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STRUCTURE–ACTIVITY RELATIONSHIP OF LIGANDS OF THE PYRIMIDINE NUCLEOSIDE PHOSPHORYLASES*

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Abstract—Eighty-seven pyrimidine base and nucleoside analogs were evaluated as inhibitors of uridine phosphorylase (UrdPase) and thymidine phosphorylase (dThdPase). These findings, together with an extensive literature review, have allowed construction of structure–activity relationships for the binding of ligands to UrdPase and dThdPase and provide a basis for the rational design of new inhibitors of these enzymes. Additionally, 2,6-pyridinediol and 6-benzyl-2-thiouracil have been identified as being potent inhibitors of UrdPase and dThdPase respectively.

Two separate pyrimidine nucleoside phosphorylases, uridine phosphorylase (UrdPase‡, EC 2.4.2.3) and thymidine phosphorylase (dThdPase, EC 2.4.2.4), are known to occur in the cytosol of mammalian cells [1]. These enzymes catalyze the reversible phosphorolysis of a number of naturally occurring and synthetic pyrimidine nucleosides [1–5] as follows:

$$\text{Pyr(d)R} + \text{P}_\text{i} \rightleftharpoons \text{Pyr} + \text{(d)R-1-P}$$

It has been speculated that inhibitors of the pyrimidine nucleoside phosphorylases might be useful as chemotherapeutic agents by interfering with the salvage of pyrimidine bases [6] or by enhancing the efficacy of pyrimidine analogs such as 5-fluorouridine, 5-fluoro-2′-deoxyuridine, or 5-iodo-2′-deoxyuridine which are cleaved and thereby inactivated by the pyrimidine nucleoside phosphorylases [3, 6–10]. Unfortunately, few potent pyrimidine nucleoside phosphorylase inhibitors have been found [6, 9–14].

In the present study, eighty-seven pyrimidine base and nucleoside analogs have been evaluated as inhibitors of the pyrimidine nucleoside phosphorylases. 2,6-Pyridinediol and 6-benzyl-2-thiouracil have been identified as potent inhibitors of UrdPase and dThdPase respectively. Additionally, our findings together with an extensive literature review have allowed us to construct structure–activity relationships for the binding of pyrimidines and pyrimidine analogs to the pyrimidine nucleoside phosphorylases. This report proposes a basis for the rational design of new inhibitors of these enzymes. A preliminary report has been presented [15].

MATERIALS AND METHODS

Chemicals

The sources of the pyrimidine base and nucleoside analogs used in this study are indicated in Table 4 by the following abbreviations: ALD, Aldrich Chemical Co., Milwaukee, WI; APG, Dr. Arthur P. Grollman, SUNY-Stonybrook, Long Island, NY; CAL, Calbiochem-Behring Corp., La Jolla, CA; JJF, Dr. Jack J. Fox, Sloan–Kettering Institute for Cancer Research, New York, NY; K & K, K & K Laboratories, Inc., Plainview, NY; LBT, Dr. Leroy B. Townsend, University of Michigan, Ann Arbor, MI; MAL, Mallinckrodt Chemical Works, St. Louis, MO; NCI, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD; PL, P-L Biochemicals, Milwaukee, WI; RKR, Dr. Roland K. Robins, Brigham Young University, Provo, UT; SIGMA, Sigma Chemical Co., St. Louis, MO; SYN, synthesized by the authors (see below); and VEGA, Vega Biochemicals, Tucson, AZ. Additionally, [2-^{14}C]uridine (52.4 mCi/mmole), [2-^{14}C]thymidine (51.6 mCi/mmole), and Omnifluor were obtained from the New England Nuclear Corp., Boston, MA;

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‡ Abbreviations: UrdPase, uridine phosphorylase; dThdPase, thymidine phosphorylase; Pyr(d)R, pyrimidine ribonucleoside or pyrimidine 2′-deoxyribonucleoside; P_i, orthophosphate; Pyr, pyrimidine base; (d)R-1-P, α-D-deoxyribose-1-phosphate or α-D-ribose-1-phosphate; acyclouridine, 1-(2′-hydroxyethoxymethyl)uracil; acyclothymidine, 5-methyl-1-(2′-hydroxyethoxymethyl)uracil; acyclocytidine, 1-(2′-hydroxyethoxymethyl)cytosine; 5-fluoroacyclouridine, 5-fluoro-1-(2′-hydroxyethoxymethyl)-uracil; 5-bromoacyclouridine, 5-bromo-1-(2′-hydroxyethoxymethyl)uracil; 5-benzylacyclouridine, 5-benzyl-1-(2′-hydroxy-ethoxymethyl)uracil; 5-benzyloxybenzylacyclouridine, 5-(*m*-benzyloxybenzyl)-1-(2′-hydroxyethoxymethyl) uracil; 5-benzyloxybenzyluracil, 5-(*m*-benzyloxybenzyl)uracil or 5-{[3-(phenylmethoxy)-phenyl]methyl}uracil; 5-benzylacyclocytidine, 5-benzyl-1-(2′-hydroxyethoxymethyl)cytosine; 5-benzyloxybenzylacyclocytidine, 5-(*m*-benzyloxybenzyl)-1-(2′-hydroxyethoxymethyl)cytosine; 5-benzyl-2-thioacyclouridine, 5-benzyl-2-thio-1-(2′-hydroxyethoxymethyl)uracil; 3-deazauracil, 2,4-dihydroxypyridine; 3-oxauracil, 2*H*-1,3-oxazine-2,6-(3*H*)-dione; barbituric acid, 2,4,6-trihydroxypyrimidine; thymine-propenal, 3-(thymin-1′-yl)2-propenal; uramil, 5-amino-2,4,6-trihydroxypyrimidine or 5-aminobarbituric acid; 2′-deoxyglucosylthymine, 1-(2′-deoxy-β-D-glucopyranosyl)thymine; showdomycin, 3-β-D-ribofuranosylmaleimide; FdUrd, 5-fluoro-2′-deoxyuridine; Urd, uridine; and dThd, thymidine.

silica gel G UV254 Polygram TLC plates were from Brinkmann, Westbury, NY; and DEAE-cellulose (DE-23) was from Whatman Biochemicals, Maidstone, Kent, UK. All other chemicals were obtained from the Sigma Chemical Co.

Chemical synthesis

The following compounds were synthesized by methods previously published: 5-benzylacyclouridine [6], 5-benzyloxybenzylacyclouridine [6], acyclouridine [16], acyclothymidine [16], acyclocytidine [16], 5-fluoroacyclouridine [16], 5-bromoacyclouridine [16], 4-thiouracil [17], 2-selenocytidine [18], 4-selenouridine [18], 6-benzyluracil [19], 5-benzyloxybenzyluracil [13, 20], and 5-benzyluracil [13, 20]. 5-Benzylacyclocytidine, 5-benzyloxybenzylacyclocytidine, and 2-thio-5-benzylacyclouridine were prepared employing methods to be published elsewhere (S. H. Chu, manuscript in preparation).

Animal tissues

Sarcoma S-180 cells were grown in the peritoneal cavity of male CD-1 mice (Charles River Laboratories, Wilmington, MA). This strain of mouse (uninoculated) was also used as the source of liver tissue.

Preparation of cytosol extracts for pyrimidine nucleoside phosphorylase assay

S-180 cells were collected in normal saline and washed as described previously [21]. Livers were obtained from freshly killed CD-1 mice. Tissues were then homogenized using a motor-driven Teflon pestle in 3 vol. of 20 mM potassium phosphate buffer (pH 8.0) which also contained (except where indicated otherwise) 1 mM EDTA and 1 mM mercaptoethanol (Buffer A). Homogenates were centrifuged at 4° for 1 hr at 105,000 *g*, and the pellets were discarded. S-180 cytosol was used as the source of UrdPase because it contains high levels of this enzyme but no dThdPase [10] and was stored frozen at −14° until ready for use. The UrdPase activity of frozen S-180 cytosol was stable for several days. Mouse liver cytosol was further purified as described below.

Partial purification of mouse liver dThdPase

DEAE-cellulose was equilibrated with Buffer A, packed into a column (2.5 × 5 cm), and 3 ml of mouse liver cytosol was applied. The column was then eluted with 50–75 ml of Buffer A. The eluent was brought to 80% saturation with ammonium sulfate, centrifuged for 20 min at 20,000 *g*, and the pellet was stored for up to 2 weeks at 4°. Immediately prior to use, the pellet was dissolved in 4 ml of Buffer A and used as the source of dThdPase (subsequently referred to as "partially purified dThdPase"). Mouse liver UrdPase binds to DEAE-cellulose under these conditions whereas dThdPase does not [1, 10]. Thus, this procedure removed any contaminating UrdPase from our partially purified dThdPase preparation.

The partial purification of dThdPase was necessary to eliminate artifactual results arising from the presence of UrdPase. Many pyrimidine nucleosides (e.g. uridine and 5-fluorouridine) which do not bind to dThdPase are good substrates for UrdPase [1–3, 10]. Thus, if we had not removed UrdPase contamination from our dThdPase preparation, inhibition brought about by products of UrdPase reactions (i.e. pyrimidine bases which bind to dThdPase) might have been incorrectly attributed to non-inhibitory nucleosides. Additionally, mouse liver UrdPase is known to cleave thymidine (the substrate used in our dThdPase assay). Thus, for inhibition studies to be specific for dThdPase, UrdPase should not be present.

Pyrimidine nucleoside phosphorylase assay

In a volume of 140 μl, the standard assay mixture contained 20 mM potassium phosphate, 1 mM EDTA, 1 mM mercaptoethanol, a substrate (either 0.15 mM uridine or 0.15 mM thymidine, each of a specific activity of 6 mCi/mmole), and, when indicated, an appropriate amount of inhibitor was included. As described below, modified reaction mixtures were used in certain cases. Typically, inhibitors were screened at concentrations of 0.05, 0.15, 0.45, and 0.90 mM; however, lower concentrations had to be used for very potent inhibitors (1-benzyluracil, 2,6-pyridinediol, 5-benzyloxybenzyluracil, 5-benzyluracil, 5-nitrouracil, 5-benzylacyclouridine, and 5-benzyloxybenzylacyclouridine) and for inhibitors with low water solubility (1-cyclohexyluracil, 1-cyclohexylthymine, 6-benzyluracil, 5-benzylacyclocytidine, 5-benzyloxybenzylacyclocytidine, and 2-thio-5-benzylacyclouridine). Also, inhibitor concentrations greater than 0.90 mM were used in assays of certain poor enzyme inhibitors (barbituric acid, 6-methyluracil, and 5-bromo-1-methyluracil) where accurate determination of an apparent K_i value was desired. Reactions were started by the addition of S-180 cytosol (0.01 to 0.04 mg protein) or partially purified dThdPase (0.03 to 0.09 mg protein) for assays of UrdPase and dThdPase respectively. Assays for UrdPase and dThdPase were carried out as previously described [10].

Pyrimidine nucleoside phosphorylase assays carried out under special conditions

Thymine-propenal [22] and showdomycin [23] were reported to react readily with sulfhydryl groups and, therefore, mercaptoethanol was routinely omitted from Buffer A in the preparation of homogenates, reaction mixtures, and dialysis buffers for the testing of these compounds. Also, since it was suspected that one of the components of Buffer A might be reacting with 2,6-pyridinediol, additional assays were carried out with this compound in which either mercaptoethanol or EDTA was omitted from the homogenization buffers and reaction mixtures.

Testing of compounds as tight- or covalently-binding inhibitors of the pyrimidine nucleoside phosphorylases

Enhancement of the degree of enzyme inhibition by preincubation is the hallmark of so-called "tight-binding" inhibitors [24]. In addition to screening procedures described above, 2,6-pyridinediol, showdomycin and thymine-propenal were tested for the possibility of being tight-binding inhibitors of UrdPase and dThdPase by preincubating these compounds for 20 min at 37° with the enzyme-containing extracts. Reactions were then started by the addition

of substrate, and assays for uridine and thymidine phosphorolytic activity were carried out as described above.

Possible covalent bonding of 2,6-pyridinediol, showdomycin and thymine-propenal was studied as follows. One-ml aliquots of S-180 cytosol and partially purified dThdPase were incubated for 20 min at 37° with and without 0.9 mM inhibitor, followed by dialysis against 2 liters of appropriate buffer with four changes over a 24-hr period. Assays for pyrimidine nucleoside phosphorolytic activity were carried out as above.

Determination and significance of apparent K_i *values*

Apparent K_i values were determined from Dixon plots ($1/v$ vs $[I]$) of the data by a computer program with least-squares fitting. The phosphate concentration used in our assays (20 mM) is saturating for both UrdPase [2] and dThdPase [25]. Apparent K_i values are related to K_i values by the following equation:

$$\text{apparent } K_i = \frac{K_i(1 + [S]/K_m)}{1 + ([S]/K_m)(K_{is}/K_{ii})}$$

where K_{is} and K_{ii} are the inhibition constants that would have been estimated from the replots of slope and intercept, respectively, of a Lineweaver–Burk plot vs $[I]$. If a compound is a competitive inhibitor with respect to a nucleoside substrate, $K_{ii} = \infty$ and $K_{is} = K_i$.

Therefore:

$$\text{apparent } K_i = K_i(1 + [S]/K_m)$$

Thus, for UrdPase, which has a K_m value for uridine of 0.05 to 0.15 mM [1, 2, 10], the apparent K_i of a competitive inhibitor is approximately 2- to 4-fold higher than the K_i. It must be stressed, however, that inhibitors were not characterized with regard to type of inhibition produced (i.e. competitive, non-competitive, or uncompetitive).

Protein determination

Protein concentrations were determined by the Bio-Rad Laboratories assay procedure, using bovine γ-globulin as a standard.

RESULTS

In order to have a broad basis to formulate theories on structure–activity relationship, an extensive literature survey has been conducted. The substrate specificities of UrdPase and dThdPase from the literature are summarized in Tables 1 and 2 respectively. The more potent inhibitors of the pyrimidine nucleoside phosphorylases are listed in Table 3.

Apparent K_i values (appK_i) for all compounds which showed activity as pyrimidine nucleoside phosphorylase inhibitors appear in Table 4. Roman numerals cited in the following text refer to compounds listed in Table 4.

Showdomycin (LXXVII), thymine-propenal (XXXVIII) and 2,6-pyridinediol (VII) are not tight-binding inhibitors of UrdPase or dThdPase since preincubation for 20 min with the enzyme preparations had no effect on inhibition. Also, dialysis after preincubation with these compounds reversed inhibition and, therefore, we conclude that these compounds do not bind covalently to either enzyme.

The stock solution (3.15 mM) of the potent UrdPase inhibitor 2,6-pyridinediol prepared in Buffer A turned to a deep purple color after standing for 1 hr at room temperature. The presence of EDTA in the buffer appears to have been involved in this color change, since no purple color developed even after 1 day when this compound was dissolved in 20 mM potassium phosphate buffer (pH 8.0) with or without 1 mM mercaptoethanol. However, the omission of either EDTA or mercaptoethanol from the homogenization buffer and reaction mixtures had no effect on the inhibition of UrdPase and dThdPase by 2,6-pyridinediol, thus making it unlikely that an EDTA or mercaptoethanol adduct was involved. Furthermore, there was no difference in enzyme inhibitory potency of a freshly prepared solution of 2,6-pyridinediol in Buffer A and that of a deeply colored solution that had been stored for 1 week at room temperature.

DISCUSSION

Binding of ligands to uridine phosphorylase

Hydrophobic region on UrdPase. Present findings support the hypothesis that a hydrophobic region exists on UrdPase, which is situated adjacent to the binding site of the 5-position of uracil [10, 13]. The enhanced binding of thymine (XXIV) over uracil (I), acyclothymidine (XLIII) over acyclouridine (XLIV), and 5-methyluridine (LXXII) over uridine (LXXX) all may be explained by the interaction of the 5-methyl groups of these more potent analogs with the postulated hydrophobic region of UrdPase. Certain larger hydrophobic groups substituted at the 5-position of uracil dramatically enhance binding to UrdPase. Specifically, 5-benzyluracil (XVII), 5-benzyloxybenzyluracil (XVI), 5-benzylacyclouridine (LIII), and 5-benzyloxybenzylacyclouridine (LV) all bind very tightly to UrdPase, and, indeed, the last compound (LV) is the most potent inhibitor known for this enzyme [6].

Baker and Kelley [13] have made an extensive study of the nature and dimensions of the hydrophobic region of UrdPase, and their conclusions may help explain some of our findings. These authors reported that various 5-benzyluracils, including XVI and XVII, bound well to UrdPase, whereas 5-phenyluracil was non-inhibitory [13]. Therefore, they concluded that steric factors play an important role in interactions involving the hydrophobic region of UrdPase [13]. They have further hypothesized that the phenyl moiety of 5-benzyluracil binds to UrdPase in a conformation coplanar with uracil [13]. On the other hand, 5-phenyluracil, in which the phenyl group exists in a relatively rigid conformation perpendicular to the plane of the uracil ring, does not bind because the 5-position substituent cannot properly interact with the hydrophobic pocket and sterically hinders binding [13]. In the present study, we found that the 5-substituted pyrimidine analogs pentobarbital (LXXXVI) and phenobarbital

Table 1. Substrate specificity of uridine phosphorylase

Compound*	Enzyme source	Ref.
Substrates for nucleoside cleavage		
Arabinosyluracil	PRK cells	26
5-Bromo-2′-deoxyuridine	Ehrlich ascites cells	3
5-Chloro-2′-deoxyuridine	Ehrlich ascites cells	3
2′-Deoxyuridine	Ehrlich ascites cells	1–3
	Rat brain	27
	PRK cells	26
5′-Deoxy-5-fluorouridine	Ehrlich ascites cells	28
5-Ethyluridine	PRK cells	26
5-Fluoro-2′-deoxyuridine	Ehrlich ascites cells	2, 3, 28, 29
	Walker 256 carcinoma	13
	PRK cells	26
5-Fluorouridine	Ehrlich ascites cells	2, 28, 29
	PRK cells	26
5-Iodo-2′-deoxyuridine	Ehrlich ascites cells	2, 3
2′-*O*-Methyluridine	PRK cells	26
5-Methyluridine	Ehrlich ascites cells	2
	PRK cells	26
6-Methyluridine	PRK cells	26
5-Nitro-2′-deoxyuridine	Rat liver	30
5-Propynyloxyuridine	PRK cells	26
2-Thiouridine	Rat liver	31
Thymidine	Ehrlich ascites cells	1–3, 32
	Rat brain	27
	Mouse liver	1, 10
	Sarcoma S-180 cells	10
	L5178Y cells	10
	L1210 cells	10
5-Trifluoromethyl-2′-deoxyuridine	Ehrlich ascites cells	32, 33
Uric acid riboside	Beef erythrocytes	34
	Rat small intestine	34
Uridine	Ehrlich ascites cells	1, 2, 29
	Rat brain	27
	Mouse liver	1, 10
	Sarcoma S-180 cells	10
	L5178Y cells	10
	L1210 cells	10
	PRK cells	26
Substrates for nucleoside synthesis with ribose-1-phosphate as the pentose donor		
6-Azathymine	Ehrlich ascites cells	2, 35
6-Azauracil	Ehrlich ascites cells	2, 35
Oxypurinol	Guinea pig small intestine	36
Thiouracil	Rat liver	31
Thymine	Ehrlich ascites cells	2
	Rat liver	37
Uracil	Ehrlich ascites cells	2
Substrates for nucleoside synthesis with 2′-deoxyribose-1-phosphate as the pentose donor		
6-Azathymine	Ehrlich ascites cells	2
5-Bromouracil	Ehrlich ascites cells	2
Thymine	Ehrlich ascites cells	2
Uracil	Ehrlich ascites cells	2
Non-substrates for nucleoside cleavage		
Acyclothymidine	Sarcoma S-180 cells	10
Acyclouridine	Sarcoma S-180 cells	10
Arabinopyranosyl-5-bromouracil	Rat liver	38
Arabinopyranosyl thymine	Rat liver	38
Arabinopyranosyl uracil	Rat liver	38
Arabinosyl thymine	Hamster liver	39
	PRK cells	26
6-Azathymidine	Ehrlich ascites cells	35
6-Azauridine	Ehrlich ascites cells	2, 29, 35
	Mouse liver	40
	L5178Y cells	40
	Sarcoma S-180 cells	40
Cytidine	Ehrlich ascites cells	2, 35, 41
	Rat liver	37, 42
	Rat brain	27

Table 1 (*continued*). Substrate specificity of uridine phosphorylase

Compound*	Enzyme source	Ref.
2',3'-Dehydro-5-fluoro-2'-deoxyuridine	Ehrlich ascites cells	43
2'-Deoxyallosylthymine	Ehrlich ascites cells	44
2'-Deoxycytidine	Ehrlich ascites cells	2
	Rat liver	37
2'-Deoxyglucosylthymine	Ehrlich ascites cells	44, 45
2'-Deoxyxylofuranosyl-5-fluorouracil	Ehrlich ascites cells	3
2'-Deoxyribopyranosylthymine	Ehrlich ascites cells	45
2'-Deoxyxylopyranosylthymine	Ehrlich ascites cells	45
3',5'-Diacetyl-5-fluoro-2'-deoxyuridine	Ehrlich ascites cells	3
3'-4-Di-*O*-methyl-5-fluoro-2'-deoxyuridine	Ehrlich ascites cells	46
Digitoxosylthymine	Ehrlich ascites cells	44
5-Ethyl-2'-deoxyuridine	PRK cells	26
α-5-Fluoro-2'-deoxyuridine	Ehrlich ascites cells	3
5-Fluoro-2'-fluoro-2'-deoxyuridine	Ehrlich ascites cells	33
Glucopyranosyl-5-bromouracil	Rat liver	38
Glucopyranosyl thymine	Rat liver	38
Glucopyranosyl uracil	Rat liver	38
5-Methyl-2'-deoxycytidine	Ehrlich ascites cells	2
3'-*O*-Methyl-5-fluoro-2'-deoxyuridine	Ehrlich ascites cells	46
4-*O*-Methyl-5-fluoro-2'-deoxyuridine	Ehrlich ascites cells	46
5'-*O*-Methyl-5-fluorodeoxyuridine	Ehrlich ascites cells	46
3'-*O*-Methyluridine	PRK cells	26
5'-*O*-Methyluridine	PRK cells	26
3'-Monoacetyl-5-fluoro-2'-deoxyuridine	Ehrlich ascites cells	3
Orotidine	Ehrlich ascites cells	2, 41
	L5178Y cells	38
	Sarcoma S-180 cells	40
	Mouse liver	40
5-Propynyloxy-2'-deoxyuridine	PRK cells	26
Pseudouridine	PRK cells	26
Showdomycin	Ehrlich ascites cells	23
Tetrahydropyranosyl thymine	Ehrlich ascites cells	45
Thymidine	PRK cells	26
Xylopyranosyl-5-bromouracil	Rat liver	38
Xylopyranosyl thymine	Rat liver	38
Xylopyranosyl uracil	Rat liver	38
Non-substrates for nucleoside synthesis with ribose-1-phosphate as the pentose donor		
5-Azauracil	Mouse liver	47
Uric acid	Beef erythrocytes	34
	Rat small intestine	34

* Unless indicated otherwise, all nucleosides and nucleoside analogs have the β-D-configuration.

Table 2. Substrate specificity of thymidine phosphorylase

Compound*	Enzyme source	Ref.
Substrates for nucleoside cleavage		
5-Allyl-2'-deoxyuridine	Horse liver	5
5-Bromo-2'-deoxyuridine	Human spleen	4
	Horse liver	5
5-Chloro-2'-deoxyuridine	Horse liver	5
5-Cyano-2'-deoxyuridine	Horse liver	5
2'-Deoxyuridine	Mouse liver	1
	Horse liver	5
	Human platelet	25

Table 2 (*continued*). Substrate specificity of thymidine phosphorylase

Compound*	Enzyme source	Ref.
5-Ethyl-2′-deoxyuridine	Horse liver	5
5-Fluoro-2′-deoxyuridine	Human spleen	4
	Horse liver	5
5-Formyl-2′-deoxyuridine	Horse liver	5
5-Hydroxymethyl-2′-deoxyuridine	Horse liver	5
5-Iodo-2′-deoxyuridine	Human spleen	4
	Horse liver	5
N-3-Methyl-2′-thymidine	Horse liver	48
5-Nitro-2′-deoxyuridine	Horse liver	30
5-Propenyl-2′-deoxyuridine	Horse liver	5
Thymidine	Human spleen	4
	Mouse liver	1, 10
	Horse liver	5
	Human platelet	25
5-Trifluoromethyl-2′-deoxyuridine	Horse liver	5
Substrate for nucleoside synthesis with 2-deoxyribose-1-phosphate as the pentose donor		
5-Aminouracil	Horse liver	49
5-Bromo-2-thiouracil	Horse liver	49
5-Bromouracil	Horse liver	49
5-Chlorouracil	Horse liver	49
8-Chloroxanthine	Human spleen	50
5-Iodo-2-thiouracil	Horse liver	49
5-Nitrouracil	Horse liver	49
2-Thiouracil	Horse liver	49
6-Thioxanthine	Human spleen	50
Thymine	Horse liver	49
	Human spleen	51
Uracil	Horse liver	49
Xanthine	Human spleen	50, 51
Non-substrate for nucleoside cleavage		
Acyclothymidine	Mouse liver	10
Acyclouridine	Mouse liver	10
Arabinopyranosyl-5-bromouracil	Rat liver	38
Arabinopyranosyl thymine	Rat liver	38
Arabinopyranosyl uracil	Rat liver	38
Arabinosyl thymine	Human spleen	4
	Hamster liver	39
Arabinosyl uracil	Human spleen	4
6-Azauridine	Mouse liver	40
Cytidine	Rat liver	37
	Human platelet	25
2′-Deoxycytidine	Rat liver	37
	Human platelet	25
Glucopyranosyl-5-bromouracil	Rat liver	38
Glucopyranosyl thymine	Rat liver	38
Glucopyranosyl uracil	Rat liver	38
Uric acid riboside	Mouse liver	52
Uridine	Mouse liver	1, 10
	Human platelet	25
Xylopyranosyl-5-bromouracil	Rat liver	38
Xylopyranosyl thymine	Rat liver	38
Xylopyranosyl uracil	Rat liver	38
Non-substrates for nucleoside synthesis with 2-deoxyribose-1-phosphate as the pentose donor		
Adenine	Human spleen	50
Guanine	Human spleen	50
Hypoxanthine	Human spleen	50, 51
Orotic acid	Horse liver	49
	Human spleen	50
2-Thioxanthine	Human spleen	50
Uric acid	Human spleen	50

* All nucleosides and nucleoside analogs have the β-D-configuration.

Table 3. Potent inhibitors of the pyrimidine nucleoside phosphorylases

Compound	Enzyme source	K_i (μM)	Inhibition			Ref.
			Substrate (mM)	Inhibitor (μM)	% Inhibition	
Uridine phosphorylase						
Acyclothymidine	Sarcoma S-180 cells	3.0				10
Acyclouridine	Sarcoma S-180 cells	15.0				10
5-*m*-Benzyloxybenzylacyclouridine	Sarcoma S-180 cells	0.032				6
5-*m*-Benzyloxybenzyluracil	Walker 256 carcinoma		FdUrd, 0.4	0.5	50	13
	Sarcoma S-180 cells	0.270				6
	Ehrlich ascites cells		FdUrd, 0.4	1.6	50	9
	Novikoff hepatoma		FdUrd, 0.4	1.0	50	9
5-Benzylacyclouridine	Sarcoma S-180 cells	0.098				13
5-Benzyluracil	Walker 256 carcinoma		FdUrd, 0.4	5.3	50	13
	Sarcoma S-180 cells	1.575				6
	Ehrlich ascites cells		FdUrd, 0.4	9.0	50	9
	Novikoff hepatoma		FdUrd, 0.4	10.0	50	9
2'-Deoxyglucosylthymine	Ehrlich ascites cells	40.0				11
	Sarcoma S-180 cells		Urd, 0.15	15.0	50	10
	Ehrlich ascites cells		FdUrd, 0.4	23.0	50	9
	Novikoff hepatoma		FdUrd, 0.4	24.0	50	9
1-*m*-Methoxybenzyluracil	Walker 256 carcinoma		FdUrd, 0.4	1.9	50	13
5-Nitrouracil	Walker 256 carcinoma		FdUrd, 0.4	15.0	50	15
Showdomycin	Ehrlich ascites cells		Urd, 1.0	1200.0*	50	23
	Ehrlich ascites cells		Urd, 1.0	1200.0†	100	23
Thymidine phosphorylase						
6-Amino-5-bromouracil	Horse liver		dThd, 1.0	30.0	50	12
	HeLa cells		FdUrd, 0.4	46.0	50	9
6-Amino-5-iodouracil	Horse liver		dThd, 1.0	40	50	12
6-Amino-5-fluorouracil	Horse liver		dThd, 1.0	370	50	12
6-Aminothymine	Horse liver		dThd, 1.0	70	50	12
5-Nitrouracil	Rabbit liver		FdUrd, 0.4	43	50	14
5-Bromo-6-benzylethylaminouracil	Rabbit liver		FdUrd, 0.4	25	50	14

* No preincubation.
† Inhibitor was preincubated for 10 min with enzyme.

Table 4. Apparent K_i (appK_i) values for inhibition of UrdPase and dThdPase by pyrimidines and pyrimidine analog

	Compound	Source	UrdPase appK_i (μM)	dThdPase appK_i (μM)
I	Uracil	SIGMA	193 ± 7	925 ± 42
1-Position				
II	1-Benzyluracil	VEGA	26 ± 4	†
III	1-Cyclohexylthymine	VEGA	*	*
IV	1-Cyclohexyluracil	VEGA	*	*
V	1-Ethyluracil	VEGA	372 ± 54	†
VI	1-Methyluracil	VEGA	436 ± 5	†
VII	2,6-Pyridinediol (1-deazauracil)	ALD	3.4 ± 0.4	433 ± 77
2-Position				
VIII	2-Thiouracil	SIGMA	592 ± 137	†
3-Position				
IX	3-Deazauracil	SIGMA	†	†
X	3-Methyluracil	SIGMA	†	†
XI	3-Oxauracil	SIGMA	1686 ± 119	†
4-Position				
XII	Cytosine	SIGMA	†	†
XIII	4-Thiouracil	SYN	83 ± 25	†
5-Position				
XIV	5-Aminouracil	SIGMA	1100 ± 392	†
XV	5-Azauracil	SIGMA	1312 ± 221	1003 ± 100
XVI	5-Benzyloxybenzyluracil	SYN	1.5 ± 0.3	‡
XVII	5-Benzyluracil	SYN	3.7 ± 0.4	*
XVIII	5-Bromouracil	SIGMA	9 ± 1	33 ± 9
XIX	5-Chlorouracil	SIGMA	11 ± 2	36 ± 8
XX	5-Flourouracil	SIGMA	59 ± 6	421 ± 123
XXI	5-Hydroxymethyluracil	SIGMA	1217 ± 174	†
XXII	5-Iodouracil	SIGMA	21 ± 3	55 ± 14
XXIII	5-Nitrouracil	SIGMA	1.7 ± 0.3	73 ± 7
XXIV	Thymine	SIGMA	30 ± 6	347 ± 91
6-Position				
XXV	6-Aminouracil	SIGMA	235 ± 77	275 ± 25
XXVI	6-Azathymine	SIGMA	†	†
XXVII	6-Azauracil	SIGMA	†	†
XXVIII	Barbituric acid	SIGMA	1360 ± 127	†
XXIX	6-Benzyluracil	SYN	*	*
XXX	6-Methyluracil	SIGMA	1169 ± 42	6388 ± 2698
XXXI	Orotic acid	SIGMA	†	†
More than one position substituted				
XXXII	6-Benzyl-2-thiouracil	SIGMA	†	181 ± 95
XXXIII	5-Bromo-1-methyluracil	SIGMA	1146 ± 239	6401 ± 2914
XXXIV	5,6-Diaminouracil	SIGMA	214 ± 23	2251 ± 289
XXXV	2,4-Dithiouracil	SIGMA	748 ± 109	†
XXXVI	1-Ethylthymine	VEGA	157 ± 43	†
XXXVII	1-Methylthymine	VEGA	371 ± 36	†
XXXVIII	Thymine-propenal	APG	969 ± 83	†
XXXIX	6-*n*-Propyl-2-thiouracil	SIGMA	273 ± 19	†
XL	4-Thiothymine	VEGA	49 ± 4	†
XLI	Uramil	SIGMA	†	†
Nucleosides and nucleoside analogs				
XLII	Acyclocytidine	SYN	†	†
XLIII	Acyclothymidine	SYN	15 ± 2	†
XLIV	Acyclouridine	SYN	51 ± 1	†
XLV	5′-Amino-5′-deoxythymidine	CAL	†	†
XLVI	Arabinosyl thymine	CAL	†	†
XLVII	Arabinosyl uracil	CAL	†	†
XLVIII	6-Aza-3-deazacytidine	LBT	†	†
XLIX	6-Aza-3-deazauridine	LBT	†	†
L	6-Azauridine	SIGMA	†	†

Table 4 (*continued*). Apparent K_i (appK_i) values for inhibition of UrdPase and dThdPase by pyrimidines and pyrimidine analogs

	Compound	Source	UrdPase appK_i (μM)	dThdPase appK_i (μM)
LI	Barbituric acid riboside	SIGMA	226 ± 14	*
LII	5-Benzylacyclocytidine	SYN	64 ± 6	*
LIII	5-Benzylacyclouridine	SYN	1.0 ± 0.3	*
LIV	5-Benzyloxybenzylacyclocytidine	SYN	§	‡
LV	5-Benzyloxybenzylacyclouridine	SYN	0.17 ± 0.03	‡
LVI	5-Benzyl-2-thioacyclouridine	SYN	54 ± 6	†
LVII	5-Bromoacyclouridine	SYN	42 ± 6	†
LVIII	Cytidine	SIGMA	†	†
LIX	3-Deazauridine	SIGMA	†	†
LX	3′-Deoxy-3′-aminothymidine	CAL	†	†
LXI	2′-Deoxyglucosylthymine	NCI	12 ± 2	931 ± 129
LXII	2′-Deoxyuridine	SIGMA	1720 ± 316	†
LXIII	5′-Deoxyoxazinomycin	RKR	†	†
LXIV	5-Fluoroacyclouridine	SYN	44 ± 7	†
LXV	2′-Fluoro-arabinosylcytosine	JJF	†	†
LXVI	2′-Fluoro-arabinosyl-5-fluorouracil	JJF	†	†
LXVII	5-Fluoro-2′-deoxyuridine	SIGMA	995 ± 38	714 ± 120
LXVIII	5-Fluorouridine	CAL	197 ± 98	†
LXIX	2′-Fluoro-arabinosyl-5-methyluracil	JJF	†	†
LXX	5′-Iodo-5′-deoxyoxazinomycin	RKR	†	†
LXXI	3-Methyluridine	SIGMA	†	†
LXXII	5-Methyluridine	PL	43.3 ± 0.4	†
LXXIII	Orotidine	CAL	†	†
LXXIV	Pseudouridine	SIGMA	†	†
LXXV	2-Selenocytidine	SYN	†	†
LXXVI	4-Selenouridine	SYN	1226 ± 279	†
LXXVII	Showdomycin	LBT	154 ± 17	†
LXXVIII	4-Thiouridine	VEGA	89 ± 5	†
LXXIX	Thymidine	SIGMA	204 ± 13	323 ± 64
LXXX	Uridine	SIGMA	229 ± 13	†
Miscellaneous				
LXXXI	Dihydrothymine	SIGMA	†	†
LXXXII	Dihydrouracil	SIGMA	†	†
LXXXIII	Hydantoin	SIGMA	†	†
LXXXIV	2-Hydroxypyrimidine	ALD	†	†
LXXXV	4-Hydroxypyrimidine	ALD	†	†
LXXXVI	Pentobarbital	K&K	†	†
LXXXVII	Phenobarbital	MAL	†	†

* Less than 5% inhibition of substrate cleavage at the maximal inhibitor concentration of 0.09 mM.
† Less than 5% inhibition of substrate cleavage at the maximal inhibitor concentration of 0.90 mM.
‡ Less than 5% inhibition of substrate cleavage at the maximal inhibitor concentration of 0.009 mM.
§ A reliable appK_i value for 5-benzyloxybenzylacyclocytidine could not be obtained because precipitates of this poorly water-soluble compound formed at inhibitory concentrations. However, some inhibition (approximately 40%) of uridine cleavage was detected with concentrations of 5-benzyloxybenzylacyclocytidine in the range of 0.045 to 0.090 mM, indicating that this compound can bind to UrdPase.

(LXXXVII) did not inhibit UrdPase whereas the unsubstituted analog barbituric acid (XXVIII) did. We propose the lack of UrdPase inhibition by LXXXVI and LXXXVII to be due to the presence of the non-coplanar groups substituted at the 5-position of these compounds which, like the phenyl group of 5-phenyluracil, sterically preclude binding to UrdPase. Potvin *et al.* [53] have similarly reported poor binding of other 5,5-disubstituted barbituric acids to UrdPase.

Present studies, in agreement with previous reports [9, 13, 54], indicate that both 1-benzyluracil (II) and 5-benzyluracil (XVII) bind well to UrdPase. In an attempt to produce a more potent inhibitor of UrdPase, Baker and Kelley [13] synthesized and evaluated 1,5-dibenzyluracil. However, this compound was only 2-fold more potent than 5-benzyluracil [13]. Since a much greater enhancement of UrdPase inhibition would be expected if there were separate binding sites for each of the benzyl groups of 1,5-dibenzyluracil, it was concluded that a single hydrophobic region exists on UrdPase adjacent to the point of binding of *C*-5 or uracil [13]. Thus, the inhibitory potency of 1-benzyluracil may be

explained by the binding of this compound to UrdPase predominantly in an orientation where the pyrimidine base is rotated 180° about the *N*3-*C*6 axis so that the 1-benzyl moiety is interacting with the hydrophobic pocket [13].

We tested 1-cyclohexylthymine (III) and 1-cyclohexyluracil (IV) because it was conceivable that these compounds might bind to UrdPase in a rotated conformation as does 1-benzyluracil. Also, certain pyrimidine glucopyranosides [most notably 2′-deoxyglucosylthymine (LXI) and 2′-deoxyglucosyluracil] are known to inhibit UrdPase [9–11, 43, 44, 45, 55], and it was thought that the 1-cyclohexyl side chains of III and IV might have sufficient structural resemblance to a glucopyranose to bind to the enzyme. However, neither III nor IV inhibited UrdPase. In retrospect, this is not surprising since a requirement for the binding of nucleoside analogs to UrdPase is a hydroxyl group in the proper orientation to mimic the 3′-hydroxyl group of the substrate uridine (see below) [10, 45]. In addition, the steric limitations of the hydrophobic region on UrdPase probably preclude the binding of the non-planar cyclohexyl moieties of III and IV.

Several 1-alkyl substituted uracils were screened as inhibitors of UrdPase. 1-Methyluracil (VI) bound only half as well as uracil (I), and a slight increase in binding was observed when the chain length was extended by one carbon to give 1-ethyluracil (V). Similarly, 1-methylthymine (XXXVII) bound only about one-twelfth as well as thymine (XXIV), but 1-ethylthymine (XXXVI) bound 2-fold better than XXXVII. Baker and Kelley [54] have observed that elongation of the carbon chain of 1-alkyl uracils enhances binding to UrdPase presumably by interaction of the rotated pyrimidine with the hydrophobic region [13], and this might explain the enhanced potency of V over VI and XXXVI over XXXVII.

Effect of electron-withdrawing groups at the 5-position of uracil analogs. Various electron-withdrawing groups (i.e. a nitro-group or halogens), substituted at the 5-position of uracil, enhanced the binding of these analogs to UrdPase (see Table 4). Similar findings have been reported by Baker and Kelley [13]. Since such electron-withdrawing substituents enhanced the acidity of these compounds, it is likely that the dissociated anionic forms bound more tightly to UrdPase than the uncharged species. The pK_a values of selected uracil analogs, together with the percent ionization of each under our assay conditions, are presented in Table 5. The electronegativity of the 5-substituent (and thus acidity of the uracil), however, does not appear to have been the only factor which influenced the binding of these analogs to UrdPase because the order of electronegativity of substituents (i.e. nitro- > fluoro- > chloro- > bromo- > iodo- > hydrogen) [56] does not correspond exactly with the observed potency of UrdPase inhibition, i.e. 5-nitrouracil (XXIII) > 5-bromouracil (XVIII) > 5-chlorouracil (XIX) > 5-iodouracil (XXII) > 5-fluorouracil (XX) > uracil (I). One factor which may play an important role is the hydrophobicity of the 5-substituent. There is considerable variation in the hydrophobic character of the different halogens, and this has been approximated by Fujita *et al.* [58] by the logarithmic measure of hydrophobicity (Table 5). Thus, the enhanced binding of 5-bromouracil (XVIII, $\pi = 0.86$) over 5-chlorouracil (XIX, $\pi = 0.71$) and especially over 5-fluorouracil (XX, $\pi = 0.14$) may be explained on the basis of stronger interaction of the bromine substituent of XVIII with the hydrophobic pocket on UrdPase.

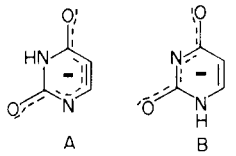
We propose that the electron-withdrawing properties of small 5-position substituents of uracils have a greater effect on binding than hydrophobic character. 5-Iodouracil (XXII) bound only about one-half as well to UrdPase as 5-chlorouracil (XIX) and 5-bromouracil (XVIII) despite the larger π value of an iodo-group relative to chloro- or bromo-groups (Table 5). Since XIII would be expected to bind more tightly than XVIII and XIX, if interaction with

Table 5. Physical properties of selected uracil analogs

Compound	pK_a*	% Ionized at pH 8.0	π Value for 5-position substituent†
5-Aminouracil			−1.23
5-Bromo-1-methyluracil	7.84	59	0.86
5-Bromouracil	8.05	47	0.86
5-Chlorouracil	7.95	53	0.71
3-Deazauracil	6.50	97	0
5-Fluorouracil	7.98	51	0.14
5-Hydroxymethyluracil			−1.03
5-Iodouracil	8.25	36	>1
1-Methyluracil	9.75	2	0
3-Methyluracil	9.95	1	0
5-Nitrouracil	5.30	100	−0.28
2-Thiouracil	7.65	69	0
Thymine	9.90	1	0.56
Uracil	9.50	3	0

* pK_a values are taken from Ref. 57.

† π Values are a logarithmic measure of hydrophobicity calculated from 1-octanol-water partition coefficients of substituted benzenes by Fujita *et al.* [58].



A B

Fig. 1. Tautomeric forms of uracil mono-anion.

the hydrophobic region were of prime importance, we attribute the relatively poor binding of XXII to the fact that this compound was less ionized than XVIII or XIX under our assay conditions (see Table 5). Also consistent with this hypothesis is our finding that 5-nitrouracil (XXIII) bound very well to UrdPase despite the very polar nature of the nitro-substituent ($\pi = 0.28$).

Binding of mono-anionic uracil analogs to UrdPase. As discussed above, many uracil analogs which exist to a great extent in solution as mono-anions [e.g. 5-bromouracil (XVIII), 5-nitrouracil (XXIII), etc.] bind better to UrdPase than uncharged compounds such as uracil (I). It has been shown that the mono-anionic species of uracil and many uracil analogs in solution are mixtures of tautomers with either the *N*-1 hydrogen or the *N*-3 hydrogen dissociated [59–63]. In general, the negative charge of uracil mono-anions does not remain localized; rather, it is spread over the π-electron system of the pyrimidine ring, and the pattern of dispersion differs depending upon which hydrogen (*N*-1 or *N*-3) is dissociated (Fig. 1). As will be discussed below, our studies indicate that the position at which ionization occurs is an important factor in the binding of these compounds to UrdPase.

We hypothesize that the mono-anionic tautomers of these compounds which bind preferentially, if not exclusively, to UrdPase are those with the *N*-1 hydrogen rather than the *N*-3 hydrogen dissociated. 1-Methyluracil (VI) bound 2-fold less tightly to UrdPase than uracil (I). Since both uracil and 1-methyluracil were largely unionized under our assay conditions (Table 5), it is unlikely that any shift in ionization caused this difference. Therefore, the small decrease in affinity of 1-methyluracil as compared to uracil might have been due to a slight steric effect of the 1-methyl group. However, the introduction of a methyl group at the 1-position of 5-bromouracil [i.e. 5-bromo-1-methyluracil (XXXIII)] resulted in a 127-fold decrease in binding relative to 5-bromouracil (XVIII). The magnitude of this change in affinity obviously cannot be explained by steric factors alone. Unlike uracil and 1-methyluracil, 5-bromouracil and 5-bromo-1-methyluracil were both significantly ionized (approximately 50%) under our assay conditions (Table 5). Additionally, only the *N*-3 hydrogen could dissociate from 5-bromo-1-methyluracil, whereas 5-bromouracil (like most other 5-halogenated uracils) ionizes predominately through dissociation of the *N*-1 hydrogen [59, 60, 62, 63]. We propose, therefore, that the strong UrdPase inhibition produced by 5-bromouracil (and other anionic uracils not substituted at the 1-position) was largely due to the tight-binding of charged species formed by ionization at the 1-position.

Consistent with the foregoing proposal, Baker and Kelley [13] have shown that substitution of the potent UrdPase inhibitor 5-nitrouracil with a methyl group at the 1-position (i.e. 5-nitro-1-methyluracil) abolishes binding to UrdPase [13]. 5-Nitrouracil is highly acidic (Table 5) and, although no pK_a value is presently available for 5-nitro-1-methyluracil, it is reasonable to assume that this compound is also very acidic because of the powerful electron-withdrawing properties of the 5-position substituent [56]. However, 5-nitrouracil ionizes exclusively by dissociation of the *N*-1 hydrogen [60], whereas only the *N*-3 hydrogen can dissociate from 5-nitro-1-methyluracil. Employing the same reasoning presented above to explain the poor binding to UrdPase by 5-bromo-1-methyluracil, relative to 5-bromouracil, we propose that the loss of UrdPase inhibition which occurs when a methyl group is introduced at the 1-position of 5-nitrouracil may be attributed, at least in part, to a shift in the locus of ionization from *N*-1 to *N*-3. Another factor which probably contributes to this observation is the poor interaction of the hydrophilic 5-nitro group (Table 5) with the hydrophobic region.

Hydrogen-bonding to UrdPase by the N-3 *hydrogen.* We propose that an undissociated *N*-3 hydrogen greatly enhances the binding of uracil analogs to UrdPase, possibly by participating in hydrogen-bonding to the enzyme. 3-Oxauracil (XI) lacks a hydrogen analogous to the *N*-3 hydrogen of uracil, and presumably it is this property which is responsible for the poor binding of this compound to UrdPase.

Another line of evidence in support of the foregoing hypothesis comes from the studies with various acidic 1-substituted uracils. Substitution of the 1-position of 5-bromouracil (XVIII) shifts the locus of ionization from the 1- to the 3-position of 5-bromo-1-methyluracil (XXXIII) and 5-bromoacyclouridine (LVII), and certainly (by evidence provided above) the lack of an anionic charge resulting from dissociation of the *N*-1 hydrogen is partially responsible for the poor binding relative to XVIII. However, 5-bromo-1-methyluracil (XXXIII) bound 3-fold less tightly to UrdPase than 1-methylthymine (XXXVII) and, similarly, 5-bromoacyclouridine (LVII) bound 13-fold less tightly to UrdPase than acyclothymidine (XLIII), despite the greater hydrophobicity in each case of the 5-bromo substituent relative to the 5-methyl substituent (Table 5). Therefore, it appears that another important factor involved in the poor binding to UrdPase of 5-bromo-1-methyluracil (XXXIII) and 5-bromoacyclouridine (LVII) relative to 5-bromouracil (XVIII) was the fact that the ionized species of these compounds do not possess an *N*-3 hydrogen capable of participating in hydrogen-bonding. Thus, the inhibition of UrdPase produced by XXXIII and LVII may have been largely or entirely due to the binding of the unionized fraction of these compounds in which a hydrogen bond is involved.

Also consistent with the involvement of the *N*-3 hydrogen of uracil analogs in hydrogen-bonding to UrdPase are the findings that neither 3-methyluracil (X) nor 3-methyluridine (LXXI) inhibited this enzyme. While it is possible that steric factors preclude the binding of *N*-3 methylated uracils, these

non-inhibitory compounds also lack an *N*-3 hydrogen for hydrogen bonding.

Participation of the 2- *and* 4-*position oxo-groups in binding to UrdPase.* Prior to a discussion of the participation of the oxo-groups of uracil in binding to UrdPase, some clarification is appropriate concerning the tautomerism of uracil and derivatives thereof. The 2- and 4-position oxo-groups of uracil can theoretically undergo lactam–lactim tautomerization and, thereby, six tautomeric forms are possible. However, as reviewed by Kwiatkowski and Pullman [62], the monolactim and dilactim tautomeric forms of uracil are not detectable in solution at any pH. The same holds true for 1- and/or 3-position alkyl-substituted uracils [59, 60, 62], uracils substituted at the 5- and/or 6-positions [62], uridine [62], thymidine [62], analog uracil nucleosides [62], 2,4-dithiouracil [64], 4-thiouridine [65], and the neutral species of 2-thiouracil [64]. Although uracil and uracil analogs exist predominantly, if not exclusively, as dilactam tautomers in solution, the possibility that monolactim or dilactim tautomers of these compounds (undetectable by current techniques) bind well to uridine phosphorylase cannot be excluded at the present time.

2-Thiouracil (VIII) bound 3-fold less tightly to UrdPase than uracil (I). This observation indicates that the 2-position oxo-group of uracil is required for binding to the enzyme. The diminished binding of VIII relative to I may reflect a lack of steric tolerance of the enzyme for the 2-thione group which has a larger van der Waals radius than an oxo-group. Also, thione groups do not participate as strongly as do oxo-groups in hydrogen-bonding [66], and this fact may explain the foregoing findings if hydrogen-bonding to UrdPase does occur through the 2-position oxo-group of uracil. Additionally, it should be noted that the mono-anion of 2-thiouracil, which was the predominant species under our assay conditions (Table 5), is unique in that the negative charge is localized on the exocyclic 4-position oxygen [67], rather than delocalized as in Fig. 1. Thus, another possible basis for the poorer binding of 2-thiouracil (VIII) to UrdPase may have been a lack of tolerance for an anionic charge on the 4-position oxygen. Also consistent with the notion that optimal binding to UrdPase is achieved with 2-oxo rather than 2-thio uracils is the finding that 5-benzyl-2-thioacyclouridine (LVI) bound 50-fold less tightly to this enzyme than did 5-benzylacyclouridine (LIII).

On the other hand, it appears that the region on UrdPase which binds the 4-position oxo-group of uracil is somewhat non-specific. 4-Thiothymine (XL) bound almost as well to the enzyme as thymine (XXIV), and the same was true of 4-thiouracil (XIII) as compared to uracil (I). Likewise, 4-thiouridine (LXXVIII) bound almost 3-fold better than uridine (LXXX). It is also of interest that the large 4-position selenium group of 4-selenouridine (LXXVI) did not preclude binding. However, it should also be mentioned that the substitution of thione groups for oxo-groups on uracil generally increases the acidity of such compounds, and that the observed binding may be the result of two opposing forces, namely, enhancement of binding by facilitation of the dissociation of the *N*-1 hydrogen versus lack of steric tolerance or the weak hydrogen-bonding properties of the 4-position thione group.

Using a different series of uracil analogs, Baker and Kelley [13] have drawn similar conclusions regarding the participation of the 2- and 4-position oxo-groups in binding to UrdPase.

Non-inhibition of UrdPase by cytidine and cytosine. Our studies show that neither cytidine (LVIII) nor cytosine (XII) inhibited the phosphorolysis of uridine by UrdPase. Although the binding site on UrdPase which accepts the 4-position oxo-group is at least somewhat non-specific (see above), it is possible that the larger amino group at the 4-position of XII and LVIII may sterically hinder binding. Also, both cytosine (XII) and cytidine (LVIII) in solution exist exclusively as the amino (rather than imino) tautomer at the 4-position [59, 62, 68]; thus, the lack of an *N*-3 hydrogen for hydrogen-bonding (see above) may also be an important factor in the non-inhibition of UrdPase by XII and LVIII.

Interestingly, the cytidine analogs 5-benzylacyclocytidine (LII) and 5-benzyloxybenzylacyclocytidine (LIV) inhibited UrdPase, although the apparent K_i values for these compounds were at least an order of magnitude higher than those for the corresponding uridine analogs 5-benzylacyclouridine (LIII) and 5-benzyloxybenzylacyclouridine (LV). These findings cannot be the result of enzymatic deamination of LII and LIV to LIII and LV, respectively, since our UrdPase source (S-180 cells) has been shown to be devoid of cytidine deaminase [22]. The fact that the cytidine analogs LII and LIV do bind to UrdPase underscores the lack of absolute specificity of the enzyme in the region of the enzyme which accepts the 4-position oxo-group of uracil (see above). We attribute the UrdPase binding to strong interaction of the 5-position hydrophobic groups of these compounds with the hydrophobic region and hydrogen-bonding by the terminal hydroxyl group of the 2′-hydroxyethoxymethyl side chains of LII and LIV; evidently, these binding forces are sufficient to overcome the forces which preclude the binding of cytosine (XII) and cytidine (LVIII).

Non-inhibition of UrdPase by 3-*deazauracil and* 3-*deazauridine.* It should be noted that 3-deazauracil (IX) and 3-deazauridine (LIX) are pyridine rather than pyrimidine analogs in spite of their misleading "common names". Unlike uracils which have the 2,4-diketo structure (see above), IX [69] and LIX [70] undergo keto–enol tautomerization at the 4-position. Unfortunately, the relative proportions of the keto and enol tautomers of these compounds in solution are not presently known. The failure of 3-deazauracil (IX) and 3-deazauridine (LIX) to inhibit UrdPase may have been due to one or more of the following: (a) lack of tolerance for the hydroxyl group of the enol tautomer; (b) lack of a hydrogen at the 3-position available for participation in hydrogen-bonding; or (c) lack of tolerance for an endocyclic methylene group (in the case of the keto tautomer) or —CH= group (in the case of the enol tautomer) at the 3-position.

Binding of barbituric acid, barbituric acid riboside, and other 6-*substituted pyrimidine analogs to UrdPase.* Barbituric acid (XXVIII) and barbituric acid riboside (LI) each bound weakly to UrdPase relative

to uracil. Consistent with these results, Potvin *et al.* [53] have estimated a K_i value of 0.8 mM for barbituric acid as an inhibitor of rat brain UrdPase. Relevant points concerning the tautomerism of barbituric acids are essential in evaluating these findings. Uncharged barbituric acid [71] and barbituric acid riboside [72] both have been clearly shown to exist as the 2,4,6-triketo tautomers. Under our assay conditions, however, the mono-anions of XXVIII and LI are the only species present [71, 73]. Evidence presently available is inconclusive [71], but it appears that the anions of barbituric acid and (presumably) barbituric acid riboside are formed via loss of a proton from the 5-position methylene group with subsequent enolization involving the 6-position oxo-group [73, 74]. Localization of the resulting negative charge occurs on the exocyclic 6-position oxygen [71, 73], and the lack of tolerance by UrdPase for such a group at the 6-position of uracils presumably is the basis of the poor binding of barbituric acid (XXVIII) and barbituric acid riboside (LI). Of course, a clearer understanding of the binding of barbituric acid mono-anions to UrdPase must await more definitive studies on the tautomerism of these compounds.

Of the remaining 6-position substituted pyrimidine analogs tested as inhibitors of UrdPase, only 6-aminouracil (XXV) produced noteworthy inhibition; 6-methyluracil (XXX) bound weakly; and 6-azathymine (XXVI), 6-azauracil (XXVII), 6-benzyluracil (XXIX), orotic acid (XXXI), 6-azauridine (L) and orotidine (LXXIII) did not bind at all. Thus, UrdPase is highly specific for uracil in the region adjacent to the point of binding of the 6-position of this compound.

Inhibition of UrdPase by 2,6-pyridinediol. We found that 2,6-pyridinediol (VII) inhibited UrdPase 57-fold better than uracil (I). 2,6-Pyridinediol (VII) has been shown to exist in solution as 1-deazauracil and the mono–keto tautomer thereof in a 1:4 molar ratio [75]. We propose that it is the 1-deazauracil tautomer which binds to UrdPase, because it possesses the necessary functional groups analogous to the 2-position oxo-group and *N*-3 hydrogen of uracil. The factors responsible for the strong binding to UrdPase of 2,6-pyridinediol (VII) relative to uracil (I) are not presently understood.

Binding of thiotropic analogs to UrdPase. Showdomycin (LXXVII), a C-nucleoside derivative of maleimide, has been shown to inhibit UrdPase from Ehrlich ascites cells [23]. This effect is reportedly enhanced by preincubation of showdomycin with enzyme [23], and thus it has been speculated that covalent binding to a thiol group in the active site may occur by reaction with the maleimide moiety (a known thiotropic group) [76]. Our findings do not support this proposal, because the relatively weak inhibition produced by showdomycin was unchanged by preincubation and was lost after dialysis.

The propenal moiety of thymine-propenal (XXXVIII) is highly reactive and will readily alkylate thiol groups causing the release of thymine [22]. We thought that XXXVIII might bind to UrdPase and irreversibly inhibit this enzyme by alkylating an essential thiol group in the active site; however, our results show that this does not occur. Since uracils substituted at the 1- or 3-positions with alkyl groups generally do not bind well to UrdPase (see above), we suspect that likewise XXXVIII does not bind to the enzyme. The observed weak inhibition of UrdPase by XXXVIII may have been an artifact brought about by thymine formed from the propenal derivatives after non-specific reaction with thiol groups from our enzyme preparation.

Binding of miscellaneous compounds to UrdPase. Previous studies have shown that several compounds which are not pyrimidine analogs bind weakly to UrdPase. Specifically, sulfinpyrazone, colchicine, and phenylbutazone all have been shown to inhibit UrdPase competitively ($K_i > 1$ mM) [34]. The nature of the binding of these compounds to the active site of UrdPase is anomalous since they bear no structural resemblance to uracil; however, it is possible that the large hydrophobic substituent groups (e.g. benzene rings, etc.) of sulfinpyrazone, colchicine, and phenylbutazone may interact with the hydrophobic region.

Several purines have been shown to bind very weakly to UrdPase, most notably, 2-thio-6-oxypurine [34]. 3-Ribosyl uric acid has been reported to be cleaved by an intestinal phosphorylase [34] later proven to be UrdPase [52]. Also, UrdPase-catalyzed synthesis of 7-ribosyloxypurinol from oxypurinol and ribose-1-phosphate has been demonstrated [36]. The pertinent chemical feature common to all of these purines is that they may be regarded as 5,6-substituted uracils or uridines [36]. Thus, the binding of these compounds to the active site of UrdPase may be rationalized on the basis of interaction with the binding sites (discussed above) for the oxo-groups and *N*-3 hydrogen of uracil or uridine.

Binding of nucleoside analogs to UrdPase. In general, UrdPase has strict structural and conformational requirements for binding with regard to the sugar portion of nucleoside analogs.

2′-Deoxyribonucleosides, i.e. 2′-deoxyuridine (LXII), thymidine (LXXIX), and 5-fluoro-2′-deoxyuridine (LXVII), bound 4- to 8-fold less tightly to UrdPase than the corresponding ribonucleosides, i.e. uridine (LXXXI), 5-methyluridine (LXXII), and 5-fluorouridine (LXVIII) respectively. The low substrate activity of these compounds relative to the corresponding ribonucleosides is consistent with the poor affinity of UrdPase for 2′-deoxyribonucleosides. Bose and Yamada [77] have proposed that the 2′-hydroxyl group of uridine participates in hydrogen-bonding with a histidyl residue on UrdPase, and the lack of hydrogen-bond formation may explain the observed poor binding of pyrimidine-2′-deoxyribonucleosides.

2′-Deoxyribonucleoside-type analogs, i.e. arabinosyl thymine (XLVI), arabinosyl uracil (XLVII), 2′-fluoro-arabinosyl-5-fluorouracil (LXVI), and 2′-fluoro-arabinosyl-5-methyluracil (LXIX), did not bind to UrdPase, possibly because of the rather drastic conformational changes in the sugar moiety of pyrimidine arabinosyl nucleosides [78] and 2′-fluoro-2′-deoxy-arabinosyl nucleosides [79] relative to uridine and 2′-deoxyuridine.

Replacement of hydroxyl groups with amino groups at the 3′- or 5′-positions of thymidine [i.e. 3′-deoxy-3′-aminothymidine (LX) and 5′-amino-5′-

deoxythymidine (XLV) produced compounds which do not bind to UrdPase. The 3′-hydroxyl group of pyrimidine nucleosides appears to be essential for binding to UrdPase from this and other studies [10, 45]. On the other hand, the 5′-hydroxyl group of this class of compounds does not appear to be essential for binding as 5′-deoxy-5-fluorouridine has been shown to be a good substrate for UrdPase (Table 1).

Some structural requirements for the binding of 2′-deoxyglucosylthymine are known. The introduction of an equatorial hydroxyl group at the 2′-position of 2′-deoxyglucosylthymine (i.e. β-D-glucosylthymine) abolishes binding to UrdPase [45]. Evidently, the 2′-hydroxyl group of β-D-glucosylthymine is not properly oriented so as to mimic the 2′-hydroxyl group of uridine. On the other hand, the equatorial 3′-hydroxyl group of 2′-deoxyglucosylthymine is essential for binding to UrdPase since inversion of configuration at *C*-3′ of 2′-deoxy-β-D-xylopyranosylthymine (which is equipotent with 2′-deoxyglucosylthymine) yields a compound (2′-deoxy-β-D-ribopyranosylthymine) which is inactive [45]. The 6′-hydroxymethyl group of 2′-deoxyglucosylthymine is non-essential since, as mentioned above, no loss in binding is observed as compared to 2′-deoxy-β-D-xylopyranosylthymine which lacks this group [45]. It is interesting that 2′-deoxyglucosylthymine and derivatives thereof which bind well to UrdPase are not cleaved by this enzyme; evidently some aspect of the 6-membered pyranose ring renders these nucleosides inactive as substrates [44, 45].

The terminal side chain hydroxyl group of the pyrimidine acyclonucleosides [1-(2′-hydroxyethoxymethyl)uracils] has been suggested to form a hydrogen bond to UrdPase, possibly to the same site as presumably do the 3′-hydroxyl groups of uridine and 2′-deoxyglucosylthymine [10]. This proposal is based on the finding that substitution of the terminal side chain hydroxyl group of acyclouridine or acyclothymidine with functional groups that lack a hydrogen available for hydrogen bonding (chloro- or *O*-nitro) yields compounds which do not bind to UrdPase whereas an amino group so substituted gives a compound [1-(2′-amino-ethoxymethyl)uracil] which does inhibit this enzyme [10].

Included in this study are a number of pyrimidine nucleoside analogs that have not been tested previously as inhibitors or substrates of UrdPase. The non-inhibition of UrdPase by 5′-amino-5′-deoxythymidine (XLV), 6-aza-3-deazacytidine (XLVIII), 6-aza-3-deazauridine (XLIX), 3-deazauridine (LIX), 3′-deoxy-3′-aminothymidine (LX), 5′-deoxyoxazinomycin (LXIII), 2′-fluoro-arabinosylcytosine (LXV), 2′-fluoro-arabinosyl-5-flourouracil (LXVI), 2′-fluoro-arabinosyl-5-methyluracil (LXIX), 5′-iodo-5′-deoxyoxazinomycin (LXX), and 2-selenocytidine (LXXV) indicates that these compounds did not bind to the enzyme and, consequently, low or absent UrdPase substrate activity may be inferred. Likewise, the failure of arabinosyl thymine (XLVI), arabinosyl uracil (XLVII), 6-azauridine (L), cytidine (LVIII), orotidine (LXXIII) and pseudouridine (LXXIV) to inhibit UrdPase concurs with the lack of substrate activity of these compounds for UrdPase reported by others (Table 1).

Binding of ligands to thymidine phosphorylase

Hydrophobic region on dThdPase. We postulate the existence of a hydrophobic region on dThdPase situated adjacent to the point of binding of the 5-position of uracil. Presumably, the 3-fold enhancement of dThdPase inhibition by thymine (XXIV) and thymidine (LXXIX), relative to uracil (I) and 2′-deoxyuridine (LXII), respectively, is due to interaction of the 5-position methyl groups of XXIV and LXXIX with the hydrophobic region. Also, the lack of dThdPase inhibition by 5-aminouracil (XIV) and 5-hydroxymethyluracil (XXI) is consistent with the notion of a hydrophobic region rather than a non-specific "pocket" because of the polar (i.e. hydrophilic) nature of the 5-position substituents of these compounds (Table 5).

The dimensions of the hydrophobic region on dThdPase are not clear. While uracils with small 5-position substituents (e.g. methyl, bromo-, iodo-, etc.) bind well to dThdPase (see above), the larger substituents of 5-benzyluracil (XVII) and 5-benzyloxybenzyluracil (XVI) prevent binding. Nakayama *et al.* [5] have shown that horse liver dThdPase cleaves 5-ethyl-2′-deoxyuridine, 5-allyl-2′-deoxyuridine, and 5-propenyl-2′-deoxyuridine, indicating that 5-position substituents larger than methyl groups are tolerated by the enzyme; however, the best substrates had 5-position substituents approximately the size of a methyl group. Clearly, further studies on the binding of 5-substituted uracil analogs are needed for a better understanding of this hydrophobic region.

The relative inhibitory potencies of 5-halogenated uracils may be explained, in part, by interaction with the hydrophobic region on dThdPase. 5-Chlorouracil (XIX), 5-bromouracil (XVIII), and 5-iodouracil (XXII), all of which have hydrophobic 5-position substituents (Table 5) with van der Waals radii in the range of a methyl group (i.e. 1.80 to 2.15 Å), bound 17- to 28-fold better to dThdPase than uracil. Although the extensive ionization of these compounds (Table 5) is probably a major factor in their tight-binding to dThdPase (see above), the equally highly ionized 5-fluorouracil (XX) (Table 5) bound only 2-fold better than uracil (I). Thus, consistent with the existence of a region on dThdPase that interacts most strongly with hydrophobic substituents the size of a methyl group, we attribute the poor relative binding of 5-fluorouracil (XX) to the lower hydrophobicity (Table 5) and smaller van der Waals radius (1.35 Å) of the fluorine substituent.

Given this proposed hydrophobic region, it is surprising that 5-nitrouracil (XXIII) inhibited dThdPase very strongly despite the hydrophilic character of the 5-position nitro-group (Table 5). 5-Nitrouracil also strongly inhibits rabbit liver dThdPase [14]. Although the tight-binding of 5-nitrouracil may be attributed to the complete ionization of this compound under our assay conditions (Table 5), the fact that it does bind at all illustrates that a multiplicity of factors may be involved in the binding of a given compound to dThdPase.

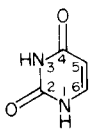
Binding of uracil mono-anions to dThdPase. In agreement with other reports [12, 14], we found that electron-withdrawing groups substituted at the 5-position of uracils enhance binding to dThdPase.

Specifically, 5-fluorouracil (XX), 5-chlorouracil (XIX), 5-bromouracil (XVIII), 5-iodouracil (XXII), and 5-nitrouracil (XXIII) bound to the enzyme 2- to 28-fold better than uracil (I). Since these 5-substituted uracils are highly acidic and, therefore, highly ionized under our assay conditions (Table 5), we conclude that uracils bearing a negative charge bind well to dThdPase. Unfortunately, our studies do not permit determination of preferential binding (if any) of the tautomers of uracil mono-anions to dThdPase.

Uracil analogs substituted at the 1*-position.* 2,6-Pyridinediol (VII), which exists in solution as 20% "1-deazauracil" [75], bound to dThdPase 2-fold better than uracil. 1-Methyluracil (VI), 1-ethyluracil (V), and other 1-substituted uracils (II, III, IV, XXXVI, and XXXVII), on the other hand, did not inhibit dThdPase, indicating a low tolerance for the binding of analogs substituted at the 1-position.

Uracil analogs substituted at the 2- *or* 4*-positions.* dThdPase is apparently specific for the 2- and 4-position oxo-groups of uracils, as 2-thiouracil (VIII), 2,4-dithiouracil (XXXV) and 4-thiothymine (XL) did not bind to this enzyme, whereas uracil (I) and thymine (XXIV) did. Consistent with these findings, Lindsay and Yu [80] have reported that 2-thiouracil does not inhibit horse liver dThdPase. The larger van der Waals radius and/or weaker hydrogen-bonding capability of the 2-position thione group [66], as compared to an oxo-group, may be responsible for the observed poor binding of 2-thiouracil (VIII) to dThdPase. It should be mentioned, however, that the 2-thione group does not totally prevent binding to this enzyme, since horse liver dThdPase has been shown to catalyze the synthesis of 2-thio-2′-deoxyuridine from 2-thiouracil and 2′-deoxyribose-1-phosphate [49].

The 4-amino-substituted uracil analog cytosine (XII) did not inhibit dThdPase. Likewise, 2′-deoxycytidine has been shown not to be a substrate for dThdPase from rat liver [37] and human platelets [25].



POSITION	URIDINE PHOSPHORYLASE	THYMIDINE PHOSPHORYLASE
N-1	Replacement of *N*-1 with a methylene group enhances binding 57-fold	Replacement of *N*-1 with a methylene group enhances binding 2-fold
	Substitution with 2′-(hydroxyethoxymethyl) or 2-deoxy-glucose enhances binding about 4-fold	
C-2	2-Oxo group required	2-Oxo group required
N-3	Undissociated *N*-3 hydrogen required	*N*-3 required
C-4	4-Oxo group may be replaced by a thione group	4-Oxo group required
C-5	Certain hydrophobic groups enhance binding: methyl, 6-fold; benzyl, 52-fold; benzyloxybenzyl, 129-fold	Hydrophobic groups enhance binding: methyl, 3-fold
	Halogens enhance binding through hydrophobic interaction and enhancement of dissociation of *N*-1 hydrogen: fluorine, 3-fold; chlorine, 18-fold; bromine, 21-fold; iodine, 9-fold	Halogens enhance binding through hydrophobic interaction and facilitation of ionization; fluorine, 2-fold; chlorine, 26-fold; bromine, 28-fold; iodine, 17-fold
	Nitro group enhances binding 114-fold by facilitating dissociation of *N*-1 hydrogen	Nitro group enhances binding 13-fold by enhancing ionization
C-6	Substitution generally diminishes binding	Amino group enhances binding 3-fold; other substitutions generally diminish binding

Fig. 2. Structure–activity relationships for the binding of ligands to the pyrimidine nucleoside phosphorylases.

Uracil analogs substituted at the 3-position. 3-Deazauracil (IX), 3-methyluracil (X), and 3-oxauracil (XI) did not bind to dThdPase, indicating a lack of tolerance for uracil analogs substituted at this position. Consistent with these findings is the reported low substrate activity of *N*-3-methylthymidine for horse liver dThdPase [48].

Uracil analogs substituted at the 6-position. 6-Aminouracil (XXV) inhibited dThdPase 3-fold better than uracil and was the only 6-substituted uracil [with the exception of 6-benzyl-2-thiouracil (XXXII) which will be discussed below] which had activity. 6-Aminouracil and various 5-substituted derivatives thereof represent the only known class of dThdPase inhibitors, the most potent of the series being 5-bromo-6-aminouracil [12].

6-Benzyl-2-thiouracil, a new inhibitor of dThdPase. 6-Benzyl-2-thiouracil (XXXII) inhibited dThdPase 5-fold better than uracil (I). The strong binding of 6-benzyl-2-thiouracil (XXXII) to dThdPase is surprising since neither 2-thiouracil (VIII) nor 6-benzyluracil (XXIX) bound to the enzyme.

Binding of nucleoside analogs to dThdPase. dThdPase is apparently highly specific for the 2′-deoxyribosyl moiety of nucleoside ligands. Of the thirty-three nucleoside analogs tested, only the 2′-deoxyribosyl nucleosides, 2′-deoxyuridine (LXII), 5-fluoro-2′-deoxyuridine (LXVII), and thymidine (LXXIX) bound to dThdPase. Non-inhibitory compounds included the ribonucleosides 5-fluorouridine (LXVIII), 5-methyluridine (LXXII), and uridine (LXXX), the arabinonucleosides arabinosyl thymine (XLVI) and arabinosyl uracil (XLVII), the acyclonucleosides acyclothymidine (XLIII) and acyclouridine (XLIV), and several nucleoside analogs modified in the sugar portion of the molecule (XLV, LX, LXI, LXVI, and LXIX). Consistent with these findings, it has also been shown that dThdPase accepts only 2′-deoxyribosyl nucleosides as substrates; ribonucleosides and arabinonucleosides are not cleaved (Table 2).

Design of new pyrimidine nucleoside phosphorylase inhibitors

The structure–activity relationships for ligands of UrdPase and dThdPase are summarized in Fig. 2. These findings provide a basis for the rational design of inhibitors of these enzymes, as discussed below.

Potentiation of UrdPase or dThdPase inhibition may occur when two or more functional groups that individually enhance uracil binding are substituted simultaneously. Indeed, this assumption was the basis for our recent successful design and synthesis of 5-benzylacyclouridine and 5-benzyloxybenzylacyclouridine as inhibitors of UrdPase [6]. By extrapolation, it is worthwhile to synthesize and evaluate compounds such as 5-benzyl-2′-deoxyglucosyluracil, 5-benzyloxybenzyl-2′-deoxyglucosyluracil, and 3-benzyl-2,6-pyridinediol (i.e. "5-benzyl-1-deazauracil") for UrdPase inhibition.

Also, we have clearly delineated the critical functional groups of uracil for binding to UrdPase and dThdPase, and these findings have great importance in studies aimed at identifying new inhibitors of these enzymes. For example, *C*-5 substituted uracils and 6-aminouracils offer the best promise in the search for new dThdPase inhibitors since uracils substituted at other positions generally do not bind well.

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