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STRUCTURE–ACTIVITY RELATIONSHIP OF LIGANDS OF DIHYDROURACIL DEHYDROGENASE FROM MOUSE LIVER*

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Abstract—One hundred and five nucleobase analogues were screened as inhibitors of dihydrouracil dehydrogenase (DHUDase, EC 1.3.1.2) from mouse liver. 5-Benzyloxybenzyluracil, 1-deazauracil (2,6-pyridinediol), 3-deazauracil (2,4-pyridinediol), 5-benzyluracil, 5-nitrobarbituric acid and 5,6-dioxyuracil (alloxan) were identified as potent inhibitors of this activity, with apparent K_i values of 0.2, 0.5, 2.1, 3.4, 3.8 and 6.6 μM respectively. Both 5-benzyloxybenzyluracil and 1-deazauracil were also potent inhibitors of DHUDase from human livers. These findings along with an extensive review of literature allowed the formulation of a structure–activity relationship. The binding to DHUDase required intact *C*2 and *C*4 oxo groups. Replacement of *N*1 or *N*3 by an endocyclic carbon enhanced binding. In contrast, replacement of *C*5 or *C*6 by an endocyclic nitrogen abolished binding. Addition of a charged group to *C*5 and/or *C*6, and of a hydrophobic group to *C*5 but not *C*6 improved the binding.

Dihydrouracil dehydrogenase (DHUDase‡, EC 1.3.1.2) is the first of a chain of three enzymes concerned with pyrimidine base catabolism in mammals. This enzyme catalyzes the reversible conversion of pyrimidine bases (uracil, thymine but not cytosine) and their analogues to their corresponding dihydropyrimidines as follows:

$$\text{pyrimidine base} + \text{NADPH} + \text{H}^+ \rightleftharpoons \text{dihydropyrimidine} + \text{NADP}^+$$

Until recently, the presence of DHUDase in various tissues and its potential importance as a target for chemotherapy were debatable [cf. Ref. 1]. The importance of DHUDase for chemotherapy stems from the fact that the widely used anticancer drug 5-fluorouracil (FUra) or the radiosensitizing drugs 5-iodo- and 5-bromouracils are better substrates for DHUDase than the naturally occurring nucleobases, uracil and thymine [1–7], and hence are inactivated by this enzyme. In addition, recent reports associated the host toxicity of FUra including cholestasis [8] and neurological disorders (cf. Ref. 9) to derivatives of its catabolite α-fluoro-β-alanine. Therefore, inhibitors of this enzyme may be useful as chemotherapeutic agents by preventing the host-toxicity of FUra as well as enhancing its efficacy. Indeed, DHUDase inhibitors were shown to potentiate the effect of FUra *in vitro* and *in vivo* [7, 10–12]. Nevertheless, little is known about the structural requirement for ligands to bind to DHUDase, and few potent inhibitors, which are also devoid of substrate activity for DHUDase, are presently known [2–7, 11, 13–21].

In the present study, we have evaluated 105 nitrogen heterocycles, mostly pyrimidines, for their capacity to inhibit DHUDase. 5-Benzyloxybenzyluracil and 1-deazauracil (2,6-pyridinediol) were identified as the most potent inhibitors of this enzyme known to date. The present results along with an extensive literature review enabled us to formulate a structure–activity relationship and to propose the basis for a rational design of new inhibitors of DHUDase. Preliminary reports have been presented [18, 21].

MATERIALS AND METHODS

Chemicals. The sources of tested compounds are indicated in Table 1 by the following abbreviations: ALD, Aldrich Chemical Co., Milwaukee, WI; AP, Alfa Products, Danvers, MA; CAL, Calbiochem-Behring Corp., La Jolla, CA; CDC, Chemical Dynamics Corp., South Plainfield, NJ; GZ, Dr. Gury Zvilichovsky, Hebrew University, Jerusalem, Israel; K&K, K&K Laboratories, Inc., Plainview, NY; MAL, Mallinckrodt Chemical Works, St. Louis, MO; PL, P-L Biochemicals, Milwaukee, WI; RFS, Dr. Raymond F. Schinazi, Emory University, Atlanta, GA; SHC, Dr. Shih Hsi Chu, Brown University, Providence, RI; SIGMA, Sigma Chemical Co., St. Louis, MO and VEGA, Vega Biochemicals, Tucson, AZ. [6-^{14}C]Uracil (56 mCi/mmol) was purchased from Moravek Biochemicals, Brea, CA; and [6-^{14}C]5-fluorouracil (55 mCi/mmol), from Amersham Corp., Arlington Heights, IL. Uracil, dihydrouracil, carbamyl-β-alanine, β-alanine, NADPH, and ninhydrin were from the Sigma Chemical Co.; Polygram CEL 300 UV_{254} TLC plates were from Brinkmann, Westbury, NY. Omnifluor was from New England Nuclear Corp., Boston, MA;

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‡ Abbreviations: DHUDase, dihydrouracil dehydrogenase; and FUra, 5-fluorouracil.

and dimethylaminobenzaldehyde from the Aldrich Chemical Co. All other chemicals were from the Fisher Scientific Co., Boston, MA.

Animal tissues. Livers were obtained from Swiss Albino (CD1) mice (Charles River Laboratories, Boston, MA). Mice were killed by cervical dislocation and the livers were removed. Livers were then washed with ice-cold normal saline (0.9%) before any further manipulation.

Preparation of extracts. The livers were homogenized in ice-cold homogenization buffer (3:1, v/w) which contained 20 mM potassium phosphate (pH 8), 1 mM EDTA, and 1 mM mercaptoethanol with a polytron homogenizer (Brinkmann). The homogenates were centrifuged at 105,000 *g* for 1 hr at 4°. The supernatant fluid (cytosol) was collected and used as the enzyme source [1].

Dihydrouracil dehydrogenase assay. All assays were carried out under conditions in which activity was linear with time and enzyme concentration, and as described previously [1]. The reaction mixture, in capped Eppendorf tubes, contained 10 mM potassium phosphate (pH 8), 0.5 mM EDTA, 0.5 mM mercaptoethanol, 2 mM dithiothreitol (DTT), 5 mM $MgCl_2$, 25 μM [6-^{14}C]uracil (56 Ci/mol), 100 μM NADPH, various concentrations of inhibitor (0–50 mM), and 25 μl of cytosol in a final volume of 50 μl. Typically, putative inhibitors were screened at 0.625, 1.25, 2.5, and 5.0 μM. Higher or lower concentrations were used on compounds that did not inhibit or that proved too potent respectively. The reaction was started by the addition of cytosol. Incubations were carried out at 37° for 15 min. The reaction was stopped by boiling for 1 min, and then freezing for 20 min. Precipitates were removed by centrifugation, and 5 μl of supernatant fluid was spotted on cellulose TLC plates which were prespotted with 5 μl of a standard mixture of uracil (10 mM), dihydrouracil (25 mM), carbamyl-β-alanine (10 mM), and β-alanine (10 mM). Plates were then developed overnight in the top phase of a mixture of *n*-butanol:water:ammonia (90:45:15, by vol.). R_f values for dihydrouracil, uracil and carbamyl-β-alanine plus β-alanine were 0.46, 0.23 and 0.09 respectively. Uracil was identified by UV quenching and β-alanine by spraying with 0.2% ninhydrin in 85% ethanol. Dihydrouracil and carbamyl-β-alanine were visualized by spraying with 5% dimethylaminobenzaldehyde in 1 N HCl in 50% ethanol after dihydrouracil was hydrolyzed to carbamyl-β-alanine by KOH (0.5 N in 50% ethanol). Spots were cut out and counted in Omnifluor. Dihydrouracil dehydrogenase activity was determined by measuring the sum of the products dihydrouracil, carbamyl-β-alanine and β-alanine.

Protein determination. Protein concentrations were estimated by the method of Bradford [22] using gamma-globulin as a standard.

Determination of apparent K_i *values.* The apparent K_i values (i.e. at 100 μM NADPH) were determined from the plot of 1/v versus [I] (Dixon plot, [23]), using a computer program based on least-squares fitting. We did not try to delineate whether or not an analogue is also a substrate. It should be noted, however, that the apparent K_i values determined for analogues, including thymine (5-methyluracil), that are also alternate substrates actually represent apparent K_m values. Theoretically, it is possible to estimate the inhibition constant of uracil by initial velocity and product inhibition studies; however, such measurements are impractical, as over 90% of the dihydrouracil formed in the present assay system is converted instantly to carbamyl-β-alanine [1]. Hence, the apparent K_m for uracil, 9 μM, was used as the reference value, with the understanding that this value could be larger or smaller than the actual apparent K_i.

RESULTS AND DISCUSSION

One hundred and five nitrogen heterocycles were tested for their abilities to bind DHUDase from mouse liver (Table 1). Benzyloxybenzyluracil and 1-deazauracil proved to be among if not the most potent inhibitors of this activity known to date, with apparent K_i values of 0.2 and 0.5 μM respectively. The best reported K_i is 0.2 μM for 3-cyano-2,6-dihydroxypyridine inhibiting rat liver DHUDase [20]. Benzyloxybenzyl- and 1-deazauracils were also potent inhibitors of DHUDase from human liver with apparent K_i values of 2.8 ± 0.5 and 0.6 ± 0.08 μM respectively. To formulate a comprehensive structure–activity relationship for the binding of ligands to DHUDase, it was necessary, in addition to the above study, to conduct an extensive review of literature on inhibitors or alternate substrates of mammalian DHUDase as listed in Tables 2–4.

Uracil was chosen as the reference compound because it is the preferred natural substrate ([6], Table 2 and unpublished results). Hence, all compounds assessed in this study were considered as modified analogues of uracil. It is noteworthy that uracil in solution can undergo keto/enol tautomerism. The predominant species has been reported to be the keto tautomeric form [24]. This tautomer is also predominant with certain 1- and/or 3-alkylated substituents [24–26], as well as uracils with various substitutions at the 5- and/or 6-positions [24]. Moreover, when ionized, uracils assume an anion form in which the negative charge resonates between the *C*4 and *C*2 positions [24]. Uracils may also yield an equilibrium mixture of monoanions by dissociation of the *N*1 or *N*3 protons [24–26]. Alternatively, uracils may protonate at oxygen to yield mono- or dications, the protonation of the oxygen attached to *C*4 being considerably more stable than that of the oxygen attached to *C*2 [24]. In the following we will attempt to assign the effect of the various substitutions on binding to DHUDase.

Substitutions at the 1-position. Several substitutions (Table 1) at position 1 of uracil were tested. Uridine (II), which has been reported to be an inhibitor of the rat liver enzyme (Table 3), did not inhibit DHUDase from mouse liver (Table 1). Thus, a pentose attached to the *N*1 of uracil abolishes binding to DHUDase. Acyclouridine (III), 5-fluoroacyclouridine (XCVIII) and ftorafur (LXI) (Table 1), which can also be considered as *N*1 nucleoside analogues, were not inhibitory.

Attaching a hydrophobic group to *N*1 decreased the ability of such analogues to bind to DHUDase

as compared to uracil. However, the binding capacity diminished with decreasing hydrophobicity, e.g. 1-benzyl- (V) > 1-ethyl- (VI) > 1-methyl- (VII) uracil. It is interesting that the major difference between a benzyl (V) and a cyclohexyl (IV) group is planarity, benzyl being planar whereas cyclohexyl is not; hence, steric factors may play a significant role in the reactivity of these two compounds with the enzyme, thus the larger apparent K_i value for 1-cyclohexyluracil. The fact that the nature of the hydrophobic substituent at *N*1 affects binding to the enzyme, nevertheless, suggests some kind of interaction between the hydrophobic group at *N*1 of the ligand and enzyme.

Substituting an endocyclic carbon for *N*1 of uracil, i.e. 1-deazauracil (VIII), increased the binding to DHUDase *ca.* 20-fold. Although this increase in binding capacity could be partly due to the difference between the apparent K_i and the apparent K_m values of uracil, this finding, nevertheless, indicates that the hydrogen at position *N*1 of uracil is not required for binding to the enzyme. It has been suggested [27] that 1-deazauracil may bind orotate phosphoribosyltransferase in an orientation rotated by 180° around its *C*2–*C*5 axis. Since both enzymes accept uracil as substrate or product, it is possible that this also occurs when 1-deazauracil binds DHUDase. Interestingly, at pH 8, uracil ($pK_a = 9.5$) is considerably more basic than 1-deazauracil ($pK_a = 1.2$) [27, 28]. Whether or not this difference in acidity is responsible for the better binding of 1-deazauracil remains to be seen.

Substitutions at the 2-position. None of the compounds tested, 2-amino- (IX), 2-deoxy- (X) and 2-thio- (XI) uracils, inhibited DHUDase (Table 1), indicating that an intact -oxo group attached to *C*2 of uracil is necessary for binding the enzyme. This speculation is corroborated by the fact that, with the exception of 4,6-dihydroxy-2-methylpyrimidine (LXX), all the compounds, i.e. 4,6-dihydroxy-2-mercaptopyrimidine (LXXI), 4,6-dihydroxy-2-aminopyrimidine (LXXII) (Table 1) and 2-thio-5-methyluracil (Table 4), with a substituent at *C*2 other than oxo did not bind or weakly bound DHUDase. The possibility exists that LXX binds as 3-deaza-5-aza-6-methyluracil. Indeed, 5-aza-3-deazauracil (LXIX) bound DHUDase well, whereas 6-methyluracil (XLV) did not bind at all.

Substitutions at the 3-position. 3-Methyl, (XIII) and 3-oxa- (XIV) uracils did not bind at all (Table 1). Neither did 1,3-dimethyl- (LXII) (Table 1), 3-methyl-5-nitro- or 1,3-dimethyl-5-nitrouracils (Table 4).

3-Deazauracil (XII), on the other hand, bound DHUDase five times better than uracil. This fact suggests that the proton on *N*3 of uracil is not necessary for binding to the enzyme. 3-Deazauracil has a pK_a value of 6.5 [27] while that of 1-deazauracil is 1.2 [28]. Whether or not this difference in acidity of the ring is responsible for the better binding of 1-deazauracil is an open question.

Substitutions at the 4-position. Cytosine (XV), 4-methylpyrimidine-2-one (XVI) and pyrimidine-2-one (XVII) did not bind DHUDase (Table 1). Although 4-thiouracil (Table 3) is listed as an inhibitor of this activity, the inhibition at twice the concentration of the substrate was marginal. Furthermore, 4-thiothymine (LXVII) did not inhibit at all (Table 1). A further examination of the substitutions at more than one position involving position 4 of uracil indicates than an oxo group at this position is necessary for binding to DHUDase. Indeed, both 4-amino-5-bromo-2-hydroxypyrimidine (LXXIX), 4-amino-5-fluoro-2-hydroxypyrimidine (LXXX) (Table 1) and 4-amino-5-methyluracil- (Table 4) did not inhibit this activity. These results suggest that the *C*4 like the *C*2 oxo group of uracil is essential for binding to DHUDase. This finding rules out the possibility that 1-deazauracil binds in a configuration rotated by 180° around its *C*2–*C*5 axis.

Substitutions at the 5-position. With the exception of 5-aza- (XVIII), 5-carboxy- (XXIII) and 5-hydroxymethyl- (XXIX) uracils, all of the tested heterocycles with various substituents at *C*5 were inhibitors of DHUDase (Table 1).

The 5-hydroxy- of 5-hydroxy- (XXX) or 5-dihydroxyboryl- (XXVII) as well as 5-mercapto- (XXXII) and 5-carboxy- (XXIII) uracils carry groups that are largely negatively charged [27, 29]. 5-Hydroxy-, 5-dihydroxyboryl- and 5-mercaptouracils bound well to DHUDase. The inability of 5-carboxyuracil to bind DHUDase, on the other hand, could arise from the bulky carboxyl group interfering with binding to the enzyme.

Uracils substituted with electron-withdrawing groups at the 5-position, e.g. 5-fluoro- (XXVIII), 5-bromo- (XXII), 5-chloro- (XXIV), 5-iodo- (XXXI) and 5-nitro- (XXXIII) uracils, all bound well. These substitutions are known to increase the acidity of the uracil ring [27, 29]. It is to be noted here, however, that the halogenated compounds are better substrates for this enzyme than either uracil or thymine ([1, 6] and Table 2). This fact bars any assessment of local substituent effects, since the reported K_i values are gross approximations.

5-Aminouracil (XIX) also bound DHUDase well. Since the methyl group of thymine [4] and perhaps also the amino (pK_a of aniline is 9.3 [30]) group of 5-aminouracil are electron-donating groups while hydroxy and mercapto are electron-accepting groups, it would seem that what affects the binding to the enzyme is not the nature of the group at *C*5, i.e. electron-accepting or electron-donating, but rather the effect of localized electron density at *C*5 and its subsequent effect on the oxygen atoms at *C*2 and *C*4, which are shown in this study to be essential for a ligand to bind the enzyme. It is to be noted that, as mentioned above, ionized uracils assume an anion form in which the negative charge resonates between the *C*2 and *C*4 positions [24]. Moreover, uracils protonate at oxygen to yield mono- or dications, the protonation at the *C*4-oxygen being considerably more stable than that at the *C*2-oxygen [24]. In either case, the electron density at oxygens *C*2 and *C*4 are presumably perturbed. We propose that these perturbations modulate binding to the enzyme. In this context, substituting a carbon, i.e. a less electronegative atom, for *N*1 or *N*3 differentially improved the binding to the enzyme. Indeed, replacement of *N*3 which is adjacent to both oxo functions with a carbon atom must create a localized electron density different from that brought about

Table 1. Inhibitory potencies of pyrimidine base analogues for dihydrouracil dehydrogenase from mouse liver cytosol

	Compound	Source	Apparent K_i (μM)
I	Uracil	SIGMA	9.3 ± 9.0*
1-Position			
II	Uridine	SIGMA	336.9 ± 165.6
III	Acyclouridine [1-(2'-hydroxyethoxymethyl)uracil]	SIGMA	272 ± 98
IV	1-Cyclohexyluracil	VEGA	77.6 ± 28.6
V	1-Benzyluracil	VEGA	19.7 ± 5.2
VI	1-Ethyluracil	VEGA	43.9 ± 0.9
VII	1-Methyluracil	VEGA	163 ± 27.1
VIII	2,6-Pyridinediol ("1-deazauracil")	ALD	0.5 ± 0.005
2-Position			
IX	1-Amino-4-hydroxypyrimidine (isocytosine)	SIGMA	>1000
X	4-Hydroxypyrimidine ("2-deoxyuracil")	VEGA	172 ± 19
XI	2-Thiouracil	SIGMA	319 ± 121
3-Position			
XII	2,4-Pyridinediol (3-deazauracil)	SIGMA	2.1 ± 0.2
XIII	3-Methyluracil	SIGMA	>1000
XIV	3-Oxauracil	SIGMA	815 ± 175
4-Position			
XV	Cytosine (4-aminouracil)	SIGMA	544 ± 285
XVI	4-Methylpyrimidine-2-one (4-methyluracil)	CDC	139 ± 32
XVII	Pyrimidine-2-one ("4-deoxyuracil")	VEGA	>1000
5-Position			
XVIII	5-Azauracil	SIGMA	>1000
XIX	5-Aminouracil	SIGMA	4.0 ± 0.3
XX	5-Benzyluracil	SHC	3.4 ± 0.042
XXI	5-Benzyloxybenzyluracil	SHC	0.2 ± 0.005
XXII	5-Bromouracil	SIGMA	8.6 ± 1.0
XXIII	5-Carboxyuracil (isoorotic acid)	SIGMA	>1000
XXIV	5-Chlorouracil	SIGMA	18.5 ± 1.0
XXV	5-Ethyluracil	SHC	15.1 ± 3.9
XXVI	5-Diazouracil	SIGMA	43.0 ± 24
XXVII	5-Dihydroxyboryluracil	RFS	22.0 ± 2.8
XXVIII	5-Fluorouracil	SIGMA	23.5 ± 2.0
XXIX	5-Hydroxymethyluracil	VEGA	200 ± 104
XXX	5-Hydroxyuracil (isobarbituric acid)	SIGMA	20.1 ± 1.8
XXXI	5-Iodouracil	SIGMA	2.8 ± 0.3
XXXII	5-Mercaptouracil	CDC	15.2 ± 1.0
XXXIII	5-Nitrouracil	SIGMA	6.3 ± 0.6
XXXIV	Thymine (5-methyluracil)	SIGMA	7.4 ± 0.3
6-Position			
XXXV	6-Aminouracil	ALD	22.8 ± 1.1
XXXVI	6-Azauracil	SIGMA	111 ± 32
XXXVII	Barbituric acid (6-hydroxyuracil)	SIGMA	120 ± 32
XXXVIII	6-Benzyluracil	SHC	497 ± 342
XXXIX	6-Carboxymethyluracil	SIGMA	78.6 ± 20.2
XL	6-Chloromethyluracil	ALD	>1000
XLI	6-Chlorouracil	VEGA	13.8 ± 0.8
XLII	6-Dihydroxyboryluracil	RFS	26.6 ± 1.8
XLIII	6-Iodouracil	VEGA	187 ± 37
XLIV	Methylorotate (orotic acid methyl ester)	ALD	78.1 ± 34
XLV	6-Methyluracil	SIGMA	259 ± 32
XLVI	Orotic acid (6-carboxyuracil)	SIGMA	>1000
XLVII	Uracil-6-methylsulfone	SIGMA	>1000
More than one position substituted			
XLVIII	5-Aminobarbituric acid (5-amino-6-hydroxyuracil or uramil)	SIGMA	139 ± 62
XLIX	5-Azabarbituric acid (cyanuric acid)	ALD	>1000
L	1,3-Dimethylbarbituric acid	VEGA	>1000
LI	1-Methylbarbituric acid	VEGA	60.8 ± 10.0
LII	5-Nitrobarbituric acid	AP	3.8 ± 0.3
LIII	5-Nitrosobarbituric acid	VEGA	82 ± 11
LIV	Pentobarbital [5-ethyl-5-(1-methylbarbituric acid)]	K&K	>1000
LV	Phenobarbital (5-ethyl-5-phenylbarbituric acid)	MAL	682 ± 307
LVI	2-Thiobarbituric acid	CDC	>1000
LVII	Violuric acid (5-hydroxyiminobarbituric acid)	AP	295 ± 194
LVIII	5-Aza-1-methyluracil	CDC	691 ± 267

Table 1—*continued*

	Compound	Source	Apparent K_i (μM)
LIX	5-Bromo-1-methyluracil	SIGMA	>1000
LX	5-Fluoro-1-methyluracil	VEGA	>1000
LXI	Ftorafur [5-fluoro-1-(tetrahydro-2-furyl-uracil)]	CAL	>1000
LXII	1,3-Dimethyluracil	SIGMA	532 ± 190
LXIII	6-Chloro-1,3-dimethyluracil	VEGA	>1000
LXIV	1,5-Dimethyluracil	VEGA	>1000
LXV	5-Methyl-1-ethyluracil	VEGA	>1000
LXVI	5-Methyl-1-cyclohexyluracil	VEGA	390 ± 154
LXVII	4-Thio-5-methyluracil	VEGA	>1000
LXVIII	6-Aza-5-methyluracil	SIGMA	53.1 ± 14
LXIX	4,6-Dihydroxypyrimidine ("5-aza-3-deazauracil")	ALD	20.5 ± 4.2
LXX	4,6-Dihydroxy-2-methylpyrimidine	CDC	63.0 ± 16
LXXI	4,6-Dihydroxy-2-mercaptopyrimidine	CDC	>1000
LXXII	4,6-Dihydroxy-2-aminopyrimidine	VEGA	169 ± 35
LXXIII	4,5-Dihydroxy-2-methylpyrimidine	VEGA	126 ± 68
LXXIV	5-Bromo-2,4-dimethoxypyrimidine	VEGA	>1000
LXXV	6-*n*-Propyl-2-thiouracil	VEGA	>1000
LXXVI	6-Benzyl-2-thiouracil	SIGMA	378 ± 261
LXXVII	2,4-Dithiouracil	SIGMA	>1000
LXXVIII	4-Amino-6-hydroxy-2-mercaptopyrimidine	ALD	>1000
LXXIX	4-Amino-5-bromo-2-hydroxypyrimidine	VEGA	132 ± 43
LXXX	4-Amino-5-fluoro-2-hydroxypyrimidine	VEGA	>1000
LXXXI	4-Oxo-2,6-dicarboxypyridine (chelidamic acid)	ALD	>1000
LXXXII	2-Hydroxy-6-methylpyridine	ALD	236 ± 85
LXXXIII	5-Aminoorotic acid	SIGMA	>1000
LXXXIV	5-Azaorotic acid (oxonic acid)	ALD	>1000
LXXXV	5-Bromoorotic acid	CDC	124 ± 72
LXXXVI	5-Chloroorotic acid	CDC	52.6 ± 13.3
LXXXVII	5.Fluoroorotic acid	PL	169 ± 148
LXXXVIII	5-Iodoorotic acid	SIGMA	79.4 ± 36
LXXXIX	5-Nitroorotic acid	ALD	72.0 ± 38
XC	DL-5,6-Dihydroorotate	SIGMA	>1000
XCI	L-5,6-Dihydroorotate	VEGA	>1000
XCII	2-Thioorotic acid	VEGA	>1000
XCIII	4-Hydroxy-2-methylthioorotate	VEGA	362 ± 159
XCIV	5,6-Diaminouracil	SIGMA	135 ± 122
XCV	5,6-Dioxyuracil (alloxan)	SIGMA	6.6 ± 4.2
XCVI	5,6-Dihydrouracil	SIGMA	272 ± 216
XCVII	6-Amino-5-nitrouracil	GZ	64.4 ± 4.1
XCVIII	5-Fluoroacyclouridine [5-fluoro-1-(2'-hydroxyethoxymethyl)uracil]	SHC	544 ± 498
Miscellaneous			
XCIX	Adenine	PL	>1000
C	Guanine	SIGMA	>1000
CI	Hypoxanthine	SIGMA	>1000
CII	Xanthine	SIGMA	256 ± 73
CIII	Allopurinol	SIGMA	>1000
CIV	Hydantoin	SIGMA	453 ± 270
CV	Maleimide	ALD	286 ± 89

Values are means ± SD, N = 3.
* Apparent K_m value for uracil was determined at [NADPH] = 100 μM.

by the replacement of *N*1 which is adjacent only to the oxo function at *C*2.

On the other hand, the halogens at *C*5 of uracils also possess hydrophobic characteristics [29, 31], so that hydrophobicity may play a role in the binding to DHUDase. Indeed, when compared to the binding of 5-methyluracil (XXXIV), lack of hydrophobicity [29, 32] apparently is the factor affecting the binding of 5-hydroxymethyluracil (XXIX). Moreover, DHUDase like uridine phosphorylase accepts uracil as substrate or product. It has been suggested [29, 31] that a hydrophobic pocket exists on uridine phosphorylase adjacent to the binding site of the *C*5 of uracil. A similar pocket appears to exist on DHUDase around the binding site of the *C*5 of uracil. Such a pocket would explain the binding of 5-halogenated compounds regardless of their charge characteristics as well as that of 5-ethyl- (XXV) (Table 1), 5-propyl- and 5-isopropyluracils (Table 3). Indeed 5-ethyl- (XXV), 5-benzyl- (XX) and 5-benzyloxybenzyl- (XXI) uracils proved inhibitory, indicating the existence of such a pocket.

In light of these findings, it is possible to explain the binding of 1-benzyl- (V) and 1-ethyl- (VI) uracil

Table 2. Alternate substrates of dihydrouracil dehydrogenase from various mammalian sources

Alternate substrate	% Activity	Reference substrate*	[mM]†	Enzyme source	Ref.
Uracil	125	Thy	0.02	Rat liver	6
5-Bromouracil	122‡	Ura	0.10	Rat liver	13
	85	Ura	0.17	Rat liver	4
	134	Ura	0.17	Hamster liver	4
	168	Thy	0.020	Rat liver	6
5-Chlorouracil	87	Ura	0.17	Rat liver	
	118	Ura	0.17	Hamster liver	4
5-Cyanouracil	16	Ura	0.17	Rat liver	
	236	Ura	0.17	Hamster liver	4
5-Diazouracil	77	Thy	0.02	Rat liver	6
5-Fluorouracil	108	Ura	0.17	Rat liver	4
	150	Ura	0.17	Hamster liver	4
	170	Thy	0.02	Rat liver	6
5-Iodouracil	87	Ura	0.17	Rat liver	
	103	Ura	0.17	Hamster liver	4
	75	Thy	0.02	Rat liver	6
5-Nitrouracil	"Little"	Ura	0.10	Rat liver	13
	17	Thy	0.02	Rat liver	6
Thymine (5-methyluracil)	75	Ura	0.17	Rat liver	4
	59	Ura	0.17	Hamster liver	4

* Substrate to which 100% activity is assigned, when assayed under the same conditions and concentration as the alternate substrate.

† Concentration of alternate substrate and reference substrate.

‡ As calculated from cited reference.

to DHUDase. It has been suggested that the binding of 1-benzyl- or 1-ethyluracil to uridine phosphorylase [31] occurs in the configuration 1-deaza,5-aza, i.e. with the analogue rotated 180° about the *N*3–*C*6 axis so as to interact with the hydrophobic pocket of the 5-position. A similar situation could hold true for DHUDase. Alternatively, the hydrophobic pocket where the *C*5 of uracil binds DHUDase may accommodate the binding of large hydrophobic substituents at *N*1 (i.e. 1-benzyl- and 1-ethyl-). The relatively small 1-methyluracil. (VII) would be too short to reach into that pocket, without involving a rotational movement of the ligand. This speculation is strengthened by the fact that while 5-methyluracil (XXXIV) bound well, 1-methyl abolished the binding of uracil.

Substitutions at the 6-position. Of the pyrimidines with substituents at *C*6, only 6-amino (XXXV), 6-dihydroxyboryl- (XLII) and 6-chloro- (XLI) uracils bound well. Surprisingly, 6-iodouracil (XLIII) did not inhibit DHUDase. It is suggestive that the chloro group is more electronegative but less hydrophobic than an iodo group [29, 32]. It is also possible, however, that the larger iodo (2.2 Å) interferes with the binding while the chloro (1.8 Å) does not. Interestingly, neither orotic acid (XLVI) nor 6-methyluracil (XLV) bound to DHUDase, yet both 6-carboxymethyluracil. (XXXIX) and methylorotate (XLIV) inhibited this enzyme to the same extent. 6-Chloromethyluracil (XL), 6-benzyluracil (XXXVIII) and uracil-6-methylsulfone (XLVII), on the other hand, did not bind at all. With respect to more than one substitution, a 5-methyl- improved the binding of 6-azauracil (XXXVI, LXVIII) and a 5-nitro- that of barbituric acid (XXXVII, LII), while it impaired the binding of 6-aminouracil (XXXV, XCVII).

Substitutions at more than one position. Among this group of substituents, 5-nitrobarbituric acid (LII) and alloxan (XVC) bound best, whereas 5,6-diaminouracil (XCIV) did not bind well.

In conclusion: (1) replacement of *N*1 or *N*3 by an endocyclic carbon enhanced binding, (2) additions to *N*1 and *N*3, however, were not generally tolerated by DHUDase, (3) binding to DHUDase required intact *C*2 and *C*4 (*C*6) oxo groups, (4) replacement of *C*5 or *C*6 by an endocyclic nitrogen decreased binding, (5) addition of a hydrophobic group to *C*5 but not *C*6 increased the binding, (6) amino- and chloro- substituents at *C*6 were acceptable, (7) certain 5,6 combinations, especially those involving largely negatively or positively charged but not hydrophobic groups at *C*6, with hydrophobic groups at *C*5 improved the binding.

Designs of new inhibitors of DHUDase. Based on the structure–activity relationship formulated in the present study and the assumption that combining functional groups that individually enhance the binding of uracil to DHUDase may have an additive effect, we propose the synthesis and evaluation of 5-benzyloxybenzyl-1-deazauracil and 5-benzyloxybenzyl-3-deazauracil as inhibitors of DHUDase. Similarly, since attaching a nitro group to barbituric acid increased the binding to DHUDase, 5-benzyloxybenzylbarbituric acid may display potent inhibitory activity towards this enzyme. The simi-

Table 3. Inhibitors of dihydrouracil dehydrogenase from various mammalian sources

Inhibitor	[mM]	% Inhibition	Substrate*	[mM]	Enzyme source	Ref.
Uracil	1.0	42	IUra	0.2	Rat liver	17
	0.018	50	FUra	0.01	Rat liver	20
	0.156	20	Thy	0.078	Rat liver	15
1-Position						
1-Deazauracil (2,6-dihydroxypyridine)	0.0007	50	FUra	0.01	Rat liver	20
	0.0002	50†	Ura	0.025	Mouse liver	18
Uridine (1-Ribosyluracil)	0.5	48	Ura	0.5	Rat liver	14
	0.156	48	Ura	0.078	Rat liver	15
2-Position						
3-Position						
3-Deazauracil (2,4-dihydroxypyridine)	0.009	50	FUra	0.01	Rat liver	20
	0.002	50†	Ura	0.025	Mouse liver	18
4-Position						
4-Thiouracil	0.156	20	Ura	0.078	Rat liver	15
5-Position						
5-Aminouracil	0.5	41	Ura	0.5	Rat liver	14
	0.01	16	Thy	0.01	Pig liver	16
	0.019	50	FUra	0.01	Rat liver	20
	0.156	68	Ura	0.078	Rat liver	15
5-Bromouracil	0.01	31	Thy	0.01	Pig liver	16
	0.6	63	IUra	0.2	Rat liver	17
	0.1	75	Ura	0.1	Mouse liver	11
	0.018	50	FUra	0.01	Rat liver	20
	0.156	87	Ura	0.078	Rat liver	15
(*E*)-5-(2-bromovinyl)uracil	0.004	17	FUra	0.02	Rat liver	7
		76‡				7
	>0.1	50	FUra	0.01	Rat liver	20
5-Chlorouracil	0.026	50	FUra	0.01	Rat liver	20
	0.156	85	Ura	0.078	Rat liver	15
5-Cyanouracil	0.025	47†	IUra	0.2	Rat liver	17
		50‡				17
Diazouracil	0.01	27	Thy	0.01	Pig liver	16
	0.025	38†	IUra	0.2	Rat liver	17
	0.025	97‡	IUra	0.2	Rat liver	17
	0.003	50	FUra	0.19	Human liver	19
5-Ethyluracil	0.5	50	Ura	0.5	Rat liver	14
	0.156	46	Ura	0.078	Rat liver	15
5-Fluorouracil	0.01	19	Thy	0.01	Pig liver	16
	1.0	58	IUra	0.2	Rat liver	17
	0.156	83	Ura	0.078	Rat liver	15
5-Hydroxymethyluracil	>0.1	50	FUra	0.01	Rat liver	20
	0.01	15	Thy	0.01	Pig liver	16
5-Hydroxyuracil	0.5	38	Ura	0.5	Rat liver	14
	0.01	17	Thy	0.01	Pig liver	16
	0.156	22	Ura	0.078	Rat liver	15
5-Iodouracil	0.01	29	Thy	0.01	Pig liver	16
	0.033	50	FUra	0.01	Rat liver	20
	0.156	72	Ura	0.078	Rat liver	15
5-Isopropyluracil	0.156	20	Ura	0.078	Rat liver	15
5-Nitrouracil	0.5	61	Ura	0.5	Rat liver	14
	0.01	28	Thy	0.01	Pig liver	16
	0.156	55	Ura	0.078	Rat liver	15
5-Propyluracil	0.156	61	Ura	0.078	Rat liver	15
5-Thiouracil	0.156	48	Ura	0.078	Rat liver	15
Thymine (5-methyluracil)	1.0	75	IUra	0.2	Rat liver	17
	0.08	50	FUra	0.19	Human liver	19
	0.008	50	FUra	0.01	Rat liver	20
	0.156	90	Ura	0.078	Rat liver	15
6-Position						
Barbituric acid (6-hydroxyuracil)	>0.05	50	FUra	0.01	Rat liver	20
Orotic acid (6-carboxyuracil)	0.5	35	Ura	0.5	Rat liver	14

Table 3—*continued*

Inhibitor	[mM]	% Inhibition	Substrate*	[mM]	Enzyme source	Ref.
More than one position substitutions						
5-Bromo-6-azauracil	0.5	17	Ura	0.5	Rat liver	14
5-Methyl-6-azauracil	0.6	17	Ura	0.5	Rat liver	14
	0.1	20	Ura	0.1	Mouse liver	11
	0.156	13	Ura	0.078	Rat liver	15
5-Aldehydobarbituric acid	0.004	50	FUra	0.01	Rat liver	20
5-Aminobarbituric acid	>0.05	50	FUra	0.01	Rat liver	20
5-Bromobarbituric acid	0.005	50	FUra	0.01	Rat liver	20
5-Chlorobarbituric acid	0.002	50	FUra	0.01	Rat liver	20
5-Hydroxybarbituric acid	>0.05	50	FUra	0.01	Rat liver	20
5-Methylbarbituric acid	0.007	50	FUra	0.01	Rat liver	20
5-Nitrobarbituric acid	0.003	50	FUra	0.01	Rat liver	20
5-Bromoorotic acid	0.5	14	Ura	0.5	Rat liver	14
5-Bromo-2,4-dihydroxypyridine	0.00008	50	FUra	0.01	Rat liver	20
5-Chloro-2,4-dihydroxypyridine	0.0001	50	FUra	0.01	Rat liver	20
5-Methyl-2,4-dihydroxypyridine	0.003	50	FUra	0.01	Rat liver	20
5-Chloro-2,6-dihydroxypyridine	0.00002	50	FUra	0.01	Rat liver	20
5-Cyano-2,6-dihydroxypyridine	0.00006	50	FUra	0.01	Rat liver	20
5-Nitro-2,6-dihydroxypyridine	0.00006	50	FUra	0.01	Rat liver	20
4,6-Dihydroxypyrimidine	0.04	50	FUra	0.01	Rat liver	20
5-Bromo-4,6-dihydroxypyrimidine	0.024	50	FUra	0.01	Rat liver	20
5-Chloro-4,6-dihydroxypyrimidine	0.032	50	FUra	0.01	Rat liver	20
5-Ethyl-4,6-dihydroxypyrimidine	>0.05	50	FUra	0.01	Rat liver	20
5-Methyl-4,6-dihydroxypyrimidine	>0.05	50	FUra	0.01	Rat liver	20
5-Aminomethyl-2,6-dihydroxypyrimidine	>0.05	50	FUra	0.01	Rat liver	20
5,6-Dihydrothymine	1.0	5	IUra	0.2	Rat liver	17
5,6-Dioxyuracil (alloxan)	0.002	50†	Ura	0.025	Mouse liver	18
5-Bromo-5,6-dihydrouracil	0.156	23	Ura	0.078	Rat liver	15
6-Aminothymine	0.1	45	Ura	0.1	Mouse liver	11
6-Methoxy-5-diazouracil	0.003	50	FUra	0.19	Human liver	19
5-Methyl-6-azauridine	0.5	11	Ura	0.5	Rat liver	14
5-Bromouridine	0.156	42	Ura	0.078	Rat liver	15
5-Fluorouridine	0.156	38	Ura	0.078	Rat liver	15
5-Methyluridine	0.08	50	FUra	0.19	Human liver	19
5-Nitrouridine	0.5	33	Ura	0.5	Rat liver	14
5-Bromo-2′-deoxyuridine	1.0	73	IUra	0.2	Rat liver	17
Thymidine	0.156	48	Ura	0.078	Rat liver	15

* Substrate used and its concentration.
† As calculated from cited reference.
‡ With preincubation.

Table 4. Analogues reported to be non-inhibitory to dihydrouracil dehydrogenase from various mammalian sources

Compound	[mM]	Enzyme source	Substrate*	[mM]	Ref.
1-Position					
1-Methyluracil	0.5	Rat liver	Ura	0.5	14
	0.156	Rat liver	Ura	0.078	15
	0.156	Rat liver	Thy	0.078	15
2-Position					
2-Methylthiouracil	0.156	Rat liver	Ura	0.078	15
2-Thiouracil	0.01	Pig liver	Thy	0.01	16
	0.156	Rat liver	Ura	0.078	15
3-Position					
3-Methyluracil	0.5	Rat liver	Ura	0.5	14
	0.01	Pig liver	Thy	0.01	16
	0.156	Rat liver	Ura	0.078	15
4-Position					
Cytosine (4-aminouracil)	0.01	Pig liver	Thy	0.01	16
	0.156	Rat liver	Ura	0.078	15
4(6)-Methyluracil	0.01	Pig liver	Thy	0.01	16
Uracil 4(6)-acetic acid	0.01	Pig liver	Thy	0.01	16

Table 4—*continued*

Compound	[mM]	Enzyme source	Substrate*	[mM]	Ref.
5-Position					
5-Azauracil	0.5	Rat liver	Ura	0.5	14
5-Benzyluracil	0.156	Rat liver	Ura	0.078	15
5-Carboxyuracil	0.01	Pig liver	Thy	0.01	16
	0.156	Rat liver	Ura	0.078	15
5-Hydroxymethyluracil	0.156	Rat liver	Ura	0.078	15
5-Ribosyluracil	0.5	Rat liver	Ura	0.5	14
6-Position					
6-Azauracil	0.5	Rat liver	Ura	0.5	14
	0.01	Pig liver	Thy	0.01	16
	0.156	Rat liver	Ura	0.078	15
6-Methyluracil	0.156	Rat liver	Ura	0.078	15
Orotic acid (6-carboxyuracil)	0.01	Pig liver	Thy	0.01	16
	0.156	Rat liver	Ura	0.078	15
More than one position substitutions					
4-Amino-6-azauracil	0.01	Pig liver	Thy	0.01	16
5-Methyl-6-azauracil	0.01	Pig liver	Thy	0.01	16
1-Methyl-6-azauracil	0.5	Rat liver	Ura	0.5	14
3-Methyl-6-azauracil	0.5	Rat liver	Ura	0.5	14
1,3-Dimethyl-6-azauracil	0.5	Rat liver	Ura	0.5	14
1-Methyl-5-azauracil	0.5	Rat liver	Ura	0.5	14
3-Methyl-5-azauracil	0.5	Rat liver	Ura	0.5	14
2-Thio-4-aminouracil	0.01	Pig liver	Thy	0.01	16
	0.156	Rat liver	Ura	0.078	15
4-Amino-5-methyluracil	0.156	Rat liver	Ura	0.078	15
4-Amino-5-hydroxymethyluracil	0.01	Pig liver	Thy	0.01	16
	0.156	Rat liver	Ura	0.078	15
4-Amino-5-carboxyuracil	0.01	Pig liver	Thy	0.01	16
1-Methyl-5-nitrouracil	0.5	Rat liver	Ura	0.5	14
3-Methyl-5-nitrouracil	0.5	Rat liver	Ura	0.5	14
1,3-Dimethyl-5-nitrouracil	0.5	Rat liver	Ura	0.5	14
5-Fluoroorotic acid	0.01	Pig liver	Thy	0.01	16
1,3-Dimethyluracil	0.5	Rat liver	Ura	0.5	14
1,6-Dimethyluracil	0.5	Rat liver	Ura	0.5	14
5,6-Dihydrothymine	0.156	Rat liver	Ura	0.078	15
5,6-Dihydrouracil	1.0	Rat liver	IUra	0.2	17
	0.156	Rat liver	Ura	0.078	15
5-Bromo-5,6-dihydrouracil	0.156	Rat liver	Ura	0.078	15
5-Chloro-5,6-dihydrouracil	0.156	Rat liver	Ura	0.078	15
6-Methyl-5,6-dihydrouracil	0.156	Rat liver	Ura	0.078	15
6-Methyl-5-bromo-5,6-dihydrouracil	0.156	Rat liver	Ura	0.078	15
5,6-Dihydro-2-thiouracil	0.156	Rat liver	Ura	0.078	15
5-Bromo-5,6-dihydro-2-thiouracil	0.156	Rat liver	Ura	0.078	15
5-Todo-5,6-dihydro-2-thiouracil	0.156	Rat liver	Ura	0.078	15
Propyl-thiouracil	0.01	Pig liver	Thy	0.01	16
2-Thiouracil-5-carbonic acid	0.01	Pig liver	Thy	0.01	16
2-Thio-5-methyluracil	0.01	Pig liver	Thy	0.01	16
	0.156	Rat liver	Ura	0.078	15
6-Methyl-2-methylthiouracil	0.156	Rat liver	Ura	0.078	15
5-Chloro-6-methyl-2-thiouracil	0.156	Rat liver	Ura	0.078	15
5-Iodo-6-methyl-2-thiouracil	0.156	Rat liver	Ura	0.078	15
5-Hydroxy-6-methylpyrimidine	0.01	Pig liver	Thy	0.01	16
6-Carboxy-4-hydroxy-2-mercaptopyrimidine	0.01	Pig liver	Thy	0.01	16
6-Azauridine	0.5	Rat liver	Ura	0.5	14
	0.01	Pig liver	Thy	0.01	16
5-Ethyluridine	0.156	Rat liver	Ura	0.078	15
	0.156	Rat liver	Thy	0.078	15
Cyclo-5-diazouridine	2.0	Human liver	FUra	0.19	19
Thymine riboside	0.01	Pig liver	Thy	0.01	16
5-Hydroxyuridine	0.01	Pig liver	Thy	0.01	16
3-Methyluridine	0.5	Rat liver	Ura	0.5	14
β-Alanine	0.156	Rat liver	Ura	0.078	15
N-Carbamyl-β-alanine	0.156	Rat liver	Ura	0.078	15

* Substrate used and its concentration.

larities between the structure–activity relationship of DHUDase and uridine phosphorylase [29] suggest a close resemblance between the active sites of these two enzymes. Therefore, the proposed new compounds may also prove inhibitory to uridine phosphorylase. In addition, the similarities between their active sites suggest that DHUDase and uridine phosphorylase, two consecutive enzymes on the pyrimidine salvage/catabolism pathway, may have evolved from the same ancestral protein.

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