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ENANTIO-SELECTIVITY OF HUMAN NUCLEOSIDE MONOPHOSPHATE KINASES

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□ *Over recent years, there has been a renewed interest in the development of* L*-nucleosides as safe and efficacious drugs for the treatment of viral infections. Biological activity of these compounds requires phosphorylation to their triphosphate form, involving nucleoside monophosphate kinases in the second step. In order to characterize the activation pathway of* L*-nucleosides of the pyrimidine series, we studied the enantio-selectivity of human uridylate-cytidylate and thymidylate kinases. The results showed that these enzymes are only weakly enantio-selective and are thus probably involved in the activation of* L*-nucleosides* in vivo. *An activation pathway for telbivudine (*L*-dT) was therefore proposed.*

Keywords Structure activity relationship; human NMP kinases; HBV; L-dT

INTRODUCTION

Following FDA approval of lamivudine (3TC) for the treatment of HIV and HBV, L-nucleosides have become the focus of a number of studies to assess their anti-viral potential.[1] On overall, L-nucleosides are more resistant

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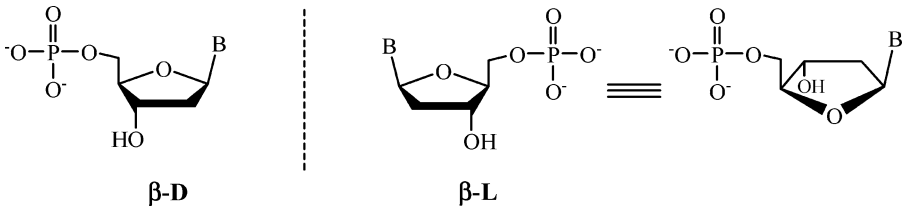

FIGURE 1 Structure of D- and L-deoxyribonucleoside 5′-monophosphates (B = thymine, uracil, cytosine).

to degrading enzymes and show better toxicological profiles than their D-counterparts.[1] Additionally, derivatives of the L series not only possess activity against viruses but some of them also act as anti-cancer[2] and anti-malarial agents.[3] In vivo, activation of L-nucleosides necessitates phosphorylation to the triphosphate.[4] While the first phosphorylation step has been extensively studied, less attention has been drawn to the second step.[4] This step, i.e., the phosphorylation of nucleosides monophosphate and their analogues into their diphosphate derivatives, involves nucleoside monophosphate kinases in both the de novo and salvage pathways.[4,5] Human cells contain a thymidylate kinase (TMPK) and a uridine monophosphate-cytidine monophosphate kinase (UMP-CMPK) that are only 21% identical in sequence but that adopt the same fold characterised by a P loop, a NMP binding domain and a LID domain.[6] The recent interest in L-nucleosides of therapeutic potential has raised the need to assess the enantio-selectivity of NMP kinases. In this study, we therefore examined the possibility to phosphorylate L-dTMP, L-dUMP and L-dCMP (Figure 1) using hTMPK and hUMP-CMPK, respectively.

MATERIALS AND METHODS

All D-nucleotides were purchased from Sigma Chemicals (St. Louis, MO, USA).

L-Nucleoside 5′-monophosphates (NMP) were prepared from L-nucleosides following Yoshikawa's phosphorylation procedure.[7] Nucleosides were dissolved in triethylphosphate and 1.2–2 eq. phosphorus oxychloride ($POCl_3$) was added. After several hours of stirring at room temperature, the intermediate phosphorodichloridates were hydrolyzed with triethylammonium hydrogencarbonate buffer (TEAB) 1 M pH 7.5 to afford the corresponding nucleoside monophosphates. Nucleoside 5′-monophosphates were purified on DEAE-Sephadex A-25 (elution: linear gradient of TEAB pH 7.6 from 10 to 300 mM) followed by chromatography on RP18 (elution: water to methanol 50%). The triethylammonium counter ions were exchanged to sodium by passing the nucleotide solution through a DOWEX-AG 50WX2-400 column. Yields were 43–80%. The structures and purities

TABLE 1 Catalytic efficiencies of the human UMP-CMP and TMP kinases for the natural nucleoside monophosphates and their corresponding L-enantiomers

Enzyme	Substrate	K_m (mM)	V (U.mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	K_i (mM)
	D-UMP	*0.05 ± 0.01*	*350 ± 30*	*130 ± 10*	*2.8 × 10^6*	*1.5 ± 0.4*
	D-dUMP	1.3 ± 0.3	18 ± 1	8.0 ± 0.7	6.2 × 10^3	
hUMP/	L-dUMP	0.70 ± 0.09	0.92 ± 0.04	0.37 ± 0.02	5.3 × 10^2	
CMPK	*D-CMP*	*0.020 ± 0.005*	*350 ± 30*	*130 ± 10*	*6.5 × 10^6*	*0.5 ± 0.1*
	D-dCMP	0.9 ± 0.1	198 ± 13	80 ± 5	8 × 10^4	
	L-dCMP	0.73 ± 0.06	2.00 ± 0.05	0.82 ± 0.02	1 × 10^3	
hTMPK	D-dTMP	0.024 ± 0.002	6.8 ± 0.2	3.0 ± 0.1	1.2 × 10^5	
	L-dTMP	0.38 ± 0.07	0.86 ± 0.07	0.34 ± 0.03	9 × 10^2	5 ± 1

of L-NMP were assessed by nuclear magnetic resonance (^{1}H, ^{13}C, ^{31}P), fast-atom-bombardment MS, UV spectroscopy, HPLC and polarimetry. In some cases, it was necessary to separate nucleoside 5′-monophosphate from traces of nucleoside 3′-monophosphate. This was done by HPLC using Hypercarb, a porous graphic carbon stationary phase.[8]

After transformation of *E. coli* Rosetta(DE3)pLysS cells with the appropriate expression plasmid, recombinant hTMPK and hUMP-CMPK were produced and purified as already reported.[5] The NMP kinases activity was mesured as previously described.[5]

RESULTS

The reaction of recombinant human UMP-CMPK for L-dCMP and L-dUMP was detected using a coupled spectrophotometric assay.[5] Human UMP-CMPK showed an apparent affinity (K_m) to L-dUMP and L-dCMP slightly better than that to D-dUMP and D-dCMP, i.e., 0.70 mM and 0.73 mM compared to 1.3 mM and 0.9 mM, respectively (Table 1). The L-derivatives were however much slower substrates than the natural nucleotides with a 20-fold decrease in velocity from D-dUMP to L-dUMP and a 100-fold decrease from D-dCMP to L-dCMP. Similarly, the reaction rate of hTMPK for L-dTMP was 7-fold lower than for D-dTMP. Excess of L-dTMP inhibited the enzyme whereas no inhibition was observed with the natural enantiomer. Inhibition occurred at 1.2 mM of L-dTMP leading to an inhibition constant of 5 mM (data not shown). The Michaelis constant of hTMPK for L-dTMP was 0.38 mM compared to 0.025 mM for D-dTMP. The catalytic efficiency for L-dTMP was thus approximately 100-fold lower than for D-dTMP (Table 1).

DISCUSSION

From the results presented here it appears that recombinant hTMPK and hUMP-CMPK both showed relaxed specificity. The activities for the

L-derivatives were however lower than those for the corresponding D-enantiomers with a decrease in catalytic efficiency within the 10–100-fold range. A previous study reported on the phosphorylation of L-deoxycytidine monophosphate bearing modifications on the sugar ring or the cytidine base.[9] The study showed that a substitution at the 5 position of the base had a negative impact on the catalytic efficiency. It also demonstrated that the replacement of C-2′ of the ribose by a sulphur atom improved the catalytic efficiency contrary to the replacement by an oxygen atom. Among the L-nucleotides tested only 3TCMP was almost as good as the natural substrate, dCMP. However, this study did not evaluate the enantio-selectivity of the enzyme as L-dCMP was not tested. Our study therefore concludes on the relaxed enantio-selectivity of hUMP-CMP kinase.

Among the L-nucleosides of therapeutic potential, L-dT, the enantiomer of thymidine, is currently in phase III clinical trials.[1] This compound possesses specific anti-HBV activity and is hoped to minimise the risk of resistance development as, contrary to the nucleoside analogues accepted for the treatment of HBV, it targets the positive strand of HBV DNA. Additionally, L-dT exhibits an exceptional safety profile with no serious adverse effects observed in patients. Biological activity of L-dT requires phosphorylation to its triphosphate form. Although phosphorylation to the triphosphate form has been demonstrated in HepG2 cells, no phosphorylation pathway for L-dT has been characterised so far. In the light of the present and previous studies, an activation pathway for L-dT was proposed. The first step is thought to involve either the mitochondrial thymidine kinase (dTK2) or the cytosolic deoxycytidine kinase (dCK) as the cytosolic thymidine kinase (dTK1) was found to be strictly enantio-selective.[10] The second phosphate is most likely added by thymidylate kinase (TMPK) as we showed in this study that this enzyme accepts L-dTMP as acceptor substrate. Finally, the triphosphate form is probably produced by phosphoglycerate kinase (PGK) as NDPK was found not to accept L-derivatives.[11] L-dT therefore follows an activation pathway similar to L-FMAU, another anti-HBV agent in phase III clinical trials.[12] Interestingly, for both compounds, the efficiency of phosphorylation by hTMPK was only 0.9–1% that of D-dTMP, reflecting a preference of the active site for the natural configuration.

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