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RAPID COMMUNICATION

SUBSTRATE SPECIFICITY OF *ESCHERICHIA COLI* THYMIDINE PHOSPHORYLASE FOR PYRIMIDINE NUCLEOSIDES WITH ANTI-HUMAN IMMUNODEFICIENCY VIRUS ACTIVITY

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ABSTRACT. Various nucleoside antiviral agents and their metabolites were examined for their ability to be cleaved across the glycosidic bond by *Escherichia coli* thymidine phosphorylase. The increasing order of susceptibility to cleavage was U > T >> C derivatives. Nucleosides that were unsaturated in the sugar moiety were more susceptible than saturated ones. 3'-Deoxy-2',3'-didehydrothymidine was a substrate, whereas 3'-azido-, 3'-fluoro-, 3'-oxo- and 3'-thiapyrimidine nucleosides were resistant to this enzyme.

Nucleosides remain one of the most promising class of compounds for the treatment of acquired immunodeficiency syndrome (AIDS) and AIDS-related complex (ARC) [1]. Most of these compounds are metabolized to their 5'-triphosphate form and are potent competitive inhibitors of natural nucleoside-5'-triphosphate against human immunodeficiency virus (HIV) reverse transcriptase (RT). Their selectivity is based on their weak inhibition of human DNA polymerases [1]. These nucleotides are thought to act as chain terminators of the viral DNA catalyzed by the viral RT. Although much emphasis has been placed on the metabolism of these compounds in cell culture, few studies have addressed their potential catabolism by various enzymes present in certain human cells. Indeed, as recently reported, phosphorylases may have an impact on the efficacy and toxicity of certain 2',3'-dideoxynucleosides [2-5]. In this study, we evaluated a variety of pyrimidine nucleosides with anti-HIV activity for their resistance to bacterial thymidine phosphorylase (dThdPase).

The compounds studied were 3'-azido-3'-deoxythymidine (AZT; Zidovudine; Retrovir) [6], 2',3'-dideoxycytidine (D2C), 3'-deoxythymidine (D2T), 2',3'-dideoxyuridine (D2U) [7], 3'-azido-2',3'-dideoxyuridine (AzddU, CS-87) [8-10], 2',3'-dideoxy-2',3'-didehydrocytidine (D4C) [7,11], 3'-deoxy-2',3'-didehydrothymidine (D4T) [7,12,13], 2',3'-dideoxy-2',3'-didehydrouridine (D4U) [7], 3'-fluoro-3'-deoxythymidine (FLT) [14], dioxolane-T [15,16], 2',3'-dideoxy-5-fluoro-3'-thiauridine (FTU), and 2',3'-dideoxy-5-fluoro-3'-thiacytidine (FTC) [16].¶ In addition, the nucleoside 3'-amino-3'-deoxythymidine (AMT) was evaluated, since this compound is a recently discovered catabolite of AZT that was found in monkeys and humans treated with AZT [17,18]. Studies using rat and human liver microsomes have demonstrated that the rate of formation of AMT and AMT-glucuronide increases in the presence of NADPH, suggesting the involvement of an NADPH-dependent enzyme system [3]. Of significance was the finding that AMT was 5- to 7-fold more toxic to granulocyte-macrophage and erythroid bone precursor cells derived from human bone marrow when compared to AZT. Similarly, 3'-amino-2',3'-dideoxyuridine

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(AMDU) was evaluated, since small amounts of this compound are formed in liver microsomes and rhesus monkeys that were treated with AzddU [19; Schinazi R, unpublished results].

Racemic FTC is a potent anti-HIV-1 agent in human lymphocytes, which was recently reported to also have potent anti-hepatitis B virus activity in 2.2.15 cells [20].¶ Only the (+)-enantiomer of FTC was converted to (+)-FTU by cytidine deaminase.¶ A concern is that if FTC and FTU are substrates for thymidine phosphorylase, then 5-fluorocytosine (5-FCyt) and 5-fluorouracil (5-FUra) would be produced. 5-FUra is a highly cytotoxic compound. Therefore, the susceptibility of FTC and FTU to thymidine phosphorylase was examined in this paper.

MATERIALS AND METHODS

Materials. The 2',3'-dideoxy analogues reported herein and racemic dioxolane-T, FTC, and FTU were prepared according to published procedures [7-16]. All the compounds were characterized by melting point, ^{1}H-NMR spectroscopy and elemental analysis. dThdPase from *E. coli* was obtained from the Sigma Chemical Co. (St. Louis, MO). HPLC grade solvents and KH_2PO_4, and all other chemicals, analytical grade, were obtained from J.T. Baker (Phillipsburg, NJ).

Enzyme assays. Stock solutions of the compounds were prepared in deionized water. The enzyme reaction was performed in a buffer consisting of 100 mM KH_2PO_4, pH 7.4, at room temperature. The reaction was initiated by adding 1.1 units of enzyme to 100 μM compound in the buffer. The final volume was 200 μL. Since the commercial enzyme already contains 2 mM uracil as a stabilizer, the final concentration of uracil present in the enzyme mixture was 10 μM. Reactions were terminated at 30 min and 60 min after addition of the enzyme by filtering the sample through a 0.45 μm Acrodisc® LC13 PVDF filter (Gelman Sciences, Ann Arbor, MI); then the eluent was stored frozen at -70° until analysis by HPLC.

HPLC analysis. A 40 μL portion of each sample was analyzed by HPLC using a Hewlett Packard 1050 instrument. A Hypersil ODS-18 5 μm column (Jones Chromatography, Lakewood, CO) was used for separation of the various nucleosides and bases. The flow rate was 1 mL/min, and the compounds were detected at a UV wavelength of 262 nm. Different mobile phases and conditions used in these analyses are listed below as Methods A through E. Method A: isocratic elution with 10 mM KH_2PO_4 (pH 6.6) and MeOH (93:7). Method B: isocratic elution for 6 min with 10 mM KH_2PO_4 and MeOH (93:7), followed by a 1 min linear gradient from 7 to 20% MeOH. Method C: isocratic elution for 10 min with 10 mM KH_2PO_4 and MeOH (93:7), followed by a 10 min linear gradient from 7 to 30% MeOH. Method D: isocratic elution with 10 mM KH_2PO_4. Method E: isocratic elution for 5 min with 10 mM KH_2PO_4, then a 10 min linear gradient from 0 to 30% MeOH. For consistency, all HPLC peaks were compared relative to the uracil peak in order to obtain the approximate concentration of compound at different time points. In the case of uracil and cytosine containing nucleosides, thymine (10 μM) was used as an internal standard in order to determine the concentrations of the base and nucleoside.

RESULTS AND DISCUSSION

dThdPase is an enzyme that catalyzes the reversible phosphorolysis of several natural and synthetic pyrimidine nucleosides. Niedzwicki *et al.* [21] have studied the structure-activity relationship of a number of nucleosides as substrates or inhibitors of dThdPase derived from mouse liver. They determined that compounds with electron-withdrawing or hydrophobic groups at the 5-position of the pyrimidine moiety

enhance binding to dThdPase. The authors indicate that dThdPase is highly specific for the 2'-deoxyribosyl moiety of nucleoside ligands, and that ribo- or arabino-nucleosides are not cleaved by dThdPase. Similarly, Kono *et al.* [22] determined the substrate specificity of dThdPase derived from human liver tumor using a variety of uridine and thymidine modified compounds. It was found that an intact 3'-hydroxyl function was essential for cleavage. 5-Bromo, -chloro, and -fluorouridine were substrates for dThdPase, whereas 2'-, 3'- or 5'-deoxyuridine were not. 5'-Deoxy-5-fluorouridine was also a better substrate than 5-fluoro-2'-deoxyuridine (FdUrd) for this enzyme. However, no 2',3'-dideoxypyrimidine nucleosides were studied by these groups. Since this class of compounds is currently clinically important for the treatment of HIV infections, it was essential to determine if these compounds are cleaved by dThdPase that is present in various cells. We have successfully applied a relatively simple HPLC system to determine the degree of glycosidic bond cleavage of pyrimidine nucleosides (Table 1). The results of the enzyme assays and the HPLC method are shown in Table 1. The data demonstrated that among the 2',3'-dideoxypyrimidine nucleosides, the U derivatives were better substrates than either the T or C derivatives. The increasing order of susceptibility to cleavage was U > T >> C derivatives. Consistent with previous findings with cytosine containing nucleosides [23], D4C was markedly less susceptible than D4T to dThdPase (Table 1). The 2',3'-dideoxy derivatives (D2U, D2C or D2T) were more resistant than the D4 compounds (D4U and D4T). D2C, AZT, and AzddU were completely resistant to dThdPase. The 3'-amino derivatives, AMT and AMDU, were susceptible to cleavage by *E. coli* dThdPase. Small amounts (<3%) of thymine were detected with FLT. The 3'-oxo derivative related to thymidine was also a poor substrate for this enzyme and substitution of a 3'-hydroxy function by a 3'-fluoro or 3'-azido group in thymidine markedly increased resistance to phosphorylases. FTC and FTU were not substrates for this enzyme, suggesting that the potential generation of 5-FUra from FTC is unlikely in cell culture or *in vivo*. Studies with purified human liver dThdPase and in rhesus monkeys, as well as metabolic studies using radiolabeled FTC in human peripheral blood mononuclear cells and HepG2 cells, suggest that cleavage to 5-FUra is unlikely in mammalian cells [Schinazi R, unpublished results].¶ As expected, 99% of the FdUrd (used as a positive control) was cleaved by dThdPase after 1 hr, whereas 2'-deoxycytidine (used as a negative control) was not (Table 1).

We have demonstrated that certain D4-pyrimidine derivatives are substrates for dThdPase. It is significant that D4T, a compound undergoing Phase I/II clinical trials in HIV-infected individuals, is a good substrate for this enzyme. D4T has a good oral bioavailability in animals [14]; however, only about 50% of the dose is recovered in urine [24]. It is conceivable that a portion of this drug could be converted to thymine and its metabolites in humans. Of significance is the finding that intracellular metabolic studies in bone marrow cells indicated that 70% of the radioactivity derived from [^{3}H]D4T is associated with thymidine in nucleic acids [4]. This conversion could be mediated by dThdPase and may have important consequences since the intracellular levels of this enzyme vary in different cells. Furthermore, the thymidine produced by the degradative enzyme may compete for the phosphorylation and degradation of D4T. Lower levels of dThdPase are found in T-lymphocytes than in bone marrow cells, which may explain its selective antiviral activity in human lymphocytes and lack of bone marrow toxicity in humans [4]. It is significant that both D4T and 2',3'-dideoxyinosine are cleaved by dThdPase and purine nucleoside phosphorylase, respectively, and also do not cause bone marrow toxicity *in vitro* and *in vivo*.

It should be stressed that the bacterial enzyme used in these studies may not have the same substrate specificity requirement for cleavage as the human-derived enzyme. AMT is a good substrate for *E. coli* dThdPase (Table 1), but a poor substrate for mouse liver enzyme [21]. Studies are in progress to determine the substrate specificity of human liver dThdPase towards AMT. Similar studies on the effect of human dThdPase on D4T are consistent with our findings, suggesting a good correlation between bacterial and mammalian enzymes.

Table 1. Effect of *E. coli* thymidine phosphorylase on various pyrimidine nucleosides

Nucleoside	Base	HPLC method	Retention time (min)	% Nucleoside and base at: 0 hr*	0.5 hr	1 hr	Relative %+
Urd		E	11.1	100.0	0.0	0.0	
	Uracil		5.7	0.0	100.0	100.0	101.5
FdUrd		A	22.5	100.0	1.0	1.0	
	5-FUra		4.14	0.0	99.0	99.0	100.4
D2U		C	15.4	100.0	67.8	44.7	
	Uracil		3.8	0.0	32.2	55.3	56.1
D4U		C	12.0	97.3	43.0	14.6	
	Uracil		3.8	2.7	57.0	85.4	86.7
AMDU		D	10.1	97.3	29.5	9.0	
	Uracil		5.5	2.7	70.5	91.0	92.3
AzddU		C	21.0	100.0	100.0	100.0	
	Uracil		3.75	0.0	0.0	0.0	0.0
FTU		A	22.5	100.0	100.0	100.0	
	5-FUra		4.14	0.0	0.0	0.0	0.0
dThd		C	13.6	99.3	1.4	1.4	
	Thymine		6.6	0.7	98.6	98.6	100.0
D2T		C	20.2	100.0	87.1	68.1	
	Thymine		6.9	0.0	12.9	31.9	32.4
D4T		A	28.5	100.0	43.1	27.1	
	Thymine		6.9	0.0	57.9	72.9	74.0
AMT		A	7.9	100.0	62.6	43.2	
	Thymine		6.6	0.0	37.4	56.8	57.6
FLT		C	21.8	100.0	98.7	97.4	
	Thymine		6.9	0.0	1.3	2.6	2.6
AZT		B	25.0	100.0	100.0	100.0	
	Thymine		6.6	0.0	0.0	0.0	0.0
Dioxolane-T		C	16.1	98.4	98.4	98.4	
	Thymine		6.8	1.6	1.6	1.6	0.0
dCyd		E	11.2	100.0	100.0	100.0	
	Cytosine		4.2	0.0	0.0	0.0	0.0
D2C		E	13.8	100.0	100.0	100.0	
	Cytosine		4.3	0.0	0.0	0.0	0.0
D4C		E	13.2	99.5	99.5	99.5	
	Cytosine		4.3	0.5	0.5	0.5	0.0
FTC		E	15.8	100.0	100.0	100.0	
	5-FCyt		4.4	0.0	0.0	0.0	0.0

* None of the compounds were cleaved after incubation without enzyme for 60 min at room temperature.

+ Relative to thymidine at 1 hr (minus background).

Thus, it appears that the mammalian enzyme has a greater substrate specificity than the bacterial isozyme and that data obtained with the bacterial enzyme should be interpreted cautiously. Consequently, nucleosides that are cleaved by the bacterial enzyme should also be studied with the human isozyme. Studies with other related nucleosides using a human liver enzyme preparation are ongoing. Such studies could provide an understanding of the selective activity and lack of toxicity of these compounds and a rationale for designing other cell-specific inhibitors of HIV replication with minimal mammalian toxicity. All the anti-HIV nucleosides described herein are given orally to adult patients and could interact with degradative bacterial enzymes found in the gut to produce inactive compounds or competing natural nucleosides. This possibility appears unlikely based upon the finding that most of the currently available clinically relevant orally bioavailable pyrimidine nucleosides such as AZT, D2C, AzddU, and FLT are very poor substrates for bacterial dThdPase.

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