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5-Phenylthioacyclouridine: A Potent and Specific Inhibitor of Uridine Phosphorylase

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ABSTRACT. 5-Phenylthioacyclouridine (PTAU or 1-[(2-hydroxyethoxy)methyl]-5-phenylthiouracil) was synthesized as a highly specific and potent inhibitor of uridine phosphorylase (UrdPase, EC 2.4.2.3). PTAU has inhibition constant (K_{is}) values of 248 and 353 nM towards UrdPase from mouse and human livers, respectively. PTAU was neither an inhibitor nor a substrate for thymidine phosphorylase (EC 2.4.2.4), uridine–cytidine kinase (EC 2.7.1.48), thymidine kinase (EC 2.7.1.21), dihydrouracil dehydrogenase (EC 1.3.1.2), orotate phosphoribosyltransferase (EC 2.4.2.10), or orotidine 5′-monophosphate decarboxylase (EC 4.1.2.23), the enzymes that could utilize the substrate (uridine or thymidine) or products (uracil or thymine) of UrdPase. Different isomers of 5-tolylthiouracil also were synthesized and tested as inhibitors of UrdPase. The meta-substituted isomer was 3- to 4-fold more potent as an inhibitor of UrdPase than the *para-* or *ortho*-substituted isomers. These data indicate that the hydrophobic pocket in the active site of UrdPase adjacent to the 5-position of the pyrimidine ring can accommodate the *meta*-substituted 5-phenyluracils better than the other isomers, leading to improved inhibition. Therefore, it is anticipated that the potency of PTAU can be increased further by the addition of certain hydrophobic groups at the *meta* position of the phenyl ring. PTAU has potential usefulness in the therapy of cancer and AIDS as well as other pathological and physiological disorders that can be remedied by the administration of uridine. BIOCHEM PHARMACOL **60**;6:851–856, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. 5-phenylthioacyclouridine; inhibitor; uridine phosphorylase; chemotherapy

The enzyme UrdPase¶ (EC 2.4.2.3) plays a crucial role in the chemotherapy of cancer and AIDS. The importance of UrdPase in cancer chemotherapy stems from the fact that UrdPase is the enzyme responsible for the activation and deactivation of several chemotherapeutic analogues, most notably the 5-fluoropyrimidines [1–7], as several human tumors are devoid of dThdPase (EC 2.4.2.4) activity [1, 5, 7]. Moreover, UrdPase exhibits a circadian rhythm in mice [8, 9], which is opposite to that observed for the anticancer efficacy of FdUrd [10]. In addition, host toxicities of some of these anticancer (e.g. FUra) [11–14] as well as anti-HIV (e.g. AZT) [15, 16] drugs are antagonized by uridine, the availability and concentration of which are controlled by UrdPase [17–25]. The important role played by UrdPase in the chemotherapy of cancer and AIDS has generated a strong interest in developing inhibitors for this enzyme. Such inhibitors would enhance the chemotherapeutic efficacy of these drugs by preventing their degradation and/or host toxicity.

Acyclouridines were shown to be potent inhibitors of UrdPase from various sources [1, 5–7, 26–32]. They were shown to potentiate the efficacy of FdUrd *in vitro* and *in vivo* [5, 7], to increase the level and duration of uridine in plasma [17–25] and heart perfusate [33], to enhance the salvage of uridine by various tissues [18–20], and to reduce host toxicity of FUra [18, 20, 34], FdUrd [35], and AZT [15, 36]. In this study we report the synthesis of PTAU as an inhibitor of UrdPase. PTAU is lipophilic, and therefore it is anticipated that its uptake by the liver and intestine, the principal organs involved in pyrimidine catabolism, would be facilitated. A preliminary report has been presented [37].

MATERIALS AND METHODS

Chemicals

[2-^{14}C]Uridine (56 Ci/mol) and [2-^{14}C]thymidine (56 Ci/mol) were obtained from Moravek Biochemicals, Inc., and silica gel G/UV$_{254}$ polygram TLC plates from Brinkmann.

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¶ *Abbreviations*: AZT, 3′-azido-3′-deoxythymidine; BAU, 5-benzylacyclouridine; BBAU, 5-(benzyloxybenzyl)acyclouridine; dThdPase, thymidine phosphorylase; DTT, dithiothreitol; FdUrd, 5-fluoro-2′-deoxyuridine; FUra, 5-fluorouracil; PTAU, 5-phenylthioacyclouridine or 1-[(2-hydroxyethoxy)methyl]-5-phenylthiouracil; and UrdPase, uridine phosphorylase.

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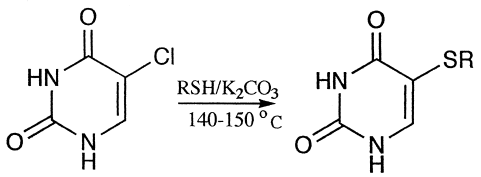


SCHEME 1.

The protein assay kit was purchased from Bio-Rad Laboratories. All other chemicals were obtained from the Sigma Chemical Co. or the Aldrich Chemical Co.

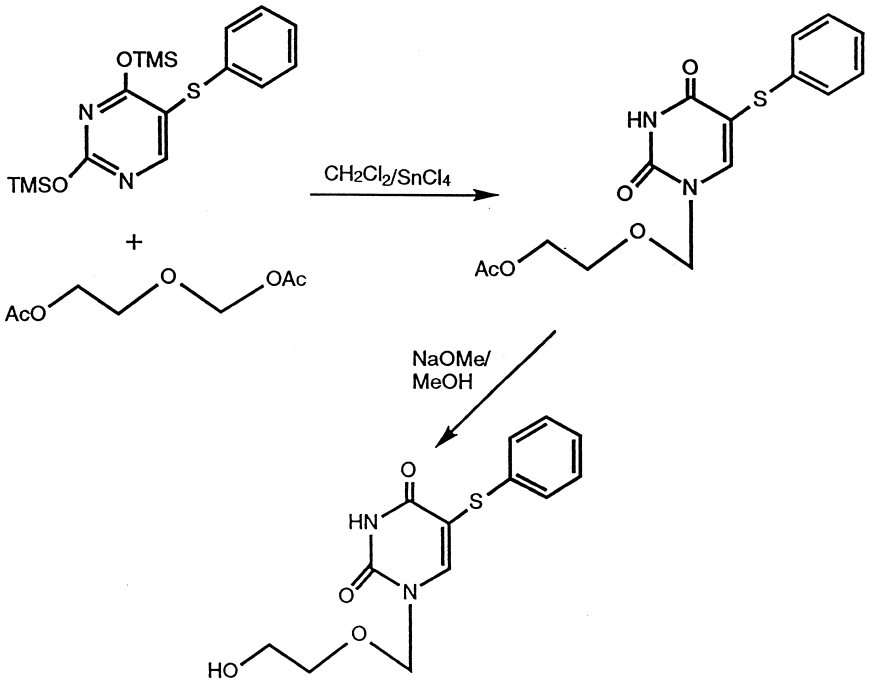
Chemical Synthesis

5-Phenylthiouracil and the other 5-substituted uracils were prepared from 5-chlorouracil by the method outlined in Scheme 1. PTAU or 1-[(2-hydroxyethoxy)methyl]-5-phenylthiouracil was prepared by the method outlined in Scheme 2. Melting points were determined on an Electrothermal melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a General Electric QE-300 (300 MHz) spectrometer. Experiments were monitored using TLC analysis performed on Kodak Chromatogram sheets precoated with silica gel and a fluorescent indicator; column chromatography employing silica gel (60–200 mesh; Fisher Scientific) was used for the purification of products. Microanalyses were performed at Galbraith Laboratories, Inc.

5-PHENYLTHIOURACIL. 5-Phenylthiouracil was synthesized as described previously [31, 38]. ^{1}H NMR ($CDCl_3$) δ 11.35 (brs, 2H, NH), 7.88 (s, 1H, 6-H), 7.12–7.30 (m, 5H, SPh).

2-ACETOXYETHYL ACETOXYMETHYL ETHER. 2-Acetoxyethyl acetoxymethyl ether was synthesized as previously described [39]. ^{1}H NMR ($CDCl_3$) δ 5.3 (s, 2H, OCH_2O), 4.08 (A_2B_2 pattern, 4H, OCH_2CH_2O), 3.08 (s, 6-H, $COCH_3$).

1-[(2-ACETOXYETHOXY)METHYL]-5-PHENYLTHIOURACIL. *N,O*-Bis(trimethylsilyl)-acetamide (6.40 mL, 25 mmol) was added to a mixture of 5-phenylthiouracil (2.20 g, 10 mmol) and 2-acetoxyethyl acetoxymethyl ether (2.15 g, 12 mmol) in dry CH_2Cl_2 (40 mL) with stirring under a nitrogen atmosphere. After 2 hr of stirring at room temperature, the clear solution was cooled to 0°, and $SnCl_4$ (10 mL, 1.0 M CH_2Cl_2 solution, 10 mmol) was added. Then the reaction mixture was warmed to room temperature and stirred overnight. The solution was poured slowly into a mixture of $CHCl_3$ (100 mL) and saturated aqueous $NaHCO_3$ (100 mL) solution. The resulting emulsion was separated by filtration, the aqueous fraction was extracted further with EtOAc (3 × 50 mL), and the combined organic fractions were dried over Na_2SO_4 and concentrated under reduced pressure. Trituration of the oily residue with diethyl ether (40 mL) afforded the product as a white solid (2.05 g, 61%), m.p. 96–99°. ^{1}H NMR ($CDCl_3$) δ 1.87 (s, 3H, $COCH_3$), 3.61–4.05 (A_2B_2 pattern, 4H, OCH_2CH_2O), 5.02 (s, 2H, OCH_2O), 7.05–7.19 (m, 5H, SPh), 7.50 (s, 1H, 6-H), 8.88 (s, 1H, NH, D_2O exchange-



SCHEME 2.

able). Anal. Calc. for $C_{15}H_{16}N_2O_5S$: C, 53.94; H, 4.79; N, 8.33. Found: C, 53.36; H, 4.73; N, 8.50.

1-[(2-HYDROXYETHOXY)METHYL]-5-PHENYLTHIOURACIL (5-PHENYLTHIOACYCLOURIDINE OR PTAU). To a solution of 1-[(2-acetoxyethoxy)methyl]-5-phenylthiouracil (1.68 g, 5 mmol) in MeOH (20 mL) was added 10 mL of 1 N NaOMe in MeOH. After 4 hr at room temperature, the pH was adjusted to 4.0 with 1 N HCl, and the solvent was removed under reduced pressure to obtain a solid. The residue was loaded onto a silica gel column, and the product was obtained by elution with CH_2Cl_2/MeOH (9:1). The TLC pure fractions were pooled and concentrated, and the residue was recrystallized from absolute ethanol to yield the desired title product as a white crystalline solid (1.18 g, 80%), m.p. 131–133°. ^{1}H NMR (DMSO-d_6) δ 3.31–3.57 (m, 4H, OCH_2CH_2O), 4.66 (t, 1H, OH, D_2O exchangeable), 5.16 (s, 2H, NCH_2O), 7.13–7.31 (m, 5H, SPh), 8.29 (s, 1H, 6-H), 11.67 (s, 1H, NH, D_2O exchangeable). Anal. Calc. for $C_{13}H_{14}N_2O_4S$: C, 53.03; H, 4.79; N, 9.52. Found: C, 52.64; H, 4.80; N, 9.52.

5-*O*-TOLYLTHIOURACIL (5-*ORTHO*-TOLYLTHIOURACIL OR 2-METHYL-5-PHENYLTHIOURACIL). Reaction of 5-chlorouracil (730 mg, 5 mmol) with *o*-thiocresol (620 mg, 5 mmol) in 25 mL of ethylene glycol in the presence of potassium carbonate (700 mg, 5 mmol) as described for the preparation of 5-phenylthiouracil yielded the title compound (680 mg, 67%), m.p. 263–265°. ^{1}H NMR ($CDCl_3$) δ 11.32 (brs, 2H, NH), 7.82 (s, 1H, 6-H), 6.90–7.20 (m, 4H, SPh), 2.32 (s, 3H, $PhCH_3$). Anal. Calc. for $C_{11}H_{10}N_2O_2$: C, 56.31; H, 4.30; N, 11.96. Found C, 56.11; H, 4.30; N, 11.96.

5-*M*-TOLYLTHIOURACIL (5-*META*-TOLYLTHIOURACIL OR 3-METHYL-5-PHENYLTHIOURACIL). Reaction of 5-chlorouracil (730 mg, 5 mmol) with *m*-thiocresol (620 mg, 5 mmol) in 25 mL of ethylene glycol in the presence of potassium carbonate (700 mg, 5 mmol) as described for the preparation of 5-phenylthiouracil yielded the title compound (570 mg, 56%), m.p. 265–268°. ^{1}H NMR ($CDCl_3$) δ 11.30 (brs, 2H, NH), 7.85 (s, 1H, 6-H), 6.95–7.18 (m, 4H, SPh), 2.25 (s, 3H, $PhCH_3$). Anal. Calc. for $C_{11}H_{10}N_2O_2$: C, 56.31; H, 4.30; N, 11.96. Found C, 55.76; H, 4.26; N, 12.03.

5-*P*-TOLYLTHIOURACIL (5-*PARA*-TOLYLTHIOURACIL OR 4-METHYL-5-PHENYLTHIOURACIL). Reaction of 5-chlorouracil (730 mg, 5 mmol) with *p*-thiocresol (620 mg, 5 mmol) in 25 mL of ethylene glycol in the presence of potassium carbonate (700 mg, 5 mmol) as described for the preparation of 5-phenylthiouracil yielded the title compound (610 mg, 60%), m.p. 270–273°. ^{1}H NMR ($CDCl_3$) δ 11.30 (brs, 2H, NH), 7.85 (s, 1H, 6-H), 6.90–7.22 (m, 4H, SPh), 2.25 (s, 3H, $PhCH_3$). Anal. Calc. for $C_{11}H_{10}N_2O_2$: C, 56.31; H, 4.30; N, 11.96. Found C, 56.11; H, 4.40; N, 11.92.

Enzyme Studies

PREPARATION OF ENZYME EXTRACTS. Human liver samples were obtained from the Tissue Procurement Facility of the Comprehensive Cancer Center of the University of Alabama at Birmingham. Mouse livers were obtained from female CD-1 mice (18–20 g) (Charles River Laboratories). The samples were minced and homogenized in ice-cold (3:1, v/w) buffer [20 mM potassium phosphate (pH 8.0), 1 mM DTT, 1 mM EDTA] using a Polytron homogenizer (Brinkmann Instruments). The homogenates were centrifuged at 105,000 *g* for 1 hr at 4°. The supernatant fluids (cytosol) were collected. UrdPase and dThdPase were isolated from the cytosol by column chromatography as previously described [6].

URDPASE AND DTHDPASE ASSAYS. Assays were conducted at 37° under conditions where enzyme activity was linear with respect to time and enzyme concentration. Nucleoside (uridine or thymidine) cleavage was measured isotopically by following the formation of nucleobases from their respective nucleosides as previously described [6]. Five concentrations of the inhibitor were used, ranging from 8 to 900 μM. The reaction mixture contained 20 mM potassium phosphate (pH 8), 1 mM EDTA, 1 mM DTT, 1 mM [2-^{14}C]uridine or [2-^{14}C]thymidine (6 Ci/mol), and 25 μL cytosol in a final volume of 50 μL. Reactions were started by the addition of extract and stopped by boiling in a water bath for 2 min followed by freezing. Precipitated proteins were removed by centrifugation. Uridine and thymidine were separated from their respective nucleobases on silica gel TLC plates developed with chloroform:methanol:acetic acid (90:5:5, by vol.), and the radioactivity in the spots was determined on a percentage basis using a Berthold LB-2821 Automatic TLC-Linear Analyzer. The R_f values were: uridine, 0.07; uracil, 0.43; thymidine, 0.14; and thymine, 0.62.

Kinetic Studies

Inhibition constants (K_{is}) and apparent K_i values were estimated from the data using computer programs with least squares fitting. The program was written by Dr. Sungman Cha and modified to fit IBM BASIC by Dr. F. N. M. Naguib.

DETERMINATION OF K_{IS} VALUES. K_{is} values were estimated from the slopes and intercepts of the double-reciprocal plots by the methods of Wilkinson [40] and Cleland [41], using uridine concentrations ranging from 30 to 700 μM.

DETERMINATION OF APPARENT K_I VALUES. Using uridine (125 μM) and five different concentrations of the inhibitor ranging from 50 to 900 μM, apparent K_i values were estimated from Dixon plots (1/v vs [I]) [42] of the data.

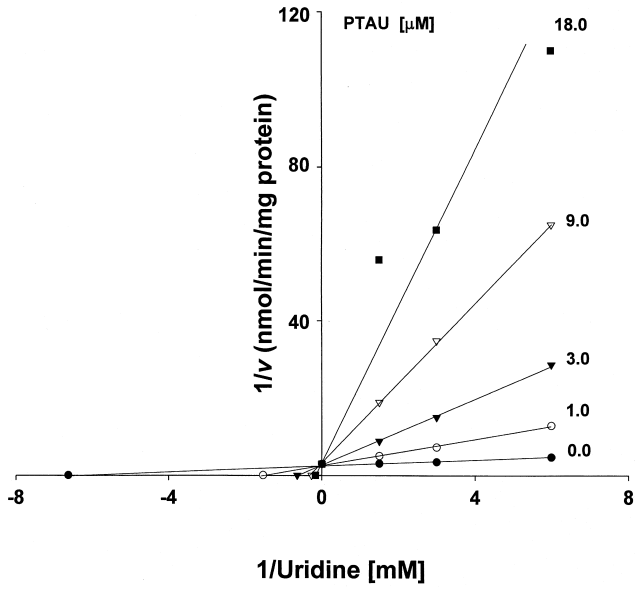


FIG. 1. Inhibition of human liver UrdPase by PTAU. Plots of 1/velocity versus 1/[uridine] at various fixed concentrations of PTAU. Each point represents at least three determinations.

Protein Determination

Protein concentrations were determined spectrophotometrically by the method of Bradford [43], using bovine γ-globulin as a standard.

RESULTS AND DISCUSSION

In the present study, we synthesized PTAU as a new, highly specific, and potent inhibitor of UrdPase. PTAU was subjected to kinetic studies to determine the mechanism of inhibition and the K_{is} value, and also was compared with BAU and BBAU. All three inhibitors (PTAU, BAU, and BBAU) showed competitive inhibition with UrdPase from mouse and human livers. The liver was chosen as the source of UrdPase because it is generally believed that it is the organ that regulates pyrimidine metabolism in the body [17, 44–48]. Figure 1 shows the Lineweaver–Burk plot of PTAU with human liver UrdPase. PTAU had K_{is} values of 248 and 353 nM towards UrdPase from mouse and human livers, respectively (Table 1). Table 1 also compares the K_{is} values of PTAU with those of its 5-benzyl-substituted counterparts (BAU and BBAU) as inhibitors of UrdPase from mouse and human livers. The results in Table 1 demonstrate that PTAU was more potent than its benzylacyclouridine counterpart, BAU, and comparable in potency to BBAU, a more potent inhibitor of UrdPase than BAU [26, 27].

TABLE 1. Inhibition constants (K_{is}) of PTAU compared with those of BAU and BBAU for UrdPase from mouse and human livers

	K_{is}* (nM)	
Inhibitor	**Mouse liver**	**Human liver**
PTAU	248 ± 46	353 ± 76
BAU	420 ± 40	1190 ± 200
BBAU	170 ± 00	220 ± 29

*Values are means ± standard error of estimation from at least three determinations measured at 20 mM inorganic phosphate.

TABLE 2. Apparent K_i values of 5-thio-substituted uracil analogues for UrdPase from human liver

Compound	Apparent K_i* (μM)
5-(Phenylthio)uracil	175.2 ± 66.3
meta-Tolylthiouracil	28.6 ± 4.3
para-Tolylthiouracil	100.1 ± 48.5
ortho-Tolylthiouracil	119.9 ± 53.2

*Values are means ± standard error of estimation from at least three determinations measured at 125 μM uridine and 20 mM inorganic phosphate.

To determine the specificity of PTAU, the compound was tested against various enzymes that could utilize the substrate (uridine or thymidine) or products (uracil or thymine) of UrdPase as previously described [28]. PTAU was neither an inhibitor nor a substrate for dThdPase, uridine–cytidine kinase (EC 2.7.1.48), thymidine kinase (EC 2.7.1.21), dihydrouracil dehydrogenase (EC 1.3.1.2), orotate phosphoribosyltransferase (EC 2.4.2.10), or orotidine 5′-monophosphate decarboxylase (EC 4.1.2.23), as there was no significant effect on their activity in the presence of these compounds. These experiments indicate that PTAU is a specific inhibitor of UrdPase.

Consistent with the notion that increased hydrophobicity at the 5-position of the pyrimidine ring enhances binding of pyrimidines to UrdPase by interacting with a hydrophobic pocket on the enzyme adjacent to the 5-position of the pyrimidine ring [1, 26–30, 32, 49, 50], we synthesized and tested different isomers of 5-tolylthiouracil as inhibitors of UrdPase. Table 2 compares the apparent K_i values of these different isomers of tolylthiouracil. The *meta*-substituted isomer was 3- to 4-fold more potent as an inhibitor of UrdPase than the *para*- or *ortho*-substituted isomers. Similar results were observed with *meta* isomers of 5-benzyluracil [51] and 5-(benzyloxy)benzylbarbituric acid [52]. These data clearly indicate that the *meta* isomer of 5-phenyl-substituted uracils provides a significant inhibitory effect on UrdPase over the *ortho* or *para* isomers. It also provides evidence that the hydrophobic pocket in the active site of UrdPase [1, 26–30, 32, 49, 50] can accommodate the *meta*-substituted 5-phenyluracils better than the other isomers, leading to better inhibition. Therefore, it is expected that further hydrophobic substitutions (e.g. alkyl, aryl groups) at the *meta* position of the phenyl ring of PTAU would yield even more potent inhibitors of UrdPase than PTAU itself.

PTAU has an excellent potential in various aspects of chemotherapy. The usefulness of UrdPase inhibitors has already been established in the field of experimental chemotherapy of cancer and AIDS. We previously developed various 5-substituted derivatives of acyclouridine and acyclobarbituric acid as specific inhibitors of UrdPase [1,

26–28, 31]. These inhibitors were shown to enhance the efficacy of FdUrd against human tumors *in vitro* and *in vivo* [5, 7]. Furthermore, the compounds were shown to elevate the concentration and prolong the half-life of uridine in the plasma [17–25], as well as increase the salvage of uridine by various tissues [18, 19]. Therefore, these inhibitors were used to protect against or rescue from the host toxicity of anticancer (i.e. FUra) [18, 20, 34) and anti-HIV (i.e. AZT) [16, 36] drugs, the toxicities of which were shown to be antagonized by administration of exogenous uridine. The new acyclonucleoside PTAU is likely to share the various chemotherapeutic characteristics of the older inhibitors. Furthermore, it is anticipated that the lipophilicity of PTAU will enhance its uptake by the liver and intestine, the main organs responsible for pyrimidine metabolism, and hopefully its chemotherapeutic efficacy.

In conclusion, we have synthesized PTAU as a specific and potent inhibitor of UrdPase. Its potency may be increased further by the addition of hydrophobic groups at the *meta* position of its phenyl ring. PTAU could have a wide application in the therapy of cancer, AIDS, as well as other pathological and physiological disorders where administration of uridine has been shown to be useful [cf. Refs. 23 and 25 and references therein].

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