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Synthesis and Evaluation of Thymidine-5′-*O*-monophosphate Analogues as Inhibitors of *Mycobacterium tuberculosis* Thymidylate Kinase

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Abstract—A number of 2′- and 3′-modified thymidine 5′-*O*-monophosphate analogues were synthesized as potential leads for new anti-mycobacterial drugs. Evaluation of their affinity for *Mycobacterium tuberculosis* thymidine monophosphate kinase showed that a 2′-halogeno substituent and a 3′-azido function are the most favorable leads for further development of potent inhibitors of this enzyme.

Tuberculosis (TB) is frighteningly on the rise. Once nearly vanquished by antibiotics, at least in the developed world, tuberculosis resurged in the late 1980's and now kills more than 2 million people a year—second only to AIDS among infectious diseases.[1]

Mycobacterium tuberculosis, the causative agent of this disease, is primarily transmitted via the respiratory route and mostly causes pulmonary tuberculosis. Estimates are that one third of the world′s population is infected with this organism, but infection does not usually lead to the active disease. Reactivation of this latent infection can be caused by immunodeficiency, HIV infection, use of corticosteroids, aging, alcohol and drug abuse.

TB has re-emerged as a serious public health threat worldwide because of a significant increase in multiple-drug-resistant TB and synergism between HIV and *M. tuberculosis* infection.[2] Resolution of the current TB epidemic will require prevention of new TB infections as well as improved methods for treating existing ones. All this has stimulated the search for new targets and the development of new antibiotic drugs to meet the global emergency.

M. tuberculosis thymidine monophosphate kinase (TMPKmt), which phosphorylates dTMP to dTDP, is believed to be an attractive potential target for chemotherapeutic intervention because: (i) the enzyme lies at the junction of the de novo and salvage pathways for thymidine triphosphate (dTTP) and is the last specific enzyme for its synthesis,[3] (ii) in vivo studies with the *cdc*8 mutant in *Saccharomyces cerevisiae* have demonstrated that it is essential for DNA synthesis,[4,5] and (iii) sequence comparison of TMPKmt with the human enzyme reveals only a 22% sequence identity which should make the design of selective inhibitors possible.[6]

This enzyme has recently been crystallized by Li de la Sierra et al.[6] Examination of its X-ray structure (Fig. 1) reveals that the main binding forces between dTMP and the enzyme are: (i) a stacking interaction between the pyrimidine ring and Phe70, (ii) a hydrogen bond between *O*4 of thymine and the Arg74 side chain which results in a preference for thymine over cytosine, (iii) a

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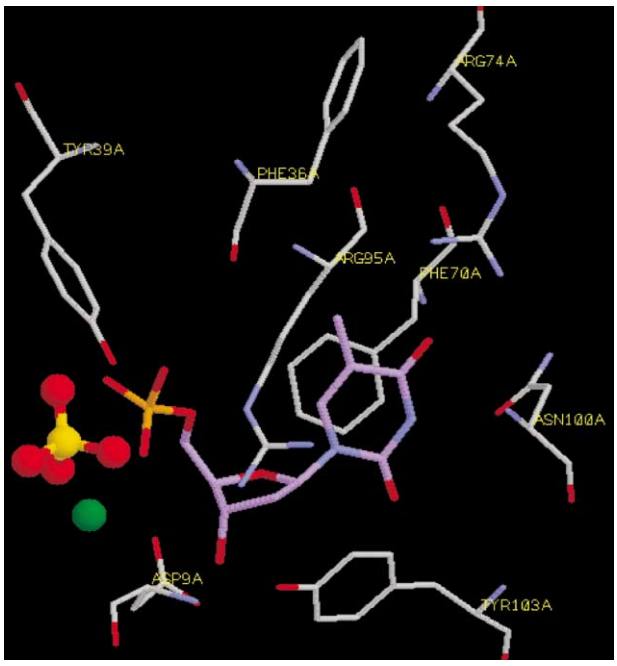


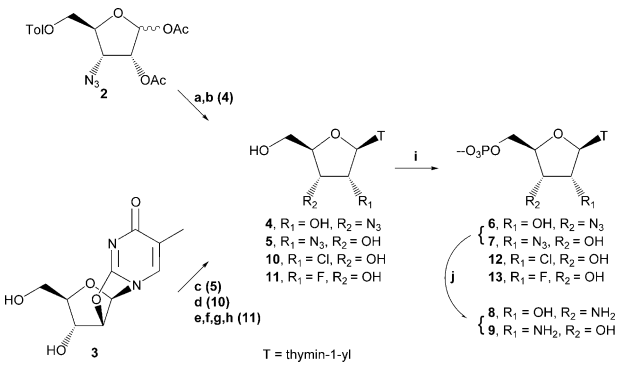
Figure 1. Simplified image of the dTMP binding site of *M. tuberculosis*, complexed with dTMP, showing the most important amino acids interacting with dTMP. dTMP is pictured in purple and the magnesium ion as a green sphere. A sulphate ion (depicted in ball and stick format) is located at the place normally occupied by the β phosphate of ATP.

hydrogen bond between Asn100 and *N*3 of the thymine ring, (iv) a hydrogen bond between the 3′-hydroxyl of dTMP and the terminal carboxyl of Asp9, that in its turn interacts with the magnesium ion that is responsible for positioning the phosphate oxygen of dTMP, and (v) hydrogen bonds and an ionic interaction between the 5′-*O*-phosphoryl group and Tyr39, Phe36, Arg95 and Mg^{2+}, respectively.[6]

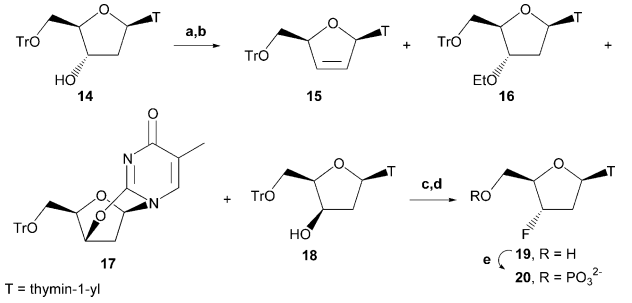
The presence of Tyr103 close to the 2′-position is believed to render the enzyme catalytically selective for 2′-deoxynucleotides versus ribonucleotides.

Considering these interactions and the available K_i and K_m values of some nucleotides for TMPKmt, we decided to make a series of nucleotides with modifications at the 2′- and 3′-positions of the dTMP scaffold in order to establish a preliminar structure activity relationship (SAR), that should be directive for the further design of inhibitors of TMPKmt.

3′-Azido-3′-deoxythymidine 5′-*O*-monophosphate (AZT-MP) was the best known inhibitor so far with a K_i value of 10 μM.[7] Remarkably, the presence of the 3′-azido group totally abolishes its conversion by TMPKmt without significantly altering the affinity in comparison to dTMP. We assume that the azide moiety interacts directly with Asp9 and displaces the magnesium ion, which seriously disturbs the geometry of the active site.[6] To probe the effect of an amino group at the 3′-position, AZT-MP was reduced to 3′-amino-3′-deoxythymidine 5′-*O*-monophosphate (**1**). The corresponding ribo analogue **8**, was synthesized to examine the capability of



Scheme 1. Synthetic pathways of nucleotides **8**, **9**, **12** and **13**. Reagents and conditions: (a) 5-methyl-2,4-bis(trimethylsilyl)oxypyrimidine, $(CH_3)_3SiOSO_2CF_3$, $Cl_2(CH_2)_2$; (b) NH_3, MeOH; (c) NaN_3, DMF; (d) HCl, dioxane; (e) 3,4-dihydropyran, *p*-toluenesulfonic acid, DMF; (f) 1 N NaOH, MeOH; (g) DAST, CH_2Cl_2, pyridine; (h) *p*-toluenesulfonic acid, MeOH; (i) $POCl_3$, $(MeO)_3PO$; (j) Ph_3P, NH_4OH, pyridine.



Scheme 2. Synthetic pathway of nucleotide **20**. Reagents and conditions: (a) MsCl, pyridine; (b) NaOH, EtOH; (c) DAST, CH_2Cl_2, pyridine; (d) CH_3COOH, H_2O, 90 °C; (e) $POCl_3$, $(MeO)_3PO$.

Tyr103 to discriminate between ribo- and deoxynucleotides. Introduction of an amino group at the 2′-position in **9**, on the other hand, could indicate if the positively charged nitrogen is able to interact with Tyr103 by a cation–π interaction.

To sort out the influence of the size and the electronegativity of groups at the 2′- and 3′-positions on the affinity for TMPKmt, a fluorine (**13**) and a chlorine (**12**) were introduced at C-2′, and the 3′-hydroxyl was replaced by a fluorine (**20**). ^1H NMR studies have demonstrated a relation between the electronegativity and the size of the substituents and the sugar puckering in a series of 2′-substituted-2′-deoxyadenosines.[8] Introduction of a fluorine increased the percentage of sugar that exhibits the 2′-*exo*-3′-*endo* conformation, while a 2′-chloro substitution favoured the 2′-*endo*-3′-*exo* conformation. The latter puckering is the one adopted by the sugar part of dTMP when bound to TMPKmt. It could be expected that nucleotide analogues that exhibit a similar S-type conformation would have a higher affinity for TMPKmt, than those that have to be forced into that configuration by the enzyme.

The synthetic methods used to prepare the desired dTMP analogues are outlined in Schemes 1 and 2. All phosphorylation steps were performed using the procedure of Yoshikawa et al.,[9] that is treatment with $POCl_3$

(3 equiv) in $(MeO)_3PO$. The obtained compounds were purified by column chromatography (*i*PrOH/NH_4OH/H_2O: 77.5/15/2.5→60/30/5), followed by HPLC (C-18, CH_3CN/MeOH/0.05% HCOOH in H_2O: 45/45/10, 3 mL/min).

1-(3′-Azido-3′-deoxy-β-D-ribofuranosyl)thymine (**4**) was prepared by Vorbrüggen coupling[10] of sugar synthon **2**[11] with 5-methyl-2,4-bis(trimethylsilyl)oxypyrimidine followed by alkaline deprotection, while its 2′-azido isomer **5** was obtained via opening of 2,2′-anhydrothymidine (**3**) with NaN_3 in DMF according to the method of Verheyden (Scheme 1).[12] Azidonucleosides **4** and **5** were converted to the corresponding aminonucleoside monophosphates **8** and **9** by sequential 5′-*O*-phosphorylation and reduction of the azido group with Ph_3P and NH_4OH.[13]

Compound **3** also served as synthon for the preparation of 2′-chlorothymidine[14] (**10**) and 2′-fluorothymidine (**11**). For the synthesis of the latter, **3** was treated with 3,4-dihydropyran in DMF, followed by saponification to yield its 3′,5′-bisprotected arabinosyl derivative. Fluorination with DAST, followed by deprotection with *p*-toluenesulfonic acid in MeOH, afforded **11**.[15] Nucleosides **10** and **11** were converted to their monophosphates **12** and **13** in 53 and 55% yield, respectively.

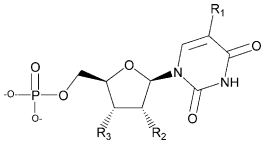
3′-Deoxy-3′-fluorothymidine (**19**) was synthesized from 5′-*O*-tritylthymidine (**14**), using the method of von Janta-Lipinski.[16] In our hands, treatment of the intermediate 3′-mesyl ester of **14** with NaOH in ethanol resulted in the formation of four reaction products: the desired SN2 product **18** (17% yield), as well as the 5′-tritylethers of 3′-*O*-ethylthymidine (**16**, 26%), 3′-deoxy-2′,3′-didehydrothymidine (**15**, 12%) and 2,3′-anhydrothymidine (**17**, 33%). Compound **18** was then converted into **19** via treatment with DAST[17] and subsequent detritylation, and phosphorylation of **19** yielded **20** (Scheme 2).

All compounds were tested on TMPKmt by the reported spectrophotometric assay.[18] The results are given in Table 1.

The K_i value of **1** increases 20-fold compared to AZT-MP. This strongly suggests that the 3′-amino group does not interact with Asp9 as strong as AZT-MP. To our surprise the ribo-analogue **8** shows good affinity (27 μM) for TMPKmt. A modelling experiment was suggestive for an interaction between the 3′-amino group and the carboxyl function of Asp9. According to the model, Tyr103, believed to discriminate between deoxy- and ribonucleotides, remained further away from the C-2′ compared to the X-ray structure of TMPKmt with dTMP. Analogue **8** represents a first example of a ribonucleotide with good affinity for TMPKmt. The higher K_i value of 2′-amino dTMP (**9**) suggests that the positively charged nitrogen is not capable of interacting optimally with Tyr103 or Asp9. The monophosphate of d_4T (d_4T-MP) behaves as substrate for TMPK-tub, although its K_m value is 30 times larger than that of the natural substrate.[6]

The introduction of halogens at the 2′-position appears to be slightly better tolerated than an amino group on that position. **12** exhibits appreciable affinity with a K_i value of 19 μM. Changing the chlorine of **12** for a fluorine (**13**) leads to a 2-fold affinity drop. A modelling experiment of **12** (results not shown) showed that introduction of the chlorine affects the relative position of the sugar ring. As a result, the 2′-chlorine occupies the pocket where normally the 3′-hydroxyl group resides. Indeed, the 3′-analogues indicate that this particular domain can accommodate larger substituents. The somewhat lower affinity of the 2′-fluoro nucleotide could be due to the fact that the smaller and more electronegative fluorine is not able to fill up that cavity, perhaps as a result of a different preferential sugar pucker. Replacement of the 3′-OH of dTMP by a 3′-F in **20** affords an analogue that behaves as a substrate with a K_m of 30 μM.

Table 1. Kinetic parameters of TMPKmt with various nucleoside monophosphates



Compd	R1	R2	R3	K_m (μM)	V_m (μmol/min mg of protein)	K_i (μM)
dTMP	CH_3	H	OH	4.5	10.6	
DUMP[7]	H	H	OH	2100	3.5	
5F-dUMP[7]	F	H	OH	420	4.7	
5Br-dUMP[7]	Br	H	OH	33	9.8	
5I-dUMP[7]	I	H	OH	140	7.5	
AZTMP[7]	CH_3	H	N_3			10
1	CH_3	H	NH_2			235
8	CH_3	OH	NH_2			27
9	CH_3	NH_2	OH			55
12	CH_3	Cl	OH			19
13	CH_3	F	OH			43
d_4T-MP[7]	CH_3	Dehydro	Dehydro	140	0.16	
20[7]	CH_3	H	F	30	0.11	

In conclusion, only a few dTMP analogues, that is the 5-halogeno derivatives, d_4T-MP, dUMP and 3′-deoxy-3′-fluorothymidine 5′-*O*-monophosphate (**20**), remain substrates for TMPKmt, while other modifications lead to competitive inhibitors. The introduction of a bromine, the best known 5-modification so far, does not drastically lower the affinity in comparison to dTMP.

Among the tested sugar-modified dTMP analogues, the best inhibition is obtained by the introduction of a 2′-chloro or the replacement of the 3′-OH by an azido group. From these results it seems interesting to combine a 2′-chlorine and 3′-azido group and to explore other substitution patterns at the 3′-position. Because modelling experiments show that there is a large space at the 3′-position, introduction of larger nitrogen containing compounds that could interact with Asp9 will be the basis for further development of more potent ligands.

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