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13. ABSTRACT ***(Maximum 200 Words)***

Thymidine phosphorylase (TP) catalyzes the phosphorolysis of thymidine and other pyrimidine 2'-deoxyribonucleosides. In addition, TP has been shown to possess angiogenic activity in a number of *in vitro* and *in vivo* assays, and its angiogenic activity has been linked to its catalytic activity. Several 5- and 6-substituted uracil derivatives were synthesized and evaluated for their ability to inhibit TP activity. In the past year we have identified a 6-amino-substituted uracil analog, 6-(2-aminoethyl)amino-5-chlorouracil (AEAC) to be one of the most active compounds. AEAC was found to be a competitive inhibitor of TP with a K_i of 165 nM. Human recombinant TP induced human umbilical vein endothelial cell (HUVEC) migration in a modified Boyden chamber assay *in vitro*, and this action could be abrogated by the AEAC. This was specific for TP, as the inhibitor had no effect on the chemotactic actions of vascular endothelial growth factor (VEGF). HUVEC migration was also induced when TP-transfected human breast carcinoma cells were used in a co-culture assay in place of the purified angiogenic factors, and a TP inhibitor nearly completely blocked the tumor cell-mediated migration. These studies suggest that inhibitors of TP may be useful in breast cancers which are dependent upon TP-driven angiogenesis.

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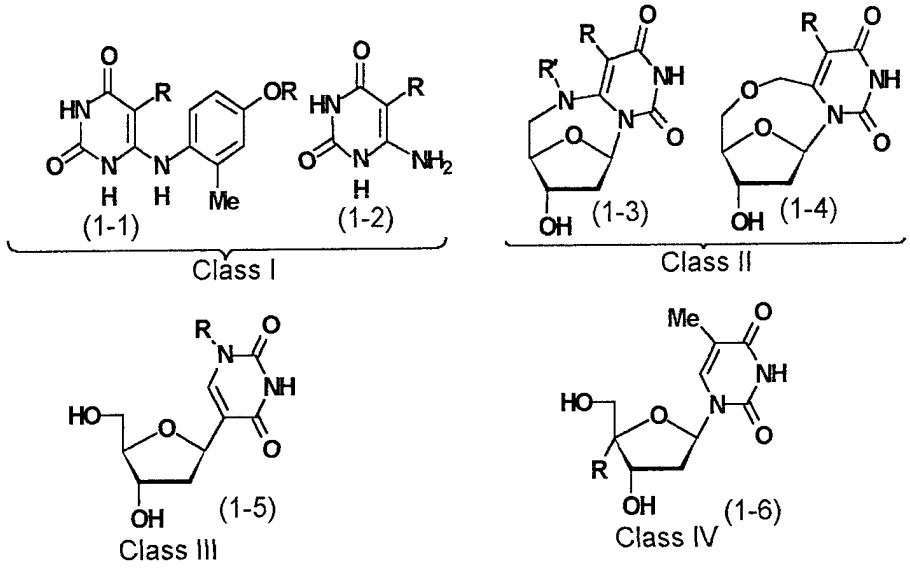
Introduction. There is a need for alternative approaches to treat metastatic breast cancer. One rapidly developing area of investigation is the study of tumor angiogenesis, the process by which a growing tumor mass recruits the new blood vessels required for its continued growth, and through which the tumor can spread to distant sites. Indeed the neovascularization process is thought to be one of the rate limiting steps for the growth of primary and metastatic tumors (1-2). Most studies have demonstrated the importance of angiogenesis in the progression of human breast malignancies and found the extent of vascularization to be correlated with tumor size and an indicator of node metastasis (3-8). Several polypeptides and growth factors that are produced by breast cancer epithelial and stromal cells have been identified as having endothelial cell mitogenicity and angiogenic activity (1,9). Our studies are focusing on the angiogenic factor PD-ECGF, based on evidence demonstrating its role in experimental and human cancer, and the finding that PD-ECGF is identical to human thymidine phosphorylase (TP), an enzyme that catalyzes the reversible conversion of thymidine to thymine and 2'-deoxyribose-1-phosphaste (10-12). When transfected into NIH 3T3 cells, TP was found to increase the vascularization of tumors growing in nude mice after sc inoculation (13). Similarly, overexpressing TP in MCF7 breast carcinoma cells markedly increased tumor growth *in vivo*, although it had no effect on the growth of the cells *in vitro* (14). Western blot analysis of primary human breast tissue showed that TP expression was elevated in the tumors compared to the normal tissue, a finding which provides clinical relevance to the transfection experiments. Studies suggest that the angiogenic and endothelial cell chemotactic activities of PD-ECGF are dependent upon its enzymatic activity, and this has been confirmed with site-directed mutagenesis studies (14,15-17). Of the angiogenic factors identified to date, TP is the only one in which an enzymatic activity of the factor is required for angiogenic activity. These observations serve as the basis for our hypothesis that inhibition of the catalytic activity of TP will also block its angiogenic properties. By synthesizing inhibitors of TP, we will be able to test this hypothesis and provide a basis for the development of a novel class of antitumor agents.

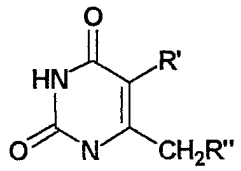
Statement of Work Objective 1: *Synthesize inhibitors of the tumor angiogenesis factor PD-ECGF/TP by targeting the catalytic site of the growth factor/enzyme. Four classes of potential inhibitors will be made (Fig. 1), including 6-aminouracils (class I), anti-cyclonucleosides (class II), C-nucleosides comprising N-1 substituted-2'-deoxy-pseudouridines (class III), and thymidine analogues substituted at C-4' with an alkyl chain terminating in an anionic group (class IV).*

Fig. 1



Subsequent developments in the area of TP inhibitors as potential antiangiogenic agents which had taken place since the grant was written led us to incorporate a fifth class of analogs in our studies.

Fig. 2



Class V

R' = H, Me, Cl

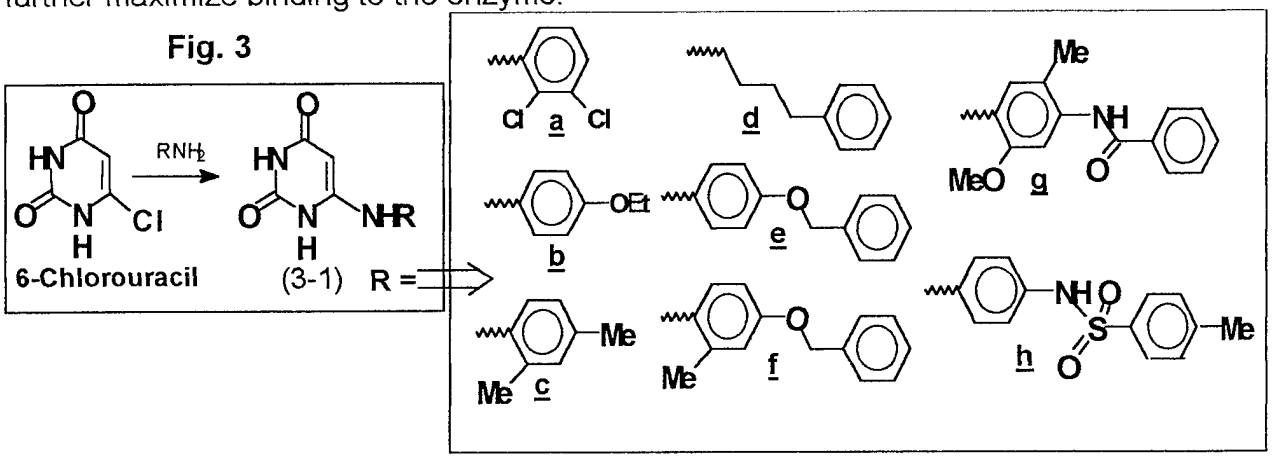
R" = Guanidino derivs. or other strongly basic multifuntionalities

The report of the synthesis of each of the five classes are provided below.

Class 1. As originally proposed, several variously substituted derivatives of 6-aminouracils (Class I inhibitors) were synthesized to maximize binding to the enzyme and thereby identify compounds with greater potency and specificity. One of our synthetic routes is illustrated in Fig.3 and makes use of the direct nucleophilic displacement of Cl from 6-chlorouracil by a variety of amines. Of the compounds obtained, **3-1a** - **3-1f**, the most active appear to be those where the substitution at C-6 incorporates two phenyl rings connected by a two atom bridge (**3-1e** and **3-1f** with IC_{50s}

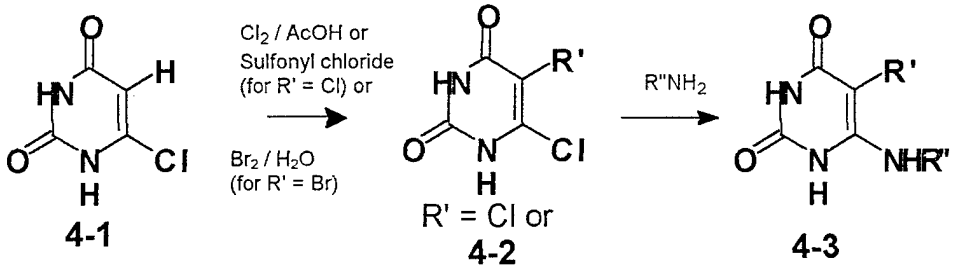
of 114 μM and 30 mM, respectively). Additional compounds with this same substitution pattern (**3-1g** and **3-1h** are two examples) were synthesized in order to fine tune and further maximize binding to the enzyme.

Fig. 3



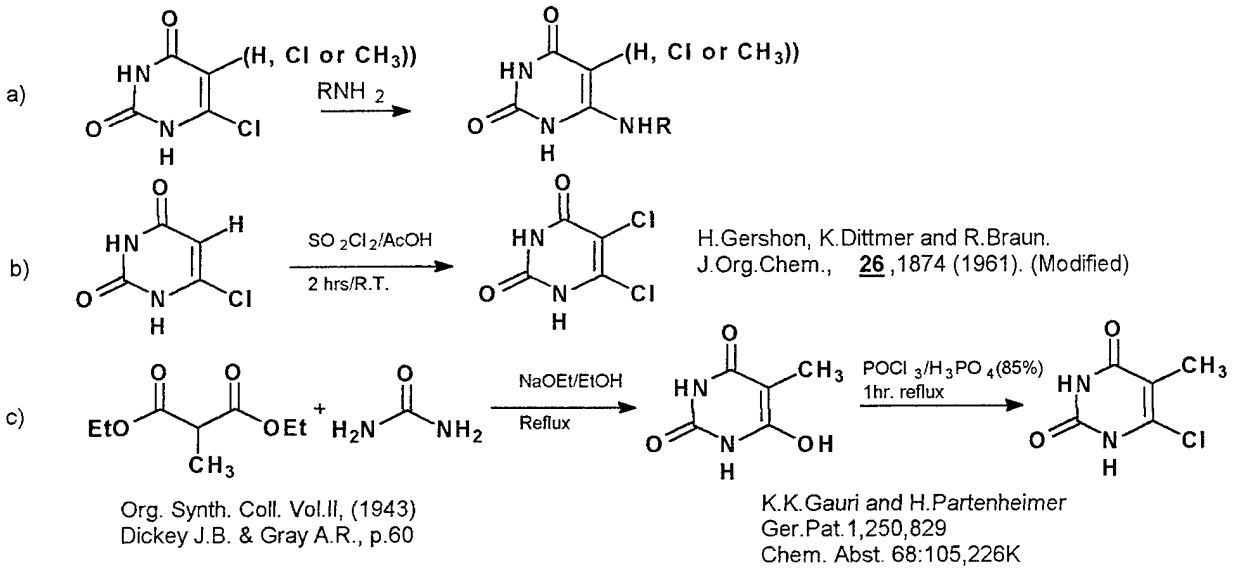
These substituted phenyl derivatives exhibited moderate TP inhibitory activities, with IC_{50} values in the range of 30 - 275 μM. Halogenation of the 5-position of these compounds was attempted in order to obtain those targets where R' = Cl or Br since, as was described in the original application, it is anticipated that such derivatives would exhibit enhanced activities because of their closer stereoelectronic resemblance to thymine. In most instances, chlorination or bromination at C-5 was also accompanied by partial halogenation of the phenyl substituent at C-6 thus affording complex mixtures of products. It was decided therefore to prepare the desired 5-halogenated derivatives by reaction of already 5-halogenated 6-chlorouracil intermediates (such as **4-2**, in fig 4) with the appropriate amines as we originally suggested as a back-up approach. As illustrated in figure 4, halogenation of 6-chlorouracil **4-1** (readily accomplished via known procedures) and the displacement of the 6-chloro function by various anilino and other amines (as already carried out for derivatives with R' = H) was next undertaken.

Fig. 4



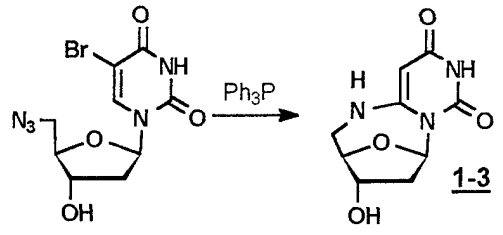
Additional compounds were prepared as shown in Fig. 5 (a) by substitution of the 6-chloro group of a variety of uracil derivatives by appropriate primary amines of commercial sources of readily available through procedures reported in the chemical literature. The 6-chloro substituted pyrimidine precursors were themselves obtained by adaptations (or modifications) of published procedures.

Fig. 5



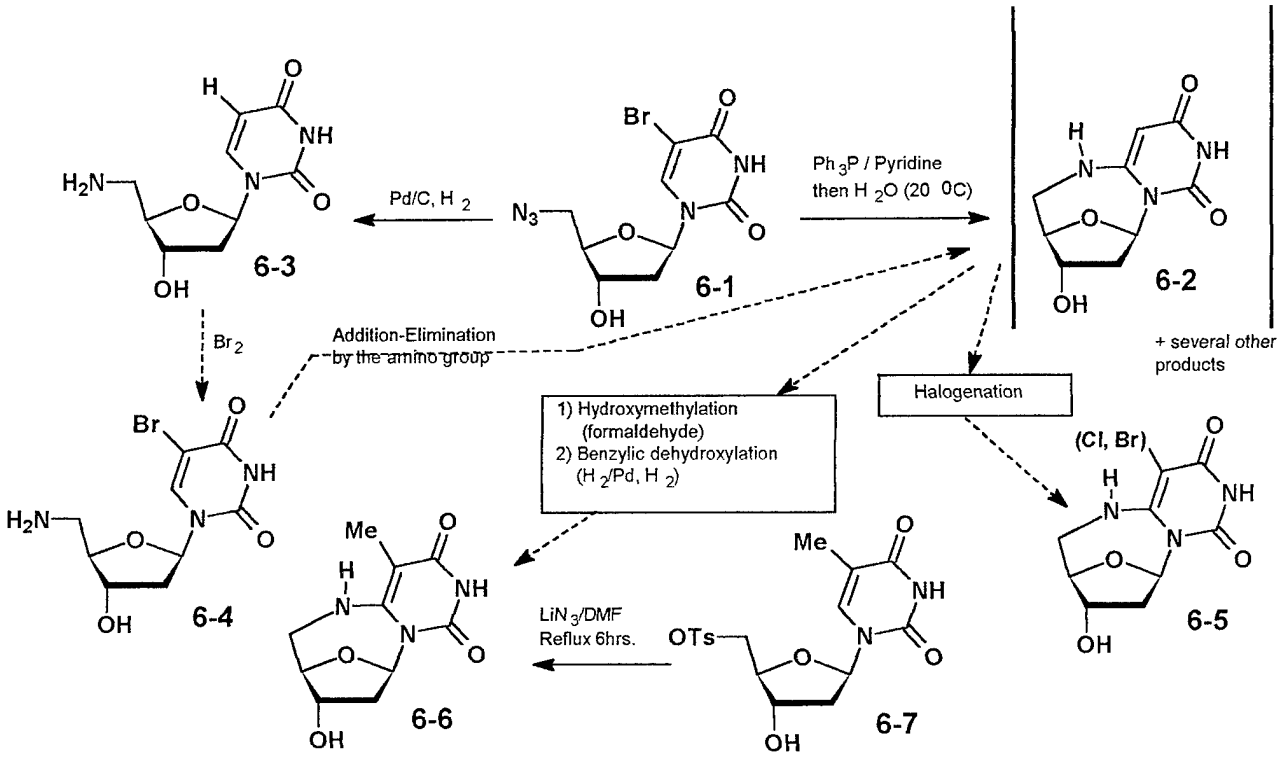
The precursor 5,6-dichlorouracil was obtained by the chlorination of commercially available 6-chlorouracil with sulfuryl chloride in acetic acid (Fig. 5b). The 5-methyl substituted derivative was obtained by susbtitution of the 6-OH group of 5-methylbarbituric with Cl using phosphorous oxychloride in 85% phosphoric acid (Fig. 5c).

Class 2. In our investigation of new analogs belonging to class II, we have synthesized key intermediates which are, as illustrated below, only one step removed from target analog **1-3** (where R'=R=H in fig. 1). The remaining step involves a final cyclization via reduction of the azido groups to NH_2 with triphenylphosphine to afford the desired product.



Access to the targeted 5',6-cyclouridine derivatives of class II by selective reduction of

Fig. 6

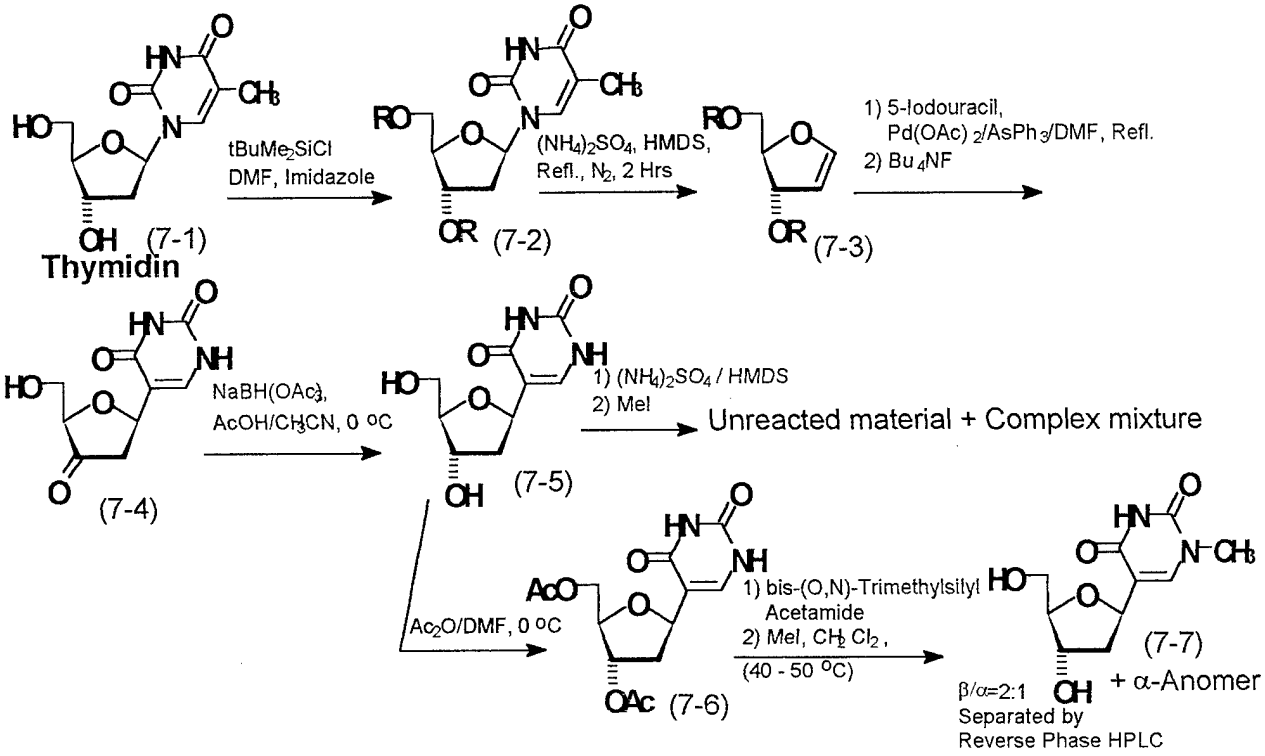


the azido function of intermediate **6-1** (Fig. 6) to give **6-4** has also been attempted in the expectation that this product could be made to undergo addition-elimination to the final desired product **6-2**. Catalytic hydrogenation over Pd/C however was found to give **6-3** undoubtedly produced by the concurrent hydrogenolytic debromination at C-5. The desired product **6-2** could be obtained, albeit in poor yields, by reduction of **6-1** with triphenyl phosphine. Its formation, however, was accompanied by several undesirable by-products. A more promising strategy, therefore, was the bromination of **6-3** (more readily obtained by the direct 5'-azidation of 2'-deoxyuridine) to afford **6-4** and, after ring closure, **6-2**. The latter would then serve as the major intermediate to all remaining targets (**6-5** or **6-6**) as outlined in fig. 6. An attractive alternate route to the desired 5-Me derivative **6-6** (reported in the literature by A. Matsuda and associates) was also employed. It is based on displacement of the 5'-O-tosyl function of thymidine derivative **6-7** by azide followed by an *in situ*, thermally induced 1,3-dipolar cycloaddition of the azido function onto the 5,6 pyrimidine double bond and spontaneous elimination of N_2. None of these approaches, however, resulted in the production of the desired compound.

Class 3. Our choice of this class of C-nucleoside 2'-deoxypseudouridines analogues as potential inhibitors of TP was based on the stereo electronic similarities that exist between these and thymidine, a natural substrate for the enzyme. The synthetic route originally envisaged had to be modified substantially and the method finally adopted is illustrated in Fig. 7. A key modification involved the protection of the two hydroxyl groups in compounds **7-5** by acetylation without which a complex mixture of products (as well as unreacted material) was obtained. The first member of this class, 1-methyl-

2'-deoxyuridine (**7-7**), unexpectedly was found to have little inhibitory activity (IC50 >4 mM). The enzyme, however, can accommodate 2'-deoxyuridines with 5-substituents as large as 2-bromovinyl and propynyloxy groups.

Fig. 7



We have also begun the synthesis (see Fig. 8) of a new series of analogs to find out whether structural extension of the aminosubstituents on C-6 of uracil by a short distance might not enhance the inhibitory activity of Class III inhibitors. The new derivatives obtained to date (**8-1c**, **8-1d**, and **8-1e**) differ from their homologues of Fig.3 only by a single methylene group inserted between C-6 and the amino substituent. We had shown that 5-bromo-6-aminouracil is a weak inhibitor of human TP, with an Ki of 13 mM. It would be of interest to find out therefore whether 6-aminouracil derivatives with 5-substituents other than Br might exhibit better activities. Synthetic studies in our laboratory would seem to indicate that (as shown in Fig. 9) 6-aminouracil reacts with aromatic isothiocyanates to give the C-5 substituted thioamide product (and not, as might be expected, the C-6 thioureido derivative). This synthetic method might open access to many new analogs of 5-bromo-6-aminouracil with groups other than the halogens and with various steric requirements. Products obtained were **9-1a**, **9-1b** and **9-1c**.

Fig. 8

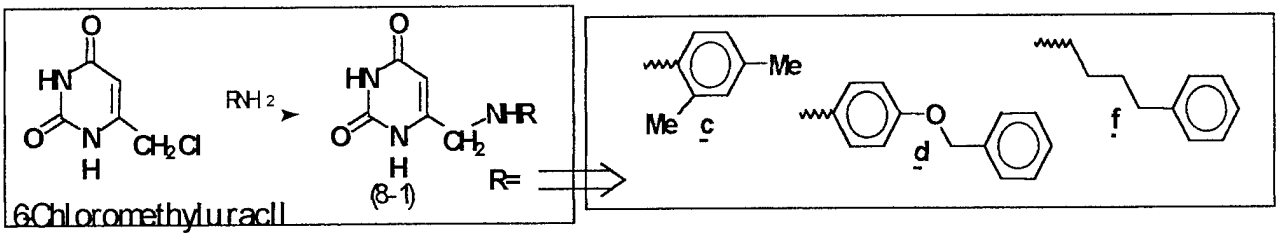
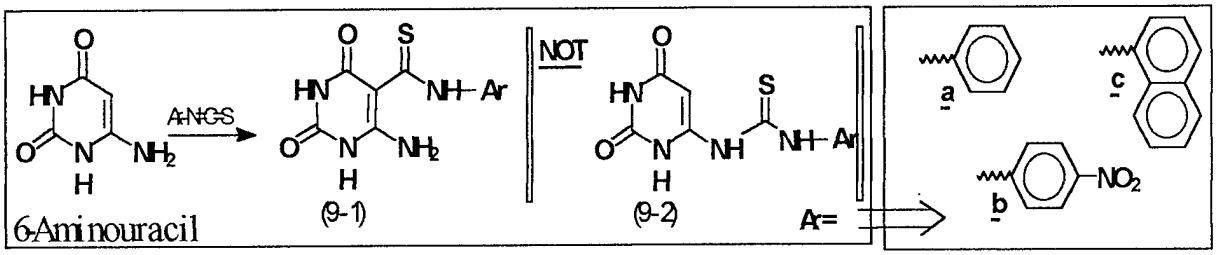
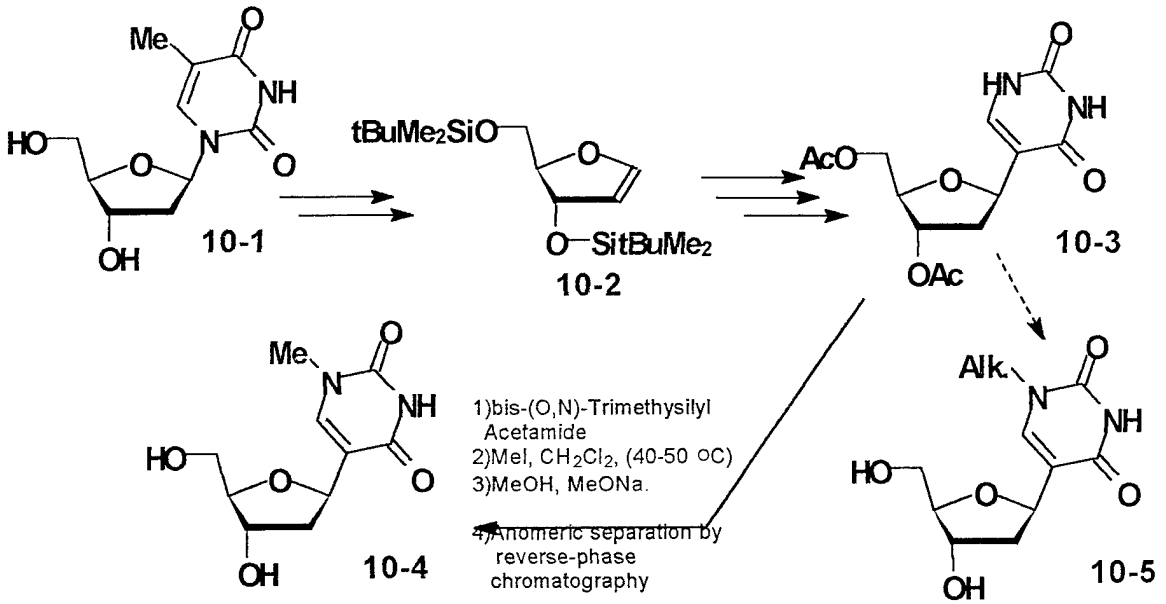


Fig 9



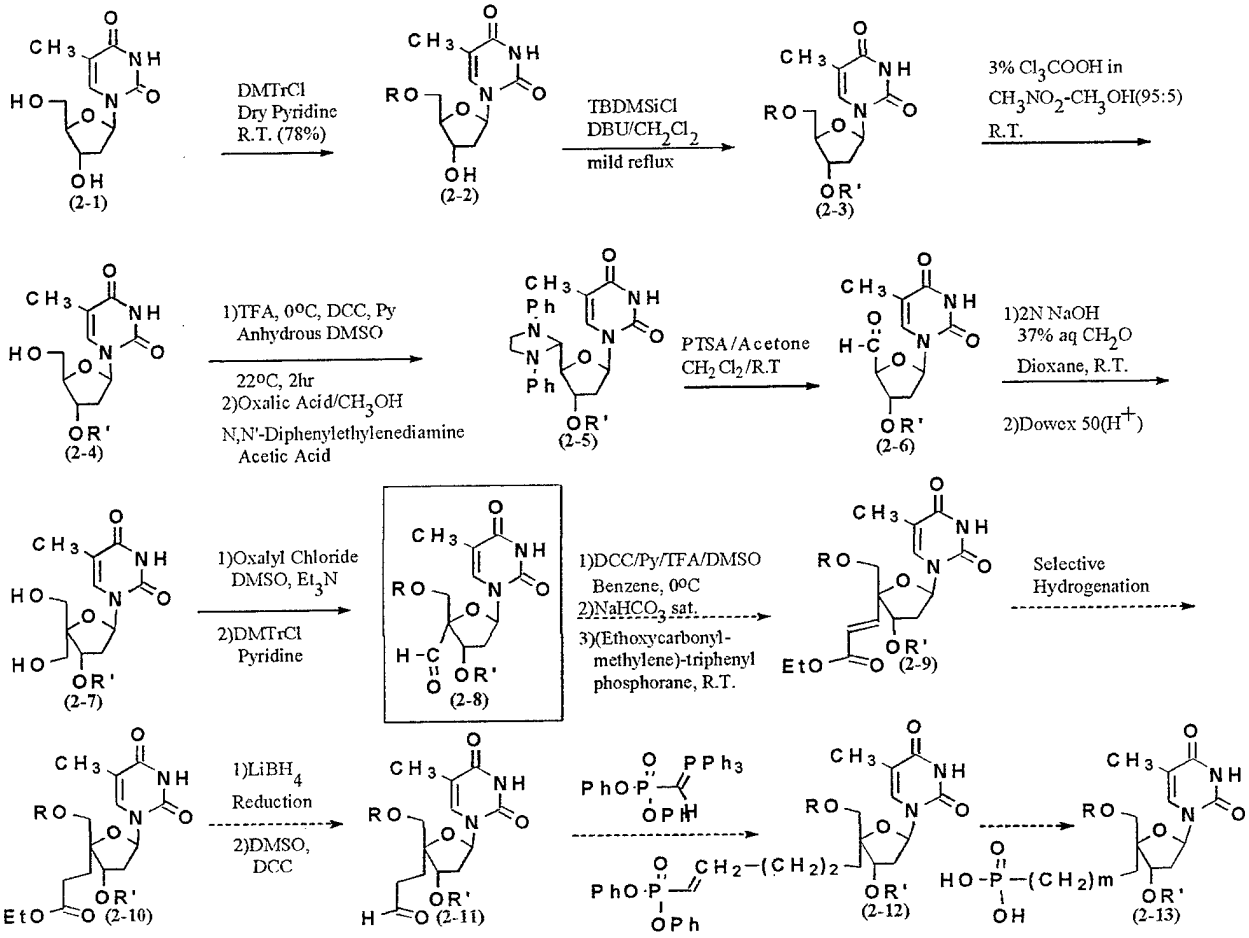
The thymidine analog **10-4** which is the major representative of class III was synthesized as outlined in fig. 10 and was found to be inactive. Efforts were expended to resynthesize key intermediate **10-3** so as to be able to make new derivatives with groups other than the N-methyl to be assayed for enhanced activity. This work was discontinued, however, in light of the activity seen in some of the other classes.

Fig. 10



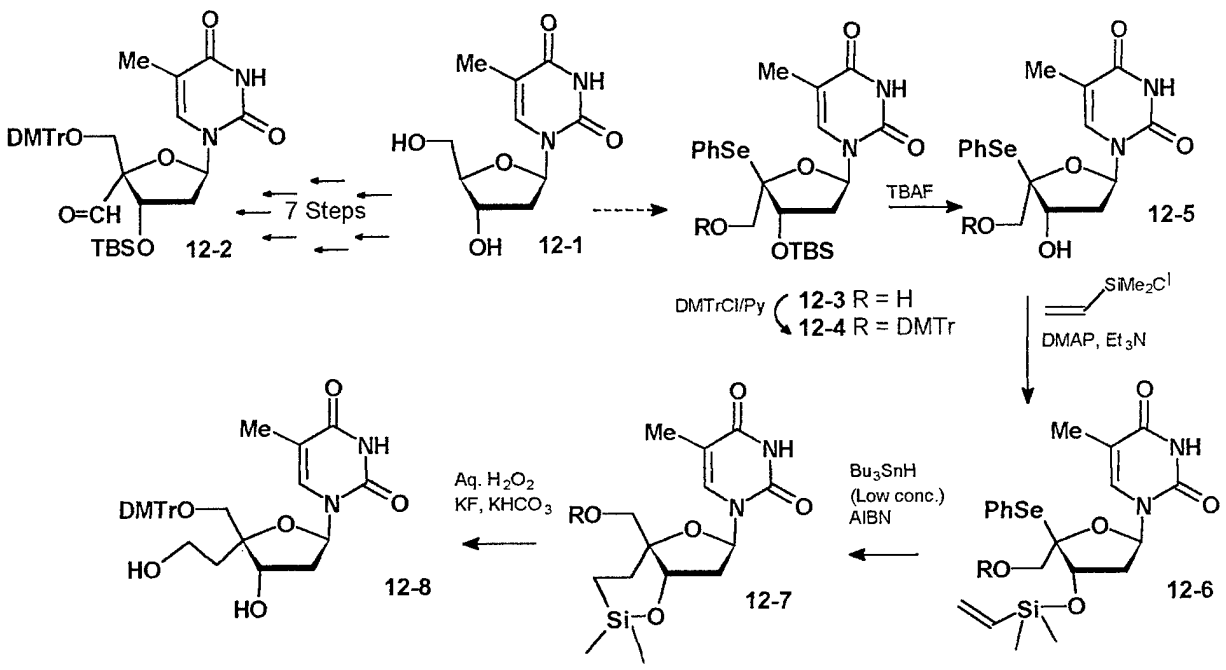
Class 4. Progress was achieved in the synthesis of derivatives of class IV (see Fig. 11) where a series of synthetic conversions starting with thymidine (**2-1**) have resulted, as originally planned, in the synthesis of key intermediate **2-8**. The latter should be readily convertible to the desired potential bisubstrate inhibitors **2-13** as shown.

Fig. 11

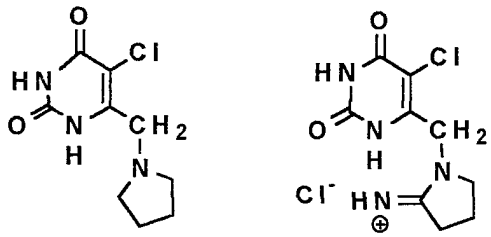


Further efforts to utilize compound **12-2** (fig. 12), a key intermediate in the projected synthesis of the members of class IV it for subsequent functionalization of the side chain at C-4' have been thwarted by its relative instability and the length of the multistep procedure required to obtain it. We have tried to exploit an alternative approach based on the recently reported regio- and stereospecific method for introducing a 2-hydroxyethyl group as a C-4' carbon-branched function in thymidine (**12-1**). The method (outlined in fig. 12) utilizes the 4'-(phenylseleno)thymidine derivative **12-3** (prepared in several high yielding steps from thymidine) for introducing a dimethylvinylsilyl group as a radical acceptor tether (as in **12-6**) followed by a free radical reaction (Bu_3SnH/AIBN) and a Tamao oxidation to give the desired product **12-8**. The latter is ideally suited as synthetic substitute for intermediate **12-2** since its primary hydroxyl function can undergo selective functionalization either directly or indirectly to an aldehyde followed by further extension via a Wittig reaction (or a Horner modification) to afford the desired phosphonates or carboxylates proposed as targets of class IV.

Fig. 12

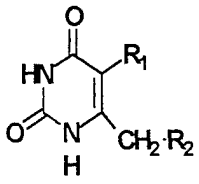


Class 5. One of the most significant recent developments in the area of TP inhibitors as antiangiogenic agents was the recent report by a Japanese pharmaceutical company (Taiho Pharmaceuticals) of a series of uracil derivatives (shown in figure to right) that had pronounced TP inhibitory activity. We decided to incorporate this important insight into our synthetic chemistry program, and therefore have established a new class of compounds to be evaluated (Class V, see Table 1, below). While somewhat similar to the uracil derivatives of class I we had suggested for investigation, these new analogs differ structurally in one significant respect, the incorporation of a methylene group at C-6 which acts as a bridge between the pyrimidine ring and the amines. We first synthesized three such C-6 methylene-containing amines (**V1**, **V2**, **V3**, Table 1) and determined their activity. All three were poor inhibitors, with K_i greater than 0.3 mM. Suspecting that this may have been due to the lack of a 5-substitution on the analogs, we next prepared compound **V5** with a 5-Cl to mimic the Taiho compound.

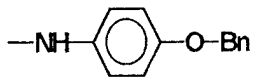
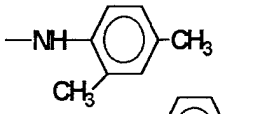
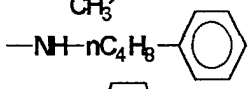
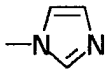
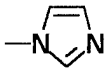
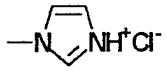


The activity of this compound was 1.5 μM, making it the most potent inhibitor of TP we had synthesized to date, surpassing the activity of our previously synthesized 5-bromo-6-aminouracil (K_i = 13 μM) by an order of magnitude. Removal of the 5-Cl moiety (compound **V4**) reduced the K_i to 120 μM, confirming the importance of this substituent. We also synthesized the HCl salt (**V6**) and found it had equivalent inhibitory activity on TP as did **V5**, thereby providing a readily water soluble analog.

Table 1.

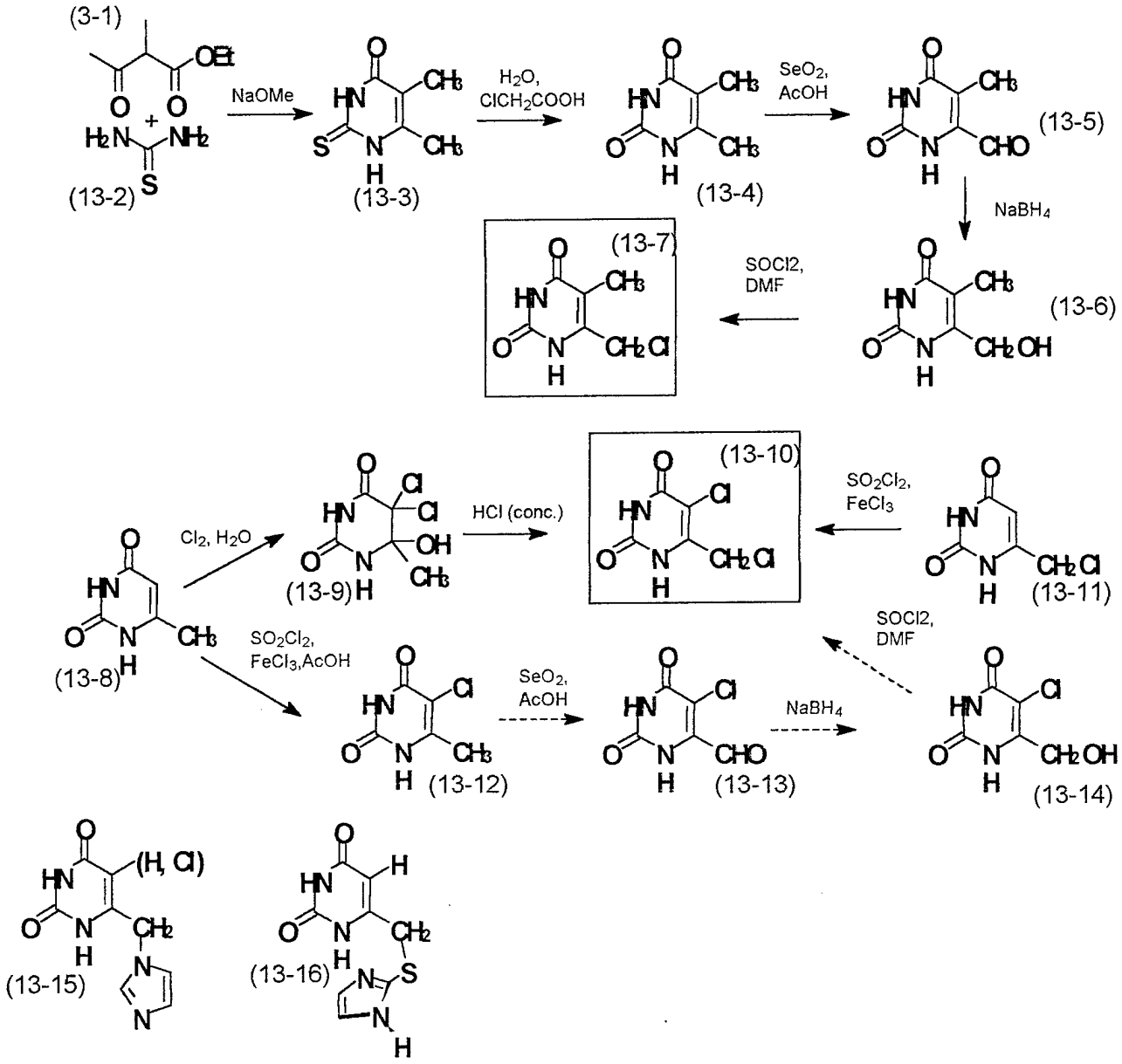


Class V

Compound	R_1	R_2
V1	H	
V2	H	
V3	H	
V4	H	
V5	Cl	
V6	Cl	

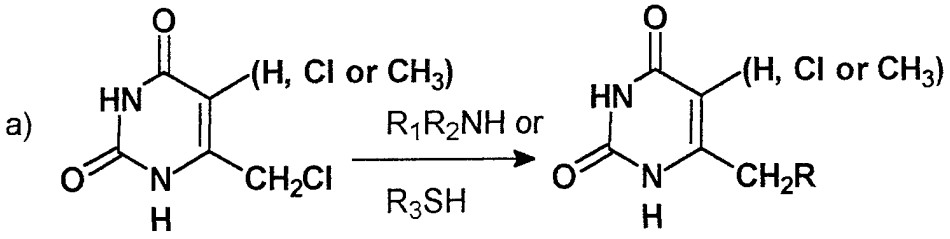
In light of these developments, we focused our efforts on the synthetic investigation of 5-substituted 6-chloromethyluracils for use as precursors to this new type of TP inhibitors. The results of our work in this area are summarized in Fig. 13. We selected **13-7** and **13-10** as the two precursors of highest priority and most likely to afford highly active analogs. **13-7** was prepared through the sequence shown starting with the reaction of 2-methyl ethylacetoacetate and thiourea. The corresponding 5-Cl derivative **13-10** was obtained originally by chlorination of the commercially available 6-chloromethyluracil **13-11**. In view of its excessive cost, other synthetic approaches to **13-10** were investigated. We were able to prepare it from the more economical and commercially available 6-methyluracil **13-8**. A somewhat longer but possibly better yielding approach should also be possible as shown (**13-8 13-12 13-13 13-14 13-10**) which makes use of the same methodology we employed for the 5-Me series. Initial conversions of these precursors to **13-15** and **13-16** have already been carried out.

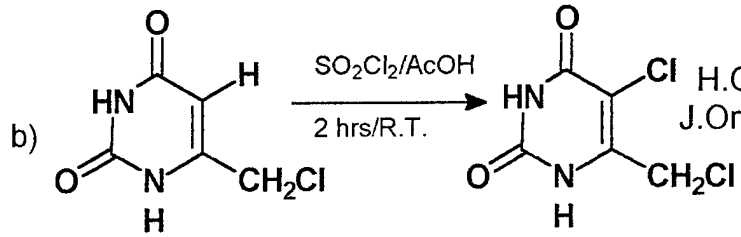
Fig. 13



Compounds shown in Fig. 14 (a) were prepared by substituting the Cl group of variously 5-substituted 6-chloromethyluracils with the appropriate amine or thiono- derivative. Precursor 5-chloro-6-chloromethyluracil was obtained by direct chlorination of the 5-position of 6-chloromethyluracil (commercially available) with sulfuryl chloride in acetic acid (Fig. 14b). The 5-methyl-6-chloromethyluracil precursor was obtained from readily available 5,6-dimethyluracil by oxygenation to the 6-aldehydo derivative, reduction to the 6-hydroxymethyl intermediate and chlorination with thionyl chloride and dimethylformamide (Fig. 14c).

Fig. 14





H.Gershon, K.Dittmer and R.Braun.
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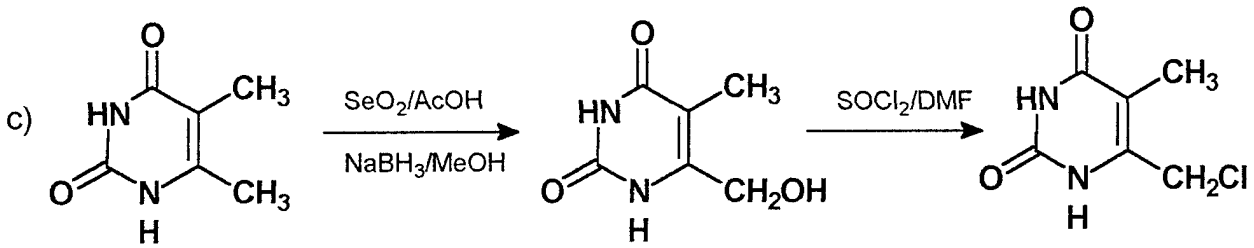

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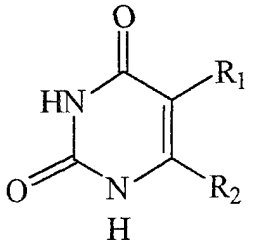
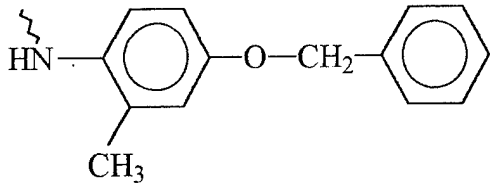
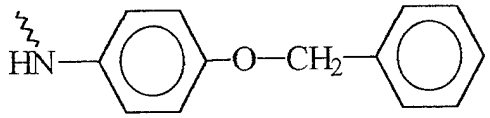
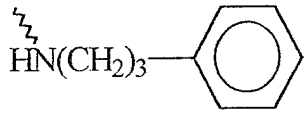
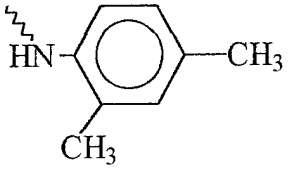
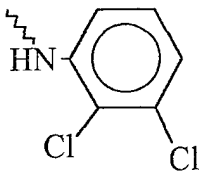
J.Org.Chem.,**37**,4381 (1972)

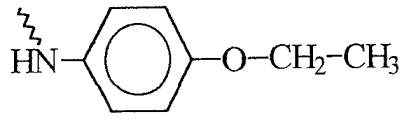
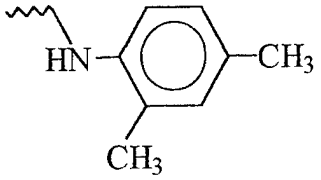
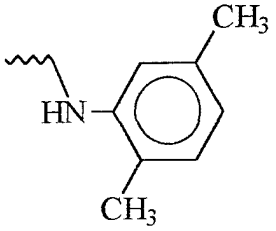
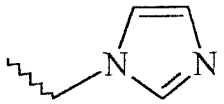
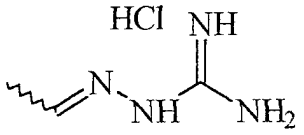
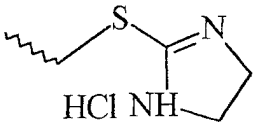
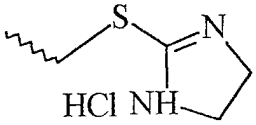
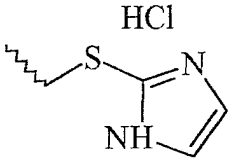
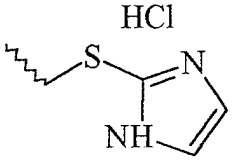
R.S.Klein and J.J.Fox
J.Org.Chem.,**37**,4381 (1972)

Statement of Work Objective 2: *Evaluate the target compounds for their ability to inhibit the enzyme activity of human TP and to block TP-mediated endothelial cell migration in vitro.*

Table 2: Inhibition of TP activity by 5-, 6-substituted uracil analogs.



Compound	R_1	R_2	IC_{50}
1	Br	$-NH_2$	17 μM
2	Cl	$-NHCH_2CH_2NH_2$ HCl	0.25 μM
3	CH_3	$-NHCH_2CH_2NH_2$ HCl	1.7 μM
4	Cl	$-NHCH_2CH_2OH$	100 μM
5	CH_3	$-NHCH_2CH_2OH$	> 1000 μM
6	Cl	$-NHCH_2CH_2CH_2OH$	650 μM
7	H		30 μM
8	H		114 μM
9	H		122 μM
10	H		160 μM
11	H		220 μM

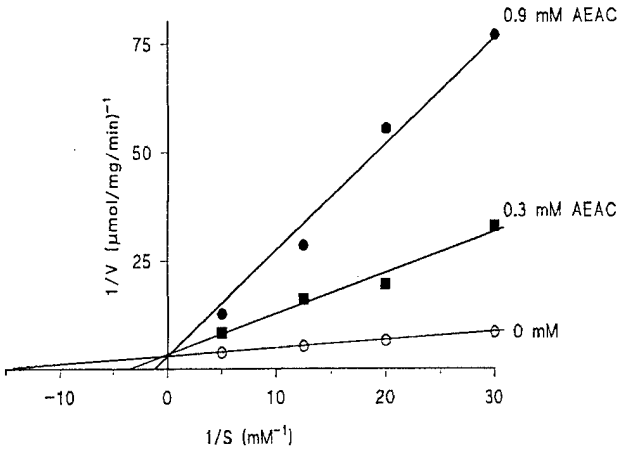
12	**H**		275 µM
13	**H**		>300 µM
14	**H**		10 µM
15	**Cl**		0.30 µM
16	**Cl**		500 µM
17	CH_3		200 µM
18	**Cl**		15 µM
19	**H**		> 300 µM
20	**Cl**		300 µM

TP activity was measured in assays which contained 0.2 M KH_2PO_4 (pH 7.8), 0.2 mM [5'-^{3}H] thymidine (1 µCi), human TP (5 ng), and several different concentrations of the

inhibitor being tested. Reactions were stopped after 30 min at 37 by the addition of ice-cold TCA containing activated charcoal. After centrifugation, an aliquot of the supernatant was counted in a liquid scintillation counter. IC_{50} values for each compound shown in Table 1 were calculated from data from at least 3 determinations.

We next further the mechanism of inhibition of compound **2**, 6-aminoethylamino-5-chlorouracil (AEAC) on TP inhibition by varying both the concentration of inhibitor, and that of the substrate thymidine.

FIG. 15: Double reciprocal analysis of TP inhibition by AEAC.



Assays of TP activity were carried out as described in Table 2 except that the concentration of substrate thymidine was varied between 33 and 200 µM. AEAC was used at concentrations of 0 mM (O), 0.3 mM (■), and 0.9 mM (●). Data are means of two experiments.

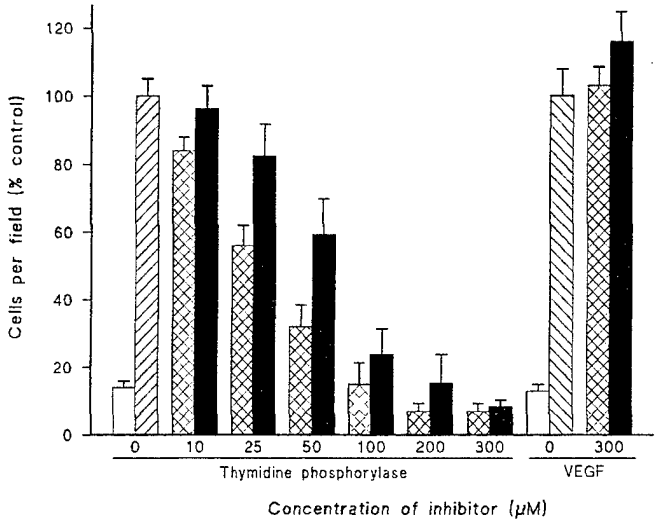
Conclusion: Compound **2** (Table 2) (AEAC) is a competitive inhibitor of TP.

Additional studies showed that AEAC was a selective inhibitor, as it had no effect on uridine phosphorylase or purine nucleoside phosphorylase activities (data not shown).

We next examined the effect of AEAC and a second potent TP inhibitor, 5-chloro-6(1-imidazolyl-methyl) uracil (CIMU; compound **15** (Table 2) on TP-mediated endothelial cell migration. Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords obtained less than 5 hours after delivery. After rinsing, veins were incubated with collagenase (100 U/ml) in Medium 199 with Earles salts for 10 min. Primary cultures were seeded into flasks precoated with 0.02% (w/v) gelatin in PBS. Culture medium consisted of M199 containing 20% newborn calf serum, 5% pooled human serum, 2 mM L-glutamine, 5 U/ml penicillin and 5 µg/ml streptomycin. Cells were incubated at 37° in a humidified incubator with 5% CO_2 with a medium change after 24 hours and every 2 days thereafter until confluent. Primary cultures of HUVEC were harvested by incubation with 0.05% trypsin/0.02% EDTA, and the cells collected and cell number determined using a Coulter counter.

Modified Boyden chamber migration assay. HUVEC (passages 1-4) were harvested using cell dissociation solution (Sigma) and pelleted by centrifugation. Cells (10^5) were placed into transwell inserts (Becton Dickinson; 8 μm pore) precoated with fibronectin (10 μg/ml). Inserts containing cells were placed into a 24 well plate containing 0.7 ml M199 with 1% serum and incubated (37°) for 1 hour. A chemotactic stimulus, recombinant human TP (200 ng/ml) or human VEGF (10 ng/ml; R&D Systems) was added to the lower wells in the presence and absence of various concentrations of inhibitors, and the cells incubated for 5 hours at 37°. At the end of the incubation period, the surface of the upper membrane was swabbed with a cotton-tipped applicator to remove non-migrating endothelial cells. Wells and inserts were washed 3 times with PBS and stained for 30 min. in medium with 10 μM Cell Tracker Green (Molecular Probes). The cluster plate was washed 3X with PBS, cells fixed in 3.7% formaldehyde for 10 min. and mounted on microscope slides. For quantitation, five random fields were photographed (100x total magnification) and the number of HUVEC in each field counted.

FIG. 16: Effect of TP inhibitors on endothelial cell migration.



Human endothelial cells, isolated as described above, were used in a modified Boyden chamber assay. Cells (10^5) were placed into fibronectin-coated transwell inserts, which were then placed into 24 well plates in which the lower wells contained 0.7 ml M199 with 1% serum. After 1 hour, recombinant human TP (200 ng/ml) or human VEGF (10 ng/ml) were added to the lower wells, as indicated (single hatched bars). Control wells (open bars) had no added angiogenic factor. Wells also contained the indicated concentrations (0 to 300 μM) of either CIMU (double hatched bars) or AEAC (solid bars). Cells were incubated for 5 hours at 37°, at which time non-migrating endothelial cells were removed from the upper surface of the membrane. Cells were stained with Cell Tracker Green, washed with PBS, fixed in formaldehyde, and mounted on microscope slides. Migration was quantitated by counting five random photographed (100x total magnification) fields, and the number of HUVEC in each field counted.

Technical objective 3: *Determine the role of TP expression on tumor growth in vivo by transfecting human carcinoma cells with a construct containing the TP cDNA. The*

transfected cells will be grown as xenografts in nude mice and tumor growth and metastasis will be compared to control-transfected cells. Transfectants exhibiting enhanced tumorigenicity will be further used as a model for determining the antitumor activities of the new inhibitors in vivo.

We have had difficulty in establishing *in vivo* assays for assessing the activity of our TP inhibitors, in particular, the establishment of tumors in nude mice from a MCF7 xenograft, despite attempting a number of different modifications to the protocol.

As an alternative to these approaches, we have adapted the Boyden chamber assay with HUVECs to incorporate human mammary tumor cells in a co-culture assay. These assays utilized tumor cells in place of the purified angiogenic factors; no other angiogenic stimulus was added to the incubation. Wild type MCF7 breast carcinoma cells and cells which had been stably transfected with a human TP cDNA (MCF7/TPneo) were used. 10^5 cells were added to the lower wells and were allowed to attach for 24 hours. The medium was then replaced with M199 medium with and without 0.3 mM of the inhibitor CIMU (see above), combined with HUVEC-containing transwells, and migration over a 5 hour period was analyzed as described above.

TABLE 3: Inhibition of HUVEC migration by a TP inhibitor in a co-culture assay.

Tumor Cells	CIMU	HUVEC cells per field	Inhibition
None	-	11 ± 1.7	
MCF7/TPneo	-	115 ± 9.0	
MCF7/TPneo	+	28 ± 2.6	84%

Data are means ± SEM.

Key Research Accomplishments

- Synthesized members of three different classes of uracil derivatives.
- Tested all synthesized compounds for their ability to inhibit thymidine phosphorylase catalytic activity. Observed a wide range of inhibitory activity, and identified two compounds, AEAC (6-(2-aminoethyl)amino-5-chlorouracil) and CIMU (5-chloro-6(1-imidazolyl-methyl) uracil), as potent competitive inhibitors of TP.
- Found that AEAC and CIMU inhibited TP-mediated endothelial cell migration *in vitro.* The compounds were selective for TP-mediated migration, i.e. they did not inhibit VEGF (vascular endothelial growth factor)-mediated migration.
- Transfected MCF7 breast cancer cells with TP and found these cells induced greater endothelial cell migration than control breast cancer cells. This increase in breast cancer cell-mediated angiogenesis could be blocked by a TP inhibitor.

Reportable Outcomes

1. Manuscript: Klein, RS, Lim, T, Lenzi, M, Hotchkiss, K.A. and Schwartz, E.L. (2001) Novel 6-substituted uracil analogs as inhibitors of the angiogenic actions of thymidine phosphorylase. Biochem. Pharmacol. 62:1257-1263.

2. Synthesis of TP inhibitors as potential anti-angiogenic and anti-cancer agents. One of these compounds, AEAC, is currently undergoing further pre-clinical evaluation by the Developmental Therapeutics Program of the NCI.

Conclusion

An inhibitor of the catalytic activity of thymidine phosphorylase blocks migration of endothelial cells *in vitro*, a key component of angiogenesis, included when mediated by breast cancer cells expressing high levels of TP. This or related inhibitors could potentially be used clinically as a treatment for breast cancer or other solid tumors which express TP, and which are dependent upon TP-mediated angiogenesis.

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Biochemical Pharmacology

Novel 6-substituted uracil analogs as inhibitors of the angiogenic actions of thymidine phosphorylase

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Abstract

Thymidine phosphorylase (TP) catalyzes the reversible phosphorolysis of thymidine and other pyrimidine 2′-deoxyribonucleosides. In addition, TP has been shown to possess angiogenic activity in a number of *in vitro* and *in vivo* assays, and its angiogenic activity has been linked to its catalytic activity. A series of 5- and 6-substituted uracil derivatives were synthesized and evaluated for their abilities to inhibit TP activity. Among the most active compounds was a 6-amino-substituted uracil analog, 6-(2-aminoethyl)amino-5-chlorouracil (AEAC), which was a competitive inhibitor with a K_i of 165 nM. The inhibitory activity of AEAC was selective for TP, as it did not inhibit purine nucleoside phosphorylase or uridine phosphorylase at concentrations up to 1 mM. Human recombinant TP induced human umbilical vein endothelial cell (HUVEC) migration in a modified Boyden chamber assay *in vitro*, and this action could be abrogated by the TP inhibitors. The actions of the inhibitors were specific for TP, as they had no effect on the chemotactic actions of vascular endothelial growth factor (VEGF). HUVEC migration was also induced when TP-transfected human colon and breast carcinoma cells were co-cultured in the Boyden chamber assay in place of the purified angiogenic factors, and a TP inhibitor blocked the tumor cell-mediated migration almost completely. These studies suggest that inhibitors of TP may be useful in pathological conditions that are dependent upon TP-driven angiogenesis.

Keywords: Thymidine phosphorylase; Angiogenesis; Endothelial cells

1. Introduction

A number of polypeptide factors that are produced by tumor epithelial and stromal cells and that promote angiogenic activity have been identified, including VEGF, PD-ECGF, FGF, TGF, and TNF-α [1]. The angiogenic factor PD-ECGF has been found to be identical to human TP, an enzyme that catalyzes the reversible conversion of thymidine to thymine [2–5]. Several lines of evidence have established the role of TP in experimental and clinical tumor angiogenesis. TP was found to have a chemotactic effect on endothelial cells and had angiogenic activity in several *in vivo* assays [2–6]. When transfected into 3T3 or MCF7 carcinoma cells, the TP gene increased the vascularization and growth of tumors growing in nude mice, but it had no effect on the growth of the cells *in vitro* [6,7]. Western blot analysis of primary human malignancies, including colon, esophageal, gastric, breast, bladder, ovarian, and lung cancers, showed that TP expression was elevated up to 10-fold in the tumors when compared with the corresponding non-neoplastic regions of the same organs. In the majority of these studies, higher TP expression was linked to increases in angiogenesis, invasion, metastasis, and to shorter patient survival [8–21]. In studies of GI cancers in which multiple angiogenic factors were examined, TP was found to be an independent prognostic marker of tumor aggressiveness [18,19].

TP has a well documented role in the salvage biosynthesis of pyrimidines. It also readily catabolizes and inactivates a number of 5-substituted 2′-deoxyuridines, including sev-

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Abbreviations: AEAC, 6-(2-aminoethyl)amino-5-chlorouracil; CIMU, 5-chloro-6-(1-imidazolyl-methyl) uracil; FGF, fibroblast growth factor; HUVEC, human umbilical vein endothelial cell(s); PD-ECGF, platelet-derived endothelial cell growth factor; PNP, purine nucleoside phosphorylase; TGF, transforming growth factor; TNF-α, tumor necrosis factor-α; TP, thymidine phosphorylase; UP, uridine phosphorylase; and VEGF, vascular endothelial growth factor.

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PII: S0006-2952(01)00783-3

eral with antiviral or anticancer activities; therefore, the identification of a TP inhibitor as a potential therapeutic agent has been a medicinal chemistry objective for over 30 years [22–26]. Despite these efforts, only compounds with modest potency, IC_{50} values $\geq$ 30 μM, were identified [27–30]. Recent studies using site-directed mutagenesis analysis suggested that the angiogenic activity of TP was also linked to its enzymatic activity [7,27]. This has prompted a renewed interest in the identification of TP inhibitors to be used as potential anti-angiogenic agents, and several new classes of TP inhibitors have been reported [28–31], including one class with IC_{50} values 1000-fold lower than those previously described [30,31]. In this study, we describe the synthesis of several new potent TP inhibitors, and further demonstrate that these compounds block the *in vitro* pro-angiogenic actions of both purified human recombinant TP, and human tumor cells expressing high levels of TP.

2. Materials and methods

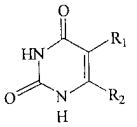
2.1. Synthesis of 5,6-disubstituted and 6-substituted uracil derivatives

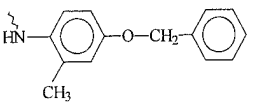
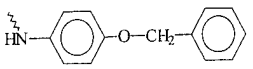
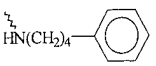
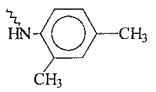
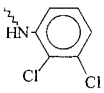
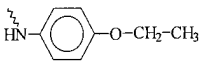
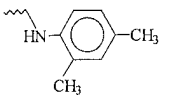
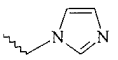
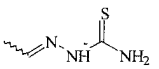
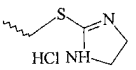
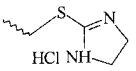
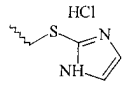
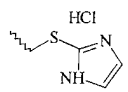
Compounds **2** through **6** (Table 1) were obtained by treatment of appropriately 5-substituted 6-chlorouracil derivatives with an excess of the diamine or hydroxylamine (15- to 20-fold molar excess) at 100° without solvent for 1–4 hr. Derivatives **7–12** were obtained by treatment of the corresponding 5-substituted 6-chlorouracil derivatives with the appropriate amine in 1.5- to 4-fold molar excess, using the general methods previously described [32]. Reactions were conducted with the reactants in the neat at 200° (for **11**) or in water at reflux (for **7–10** and **12**).

The 6-chlorouracils used as starting materials for **2-12** were obtained as follows: the 5,6-dichlorouracil precursor of **2, 4,** and **6** was obtained by C-5 chlorination of commercially available 6-chlorouracil with sulfuryl chloride in acetic acid by a modification of a reported procedure [33]. 6-Chloro-5-methyluracil, which served as the precursor of **3** and **5** [34], was obtained by treatment of 5-methylbarbituric acid [35] with $POCl_3$ in concentrated H_3PO_4 [36]. Derivatives **13, 14,** and **16-19** were prepared by treatment of appropriately 5-substituted 6-chloromethyluracils with the corresponding amine (in 4–10 molar excess in refluxing methanol for 16 hr) or with the corresponding thiono derivative (in 1.2 molar excess in refluxing methanol for 2 hr). Commercially available 6-chloromethyluracil (used directly as a synthetic precursor for **13** and **18**) was chlorinated as described above to give 5-chloro-6-chloromethyluracil, the synthetic precursor for **14, 17,** and **19.** The thiosemicarbazone derivative of thymine-6-carboxaldehyde, compound **15,** was prepared as described [37]. Thymine-6-carboxaldehyde was also used to prepare 5-methyl-6-chloromethyluracil (the synthetic precursor of **16**) by reduction to the alcohol with sodium borohydride followed by chlorination with thionyl chloride as we reported previously [38].

All the amines used for the synthesis of **2-12** were commercially available except for 4-benzyloxy-2-methylaniline

Table 1
Inhibition of TP activity by 5- and 6-substituted uracil analogs



Compound	R_1	R_2	IC_{50} (μM)
1	Br	$-NH_2$	17
2	Cl	$-NHCH_2CH_2NH_2$ HCl	0.25
3	CH_3	$-NHCH_2CH_2NH_2$ HCl	1.7
4	Cl	$-NHCH_2CH_2OH$	90
5	CH_3	$-NHCH_2CH_2OH$	>1000
6	Cl	$-NHCH_2CH_2CH_2OH$	650
7	H		30
8	H		114
9	H		122
10	H		160
11	H		220
12	H		275
13	H		>300
14	Cl		0.30
15	CH_3		500
16	CH_3		200
17	Cl		15
18	H		>300
19	Cl		300

(for compound **7**), which was prepared, using standard methodologies, by benzylation of commercially available 3-methyl-4-nitrophenol (heating to reflux in ethanol for 3 hr with benzylbromide and potassium carbonate). The 4-benzyloxy-2-methyl-nitrobenzene thus obtained was then reduced with iron powder in tetrahydrofuran/water at 0° under nitrogen to give the desired 4-benzyloxy-2-methylaniline.

All products were fully characterized by elemental analysis and ^{1}H-NMR techniques.

2.2. Assay of TP, UP, and PNP activities

The effects of the uracil analogs on purified recombinant human TP activity were evaluated. In addition to the inhibitors, the assays contained 200 mM KH_2PO_4 (pH 7.8), 0.2 mM [5′-^{3}H]thymidine (50 mCi/mmol; Moravek Biochemicals), and 50 ng TP. Reactions were stopped after 20 min at 37° by the addition of 0.5 mL of ice-cold activated charcoal in 5% trichloroacetic acid. After centrifugation, radioactivity in an aliquot of the supernatant was determined by liquid scintillation counting.

UP activity was measured as previously described [39]. Reactions contained, in 0.1 mL, 50 mM Tris–HCl (pH 7.5), 1 mM potassium phosphate, 200 μM uridine, 1 μCi [5-^{3}H]uridine, 100 ng purified recombinant human UP (provided by Dr. G. Pizzorno), and increasing concentrations of inhibitor. After 30 min at 37°, aliquots of the reactions were analyzed on silica gel TLC plates using chloroform:methanol:acetic acid (85:15:5, by vol.). Spots co-migrating with authentic uracil and uridine were scraped, and the amount of [^{3}H]uracil formed from [^{3}H]uridine was determined by liquid scintillation counting. The effect of the TP inhibitors on bovine PNP activity was assessed in a coupled spectrophotometric assay in which inosine was converted to hypoxanthine by PNP, and subsequently to uric acid by xanthine oxidase [40]. Reactions contained 10 mM KH_2PO_4 (pH 7.7), 40 μM inosine, 19 ng PNP (Sigma), 0.01 U xanthine oxidase (Sigma), and increasing concentrations of a uracil analog. The change in absorbance of uric acid at 293 nm was monitored continuously, and enzyme activity was calculated using an extinction coefficient of 12.9 mM^{-1} cm^{-1}.

2.3. Isolation of HUVEC

HUVEC were isolated from umbilical cords obtained less than 5 hr after delivery. After rinsing, veins were incubated with collagenase (100 U/mL, Worthington) in Medium 199 with Earle's salts (GIBCO) for 10 min. Primary cultures were seeded into flasks precoated with 0.02% (w/v) gelatin in PBS. Culture medium consisted of M199 containing 20% newborn calf serum, 5% pooled human serum, 2 mM l-glutamine, 5 U/mL of penicillin, and 5 μg/mL of streptomycin. Cells were incubated at 37° in a humidified incubator with 5% CO_2 with a medium change after 24 hr and every 2 days thereafter until confluent. Primary cultures of HUVEC were harvested by incubation with 0.05% trypsin/0.02% EDTA, and the cell number was determined using a Coulter counter.

2.4. Modified Boyden chamber migration assay

HUVEC (passages 1–4) were harvested using a cell dissociation solution (Sigma) and pelleted by centrifugation (250 *g* for 5 min). Cells (10^5) were placed into transwell inserts (Corning-Costar; 8 μm pore) precoated with fibronectin (10 μg/mL). Inserts containing cells were placed into a 24-well plate containing 0.7 mL M199 with 1% serum and incubated (37°) for 1 hr. A chemotactic stimulus, recombinant human TP (100 ng/mL) or human VEGF (10 ng/mL; R&D Systems), was added to the lower wells in the presence and absence of various concentrations of inhibitors, and the cells were incubated for 5 hr at 37°. At the end of the incubation period, the surface of the upper membrane was swabbed with a cotton-tipped applicator to remove non-migrating endothelial cells. Wells and inserts were washed three times with PBS and stained for 30 min in medium containing 10 μM Cell Tracker Green (Molecular Probes). The cluster plate was washed three times with PBS, and cells were fixed in 3.7% formaldehyde for 10 min and mounted on microscope slides. For quantitation, five random fields were photographed (100× total magnification), and the number of HUVEC in each field was counted.

Boyden chamber co-culture experiments utilized tumor cells in place of the purified angiogenic factors; no other angiogenic stimulus was added to the incubation. HT29 colon and MCF7 breast carcinoma cells that had been stably transfected with a human TP cDNA (HT29/TPneo and MCF7/TPneo) were used. Cells (2×10^5) were added to the lower wells and were allowed to attach for 24 hr. The medium was then replaced with M199 medium containing 1% serum with and without inhibitor, combined with HUVEC-containing transwells, and migration over a 5-hr period was analyzed as described above.

3. Results and discussion

The ability of TP to inactivate pyrimidine 2′-deoxynucleosides of chemotherapeutic interest provided medicinal chemists with an early incentive for developing TP inhibitors. Consequently, there existed a rich background of previous structure–activity relationships on which the current studies were based [24]. One common theme that emerged from a survey of the previous work on inhibition of TP was that 5-substituted 6-aminouracil-based compounds were effective inhibitors [22,23,32]. Thus, we explored this class of TP inhibitors further. 5-Bromo-6-aminouracil was synthesized and found to be a modestly active inhibitor of human TP, with an IC_{50} of 17 μM (compound **1**, Table 1). This was in good agreement with a previous report of an IC_{50} of 30 μM for this compound when tested against horse liver TP [22] and an IC_{50} of 15 μM for the inhibition of the human

enzyme by the closely related 5-chloro-6-aminouracil derivative [31]. The latter compound has also been shown to have inhibitory activity when tested in TP-mediated angiogenesis models [27]. Introduction of an ethylamino extension on the 6-amino position along with a 5-halogen substitution (compound **2**) produced a greater than 65-fold increase in potency for TP inhibition when compared with 5-bromo-6-aminouracil (**1**). The inhibitory activity of **2** was reduced 7-fold when a 5-methyl group was substituted for the 5-chloro constituent (**3**), and 360- to 2600-fold when the 6-aminoethylamino side chain was replaced with an aminoethanol substitution (**4**) or an amino-propanol substitution (**6**).

It is known from previous work that inhibition of TP is very sensitive to both the precise substitution pattern of different 6-aminouracils [22,23,32], and to the source of the TP enzyme being examined, with some compounds found to have up to 900-fold differences in inhibition of the *Escherichia coli* enzyme compared with the rabbit liver enzyme [23]. The basis for both of these differences likely relates to variations in the hydrophobic regions that surround the active sites of the enzyme and which interact with the *N*-substituent groups. Because less is known of the specificity of the human recombinant TP that we utilized, we decided to reevaluate some of the previously identified inhibitors that had been tested with TP from different sources. Therefore, several of the 6-anilino and alkylamino uracil derivatives identified earlier as the best inhibitors of TP [22,23,38] were synthesized (compounds **7-12**). They all were found to have relatively modest inhibitory activity. A more recent study [31] had reported that a number of 5-halogeno-amino-substituted 6-methyluracil derivatives were more potent inhibitors of TP than those described earlier. To explore this possibility further, several of these were resynthesized (**14, 17,** and **19**) together with new derivatives bearing different substitutions at position C-5 of the uracil moiety (**13, 15, 16,** and **18**) for study as potential antiangiogenic agents. Only one these, compound **14,** was noteworthy as a TP inhibitor, with an IC_{50} of 0.30 μM.

Two conclusions can be drawn from these studies: (a) the replacement of the C-5 methyl group of thymine by chlorine consistently resulted in significantly better inhibitors of TP for this particular class of derivatives (compare **2** vs **3,** and **4** vs **5**); and (b) potency increased with the replacement of the aminoethanol group of compounds **4** and **5** by the considerably more basic aminoethylamine in compounds **2** and **3,** an observation that generally paralleled the increase in potency seen with increasing basic strength of the functionalities linked to the C-6 methylene group of the 5-halogeno-amino-substituted 6-methyluracil derivatives [31]. Indeed, the observed potency of compounds **2** and **3** was quite striking and suggests that future investigations should focus on uracil analogs bearing a halogen at C-5 and other strongly basic polyamine (or amidine) functionalities directly attached to C-6 of the uracil moiety via a C—N bond.

The inhibition of TP, UP, and PNP activities by the two most potent of the compounds tested, AEAC (**2**) and CIMU (**14**) was directly compared, as shown in Fig. 1. Although the two compounds had nearly identical inhibitory effects on TP activity, they could be distinguished by their ability to inhibit UP activity. CIMU inhibited UP activity with an IC_{50} of 100 μM, a concentration approximately 300-fold higher than required to inhibit TP, whereas AEAC remained completely selective for TP inhibition up to a concentration of 1 mM. Neither AEAC nor CIMU had an effect on PNP activity up to a concentration of 0.1 mM. Further analysis of AEAC with various concentrations of the substrate thymidine demonstrated that AEAC was a competitive inhibitor of TP with a calculated K_i of 165 nM (Fig. 2). Both TP and VEGF induced HUVEC migration in a modified Boyden chamber assay (Fig. 3), although only the migration induced by TP was dependent upon the presence of thymidine (data

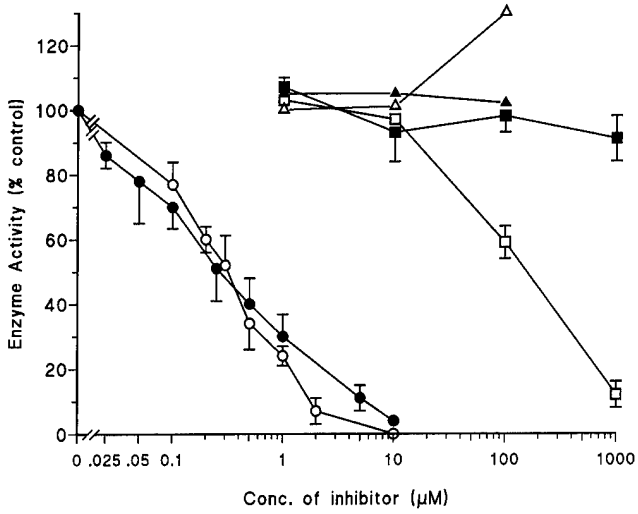


Fig. 1. Inhibition of TP, UP, and PNP activities by 5,6-substituted uracil analogs. The effects of the uracil analogs CIMU (○△□) and AEAC (●▲■) on TP (○●), UP (□■), and PNP (△▲) activities were evaluated. In addition to the inhibitors at the indicated concentrations, TP assays contained 200 mM KH_2PO_4 (pH 7.8), 0.2 mM [5′-^{3}H]thymidine (50 mCi/mmol), and 50 ng recombinant human TP. Reactions (20 min at 37°) were stopped with ice-cold activated charcoal in 5% trichloroacetic acid. After centrifugation, radioactivity in an aliquot of the supernatant was determined by liquid scintillation counting; TP activity in the absence of inhibitor (100% value) was 222 nmol/mg/min. Data are means ± SEM of four experiments. UP reactions contained, in 0.1 mL, 50 mM Tris–HCl (pH 7.5), 1 mM potassium phosphate, 200 μM uridine, 1 μCi [5-^{3}H]uridine, 100 ng purified human UP, and the indicated concentrations of AEAC and CIMU. After 30 min at 37°, aliquots of the reactions were analyzed by silica gel TLC as described in "Materials and methods." Radioactivity in spots co-migrating with authentic uracil and uridine were determined by liquid scintillation counting; activity in the absence of inhibitor was 3.92 μmol/mg/min. Data are means ± SEM of three experiments. PNP activity was assessed in a coupled spectrophotometric assay in which inosine was converted to uric acid by the combined actions of PNP and xanthine oxidase. Reactions contained 10 mM KH_2PO_4 (pH 7.7), 40 μM inosine, 19 ng PNP, 0.01 U xanthine oxidase, and increasing concentrations of AEAC and CIMU. The change in absorbance of uric acid at 293 nm was monitored continuously, and enzyme activity was calculated using an extinction coefficient of 12.9 mM^{-1} cm^{-1}. Data are from a single experiment; activity in the absence of inhibitor was 102 μmol/mg/min.

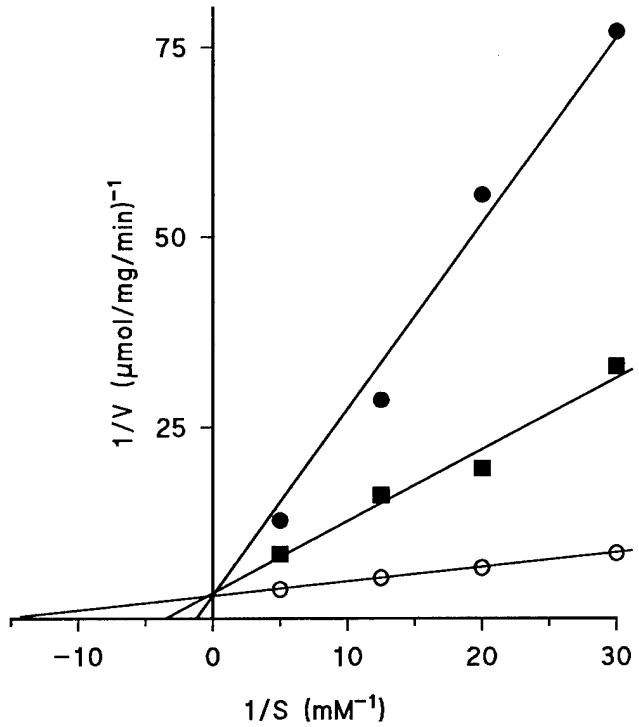


Fig. 2. Double-reciprocal plot of TP inhibition by AEAC. Assays of TP activity were carried out as described in Fig. 1 except that the concentration of the substrate thymidine was varied between 33 and 200 μM. AEAC was used at concentrations of 0 mM (○), 0.3 mM (■), and 0.9 mM (●). Data are means of two experiments.

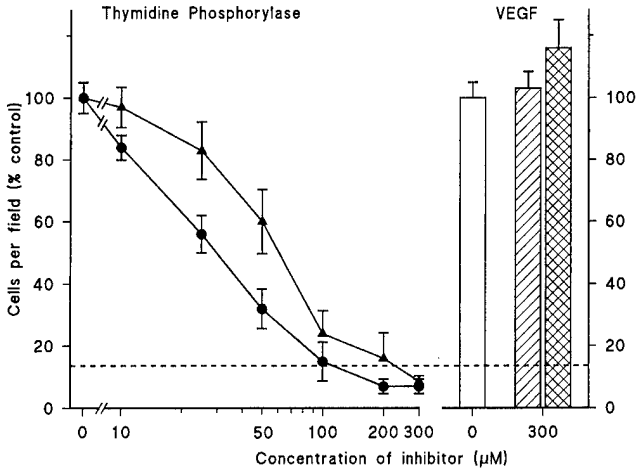


Fig. 3. Stimulation of HUVEC migration by TP and VEGF, and inhibition by uracil analogs. Human endothelial cells, isolated as described in "Materials and methods," were used in a modified Boyden chamber assay. Cells (10^5) were placed into fibronectin-coated transwell inserts, which were then placed into 24-well plates in which the lower wells contained 0.7 mL M199 with 1% serum. After 1 hr, recombinant human TP (200 ng/mL) (left) or human VEGF (10 ng/mL) (right) was added to the lower wells. Control wells (dashed line) had no added angiogenic factor. Wells also contained the indicated concentrations (0–300 μM) of either CIMU (● and single hatched bar) or AEAC (▲ and double hatched bar). Cells were incubated for 5 hr at 37°, at which time non-migrating endothelial cells were removed from the upper surface of the membrane. Cells were stained with Cell Tracker Green, washed with PBS, fixed in formaldehyde, and mounted on microscope slides. Migration was quantitated by counting five random photographed (100× total magnification) fields, and the number of HUVEC in each field was counted. Data are means ± SEM of three experiments.

not shown). This confirmed that the effect of TP on endothelial cells was dependent upon its catalytic activity. TP-mediated HUVEC migration was inhibited by both CIMU and AEAC in a concentration-dependent manner, with 50% inhibition at 30 and 60 μM, respectively, and with complete inhibition at concentrations of 200 μM (Fig. 3). In contrast, neither compound had an inhibitory effect on VEGF-mediated HUVEC migration, even at concentrations of 300 μM (Fig. 3).

An elevated level of TP expression, when compared with surrounding uninvolved tissue, is a common finding in a wide range of human solid tumors. We have transfected human colon and breast cancer cells with the TP cDNA as a means of evaluating the angiogenic properties of TP-overexpressing tumor cells. These cells were strong inducers of HUVEC migration in the Boyden chamber assay (Table 2), and were 2- to 4-fold more chemotactic for HUVEC than HT29 or MCF7 cells that had been transfected with a control vector (data not shown). Consistent with its inhibitory activities against purified TP, CIMU also strongly inhibited HUVEC migration induced by TP-expressing tumors cells (Table 2), an observation that supports further investigation into the potential use of these inhibitors in pathological conditions that are dependent upon TP-mediated angiogenesis.

Immunohistochemical studies of certain human tumors have shown a consistent positive correlation between the level of TP expression and tumor angiogenesis and aggressiveness. The development of TP inhibitors would provide a means to evaluate the extent to which TP overexpression contributes to the early development, progression, and maintenance of experimental and human cancers, particularly in relation to other angiogenic factors whose expres-

Table 2
Inhibition of HUVEC migration by a TP inhibitor in a co-culture assay

Tumor cells	CIMU	HUVEC cells/field	% Inhibition
None	−	11 ± 1.7	
HT29/TPneo	−	265 ± 32	
HT29/TPneo	+	69 ± 9.4	77
MCF7/TPneo	−	115 ± 9.0	
MCF7/TPneo	+	28 ± 2.6	84

HUVEC migration was assessed in a modified Boyden chamber assay in which human cancer cells were used in place of added angiogenic stimuli; HT29 colon and MCF7 breast carcinoma cells that had been stably transfected with a human TP cDNA (HT29/TPneo and MCF7/TPneo) were used. Tumor cells (10^5) were added to the lower wells of a 24-well plate and were allowed to attach for 24 hr. The medium was then replaced with M199 medium with and without 0.3 mM CIMU, combined with HVUEC-containing transwells, and migration over a 5-hr period was analyzed as described in "Materials and methods." Data are means ± SEM of two experiments, each done in triplicate.

sion has also been shown to be correlated with increased neovascularization. The possibility that a TP inhibitor could have therapeutic utility as an anticancer drug is supported by studies which found that elevated TP expression increased the growth of tumor cells as xenografts *in vivo* without altering their growth *in vitro* [6,7,30]. This enhancement of tumor growth *in vivo* was reversed when the mice were treated with a TP inhibitor [27,30]. TP has also been implicated as an angiogenic factor contributing to the pathology of other diseases, including (a) rheumatoid arthritis, where TP was found to be highly elevated in synovial fluid, (b) psoriasis, in which increased TP expression was seen in psoriatic lesions, and (c) gastric ulcers, where TP was found to be elevated near gastric ulcer margins when compared with uninvolved fundic and pyloric stomach [41–44]. Plasma TP levels were higher in intractable gastric ulcer patients compared with normal individuals, duodenal ulcer patients, and patients whose gastric ulcer had undergone significant resolution [44].

An increasing appreciation of the critical role angiogenesis plays in tumors and in pathologies associated with inflammatory responses has prompted the pursuit of a variety of different approaches to block neovascularization, and a number of therapeutic agents are currently in clinical trials. Approaches that target the actions of angiogenic factors have focused on the development of antibodies, small molecules, or gene therapy to directly neutralize either the angiogenic factor, or to block the receptors for the angiogenic factors on endothelial cells [45–48]. TP is distinct from other angiogenic factors in that, unlike classical factors which bind to a cell surface receptor, TP exerts its actions through its catalytic activity, the products of which are presumed to be responsible for the angiogenic activities of TP. Therefore, TP provides unique opportunities for the design of angiogenesis inhibitors, and, in addition to their therapeutic potential, these compounds should be useful in evaluating the contribution of TP to the progression of a number of pathological conditions.

Acknowledgments

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