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Effect of the *N*-Glycosidic Bond Conformation and Modifications in the Pentose Moiety on the Binding of Nucleoside Ligands to Uridine Phosphorylase

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SUMMARY

Several arabinosyl-, xylosyl-, lyxosyl, 5′-deoxy-, acyclo-, 2,2′-anhydro-, 2,3′-anhydro-2′-deoxy-, 2,5′-anhydro-, 6,5′-cyclo-, and carbocyclic analogues of uridine with various 5-substitutions (fluoro, methyl, bromo, ethyl, benzyl, or benzyloxybenzyl) have been tested and compared with their corresponding ribo- and 2′-deoxyribosides for their potency to inhibit uridine phosphorylase (UrdPase) from both mouse and human livers. The effect of the α- and β-configurations of the glycosidic bond was also tested. Xylo-, lyxo-, 2,3′-anhydro-2′-deoxy-, 6,5′-cyclo-, and carbocyclic uridines did not bind to the enzyme. Ribosides bound better than the corresponding 2′-deoxyribosides, which were better than the 5′-deoxyribosides. 2′-α-Deoxyribosides bound to the enzyme, albeit less tightly than the corresponding β-anomers. The acyclo- and 2,2′-anhydrouridines were all potent inhibitors with the 2,2′-anhydro- derivatives being the most potent. 2,5′-Anhydrouridine bound to UrdPase less effectively than 2,2′-anhydrouridine and acyclouridine. Arabinosyl uracil was at best a very poor inhibitor but binds better if a benzyl group is added at the 5-position of the pyrimidine ring. This binding was enhanced further by adding a 5-benzyloxybenzyl group. A similar enhancement of the binding with increased hydrophobicity at the 5-position of the pyrimidine ring was observed with ribosides, α- and β-anomers of the 2′-deoxyribosides, acyclonucleosides, and 2,2′-anhydronucleosides. The inhibitory potencies of these compounds with UrdPase from human liver roughly parallel those obtained with UrdPase from mouse liver. It is concluded that the presence of a *N*-glycosidic bond as well as a properly oriented 3′-hydroxyl group are prerequisites for a nucleoside ligand to bind to UrdPase. On the other hand, the presence of a 2′- or 5′-hydroxyl group or an *N*-glycosidic bond in the β-configuration enhances but is not essential for binding. Furthermore, the potency of the binding of 2,2′-anhydrouridines (fixed *syn*-isomers) in contrast to the complete lack of binding of the 6,5′-cyclouridines (fixed *anti*-isomers) to UrdPase indicates that the binding of ligands to this enzyme is in the *syn*-conformation around the *N*-glycosidic bond.

UrdPase (EC 2.4.2.3) plays an important role in the metabolism of pyrimidines. It modulates the salvage pathway by anabolizing or catabolizing various pyrimidine nucleosides and their analogues. It is also involved in the regulation of the levels of uridine in the plasma (1, 2). The enzyme catalyzes the following reversible phosphorolysis of pyrimidine nucleosides:

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

Although UrdPase from most mammalian sources primarily cleaves pyrimidine (except cytosine) ribosides, it is relatively nonspecific because it also cleaves their corresponding 2′- and 5′-deoxyribosides (3–9).

Interest in UrdPase as a target for chemotherapy arises from the fact that this enzyme is active in the degradation of several chemotherapeutic pyrimidine nucleoside analogues, e.g., 5-fluorouridine and 5-fluoro-2′-deoxyuridine, thus reducing their effectiveness (10–12). Benzylacyclouridines were developed as potent and specific inhibitors of UrdPase (13–19). 2,2′-Anhydrouridines were also found to be very potent inhibitors of this enzyme (19–22). Inhibitors of UrdPase were shown to enhance the efficacy of 5-fluoro-2′-deoxyuridine (14, 22–25), increase the levels of uridine in plasma (1, 2) and heart perfusate (26), increase the salvage of uridine by various tissues (2, 26), and protect against 5-fluorouracil toxicity (2).

In a systematic search for new UrdPase inhibitors, a structure-activity relationship of the binding of ligands to UrdPase was formulated by Niedzwicki *et al.* (9). However, that study was primarily concerned with modifications in the aglycon pyrimidine rather than in the pentose moiety. In this report we examine the role of the ribose moiety as well as the effect of α- and β-anomers in the binding of nucleoside ligands to UrdPase. We compared the binding of uridine, 2′-deoxyuridine (α- and β-anomers), and 5′-deoxy-, acyclo-, 2,2′-anhydro-, 2,3′-

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ABBREVIATIONS: UrdPase, uridine phosphorylase; acyclo-, 1-[(2-hydroxyethoxy)methyl]-; acyclouridine, 1-[(2-hydroxyethoxy)methyl]uracil; 2,2′-anhydro-, 2,2′-anhydro-1-(β-D-arabinofuranosyl)-; 2,2′-anhydro-5-(benzyloxybenzyl)uridine, 2,2′-anhydro-1-(β-D-arabinofuranosyl)-5-(3-benzyloxybenzyl)uracil; 2,2′-anhydro-5-benzyluridine, 2,2′-anhydro-1-(β-D-arabinofuranosyl)-5-benzyluracil; 2,2′-anhydro-5-ethyluridine, 2,2′-anhydro-1-(β-D-arabinofuranosyl)-5-ethyluracil; 2,2′-anhydrouridine, 2,2′-anhydro-1-(β-D-arabinofuranosyl)uracil; 2,3′-anhydro-2′-deoxy-, 2,3′-anhydro-1-(2′-deoxy-β-D-xylofuranosyl)-; 2,3′-anhydro-2′-deoxy-5-(benzyloxybenzyl)uridine, 2,3′-anhydro-1-(2′-deoxy-β-D-xylofuranosyl)-5-(3-benzyloxybenzyl)uracil; 2,3′-anhydro-2′-deoxy-5-benzyluridine, 2,3′-anhydro-1-(2′-deoxy-β-D-xylofuranosyl)-5-benzyluracil; 2,5′-anhydro-, 2,5′-anhydro-1-(β-D-ribofuranosyl)-; 2,5′-anhydrouridine, 2,5′-anhydro-1-(β-D-ribofuranosyl)uracil; arabinoxyl-, 1-(β-D-arabinofuranosyl)-; arabinosyl 5-benzyluracil, 1-(β-D-arabinofuranosyl)-5-benzyluracil; arabinosyl 5-(benzyloxybenzyl)uracil, 1-(β-D-arabinofuranosyl)-5-(3-benzyloxybenzyl)uracil; arabinosyl uracil, 1-(β-D-arabinofuranosyl)uracil; BAU, benzylacyclouridine or 5-benzyl-1-[(2-hydroxyethoxy)methyl]uracil; BBAU, benzyloxybenzylacyclouridine or 5-(3-benzyloxybenzyl)-1-[(2-hydroxyethoxy)methyl]uracil; 5-(benzyloxybenzyl)uridine, 1-(β-D-ribofuranosyl)-5-(3-benzyloxybenzyl)uracil; 5-benzyluridine, 1-(β-D-ribofuranosyl)-5-benzyluracil; 6,5′-cyclo, 5′-deoxy-6,5′-cyclo-; 6,5′-cyclouridine, 5′-deoxy-6,5′-cyclo-1-(β-D-ribofuranosyl)-uracil; 6,5′-cyclo-2′-deoxyuridine, 5′-deoxy-6,5′-cyclo-1-(2-′deoxy-β-D-ribofuranosyl)uracil; 2′-α-deoxy-5-(benzyloxybenzyl)uridine, 1-(2′-deoxy-α-D-ribofuranosyl)-5-(3-benzyloxybenzyl)uracil; 2′-β-deoxy-5-(benzyloxybenzyl)uridine, 5-(3-benzyloxybenzyl)-1-(2′-deoxy-β-D-ribofuranosyl)uracil; 2′-α-deoxy-5-benzyluridine, 1-(2′-deoxy-α-D-ribofuranosyl)-5-benzyluracil; 2′-β-deoxy-5-benzyluridine, 1-(2′-deoxy-β-D-ribofuranosyl)-5-benzyluracil; 5′-β-deoxy-5-fluorouridine, 1-(5′-deoxy-β-D-ribofuranosyl)-5-fluorouracil; (d)Rib-1-P, α-D-(2′- or 5′-deoxy)ribose-1-phosphate; 5-ethylacyclouridine, 5-ethyl-1-[(2-hydroxyethoxy)methyl]uracil; 5-fluoro-2-deoxyuridine, 1-(2′-deoxy-β-D-ribofuranosyl)-5-fluorouracil; 5-fluorouridine, 1-(β-D-ribofuranosyl)-5-fluorouracil; 5′-(hydroxymethyl)-6,5′-(*S*)-cyclouridine, 5′-deoxy-5′-(hydroxymethyl)-6,5′-(*S*)-cyclo-1-(β-D-ribofuranosyl)uracil; 5′-(hydroxymethyl)-6,5′-(*R*)-cyclouridine, 5′-deoxy-5′-(hydroxymethyl)-6,5′-(*R*)-cyclo-1-(β-D-ribofuranosyl)uracil; α-lyxothymine, 1-(α-D-lyxofuranosyl)-5-methyluracil; β-lyxothymine, 1-(β-D-lyxofuranosyl)-5-methyluracil; P_i, orthophosphate; Pyr, pyrimidine base; Pyr(d)Rib, pyrimidine riboside or deoxyriboside; α-xylothymine, 1-(α-D-xylofuranosyl)-5-methyluracil; β-xylothymine, 1-(β-D-xylofuranosyl-5-methyluracil.

anhydro-2′-deoxy-, 2,5′-anhydro- uridines as well as α- and β-xylosyl-, α- and β-lyxosyl-, arabinosyl-uracils with a fluoro, bromo, methyl, ethyl, benzyl, or benzyloxybenzyl group at the 5-position of the pyrimidine ring. These 5-position substitutions are known to enhance the binding of pyrimidine aglycons to UrdPase (9). Furthermore, the binding of the 2,2′-anhydrouridines (fixed *syn*-isomers) was compared with that of various 6,5′-cyclo- (fixed *anti*-isomers) and carbocyclic uridines (lack the *N*-glycosidic bond) to determine the role and the effect of the *N*-glycosidic bond configuration in the binding of nucleosides to UrdPase.

Materials and Methods

Chemicals. The 5-position-substituted uridines and their acyclo-, arabinosyl-, 2,2′-anhydro-, 2,3′-anhydro-2′-deoxy- derivatives (13, 22, 24, 25, 27), 6,5′-cyclouridine, 6,5′-cyclo-2′-deoxyuridine, and their 5-bromo- and 5-methyl- derivatives (28, 29), 5′-(hydroxymethyl)-6,5′(*S*)-cyclouridine, 5′-(hydroxymethyl-6,5′(*R*)-cyclouridine (30), α-xylothymine, β-xylothymine (31), α-lyxothymine, β-lyxothymine (32), carbocyclic uridine (33), and carbocyclic 5-methyluridine (34) were synthesized as previously described. Carbocyclic 2,2′-anhydrouridine and carbocyclic 2,2′-anhydro-5-methyluridine[1] were prepared by a procedure similar to that of Hampton and Nichol (35) for the preparation of 2,2′-anhydrouridine. 2,5′-Anhydrouridine was prepared from its 2′,3′-diacetate (36) with methanol and triethylamine.[2] The chemical structure of these compounds are shown in Figs. 1 and 2. All other compounds were obtained from Sigma Chemical Co. (St. Louis, MO); [2-^{14}C]uridine (50 Ci/mol) from Moravek Biochemicals, Inc. (Brea, CA); silica gel G/UV$_{254}$ Polygram thin layer chromatography plates, from Brinkmann Instruments, Inc. (Westbury, NJ); Omnifluor scintillant, from New England Nuclear (Boston, MA); and bovine γ-globulin and dye reagent for protein estimation, from Bio-Rad Laboratories (Richmond, CA).

Animal tissues. Human liver specimens were from donors and were obtained through the Alabama Regional Organ Bank, when they were not used for medical purposes, as a courtesy of Dr. Jean-Pierre Sommadossi, Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, AL). The gall bladder was removed and the liver was sliced, perfused with cold normal saline, and stored at −70° until it was used. Mouse livers were obtained from Swiss Albino (CD1) mice (Charles River Laboratories, Boston, MA). Mice were killed by cervical dislocation and the livers were removed.

Preparation of extracts. Organs were weighed then homogenized in ice-cold (3:1, v/w) 20 mM potassium phosphate buffer (pH 8) containing 1 mM EDTA, and 1 mM dithiothreitol with a Polytron homogenizer (Brinkmann) and the homogenates were centrifuged at 105,000 × *g* for 1 hr at 4°. The supernatant fluids (cytosol) were collected and used as the enzyme source.

Enzyme assay. The assay mixture contained saturating concentration of phosphate (20 mM potassium phosphate, pH 8), 1 mM EDTA, 1 mM dithiothreitol, 1 mM [2-^{14}C]uridine (9 mCi/mmol), five different concentrations of the inhibitor when present, and 40 μl of enzyme preparation in a final volume of 80 μl. Incubation was carried out at 37° for 30 min. The reaction was terminated by boiling for 1 min, followed by freezing for at least 20 min. Proteins were removed by centrifugation and 5 μl of the supernatant fluid were spotted on silica gel thin layer chromatography plates that had been prespotted with 5 μl of a standard mixture of 10 mM each uridine and uracil. The plates were then developed in a mixture of chloroform and methanol (90:10, v/v). Uridine and uracil were identified by UV quenching. R_F values for uridine and uracil were 0.09 and 0.39, respectively. Spots were cut out and counted in 20 ml of Omnifluor-based scintillant. In the absence of inhibitor, the activity was linear with time and amount of enzyme.

Determination and significance of apparent K_i values. Apparent K_i values were estimated from Dixon plots ($1/v$ versus $[I]$) (37) of the data by a computer program with least square fitting. Apparent K_i values are related to K_i values by the following equation:

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

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

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

Thus, for UrdPases from mouse and human liver, which have K_m values of 143 and 242 μM uridine, respectively (18), the apparent K_i of a competitive inhibitor, measured at uridine concentrations of 1 mM, is approximately 8- and 5-fold higher than their respective K_i values. It should be noted, however, that we have not characterized the compounds used in this study with regard to the type of inhibition (competitive, noncompetitive, or uncompetitive) or whether or not they are substrates for the enzyme. Nevertheless, the acyclo-, 2,2′-anhydro-,

[1] F. Y. Shealy and C. A. O'Dell, unpublished observations.
[2] B. A. Otter and E. A. Falco, unpublished observations.

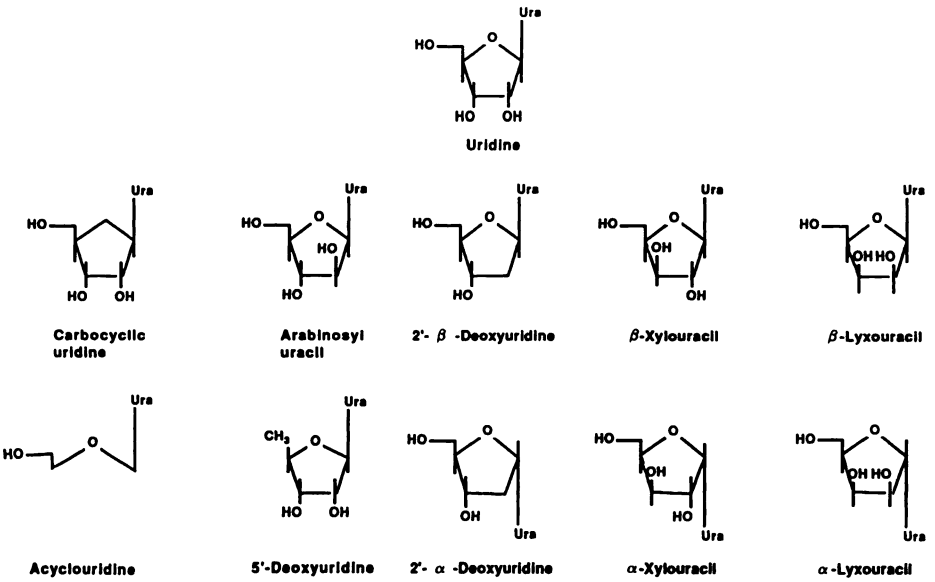

Fig. 1. The chemical structures of uridine and various types of analogues modified in the pentose moiety.

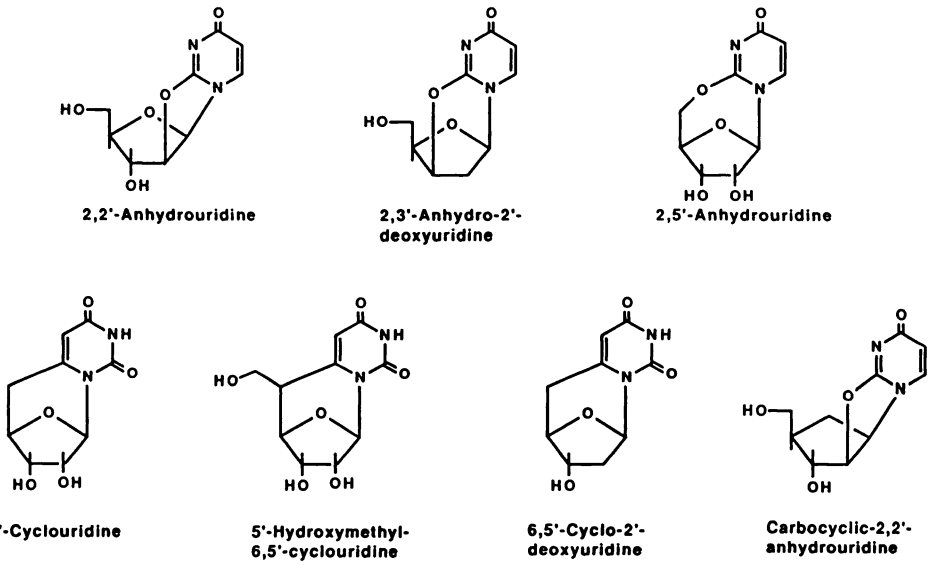

Fig. 2. The chemical structures of various types of anhydrouridines, cyclouridines, and carbocyclic anhydrouridine.

and deoxyriboside derivatives of uridine are known competitive inhibitors of UrdPase (9, 13, 15–21, 38) and all the 5-position substituents included in the present study do not alter the competitive inhibition of any of the compounds studied so far (9, 13, 15–21, 38). Therefore, it is assumed that the compounds that bind to UrdPase in the present investigation are competitive inhibitors of the enzyme, i.e., bind to the same site as does uridine. Under the present method of screening, if a compound is an alternative substrate for the enzyme (e.g., 2′-β-deoxyuridine), the apparent K_i would be its apparent K_m (39).

Determination of K_i for uridine. The K_i values for uridine for UrdPase from mouse and human livers were estimated from the data of Naguib *et al.* (18) as the coordinates of the common intersection point in Lineweaver-Burk plots of 1/velocity versus 1/[uridine]. Detailed kinetics studies on UrdPase from mouse liver have established that uridine leaves the enzyme-substrate complex first followed by P_i.[3] Therefore, the K_{ia} (the inhibition constant for the first substrate to bind) is the dissociation constant (K_i) for uridine (40). We are assuming that similar kinetics apply to UrdPase from human liver.

Protein estimation. Protein concentrations were determined by the method of Bradford (41) using bovine γ-globulin as a standard.

Results

Figures 1 and 2 show the chemical structures of uridine and the various types of derivatives studied in the present investigation. The apparent K_i values of these compounds for UrdPase from mouse and human livers are shown in Table 1.

Xylo-, lyxo-, 2,3′-anhydro-2′-deoxy-, 6,5′-cyclo-, and carbocyclic analogues of uridine did not inhibit the enzyme, regardless of the 5-position substituents, indicating that these compounds do not bind to the enzyme. Arabinosyl uracil was at best a very poor inhibitor of UrdPase. However, its binding to the enzyme was enhanced markedly by the addition of a benzyl or benzyloxybenzyl group at the 5-position of the pyrimidine

[3] K. S. Park, M. H. el Kouni, F. N. M. Naguib, and S. Cha, manuscript submitted for publication.

TABLE 1

Inhibitory potencies of various uridines and their modified sugar congeners for UrdPase from the cytosol of mouse and human livers at 1 mM uridine and saturating concentration of phosphate (20 mM)

Compound	Apparent K_i[a]			
	Mouse		Human	
	μM			
Uridine	577	± 53[b]	425	± 98[b]
5-Fluorouridine[c]	445	± 54.4	214	± 14.8
5-Benzyluridine	52.9	± 5.5	67.9	± 4.8
5-(Benzyloxybenzyl)uridine	18.1	± 0.74	12.5	± 1.9
2′-β-Deoxyuridine[c]	976	± 117	820	± 85.8
2′-β-Deoxy-5-benzyluridine	108	± 9.2	140	± 15.6
2′-β-Deoxy-5-(benzyloxybenzyl)uridine	21.5	± 2.3	14.0	± 1.48
2′-α-Deoxy-5-benzyluridine	539	± 128	896	± 80.7
2′-α-Deoxy-5-(benzyloxybenzyl)uridine	70.7	± 8.81	80.8	± 7.52
5′-β-Deoxy-5-fluorouridine[c]	1667	± 242	278	± 50.3
Arabinosyl uracil	1900	± 275	—[d]	
Arabinosyl 5-benzyluracil	472	± 24	1000	± 121
Arabinosyl 5-(benzyloxybenzyl)uracil	37.2	± 15.4	39.4	± 11.5
β-Xylothymine	—		ND[e]	
α-Xylothymine	—		ND	
β-Lyxothymine	—		ND	
α-Lyxothymine	—		ND	
Acyclouridine	113	± 9.8	68.7	± 6.9
5-Ethylacyclouridine	15.9	± 3.8	18.9	± 1.8
BAU	3.12	± 0.21	3.97	± 0.30
BBAU	1.25	± 0.10	1.95	± 0.23
2,2′-Anhydrouridine	131	± 1.6	245	± 12.4
2,2′-Anhydro-5-ethyluridine	0.19	± 0.091	0.47	± 0.03
2,2′-Anhydro-5-benzyluridine	0.81	± 0.17	2.39	± 0.10
2,2′-Anhydro-5-(benzyloxybenzyl)uridine	0.29	± 0.036	0.74	± 0.05
2,3′-Anhydro-5-benzyl-2′-deoxyuridine	—		—	
2,3′-Anhydro-5-(benzyloxybenzyl)-2′-deoxyuridine	—		—	
2,5′-Anhydrouridine	635	± 100	—	
6,5′-Cyclouridine	—		—	
6,5′-Cyclo-5-bromouridine	—		—	
6,5′-Cyclo-5-methyluridine	—		—	
5′-(Hydroxymethyl)-6,5′(*R*)-cyclouridine	—		—	
5′-(Hydroxymethyl)-6,5′(*S*)-cyclouridine	—		—	
6,5′-Cyclo-2′-deoxyuridine	—		—	
6,5′-Cyclo-2′-deoxy-5-bromouridine	—		—	
Carbocyclic uridine	—		ND	
Carbocyclic 5-methyluridine	—		ND	
Carbocyclic 2,2′-anhydrouridine	—		ND	
Carbocyclic 2,2′-anhydro-5-methyluridine	—		ND	

[a] Values ± standard error of estimation from at least three determinations.
[b] Values represent K_i determined as K_m as described under Materials and Methods.
[c] These compounds are known substrates for UrdPase, therefore their data represent K_m values (39) and should be compared with the K_m values of uridine, which are 143 and 242 μM for mouse and human liver enzymes, respectively (18).
[d] —, Apparent $K_i > 2$ mM.
[e] ND, Not determined.

ring, i.e., arabinosyl uracil < arabinosyl 5-benzyluracil < arabinosyl 5-(benzyloxybenzyl)uracil. 2,5′-Anhydrouridine bound to UrdPase from mouse liver.

All ribosides, 2′-deoxyribosides, and 5′-deoxyribosides tested bound to UrdPase, albeit to different degrees. The ribosides bound better than their corresponding 2-deoxyribosides, which were better than the 5′-deoxyribosides. The α-anomers bound to the enzyme; however, the corresponding β-anomers bound 3- to 6-fold better. The binding of the ribosides and 2′-deoxyribosides (both α- and β-anomers) was increased by addition of a benzyl group at the 5-position of the pyrimidine ring and even further by the addition of a 5-benzyloxybenzyl group.

The acyclo- and 2,2′-anhydro-uridines were better inhibitors than both ribosides and 2′-deoxyribosides. For the same modification, the 2,2′-anhydro- derivatives were better inhibitors than the corresponding acyclo- derivatives. Addition of the 5-ethyl group enhanced the binding of acyclo-5-ethyluridine over acyclouridine. This binding was further enhanced by 5-benzyl (BAU) or a 5-benzyloxybenzyl (BBAU) substitutions. Similarly the 5-ethyl group enhanced the binding of 2,2′-anhydro-5-ethyluridine over 2,2′-anhydrouridine. However, 2,2′-anhydro-5-ethyluridine was a more potent inhibitor than 2,2′-anhydro-5-benzyluridine or 2,2′-anhydro-5-(benzyloxybenzyl)uridine. Compounds with 5-benzyloxybenzyl group were more inhibitory than those with 5-benzyl substituents. With the exception of 2,5′-anhydrouridine, all compounds that inhibited mouse liver UrdPase were also inhibitory for the human liver enzyme. Although the apparent K_i values differed for the same compound, the relative inhibitory potencies of these compounds were roughly parallel in the two species.

Discussion

Inhibitors of UrdPase were shown to potentiate the efficacy and selective toxicity of 5-fluoro-2′-deoxyuridine (14, 22–25) and to increase uridine levels in plasma (1, 2) and heart perfusate (26) as well as its salvage by various tissues (2, 26). They also protect against 5-fluorouracil toxicity (2). In addition to their potential usefulness in cancer chemotherapy, inhibitors of UrdPase can also be used to elucidate the structure of the binding site of this enzyme. Recently, Niedzwicki *et al.* (9) formulated a structure-activity relationship of the binding of aglycon ligands to UrdPase. In the present study, we have extended this structure-activity relationship and examined the binding to UrdPase of various pentose derivatives of uridine, including α- and β-anomers. We have also tested the effect of the conformation around the *N*-glycosidic bond.

Effect of the conformation around the *N*-glycosidic bond. The uridine derivatives shown in Figs. 1 and 2 can be divided into four general categories according to the conformation around the *N*-glycosidic bond as follows: derivatives that are fixed in the *syn*-range (2,2′-anhydro-, 2,3′-anhydro-2′-deoxy-, and 2,5′-anhydro-), derivatives that are fixed in the *anti*-range (6,5′-cyclo-), derivatives that are not fixed in either position (ribosyl-, 2′-deoxyribosyl-, 5′-deoxyribosyl-, arabinosyl- xylosyl-, lyxosyl-, and acyclo- derivatives), and derivatives that lack the *N*-glycosidic bond of a true nucleoside (carbocyclic derivatives). The present results show that the 2,2′-anhydrouridines strongly bind to UrdPase, followed by the acyclic nucleosides, ribosides, 2,5′-anhydronucleosides, 2′-deoxyribosides, 5′-deoxyribosides, and finally the arabinosides. All other compounds tested did not bind. The lack of binding of the carbocyclic uridines is likely to be due to the presence of a cycloalkyl-heterocycle bond in these compounds instead of an *N*-glycosidic bond as in true nucleosides. The lack of binding of the 6,5′-cyclonucleosides, on the other hand, can be attributed to the *anti*-conformation of their *N*-glycosidic bond. The lack of binding in these compounds is not due to the absence

of the 5′-hydroxyl group. As will be discussed below, the 5′-hydroxyl group enhances but is not essential for binding to UrdPase. Furthermore, the hydroxymethyl- derivatives of the 6,5′-cyclo- compounds, which provide such a group, also did not bind to UrdPase.

In contrast to the complete lack of binding of the 6,5′-cyclouridines, which are fixed *anti*-forms, the 2,2′-anhydrouridines were the best inhibitors of UrdPase. Because the conformation around the *N*-glycosidic bond in the 2,2′-anhydrouridines is fixed in the *syn*-range, we conclude that nucleosides bind to UrdPase as *syn*-rotamers. This conclusion is further supported by the finding that 2,5′-anhydrouridine, which is fixed as a *syn*-rotamer, also binds to UrdPase. Furthermore, acyclonucleosides bind better to UrdPase than do the corresponding nucleosides (9, 13–19, 38; Table 1). Uracil nucleosides are predominantly present as *anti*-rotamers (42). In contrast, the torsion angle around the *N*-glycosidic bond in their acyclonucleosides is ~90° (43), i.e., borderline between *syn*- and *anti*-conformation, and is more flexible to rotate to the *syn*-position (43) than nucleosides. This can explain the better binding of the acyclonucleosides. These results indicate the requirement for the presence of a true *N*-glycosidic bond conformed in the *syn*-position in order for a nucleoside to bind to UrdPase. A similar conclusion was reached by Krajewska and Shugar (44). They demonstrated that 6-methyluridine, a nucleoside constrained as a *syn*-rotamer, is a substrate for UrdPase from *Salmonella typhimurium* and postulated that uridine and other substrates of UrdPase are converted to the *syn*-conformation during interaction with the enzyme (44).

Effect of the 5′-hydroxyl group. The present results indicate that the 5′-hydroxyl group enhances but is not essential for binding of nucleoside ligands to UrdPase. Table 1 shows that 5′-deoxy-5-fluorouridine and 2,5′-anhydrouridine, which lack the 5′-hydroxyl group, do bind to the enzyme. Nevertheless, the absence of the 5′-hydroxyl group in 5′-deoxy-5-fluorouridine reduces its binding from that of its riboside analogue (5-fluorouridine). The absence of the 5′-hydroxyl group can also explain, at least in part, the lack of strong binding expected from 2,5′-anhydrouridine, an analogue constrained in the favored *syn*-conformation. However, there may be other factor(s), in addition to the absence of 5′-hydroxyl group, responsible for the lack of strong binding of 2,5′-anhydrouridine. Among these factors are changes in conformation of the pentose moiety and/or in the orientation of the 3′-hydroxyl group (see below).

Effect of the 3′-hydroxyl group. It was previously shown that the presence of a hydroxyl group in the proper *ribo*-configuration at the 3′-position is essential for the binding of β-nucleosides to UrdPase (9, 38, 45). Our results confirm and further emphasize the importance of the 3′-hydroxyl group on the binding of nucleoside ligands to UrdPase. The presence and proper orientation of the 3′-hydroxyl group can explain the lack of binding of the 2,3′-anhydro-2′-deoxyuridines. Although the 2,3′-anhydro-2′-deoxyuridines are fixed in the *syn*-range required for binding, these compounds lack the 3′-hydroxyl group necessary for binding to UrdPase. A similar argument can explain the lack of binding of the β-lyxo- and the β-xylo-compounds. Both types of derivatives lack the 3′-hydroxyl group in the proper *ribo*-orientation required for hydrogen bonding. Etzold *et al.* (45) have also reported that 2′-deoxyxylothymine (1-(2′-deoxy-β-D-xylofuranosyl)thymine) did not bind to UrdPase and attributed that to the absence of a properly oriented 3′-hydroxyl group in this compound.

Effect of 2′-hydroxyl group. The better binding of ribosides over 2′-deoxyribosides to UrdPase can presumably be explained by the suggestion of Bose and Yamada (46) that the 2′-hydroxyl group in ribosides is involved in hydrogen bonding with a histidine residue on UrdPase. However, this suggestion does not explain the very strong binding of the 2,2′-anhydro-compounds, which lack the 2′-hydroxyl group. On the other hand, arabinosyl uracil, which has a 2′-hydroxyl group but in the *ara*-configuration, binds poorly to UrdPase. The strong binding of the 2,2′-anhydrouridines suggests that the contribution of their *syn*-configuration to the binding is stronger than that of the 2′-hydroxyl group. As for the poor binding of the arabinosides, it can be attributed to the significant changes in their conformation from that of the ribosides (9, 47) or the intolerance of the enzyme for a 2′-hydroxyl group in the *ara*-configuration, which also stabilizes the *anti*-form of the arabinosides (47).

Binding of the α- and β-anomers. The present results clearly demonstrate that the α-anomers of the 2′-deoxyuridines can bind to UrdPase, albeit to a lesser degree than their β-anomers. The binding of the 2′-α-deoxyribosides may be explained in terms of the role of the 3′-hydroxyl group in the binding of β-nucleosides to UrdPase (see above). We postulate that the 5′-hydroxyl group in the 2′-α-deoxyribosides can occupy a similar position to that occcupied by the 3′-hydroxyl group in the 2′-β-deoxyribosides, thus allowing the hydrogen bonding and binding of the α-anomers to the enzyme. Fig. 3 illustrates that indeed such configuration of the 5′-hydroxyl group can take place in the α-anomers of 2′-deoxyuridine. This proposition is further enhanced by the observed lack of binding of α-xylothymine and α-lyxothymine to UrdPase. In contrast to 2′-α-deoxyribosides, the 5′-hydroxyl group in the α-xylo- and α-lyxo- compounds cannot be properly oriented to simulate the essential role played by the 3′-hydroxyl group in the binding of β-nucleosides to UrdPase. This is due to the steric hindrance imposed on the freedom of rotation of the 5′-hydroxyl group in the α-xylo- and α-lyxo- compounds by the *xylo*-configuration of their 3′-hydroxyl group. Such restriction obstructs the 5′-hydroxyl group from occupying the proper position required for hydrogen bonding, thus preventing the binding of the α-xylo- and α-lyxo- compounds to UrdPase.

Effect of 5-position substitution. The enhancement of binding of ligands by increasing the hydrophobicity of the 5-position substituents (ethyl < benzyl < benzyloxybenzyl) of the ribosides, 2′-deoxyribosides (both α- and β-anomers), arabinosides, and acyclonucleosides is in agreement with the suggestion that a hydrophobic pocket exists on UrdPase, adjacent to the binding site of the 5-position of the pyrimidine ring (9, 19, 48). Although addition of a 5-ethyl, 5-benzyl, or 5-benzyloxybenzyl group also enhanced the binding of 2,2′-anhydrouridine to UrdPase, the degree of binding (2,2′-anhydrouridine < 2,2′-anhydro-5-benzyluridine < 2,2′-anhydro-5-(benzyloxybenzyl) uridine < 2,2′-anhydro-5-ethyluridine) did not follow the increase in hydrophobicity of these groups (ethyl < benzyl < benzyloxybenzyl) seen with acyclonucleosides with the same 5-position substitution (9, 17–19, 21; Table 1). Veres *et al.* (20) also reported that addition of 5-propyl or 5-isopropyl to 2,2′-anhydrouridine, reduced its binding to UrdPase relative to that of 2,2′-anhydro-5-ethyluridine. Therefore, it appears that in

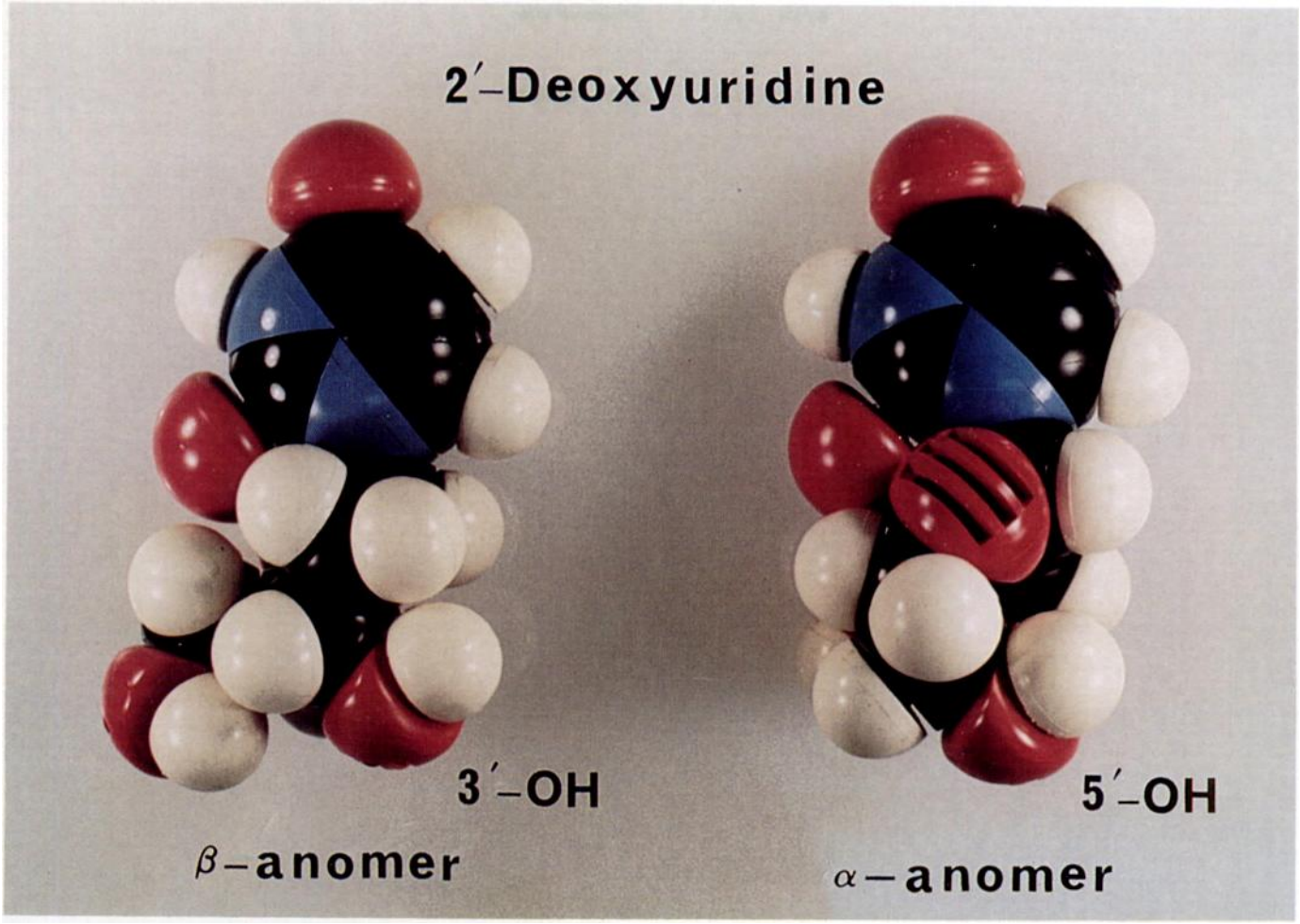

Fig. 3. Space filling models of the α- and β-anomers of 2′-deoxyuridine. Note that the 3′-hydroxyl group of 2′-β-deoxyuridine and the 5′-hydroxyl group of 2′-α-deoxyuridine occupy similar positions when the α-anomer is rotated 180° around the C1′-C4′ axis of the pentose moiety.

the case of the 2,2′-anhydrouridines, the fixed conformation may interfere with the proper orientation of 5-position substituents larger than an ethyl group, rendering these compounds less accessible to the active site of the enzyme whereas, in the acyclonucleosides, such interference does not occur because of the freedom of rotation around their *N*-glycosidic bond. It follows that, for the binding of ligands with large 5-position substituents, a greater degree of freedom of rotation around the *N*-glycosidic bond is required to allow the fitting of 5-position substituents into the hydrophobic pocket.

The contribution of the 5-position to the binding of ligands to UrdPase seems to be of far greater importance than the role of the 2′-hydroxyl group in the *ribo*-configuration. This is demonstrated when the lack of binding of arabinosyl uracil was overcome by adding a hydrophobic group at the 5-position [arabinosyl 5-benzyluracil and arabinosyl 5-(benzyloxybenzyl)uracil]. It is also evidenced by the fact that whereas the unsubstituted 2′-deoxyriboside (2′-deoxyuridine) binds approximately 5-fold less than the riboside (uridine), 2′-β-deoxy-5-(benzyloxybenzyl)uridine bound equally as well as 5-(benzyloxybenzyl)uridine (Table 1).

In conclusion, the present results indicate that the presence of a true *N*-glycosidic bond in the *syn*-conformation and a 3′-hydroxyl group in the proper orientation are among the strict structural and conformational requirements for a nucleoside ligand to bind to UrdPase. The presence of a 2′-, 5′-hydroxyl group and an *N*-glycosidic bond in the β-configuration enhances but is not essential for binding.

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