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SHORT COMMUNICATION

A Comparison of the Enantioselectivities of Human Deoxycytidine Kinase and Human Cytidine Deaminase*

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ABSTRACT. The stereoselectivities of recombinant human deoxycytidine kinase (EC 2. 7.1.74) (dCK) and of recombinant human cytidine deaminase (EC 3.5.4.5) (CDA) were investigated with respect to a series of cytidine analogs, most of them having the unnatural L-stereochemistry. The enantioselectivity of dCK was always low and generally favored the L-enantiomers in the case of β-2′,3′-dideoxycytidine (β-ddC), 5-fluoro-β-2′,3′-dideoxycytidine (β-FddC) and β-cytidine (β-riboC). Concerning β-2′-deoxycytidine, dCK showed a preference for the D-enantiomer. All other examined β-L-cytidine analogs, [1-β-L-lyxofuranosyl cytosine (β-L-lyxoC), 1-β-L-xylofuranosyl cytosine (β-L-xyloC), and 5-fluoro-1-β-L-xylofuranosyl cytosine (β-L-Fxylo C)], were substrates of dCK regardless of the nature of the pentose. None of the studied α-L-anomers (α-L-riboC, α-L-araC, α-L-lyxoC, or α-L-xyloC) was a substrate of dCK. Contrasting with the relaxed enantioselectivity of dCK, CDA had a strict requirement for D-cytidine analogs since none of the already listed β-L- or α-L analogs was a substrate or an inhibitor of the enzyme. The conjunction of the preceding stereochemical properties of dCK and CDA confers to L-cytidine analogs important potentialities in antiviral and anticancer therapies. BIOCHEM PHARMACOL **56**;9:1237–1242, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. L-cytidine analogs; enzyme enantioselectivity; deoxycytidine kinase; cytidine deaminase; kinetics

Until recently, it has been assumed that the cellular and viral enzymes would recognize only the natural D-enantiomers of the nucleoside analogs in antiviral chemotherapy. This view has changed in the last 4 years following the discovery that 3TC¶ and FTC (both L-enantiomers) have strong anti-HIV and anti-HBV activities [1–3]. Other L-enantiomers of cytidine analogs have shown pronounced antiviral or anticancer activities. Thus, β-L-2′,3′-dideoxycytidine and β-L-2′,3′-dideoxy-5-fluorocytidine are potent inhibitors of HIV and HBV [4, 5].

Most of the L-nucleoside analogs with activity so far discovered are cytosine nucleoside derivatives, although unnatural enantiomers of uridine analogs have been demonstrated to be active against HSV-1 [6] or against HBV replication [7]. Analogs of L-adenosine, such as β-L-2′,3′-dideoxyadenosine and β-L-2′,3′-didehydro-2′,3′-dideoxyadenosine, also have anti-HBV activities [8, 9]. To justify the observed anti-HIV activities of L-nucleoside analogs, the inhibitory properties of the corresponding 5′-triphosphates with respect to HIV-1 reverse transcriptase were studied [3, 10, 11]. L-nucleoside analog activities also depend on the enantioselectivities of activating enzymes (nucleoside kinases) or deactivating enzymes (deaminases and phosphorylases). The enantioselectivities of these enzymes are not well known, the existing investigations only concerning a very small number of substrates. The results of these studies suggest that D-enantiomers are strongly fa-

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¶ *Abbreviations:* CDA, cytidine deaminase; dCK, deoxycytidine kinase; β-D-ddC, β-D-2′,3′-dideoxycytidine; β-D-FddC, β-D-2′,3′-dideoxy-5-fluorocytidine; β-D-riboC, β-D-cytidine; FTC, (2*R*,5*S*)-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolane-5-yl]cytosine; HBV, hepatitis B virus; α-L-araC, 1-α-L-arabinofuranosyl cytosine; β-L-araC, 1-β-L-arabinofuranosyl cytosine; β-L-ddC, β-L-2′,3′-dideoxycytidine; β-L-FddC, β-L-2′,3′-dideoxy-5-fluorocytidine; α-L-lyxoC, 1-α-L-lyxofuranosyl cytosine; β-L-lyxoC, 1-β-L-lyxofuranosyl cytosine; β-L-riboC, β-L-cytidine; α-L-xyloC, 1-α-L-xylofuranosyl cytosine; β-L-xyloC, 1-β-L-xylofuranosyl cytosine; β-L-FxyloC, 5-fluoro-1-β-L-xylofuranosyl cytosine; 3TC, (2*R*, 5*S*)-1-[2-(hydroxymethyl)-1,3-oxathiolane-5-yl]cytosine.

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vored over L-enantiomers except in the case of human dCK, *Herpes simplex* virus thymidine kinase and, by inference, cellular nucleotide kinases and nucleoside diphosphate kinase [3]. Moreover, it is remarkable that almost all active L-nucleoside analogs so far discovered are substrates of either dCK or *Herpes simplex* virus thymidine kinase [5, 8, 11], enzymes which present partial sequence homology [12].

Following our finding of the antiviral properties of β-L-ddC and β-L-FddC [4, 13, 14], other work is currently in progress in our laboratory whose goal is to chemically synthesize a series of α- (or β-) D- (or L-) cytidine analogs to be evaluated against HIV and HBV replications in infected cells. The present paper is devoted to the substrate properties of some of these compounds with respect to human dCK (EC 2.7.1.74) and human CDA (EC 3.5.4.5.) and to the determinations of the enantioselectivities of these enzymes.

MATERIALS AND METHODS

β-D-dC, **1**, and β-D-riboC, **7** were obtained from Sigma. β-D-ddC, **3,** was a gift from J. Balzarini (University of Leuven, Belgium) and β-D-5FddC, **5,** a gift from V. Marquez (NIH Bethesda, USA). β-L-dC, **2** [15], β-L-ddC, **4** [16], β-L-5FddC, **6** [16], β-L-xyloC, **11** [17], and α-L-araC, **14** [18], were synthesized as previously described. β-L-riboC, **8** [19], β-L-araC, **9** [20], α-L-xyloC, **16** [21] and the hitherto unknown β-L-lyxoC, **10**, β-L-FxyloC, **12**, α-L-riboC, **13**, and α-L-lyxoC, **15** were stereospecifically synthesized by multistep reaction sequences from commercially available L-ribose, L-xylose or L-arabinose, and their structures and purities ascertained. The details of their synthesis as well as the results of their antiviral evaluation will be reported elsewhere.

Recombinant human dCK was produced and purified using the human dCK cDNA sequence [22] cloned into the pET19b (23) vector. The preparation contained a histidine tag sequence and was more than 90% pure. The specific activity of purified dCK was 240 U/mg protein, 1 unit of enzymatic activity being defined as the amount of enzyme that catalyzes the phosphorylation of 1 nmol of β-D-2′-deoxycytidine/min at 37°. Recombinant human CDA was prepared and purified as reported [24]. The specific activity of pure CDA was 500 U/mg protein. One unit of enzyme activity is the amount of enzyme catalyzing the deamination of 1 μmol of cytidine per min at 37°.

In kinetic studies, dCK activity with respect to D- and L-cytidine analogs was measured by HPLC analysis of the reaction medium which contained 50 mM of Tris HCl pH 7.5, 5 mM of ATP, 1 mM of dithiothreitol (DTT), 5 mM of $MgCl_2$, 15 mM of NaF, 10 μM to 50 μM of substrate and an appropriate amount of enzyme (135 ng/mL for most experiments). Although UTP may be the main phosphate donor *in vivo* rather than ATP [25, 26], we used the latter as in most published assays of dCK for comparison purposes. Analyses of the reaction mixture were performed by HPLC on a Hypersil ODS 3μ column under the following conditions: 10 min of isocratic elution using eluent A [5 mM of Pic A-Waters (ion pairing agent: tetrabutylammonium hydrogen sulfate and phosphoric acid)], followed by a 30-min gradient from eluent A to eluent B (5 mM of Pic A in 50% aqueous acetonitrile). Under these conditions, all studied cytosine nucleoside derivatives were separated from the corresponding 5′-monophosphate, and from ATP. The retention times in min of each substrate and its 5′-monophosphate (when observed) were, respectively: **1** (3.4, 11), **2** (3.4, 11.3), **3** (4.2, 14.1), **4** (3.9, 14.1), **5** (4.9, 9.1), **6** (4.4, 9.3), **7** (2.3, 5.8), **8** (2.3, 5.8), **9** (2.7, 6.8), **10** (2.3, 5.3), **11** (3.4, 9.8), **12** (3.9, 10.8), **13** (2.2), **14** (2.0), **15** (2.2), and **16** (2.7). The retention times of ATP and ADP were 26.7 min and 24.2 min, respectively. Identifications were achieved through coinjection of authentic samples when available or determination from UV spectra of the eluted compounds. Other identification methods included the determination of the retention times of 5′-monophosphates after incubation of the corresponding commercial 5′-triphosphates with alkaline phosphatase, controlled degradation and HPLC analysis of the reaction mixture containing di- and monophosphates. Under our conditions, the HPLC method was only fairly accurate and did not allow reliable measurements at low concentrations. For this reason, the initial concentrations of substrates were kept above 10 μM. In each case, the reaction was run until 20 to 25% of the substrate had been transformed. For each concentration of substrate, the kinetic curves were determined by at least three measurements of substrate transformation in function of time, each in duplicate. Using the GraFit program (Erithacus Software, 1992), the initial rates were obtained and used to determine the apparent V_m and K_m parameters and standard deviations according to the Lineweaver–Burk method.

In kinetic studies with CDA, the reaction mixture consisted of 100 mM of Tris-HCl pH 7.5, 100 mM of KCl, and the substrate in a 20 to 170 μM concentration range, for a final volume of 1 mL. The reaction was started by adding 0.04 enzyme unit, followed by UV spectroscopy, and was stopped after 10 min of incubation at 37°. For derivatives of cytosine, the selected wavelength was 282 nm, whereas the absorbance change was monitored at 290 nm for 5-fluorocytosine derivatives ($\Delta\epsilon$: 3600 and 2100, respectively). Deamination studies of the β-D-cytidine analogs were also completed by HPLC analysis of the reaction medium. Enzyme inhibition by L-cytidine analogs **2, 4, 6, 8,** and **9–16** was investigated under the same conditions as in substrate property studies, with 20 μM cytidine as the substrate and in the presence of a 0.26 mM or a 1 mM concentration of the compounds.

RESULTS AND DISCUSSION

dCK is an important enzyme in the salvage reactions of nucleotide synthesis since it catalyzes the phosphorylation of all three natural deoxyribonucleosides β-D-dC, β-D-2′-deoxyadenosine and β-D-2′-deoxyguanosine [27]. Conse-

TABLE 1. Substrate properties of D- and L-cytidine analogues with respect to human recombinant dCK

Compound	K_m (μM)	K_m (literature)† (μM)	V_m/K_m (Relative)	Enantioselectivity: $E_{(L/D)}$
1 (β-D-dC)	9 ± 1.6	5 (30)	1	0.34
2 (β-L-dC)	8.7 ± 2.6	—	0.34	
3 (β-D-ddC)	24 ± 14	60 (43),78.5 (32)	0.17	1.2
4 (β-L-ddC)	29 ± 12	40 (5)	0.20	
5 (β-D-FddC)	63 ± 15	—	0.19	1.3
6 (β-L-FddC)	21 ± 3.1	19 (5)	0.25	
7 (β-D-riboC)	60 ± 1.1	122 (44),270 (29)	0.13	12
8 (β-L-riboC)	47 ± 8.9	—	1.5	
9 (β-L-araC)	7.7 ± 1.1	2 (29)	3.0	
10 (β-L-lyxoC)	77 ± 37	—	0.49	
11 (β-L-xyloC)	59 ± 14	—	0.46	
12 (β-L-FxyloC)	77 ± 16	—	0.53	
13 (α-L-riboC)	*	—	—	
14 (α-L-araC)	*	—	—	
15 (α-L-lyxoC)	*	—	—	
16 (α-L-xyloC)	*	—	—	

The reaction medium contained 5 mM of ATP, 5 mM of $MgCl_2$, the substrate (10 to 50 μM) and the enzyme (135 ng/mL). Initial rates were determined by HPLC analysis of the reaction medium. Under these conditions, V_m of β-D-dC was 0.24 μmol/min·mg.

*No reaction observed under the experimental conditions used.

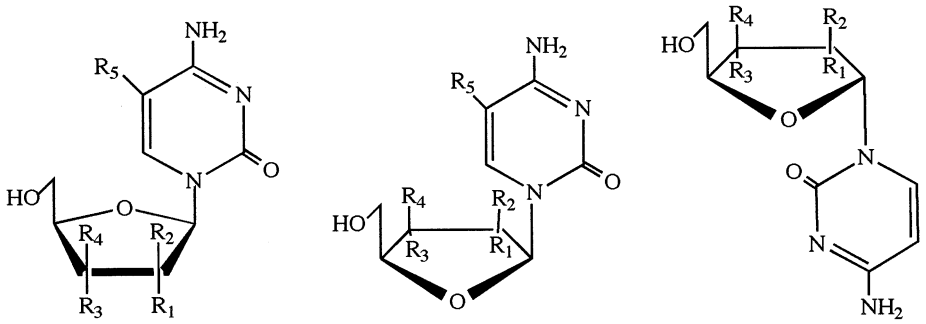
†Human or calf thymus dCK (reference numbers between parentheses).

quently, it may activate a large number of nucleoside analogs and as such may also be important in antiviral chemotherapy. Among 2′,3′-dideoxynucleoside analogs, only the anti-HIV and anti-HBV compound β-D-ddC is phosphorylated at an acceptable rate [28]. The enzyme is able to catalyze the phosphorylation of the arabino nucleoside analogs 1-β-D-arabinofuranosyl cytosine and 1-β-D-arabinofuranosyl adenine, and it accepts substituents of moderate size at positions 5 or 3′-α of β-D-ddC [28, 29]. Mechanistic studies of the human enzyme have been hampered by the occurrence of nonhyperbolic bimodal kinetic curves and negative cooperativity [23, 30]. Following a study of the quenching of intrinsic fluorescence of the enzyme induced by ligand binding, the existence of two enzymatic states or sites was suggested, one with low affinity and one with high affinity for the ligand [31]. Furthermore, dCK was reported to phosphorylate β-L-2′,3′-dideoxycytidine, 3TC and FTC, thus justifying in part the observed antiviral activities of these compounds [5, 32].

Exploring the capacity of nucleoside analogs of unnatural L-stereochemistry to inhibit HIV and HBV led us to study the enantioselectivity of deoxycytidine kinase with respect to deoxycytidine, deoxyadenosine, deoxyguanosine and their analogs [9]. In the present study, we examined the substrate properties with respect to human dCK of the D- and L-cytidine analogs shown in Fig. 1. Table 1 compares the catalytic efficiencies V_m/K_m of dCK with respect to compounds **1–16**. Among the L-enantiomers of the cytosine nucleoside derivatives that we studied, some had already been shown to inhibit HIV and HBV replication (*cf*. **4** and **6**). 3TC and FTC are apparently the only L-enantiomers of cytidine analogs which were previously tested for their substrate properties with respect to purified human dCK [32], whereas β-L-ddC, **4**, β-L-araC, **9**, and β-L-5FddC, **6** were examined as substrates of calf thymus dCK [5, 29]. Very recently, dCK purified from HeLa cells was shown to catalyze the phosphorylation of D- and L-dC with apparently the same efficiency, but no kinetic parameters were determined [33].

Our study of the enantioselectivity of human dCK with regard to four pairs of cytidine analog enantiomers (Table 1) showed that in three cases the factor $E = (V_m/K_m)_S/(V_m/K_m)_S$, [34] was near unity and that the L-enantiomer was favored. All β-L-enantiomers examined were substrates of dCK, suggesting that the enzyme has a relaxed enantioselectivity toward a broad series of β-cytidine analogs. The reason for the lack of stereospecificity of dCK is probably of structural nature and is not known since the 3D-structure of the enzyme is not yet available.

The data in Table 1 provide some structure-activity relationships regarding L-cytidine analogs. The inaptitude of the α-L-analogs **13–16** to be phosphorylated strongly suggests that a *cis* stereochemistry between the base and the hydroxymethyl group at position 4′ is necessary for enzymatic activity. The nature of the pentose was less critical than the preceding structural requirements and the four possible β-L-pentofuranosyl cytosine nucleosides **8**-**11** displayed fairly marked substrate properties. This was especially true for the L-arabino and L-ribo isomers **9** and **8**, suggesting that a 3′-hydroxyl group *trans* to the 4′-hydroxymethyl substituent may favor activity in the series of L-cytidine analogs. It is noteworthy that β-L-araC showed pronounced substrate properties as already found for calf thymus deoxycytidine kinase [29]. Previous studies indicated that dCK accepted 5-substituted-β-D-2′-deoxycytidine compounds as substrates provided the size of the 5-substituent was small [28]. Our results regarding compounds **6** and **12** and the known substrate properties of



1 : R_1=R_2=R_4=R_5=H,R_3=OH (ß-D-dC)
3 : R_1=R_2=R_3=R_4=R_5=H (ß-D-ddC)
5 : R_1=R_2=R_3=R_4=H,R_5=F (ß-D-5FddC)
7 : R_2=R_4=R_5=H,R_1=R_3=OH (ß-D-riboC)

2 : R_1=R_2=R_4=R_5=H,R_3=OH (ß-L-dC)
4 : R_1=R_2=R_3=R_4=R_5=H (ß-L-ddC)
6 : R_1=R_2=R_3=R_4=H,R_5=F (ß-L-5FddC)
8 : R_2=R_4=R_5=H,R_1=R_3=OH (ß-L-riboC)
9 : R_1=R_4=R_5=H,R_2=R_3=OH (ß-L-araC)
10 : R_1=R_3=R_5=H, R_2=R_4=OH (ß-L-lyxoC)
11 : R_2=R_3=R_5=H,R_1=R_4=OH (ß-L-xyloC)
12 : R_2=R_3=H,R_1=R_4=OH,R_5=F (ß-L-5FxyloC)

13 : R_2=R_4=H,R_1=R_3=OH (α-L-riboC)
14 : R_1=R_4=H,R_2=R_3=OH (α-L-araC)
15 : R_1=R_3=H,R_2=R_4=OH (α-L-lyxoC)
16: R_2=R_3=H,R_1=R_4=OH (α-L-xyloC)

FIG. 1. Derivatives of α- or β- (D or L) cytidines studied as substrates of human dCK or human CDA.

FTC [2] compared to **4**, **11** and 3TC suggest that a fluorine substituent at the 5-position of unnatural L-cytidine analogs is accepted without a drastic change in enzyme activity, as in the D-series.

CDA catalyzes the deamination of β-D-cytidine and β-D-2′-deoxycytidine to β-D-uridine and β-D-2′-deoxyuridine, respectively [35]. It also catalyzes the deamination of important cytidine derivatives [35] such as the antileukemic drugs β-D-araC and β-D-5-aza-2′-deoxycytidine, thereby deactivating them [36]. The 3D structure of *Escherichia coli* CDA is known [37], whereas the human enzyme has been purified and cloned [24, 36]. We studied the enantioselectivity of this recombinant human enzyme with respect to the compounds of Fig. 1 to assess the resistance of L-cytidine derivatives to deamination.

The determination of the substrate properties of compounds **1–16** with respect to recombinant human CDA (Table 2) confirms the few previous data concerning the enantioselectivity of the purified enzyme [2, 38]. Only the β-D-nucleosides **1** and **7** were deaminated with rates similar to those obtained in earlier studies using CDA from several human tissues, including placenta [35], liver [39] or granulocytes [40]. As expected, the β-D-dideoxy derivatives **3** and **5** were very poor substrates [38, 41, 42]. Without exception, all examined α-L- and β-L-cytidine derivatives proved to be completely resistant to deamination, thus suggesting that a strong enantioselectivity favoring β-D-cytidine analogs is a general property of human CDA. Very recently, the deamination of β-L-cytidine catalyzed by CDA from HeLa cells has been reported, but this result is questionable since only a crude enzyme preparation was used and the reaction product was not characterized by analytical methods other than UV spectroscopy [33]. Finally, the lack of inhibition of recombinant human CDA observed in the presence of any of the L-cytidine analogs examined in our study is also consistent with the strict enantioselectivity of the enzyme.

TABLE 2. Substrate properties of cytidine derivatives 1–16 with respect to recombinant human CDA

Compound	Relative initial rate (70 μM substrate)	K_m (μM)	V_m (μmol/min·mg)
7 (β-D-riboC)	1	44 (39)*	44 (68)*
1 (β-D-dC)	0.67	54 (39)*	55 (46)*
3 (β-D-ddC)	0.001	†	†
5 (β-D-5FddC)	<0.001	†	†
β-L-cytidine derivatives **2, 4, 6, 8, 9–12**	‡	‡	‡
α-L-cytidine derivatives **13–16**	‡	‡	‡

The reaction mixture (pH 7.5) contained the cytidine analog (20 to 170 μM) and 70 ng of enzyme per mL. Initial rates were determined from the decrease in absorbance at 282 or 290 nm.

*Ref. 24.

†Reaction too slow for K_m and V_m determinations.

‡No reaction observed under the conditions used.

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