Nucleoside Analogues as Inhibitors of Thymidylate Kinases: Possible Therapeutic Applications

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KEYWORDS:

inhibitors · nucleosides · nucleotides · thymidylate kinase · tuberculosis

Thymidylate kinase (TMPK) belongs to the nucleoside monophosphate kinase family, which catalyses the reversible transfer of a phosphoryl group from adenosine triphosphate (ATP) to a nucleoside monophosphate (NMP). TMPK is essential for cell proliferation and has been studied intensively over the last few years because of its role in the activation of antiviral drugs such as 3′-azido-3′deoxythymidine (AZT).[1–3] Characterization of *Mycobacterium tuberculosis* TMPK[4] in our laboratory revealed new structural and catalytic features, which render this enzyme a potential target for antituberculosis drugs.

Nucleotide analogues that are involved in cell growth or division, for example monophosphate derivatives, cannot enter cells and consequently cannot be delivered as active drugs. Monophosphate derivatives cross the cell membrane barrier in their nonphosphorylated form, and are then converted by intracellular kinases into the corresponding nucleotides, as occurs in HIV therapy with AZT or other nucleoside analogues. In the particular case in which TMPK is the drug target, a thymidine (dT) analogue could be activated by thymidine kinase (TK), which converts the analogue into the corresponding monophosphorylated compound (dTMP). However, a search of the *M. tuberculosis* genome[5] did not identify a gene coding for a TK. This result is in keeping with previous biochemical studies indicating a lack of TK activity in mycobacteria.[6]

The absence of TK seems on first inspection to be a severe limitation on the use of thymidine analogues or related compounds as antituberculosis drugs. However, we tested the effect of dT on *M. tuberculosis* TMPK, and were surprised to find that it is a competitive inhibitor (Table 1). It has an inhibition constant (the dissociation constant for the enzyme–inhibitor complex, K_i) of the same order of magnitude as the dissociation constant (K_m) for dTMP–enzyme complex. This property, which is common to TMPKs from various organisms (Table 1), was not observed with *M. tuberculosis* adenylate and cytidylate kinases in the presence of adenosine or cytidine, respectively (data not shown). To rationalize this observation, we set up a modeling and docking study with the MODELLER[7] and FlexX programs.[8] We made a visual inspection of the modeled complexes, and analyzed the ligand–protein contacts. The TMPKs of *Haemophilus influenzae* and *Yersinia pestis*, which are highly similar to the TMPK of *Escherischia coli* (60 and 70% identity, respectively), were automatically modeled by using an expected low root mean square deviation (RMSD) with respect to the known structure of *E. coli* TMPK.[1] *Bacillus subtilis* TMPK, which is 30% identical to *E. coli* TMPK, was modeled following our recently described approach.[4] While the catalytic residues appeared strictly conserved, several substitutions were observed in the vicinity of the bound dTMP (Table 2). Thymine, thymidine, and dTMP were successively docked in the solved crystal structures (*E. coli*[1] and *M. tuberculosis*[9] TMPKs) or in the modeled structures (*Y. pestis*, *H. influenzae,* and *B. subtilis* TMPKs). The docking program performed well and placed all the compounds similarly in the five TMPKs (the expected RMSD with the actual position was roughly 1 Å as estimated by comparison with known crystal structures). The calculated binding energies were in agreement with the experimental data, except for *B. subtilis* TMPK. The FlexX energy showed a decrease in binding energy in enterobacterial TMPKs caused by the Phe→Leu and Leu→Lys substitutions shown in Table 2. The K_i/K_m ratio was higher for *Y. pestis* and *H. influenzae* TMPKs than for *E. coli* TMPK. This result correlated well with the appearance of a net positive charge at the entrance of the active site caused by the Leu55Lys substitution. Discrepancies between the theoretical and experimental affinities for *B. subtilis* TMPK could be a result of Pro101 substitution, which might cause steric hindrance between the proline side chain and the carbonyl O4 atom of dT (O4–C_δ Pro 101 distance = 2.9 Å). A P101A mutant was modeled and tested in order to evaluate this hypothesis. An affinity similar to that of *E. coli* and *M. tuberculosis* TMPKs was found for thymine and dT with this mutant. Other factors, however, might be responsible for the unexpected lower affinity of *B. subtilis* TMPK for dT than for dTMP (Table 1). One of these factors could be the presence of an asparagine instead of an aspartic acid at position 155 (Table 2) in *B. subtilis* TMPK (see below).

Compounds other than dT were also tested on *M. tuberculosis* TMPK (Table 3) to verify the possibility that other nucleosides can also have an affinity for TMPK in the same range as the corresponding nucleoside monophosphate. Two main pairs of compounds were investigated: 5Br-dUMP and 5Br-dU, analogues modified at the 5-position of the base moiety, and 3′-azido-3′deoxythymidine monophosphate (AZTMP) and AZT, modified at the 3′-position of the pentose. In both cases, the affinity of the substrate analogue for *M. tuberculosis* TMPK was

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  1439-4227/02/03/01 $ 17.50+.50/0 CHEMBIOCHEM **2002**, No. 1

Table 1. *Comparison of the binding affinities of dTMP, dT and compounds* ***2***, ***3***, *and* ***4*** *for TMPKs from various organisms.*[a]

Organism	K_m dTMP [μM][b]	K_i dT [μM]	(K_i dT)/(K_m dTMP)	K_i **2** [μM]	K_i **3** [μM]	K_i **4** [μM]
M. tuberculosis	4.5	27	6.0	7.4	12	89
E. coli	15	19	1.3	97	33	105
Y. pestis	45	112	2.5	28.3	1530	1260
H. influenzae	20	49	2.5	13.7	1570	463
B. subtilis	10	124	12.4	261	5350	853

[a] Assay conditions are described in the Experimental Section. [b] Values for *M. tuberculosis* were taken from Munier-Lehmann et al.,[4] for *E. coli* and *Y. pestis*, from Chenal-Francisque et al.,[11] and for *H. influenzae* and *B. subtilis,* from L. Tourneux (personal communication).

Table 2. *Conservation and specific substitution within the active site of various TMPKs.*[a]

Organism	Base			Sugar			Phosphate		
M. tuberculosis	N100	S104[b]	F70	D9	D163	Y165	A49	A35	Y39
E. coli	T105	Q109	F74	E12	D157	I159	L55	R39	–
H. influenzae	S102	Q106	L71	E12	D154	I156	K55	R39	–
Y. pestis	S103	Q107	L72	E12	D155	I157	K55	R39	–
B. subtilis	P101	Q105	Y70	E12	N155	L157	L54	R38	–

[a] Single-letter nomenclature is used for the amino acids. [b] In *M. tuberculosis* TMPK, S104 is hydrogen-bonded to the substrate through a water molecule that was included during the docking. The substrate moiety, with which the given residue interacts, is indicated in the text.

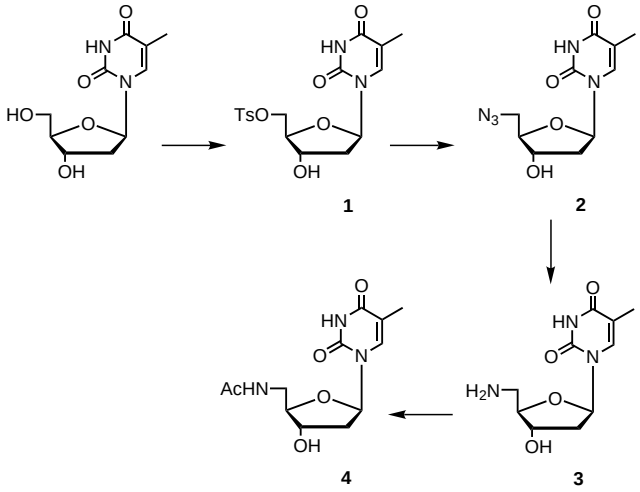
Table 3. *Nucleoside versus nucleotide affinity parameters of* M. tuberculosis *TMPK.*[a]

Compound	K_m or K_i [μM]
5Br-dUMP	33
5Br-dU	5
AZTMP	10
AZT	28

[a] Assay conditions were the same as in Table 1.

comparable to the affinity of the corresponding nucleoside (Table 3).

The absence of TK in *M. tuberculosis* might be overcome by the fact that dT itself has a good affinity for the corresponding TMPK. This unexpected property also raises the possibility of enhancing the specific delivery of compounds to bacteria within macrophages by modifying the 5′-position of dTMP. Such modifications may also decrease toxicity by reducing interaction with the host cellular TK. Synthesis of thymidine analogues modified on the 5′-position is outlined in Scheme 1. The K_i values measured for compounds **2**, **3**, and **4** with different bacterial TMPKs are given in Table 1. To test the predictive power of the modeling/docking strategy, we studied the relative affinity of compound **3** for the various TMPKs (Tables 1 and 4). Replacement of the 5′-OH group on dT by an amino group only slightly affected the affinity for *M. tuberculosis* TMPK. On the contrary, repulsion of the amino group by the specific Asn 155 substitution was expected in *B. subtilis* TMPK ($C5' - N_\delta$ Asn 155 distance = 4.7 Å), since other bacterial TMPKs possess an aspartate at position 155 (Table 2). The very marked effect observed, correlated to this particular Asp155Asn substitution in *B. subtilis* TMPK, suggested that a direct hydrogen bond is formed in other TMPKs complexed with compound **3**. Similar repulsion might be expected from the lysine side chain present in *H. influenzae* and *Y. pestis* TMPKs, but



Scheme 1. *Synthesis of compounds* ***1*** *–* ***4****. See the Experimental Section for conditions. Ts = tosyl = toluene-4-sulfonyl.*

Table 4. *FlexX scores and number of contacts (given in brackets) between the docked ligand and the protein.*

Organism	Thymine	dTMP	dT	Compound **3**
M. tuberculosis	– 25 (10)	– 35 (15)	– 29 (11)	– 26 (13)
E. coli	– 22 (10)	– 35 (17)	– 28 (11)	– 31 (12)
H. influenzae	– 18 (5)	– 23 (12)	–	– 20 (10)
Y. pestis	– 16 (6)	– 25 (10)	– 14 (6)	– 15 (8)
B. subtilis	– 14 (8)	– 23 (10)	– 15 (9)	– 16 (8)

the flexible nature of this side chain did not allow precise modeling of the interaction. FlexX energy ranks and experimental data were in good agreement.

The analogues described here are original because they are membrane-permeable and do not require phosphorylation to be active. Ongoing work aims to generate new compounds to gain

better insight into sequence – structure – function relationships. Cumulative substitutions of the various functional groups tested here would be one experimental approach. Crystallization of those complexes that largely deviate from the theoretical modeling could be another goal.

Experimental Section

Chemical synthesis: Treatment of thymidine with *p*-tolylsulfonyl chloride in pyridine at 0 °C yielded the 5′-sulfonate derivative **1** (72%). Displacement of the 5′-sulfonyl group with sodium azide in DMF at 80 °C gave the 5′-azido derivative **2** (79%). Hydrogenation of **2** in ethanol in the presence of palladium on charcoal resulted in an 83% yield of 5′-amino-2′,5′-dideoxythymidine (5′NH_2-dT, **3**). Acetylation of **3** with acetic anhydride followed by mild saponification yielded the *N*-acetylated derivative **4** (78%). Compounds **1**, **2**, and **4** were purified by silica gel chromatography. Compound **3** was purified by reversed-phase high pressure liquid chromatography. All nucleoside analogues were characterized by NMR spectroscopy.

TMPK assays: The reaction medium (0.5 mL final volume) contained 50 mM Tris(hydroxymethyl)aminomethane-HCl pH 7.4, 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM NADH, 1 mM phosphoenol pyruvate, and 2 units each of lactate dehydrogenase, pyruvate kinase, and nucleoside diphosphate kinase. Activity was determined by using the coupled spectrophotometric assay at 334 nm (0.5 mL final volume) in an Eppendorf ECOM 6122 photometer.[10] One unit of enzyme activity corresponds to 1 μmole of the product formed in 1 minute at 30 °C and pH 7.4. The concentration of ATP was kept constant at 0.5 mM. The concentrations of dT analogues and dTMP were varied between 0.05 and 0.8 mM.

This work was supported by grants from the Institut Pasteur, the Institut National de la Santé et de la Recherche Médicale, and the Centre National de la Recherche Scientifique (URA 2185 and 2128, and UMR 5048). We are grateful to S. Michelson for her fruitful comments, O. Barzu for support and constructive criticism, and A.-M. Gilles for the kind gift of some TMPKs.

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Received: August 28, 2001 [Z291]

Effect of the Protein Matrix of Cytochrome *c* in Suppressing the Inherent Peroxidase Activity of Its Heme Prosthetic Group

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KEYWORDS:

cytochrome *c* · heme proteins · oxidative stress · protein folding · radicals

Free heme is a potent peroxidase; it readily reacts with H_2O_2 and is then capable of a wide variety of oxidation reactions.[1] The heme group of cytochrome *c* (cytc) can be expected to be an equally competent producer of free radicals, but its intrinsic peroxidase activity is suppressed by the protein matrix.[2] This is of vital importance to the living cell because free radicals derived from oxygen play a key role in aging and its pathophysiology.[3] Herein, we quantify the suppression of the inherent peroxidase activity of heme by the protein matrix of cytc. We show that the peroxidase activity of unfolded cytc is similar to that of microperoxidase-8 (MP-8).[4]

The protein used here is the well-studied cytochrome *c*-550 (cytc550) from *Paracoccus versutus*.[5] Its peroxidase activity has been characterized.[2a] It is a Class I cytc, closely related both to the archetypal mitochondrial cytc proteins, and to numerous other, bacterial *c*-type cytochromes.[6]

Addition of the common denaturing agent guanidinium hydrochloride (Gdn·HCl) to ferricytc550 causes the protein to unfold, as was observed by using the following indicators (Figure 1).[7] In Figure 1A, the tryptophan fluorescence is plotted as a function of Gdn·HCl concentration. Unfolding causes the structure of cytc550 to expand and leads to an increased average distance between its tryptophan residues and the covalently bound heme group. This results in a less efficient energy transfer from the tryptophan residues to the heme moiety, and consequently the tryptophan fluorescence increases upon unfolding.[8] Secondly, unfolding leads to changes in the optical spectrum. The dominant effect is a transition of the heme iron center from low spin to high spin, caused by the loss of the native methionine ligand (Figure 1B).[9, 10] Finally, Figure 1C demonstrates that an increase in Gdn·HCl concentration causes the peroxidase activity of cytc550 to rise dramatically (about 1200-fold).[7] No such activity is seen when any of the components (Gdn·HCl, cytc550, H_2O_2, or the reducing substrate, guaiacol (gc)) are left out of the reaction mixture.[7] The activity

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