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INHIBITION OF URIDINE PHOSPHORYLASE FROM *ESCHERICHIA COLI* BY BENZYLACYCLOURIDINES*

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Abstract—The benzylacyclouridines, potent and specific inhibitors of mammalian uridine phosphorylase, were also found to be inhibitors of uridine phosphorylase but not thymidine phosphorylase from *Escherichia coli*. Competitive inhibition was observed in all cases and the most potent of these compounds was HM-BBAU (5-(3-benzyloxybenzyl)-1-[(2′-hydroxy-1′-hydroxymethyl)methyl]uracil) with a K_i value of 0.15 μM. The inhibitory potencies of these compounds parallel those obtained with enzymes from mammalian sources [Niedzwicki *et al.*, *Biochem. Pharmac.* **31**, 1857 (1982) and Naguib *et al.*, manuscript in preparation] indicating that the structure of the active site of uridine phosphorylase from *E. coli* may resemble that of the mammalian enzyme.

Pyrimidine nucleoside phosphorylases (PyrNPase)§ exist in both eukaryotes and prokaryotes [1, 2]. In most cell types, there are two distinct pyrimidine nucleoside phosphorylases, uridine phosphorylase (UrdPase, EC 2.4.2.3) and thymidine phosphorylase (dThdPase, EC 2.4.2.4), which catalyze the reversible phosphorolysis of pyrimidine nucleosides as follows:

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

Although uridine is the preferred substrate for UrdPase, the enzyme from most sources has a considerable activity toward deoxyuridine and thymidine [1, 3]. However, the enzyme from *Escherichia coli* [4] and the plasma membrane of rat liver cells [5] is more specific, as it has been reported to act almost exclusively on uridine. dThdPase, on the other hand, is highly specific for pyrimidine deoxyribonucleosides and thus it does not cleave uridine or FUrd [6, 7]. dThdPase is absent from most of the mammalian neoplastic tissues studied [1, 6, 8]. However, some human tumors contain high dThdPase activity [6, 9]. In microorganisms, such as *Haemophilus influenzae* [10] and *Bacillus stearothermophilus* [11], a single pyrimidine nucleoside phosphorylase comprises both UrdPase and dThdPase activities. The patterns of substrate specificity are quite similar to that of the UrdPase from several mammalian sources, which were reported to retain significant activity toward deoxynucleosides [10].

Benzylacyclouridine (BAU) and its analogues have been shown to be the most potent and specific inhibitors known for UrdPase from mammalian sources‖ [12–16]. Few inhibitors of UrdPase from microorganisms have been discovered. It was of interest to test whether or not these benzylacyclouridines also inhibit UrdPase from *E. coli*, in spite of the fact that the enzyme from *E. coli* differs from the mammalian enzymes in substrate specificity [1], pH optimum [9], and kinetic mechanism [17–19]. In this report, we demonstrate that several benzylacyclouridines are also potent and specific inhibitors of UrdPase from *E. coli*. Recently, Shugar [20] also reported that BAU is an inhibitor of UrdPase from *E. coli*.

MATERIALS AND METHODS

Chemicals. Benzylacyclouridines were synthesized by methods published [13] or to be published elsewhere. [2-^{14}C]Thymidine and [2-^{14}C]uridine were obtained from Moravek Biochemicals, Inc., Brea, CA; silica gel G/UV 254 polygram TLC plates were

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§ Abbreviations: BAU, 5-benzyl-1-[(2′-hydroxyethoxy)-methyl]uracil; BBAU, 5-(3-benzyloxybenzyl)-1-[(2′-hydroxyethoxy)methyl]uracil; BBBAU, 5-[3-(4-benzyloxybenzyoxy)benzyl]-1-[(2′-hydroxyethoxy)methyl]uracil; FUrd, 5-fluorouridine; *C*-methyl-BAU, 5-benzyl-1-[*C*-(2′-hydroxyethoxy)-*C*-(methyl)methyl]uracil; *C*-methyl-BBAU, 5-(3-benzyloxybenzyl)-1-[*C*-(2′-hydroxyethoxy)-*C*-(methyl)methyl]uracil; HM-BAU, 5-benzyl-1-[(2′-hydroxy-1′-hydroxymethylethoxy)methyl]uracil; HM-BBAU, 5-(3-benzyloxybenzyl)-1-[2′-hydroxy-1′-hydroxymethyl)methyl]uracil; OH-BAU, 5-(3-hydroxybenzyl)-1-[2′-hydroxyethoxy)methyl]uracil; P_i, inorganic phosphate; Pyr, pyrimidine base; Pyr(d)R, pyrimidine ribonucleoside or deoxynucleoside; PyrNPase, pyrimidine nucleoside phosphorylase; succ-BAU, 5-benzyl-1-[(2′-[3-carboxypropionyl]ethoxy)methyl]uracil, sodium salt; succ-BBAU, 5-(3-benzyloxybenzyl)-1-[(2′-[3-carboxypropionyl]ethoxy)methyl]uracil, sodium salt; dThd, thymine; dThdPase, thymidine phosphorylase; Urd, uridine; and UrdPase, uridine phosphorylase.

‖ F. N. M. Naguib, M. H. el Kouni, S. H. Chu and S. Cha, manuscript in preparation.

from Brinkmann Instruments, Westbury, NJ; the Protein Assay kit was from Bio-Rad Laboratories, Richmond, CA; and Omnifluor scintillant was from the New England Nuclear Corp., Boston, MA. all other chemicals were obtained from the Sigma Chemical Co., St. Louis, MO.

Purification of PyrNPases from E. coli. UrdPase and dThdPase were purified from *E. coli* as described elsewhere [21].

Pyrimidine nucleoside phosphorylase assay. In a final volume of 125 μl, the reaction mixture contained 0.15 mM (unless otherwise specified) [2-^{14}C]uridine or [2-^{14}C]thymidine (56 mCi/mmole), 50 mM potassium phosphate, 1 mM EDTA, 1 mM Dithiothreitol (DTT) and an appropriate amount of enzyme in 50 mM Tris–HCl buffer (pH 7.4). Incubations were carried out in a Dubnoff metabolic shaking incubator, and the reaction was terminated by boiling for 2 min. The samples were then centrifuged and 10-μl aliquots of the supernatant fraction were spotted and separated on silica gel plates with chloroform–methanol–acetic acid (90:5:5). Spots containing substrate and product were identified by u.v. quenching, and radioactivity was counted in a liquid scintillation counter.

Determination of K_i *values.* The benzylacyclouridines were first screened for inhibition of UrdPase and dThdPase at inhibitor concentrations of 1, 10, and 100 μM. Apparent K_i values were determined from Dixon plots (1/v vs [I]) of the data [12]. For compounds that exhibited apparent K_i values of less than 100 μM, the K_i values as well as the type of inhibition were also determined from the Lineweaver–Burk double-reciprocal plots. In these experiments, the concentration of phosphate was 50 mM which is saturating for both UrdPase [17, 19] and dThdPase [22].

Table 1. Inhibitory potencies of benzylacyclouridines for uridine phosphorylase from *E. coli*

Compounds	Apparent K_i* (μM)	K_i† (μM)
BAU	18.9 ± 3.7	4.3 ± 0.4
succ-BAU	69.0 ± 10.6	42.5 ± 4.8
HM-BAU	5.1 ± 0.2	3.9 ± 0.7
C-methyl-BAU	1330.0 ± 250.0	ND‡
OH-BAU	9.1 ± 1.2	6.6 ± 1.9
BBAU	0.8 ± 0.1	0.68 ± 0.03
succ-BBAU	13.9 ± 1.0	17.1 ± 8.3
HM-BBAU	0.5 ± 0.1	0.15 ± 0.01
C-methyl-BBAU	390.0 ± 45.0	ND
BBBAU	178.0 ± 20.0	ND

* Apparent K_i values were estimated from Dixon plots (1/v vs [I]) of the data.

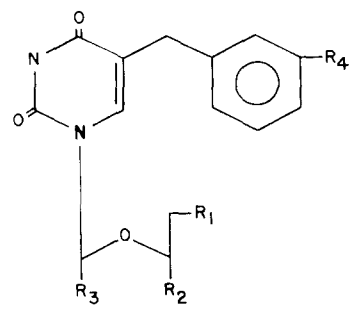
† K_i values were determined from the Lineweaver–Burk double-reciprocal plots. The concentration of inorganic phosphate was 50 mM.

‡ Not determined.

Protein determination. Protein concentrations were determined spectrophotometrically by the method of Bradford [23] using bovine gamma-globulin as a standard.

RESULTS

The structures of the ten benzylacyclouridines employed in this study are shown in Fig. 1. All compounds tested with the exception of BBBAU did not inhibit the cleavage of dThd by dThdPase from *E. coli* at concentrations up to 100 μM. BBBAU inhibited the bacterial dThdPase at 40 μM or above. On the other hand, all compounds tested, except *C*-methyl-BAU, *C*-methyl-BBAU and BBBAU, were



Compounds	R_1	R_2	R_3	R_4
BAU	-OH	-H	-H	-H
succ-BAU	$-OCOCH_2CH_2COONa$	-H	-H	-H
HM-BAU	-OH	$-CH_2OH$	-H	-H
C-methyl-BAU	-OH	-H	$-CH_3$	-H
OH-BAU	-OH	-H	-H	-OH
BBAU	-OH	-H	-H	$-OCH_2$-⬡
succ-BBAU	$-OCOCH_2CH_2COONa$	-H	-H	$-OCH_2$-⬡
HM-BBAU	-OH	$-CH_2OH$	-H	$-OCH_2$-⬡
C-methyl-BBAU	-OH	-H	$-CH_3$	$-OCH_2$-⬡
BBBAU	-OH	-H	-H	$-OCH_2$-⬡-OCH_2-⬡

Fig. 1. Chemical structures of the benzylacyclouridines.

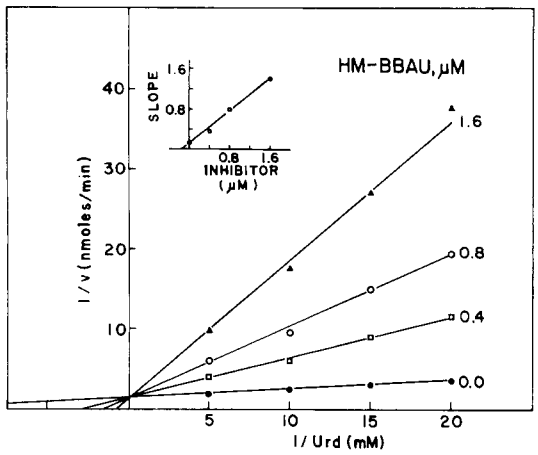


Fig. 2. Double-reciprocal plot for inhibition of uridine phosphorylase from *E. coli* by HM-BBAU.

shown to be potent inhibitors of UrdPase from *E. coli* (Table 1). Competitive inhibition was observed in all cases. A double-reciprocal plot for HM-BBAU, the most potent inhibitor of the compounds tested, is shown in Fig. 2.

DISCUSSION

UrdPase from *E. coli* has been fully characterized during recent years [4, 17, 19, 24, 25]. However, few inhibitors are known for this enzyme. 5-Azauracil was reported to inhibit both uridine synthesis and phosphorolysis by UrdPase from *E. coli* [26]. Thymidine, guanosine, inosine, adenosine, cytosine, GMP, GTP, CMP, CTP, UMP, UTP, AMP, and ATP are not inhibitors of the enzyme [4]. In this study, we have demonstrated that benzylacyclouridines are potent inhibitors of UrdPase, but not dThdPase of *E. coli*.

The UrdPase from *E. coli* has been shown to be quite different from the mammalian enzymes in several aspects. In the first place, the enzyme from *E. coli* is much more specific for the ribosyl moiety than the mammalian enzymes, e.g. rat liver [1, 4, 18]. UrdPase from various sources can be classified into two groups based on their pH optima [9]. One group has a pH optimum around 6.6. This group includes the enzyme from *E. coli*. The other group has a pH optimum around 8 and includes the enzymes from the mouse and rat livers. There is also a difference in the kinetic mechanisms of the two enzymes. The *E. coli* enzyme has a random mechanism [17]. This is in contrast to the ordered mechanism reported for the enzyme from rat liver [18].

The fact that both the mammalian UrdPase and UrdPase from *E. coli* are inhibited by the same benzylacyclouridines and that the relative inhibitory potencies of these compounds parallel those obtained with mammalian enzymes* suggest that there may exist similarity in the structure of the active sites of both enzymes. Furthermore, the fact that the benzylacyclouridines do not inhibit *E. coli* dThdPase as well as the mammalian dThdPase increases the credence in the notion that these inhibitors can be used to classify the two pyrimidine nucleoside phosphorylases. One unexpected finding is that BBBAU, which have been shown to have no effect on mammalian dThdPase even at 500 μM*, was inhibitory toward dThdPase from *E. coli* at concentrations greater than 40 μM. This difference in the effects of BBBAU against *E. coli* and mammalian dThdPase is under further investigation in this laboratory.

These benzylacyclouridines can serve as a valuable experimental tool to study the role of PyrNPase versus phosphoribosyltransferase in the metabolism of pyrimidine and their analogues in *E. coli*. In addition, these benzylacyclouridines may be useful pharmacologically in combination with other pyrimidine analogues or as a single agent in inhibiting either pyrimidine salvage or catabolism.

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