Biochemical Pharmacology, Vol. 41, No. 11, pp. 1769–1772, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

SHORT COMMUNICATIONS

Inhibition of uridine phosphorylase by some pyrimidine derivatives

(*Received* 30 *May* 1990; *accepted* 15 *December* 1990)

Uridine phosphorylase (URPase,* EC 2.4.2.3) and thymidine phosphorylase (TdRPase, EC 2.4.2.4) play an important role in the degradation of pyrimidine nucleosides as well as in the salvage of pyrimidine bases according to the equation:

$$\text{Pyr(d)R} + \text{P}_i = \text{Pyr} + \text{(d)R-1-P}.$$

The enzymes reside in the cytosol of mammalian cells [1].

Inhibitors of these enzymes might be useful as chemotherapeutic agents via diminishing the *in vivo* degradation of pyrimidine nucleoside analogs with expressed antitumor activity, such as 5-fluorouridine or 5-fluoro-2′-deoxyuridine [2–4]. A number of inhibitors of URPase, based mainly on 5-substituted uracil derivatives, have been reported [3, 5, 6]; several 5-substituted 2,2′-anhydrouridines and uracil acyclonucleosides have been recently developed as highly active substances against this enzyme [7–9].

In this study we have extended the screening of some uninvestigated differently substituted pyrimidine bases, 2,2′-anhydropyrimidine nucleoside and pyrimidine nucleoside analogs against URPase of rat liver. The most active compounds were also tested against TdRPase of mouse liver.

Materials and Methods

[2-^{14}C]Uridine (52 mCi/mmol) was obtained from Amersham International Ltd (Amersham, U.K.) and [2-^{14}C]thymidine (51 mCi/mmol) from the Institute for Radioisotope Production (Prague, Czechoslovakia). DEAE-cellulose (DE-32) and TLC plates (Silufol UV 254) were purchased from Serva Fine Biochemicals (Heidelberg, F.R.G.). Compounds III, IV, X and XII (Table 1) were obtained from Fluka AG (Buchs, Switzerland); XV (lenacil) from E.I. Du Pont de Nemours Co. (Wilmington, MA, U.S.A.); XVI from Calbiochem (Los Angeles, CA, U.S.A.); XXIV, XXV, XXVI, XXVII, XXVIII, XXIX, uridine and thymidine from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Substances I and II were synthesized by Dr C. Verkoyen (Gesamthochschule, Essen, F.R.G.). Derivatives V, VIII and IX were provided by Dr C. Wasternack (Martin Luther University, Halle, F.R.G.); VI and VII by Prof. S. Debov (First Medical Institute, Moscow, U.S.S.R.). The following compounds were synthesized in this Institute: XIV, according to [10]; IX and XIII, as described elsewhere [11, 12]; XVII, XVIII and XIX, by Dr M. Shopova according to [13, 14]. The 2,2′-anhydropyrimidine nucleosides XX, XXI and XXII were prepared by Dr E. Stankevich (Institute of Organic Synthesis, Riga, Latvian S.S.R.) as described [15, 16] and XXIII was a product of the same Institute. All other chemicals were of analytical grade.

URPase and TdRPase were essentially prepared by a combination of described methods [7, 17]. The livers of male Wistar rats (150–200 g) and male ICR mice (20–25 g) were perfused *in situ* with ice-cold 0.9% NaCl and thereafter removed. The tissues were homogenized with a glass–teflon homogenizer in 3 vol. of ice-cold 0.02 M potassium phosphate buffer, pH 8, containing 10 mM β-mercaptoethanol and 1 mM EDTA. The homogenates were centrifuged at 4° for 90 min at 100,000 *g* and to the supernatants solid ammonium sulfate was added. The precipitate obtained between 21 and 35% saturation [17] was resuspended in the homogenizing buffer and dialysed overnight against the same buffer. The dialysed enzyme preparations were stored at −70° showing no loss of activity over 6 months.

Purification of TdRPase. A column of DEAE-cellulose (1.6 × 10 cm, bed volume 20 mL) was equilibrated with 0.02 M potassium phosphate buffer, pH 8, containing 10 mM β-mercaptoethanol and 1 mM EDTA, and loaded with 2 mL of the crude mouse liver enzyme preparation. The column was then eluted with the same buffer and fractions of 3 mL were collected. This procedure resulted in complete separation of TdRPase from URPase. All fractions containing TdRPase activity were pooled and used in further experiments. The specific activity of URPase at 0.6 mM concentration of uridine was 22 nmol/min/mg protein. The specific activity of TdRPase at 1.0 mM concentration of thymidine was 16.6 nmol/min/mg protein. Protein concentrations were determined by the method of Lowry *et al.* [18].

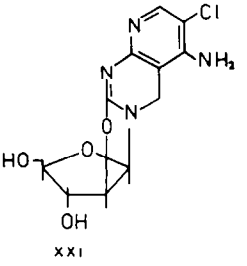
Inhibition studies. The inhibition studies with rat liver URPase and mouse liver TdRPase were performed with a radiochemical method. The incubation mixture in a final volume of 250 μL contained 0.1 M potassium phosphate buffer (pH 7.4), 2 mM β-mercaptoethanol, 0.6 mM UR or 1 mM TdR as substrates, supplemented with 0.1 μCi of [2-^{14}C]uridine or [2-^{14}C]thymidine. The inhibitor was added and the reaction was started by addition of the enzyme (10–90 μg protein). The mixtures were incubated at 37° for 10 min (URPase) or 20 min (TdRPase). The reaction rates were linear with time and enzyme concentrations under the conditions used. The reaction was stopped with 50 μL of 2 M $HClO_4$ and the mixture was centrifuged (800 *g*, 5 min). Sixty microlitres of 2 M KOH were added to the supernatant (200 μL) and the resulting pellet removed by centrifugation. Ten microlitres of the supernatant containing 2 mM uracil or thymine as standards were applied on silica gel plates (Silufol UV 254, Serva). The plates were developed with ethyl acetate–methanol–chloroform (8:1:1, by vol.). Spots corresponding to uracil or thymine were located by UV absorption, scraped into vials, eluted with 1 M HCl (200 μL) and counted in a toluene scintillator with a Beckman LS 1801 scintillation spectrometer. Apparent K_i values were determined from Dixon plots, $1/v$ vs [I], using a computer program with least-squares fitting.

* Abbreviations: URPase, uridine phosphorylase; TdRPase, thymidine phosphorylase; Pyr(d)R, pyrimidine ribonucleoside or pyrimidine 2′-deoxyribonucleoside; Pyr, pyrimidine base; (d)R-1-P, α-D-ribose-1-phosphate or α-D-deoxyribose-1-phosphate; lenacil, 3-cyclohexyl-6,7-dihydro-1*H*-cyclopentapyrimidine-2,4(3*H*,5*H*)-dione; 2,2′-anhydroorotidine hydrazide, hydrazide of 2,2′-anhydro-1-(β-D-arabinofuranosyl)-orotic acid; ACDP, 5-amino-6-chloro-2,2′-anhydro-3-(β-D-arabinofuranosyl)-3,4-dihydropyrido/2,3-*d*/pyrimidine; CCTP, 4*a*-chloro-6-cyano-2,2′-anhydro - 3 - (β - D - arabinofuranosyl) - 3,4,4*a*,5 - tetra - hydro-6*H*-pyrrolo/2,3-*d*/pyrimidine; ftorafur, 5-fluoro-1-(tetrahydro-2′-furyl)uracil.

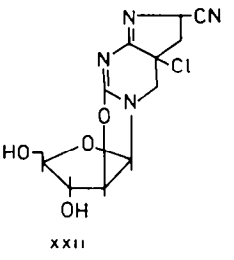
Results and Discussion

The inhibitory effect of the tested compounds on URPase from rat liver is presented in Table 1. The results revealed some new inhibitors of this enzyme. 5-Bromo-4-hydroxypyrimidine (VI) was the most potent inhibitor in this screening with an apparent K_i of 8 μM. It bound to URPase 20-fold better than did uracil (XXIX) and as well as 5-bromouracil (XXV). This is rather unexpected since the 2-positioned oxo-group of uracil seems to be essential for the binding of ligands to URPase [5, 6]. Bose and Yamada [19] have proposed that the 2-position oxo-group of uridine participates in a hydrogen-bonding with a sulfhydryl group in the active site of enzyme and for this reason, 4-hydroxypyrimidine does not bind to URPase [6]. Evidently, the strong binding of compound VI to URPase should be attributed to the effects of 5-positioned bromo-group. A reasonable explanation for the inhibitory activity of VI could be given when contrasting its pK_a value and per cent ionization under our assay conditions with those of 5-bromouracil (XXV) (Table 2). 5-Bromo-4-hydroxypyrimidine (VI) showed an enhanced acidity relative to XXV and was significantly ionized (approx. 50%). The tight binding of the anionic form of VI to URPase as well as the interaction of bromine with the hydrophobic region of the enzyme adjacent to the point of binding of 5-position of uracil [4–6] seems to compensate the lack of binding force of 2-oxo-group.

Two other compounds which inhibited considerably URPase were 6-amino-5-nitrosouracil (III) and 6-chlorothymine (VIII). The inhibitory activity of III was much higher than that reported for 6-aminouracil [3, 6]. This may be attributed to the electronegative effect of the 5-nitroso substituent which enhance the acidity of 6-aminouracil (Table 2). 6-Chlorothymine (VIII) was found to be an effective inhibitor of URPase from rat liver. Its effect was comparable with that of 6-aminothymine (XXIV). It seems that the 6-position chlorine in uracil, similarly to the 6-amino-group, is tolerated by the URPase. The enhanced acidity of 6-chlorothymine (VIII) over XXIV (Table 2) also may contribute to the activity of VIII.



XXI



XXII

Fig. 1. Structure of 2,2'-anhydropyrimidine nucleosides ACDP (XXI) and CCTP (XXII).

Two unusual 2,2'-anhydropyrimidine nucleosides: ACDP (XXI) and CCTP (XXII) (Fig. 1) were also tested as inhibitors of URPase. Compound XXII bound to the enzyme 3-fold stronger than XXI. The reasons for this

Table 1. Inhibition of URPase and TdRPase by pyrimidine derivatives

	Compound	URPase Inhibitor (μM)	URPase Inhibition (%)	TdRPase Inhibitor (μM)	TdRPase Inhibition (%)
I	6-Amino-1-benzyluracil	720	25		
II	6-Amino-1-methylthymine	500	50		
III	6-Amino-5-nitrosouracil	75	50	700	50
IV	6-Aza-4-thiouracil		*		
V	6-N-Benzylaminothymine	1000	25		
VI	5-Bromo-4-hydroxypyrimidine	40	50	1000	42
VII	5-Bromo-4-hydroxy-6-pyrimidine carboxylic acid		*		
VIII	6-Chlorothymine	70	50		*
IX	4,5-Dihydroorotic acid hydrazide		*		
X	4,6-Dihydroxy-5-nitropyrimidine	1550	25		*
XI	5,6-Dimethyl-2-thiouracil	870	50		
XII	1,3-Dimethyluracil		*		
XIII	5-Fluoroorotic acid		*		
XIV	6-Hydrazinouracil		*		
XV	Lenacil		*		
XVI	5-Methylcytosine		*		
XVII	1-Phenylorotic acid		*		
XVIII	1-Phenylorotic acid amide		*		
XIX	1-Phenylorotic acid *N*-*n*-propyl amide		*		
2,2'-Anhydropyrimidine nucleoside and pyrimidine nucleoside analogs					
XX	2,2'-Anhydroorotidine hydrazide		*		
XXI	ACDP	840	50	1000	41
XXII	CCTP	340	50		*
XXIII	Ftorafur	1000	25		*
Reference inhibitors					
XXIV	6-Aminothymine	70	50	70	50
XXV	5-Bromouracil	35	50		
XXVI	5-Fluoro-2'-deoxyuridine	900	50		
XXVII	5-Fluorouracil	150	50		
XXVIII	5-Nitrouracil	7.5	50	400	50
XXIX	Uracil	800	50		

* Less than 10% inhibition at the maximal inhibitor concentration of 2 mM.

Table 2. Acidity of selected URPase inhibitors

Compound	pK_a	% Ionized at pH 7.4	Ref.
6-Amino-5-nitrosouracil	5.2	99	*
6-Aminothymine	9.0	2.5	22
6-Aminouracil	8.7	5	22
5-Bromo-4-hydroxypyrimidine	7.4	50	*
5-Bromouracil	8.05	18	*
6-Chlorothymine	6.55	88	*
5-Fluoro-2'-deoxyuridine	7.6	39	*
Ftorafur	7.9	24	*
4-Hydroxypyrimidine	8.59	6	23

* Determined in this study by potentiometric titration.

activity are unknown. One possibility is the dihydropyrrol heterocycle of XXII to interact stronger with the hydrophobic region of URPase than the pyridine ring of XXI. The pyrimidine nucleoside analog ftorafur (XXIII) was found to be much less active against URPase than 5-fluorouridine [6] and 5-fluoro-2'-deoxyuridine (XXVI). This is not surprising since the 2'- and 3'-hydroxyl groups of pyrimidine nucleosides appear to be essential for binding to URPase [4, 6, 20].

Of the remaining compounds screened in this study only 6-amino-1-methylthymine (II) produced a noteworthy inhibition of URPase (Table 1). However, II bound to the enzyme about one-seventh as well as 6-aminothymine (XXIV). This is consistent with the findings of Baker and Kelley [5, 21] and Niedzwicki *et al.* [6] that the introduction of a methyl group at the 1-position of uracil analogs diminish their binding to URPase.

The newly evaluated active compounds were reversible inhibitors of URPase because their preincubation for 20 min with the enzyme did not increase the inhibition whilst subsequent dialysis restored the activity of the enzyme. Competitive inhibition was observed for the compounds III and VI (Fig. 2). The effect of URPase inhibitors on the phosphorolysis of thymidine in the presence of TdRPase (purified by DEAE chromatography) was studied (Table 1). 6-Amino-5-nitrosouracil (III), 5-bromo-4-hydroxypyrimidine (VI) and ACDP (XXI) were poor inhibitors of TdRPase from mouse liver, while the other compounds showed no activity even at 2 mM concentration.

It is known that orotic acid and also orotidine do not bind to URPase [6]. Likewise, the introduction of a carboxylic group in the 6-position of the potent URPase inhibitor VI (i.e. 5-bromo-4-hydroxy-6-pyrimidinecarboxylic acid, VII) abolished binding to URPase. The same holds true for 5-fluoroortic acid (XIII) relative to 5-fluorouracil (XXVII) and 2,2'-anhydroorotidine hydrazide (XX) relative to 2,2'-anhydrouridine (appK_i = 2.7 μM [7]).

In conclusion, this study revealed some new inhibitors of URPase which may be useful in cancer chemotherapy. The compounds specifically inhibit URPase having little effect on TdRPase. The demonstrated activity of 5-bromo-4-hydroxypyrimidine (VI) encourage the development of new URPase inhibitors from such pyrimidine derivatives.

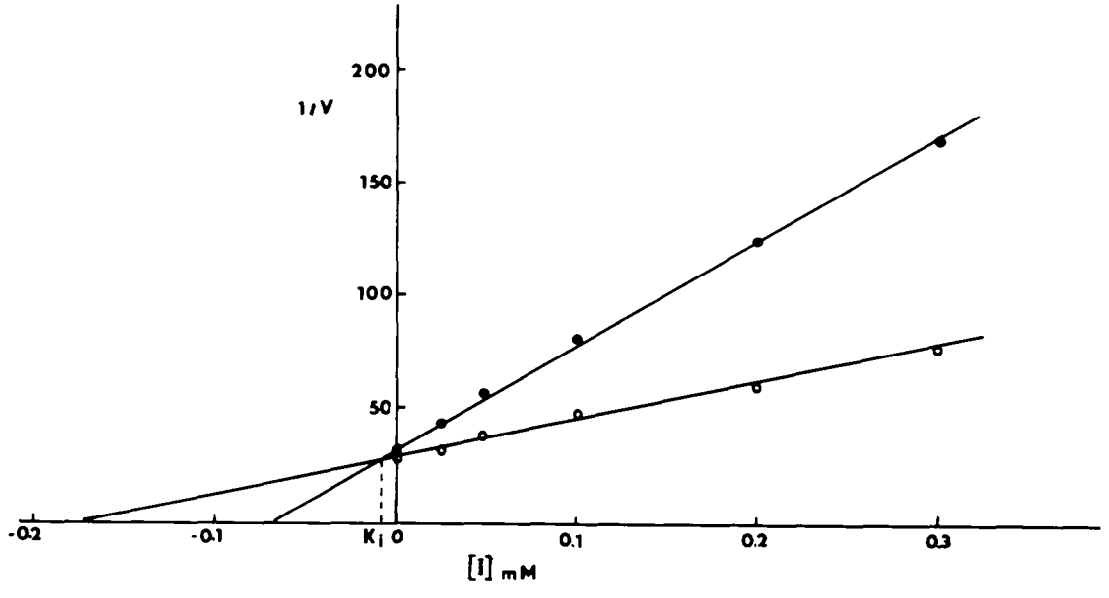


Fig. 2. Dixon plot for inhibition of URPase by 5-bromo-4-hydroxypyrimidine (VI). Substrate concentrations: (●) 0.6 mM; (○) 1.2 mM. Reactions were carried out as described in Materials and Methods, using 80 μg protein.

Acknowledgement—We are grateful to Mr A. Simeonov for his help in determination of pK_a values.

Institute of Molecular Biology
Bulgarian Academy of Sciences
1113 Sofia
Bulgaria

KONSTANTIN GRANCHAROV*
JORDANKA MLADENOVA
EVGENY GOLOVINSKY

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* Author to whom correspondence should be addressed.