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INHIBITOR PROPERTIES OF SOME 5-SUBSTITUTED URACIL ACYCLONUCLEOSIDES, AND 2,2′-ANHYDROURIDINES VERSUS URIDINE PHOSPHORYLASE FROM *E. COLI* AND MAMMALIAN SOURCES

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Abstract—Two series of 5-substituted uracil *N*(1)-acyclonucleosides, each with a different acyclic chain, were examined as inhibitors of uridine phosphorylase from rat intestinal mucosa, and several against the enzyme from Ehrlich ascites cells. In addition, several 5-substituted analogues of 2,2′-anhydrouridine were tested for their inhibitory effects vs a highly purified uridine phosphorylase from *Escherichia coli*. The results are compared with previously published data for inhibition of the *E. coli* enzyme by the acyclonucleosides, and of the rat enzyme by the anhydrouridines. In all instances, the inhibitors were active only vs the uridine, but not thymidine, phosphorylase from *E. coli*, and inhibition was competitive with respect to uridine as substrate. In general, with one or two exceptions, inhibitory effects were more pronounced against the enzyme from mammalian sources. Amongst the acyclonucleoside analogues, the most effective inhibitor of the enzyme from the rat and Ehrlich ascites cells exhibited a $K_i = 0.1\ \mu M$, comparable to that reported with the Sarcoma-180 enzyme, whereas the K_i for inhibition of the *E. coli* enzyme was 0.7 μM. By contrast, another effective inhibitor of the bacterial enzyme was 7-fold less potent against the mammalian enzyme. The 2,2′-anhydrouridines were 10- to 30-fold more effective against the rat, as compared to the *E. coli*, enzyme. The overall quantitative data provide a reasonably good basis for the further design of potent inhibitors for possible use in chemotherapy.

At least two pyrimidine nucleoside phosphorylases, uridine phosphorylase (EC 2.4.2.3) and thymidine phosphorylase (EC 2.8.2.4) are involved in the catabolism of FUrd and FdUrd, both of which are employed in tumour chemotherapy. Considerable attention has consequently been devoted to the development of effective inhibitors of both these enzymes.

There are, in addition, appreciable differences in substrate properties between the enzymes from various mammalian tissues and microorganisms. Some tumour cells have even been reported to be devoid of thymidine phosphorylase activity [1–3], and in such instances it is the uridine phosphorylase which possesses the ability to phosphorylise both ribo- and deoxyribo-pyrimidine nucleosides. Hence the interest in inhibitors of uridine phosphorylase, which may selectively inhibit phosphorolysis in tumour cells with minimal disturbances of pyrimidine nucleoside metabolism in normal cells.

One recently developed series of inhibitors vs uridine phosphorylase of Sarcoma-180 cells, with inhibition constants in the micromolar range, comprises some 5-substituted analogues of acyclouridine, 1-(2-hydroxyethoxymethyl)uracil [3, 4], depicted at the top of Table 1. We have recently reported on the inhibitory properties of some 5- and 5,6-substituted acyclouridines against highly purified uridine phosphorylase from *Escherichia coli* [5]. It was noted at that time that several of these inhibitors were appreciably less effective against the bacterial, as compared to the mammalian, enzyme.

We have now extended our findings to a direct comparison of the inhibitory properties of the acyclonucleoside analogues against the enzyme from rat intestinal mucosa, Ehrlich ascites cells and from *E. coli*. In the interim, a report appeared on the unusual inhibitory properties of some 2,2′-anhydrouridines against the rat intestinal mucosa enzyme [6] and several of these are included in the present investigation.

MATERIALS AND METHODS

Uridine was a product of BDH (Poole, U.K.) and [2-^{14}C]uridine (53 mCi/mmol) was from the Institute for Radioisotope Production (Prague, Czekoslovakia). Syntheses of the 2,2′-anhydrouridines, and of the 5- and 5,6-substituted uracil acyclonucleosides, have been described elsewhere [6].

Uridine phosphorylase from *E. coli* was purified essentially according to Vita and Magni [7], and was an electrophoretically homogeneous preparation with a specific activity for phosphorolysis of uridine of 240 μmoles/mg/min. The enzyme from rat intestinal mucosa, like that from Sarcoma-180 cells [3], was the fraction remaining following ammonium sulphate fractionation of a crude extract, with an activity of 0.14 μmoles/mg/min, and devoid of thymidine phosphorylase activity. The source of the enzyme from Ehrlich ascites cells, free of thymidine phosphorylase activity [3], was the 20,000 *g* supernatant of the cell homogenate. Thymidine phospho-

rylase from *E. coli* was the fraction remaining following column chromatography isolation of uridine phosphorylase [7], and was completely free of uridine phosphorylase activity.

Phosphorolysis of uridine by the purified *E. coli* enzyme was assayed spectrophotometrically, as previously described [5].

With the enzymes from intestinal mucosa and Ehrlich ascites cells, a radiochemical method was used. The incubation medium, 100 μl of 40 mM phosphate buffer pH 7.4, included 25, 50 or 100 μM uridine supplemented with an appropriate amount of [2-^{14}C]uridine. Inhibitor was added to successive tubes at appropriate concentrations, and the reaction initiated by addition of enzyme and incubation for 5 min at 37°. Conditions were such that the reaction was linear for 30 min. Incubation was terminated by addition of 100 μl ice-cold methanol, and 20–30 μl aliquots were spotted on silica gel plates, followed by 1 μl each of 10 mM solutions of uracil and uridine as standards. The plates were developed with ethyl acetate–methanol–chloroform (8:1:1, v/v). Spots corresponding to uracil were located with a dark u.v. lamp (R_f = 0.71 for uracil and 0.32 for uridine), cut out, transferred to a scintillation vial, and radioactivity counted in a toluene scintillator with a Beckman LS-900 instrument.

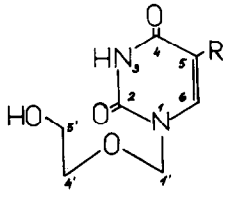
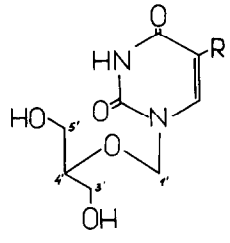
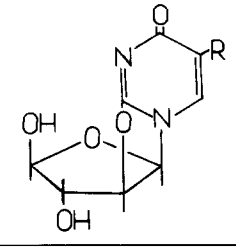
Apparent K_i values were derived from Dixon plots, $1/v$ vs $[I]$, with the aid of a linear regression program kindly prepared by Dr Z. Kaminski, using a Commodore-120 computer.

RESULTS AND DISCUSSION

Table 1 presents the experimental values of the constants, K_i, for inhibition of phosphorolysis of uridine by the rat intestinal mucosa enzyme by the previously described pyrimidine acyclonucleoside analogues [5], and for inhibition of the *E. coli* enzyme by the 2,2′-anhydrouridines. For comparison purposes the table includes previously published data for inhibition of *E. coli* [5] and Sarcoma-180 [3] enzyme, and several values for the Ehrlich ascites enzyme.

As previously noted with the *E. coli* enzyme [5], inhibition of the rat intestinal mucosa enzyme by the acyclonucleoside analogues was competitive with respect to uridine, as shown for compound **1d** in

Table 1. Inhibition constants, K_i, for inhibition of uridine phosphorylase from various sources by pyrimidine acyclonucleoside and 2,2′-anhydrouridine analogues

Inhibitor	R	K_i (μM) Rat intestinal mucosa	K_i (μM) *E. coli*	K_i (μM) Other sources
	**1a** —H	5.2 ± 0.9	35*	15†
	1b —CH_3	2.2 ± 0.5	77	3†
	1c —CH_2CH_3	0.8 ± 0.2	15	—
	1d —$CH_2CH_2CH_3$	0.5 ± 0.1	14	0.7 ± 0.2‡
	1e —$CH(CH_3)_2$	28.0 ± 6.0	58	28.0 ± 5.0‡
	1f —$(CH_2)_4$—§	32.0 ± 7.0	27	—
	1g —$CH_2C_6H_5$	—	—	0.1†
	**2a** —H	25.8 ± 5.1	23	—
	2b —CH_3	3.2 ± 0.6	21	—
	2c —CH_2CH_3	1.2 ± 0.4	5	—
	2d —$CH_2CH_2CH_3$	1.8 ± 0.3	10	—
	2e —$CH(CH_3)_2$	0.5 ± 0.1	7	0.6 ± 0.3‡
	2f —$(CH_2)_4$—§	17.8 ± 4.8	2.7	—
	2g —$CH_2C_6H_5$	0.1 ± 0.01	0.7	0.1‖
	**3a** —H	2.30¶	29.0 ± 7.0	
	3b —CH_3	0.18	—	
	3c —CH_2CH_3	0.03	1.0 ± 0.2	
	3d —$CH_2CH_2CH_3$	0.08	1.8 ± 0.3	

* Values for **1a–1f** and **2a–2g** from ref. 5; † for Sarcoma-180 enzyme from ref. 3; ‡ for Ehrlich ascites enzyme; § this is actually a 5,6-disubstituted uracil analogue, i.e. tetramethyleneuracil; ‖ for Sarcoma-180 enzyme from ref. 8; ¶ values for **3a–3d** from ref. 6.

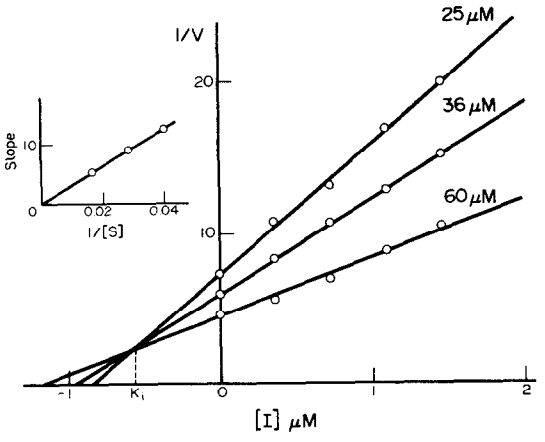


Fig. 1. Typical Dixon plot for inhibition of uridine phosphorylase from rat intestinal mucosa, in this case by compound **1d** of Table 1. Substrate concentrations were 25, 36 and 60 μM, as indicated. Reactions were carried out as described under Materials and Methods, using 3×10^{-3} mg protein.

Fig. 1. The three 2,2′-anhydrouridine analogues, previously shown to be competitive inhibitors of the rat enzyme [6], were now found to also competitively inhibit the *E. coli* enzyme. We have also found that none of the foregoing compounds detectably inhibits thymidine phosphorylase from *E. coli*.

Our attention had earlier been drawn to the fact that, amongst the series **2** analogues (Table 1), **2g** was 7-fold less effective as an inhibitor of the *E. coli* enzyme than of the crude Sarcoma-180 enzyme reported by Lin and Liu [8].

The present findings with the rat intestinal mucosa and Ehrlich enzyme further underline the differences in inhibitory properties towards the enzyme from mammalian and bacterial sources. Amongst the series **1** compounds it may be noted (Table 1) that K_i decreases systematically with an increase in length of the 5-alkyl chain from H to propyl which is related to the increase in hydrophobicity of these inhibitors, consistent with the proposal of the existence in the enzyme molecule of a hydrophobic region in the vicinity of the site which binds the inhibitor via the 5-substituent [3, 9]. By contrast, for the 5-isopropyl analogue there is a dramatic increase in K_i relative to propyl (28 μM as compared to 0.5 μM). With a 5,6-tetramethylene substituent (**1f**) the K_i is also relatively elevated, qualitatively in accord with the fact that 5,6-tetramethyleneuridine, which is a substrate, exhibits a 5-fold higher K_m and a 4-fold lower V_{max} [10]. Particularly striking are the low values of K_i with **1a–1d** for the rat, relative to the bacterial, enzyme pointing to major differences between the active sites of the two enzymes. Both **1a** and **1b** are also more effective vs the rat as compared to the Sarcoma-180 [3] enzyme.

Amongst the series **2** compounds it will be seen that **2a** is equally effective against the rat and *E. coli* enzymes. For the remaining members of the series, with the exception of **2f**, the rat enzyme is appreciably more sensitive. By contrast **2f** is 6.5-fold more effective against the *E. coli* enzyme. It is worth noting that for **2g**, the measured value of K_i with the rat enzyme (0.1 μM) is virtually identical with the value of 0.1 μM reported by others with the Sarcoma-180 enzyme [8]. Rather striking is the more than 50-fold decrease in K_i with the rat enzyme for **1e** on addition of a hydroxymethyl group to the acyclic chain to give **2e**. A similar increase, albeit only 8-fold, is observed with the *E. coli* enzyme.

The effectiveness of some of the inhibitors against the rat intestinal mucosa enzyme does not necessarily imply that they will be equally effective against the enzyme from other mammalian sources (see final paragraph of Discussion, below), although the K_i for **2g** is the same for the enzymes from the rat and Sarcoma-180 (Table 1). Several, of the inhibitors were therefore tested for activity vs the enzyme from Ehrlich ascites cells. From Table 1 it will be seen that the K_i values for **1d** and **2e** are equally low for the enzyme from this source, while the high value of **1e** with the rat enzyme is reproduced also with the Ehrlich ascites enzyme.

For the 2,2′-anhydrouridine analogues (series **3**) it will be seen, from the measured K_i values, that these are from 12- to 33-fold more effective against the rat intestinal mucosa enzyme as compared to that from *E. coli*.

The potent inhibitor properties of the 2,2′-anhydrouridines, particularly against the mammalian enzyme, call for special comment. In the case of the acyclonucleosides, the conformational flexibility of the acyclic chains is such that they are capable of mimicking the structure of the pentose ring, and may also readily adopt any of the possible conformations about the glycosidic bond. This is of interest in the light of the fact that 6-methyluridine, which is in the fixed *syn* conformation about the glycosidic bond, is a reasonable substrate for phosphorolysis by uridine phosphorylase from both bacterial and mammalian sources [10, 11] and, in fact, suggests that phosphorolysis proceeds via an intermediate of the substrate uridine in the *syn* conformation. By contrast, the 2,2′-anhydrouridines are in a fixed high-*anti* conformation, and the electronic properties of both the sugar and heterocyclic ring are appreciably different from those of uridine. It becomes, therefore, of interest to determine how these bind to the enzyme, and this aspect is under further investigation.

During the preparation of this manuscript, a report appeared by Park *et al.* [12] on the inhibitory properties of some benzylacyclouridine analogues toward the *E. coli* enzyme purified by a procedure other than ours. One of their analogues was the same as our **2g**, for which they found a K_i of 3.9 μM as compared to our value of 0.7 μM. We are at a loss as to the source of this discrepancy. Furthermore, Park *et al.* [12] report that the inhibitory potency of their compounds with the *E. coli* enzyme parallel those obtained with the enzyme from mammalian sources, and conclude therefrom that the structure of the active site of uridine phosphorylase from *E. coli* may resemble that of the mammalian enzyme. Our own findings are clearly not in accord with this conclusion, the more so in that, whereas in general most of our compounds are better inhibitors of the mammalian enzymes, one of them (**2f**) is appreciably

more effective against the bacterial enzyme. Furthermore, turning once again to our analogue **2g**, for which Park *et al.* [12] reported a K_i of 3.9 μM in the *E. coli* system (as against our value of 0.7 μM), the same compound has been shown both by us (Table 1), and independently by Lin and Liu [8], to be much more effective against both the rat and the Sarcoma-180 enzymes.

The properties of the analogues listed in the present investigation, together with those described by Park *et al.* [12] and others, constitute a reasonably good basis for the design of more potent ones for possible use in chemotherapy. The possibility nonetheless exists that the sensitivity towards a given inhibitor may vary for the enzyme from different sources. However, interest still attaches to the activity of these inhibitors vs the *E. coli* enzyme, the more so in that the latter is easily available in purified form, and has also been recently crystallized in a form suitable for X-ray diffraction studies [13]. This should now permit investigations on the mode of interaction of the bacterial enzyme with inhibitors.

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