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# 5-SUBSTITUTED-2,2′-ANHYDROURIDINES, POTENT INHIBITORS OF URIDINE PHOSPHORYLASE

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**Abstract**—5-Substituted-2,2′-anhydrouridines are a new class of competitive inhibitors of uridine phosphorylase. The most potent member of the series is 2,2′-anhydro-5-ethyluridine with an apparent $K_i$ value of 25 nM. These compounds are selective inhibitors of uridine phosphorylase and have no effect on thymidine phosphorylase. 5-Substituted-2,2′-anhydrouridines are no substrates of either uridine phosphorylase or thymidine phosphorylase.

Two different pyrimidine nucleoside phosphorylases uridine phosphorylase (URPase, E.C. 2.4.2.3) † and thymidine phosphorylase (TdRPase, E.C. 2.4.2.4) have been detected in the cytosol of mammalian cells [1–3].

These enzymes catalyze the reversible phosphorolysis of a number of natural and synthetic pyrimidine nucleosides according to the following equation:

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

TdRPase is specific for 2′-deoxyribonucleosides [1, 4]. Inhibitors of pyrmidine nucleoside phosphorylases may be useful as chemotherapeutic agents by influencing the catabolism of pyrimidine nucleoside analogs having chemotherapeutic activity. To date, only a few potent inhibitors of these enzymes have been discovered. 6-Aminothymine is the well known inhibitor of TdRPase although it also inhibits URPase activity [5]. URPase was reported to be inhibited by 2′-deoxyglucosylthymine [6] and more recently by various 5-benzyluracil derivatives [7–9] and pyrimidine acyclonucleosides [10, 11]. We have studied the effect of 5-substituted-2,2′-anhydrouridines on the activity of URPase isolated from rat intestinal mucosa and on TdRPase of mouse liver.

## MATERIALS AND METHODS

*Materials.* [2-$^{14}$C]Thymidine (53.2 mCi/mmole) and [6-$^{3}$H]uridine (21.6 Ci/mmole) were obtained from Amersham International Ltd. (Amersham, U.K.), silica gel TLC plates (SILUFOL UV 254) from Serva Fine Biochemicals (Heidelberg, F.R.G.) and DEAE-cellulose (DE-32) from Whatman Biochemicals (Maidstone, Kent, U.K.) All other chemicals were obtained from Merck (Darmstadt, F.R.G.).

*Chemical synthesis.* The 5-substituted 2,2′-anhydro - 1 - (β - D - arabinofuranosyl) - pyrimidine nucleosides (IVa-g) were prepared from the corresponding pyrimidine nucleosides [12] with the 2-acetoxy benzoyl chloride method [13] outlined in Fig. 1.

Melting points were determined with a Boetius melting point apparatus (PHMK 05) and were not corrected. Ultraviolet spectra were recorded on a Unicam SP 8000 spectrophotometer using 10-mm cuvettes, pH values were measured by precision pH meter (OP-205, Radelkis, Hungary). $^{1}$H NMR spectra were recorded at 100.1 MHz using a Varian XL-100 Fourier transform spectrometer. Chemical shifts (in p.p.m.) are relative to internal TMS. Probe temperature was maintained at 60°. Synthesis of 3′-*O*-acetyl - 2,2′ - anhydro - 1 - (β - D - arabinofuranosyl) pyrimidine nucleosides (IIIa-g).

A suspension of 2-acetoxy benzoyl chloride (II) (4.96 g, 25 mmole) and the pyrimidine nucleoside (I) (10 mmole) in acetonitrile (30 ml) was stirred at 50° for 5–7 min (until a clear solution was obtained) then at room temperature for 30 min. The solvent was removed *in vacuo* and the residue was triturated with ethylacetate. The resulting crystals were collected by filtration and recrystallized from methanol containing 3% HCl and saturated with ether to give pure IIIa-g in 70–80% yield.

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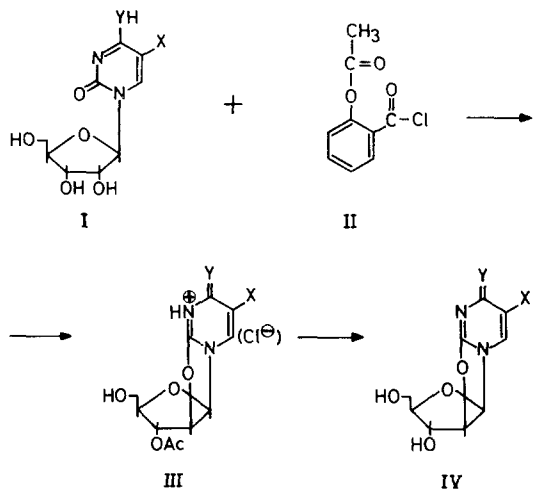
† Abbreviations used: URPase, uridine phosphorylase; TdRPase, thymidine phosphorylase; Pyr(d)R, pyrimidine ribonucleoside or pyrimidine 2′-deoxyribonucleoside; $P_i$, orthophosphate; Pyr, pyrimidine base; (d)R-1-P, α-D-ribose-1-phosphate or α-D-deoxyribose-1-phosphate; u.v., ultraviolet; app$K_i$, apparent $K_i$; 2,2′-anhydrouridine, 2,2′-anhydro-1-(β-D-arabinofuranosyl)uracil; 2,2′-anhydro-5-fluorouridine, 2,2′-anhydro-1-(β-D-arabinofuranosyl)-5-fluorouracil; 2,2′-anhydro-5-methyluridine, 2,2′-anhydro-1-(β-D-arabinofuranosyl)-5-methyluracil; 2,2′-anhydro-5-ethyluridine, 2,2′-anhydro-1-(β-D-arabinofuranosyl)-5-ethyluracil; 2,2′-anhydro-5-propyluridine, 2,2′-anhydro-1-(β-D-arabinofuranosyl)-5-*n*-propyluracil; 2,2′-anhydro-5-isopropyluridine, 2,2′-anhydro-1-(β-D-arabinofuranosyl)-5-isopropyluracil; 2,2′-anhydrocytidine, 2,2′-anhydro-1-(β-D-arabinofuranosyl)cytosine; 3′-*O*-acetyl-2,2′-anhydro-5-propyluridine, 3′ - *O* - acetyl - 2,2′ - anhydro - 1 - (β - D-arabinofuranosyl)-5-*n*-propyluracil hydrochloride; 3′-*O*-acetyl-2,2′-anhydro-5-isopropyluridine, 3′-*O*-acetyl-2,2′-anhydro - 1 - (β - D - arabinofuranosyl) - 5 - isopropyluracil hydrochloride.




| | Y | X |
|---|---|---|
| a. | -NH | -H |
| b. | -O | -F |
| c. | -O | -H |
| d. | -O | $-CH_3$ |
| e. | -O | $-CH_2-CH_3$ |
| f. | -O | $-(CH_2)_2-CH_3$ |
| g. | -O | $-CH(CH_3)_2$ |

Fig. 1. Synthetic scheme for 2,2′-anhydropyrimidine nucleosides.

*Synthesis of 2,2′-anhydro-1-(β-D-arabinofuranosyl)pyrimidine nucleosides (IVa-g).* The 3′-*O*-acetyl derivative (IIIa-g) (10 mmole) was dissolved in 0.5 N ethanolic sodium ethoxide (100 ml) and the solution was stirred at room temperature for 2 hr. After neutralization of the solution with Dowex 50 ($H^+$) the solvent was evaporated to dryness *in vacuo* and the residue recrystallized from ethanol. The crystals were collected by filtration to give pure IVa-g in 60–80% yield.

*Enzyme preparation and partial purification.* Our method is a combination of the different steps of previously described methods [19, 25]. The liver of male CFLP mice (20–25 g) and the intestinal mucosa of male CFY rats (200–250 g) were removed. Before excision the livers were perfused with 0.9% NaCl solution. Tissues were homogenized using Potter S Homogeniser (B. Braun) in 3 vol of 0.02 M potassium phosphate buffer (pH 8.0) containing 10 mM β-mercaptoethanol and 1 mM EDTA. The homogenates were centrifuged at 4° for 1.5 hr at 100,000 *g* and the cytosols were treated with ammonium sulfate. The precipitate obtained between 35 and 65% saturation was resuspended in 0.02 M potassium phosphate buffer (pH 8.0) containing 10 mM β-mercaptoethanol and 1 mM EDTA and dialysed against the same buffer. The dialysed preparations were stored at −25° showing no loss of activity over a month.

*DEAE-cellulose chromatography.* DEAE-cellulose was equilibrated with 0.02 M potassium phosphate buffer (pH 8.0) containing 10 mM β-mercaptoethanol and 1 mM EDTA, packed into a column (1.6 × 10 cm), and 3 ml of mouse liver preparation was applied. The column was then eluted with 50 ml of the potassium phosphate buffer mentioned above. The eluent was used as the source of partially purified TdRPase. Mouse liver URPase binds to DEAE-cellulose under these conditions whereas TdRPase does not [17]. This procedure removed all URPase contamination from the TdRPase preparation.

*Protein determination.* Protein concentration was determined by the method of Lowry *et al.* [18].

*Enzyme assay.* The phosphorolysis of pyrimidine nucleosides by the extract of mouse liver and rat intestinal mucosa was assayed in a 250 μl incubation mixture which contained 0.04 M potassium phosphate buffer (pH 7.4), 2 mM β-mercaptoethanol, 0.01–0.1 mg protein, a substrate at a desired concentration, and when indicated an appropriate amount of inhibitor was included. Inhibitors were screened at concentrations of 15.625 nM to 5.0 μM.

The specific activity of TdRPase at 3.33 mM substrate concentration for UdR is 4.2 nmole/min/mg protein; for TdR it is 4.4 nmole/min/mg protein at 37°. The specific activity of URPase at 3.33 mM substrate concentration for UR is 146.9 nmole/min/mg protein; for UdR it is 141.0 nmole/min/mg protein; for TdR it is 17.3 nmole/min/mg protein at 37°.

*Ultraviolet absorption spectrophotometry.* The phosphorolysis of natural pyrimidine nucleosides and 2,2′-anhydropyrimidine nucleosides was carried out according to the method of Yamada [19]. Under conditions of the assay, for a period of 20 min, the formation of free base was a linear function of time. The Yamada's method is based on the difference of u.v. absorption spectra of pyrimidines and their nucleosides at alkaline pH. The u.v. spectra of nucleosides and their bases were followed at pH 12 (Specord UV VIS). The molar extinction coefficients of nucleoside ($\varepsilon^N_{\lambda maxN}$, $\varepsilon^N_{\lambda maxB}$) and base ($\varepsilon^B_{\lambda maxN}$, $\varepsilon^B_{\lambda maxB}$) were determined at two wavelengths at λmax of the base (λmaxB) and λmax of the nucleoside (λmaxN). Concentration of the base formed in the reaction mixture was calculated by the following equation

$$c_B = \frac{(\varepsilon^N_{\lambda maxB} \cdot E_{\lambda maxN}) - (\varepsilon^N_{\lambda maxN} \cdot E_{\lambda maxB})}{(\varepsilon^B_{\lambda maxN} \cdot \varepsilon^N_{\lambda maxB}) - (\varepsilon^N_{\lambda maxN} \cdot \varepsilon^B_{\lambda maxB})}$$

where $E_{\lambda maxB}$ is the absorption of the supernatant at λmax of the base and $E_{\lambda maxN}$ is the absorption at λmax of the nucleoside.

*Thin-layer chromatography.* TLC was used in the inhibition studies. [6-$^3$H]Uridine (8 mCi/mmole, at 22.2 μM) or [2-$^{14}$C]thymidine (5.5 mCi/mmole, at 33.3 μM) was used as a substrate when the effect of 2,2′-anhydropyrimidine nucleosides on URPase or TdRPase was studied. The enzyme reaction was stopped with ice-cold methanol which also contained 2 mM of the appropriate base to facilitate visualization on TLC plates. Silica gel plates (Silufol u.v. 254 SERVA) were developed with ethylacetate–methanol–chloroform (8:1:1, v/v/v). Spots containing substrate or product were identified by u.v. quenching, cut out, eluted with 1 ml of methanol and

Table 1. Relative rates of phosphorolysis of natural pyrimidine nucleosides and their analogs by URPase and TdRPase*

| Nucleoside | Relative activity (%) | |
|---|---|---|
| | URPase | TdRPase |
| 2′-Deoxyuridine | 100 | 100 |
| Thymidine | 12 | 104 |
| Uridine | 104 | ND† |
| 2,2′-Anhydropyrimidine nucleosides (IVc; IVd; IVe; IVf; IVg; IIIf; IIIg) | ND | ND |

* Reactions were monitored spectrophotometrically. Standard reaction mixture was used and substrate concentration was 3.33 mM. Time of incubation was 20 min. Specific activities of URPase and TdRPase for TdR at 37° were 17.3 and 4.4 nmole/min/mg protein, respectively.

† ND, Not detectable.

then counted in a scintillation cocktail containing toluene, using a Packard model 2650 scintillation spectrometer. The recovery of radioactivity from the plates was 95%.

## RESULTS

2,2′-Anhydropyrimidine nucleosides are no substrates of either URPase or TdRPase (Table 1). The inhibitory effect of these compounds on URPase isolated from rat intestinal mucosa is shown in Table 2.

The introduction of a fluoro atom in position 5 of 2,2′-anhydrouridine resulted in a 3-fold decrease in app$K_i$. The app$K_i$ value greatly decreased by substitution of 2,2′-anhydrouridine with short linear alkyl chains (IVd, IVe, IVf). 2,2′-Anhydro-5-ethyluridine proved to be the most potent inhibitor of the series. The app$K_i$ value of this molecule is by two orders of magnitude lower than that of 2,2′-anhydrouridine. Introduction of a branched alkyl chain in position 5 of 2,2′-anhydrouridine (IVg) resulted in an increase in app$K_i$. Compounds having an acetyl group in position 3′ of 2,2′-anhydro-5-alkyluridines (IIIf, IIIg) and 2,2′-anhydrocytidine did not inhibit the phosphorolysis of uridine at 5 $\mu$M maximal concentration. 2,2′-Anhydrouridines are no irreversible inhibitors of URPase since preincubation of 2,2′-anhydro-5-fluorouridine for 20 min with the enzyme preparation had no effect on inhibition and dialysis after preincubation with this compound restored the activity of the enzyme. Competitive inhibition was observed in all cases. As an example a double reciprocal plot for 2,2′-anhydro-5-ethyluridine is shown in Fig. 2. We studied the effect of 2,2′-

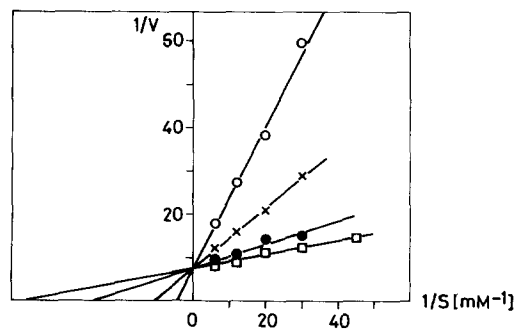



Fig. 2. Double reciprocal plot for inhibition of URPase by 2,2′-anhydro-5-ethyluridine. Key: (□) uridine alone ($K_m$ = 0.022 mM; $V_{max}$ = 0.13 $\mu$mole/min/mg protein at 37°); (●) 15.625 nM 2,2′-anhydro-5-ethyluridine; (×) 62.5 nM 2,2′-anhydro-5-ethyluridine; (○) 250 nM 2,2′-anhydro-5-ethyluridine.

Table 2. Apparent $K_i$ values for inhibition of URPase by 2,2′-anhydropyrimidine nucleosides*

| Compound | app$K_i$ (nM) |
|---|---|
| 2,2′-Anhydrouridine (IVc) | 2268 |
| 2,2′-Anhydro-5-fluorouridine (IVb) | 740 |
| 2,2′-Anhydro-5-methyluridine (IVd) | 182 |
| 2,2′-Anhydro-5-ethyluridine (IVe) | 25 |
| 2,2′-Anhydro-5-propyluridine (IVf) | 79 |
| 2,2′-Anhydro-5-isopropyluridine (IVg) | 1461 |
| 2,2′-Anhydrocytidine (IVa) | † |
| 3′-O-acetyl-2,2′-anhydro-5-propyluridine (IIIf) | † |
| 3′-O-acetyl-2,2′-anhydro-5-isopropyluridine (IIIg) | † |

* Apparent $K_i$ values were determined from Lineweaver–Burk plots of the data by a computer program with least-squares fitting.

† Less than 10% inhibition of uridine cleavage at maximal inhibitor concentration of 5 $\mu$M.

anhydropyrimidine nucleosides on the phosphorolysis of thymidine in the presence of TdRPase obtained by DEAE chromatography. We found that these compounds do not inhibit the cleavage of thymidine by TdRPase up to the inhibitor concentration of 3.33 mM.

## DISCUSSION

Inhibitors of pyrimidine nucleoside phosphorylases might be useful as chemotherapeutic agents by enhancing the efficacy of pyrimidine nucleoside analogs such as 5-fluorouridine, 5-fluoro-2′-deoxyuridine, 5-iodo-2′-deoxyuridine or 5-bromovinyl-2′-deoxyuridine which are cleaved by pyrimidine nucleoside phosphorylases [8, 20–24].

To date, only a few potent inhibitors have been found. We have observed that some 2,2′-anhydro-5-alkyluridines (IVd, IVe, IVf) are potent inhibitors of URPase. Competitive inhibition was observed in all cases. These molecules are specific inhibitors of URPase and have no effect on TdRPase. The introduction of an electron-withdrawing fluoro atom substituted in position 5 of 2,2′-anhydrouridine resulted in a decrease in app$K_i$. This notion is consistent with the finding reported previously by Barker *et al.* and Niedzwicki *et al.* who studied the effect of 5-halogenuracils [7, 25]. It has been supposed that there is a hydrophobic region on URPase adjacent to the binding site of 5-position of the pyrimidine ring [7, 10, 25]. Our observation that the app$K_i$ value of an analog having a methyl group in position 5 of 2,2′-anhydrouridine is by one order of magnitude lower than that of 2,2′-anhydrouridine, agrees with this hypothesis. The introduction of an ethyl group in position 5 of 2,2′-anhydrouridine further reduces the app$K_i$ value. 2,2′-Anhydro-5-ethyluridine is the most potent inhibitor of URPase. Analogs with longer linear or branched side chains have higher app$K_i$ values. Molecules having an acetyl group in position 3′ of 5-alkyl-2,2′-anhydouridines (IIIf, IIIg) have no inhibitory effect on URPase at concentration of 5 $\mu$M. This observation is consistent with the notion that the 3′-hydroxyl group of pyrimidine nucleosides appears to be essential for binding to URPase [10, 25, 26].

2,2′-Anhydrocytidine does not inhibit the cleavage of uridine by URPase. The finding is in agreement with that of Niedzwicki that neither cytidine nor cytosine inhibits the phosphorolysis of uridine by URPase [25].

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