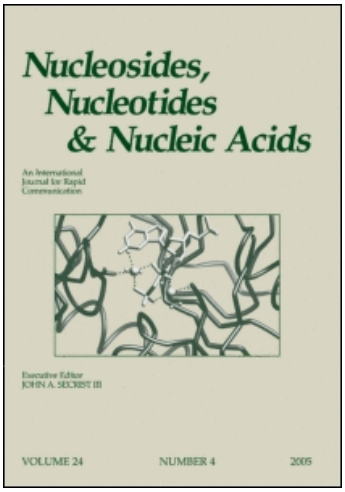

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LOOKING FOR NEW PYRIMIDINE ACYCLIC NUCLEOTIDE ANALOGUES DESIGNED FOR PHOSPHORYLATION BY HUMAN UMP-CMP KINASE

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□ *Human UMP-CMP kinase is involved in the phosphorylation of nucleic acid precursors and also in the activation of antiviral analogues including cidofovir, an acyclic phosphonate compound that mimicks dCMP and shows a broad antiviral spectrum. The binding of ligands to the enzyme was here investigated using a fluorescent probe and a competitive titration assay. At the acceptor site, the enzyme was found to accommodate any base, purine and pyrimidine, including thymidine. A method for screening analogues based on their affinity for the UMP binding site was developed. The affinities of uracil vinylphosphonate derivatives modified in the 5 position were found similar to (d)UMP and (d)CMP and improved when compared to cidofovir.*

Keywords Structure activity relationship; acyclic nucleotide phosphonate; UMP-CMP kinase; cidofovir; MABA-CDP

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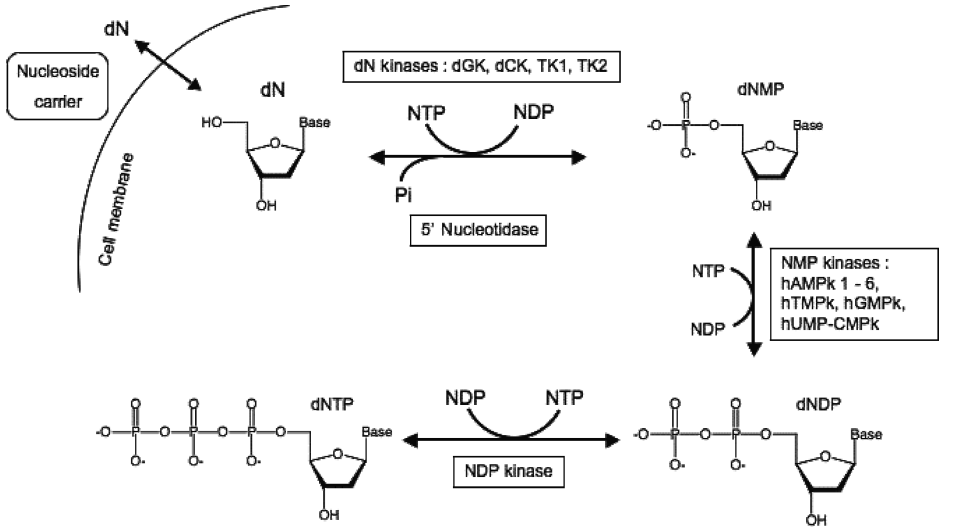
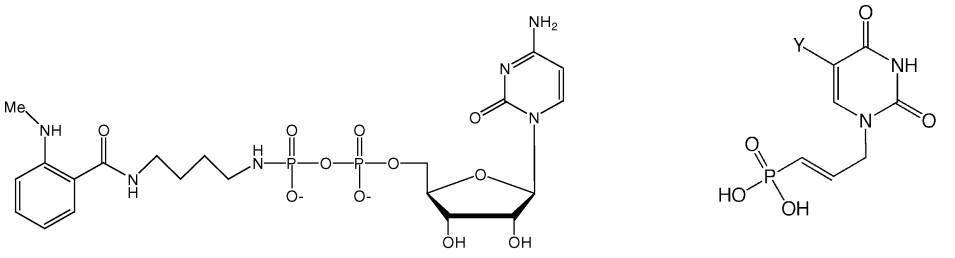


FIGURE 1 The cellular salvage pathway for nucleoside.

INTRODUCTION

Human UMP-CMP kinase plays a key role in the ribo- and deoxyribonucleosides salvage pathway as well as in the anabolic phosphorylation of nucleoside analogues used in antiviral and anticancer therapies. In cells nucleoside analogues are phosphorylated in a stepwise fashion, requiring first a deoxyribonucleoside kinase (thymidine kinase 1 and 2, deoxycytidine kinase or deoxyguanosine kinase),[1] then a NMP kinase,[2] and finally a NDP kinase,[3] and/or one of the enzymes able to synthesize ATP such as phosphoglycerate kinase,[4] pyruvate kinase, and creatine kinase[5] (Figure 1).

The first phosphorylation of nucleoside phosphonates also involves NMP kinases. Acyclic nucleoside phosphonates (ANP) have become a key class of antiviral nucleoside derivatives.[6] PMEA (adefovir) and PMPA (tenofovir), used respectively in HBV and HIV therapies, are both substrates of AMP kinases 1 and 2.[7] Here, we focus on human UMP-CMP kinase (hUCK) also known as pyrimidine nucleoside monophosphate kinase.[5] This enzyme efficiently phosphorylates several cytidine analogues such as AraC- and 3TC- monophosphate.[8,9] The acyclic phosphonate of cytidine approved in acyclovir-resistant Herpes and cytomegalovirus therapies (cidofovir, HPMPC) also is active for smallpox infections. Cidofovir, a dCMP mimic, is a very slow substrate for human hUCK.[5] As enhanced antiviral activity is correlated to a high intracellular concentration of the analogue active form (triphosphate for nucleoside analogues or diphosphate for ANP), we searched for new pyrimidine acyclic nucleotide analogues possessing improved binding and substrate properties towards hUCK.



(a) (b)

FIGURE 2 a) Formula of MABA-CDP used in the fluorescent competitive titration assay to determine dissociation constant of acyclic nucleotides; b) Formula of C5-substituted vinyl phosphonates (Y = Cl, Br, phenyl).

We investigated the binding of several ligands to the enzyme by fluorescence competition experiments against a fluorescent analogue of CDP.[10] The assay was used to screen a small library of new phosphonates analogues (Figure 2).

MATERIALS AND METHODS

Recombinant human UMP-CMP kinase was prepared using the pET-28a bacterial vector system (Novagen) and purified as previously described.[9] Fluorescent measurements were performed at 20°C on a PTI spectrofluorometer quantamaster. Nucleotide binding was observed in fluorescent competition experiments, in which a 1 mL cell contained MABA-CDP and hUCK in proportion of 1 and 3 K_D, respectively, resulting in half-saturation of the enzyme at the start of the experiments, as recommended.[10] The catalytic activity of the UCK was followed in a spectrophotometer by measuring ADP formation as described.[9] The reaction was started at 37°C by adding the enzyme, then a phosphate acceptor at the desired concentration. The absence of inhibition on the coupled system was carefully checked by measuring the reaction with 10 μM ADP in the presence and in the absence of the tested analogue.

RESULTS AND DISCUSSION

The acceptor binding site of human UMP-CMP kinase was probed by the fluorescent nucleotide, MABA-CDP (Figure 2a), in which the N-methylanthraniloyl (mant) group is bound to the β-phosphate of CDP through a butyl linker. MABA-CDP was previously reported as a specific probe for the CMP binding site of UMP-CMP kinase from *Dictyostelium*.[10] The fluorescence of free MABA-CDP increased upon addition of human UCK, shifting slightly towards blue and returning to the initial fluorescence value

of MABA-CDP upon CDP addition. From competition experiments, the concentrations of nucleotide needed to half the signal (IC_{50}) were determined. It is remarkable that CDP, CMP and dCMP were able to compete with MABA-CDP for the acceptor binding site, showing unambiguously that the same NMP site binds nucleosides and deoxynucleosides. Cytidine nucleotides have higher affinities than uridine nucleotides. AMP and even TMP were found to compete with MABA-CDP for binding with K_D below the mM range, confirming the broad specificity of this kinase. Surprisingly, despite the identity of the active site residues, NMP affinities for the human enzyme are 5–20 times higher than those for the *Dictyostelium* enzyme.

Cidofovir binds the NMP binding site with a relatively weak affinity (K_D = 300 +/− 100 μM). The recombinant hUCK was however able to slowly phosphorylate cidofovir in vitro (K_M = (1.0 +/− 0.1) mM, k_{cat} = 0.05 s^{-1}) resulting in a low catalytic efficiency, about 50 $M^{-1}s^{-1}$. With the aim of designing a cidofovir analogue that could react faster by hUCK, new acyclic phosphonates analogues of cidofovir were produced in parallel synthesis. Several 5-substituted uracil vinylphosphonates were synthesized. A primary screen using a plate-adapted assay enabled to identify compounds that competed with MABA-CDP at concentrations below 1 mM. Uracil vinylphosphonate was accepted by the enzyme with the same affinity as cidofovir (Table 1). Presence of a substitution at C5 in the vinyl series improves binding. Additionally, the smaller the size of the substituants, the better the binding : 5-Cl uracil was more tightly bound than the 5-Br derivative which was a better ligand than the 5-phenyl substituted derivative (Table 1).

When tested on the UMP-CMP kinase, no phosphorylation was detected for the vinylphosphonate series even if the enzyme showed a high affinity for some analogues. This may be due to an unproductive positioning of the phosphate moiety in the active site of hUCK. The structure of the NMP binding site of hUCK shows three arginines (R42, R93, and R137) interacting with the phosphate. The flexibility of the acyclic part of the tested

TABLE 1 The equilibrium dissociation constants (K_D) of new uracil acyclic phosphonates for human UMP-CMP kinase measured in the MABA-CDP fluorescent competition assay

Ligand	K_D in MABA-CDP competition assay (μM)
Cidofovir	300 +/− 100
Uracil vinylphosphonate	400 +/− 100
5-Cl-uracil vinylphosphonate	20 +/− 5
5-Br-uracil vinylphosphonate	50 +/− 10
5-Phe-uracil vinylphosphonate	150 +/− 50

compounds perhaps prevents these interactions, prohibiting direct phosphate transfer from the donor to the acceptor nucleotide.

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