Methyl-6-(3-phenylpropylamino)uracil, 10. Yield 56%; mp 237−240 °C (EtOH). ^{1}H NMR (DMSO-d_6): 1.61 (s, 3H, CH_3), 1.76 (m, 2H, -$CH_2CH_2CH_2$-), 2.61 (t, 2H, CH_2-Ph), 3.25 (m, 2H, NH-CH_2), 6.28 (t, 1H, NH-CH_2), 7.20 (m, 5H, Ph), 10.18 (s, 1H, 3-NH), 10.22 (s, 1H, 1-NH). Anal. ($C_{14}H_{17}N_3O_2$) C, H, N.

5-Methyl-6-(4-phenylbutylamino)uracil, 11. Yield 45%; mp 128−130 °C (EtOH). ^{1}H NMR (DMSO-d_6): 1.35−1.50 (m, 4H, 2×CH_2), 1.62 (s, 3H, CH_3), 2.65 (t, 2H, CH_2-Ph), 3.25 (m, 2H, NH-CH_2), 6.15 (t, 1H, NH-CH_2), 7.10−7.30 (m, 5H, Ph), 10.0 (s, 1H, 3-NH), 10.15 (s, 1H, 1-NH). Anal. ($C_{15}H_{19}N_3O_2$) C, H, N.

5-Ethyl-6-(2-phenylethylamino)uracil, 13. This compound was prepared as described above, except that the residue was purified by flash chromatography with $CHCl_3$:MeOH (9:1) as eluent. Yield 20%; mp 190−195 °C (EtOH). ^{1}H NMR (DMSO-d_6): 0.85 (t, 3H, CH_3), 2.19 (q, 2H, CH_2-CH_3), 2.77 (t, 2H, CH_2-Ph), 3.50 (q, 2H, NH-CH_2), 6.35 (t, 1H, NH-CH_2), 7.15−7.30 (m, 5H, Ph), 10.15 (s, 1H, 3-NH), 10.20 (s, 1H, 1-NH). Anal. ($C_{14}H_{17}N_3O_2$) C, H, N.

5-Ethyl-6-(3-phenylpropylamino)uracil, 14. Yield 26%; mp 260 °C dec (EtOH). ^{1}H NMR (DMSO-d_6): 0.85 (t, 3H, CH_3), 1.75 (m, 2H, $CH_2CH_2CH_2$), 2.21 (m, 2H, CH_2-CH_3), 2.59 (t, 2H, CH_2-Ph), 3.25 (m, 2H, NH-CH_2), 6.38 (s, 1H, NH-CH_2), 7.19−7.31 (m, 5H, Ph), 10.05 (s, 1H, 3-NH), 10.15 (s, 1H, 1-NH). Anal. ($C_{15}H_{19}N_3O_2$) C; H: calcd, 7.01; found, 7.48; N: calcd, 15.37; found, 14.96.

5-Ethyl-6-(4-phenylbutylamino)uracil, 15. Yield 59%; mp 185−190 °C (H_2O/EtOH). ^{1}H NMR (DMSO-d_6): 0.86 (t, 3H, CH_3), 1.38−1.65 (m, 4H, 2×CH_2), 2.19 (q, 2H, CH_2-CH_3), 2.61 (t, 2H, CH_2-Ph), 3.25 (m, 2H, NH-CH_2), 6.30 (t, 1H, NH-CH_2), 7.06−7.31 (m, 5H, Ph), 9.98 (br s, 1H, 1-NH), 10.15 (s, 1H, 3-NH). Anal. ($C_{16}H_{21}N_3O_2$) C, H, N.

5-Chloro-6-(4-phenylbutylamino)uracil, 16. *N*-Chlorosuccinimide (33.3 mg, 0.25 mmol) was added to a stirred suspension of PBAU (**7**) (50 mg, 0.19 mmol) in acetic acid (1 mL) at room temperature. The clear reaction mixture was stirred at the same temperature for 2.5 h until disappearance of the starting material. A few drops of water were added, and the resulting precipitate was removed by filtration under reduced pressure. Crystallization of the solid from EtOH afforded colorless crystals. Another crop of the title compound was recovered from the mother liquor to give a combined yield of 28 mg (50%), mp 210−212 °C. ^{1}H NMR (DMSO-d_6): 1.38−1.60 (m, 4H, 2×CH_2), 2.55−2.65 (t, 2H, CH_2-Ph), 3.30 (m, 2H, NH-CH_2), 6.99 (t, 1H, NH), 7.15−7.28 (m, 5H, Ph), 10.65 (s, 1H, 3-NH), 10.71 (s, 1H, 1-NH). Anal. ($C_{14}H_{16}N_3O_2Cl$) C, H, N.

1-Methyl-6-(4-phenylbutylamino)uracil, 18. A mixture of 1-methyl-6-aminouracil (**17**) (150 mg, 1.06 mmol), 4-phenylbutylamine (2 mmol) and 4-phenylbutylamine hydrochloride (2 mmol) was heated at 160 °C for 1.5 h. The reaction mixture was allowed to cool to room temperature, and the residue was boiled in chloroform (15 mL) for 20 min. The insoluble material was removed by filtration, and the solution was evaporated to dryness. The residue was purified by column chromatography on silica gel with $CHCl_3$:MeOH (95:5) as eluent to give **18** as a white solid (82 mg, 28%), mp 200−204 °C. ^{1}H NMR (DMSO-d_6): 1.41−1.68 (m, 4H, 2×CH_2), 2.59 (t, 2H, CH_2-Ph), 3.15 (m, 2H, NH-CH_2), 3.20 (s, 3H, N-CH_3), 4.50 (s, 1H, C5-H), 6.66 (m, 1H, NH-CH_2), 7.16−7.31 (m, 5H, Ph), 10.42 (s, 1H, 3-NH). Anal. ($C_{15}H_{19}N_3O_2$) C, H, N.

1-(2-Methoxyethyl)-6-(4-phenylbutylamino)uracil, 19. A mixture of 1-(2-methoxyethyl)-6-aminouracil (**26**)[28] (185 mg, 1 mmol), 4-phenylbutylamine hydrochloride (1.1 mmol) and a few drops of 4-phenylbutylamine was heated at 160 °C for 2 h. After cooling to room temperature the mixture was treated with 10% aqueous sodium bicarbonate and extracted with $CHCl_3$ (3 × 30 mL). After drying ($MgSO_4$) the collected organic extracts were evaporated to dryness, and the residue was purified by chromatography on silica gel with $CHCl_3$:MeOH (100:1 to 100:3). Crystallization from EtOH gave 203 mg (64%) of **19**, mp 148.5−150 °C. ^{1}H NMR (DMSO-d_6): 1.59 (m, 4H, 2×CH_2), 2.61 (t, 2H, CH_2-Ph), 3.04 (m, 2H, NH-CH_2), 3.22 (s, 3H, CH_3O), 3.45 (t, 2H, CH_2O), 3.98 (t, 2H, CH_2N), 4.50 (s, 1H, C5-H), 6.50 (t, 1H, NH-CH_2), 7.22 (m, 5H, Ph), 10.44 (s, 1H, NH). Anal. ($C_{17}H_{23}N_3O_3$) C, H, N.

1-(2-Hydroxyethyl)-6-(4-phenylbutylamino)uracil, 20. Trimethylsilyl iodide (0.32 mmol) was added to a stirred solution of **19** (100 mg, 0.32 mmol) in dry $CHCl_3$ (40 mL). The solution was stirred overnight, until disappearance of starting material. Methanol (10 mL) and sodium sulfite (0.5 g) were added to the solution. After stirring at room temperature for 30 min the mixture was filtered, and the filtrate was evaporated to dryness. The residue was purified by chromatography on silica gel with $CHCl_3$:MeOH (100:3 to 100:6) as eluent to give 80 mg (84%) of **20** as a white solid, mp 193−195 °C. ^{1}H NMR (DMSO-d_6): 1.60 (m, 4H, 2×CH_2), 2.60 (t, 2H, CH_2-Ph), 3.04 (m, 2H, NH-CH_2), 3.55 (m, 2H, CH_2O), 3.88 (t, 2H, CH_2N), 4.51 (s, 1H, C5-H), 5.35 (t, 1H, OH), 6.66 (t, 1H, NH-CH_2), 7.23 (m, 5H, Ph), 10.44 (s, 1H, NH). Anal. ($C_{16}H_{21}N_3O_3$) C, H, N.

3-(ω-Methoxyalkyl)-6-(4-phenylbutylamino)uracils 21 and 22. Mixtures of 3-(2-methoxyethyl)-6-chlorouracil **(27)** and 3-(3-methoxypropyl)-6-chlorouracil **(28)** (1 mmol) and 4-phenylbutylamine (5 mmol) were heated at 145 °C for 20 min. The reaction mixtures were allowed to cool to room temperature, and the residues were purified by chromatography on silica gel with $CHCl_3$:MeOH (97:3) as eluent.

3-(2-Methoxyethyl)-6-(4-phenylbutylamino)uracil, 21. Yield 30%, mp 150−155 °C (EtOH). ^{1}H NMR (DMSO-d_6): 1.41−1.64 (m, 4H, 2×CH_2), 2.60 (t, 2H, CH_2-Ph), 3.04 (m, 2H, NH-CH_2), 3.22 (s, 3H, CH_3O-), 3.41 (m, 2H, CH_3O-CH_2), 3.90 (t, 2H, CH_2-N), 4.55 (s, 1H, C5-H), 6.18 (m, 1H, NH-CH_2), 7.15−7.38 (m, 5H, Ph), 10.19 (s, 1H, NH). Anal. ($C_{17}H_{23}N_3O_3$) C, H, N.

3-(3-Methoxypropyl)-6-(4-phenylbutylamino)uracil, 22. Yield 30%, mp 148−152 °C (EtOH). ^{1}H NMR ($CDCl_3$): 1.55−1.62 (m, 4H, 2×CH_2), 1.90 (m, 2H, $CH_3OCH_2CH_2CH_2$-), 2.64 (t, 2H, CH_2-Ph), 3.10 (m, 2H, NH-CH_2), 3.28 (s, 3H, CH_3O-), 3.40 (m, 2H, CH_3O-CH_2), 3.90 (t, 2H, CH_2-N), 4.79 (s, 1H, C5-H), 4.99 (m, 1H, NH-CH_2), 7.11−7.30 (m, 5H, Ph), 10.31 (s, 1H, NH). Anal. ($C_{18}H_{25}N_3O_3$) C, H, N.

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Supporting Information Available: Inhibitory effect of **5**−**7** (SI Figure 1) and HPLC elution profile (SI Figure 2). This material is available free of charge via the Internet at http://pubs.acs.org.

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