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STRUCTURE–ACTIVITY RELATIONSHIP OF NUCLEOBASE LIGANDS OF URIDINE PHOSPHORYLASE FROM *TOXOPLASMA GONDII*

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Abstract—Seventy-nine nucleobase analogs were evaluated as potential inhibitors of *Toxoplasma gondii* uridine phosphorylase (UrdPase), and the apparent K_i (appK_i) values for these compounds were determined. Based on the inhibition data, a structure–activity relationship for the binding of nucleobase analogs to the enzyme was formulated, using uracil as a reference compound. Two compounds were identified as very potent inhibitors of *T. gondii* UrdPase, 5-benzyloxybenzylbarbituric acid and 5-benzyloxybenzyluracil, which had appK_i values of 0.32 and 2.5 μM, respectively. A comparison of the results from the present study, with similar studies on mammalian UrdPase and thymidine phosphorylase (dThdPase) (Niedzwicki *et al.*, *Biochem Pharmacol* **32**: 399–415, 1993) revealed that there are both similarities and differences between the catalytic site of *T. gondii* UrdPase and the catalytic sites of the mammalian enzymes with respect to binding of uracil analogs. One compound, 6-benzyl-2-thiouracil, was identified as a potent, specific inhibitor (appK_i = 14 μM) of *T. gondii* UrdPase, relative to mammalian UrdPase and dThdPase.

Toxoplasmosis, which is caused by the obligate intracellular protozoa *Toxoplasma gondii* (for review, see Ref. 1), is one of the most commonly recognized opportunistic infections of the central nervous system in individuals suffering from acquired immunodeficiency syndrome (AIDS)† [2]. Clinically apparent infections have been reported to occur in 3–40% of AIDS patients [2], and often result in severe or fatal central nervous system disease. Although the primary treatment for toxoplasmosis (a combination of pyrimethamine and sulfadiazine) is effective initially, treatment must often be discontinued due to the toxicity associated with this therapy [2, 3]. Therefore, new chemotherapeutic agents for the treatment of this disease need to be identified.

One potential chemotherapeutic target in *T. gondii* is pyrimidine nucleotide synthesis. *T. gondii* are capable of synthesizing pyrimidine nucleotides either *de novo*, or by salvage pathways that utilize preformed pyrimidine nucleobases or nucleosides [4–11]. In contrast to mammalian cells, the salvage of pyrimidine nucleosides in *T. gondii* does not occur by direct phosphorylation to nucleoside 5′-monophosphates, as *T. gondii* lack any detectable pyrimidine nucleoside kinase or phosphotransferase activity [5]. Therefore, nucleosides must first be converted to the nucleobase uracil in order to be salvaged by *T. gondii* [5, 11]. Cytidine and deoxycytidine are deaminated to uridine and deoxyuridine, respectively, while uridine and deoxyuridine are cleaved to uracil by pyrimidine nucleoside phosphorylase activity [5]. The pyrimidine ring is then salvaged via metabolism of uracil to uridine 5′-monophosphate by a specific uracil phosphoribosyltransferase (EC 2.4.2.9) [5, 11].

In general, pyrimidine nucleoside phosphorylases catalyze the reversible phosphorolysis of pyrimidine nucleosides and deoxynucleosides to their respective nucleobases and either ribose-1-phosphate or deoxyribose-1-phosphate. In mammalian cells, two distinct pyrimidine nucleoside phosphorylases, uridine phosphorylase (UrdPase, EC 2.4.2.3) and thymidine phosphorylase (dThdPase, EC 2.4.2.4), have been identified [12, 13]. UrdPase preferentially cleaves nucleosides, but will also accept deoxynucleosides as substrates, whereas dThdPase prefers deoxynucleosides as substrates and will not cleave nucleosides. In contrast, *T. gondii* appear to have a single, non-specific UrdPase that is responsible for the reversible phosphorolysis of both nucleosides and deoxynucleosides including uridine, deoxyuridine and thymidine [5]. Thus, inhibition of UrdPase in *T. gondii* should inhibit the salvage of all pyrimidine nucleosides in these parasites.

In the present study, seventy-nine nucleobase analogs were evaluated as potential inhibitors of *T. gondii* UrdPase, and apparent K_i (appK_i) values for these compounds were determined. Based on the inhibition data, a structure–activity relationship for the binding of nucleobase analogs to *T. gondii* UrdPase was formulated, in order to provide a basis for the rational design of more potent inhibitors of this enzyme. In addition, the results from the present study were compared with those from a similar study on mammalian UrdPase and dThdPase [14], in order

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† Abbreviations: AIDS, acquired immunodeficiency syndrome; appK_i, apparent K_i; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; dThdPase, thymidine phosphorylase; and UrdPase, uridine phosphorylase.

to identify and/or design analogs that may be specific inhibitors of the parasite enzyme.

MATERIALS AND METHODS

Materials

Chemicals and supplies. [2-^{14}C]Uridine (56 mCi/mmol) was obtained from Moravek Biochemicals, Inc. (Brea, CA); Scintilene scintillation fluid was from Fisher Scientific (Pittsburgh, PA); silica gel G/UV_{254} Polygram thin-layer chromatography plates were from Brinkmann (Westbury, NY); and Bio-Rad protein assay kits were from Bio-Rad Laboratories (Richmond, CA). 6-Amino-5-nitroso-2-thiouracil, 6-chloromethyluracil, cyanuric acid, 5,6-dihydrouracil, 2,6-dihydroxypyridine, 2-methylmercaptobarbituric acid, trithiocyanuric acid and violuric acid were obtained from the Aldrich Chemical Co., Inc. (Milwaukee, WI), and 6-aza-2-thiouracil was from American Bioorganics (North Tonawanda, NY). 1-Benzyluracil, 5-benzyluracil, 5-benzyloxybenzyluracil, 5-benzyloxybenzylbarbituric acid and 6-benzyluracil were provided by Dr. Mahmoud el Kouni, Department of Pharmacology, University of Alabama at Birmingham. Emimycin (2-hydroxypyrazine-4-oxide) was a gift from Dr. Elmer Pfefferkorn, Department of Microbiology, Dartmouth Medical School. All other chemicals and compounds were obtained from the Sigma Chemical Co. (St. Louis, MO).

Source of T. gondii. Tachyzoites of the RH strain of *T. gondii* (obtained from Dr. Jack Remington, Stanford University, Stanford, CA) were propagated by intraperitoneal passage in 18–22 g female Swiss–Webster mice (Sasco, Inc., Omaha, NE) as previously described [5]. The purity and concentration of the parasite preparations were determined microscopically using a hemacytometer and were found to contain on average 97.0% *T. gondii*, relative to the total number of cells (parasites plus host cells).

Preparation of stock solutions. In general, a stock solution of the compound to be tested was made in 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-Cl (pH 8.0)/1 mM dithiothreitol, tested the same day, and then stored at −20° for subsequent retrials of the compound. In some cases (3-oxauracil and 6-benzyl-2-thiouracil), however, stock solutions were made fresh for each trial because previously prepared stock solutions did not give reproducible results. It should also be noted that the compound 5-benzyloxybenzyluracil was prepared in 100% dimethyl sulfoxide due to its insolubility at high concentrations in aqueous systems.

Methods

Preparation of cytosol extracts. Approximately 5×10^8 *T. gondii* were suspended in 1.2 mL of 50 mM HEPES-Cl (pH 8.0)/1 mM dithiothreitol and homogenized for 30 sec at setting 10, using a Brinkmann Instruments Polytron homogenizer fitted with a PTA 7K1 probe. The homogenate was then centrifuged at approximately 116,000 *g* for 1 hr at 5°, and the supernatant (cytosol extract) was collected.

UrdPase assay. UrdPase activity was measured by following the formation of [^{14}C]uracil from [^{14}C]-uridine in the presence of inorganic phosphate, at the optimal pH (8.0) for the *T. gondii* enzyme (results not shown). The standard reaction mixture contained 50 mM HEPES-Cl (pH 8.0), 1 mM dithiothreitol, 50 μM [2-^{14}C]uridine (5 mCi/mmol), 10 mM potassium phosphate (pH 8.0), 15–30 μL of cytosol extract (approximately 5–10 μg of protein), and either 0, 0.25, 0.5, 1.0 or 2.0 mM of the compound to be tested, in a final volume of 150 μL. In some cases, lower concentrations of the compound were used for very potent inhibitors, or inhibitors that were poorly soluble. Compounds that were found to be poor inhibitors were tested at concentrations higher than 2.0 mM to more accurately determine the appK_i value. When 5-benzyloxybenzyluracil was tested, the reaction mixtures contained 6.7% dimethyl sulfoxide in addition to the standard reaction mixture.

Reactions were started by the addition of [^{14}C]-uridine, incubated at 37° for 10 min, and terminated by placing the reaction tubes in a boiling water bath for 2 min. Precipitated proteins were removed by centrifugation in a microcentrifuge (approximately 13,000 *g*) for 5 min, and a 15-μL aliquot of the resulting supernatant was mixed with 5 μL of a solution containing 10 mM each of uridine and uracil. This mixture was then spotted on silica gel TLC plates that were developed with a mixture of chloroform : methanol : acetic acid (45 : 5 : 1). The average R_f values for uridine and uracil were 0.09 and 0.39, respectively. The uridine and uracil spots were identified by UV quenching and cut out, and the radioactivity was quantified by liquid scintillation counting in 20 mL of Scintilene using a Packard 460 scintillation counter. All assays were run under conditions in which velocity was linear with respect to time and amount of cytosol extract.

Calculations and statistical analysis. Enzyme velocity was calculated by multiplying the fraction of uracil formed from uridine times the amount of uridine in the assay, and dividing by the incubation time. AppK_i values were estimated from Dixon plots of the data ($1/v$ versus $[I]$) using a computer program that employs the general principles of Cleland [15]. This program was developed by Dr. Sungman Cha (Brown University, Providence, RI) and fitted into IBM BASIC by Dr. Fardos N. M. Naguib (University of Alabama at Birmingham, Birmingham, AL). If a compound is a competitive inhibitor with respect to uridine, appK_i values are related to K_i values by the following equation [14]: $\text{app}K_i = K_i(1 + [S]/K_m)$. In the present study, the concentration of uridine (50 μM) was at its approximate K_m value [5] and the concentration of phosphate (10 mM) was at a saturating concentration (results not shown) for *T. gondii* UrdPase. Thus, the appK_i value determined for a competitive inhibitor would be about 2-fold higher than the K_i. It should be noted, however, that the type of inhibition (i.e. competitive, noncompetitive, or uncompetitive) produced by the compounds was not determined, nor were the compounds evaluated as substrates for *T. gondii* UrdPase.

Protein determinations. Protein concentrations were determined by the method of Bradford [16], using the Bio-Rad Laboratories protein assay kit and bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Determination of apparent K_i *values*

Seventy-nine nucleobase analogs were screened as potential inhibitors of *T. gondii* UrdPase. The mean and range of the appK_i values for these compounds, determined from at least two separate estimations of the appK_i, are shown in Table 1. It should be noted that appK_i values were not determined for those compounds that inhibited *T. gondii* UrdPase by less than 10% at the highest concentration tested (2 mM). Also shown in Table 1 is the ratio between the appK_i for the reference compound uracil and the appK_i value for each of the compounds screened. This ratio indicates the fold increase (values > 1) or decrease (values < 1) in binding of the compound, relative to uracil.

Binding of ligands to T. gondii *UrdPase*

Initially, uracil analogs with either endocyclic or exocyclic substitutions at only one position on the ring (see Fig. 1) were tested to determine the effect of simple changes to the uracil ring on binding to the catalytic site of *T. gondii* UrdPase. Based on the results from this initial screen, various multiple-substituted uracil analogs were then tested to determine the effect of combinations of substitutions on the uracil ring. The results shown in Table 1 will be discussed with respect to the effect of those substitutions on binding to *T. gondii* UrdPase and are summarized in Table 2.

1-Position substitutions. 2,6-Dihydroxypyridine can be considered to be an endocyclic-substituted uracil analog in which the *N1* nitrogen has been replaced with a methylene group to make 1-deazauracil [14]. This compound was found to bind to *T. gondii* UrdPase 46-fold better than uracil, indicating that neither the *N1* nitrogen of uracil not the proton associated with the *N1* nitrogen is required for binding. The factors responsible for the enhanced binding of 1-deazauracil are not clear. Given the requirements for the 2- and 4-position oxo groups (see below), it is unlikely that this compound binds to the enzyme in an orientation other than as 1-deazauracil. Furthermore, substitution of a carbon in the pyrimidine ring of uracil to make a pyridine ring (1-deazauracil) does not affect the corresponding bond angles, as both of these ring structures are planar.

Substitution of the hydrogen at the 1-position of uracil with hydrophobic groups (e.g. methyl, cyclohexyl) generally diminished binding, with the exception of a benzyl group (1-benzyluracil) that enhanced binding by about 10-fold. It has been suggested that 1-benzyluracil may bind to mammalian UrdPase in an orientation rotated 180° around the *N3-C6* axis as "5-benzyl-5-aza-1-deazauracil" [14]. Thus, the benzyl group would actually bind to a site on the enzyme which is normally adjacent to the 5-position. Since 1-deazauracil and 5-benzyluracil (see below) both bind well to this enzyme, a similar phenomenon is very likely for *T. gondii* UrdPase.

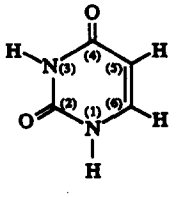

Fig. 1. Structure of the reference compound uracil with numbering system for the pyrimidine ring.

2- and 4-position substitutions. Either an oxo group or a thio group is required at both the 2- and 4-positions for binding to *T. gondii* UrdPase. Substitution of the 2- or 4-position oxo group with an amino group (2-aminouracil, 4-aminouracil), or elimination of one of the oxo groups ("2-deoxyuracil," "4-deoxyuracil"), essentially abolished binding to UrdPase. Similarly, simultaneous substitution and/or elimination of both the 2- and 4-position oxo groups ("2-chloro-4-deoxyuracil," "2-thio-4-deoxyuracil," "2,4-dideoxyuracil," 2,4-dimethyluracil) also severely decreased or abolished binding. Substitution of one or both of the oxo groups with thio groups (2-thiouracil or 2,4-dithiouracil), on the other hand, enhanced binding (about 5- and 2-fold, respectively) as compared with uracil.

The increase in binding of 2-thiouracil, as compared with uracil, may be due to two factors. First, the presence of a thio group at the 2-position increases the per cent ionization of the pyrimidine ring (from 3 to 69%) under the conditions employed [14]. Second, the monoanion of this compound has a negative charge localized on the exocyclic 4-position oxygen, rather than delocalized as is the case for uracil [14]. This is probably due to the higher electronegativity of oxygen as compared with sulfur, which results in an asymmetric distribution of electrons towards the 4-position oxygen. Therefore, it is possible that binding to the catalytic site of *T. gondii* UrdPase is increased when there is an asymmetric distribution of electrons localized on the 4-position. This is supported by the fact that 2,4-dithiouracil, which presumably has a symmetric distribution of electrons, binds about 2-fold more poorly than does 2-thiouracil. On the other hand, substitution of a thio group at the 4-position also enhances binding. Although 4-thiouracil was unavailable for testing, it was found that the analog 4-thiouridine bound 2-fold better to *T. gondii* UrdPase than did uridine (results not shown). Thus, the presence of a thio group at either the 2- or 4-position enhances binding to this enzyme.

3-Position substitutions. Two types of 3-position endocyclic-substituted uracil analogs were tested. In contrast to the *N1* nitrogen, replacement of the *N3* nitrogen with a methylene group (3-deazauracil) abolished binding to *T. gondii* UrdPase. However, the *N3* nitrogen can be replaced with an oxygen (3-oxauracil) with only a slight decrease in binding. The results indicate that the *N3* nitrogen and an undissociated proton at *N3* is preferred, but not required for binding. Substitution of the *N3* hydrogen with either methyl or butyl groups abolished binding. Since the *N3* hydrogen does not appear to be required for binding to *T. gondii* UrdPase, the lack of binding of both 3-methyluracil and 3-butyluracil is probably due to steric factors.

Table 1. AppK_i values for inhibition of *T. gondii* UrdPase by nucleobase analogs*

Compound	AppK_i (μM)	Ratio
Uracil	597 ± 240	1.00
1-Substitutions		
1-Benzyluracil	60 ± 17	9.95
1-Cyclohexyluracil	2,520 ± 110	0.24
1-Deazauracil (2,6-dihydroxypyridine)	13 ± 4	45.9
1-Methyluracil	1,890 ± 560	0.32
2-Substitutions		
2-Aminouracil (isocytosine; 2-amino-4-hydroxypyrimidine)	†	
4-Hydroxypyrimidine ("2-deoxyuracil")	†	
2-Thiouracil (2-thio-4-hydroxypyrimidine)	118 ± 30	5.06
3-Substitutions		
3-Butyluracil	†	
3-Deazauracil (2,4-dihydroxypyridine)	†	
3-Methyluracil	†	
3-Oxauracil	787 ± 260	0.76
4-Substitutions		
4-Aminouracil (cytosine)	‡	
2-Hydroxypyrimidine ("4-deoxyuracil")	†	
5-Substitutions		
5-Aminouracil	2,620 ± 880	0.23
5-Azauracil	798 ± 108	0.75
5-Benzyluracil	49 ± 1	12.2
5-Benzyloxybenzyluracil	2.5 ± 0.3	239
5-Bromouracil	517 ± 184	1.15
5-Carbethoxyuracil	7,350 ± 3,850	0.08
5-Carboxyuracil (isoorotic acid)	4,400 ± 1,000	0.14
5-Chlorouracil	279 ± 48	2.14
5-Diazouracil	1,430 ± 400	0.42
5-Ethyluracil	784 ± 205	0.76
5-Fluorouracil	270 ± 92	2.21
5-Hydroxyuracil (isobarbituric acid)	‡	
5-Hydroxymethyluracil	7,020 ± 2,100	0.09
5-Iodouracil	1,660 ± 720	0.36
5-Methyluracil (thymine)	2,460 ± 1,060	0.24
5-Nitrouracil	75 ± 36	7.96
5-*n*-Propyluracil	441 ± 105	1.35
5-Sulfaminouracil	†	
5-Trifluoromethyluracil	198 ± 76	3.02
6-Substitutions		
6-Aminouracil	1,080 ± 290	0.55
6-Azauracil	†	
Barbituric acid	476 ± 112	1.25
6-Benzyluracil	1,460 ± 80	0.41
6-Carboxyuracil (orotic acid)	12,100 ± 3,300	0.05
6-Carboxymethyluracil (uracil-4-acetic acid)	†	
6-Chlorouracil	103 ± 14	5.80
6-Chloromethyluracil	1,540 ± 130	0.39
6-Methylcarboxyuracil (methylorotate)	6,000 ± 1,860	0.10
6-Methyluracil	3,120 ± 920	0.19
6-Methylsulfoneuracil	5,310 ± 980	0.11
2,4-Substitutions		
4-Amino-2-thiouracil (2-thiocytosine)	5,510 ± 1,290	0.11
2-Chloropyrimidine ("2-chloro-4-deoxyuracil")	†	
2,4-Dimethyluracil (2,4-dimethoxypyrimidine)	‡	
2,4-Dithiouracil (2,4-dithiopyrimidine)	279 ± 105	2.14
2-Mercaptopyrimidine ("2-thio-4-deoxyuracil")	4,320 ± 770	0.14
4-Phenyl-2-thiouracil (4-phenoxy-2-thiopyrimidine)	113 ± 21	5.28
Pyrimidine ("2,4-dideoxyuracil")	†	
2,5-Substitutions		
5-Carbethoxy-2-thiouracil	1,220 ± 10	0.49
5-Carboxy-2-thiouracil	8,850 ± 450	0.07
5-Ethyl-2-thiouracil	652 ± 4	0.92
5-Methyl-2-thiouracil	783 ± 105	0.76
5-*n*-Propyl-2-thiouracil	369 ± 187	1.62

Table 1 (continued). AppK_i values for inhibition of *T. gondii* UrdPase by nucleobase analogs

Compound	AppK_i (μM)	Ratio
2,6-Substitutions		
6-Amino-2-thiouracil	879 ± 138	0.68
6-Aza-2-thiouracil	8,850 ± 780	0.07
6-Benzyl-2-thiouracil	14 ± 6	42.6
2-Methylmercaptobarbituric acid	8,030 ± 220	0.07
6-Methyl-2-thiouracil	1,180 ± 210	0.51
6-*n*-Propyl-2-thiouracil	375 ± 153	1.59
2-Thiobarbituric acid	240 ± 1	2.49
5,6-Substitutions		
5-Aminobarbituric acid (uramil)	‡	
5-Azabarbituric acid (cyanuric acid)	‡	
5-Benzyloxybenzylbarbituric acid	0.32 ± 0.03	1,866
5,6-Dihydrouracil	†	
5-Ethyl-5-(*p*-hydroxyphenyl)barbituric acid	‡	
5-Isonitrosobarbituric acid (violuric acid)	9,250 ± 1,370	0.06
5-Nitrobarbituric acid	614 ± 69	0.97
5-Nitro-6-carboxyuracil (5-nitroorotic acid)	†	
5-Nitro-6-methyluracil	55 ± 2	10.9
Other substitutions		
6-Amino-5-nitroso-2-thiouracil	210 ± 9	2.84
5-Aza-3-deazauracil (4,6-dihydroxypyrimidine)	9,830 ± 1,260	0.06
5-Bromo-1-methyluracil	5,510 ± 2,490	0.11
6-Carboxy-1-deazauracil (citrazinic acid)	†	
2-Hydroxypyrazine-4-oxide (emimycin; "4-aza-3-deazauracil")	1,370 ± 10	0.44
1,3-Benzenediol (resorcinol; "1,3-dideazauracil")	‡	
2,4,6-Trithio-5-azauracil (trithiocyanuric acid)	265 ± 4	2.25

* AppK_i values ± the range were obtained from at least two separate estimations of the appK_i. Ratio = appK_i for uracil/appK_i for the compound.
† Less than 5% inhibition at a concentration of 2 mM.
‡ Less than 10% inhibition at a concentration of 2 mM.

5-Position substitutions. Replacement of the *C5* carbon of uracil with a nitrogen (5-azauracil) decreased binding slightly. This indicates that the *C5* carbon of uracil is preferred but not required for binding to *T. gondii* UrdPase. It should be noted, however, that 5-azauracil is 97% ionized under the conditions employed [17], and the "negative" effects of this substitution may be counterbalanced by this increase in ionization. As discussed below, binding to *T. gondii* UrdPase generally increases as the per cent ionization of the pyrimidine ring increases.

Replacement of the 5-position hydrogen with groups that are more electronegative than hydrogen generally enhanced binding to *T. gondii* UrdPase. Furthermore, this enhancement in binding was in the general order of the electronegativity of the groups (i.e. nitro > fluoro > chloro > bromo > iodo > hydrogen) [14]. Thus, the compounds 5-nitro-, 5-fluoro-, 5-chloro- and 5-bromouracil all bound better to *T. gondii* UrdPase than did uracil. Substitution with an electronegative 5-trifluoromethyl group also enhanced binding (3-fold); however, its relationship with the electronegativity of the other groups is unknown. One exception to this correlation is the compound 5-iodouracil which bound more poorly (about 3-fold) than uracil, despite the fact that iodine is more electronegative than hydrogen.

One effect of substituting electron-withdrawing groups at the 5-position of uracil is to increase the per cent ionization, and therefore the acidity, of the pyrimidine ring [14]. However, there was only a partial correlation between the increase in binding of analogs substituted with these types of groups (i.e. nitro > fluoro > chloro > bromo > hydrogen > iodo) and their per cent ionization (i.e. nitro (100%) > bromo (60%) > chloro (53%) > fluoro (51%) > iodo (36%) > hydrogen (3%) [17]). Similarly, a partial correlation was observed between the hydrophobicity of these groups and their ability to bind to *T. gondii* UrdPase. It was found that an inverse relationship exists between the hydrophobicity of these substituents (i.e. iodo > bromo > chloro > fluoro > hydrogen > nitro [14]) and their effects on binding. Thus, it appears that there are two conflicting forces involved in the binding of these types of groups at the 5-position of uracil, i.e. binding increases as the electronegativity of the substituent increases, whereas binding decreases as the hydrophobicity of the substituent increases. This may explain why uracil binds to *T. gondii* UrdPase better than 5-iodouracil, despite the fact that iodine is more electronegative than hydrogen.

Replacement of the 5-position hydrogen with hydrophobic groups had mixed effects on binding to *T. gondii* UrdPase. Substitution with small hydrophobic groups (methyl, ethyl) decreased binding relative to uracil, whereas substitution with larger hydrophobic groups (propyl, benzyl, benzyloxybenzyl) increased binding. The best substitution was a benzyloxybenzyl group (5-benzyloxybenzyluracil)

Table 2. Structure–activity relationship for the binding of nucleobase analogs to *T. gondii* UrdPase

Position	Substituent effect
N1	*N1* nitrogen and undissociated hydrogen not required; replacement of *N1* nitrogen with a methylene group (1-deazauracil) enhances binding 46-fold.
	Substitution of *N1* hydrogen with a benzyl group increases binding by 10-fold. Substitution with other hydrophobic groups (methyl, cyclohexyl) diminishes binding.
C2	Either an oxo group or a thio group is required; substitution of a *C2* oxo group with a thio group enhances binding 5-fold.
N3	*N3* of uracil and undissociated *N3* hydrogen preferred but not required for binding; replacement of *N3* nitrogen with an endocyclic oxygen diminishes binding slightly (1.3-fold).
	Substitution of *N3* hydrogen with hydrophobic groups (methyl, butyl) abolishes binding.
C4	Either an oxo group or a thio group is required; substitution of a *C4* oxo group with a thio group enhances binding about 2-fold (as indicated by increased binding of 4-thiouridine relative to uridine).
C5	*C5* not required; replacement with an endocyclic imino group diminishes binding slightly (1.3-fold).
	Substitution of *C5* hydrogen with electron-withdrawing groups enhances binding: nitro, 8-fold; trifluoromethyl, 3-fold; fluoro, 2.2-fold; chloro, 2.1-fold; bromo, 1.2-fold.
	Substitution of *C5* hydrogen with small hydrophobic groups (methyl, ethyl) diminishes binding; substitution with larger hydrophobic groups enhances binding: propyl, 1.4-fold; benzyl, 12-fold; benzyoxybenzyl, 240-fold.
	Substitution of *C5* hydrogen with either charged (carboxy, diazo, sulfamino) or uncharged (amino, carbethoxy, hydroxy, hydroxymethyl) hydrophilic groups diminishes or abolishes binding.
C6	*C6* required; replacement with endocyclic imino group abolishes binding.
	Substitution of *C6* hydrogen with hydrophobic (methyl, benzyl), charged hydrophilic (carboxy) or uncharged hydrophilic (amino, carboxymethyl, carboxymethyl ester, methylsulfone) groups generally diminishes or abolishes binding.
	Substitution of *C6* hydrogen with a hydroxy or chloro group enhances binding 1.3- and 6-fold, respectively.
Multiple	Substitution of a thio group at the 2-position of uracil analogs generally enhances binding: e.g. 6-benzyl-2-thiouracil binds 104-fold better than 6-benzyluracil.
	Substitution of a nitro group at the 5-position of uracil analogs with a hydrophobic group at the 6-position enhances binding: e.g. 5-nitro-6-methyluracil binds 11-fold better than 6-methyluracil.
	Substitution of a hydroxy group at the 6-position with a hydrophobic group at the 5-position enhances binding: e.g. 5-benzyloxybenzylbarbituric acid binds 8-fold better than 5-benzyloxybenzyluracil.

that increased binding by 240-fold as compared with uracil. Although these substitutions had varying effects on binding, a general pattern was observed in which the appK_i of these compounds decreased as the size and hydrophobicity increased. A similar relationship was seen with the corresponding 2-thio derivatives (see below). These results indicate that there is a "pocket" in the catalytic site of *T. gondii* UrdPase, adjacent to the binding site of the 5-position of uracil, which is apparently large enough to accommodate a benzyloxybenzyl group. In addition, there appears to be some type of hydrophobic interaction between the 5-position benzyloxybenzyl group and hydrophobic residues in this "pocket," since this substitution significantly increases binding to the enzyme.

The increase in binding observed with the increase in the size of the hydrophobic group may be due to the distance between the hydrophobic substituents at the 5-position of uracil and the hydrophobic "pocket" of the catalytic site. It is possible that a methyl or ethyl group is not large enough to interact with the hydrophobic residues at this site. In contrast, propyl, benzyl or benzyloxybenzyl groups may be large enough to sufficiently interact with these hydrophobic residues, thereby increasing binding. This may also explain why substitution of relatively small "hydrophobic" halogen groups (e.g. iodo) at the 5-position did not result in an increase in binding. The decrease in binding of 5-methyluracil and 5-ethyluracil relative to uracil, on the other hand, is more difficult to explain. As with the larger halogen

groups, this decreased binding may be due to the poor electron-withdrawing properties of methyl and ethyl substituents. For example, only 1% of 5-methyluracil (thymine) is ionized under the conditions employed, whereas 3% of uracil is ionized [14].

Substitution of the 5-position hydrogen with either charged (e.g. carboxy, diazo, sulfamino) or uncharged (e.g. amino, carbethoxy, hydroxy, hydroxymethyl) hydrophilic groups diminished or abolished binding to *T. gondii* UrdPase. No discernable pattern was observed with respect to the size and properties of these substituent groups and binding to the enzyme. The lack of binding of these types of substituents is probably due to the postulated hydrophobic region adjacent to the binding site of the 5-position of uracil, which would tend to repel hydrophilic substituents.

6-*Position substitutions*. In contrast to the *C5* carbon, replacement of the *C6* carbon with a nitrogen (6-azauracil) abolished binding to *T. gondii* UrdPase, despite the fact that this compound is 100% ionized under the conditions employed [17]. This decrease in binding may be due to the juxtaposition of a nitrogen at the 6-position next to the *N1* nitrogen, as compared with 5-azauracil in which the nitrogen at the 5-position is separated from the *N1* or *N3* nitrogen in the ring by carbons.

Substitution of the *C6* hydrogen with hydrophobic groups (e.g. methyl or benzyl) decreased binding to the enzyme. These results tend to indicate that the region of the catalytic site of *T. gondii* UrdPase adjacent to the 6-position of uracil is either hydrophilic in nature or sterically hindered. However, with one exception (hydroxy), replacement of the 6-position hydrogen with either charged (e.g. carboxy, carboxymethyl) or uncharged (e.g. amino, carboxymethyl ester, methylsulfone) hydrophilic groups generally diminished or abolished binding. Furthermore, the compound 6-benzyl-2-thiouracil, which has a large hydrophobic group at the 6-position, had an appK_i value that was 43-fold less than that for uracil. These results indicate that there may also be a hydrophobic "pocket" in the catalytic site of *T. gondii* UrdPase adjacent to the 6-position of uracil. It should be noted, however, that benzyl groups substituted at either the 5- or 6-position can rotate, such that they would be located in approximately the same position adjacent to *C5-C6* bond of the pyrimidine ring. Therefore, it is very likely that there is a single hydrophobic region in the catalytic site of *T. gondii* UrdPase, adjacent to the binding site of the *C5-C6* region of the pyrimidine ring, which can interact with hydrophobic groups substituted at either the 5- or 6-position.

The only other substitutions at the 6-position that were tested were chloro and chloromethyl groups, which had opposite effects on binding to the enzyme. 6-Chlorouracil bound to the enzyme about 6-fold better than uracil, whereas 6-chloromethyluracil bound about 3-fold more poorly. The increased binding due to a 6-position chloro group may be due to the same electron-withdrawing effects of substituents at the 5-position (see above), since 6-chlorouracil is 100% ionized under the conditions employed [17]. The decreased binding of 6-chloromethyluracil supports this hypothesis, since the introduction of a carbon between the chloro group and the pyrimidine ring would tend to reduce the electron-withdrawing properties of the chloro group.

2-*Thio-substitutions*. The increased binding of 2-thiouracil, as compared with uracil, prompted us to test a series of 2-thio-substituted compounds to see if this increase in binding was universal. It was found that substitution of a thio group at the 2-position of uracil analogs generally increased binding, regardless of how well the original analog bound to *T. gondii* UrdPase. For example, the compounds 2-hydroxypyrimidine ("4-deoxyuracil") and 6-azauracil produced less than 5% inhibition at a concentration of 2 mM. When the 2-position oxo group on these compounds was replaced with a thio group to make 2-mercaptopyrimidine ("2-thio-4-deoxyuracil") and 6-aza-2-thiouracil, these compounds had measurable appK_i values of 4320 and 8850 μM, respectively.

The most interesting 2-thio-substituted analogs that were tested were those that had hydrophobic groups at either the 5- or the 6-position. In both cases it was observed that the appK_i values for these compounds decreased, as the size of the hydrophobic group increased (i.e. methyl > ethyl > propyl > benzyl). The most significant compounds tested were 4-phenyl-2-thiouracil and 6-benzyl-2-thiouracil. 4-Phenyl-2-thiouracil binds to *T. gondii* UrdPase about 5-fold better than uracil. It is unlikely this compound binds as 4-phenyl-2-thiouracil, however, given the requirement for an oxo or thio group at the 4-position (see above). It is more likely that this compound binds to the enzyme in an orientation rotated 180° about the *C2-C5* axis as "6-phenoxy-2-thio-4-deoxy uracil." Thus, the increased binding of this compound is probably due to the presence of a phenyl group at the 6-position, which can interact with the proposed "hydrophobic pocket" in the catalytic site of *T. gondii* UrdPase adjacent to the *C5-C6* position of the pyrimidine ring.

The strong binding of 6-benzyl-2-thiouracil (appK_i = 14 μM) was surprising given the poor binding of 6-benzyluracil and the slight increase in binding of 2-thiouracil (5-fold), relative to uracil. One would predict about a 5-fold increase in binding by the addition of a thio group at the 2-position of 6-benzyluracil; however, the increase in binding was actually about 100-fold as compared with 6-benzyluracil. Although some type of synergistic interaction is occurring between these two substitutions, it is unclear how the addition of a 2-position thio group to 6-benzyluracil results in the dramatic enhancement in binding.

5-*Nitro-substitutions*. Based on the increased binding (8-fold) of 5-nitrouracil relative to uracil, several 5-nitro-substituted analogs were tested to see if a synergistic effect on binding would occur, as was observed for the 2-thio-substituted analogs. It was found that substitution of a nitro group at the 5-position of uracil analogs that had hydrophilic groups at the 6-position (e.g. 5-nitrobarbituric acid or 5-nitro-6-carboxyuracil) diminished binding. In contrast, a strong enhancement in binding (57-fold) was seen when a 5-position nitro group was added to 6-methyluracil. As was the case for 6-benzyl-2-

thiouracil, this increase in binding was much greater than the expected increase of about 8-fold.

6-Hydroxy-substitutions. Several 6-hydroxy-substituted compounds (barbituric acid analogs) with various substitutions at the 5-position were tested. With one notable exception, all of these compounds bound to *T. gondii* UrdPase more poorly than the "parent" (5-substituted) compounds. The one exception was 5-benzyloxybenzylbarbituric acid which had an appK_i value (0.32 μM) about 1870-fold lower than uracil and was the most potent inhibitor of *T. gondii* UrdPase found in this study. The addition of a hydroxy group at the 6-position to 5-benzyloxybenzyluracil enhanced binding by about 8-fold, which is greater than the increase in binding due to substitution of a hydroxy group on uracil (barbituric acid) alone (1.3-fold). Naguib *et al.* [18] observed a similar increase (6-fold) for UrdPase from mouse liver and they postulated that it may be due to the unique ionization of 5-benzyloxybenzylbarbituric acid. This compound ionizes through the loss of a proton at *C5* which results in enolization of the 4- or 6-position oxo group. The charged oxygen may enhance binding of a preferred tautomer of this compound and/or may be involved in orienting the benzyloxybenzyl group at the 5-position in a favorable (stable) position for binding to the hydrophobic pocket in the catalytic site of the enzyme [18].

Comparison of T. gondii *UrdPase with mammalian UrdPase and dThdPase*

Table 3 shows a comparison between the structure–activity relationship determined in the present study for *T. gondii* UrdPase and those reported for mammalian UrdPase from mouse sarcoma S-180 cells and mammalian dThdPase from mouse liver [14]. As can be seen, there are both similarities and differences between the catalytic site of *T. gondii* UrdPase and the catalytic sites of mammalian UrdPase and dThdPase with respect to binding of uracil analogs substituted at various positions on the uracil ring. In general, *T. gondii* UrdPase more closely resembles mammalian UrdPase, with respect to binding of uracil analogs, than it resembles mammalian dThdPase.

T. gondii UrdPase is similar to mammalian UrdPase with respect to several types of substitutions. For example, replacement of the *N1* nitrogen of uracil with a methylene group (1-deazauracil) enhances binding significantly to *T. gondii* UrdPase and mammalian UrdPase, but only slightly to mammalian dThdPase. Three different types of substitutions enhance binding to *T. gondii* UrdPase and mammalian UrdPase, but abolish binding to mammalian dThdPase: substitution of the *N1* hydrogen with a benzyl group, substitution of the 4-oxo group with a thio group, and substitution of the *C5* hydrogen with large hydrophobic groups. It should be noted, however, that the increase in binding observed for the latter type of substitution varied between *T. gondii* UrdPase and mammalian UrdPase. For example, a benzyl group increases binding to *T. gondii* UrdPase and mammalian UrdPase by about 11- and 52-fold, respectively, whereas a benzyloxybenzyl group increases binding by about 240- and 130-fold, respectively.

The most significant similarity between *T. gondii* UrdPase and mammalian dThdPase is the ability of 6-benzyl-2-thiouracil to bind to these two enzymes, but not to mammalian UrdPase. It is interesting to note that neither of the single-substituted compounds, 2-thiouracil or 6-benzyluracil, bind significantly to mammalian dThdPase, whereas the combination of these substitutions results in a 5-fold enhancement in binding relative to uracil [14]. A similar synergism occurs with *T. gondii* UrdPase. 6-Benzyl-2-thiouracil bound 43-fold better to *T. gondii* UrdPase than did uracil, despite the fact that 2-thiouracil bound only 5-fold better and 6-benzyluracil bound about 2.5-fold more poorly. *T. gondii* UrdPase is also similar to mammalian dThdPase in that replacement of *C5* with an endocyclic imino group (5-azauracil) diminishes binding only slightly to these two enzymes, as compared with mammalian UrdPase.

T. gondii UrdPase is similar to both mammalian UrdPase and dThdPase with respect to binding of uracil analogs substituted at the 5-position with electron-withdrawing groups. Substitution of these types of groups for the *C5* hydrogen enhances binding to all of these enzymes, although the increase in binding to mammalian UrdPase and dThdPase is much greater than it is for *T. gondii* UrdPase. For example, a bromo group at the 5-position (5-bromouracil) increases binding to mammalian dThdPase and UrdPase by 28- and 21-fold, respectively, but increases binding to *T. gondii* UrdPase only about 2-fold. These enzymes are also similar with respect to substitutions at the 6-position. All three enzymes are relatively intolerant to substitutions at the 6-position, with the exception of hydroxy or chloro groups for *T. gondii* UrdPase and an amino group for mammalian dThdPase.

Several differences exist between *T. gondii* UrdPase and the mammalian enzymes with respect to binding of uracil analogs. Either an oxo or thio group can be present at the 2-position for binding to *T. gondii* UrdPase, whereas an oxo group is required (or at least preferred) at this position for binding to both mammalian UrdPase and dThdPase. Furthermore, substitution of the 2-oxo group with a thio group (2-thiouracil) increases binding to *T. gondii* UrdPase 5-fold, whereas it decreases binding to mammalian UrdPase and abolishes binding to mammalian dThdPase. *T. gondii* UrdPase is able to tolerate replacement of the *N3* nitrogen with an oxygen (3-oxauracil) better than the mammalian enzymes. This substitution decreases binding to the *T. gondii* UrdPase slightly, whereas it decreases binding to mammalian UrdPase by about 9-fold and abolishes binding to mammalian dThdPase. Finally, substitution of small hydrophobic groups (e.g. methyl) for the 5-position hydrogen diminished binding to *T. gondii* UrdPase, whereas it increases binding to both mammalian UrdPase and dThdPase.

Design of new inhibitors of T. gondii *UrdPase*

The structure–activity relationship for the binding of nucleobase analogs to *T. gondii* UrdPase is shown in Table 2. The most significant single substitutions found in this study were as follows: 1-deaza-, 2-thio-, 5-benzyl-, 5-benzyloxybenzyl-, 5-nitro and 6-chloro.

Table 3. Comparison of structure–activity relationships for *T. gondii* UrdPase with mammalian UrdPase and dThdPase

Position	Mammalian UrdPase*	*T. gondii* UrdPase	Mammalian dThdPase*
N1	Replacement of *N1* with methylene group enhances binding 57-fold	Replacement of *N1* with methylene group enhances binding 46-fold	Replacement of *N1* with methylene group enhances binding 2-fold
	Substitution of *N1* hydrogen with benzyl group increases binding 7-fold	Substitution of *N1* hydrogen with benzyl group increases binding 10-fold	Substitution of *N1* hydrogen with benzyl group abolishes binding
C2	2-Oxo group required; replacement of 2-oxo group with thio group decreases binding (3-fold)	Either 2-oxo or 2-thio group required; replacement of 2-oxo group with thio group increases binding 5-fold	2-Oxo group required; replacement of 2-oxo group with thio group abolishes binding
N3	*N3* and undissociated hydrogen required; replacement of *N3* with oxygen decreases binding (9-fold)	*N3* and undissociated hydrogen preferred but not required; replacement of *N3* with oxygen decreases binding slightly (1.3-fold)	*N3* and undissociated hydrogen required; replacement of *N3* with oxygen abolishes binding
C4	Either 4-oxo or 4-thio group required; replacement of 4-oxo group with thio group increases binding 2.5-fold	Either 4-oxo or 4-thio group required; replacement of 4-oxo group with thio group increases binding 2.1-fold	4-Oxo group required; replacement of 4-oxo group with thio group abolishes binding
C5	Replacement of *C5* with nitrogen decreases binding (7-fold)	Replacement of *C5* with nitrogen decreases binding slightly (1.3-fold)	Replacement of *C5* with nitrogen decreases binding slightly (1.1-fold)
	Substitution of *C5* hydrogen with any hydrophobic group enhances binding: methyl, 6-fold; benzyl, 52-fold; benzyloxybenzyl, 130-fold	Substitution of *C5* hydrogen with methyl or ethyl group diminishes binding. Substitution with larger hydrophobic groups enhances binding: propyl, 1.4-fold; benzyl, 11-fold; benzyloxybenzyl, 240-fold	Substitution of *C5* hydrogen with methyl group enhances binding 3-fold. Substitution with larger hydrophobic groups (benzyl, benzyloxybenzyl) abolishes binding
	Substitution of *C5* hydrogen with electron-withdrawing groups enhances binding significantly: nitro, 114-fold; bromo, 21-fold; chloro, 18-fold; iodo, 9-fold; fluoro, 3-fold	Substitution of *C5* hydrogen with certain electron-withdrawing groups enhances binding slightly: nitro, 8-fold; fluoro, 2.2-fold; chloro, 2.1-fold; bromo, 1.2-fold	Substitution of *C5* hydrogen with electron-withdrawing groups enhances binding significantly: bromo, 28-fold; chloro, 26-fold, iodo, 17-fold; nitro, 13-fold; fluoro, 2.2-fold
C6	Substitution of *C6* hydrogen diminishes or abolishes binding	Substitution of *C6* hydrogen diminishes or abolishes binding except for hydroxy and chloro groups that enhance binding 1.3- and 6-fold, respectively	Substitution of *C6* hydrogen diminishes or abolishes binding except for the amino group which enhances binding 3-fold
Multiple	6-Benzyl-2-thiouracil does not bind	6-Benzyl-2-thiouracil binds 43-fold better than uracil	6-Benzyl-2-thiouracil binds 5-fold better than uracil

* From Niedzwicki *et al.* [14].

In addition, several multiple substitutions were found to enhance binding to *T. gondii* UrdPase: 5-nitro-6-methyl-, 6-benzyl-2-thio-, and 5-benzyloxybenzyl-6-hydroxy-. These findings provide the basis for the rational design of more potent inhibitors of *T. gondii* UrdPase (see below). In addition, the comparison of the structure–activity relationships for *T. gondii* UrdPase with the corresponding mammalian enzymes (Table 3) indicates substitutions that may be useful in developing more specific inhibitors of *T. gondii* UrdPase. The most significant substitution with respect to specificity is a 2-thio substitution which increases binding to *T. gondii* UrdPase, but decreases binding to the mammalian enzymes.

It is unclear whether or not the combination of 1-

deaza-substitutions with other substitutions will enhance binding, since only one such compound (6-carboxy-1-deazauracil) was available for testing. Similarly, it is unknown if 6-chloro substitutions will be effective since no 6-chloro multiple-substituted analogs were available. The increased binding resulting from the combination of a nitro group at the 5-position with a hydrophobic group at the 6-position would suggest that a compound such as 5-nitro-6-benzyluracil may be a good inhibitor of *T. gondii* UrdPase. In addition, the substitution of a thio group at the 2-position, to analogs with large hydrophobic groups at either the 5- or 6-position, should not only increase the binding of these compounds to *T. gondii* UrdPase, but also increase their specificity. Therefore, it would be of particular interest to test analogs such as 5-benzyloxybenzyl-2-thiouracil, 6-benzyloxybenyl-2-thiouracil, or 5-benzyloxybenzyl-2-thiobarbituric acid.

The present study has identified two compounds, 5-benzyloxybenzyluracil and 5-benzyloxybenzylbar bituric acid, as very potent nucleobase inhibitors of *T. gondii* UrdPase. In addition, 6-benzyl-2-thiouracil has been identified as a potent, specific inhibitor of this enzyme relative to mammalian UrdPase and dThdPase. It should be noted, however, that nucleoside analogs appear to bind to *T. gondii* UrdPase with a greater affinity than their corresponding nucleobases. For example, uridine and 5-benzylacyclouridine bound about 6-fold better than their nucleobase counterparts (uracil and 5-benzyluracil, respectively). Therefore, we are currently conducting a structure–activity relationship for nucleoside ligands of *T. gondii* UrdPase to see if more potent inhibitors of this enzyme can be identified and/or developed.

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REFERENCES

1. Luft BJ, Toxoplasma gondii. In: *Parasitic Infections in the Compromised Host* (Eds. Walzer PD and Genta RM), pp. 179–279. Marcel Dekker, New York, 1989.
2. Luft BJ and Remington JS, Toxoplasmic encephalitis. *J Infect Dis* **157**: 1–6, 1988.
3. Mills J, *Pneumocystis carinii* and *Toxoplasma gondii* infections in patients with AIDS. *Rev Infect Dis* **8**: 1001–1011, 1986.
4. Asai T, O'Sullivan WJ, Kobayashi M, Gero AM, Yokogawa M and Tatibana M, Enzymes of the *de novo* pyrimidine biosynthetic pathway in *Toxoplasma gondii*. *Mol Biochem Parasitol* **7**: 89–100, 1983.
5. Iltzsch MH, Pyrimidine salvage pathways in *Toxoplasma gondii*. *J Euk Microbiol* **40**: 24–28, 1993.
6. O'Sullivan WJ, Johnson AM, Finney KG, Gero AM, Hagon E, Holland JW and Smithers GW, Pyrimidine and purine enzymes in *Toxoplasma gondii*. *Aust J Exp Biol Med Sci* **59**: 763–767, 1981.
7. Pfefferkorn ER, *Toxoplasma gondii*: The enzymatic defect of a mutant resistant to 5-fluorodeoxyuridine. *Exp Parasitol* **44**: 26–35, 1978.
8. Pfefferkorn ER and Pfefferkorn LC, Specific labeling of intracellular *Toxoplasma gondii* with uracil. *J Protozool* **24**: 449–453, 1977.
9. Pfefferkorn ER and Pfefferkorn LC, *Toxoplasma gondii*: Characterization of a mutant resistant to 5-fluorodeoxyuridine. *Exp Parasitol* **42**: 44–55, 1977.
10. Pfefferkorn LC and Pfefferkorn ER, *Toxoplasma gondii*: Genetic recombination between drug resistant mutants. *Exp Parasitol* **50**: 305–316, 1980.
11. Pfefferkorn ER, *Toxoplasma gondii* viewed from a virological perspective. In: *The Biology of Parasitism* (Eds. Englund PT and Sher A), MBL Lectures in Biology, Vol. 9, pp. 479–501. Alan R. Liss, New York, 1988.
12. Krenitsky TA, Barclay M and Jacquez JA, Specificity of mouse uridine phosphorylase. Chromatography, purification, and properties. *J Biol Chem* **239**: 805–812, 1964.
13. Niedzwicki JG, Chu SH, el Kouni MH, Rowe EC and Cha S, 5-Benzylacyclouridine and 5-benzyloxybenzylacyclouridine, potent inhibitors of uridine phosphorylase. *Biochem Pharmacol* **31**: 1857–1861, 1982.
14. Niedzwicki JG, el Kouni MH, Chu SH and Cha S, Structure–activity relationship of ligands of the pyrimidine nucleoside phosphorylases. *Biochem Pharmacol* **32**: 399–415, 1983.
15. Cleland WW, The statistical analysis of enzyme kinetic data. *Adv Enzymol* **29**: 1–32, 1967.
16. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**:P 248–254, 1976.
17. Niedzwicki JG, Iltzsch MH, el Kouni MH and Cha S, Structure–activity relationships of pyrimidine analogs as ligands of orotate phosphoribosyltransferase. *Biochem Pharmacol* **33**: 2383–2395, 1984.
18. Naguib FNM, Levesque DL, Wang E-C, Panzica RP and el Kouni MH, 5-Benzylbarbituric acid derivatives, potent and specific inhibitors of uridine phosphorylase. *Biochem Pharmacol.* **46**: 1273–1283, 1993.