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INHIBITION OF URIDINE PHOSPHORYLASE FROM *GIARDIA LAMBLIA* BY PYRIMIDINE ANALOGS

BARBARA M. JIMÉNEZ, PETER KRANZ, CHOY SOONG LEE, ANNETTE M. GERO and WILLIAM J. O'SULLIVAN*

School of Biochemistry, University of N.S.W., P.O. Box 1, Kensington, N.S.W. 2033, Australia

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Abstract—Fifty-six pyrimidine analogs were tested as possible inhibitors of uridine phosphorylase from *Giardia lamblia*. Values of K_i were determined for eight of these which demonstrated an inhibition greater than 60% under the standard conditions of uridine at 1 mM (approximately 1.5 times the K_m) and inhibitor at 1 mM. All were competitive with respect to uridine. The most effective inhibitors were uracil analogs substituted at the C-5 position with electron withdrawing groups (nitro groups or halogens). The inhibitory effect at the 5-position appeared to be further enhanced by substitution at the C-6 position with electron releasing groups. The order of effectiveness as inhibitors was 6-methyl-5-nitrouracil > 6-amino-5-nitrouracil > 5-benzylacyclouridine > 5-nitrouracil > 5-fluorouracil > 5-bromouracil > 6-benzyl-2-thiouracil > 1,3-dimethyluracil with K_i values of 10, 12, 44, 56, 119, 230, 190 and >1000 μM, respectively. The compounds were also effective inhibitors of the thymidine phosphorylase activity of the enzyme. The effect of the more potent compounds on *G. lamblia* in *in vitro* culture are currently under investigation.

The intestinal protozoan parasite *Giardia lamblia* is a common causative agent of serious diarrhoea in humans [1]. This parasite is unable to synthesize pyrimidines by the *de novo* pathway and is dependent on salvage for its supply of pyrimidine nucleotides [2–4]. Current therapy is less than satisfactory and there is a perceived need for new anti-giardial agents [1]. We describe here some preliminary investigations on the potential of anti-pyrimidine compounds for the chemotherapy of giardiasis.

G. lamblia lacks uridine kinase activity and synthesis of UMP and subsequent uridine nucleotides is dependent on the sequential action of uridine phosphorylase (uridine: orthophosphate-ribosyltransferase; EC 2.4.2.3) and uracil phosphoribosyltransferase (UPRTase; EC 2.4.2.9) [4]. We recently reported the purification of uridine phosphorylase from *G. lamblia* and provided evidence that the same enzyme was also responsible for deoxyuridine and thymidine phosphorylase activities [5]. The results differed from those obtained for the mammalian enzymes in which the uridine and thymidine phosphorylase activities can be separated [6] and for *Escherichia coli* [7], where uridine phosphorylase showed poor activity with deoxyuridine and thymidine.

The fact that one enzyme is responsible for the three pyrimidine nucleoside phosphorylase activities is indicative of the importance of this enzyme to the viability of the parasite, with the inference that inhibition would be deleterious to it. We have therefore carried out a systematic study of the inhibition of *G. lamblia* uridine phosphorylase by a wide range of pyrimidine compounds, analogous to the study by Niedzwicki *et al.* on mammalian uridine phosphorylase [8], with the objective of providing a basis for the development of potential chemotherapeutic agents.

MATERIALS AND METHODS

Materials

[2-^{14}C]Uridine (55 mCi/mmol) and [2-^{14}C]thymidine (59.7 mCi/mmole) were obtained from the Radioactive Centre (Amersham, U.K.). Hepes† (*N*-2-hydroxyethylpiperazine-*N*-2-ethane sulphonic acid) and ribose-1-phosphate were obtained from Sigma Chemical Co. (St. Louis, MO). Polygram Silica gel UV 254 sheets (20 × 20 cm) were obtained from Macherey–Nagel and Co. The Mono Q HR 5/5 column was from Pharmacia (Sydney, Australia). Centricon 10 and 30 were obtained from Amicon (Melbourne, Australia). 5-Benzylacyclouridine (BAU) was a gift from Drs Sungman Cha and M. H. el Kouni, Brown University (Providence, RI) [8]. Other pyrimidine analogs were either obtained commercially or were supplied by Dr D. J. Brown (Australian National University, Canberra, Australia). The sources of these compounds or the methods for their chemical synthesis are described in Gero *et al.* [9].

All other chemicals and solvents were of analytical reagent grade and were used without further purification. Deionized-distilled water was used throughout.

Methods

Preparation of parasite extracts. Extracts were prepared substantially as described previously [5]. Trophozoites from *G. lamblia* (Portland 1 Stock ATCC 30888; [10]) were obtained from Dr P. Boreham

* To whom correspondence should be addressed.

† Abbreviations used: UPRTase, uracil phosphoribosyltransferase; Hepes, *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulphonic acid; FPLC, Fast Protein Liquid Chromatography; BAU,5-benzylacyclouridine; PBS, phosphate buffered saline.

(Queensland Institute of Medical Research, Brisbane, Australia). The trophozoites were grown axenically under microaerobic conditions at 37° using TYI-S-33 medium [11] with the modification that pooled human serum replaced foetal calf serum [4]. Trophozoites were harvested after 48 hr of growth, which coincided with mid-log phase. Cultures reached stationary phase after 72 hr with a final cell density of approximately 3×10^6 cells/ml. Cell numbers were assessed microscopically, using a haemocytometer. Twenty vessels, each containing 50 ml of culture of mid-log phase *G. lamblia* trophozoites, were put on ice for 20 min, shaken and the trophozoites collected by centrifugation. The pellets of cells were washed twice with approximately 40 ml of 0.02 M potassium phosphate, 0.155 M NaCl, pH 7.4 (PBS). One litre of culture yielded approximately 1 g (wet weight) of cells and this was resuspended in 10 ml of 20 mM Tris–HCl, pH 7.4, containing 0.1 mM EDTA and 5 mM 2-mercaptoethanol. The trophozoites were disrupted using a Sonifier Disrupter B-12 with three 15 sec bursts at 4°. The crude homogenate was then centrifuged at 40,000 *g* for 15 min at 4° and the supernatant fluid used for subsequent procedures.

Partial purification of uridine phosphorylase. Details of the enzyme purification have been described previously [5]. In brief, for a particular preparation, 2 ml of the crude high speed supernatant containing approximately 5 mg of protein, was applied to a Mono Q HR 5/5 column (5×0.5 cm) previously equilibrated with 20 mM Tris–HCl, pH 7.3, containing 0.1 mM EDTA and 10 mM mercaptoethanol. The column was washed with starting buffer until protein could no longer be detected in the eluant. The enzyme was eluted at between 65 and 75 mM KCl, with a programmed linear KCl gradient (0–0.5 M) in Tris–HCl, pH 7.3, with segments of different slopes at a flow rate of 0.5 ml/min. The effluent was monitored at 280 nm in a LKB UV-absorbance detector set at 0.05 full scale deflection. The above preparative procedure was repeated (usually 3 times) until sufficient enzyme was collected to proceed to the next step. Active fractions were pooled and concentrated with Centricon 30 with a molecular weight cut off of 30,000 [5]. Preparations used had specific activities that ranged from 2.5 to 4.0 μmoles/mg protein/min.

Uridine (thymidine) phosphorylase assays. The standard assay contained, 5 mM Na_2HPO_4, 0.1 M Hepes–KOH, pH 7.0, various concentrations of [2-^{14}C]uridine or [2-^{14}C]thymidine and enzyme (<40 μg protein) in a volume of 200 μl. The reaction mixture was incubated at 30° for 10 min and terminated by spotting 10 μl aliquots onto a Silica gel UV 254 TLC plate. The product and substrate were separated using chloroform : methanol (90 : 10, v/v) as solvent; R_f values were 0.11 and 0.36 for uridine and uracil, respectively. The analogs were first screened at a concentration of 1 mM for their inhibitory effect, with uridine at 1 mM, i.e. at approximately 1.5 times the K_m [5]. The compounds which demonstrated more than 60% inhibition under these conditions, were further investigated. Apparent K_i values were determined either from Dixon plots ($1/v$ vs $[I]$) [12] or from a computer fit to the rate equation for competitive inhibition, using an adaptation of the FORTRAN program described by Cleland [13]. Protein concentrations were determined using a modification of the Bradford method with bovine serum albumin as standard [14, 15].

RESULTS

Inhibition of uridine phosphorylase by pyrimidine analogs

A systematic study of the inhibition of the partially purified uridine phosphorylase was carried out with 56 pyrimidine analogs. The results of the inhibition assays at 1 mM concentration of each compound and with uridine also at 1 mM, are summarized in Table 1 together with the published anionic pK_a values where available [9]. The compounds most active as inhibitors were shown to be those that were partially ionized as anions at pH 7.0. Most contained an electron-withdrawing group in the molecule.

Determination of K_i *values*

The eight analogs which demonstrated more than 60% inhibition, were subjected to more detailed kinetic analysis. The K_i values are tabulated in Table 2 and compared with apparent K_i values with uridine as substrate at 0.15 mM concentration, obtained by Niedzwicki *et al.* [8] for the mouse uridine phosphorylase. Compounds which inhibited the *G. lamblia* uridine phosphorylase less than 60% under the standardized experimental conditions were not further investigated.

The K_i values further separated the compounds into a rank order, the best inhibitors being uracil analogs substituted at the 5 position with electron-withdrawing groups and at the 6 position with electron-releasing groups. The most effective were 6-methyl-5-nitrouracil, 6-amino-5-nitrouracil, 5-benzylacyclouridine, 5-nitrouracil and 5-fluorouracil with K_i values of 10, 12, 44, 56 and 119 μM respectively. Values of K_i for 1,3-dimethyluracil and 6-benzyl-2-thiouracil were considerably higher (Table 2). All inhibitors were competitive with respect to uridine. The inhibition constants for the *G. lamblia* uridine phosphorylase are generally similar, although slightly higher, than those reported for the mouse enzyme [8].

Inhibition by BAU

Due to the reported potency of 5-benzylacyclouridine against uridine phosphorylase from both mouse tissues [8] and *E. coli* [16], the K_i for this inhibitor with the *G. lamblia* enzyme was determined from a family of double reciprocal plots (Fig. 1). BAU was confirmed as a competitive inhibitor. Analysis of the data yielded a value of 0.65 ± 0.06 mM for the K_m of uridine and 44 ± 5 μM for K_i of BAU.

Inhibition of thymidine phosphorylase activity

A comparison of the inhibition of the activity of the enzyme with thymidine as substrate with the eight most effective compounds from Table 1 was carried out. In general, a similar but not identical pattern as for the experiments with uridine was observed. The percentage inhibition was: 77 ± 8,

Table 1. Inhibition of *Giardia lamblia* uridine phosphorylase by pyrimidine compounds

Compound	Anionic pK_a	% Inhibition at 1 mM
6-Methyl-5-nitrouracil	6.4	100
6-Amino-5-nitrouracil	~7.5	100
5-Nitrouracil		100
5-Benzylacyclouridine		100
5-Fluorouracil	8.0	100
5-Bromouracil	7.8	93
6-Aminouracil	9.5	78
6-Benzyl-2-thiouracil		73
1,3-Dimethyluracil		67
5-Fluorodeoxyuridine		54
Oxypurinol		50
6-Methyluracil	9.7	48
4-Thiobarbituric acid		37
6-Azauracil		34
4-Amino-6-hydroxy-2-thiopyrimidine		34
3-Methyluracil	9.9	33
5-Carboxyuracil (isoorotic acid)	2.1	29
5-Aminoorotic acid	2.6	27
2-Thiouracil		24
Dihydro-DL-orotic acid		23
6-Formyluracil		21
5-Nitrobarbituric acid	<2.0	21
5,5-Diethylbarbituric acid	7.8	18
2-Amino-5-bromopyrimidine		17
Orotic acid	2.1	15
Pseudouridine		13
3-Oxauracil	~9.0	13
Dihydro-L-orotic acid		12
5-Bromoorotic acid	2.3	12
2-Amino-4-carboxy-5-chloropyrimidine		10
5-Iodoorotic acid	1.9	10
5-Amino-4-chloro-6-hydroxy-2-methylpyrimidine	~9.0	8
5-Bromo-4,6-dihydroxypyrimidine	~4.5	8
4-Amino-6-hydroxy-5-nitropyrimidine	~7.0	8
4-Amino-5-bromo-6-hydroxypyrimidine	~8.5	6
5,6-Dihydroxy-6-phenyluracil		6
5-Bromocytosine		5
5-Carboxy-3-methyluracil	~3.0	4
4-Hydroxypyrimidine	8.59	3
2-Hydroxypyrimidine	9.17	2
5-Bromo-1-methyl-5,6-dihydrouracil	~10.0	2
5-Methylorotic acid	2.5	0
5-Acetoxyuracil		0
4-Amino-5-imidazole carboxamide ribonucleoside		0
4-Amino-2-hydroxypyrimidine		0
2-Amino-4-hydroxypyrimidine	9.4	0
2-Benzyl-4,6-dihydroxy-5-nitropyrimidine		0
5-Bromo-1,3-dimethyluracil		0
4-Amino-5-imidazole carboxamide HCl		0
5-Fluorocytosine		0
4-Amino-5-bromopyrimidine		0
1,3-Dimethylbarbituric acid	4.6	0
6-Carboxy-5-hydroxy-3-methylthio-1,2,4-triazine	<3.0	0
Thymine	9.9	0
Isobarbituric acid		0
Uracil		0

94 ± 6, 94 ± 6, 80 ± 8, 52 ± 6, 63 ± 2, 37 ± 3 and 24 ± 4 for 6-methyl-5-nitrouracil, 6-amino-5-nitrouracil, 5-benzylacyclouridine, 5-nitrouracil, 5-fluorouracil, 5-bromouracil, 6-benzyl-2-thiouracil and 1,3-dimethyluracil, respectively, all with 1 mM inhibitor except for 6-methyl-5-nitrouracil, which was at 0.5 mM. The thymidine concentration was 1 mM (approx. $1.5 \times K_m$) for these experiments.

DISCUSSION

Of the fifty-six pyrimidine analogs tested against uridine phosphorylase, eight were considered worth further investigation (Table 1). The most effective compounds were the uracil analogs substituted at the C5 position with electron-withdrawing groups (i.e. nitro groups or halogens). Similar findings were

Table 2. Inhibition of *G. lamblia* uridine phosphorylase

Inhibitors	% Inhibition[a]	K_i(app.) (μM)	K_i(app.)[b] (μM)
6-Methyl-5-nitrouracil	100	10 ± 7	
6-Amino-5-nitouracil	100	12 ± 10	
5-Benzylacyclouridine	100	44 ± 5[c]	1.0 ± 0.3
5-Nitrouracil	100	56 ± 22	1.7 ± 0.3
5-Fluorouracil	100	119 ± 26	59 ± 6
5-Bromouracil	93	230 ± 26	9 ± 1
6-Benzyl-2-thiouracil	73	190 ± 32	181 ± 95[d]
1,3-Dimethyluracil	67	>1000	

[a]All inhibitors at 1 mM.
[b]Values for the mouse uridine phosphorylase at 0.15 mM uridine concentration [8].
[c]True K_i value.
[d]This value is against thymidine phosphorylase.

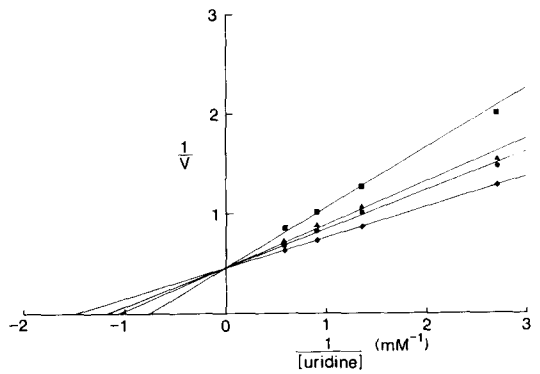

Fig. 1. Inhibition of uridine phosphorylase activity at different concentrations of BAU. Concentrations of BAU were: ◆, 0; ●, 16; ▲, 24; ■, 48 μM. Velocity is expressed as μmoles/mg protein/min.

reported by Niedzwicki *et al.* for the mouse enzyme [8]. However, the order of inhibition of the *G. lamblia* uridine phosphorylase by C5 substituted uracil analogs followed the order of electronegativity, i.e. nitro > fluoro > bromo. It appears that these electron-withdrawing groups substituted at the 5-position of uracil, enhance the binding of these analogs to uridine phosphorylase. The results support the hypothesis that there exists a hydrophobic region on uridine phosphorylase which is situated adjacent to the binding site of the 5-position of uracil [8].

The inhibitory effect of uracil analogs substituted at the 5-position appears to be further enhanced by substitution at the C6 position by electron-releasing groups (Table 2). Specifically, 6-methyl-5-nitrouracil and 6-amino-5-nitrouracil bound more tightly to uridine phosphorylase than those analogs substituted at the C5 position only. The rest of the analogs showed little or no inhibition, which reflects the view that steric factors may play an important role in the interactions involving the hydrophobic region of uridine phosphorylase.

BAU was selected for special consideration because of its established potency against uridine phosphorylase from other sources. Though a good inhibitor, the K_i value of 44 μM was considerably greater than that of 1 μM for the mouse enzyme [8] and that of 5.2 μM recently reported for uridine phosphorylase from *Schistosoma mansoni* [17]. The partially purified enzyme from the latter source appears to be similar to that from *G. lamblia*, insofar as it can also use thymidine and deoxyuridine as alternate substrates, though the pattern of inhibition with substrate analogs differed somewhat. However, the importance of uridine phosphorylase in *S. mansoni* is uncertain because of its low specific activity [17] and the fact that this parasite can also obtain its nucleotides by *de novo* synthesis [18, 19].

The analogs which were the best inhibitors of *G. lamblia* uridine phosphorylase activity were also the most effective against the thymidine phosphorylase activity. Further, the analogs which were successful inhibitors of the parasite uridine phosphorylase correlated well with their inhibitory activities against the mammalian enzyme [8]. However, there seemed to be little similarity between the analogs which inhibited the parasite thymidine phosphorylase activity and those which inhibit the mammalian thymidine phosphorylase. Similarly, the best inhibitors of mammalian uridine phosphorylase were not so effective against mammalian thymidine phosphorylase [8]. The results are not unexpected as, in *G. lamblia* the one protein is responsible for uridine, deoxyuridine and thymidine phosphorylase activities [5], while, in mammalian cells, uridine phosphorylase is a separate enzyme from thymidine phosphorylase [8].

Uridine phosphorylase has quite different roles in *G. lamblia* and mammalian systems. For the parasite, it is crucial for the synthesis of UMP from uridine, the preferred mode being via the phosphorylase and UPRTase. On the contrary, the role of the mammalian enzyme appears to be associated with the catabolic pathway, representing the principal mode of pyrimidine degradation. Synthesis of UMP is achieved either via uridine kinase or by the *de novo* pathway. The results in this paper appear to demonstrate that the parasite enzyme binds all promising compounds to a lesser extent than that from the mouse. There is, however, the possibility that inhibition of both the host and parasite enzymes would have little effect on the former but be deleterious to the latter. The effect of the four most potent inhibitors against *G. lamblia* in *in vitro* culture is currently

being investigated, with the aim of providing a basis for their testing in a mammalian system.

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