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PYRIMIDINE ACYCLONUCLEOSIDES, INHIBITORS OF URIDINE PHOSPHORYLASE*

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Abstract—A new class of nucleoside analogs, the pyridimine acyclonucleosides, are competitive inhibitors of uridine phosphorylase but have no effect on thymidine phosphorylase, uridine kinase or thymidine kinase. The most potent of the series is acyclothymidine [5-methyl-1-(2′-hydroxyethoxymethyl)uracil] with a K_i value of 3 μM. K_i values of less than 30 μM were estimated for other analogs substituted at the 5-position of the pyrimidine ring. Extracts of xenografts of six human tumors were assayed for tissue levels of uridine phosphorylase and thymidine phosphorylase and for inhibition of 5-fluoro-2′-deoxyuridine (FUdR) phosphorolytic activity by acyclouridine [1-(2′-hydroxyethoxymethyl)uracil]. FUdR cleavage was inhibited most in those tissues in which the ratio of thymidine phosphorylase to uridine phosphorylase was low. Potential usage of these uridine phosphorylase inhibitors with the chemotherapeutic agent FUdR is discussed.

Two distinct pyrimidine nucleoside phosphorylases (PyNPases)‡, uridine phosphorylase (EC 2.4.2.3) and thymidine phosphorylase (EC 2.4.2.4), have been shown to occur in the cytosol of mammalian cells [1]. Uridine phosphorylase (URPase) primarily cleaves UR, but is relatively non-specific as it will also cleave UdR, TdR, and the chemotherapeutic agents FUdR, IUdR, and BrUdR [1, 2]:

$$\text{Py(d)R} + \text{P}_i \rightleftharpoons \text{Py} + \text{(Deoxy)ribose-1-phosphate}$$

Thymidine phosphorylase (TdRPase) is apparently specific for 2′-deoxyribonucleosides, as it will cleave all of the aforementioned pyrimidine deoxyribonucleosides but not UR [1, 3–5]:

$$\text{PydR} + \text{P}_i \rightleftharpoons \text{Py} + \text{Deoxyribose-1-phosphate}$$

To date, only a few potent inhibitors of the PyNPases have been discovered. 6-Aminothymine and several closely related derivatives are among the few known inhibitors of TdRPase [6]. URPase, on the other hand, was reported to be inhibited by 2′-deoxyglucosylthymine [7] and, more recently, by various 5-benzyl derivatives of uracil [8, 9]. In this study, we report that the pyrimidine acyclonucleosides, a new series of compounds, are potent inhibitors of URPases from various murine and human tissues. A preliminary report has been presented [10].

MATERIALS AND METHODS

Chemicals. The following compounds, having the structures shown in Fig. 1, were synthesized by one of us (S. H. Chu) employing methods to be published elsewhere: AcUR (I), AcTdR (II), AcFUR (III), AcBrUR (IV), AcIUR (V), Nitro-AcTdR (VI), Chloro-AcTdR (VII), Chloro-AcUR (VIII), and Amino-AcTdR (IX). Additionally, 2′-deoxyglucosylthymine (NSC 402666) was obtained from the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute. [2-^{14}C]-Uridine (52.4 mCi/mmole) and [2-^{14}C]thymidine (51.6 mCi/mmole) were obtained from the New England Nuclear Corp., Boston, MA; [6-^{3}H]FUdR (2.3 Ci/mmole) from the Amersham/Searle Co., Arlington Heights, IL; silica gel G UV254 Polygram TLC plates from Brinkmann, Westbury, NJ; DEAE-cellulose (DE-23) from Whatman Biochemicals, Maidstone, Kent, UK; neutral Alumina (AG 7, 100–200 mesh) from Bio-Rad Laboratories, Richmond, CA; Omnifluor from the New England Nuclear Corp.; and Aquasol from the Amersham/Searle Co. All other chemicals were obtained from the Sigma Chemical Co., St. Louis, MO.

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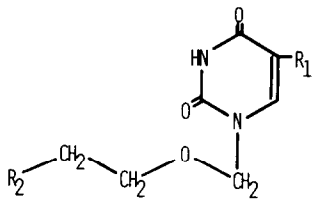
‡ Abbreviations: PyNPase, pyrimidine nucleoside phosphorylase; URPase, uridine phosphorylase; TdRPase, thymidine phosphorylase; TdR kinase, thymidine kinase (EC 2.7.1.75); UR kinase, uridine kinase (EC 2.7.1.48); UR, uridine; TdR, thymidine; UdR, 2′-deoxyuridine; FUdR, 5-fluoro-2′-deoxyuridine; BrUdR, 5-bromo-2′-deoxyuridine; IUdR, 5-iodo-2′-deoxyuridine; Py(d)R, pyrimidine ribonucleoside or deoxyribonucleoside; PydR, pyrimidine deoxyribonucleoside; P_i, inorganic phosphate; Py, pyrimidine base; AcUR, acyclouridine or 1-(2′-hydroxyethoxymethyl)uracil; AcTdR, acyclothymidine or 5-methyl-1-(2′-hydroxyethoxymethyl)uracil; AcFUR, 5-fluoroacyclouridine or 5-fluoro-1-(2′-hydroxyethoxymethyl)uracil; AcIUR, 5-iodoacyclouridine or 5-iodo-1-(2′-hydroxyethoxymethyl)uracil; AcBrUR, 5-bromoacyclouridine or 5-bromo-1-(2′-hydroxyethoxymethyl)uracil; Chloro-AcUR, chloroacyclouridine or 1-(2′-chloro-ethoxymethyl)uracil; Chloro-AcTdR, chloroacyclothymidine or 5-methyl-1-(2′-chloro-ethoxymethyl)uracil; Nitro-AcTdR, nitroacyclothymidine or 5-methyl-1-(2′-*O*-nitroethoxymethyl)uracil; Amino-AcTdR, aminoacyclothymidine or 5-methyl-1-(2′-amino-ethoxymethyl)uracil; 2′-deoxyglucosylthymine, 1-(2′-deoxy-β-D-glucopyranosyl)thymine; and DTT, dithiothreitol.



		R_1	R_2
I.	AcUR	-H	-OH
II.	AcTdR	$-CH_3$	-OH
III.	AcFUR	-F	-OH
IV.	AcBrUR	-Br	-OH
V.	AcIUR	-I	-OH
VI.	NITRO-AcTdR	$-CH_3$	$-NO_3$
VII.	CHLORO-AcTdR	$-CH_3$	-Cl
VIII.	CHLORO-AcUR	-H	-Cl
IX.	AMINO-AcTdR	$-CH_3$	$-NH_2$

Fig. 1. Chemical structures of the pyrimidine acyclonucleosides.

Animal tissues. L5178Y and L1210 leukemic cells were carried in the peritoneal cavity of male B6D2F1 mice (Cumberland Farm, Clinton, TN), and Sarcoma S-180 cells were similarly grown in CD-1 mice (Charles River Laboratories, Wilmington, MA). The latter strain of mouse (uninoculated) was also used as the source of liver tissue. Human colon tumor xenografts DLD-1, DLD-1A, DLD-1D, DLD-2, and HCT-15 and pancreatic tumor xenograft RWP-1 (all grown in nude mice) were provided by Dr. D. L. Dexter of Brown University, Providence, RI.

Preparation of cytosol extract for enzyme assay. L5178Y, L1210, and Sarcoma S-180 cells were collected in normal saline, washed as described previously [11], and homogenized in 3 vol. of 20 mM potassium phosphate buffer (pH 8.0), which contained 1 mM EDTA and 1 mM mercaptoethanol (Buffer A). Livers were obtained from freshly killed CD-1 mice and homogenized in Buffer A as above. Human tumor xenografts were homogenized in 6.25 vol. of 0.1 M Tris–HCl buffer (pH 7.5), which contained 0.25 M sucrose, 1 mM EDTA, and 1 mM mercaptoethanol (Buffer B). All homogenates were then centrifuged at 4° for 30 min at 30,000 *g* and the supernatant fractions were recentrifuged at 105,000 *g* for 1 hr. The resulting cytosol fraction was used as the source of PyNPases. Tissues to be assayed for pyrimidine nucleoside kinase activity were homogenized in 3 vol. of 0.1 M Tris–HCl buffer (pH 7.5) containing 10% glycerol and 2 mM DTT. Cytosol fractions were obtained by centrifugation as above.

PyNPase assay. In a final volume of 140 μl, reaction mixtures contained 20 mM potassium phosphate buffer (pH 8.0), 1 mM EDTA, 1 mM mercaptoethanol, 0.5 to 2.0 mg cytosol protein, a substrate at a desired concentration containing (0.13 μCi of [2-^{14}C]uridine, 0.13 μCi of [2-^{14}C]thymidine, or 2.8 μCi of [6-^{3}H]FUdR, and, when indicated, an appropriate amount of inhibitor was included. Incubations were carried out at 37° in a Dubnoff metabolic shaking incubator. At times 0 and (except where noted otherwise) 20 min, 50-μl aliquots were withdrawn from each reaction mixture and placed in a test tube containing 5 μl of 40% perchloric acid to stop the reaction. These test tubes were then centrifuged at 0° to precipitate protein, and 25 μl of the supernatant fraction was removed and neutralized with 55 μl of 0.1 N potassium hydroxide, which also contained 10 mM of the appropriate nucleoside and base to facilitate visualization on t.l.c. These samples were then centrifuged and 10-μl aliquots of the supernatant materials were spotted on silica gel G UV254 Polygram plates which were subsequently developed with chloroform–methanol (9:1). In this system, the R_f values for uridine, uracil, thymidine, thymine, FUdR, and 5-FU were 0.1, 0.26, 0.15, 0.36, 0.09, and 0.25 respectively. Spots containing substrate and product were identified by u.v. quenching and cut out. Spots containing ^{14}C were counted in Omnifluor and those containing ^{3}H were first eluted with 1 ml of methanol and then counted in Aquasol.

The spectral assay method of Krenitsky *et al.* [1] was also employed in some cases to assay PyNPase activity and was also used in testing the pyrimidine acyclonucleosides for PyNPase substrate activity. By this method, a conversion of 1% of potential substrates (1 mM) to corresponding bases could have been easily detected.

TdR kinase and UR kinase assay. The assay method used has been described previously in detail [12]. The reaction mixture contained 0.1 M Tris–HCl (pH 7.6), 0.25 mM substrate (0.4 mCi/mmole), 2.5 mM ATP, 3 mM $MgCl_2$, 20 mM NaF, 2 mM DTT, and 25–50 μl cytosol in a final volume of 100 μl. Reactions were started by the addition of cytosol and stopped by the addition of 0.2 M EDTA (pH 7.6). Nucleosides were then separated from nucleotides on Alumina columns (0.6 g in a Pasteur pipette).

DEAE-cellulose chromatography. DEAE-cellulose was equilibrated with 20 mM potassium phosphate (pH 8.0), 1 mM EDTA, and 1 mM mercaptoethanol (Buffer A), and packed into columns 2.5 × 4–6 cm. Cytosol (2–5 ml) obtained from mouse liver, Sarcoma S-180 cells, L5178Y cells, or L1210 cells was applied to the column. Each column was washed with 50–300 ml of Buffer A, and then a linear gradient to 1 M NaCl was applied over a volume of 50–70 ml. Fractions of 10 ml were collected. Each fraction was assayed for uridine (0.15 mM) and thymidine (0.15 mM) phosphorolytic activities in the presence and absence of AcUR (500 μM) using the radioisotopic technique as described above, except that reactions were allowed to proceed for 60 min rather than 20 min.

RESULTS

In the early stages of this work, we discovered that AcUR and AcTdR inhibited the cleavage of UR, but not of TdR, by cytosol extracts of mouse liver and Sarcoma S-180 cells and that neither of these compounds served as a substrate for PyNPases.

Inhibition specificity of the pyrimidine acyclonucleosides. Since two types of PyNPases are known

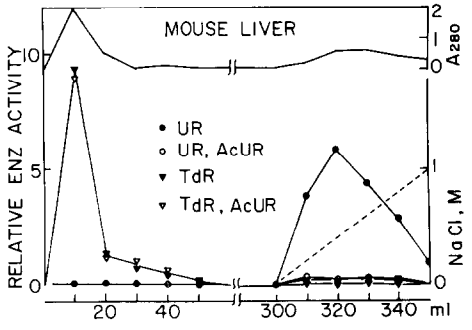


Fig. 2. DEAE-cellulose chromatograph of mouse liver cytosol.

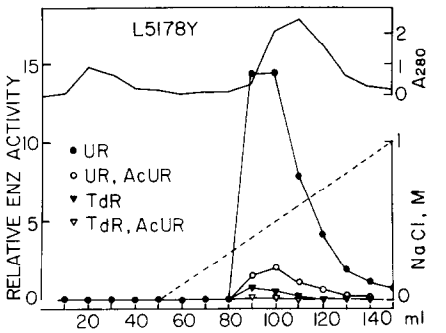


Fig. 3. DEAE-cellulose chromatography of L5178Y cell cytosol.

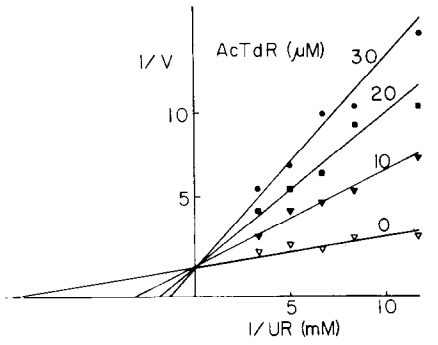


Fig. 4. Double-reciprocal plot for inhibition of URPase by AcTdR with UR as the substrate.

to occur, we decided to separate these enzymes and to determine the specificity of inhibitors for each enzyme. As shown in Fig. 2, mouse liver contained the two distinct PyNPases that can be separated by DEAE chromatography using the method of Krenitsky *et al.* [1]. The first peak (TdRPase) was eluted in the wash with Buffer A, and cleaved TdR but not UR. The second peak (URPase), which was eluted after application of the salt gradient, cleaved UR and, to a lesser extent, TdR. TdR cleavage by TdRPase was not inhibited at all by 500 μM AcUR (I), but the cleavage of both UR and TdR by URPase was markedly inhibited. These results indicate that AcUR (I) specifically inhibited URPase, but not TdRPase, and that the cleavage of TdR by the second peak was due to the non-specific URPase and not to contamination by TdRPase.

When similar studies were carried out with cytosol extracts of tumor cells of mice (Sarcoma S-180, L5178Y, and L1210), DEAE chromatographs in each case revealed the existence of only a single peak of PyNPase activity that was eluted after the application of a salt gradient. The observation that this peak had in all cases co-eluting high UR- and low TdR-cleaving activities, together with the fact that cleavage of both of these substrates was inhibited by AcUR, indicated that these tissues contained URPase only. The chromatograph of L5178Y cell extract is shown in Fig. 3.

Relative inhibitory potency of pyrimidine acyclonucleosides. Compounds I through X were screened for inhibition of TdR (0.15 mM) and UR (0.15 mM) phosphorolytic activity of mouse liver cytosol and UR (0.15 mM) phosphorolytic activity of Sarcoma S-180 cell cytosol at inhibitor concentrations of 10, 20, 30, and 40 μM. IC_{50} Values were estimated from these data (Table 1). In addition, K_i values for the pyrimidine acyclonucleosides (I–V) were estimated using Sarcoma S-180 cytosol as the source of URPase and UR as the substrate, Competitive inhibition was observed in all cases. For illustrative purposes, a double-reciprocal plot for acyclothymidine (II) is shown in Fig. 4.

Inhibition of FUdR phosphorolytic activity of human tumor cells. The effects of pyrimidine acyclonucleosides on the cleavage of FUdR in relation

Table 1. Inhibition parameters of uridine phosphorylase from mouse liver and Sarcoma S-180 cells

		IC_{50} (μM)		K_i (μM)
	Compounds	Mouse liver	S-180 cells	S-180 cells
I.	AcUR	25	40	15 ± 1
II.	AcTdR	10	15	3 ± 1
III.	AcFUR	35	40	14 ± 3
IV.	AcBrUR	40	40	13 ± 1
V.	AcIUR	40	50	30 ± 18
VI.	Nitro-AcTdR	*	*	
VII.	Chloro-AcTdR	*	*	
VIII.	Chloro-AcUR	*	*	
IX.	Amino-AcTdR	50	50	
X.	2′-Deoxyglucosylthymine	10	15	5 ± 1

* Less than 10 per cent inhibition at an inhibitor concentration of 40 μM.

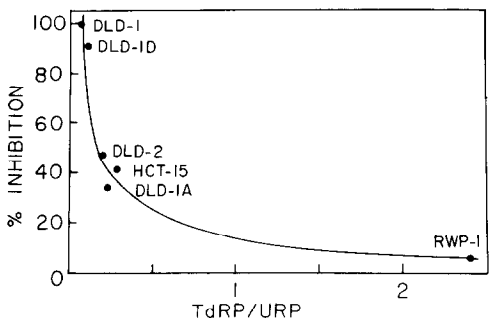


Fig. 5. Inhibition of FUdR cleaving activity of cytosol extracts of human tumor xenografts by 50 μM AcUR. The abscissa represents the ratio of phosphorylase activities with TdR and UR as substrates, both at 1 mM.

to the levels of URPase and TdRPase were investigated using cytosol extracts of xenografts of several human colon carcinomas and one human pancreatic carcinoma grown in nude mice (Fig. 5). The cleavage of FUdR (1 mM) was inhibited significantly by 50 μM AcUR (I) in all of the colon tumors (DLD-1, DLD-1A, DLD-1D, DLD-2 and HCT-15) that are relatively low in thymidine phosphorolytic activity, while, as expected, there was only a slight effect in the pancreatic tumor (RWP-1), which is high in thymidine phosphorylase.

Effect of pyrimidine acyclonucleosides on TdR kinase and UR kinase. The pyrimidine acyclonucleosides had no effect on the phosphorylation of TdR, UR, or FUdR by Sarcoma S-180 cytosol. These compounds did not inhibit either TdR kinase or UR kinase at four times the substrate concentration, and were not substrates for either enzyme. Similar results were obtained when cytosol preparations of the human tumor xenografts were used.

DISCUSSION

Despite the potential importance of modulation of URPase in the pyrimidine salvage pathway and in the metabolism of chemotherapeutically useful pyrimidine analogs [2, 13], only a few inhibitors of this enzyme are known [7–9]. We have found that the pyrimidine acyclonucleosides are specific and fairly potent inhibitors of URPase but not of TdRPase. Therefore, in addition to the obvious chemotherapeutic implications that will be discussed below, these compounds should be convenient tools to distinguish the extent of participation of each of the two PyNPases in a mixture cleaving UR, TdR, and/or other substrates.

The data presented above, together with the results of previous studies by others, allow us to postulate certain aspects of the mechanism of the active site–ligand interaction of URPase. The fact that the most potent inhibitor of the series was AcTdR (II) agrees with the finding that thymine is a better inhibitor of URPase than uracil [7]. Evidently, a hydrophobic group (e.g. methyl) at the 5-position of the pyrimidine ring enhances binding to URPase. Also consistent with this notion is the report of Woodman *et al.* [9] that a number of 5-benzyl-substituted uracil compounds are inhibitors of URPase.

The presence of a terminal side-chain hydroxyl group (i.e. R′ = OH, in Fig. 1) also seems to be an important structural requirement in the inhibition of URPase by the pyrimidine acyclonucleosides. When this hydroxyl group on AcUR (I) or AcTdR (II) is replaced with either a chloro (VII and VIII) or *O*-nitro (VI) group, the inhibitory property is lost (Table 1). As the latter groups are not capable of participating in hydrogen bonding, it appears that the terminal side-chain hydroxyl group might form a hydrogen bond in a manner critical for binding. Furthermore, the observation that replacement of the hydroxyl group with an amino group (i.e. II vs IX) diminishes, but does not completely abolish, the binding is also consistent with the possible involvement of hydrogen bonding. An equatorial 3′-hydroxyl group (presumed to participate in hydrogen bonding to URPase) has been shown by Etzold *et al.* [14] to be essential for the inhibitory action of 1-(2′-deoxy-β-D-xylopyranosyl)thymine, an equipotent analog of 2′-deoxyglucosylthymine; therefore, it is possible that the terminal hydroxyl group of the pyrimidine acyclonucleosides is serving the same function by occupying the same site as that presumably occupied by the 3′-hydroxyl group of UR and other substrates. Neither the 2′- nor the 5′-hydroxyl groups appear to be required for binding, as 2′-deoxyribonucleosides [1, 2, 15] and 5′-deoxy-5-fluorouridine [16] have been shown to serve as good substrates for URPase.

It is of interest that many of the neoplastic tissues we studied are deficient in TdRPase. We have shown that TdRPase was absent from S-180, L5178Y and L1210 cells. We have also observed that TdR phosphorolytic activity was very low in comparison to UR phosphorolytic activity in the extracts of human colon tumor xenografts. Although we have not yet separated TdRPase and URPase from these human tumors, the data in Fig. 5 suggest that some tumor cells (DLD-1 and DLD-1D) may indeed lack TdRPase, and that the cleavage of TdR detected in these cells may be due to URPase. TdRPase has also been found to be absent from Ehrlich ascites cells [1, 4], Novikoff hepatoma [1, 4], Morris 5213 and 3683 hepatomas [4], Dunning hepatoma [17], Butter Yellow-induced hepatoma in rats [4], and Walker 256 carcinoma [17]. TdR phosphorolytic activity is markedly decreased in chronic myelogenous leukemic cells, acute lymphocytic leukemic cells, and chronic lymphocytic leukemic cells, compared to normal human lymphocytes [18]. It has also been reported that acute myelogenous leukemic cells have significantly lower TdR phosphorolytic activity than non-malignant Epstein–Barr Virus-transformed lymphocytes grown in culture [19]. Thus, it appears that low or absent TdRPase may be a general property of many types of neoplasia. Exceptions to this proposition are the pancreatic tumors RWP-1 (Fig. 5) and RWP-2 (unpublished observations) which are both high in TdRPase.

The efficacy of the cancer chemotherapeutic agent FUdR is limited by its cleavage to the less toxic 5-FU by either of the PyNPases, and for many years there has been a good deal of interest in the devel-

opment of inhibitors of FUdR degradation which might enhance either the cytotoxicity or the selective toxicity of this drug, or both [5]. Our investigations with extracts of human tumor xenografts that are low in TdR phosphorolytic activity showed that FUdR cleavage can indeed be inhibited significantly by AcUR (Fig. 5). Tumors such as these, that have low or absent TdRPase, might then be expected to show an increase in sensitivity to FUdR when a pyrimidine acyclonucleoside is co-administered, as cleavage to 5-FU could thus be minimized. Furthermore, since levels of TdR kinase, the enzyme responsible for the phosphorylation of FUdR to its active metabolite, FdUMP [20], have been shown to be elevated in most tumors, as opposed to non-dividing tissues which virtually lack this enzyme [21], selective toxicity of FUdR may be enhanced by co-administration of a URPase inhibitor. This proposition is given further credence by our finding that neither TdR kinase nor UR kinase was inhibited by the pyrimidine acyclonucleosides even at concentrations four times as high as substrate.

While attempts to use URPase inhibitors to potentiate FUdR *in vivo* have met with little success [22], it is encouraging to note that Langen and Etzold [23] have shown that inhibition of URPase by 2′-deoxyglucosylthymine can increase up to 7-fold the incorporation of IUdR into the DNA of certain cat tissues (all of which contain only URPase). Since the pyrimidine acyclonucleosides (in particular, AcTdR) appear to be roughly equipotent to 2′-deoxyglucosylthymine as URPase inhibitors, it is likely that a similar degree of URPase inhibition *in vivo* may be expected from the pyrimidine acyclonucleosides, provided that these compounds cross cell membranes. If so, the pyrimidine acyclonucleosides would theoretically be able to enhance FUdR toxicity, and in man this effect should be especially pronounced in tissues such as the many tumors that contain TdR kinase and URPase but lack TdRPase.

Although direct evidence is not yet available, the inactivity of pyrimidine acyclonucleosides as substrates for any enzyme (URPase, TdRPase, and nucleoside kinases) suggests that these compounds may not be readily metabolized to give free bases or analog nucleotides. Thus, it may not be unreasonable to expect little pharmacological action (including toxicity) of these compounds when administered alone. On the other hand, as discussed above, they have a great potential as modulators of other drugs, specifically pyrimidine analogs such as FUdR.

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