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Affinity of the antiviral enantiomers of oxathiolane cytosine nucleosides for human 2′-deoxycytidine kinase

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Abstract—The two enantiomers of 2′,3′-dideoxy-3′-thiacytidine (BCH-189) and their 5-fluoro analogs (FTC) were found to be good substrates for human 2′-deoxycytidine kinase with K_m values in the 5.7 to 42.1 μM range. The affinity of the (−)-enantiomers was greater than that of the (+)-compounds. These results may explain the greater *in vitro* antiviral potency against human immunodeficiency virus and hepatitis B virus of the (−)-enantiomers when compared to their (+)-counterparts. The (+)- and (−)-enantiomers of FTC and BCH-189 are the first nucleoside analogs for which we have observed lower apparent kinetic constants for this enzyme in the presence of ATP compared to UTP.

Oxathiolane cytosine nucleosides represent a promising new class of compounds with potent and selective antiviral activity against human immunodeficiency virus (HIV)* and hepatitis B virus (HBV) [1–12]. Racemic 2′,3′-dideoxy-3′-thiacytidine (BCH-189) and 2′,3′-dideoxy-5-fluoro-3′-thiacytidine (FTC) were found to be active at submicromolar concentrations against these viruses in culture. This led to the separation or synthesis of the two possible enantiomers of these compounds by HPLC, by developing a synthesis using chiral sugars, and by using the enantioselectivity of certain enzymes such as cytosine deaminases and lipases to hydrolyze only one of the enantiomers [2–4, 9, 10]. Unexpectedly, the unnatural (−)-enantiomers were found to be the more potent enantiomers in various cell culture systems [1, 4, 9, 10]. The (−)-enantiomers were also less toxic to the lymphoblastoid CEM cells and primary human bone marrow cells than the (+)-isomers [1, 2, 12]. Metabolic studies in human cells have demonstrated the efficient and rapid conversion of the enantiomers of BCH-189 and FTC to their corresponding 5′-triphosphates [4, 5]. The 5′-triphosphate of the (−)-enantiomers are potent competitive inhibitors with respect to 2′-deoxycytidine-5′-triphosphate against HIV reverse transcriptase (RT) and are weak inhibitors of human DNA polymerases [2, 13]. These nucleotides have been shown to act as chain terminators of the viral DNA catalyzed by the viral RT in cell-free systems. The low toxicity and favorable potency of (−)-BCH-189 (3TC) and (−)-FTC compared to the (+)-compounds led to the selection of the (−)-enantiomers as candidates for the treatment of viral infections in humans.

We determined recently that racemic FTC was phosphorylated in wild-type CEM cells, but not in 2′-deoxycytidine kinase (dCydK)-deficient cells (data not shown), suggesting a unique role for dCydK in the activation of both the (+)- and (−)-enantiomers. We also ruled out 5′-nucleotidase as the phosphorylating enzyme since mycophenolic acid, which increases 5′-nucleotidase activity by elevating IMP levels [14], did not enhance phosphorylation of FTC. The anti-HIV activity of FTC and BCH-189 and their enantiomers was abolished in the presence of 2′-deoxycytidine, but not other natural 2′-deoxynucleosides [2]. Metabolic studies with radiolabeled compounds in uninfected human peripheral blood mononuclear (PBM) cells demonstrated that the total levels of nucleotides in these cells over a period of 2–48 hr were 2- to 3.2-fold greater with (−)-BCH-189 and (−)-FTC than with (+)-BCH-189 and (+)-FTC (data not shown). Whereas the (−)-enantiomers are essentially resistant to cytidine deaminase (CDA), (+)-BCH-189 and (+)-FTC are susceptible to deamination and could account for the lower levels of their phosphorylated derivatives noted in cell culture [3–5]. However, tetrahydrouridine or 2′-deoxytetrahydrouridine, potent inhibitors of CDA and cytidylate deaminase, do not enhance the anti-HIV activity of (+)-FTC in PBM cells [2]. A more likely explanation for the difference in potency of the (+)- and (−)-enantiomers may be the lower affinity of (+)-FTC and (+)-BCH-189 for human dCydK. Therefore, we determined the affinity of human dCydK for these compounds using different natural nucleosides as phosphate donors.

Materials and Methods

Materials. The pyrimidine nucleosides were synthesized in our laboratories as previously described [9, 15]. Radioactive racemic (±)-[5-^{3}H]BCH-189 (sp. act. 29 Ci/mmol), (−)-[5-^{3}H]BCH-189 (14 Ci/mmol), (−)-[6-^{3}H]-FTC (9 Ci/mmol) and (+)-[6-^{3}H]FTC (9 Ci/mmol) were synthesized by our group in collaboration with Moravek Biochemicals, Brea, CA. The detailed synthesis and characterization of these compounds will be described elsewhere. (+)-[5-^{3}H]BCH-189 (14.5 Ci/mmol) was prepared by separating it from radiolabeled racemic BCH-189 using a previously described high pressure liquid chromatographic procedure [1]. The purity of all the unlabeled and radiolabeled compounds was greater than 97.5% as determined by chiral column chromatography [1, 2]. Natural nucleosides and nucleotides were of the highest purity available (Sigma Chemical Co., St. Louis, MO). Synthetic nucleotide analogs were prepared as described previously [2, 5].

Enzyme purification. Human dCydK was isolated from actively growing MOLT-4 cells and purified by sequential steps of ammonium sulfate precipitation, HPLC-gel filtration, and DE-52 ion exchange chromatography, and further purified by dCTP-Sepharose affinity chromatography as described previously [16].

Enzyme assays. These studies utilized a preparation from MOLT-4 T lymphoblasts purified to > 80% homogeneity as described previously [16, 17]. Stock solutions of the compounds were prepared in 50 mM imidazole, pH 7.0. The enzyme assay was performed in duplicate essentially as described previously [16]. After desalting, the enzyme was incubated in a mixture containing 50 mM imidazole, pH 7.4, 25 mM dithiothreitol, 2 mM ATP or UTP, 2 mM $MgCl_2$, 5% glycerol, 100 mM KCl, 1 mg/mL bovine serum albumin, and tritiated nucleosides ranging in concentration

* Abbreviations: HIV, human immunodeficiency virus; HBV, hepatitis B virus; BCH-189, 2′,3′-dideoxy-3′-thiacytidine; FTC, 2′,3′-dideoxy-5-fluoro-3′-thiacytidine; RT, reverse transcriptase; PBM, peripheral blood mononuclear; CDA, cytidine deaminase; araC, 1-β-D-arabinofuranosylcytosine; FaraA, 9-β-D-arabinofuranosyl-2-fluoroadenine; DDC, 2′,3′-dideoxycytidine; dFdC, 2′,2′-difluoro-2′-deoxycytidine; dCydK, 2′-deoxycytidine kinase.

Table 1. Effect of phosphate donors on the kinetic constants for the phosphorylation of the enantiomers of FTC and BCH-189 by human dCydK

Compound	Phosphate donor	K_m* (μM)	V_{max}* (nmol·mL^{-1}·hr^{-1})	V_{max}/K_m
(+)-FTC	UTP	42.1	6.2	0.15
	ATP	23.9	2.6	0.11
(−)-FTC	UTP	11.8	9.3	0.79
	ATP	7.4	3.8	0.51
(+)-BCH-189	UTP	33.6	5.7	0.17
	ATP	17.6	1.7	0.09
(−)-BCH-189 (3TC)	UTP	9.9	6.5	0.66
	ATP	5.7	2.3	0.41
dCyd	UTP	0.25	24	96.0
	ATP	0.93	41	44.0
DDC	UTP	127.6	27.5	0.22
	ATP	78.5	11.6	0.15
araC	UTP	2.4	20.9	8.9
	ATP	14.8	62.4	4.2

* Double-reciprocal plots of the data were linear. The results represent the averages of at least two experiments performed in duplicate. In general, the error was less than 10%.

from 3 to 100 μM in a final volume of 30 μL. The reaction mixture was incubated at 37° for 30 min and then inactivated at 85° for 1 min. The reaction rate was linear for at least 40 min. Reaction products were applied to DE81 filter discs, which were then washed with 5 mM ammonium formate to remove unreacted substrate. After drying, reaction products were eluted from the filter discs with 0.5 mL of a solution containing 0.4 M KCl and 0.1 M HCl, 5 mL of Biosafe scintillation fluid (Research Products International Corp., Mount Prospect, IL) was added, and radioactivity was quantitated in a Beckman LS6000 SC scintillation spectrometer.

Kinetic analysis. The kinetic data were fitted to a hyperbola from which the apparent K_m and V_{max} values were calculated as described previously [16].

Results and Discussion

dCydK (EC 2.7.1.74) is an enzyme that catalyzes the phosphorylation of several natural and synthetic pyrimidine nucleosides to their corresponding 5′-monophosphates in the presence of a nucleoside-5′-triphosphate as the phosphate donor. The importance of this enzyme has been emphasized by the fact that it phosphorylates not only 2′-deoxycytidine (dCyd), but also certain clinically useful purine and pyrimidine nucleoside analogs. These include the antiviral and anticancer drugs 1-β-D-arabinofuranosylcytosine (araC), 9-β-D-arabinofuranosyl-2-fluoroadenine (FaraA), 2′,3′-dideoxycytidine (DDC), and 2′,2′-difluoro-2′-deoxycytidine (dFdC) [16, 18]. We recently purified dCydK to near homogeneity from MOLT-4 T lymphoblasts and demonstrated that UTP is the preferred physiologic phosphate donor for this enzyme with either deoxyribosyl or arabinosyl nucleoside substrates [16]. The availability of this highly purified enzyme, and the finding that certain oxathiolane nucleosides are phosphorylated only in cells expressing dCydK, prompted us to determine the kinetic constants and phosphate donor specificity of these novel enantiomeric antiviral agents. We hypothesized that these experiments could provide an insight into the enantiospecificity of dCydK and also provide a better understanding of the greater antiviral potency of the (−)-enantiomers of FTC and BCH-189.

The enantiomers of FTC and BCH-189 exhibited good affinity for human dCydK based on micromolar K_m values, although the apparent velocity was low compared to dCyd and araC (Table 1). The K_m values were consistently lower for the (−)-enantiomers than for the (+)-counterparts irrespective of the phosphate donor. In addition, the (−)-enantiomers were more efficient substrates for dCydK than DDC. In striking contrast to our previous studies on the interactions of dCydK with dCyd, araC, dFdC and FaraA [16], the enantiomers of FTC and BCH-189 had lower apparent K_m and V_{max} values when ATP was used instead of UTP. However, the efficiency ratio of V_{max}/K_m demonstrated a kinetic advantage for UTP as the phosphate donor, similar to our previous studies with other nucleoside analogs. Kinetic constants for the (+)-enantiomers were similar, while the corresponding values for the (−)-enantiomers were lower but comparable, indicating that the fluorine function at position 5 on FTC seemed to be well tolerated by dCydK. In each case, the (−)-enantiomer provided a 4- to 5-fold kinetic advantage over the (+)-stereoisomer (Table 1). These findings could account in part for the more potent antiviral activity *in vitro* of the (−)-enantiomers in human lymphocytes infected with HIV and in human liver cells transfected with HBV.

We also evaluated the ability of the 5′-triphosphates of (±)-FTC and (±)-BCH-189 to inhibit phosphorylation of the corresponding nucleosides. These studies demonstrated that 25 μM (±)-FTC-TP or 100 μM (±)-BCH-189-TP inhibited phosphorylation of the corresponding nucleoside by less than 10% when UTP was employed as the phosphate donor. In contrast, in the presence of ATP as the phosphate donor, 100 μM (±)-BCH-189-TP inhibited phosphorylation of 10 μM (−)-BCH-189 by greater than 80% (data not shown). These results are consistent with our previous report for dCTP inhibition of dCyd phosphorylation by dCydK, in which inhibition was apparent only when ATP was present as the phosphate donor [16]. Thus, under physiologic conditions, we predict that the 5′-triphosphate derivatives would not inhibit phosphorylation of (−)-FTC or (−)-BCH-189.

The results presented here are consistent with other preliminary results suggesting that cytoplasmic dCydK is responsible for the phosphorylation of (−)-BCH-189 [4]. In a recent study using calf thymus dCydK (EC 2.7.1.40), Furman *et al.* [5] reported that (−)-FTC and (+)-FTC have similar K_m values (23 vs 18 μM), but the V_{max} for (−)-FTC is approximately 4-fold greater than for (+)-FTC. The difference between these findings and those reported here may reflect different structural requirements for dCydK from calf thymus compared to the human T lymphoblast

enzyme. These results also emphasize the importance of using human enzyme in order to explain the phosphorylating profile observed in human cells.

The results described here demonstrate that the unnatural (−)-enantiomers of FTC and BCH-189 were kinetically superior to the (+)-enantiomers. Similarly, in a study using calf thymus dCydK, Krenitsky *et al.* [19] noted that the unnatural L-isomer of araC exhibits a nearly 5-fold kinetic advantage over the D-isomer, based on the relative V_{max}/K_m values. These data indicate that dCydK can accommodate substantial diversity in the sugar moiety of nucleoside substrates.

DDC and the (+)- and (−)-enantiomers of FTC and BCH-189 are the first nucleoside analogs for which we have observed lower apparent kinetic constants for dCydK in the presence of ATP compared to UTP (Table 1). This appears to be a function of the 2′,3′-dideoxy structure of these compounds. It will be important to examine the interaction between these analogs and the nucleoside active site for dCydK.

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