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

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Abstract—The affinity of a series of 2′, 3′- and 5-modified thymidine analogues for *Mycobacterium tuberculosis* thymidine monophosphate kinase (TMPKmt) was evaluated. The affinities of several non-phosphorylated analogues are in the same order of magnitude as those of their phosphorylated congeners. In view of drug delivery problems associated with phosphorylated compounds, these 'free' nucleosides seem more promising leads in the search of TMPKmt inhibitors as novel anti-tuberculosis agents.

Recently, *Mycobacterium tuberculosis* thymidine monophosphate kinase (TMPKmt) was put forward as an attractive target for the design of a novel class of anti-tuberculosis agents.[1–3]

TMPK accounts for the phosphorylation of thymidine monophosphate (dTMP) to thymidine diphosphate (dTDP), using ATP as a preferred phosphoryl donor.[4] Because TMPK lies at the junction of the de novo and salvage pathways of thymidine triphosphate (dTTP) synthesis and in view of its low (22%) sequence identity with the human isozyme,[5] it represents an attractive target for selectively blocking mycobacterial DNA synthesis. Recently, the X-ray structure of TMPKmt was solved at 1.95 Å as a complex with dTMP,[6] allowing structure-based design of TMPKmt ligands (Fig. 1).

In order to establish initial structure–activity relationships, a series of sugar- and base-modified nucleoside-5′-*O*-monophosphates were examined for TMPKmt affinity. The aim of conserving the 5′-*O*-monophosphate moiety was to preserve its hydrogen bonding and ionic interactions with Tyr39, Arg95 and Mg^{2+}. These bindings, together with the stacking interaction between the pyrimidine ring and Phe70 should account for a dTMP-like orientation of the substrate analogues under investigation, thereby rendering prediction and modelling of

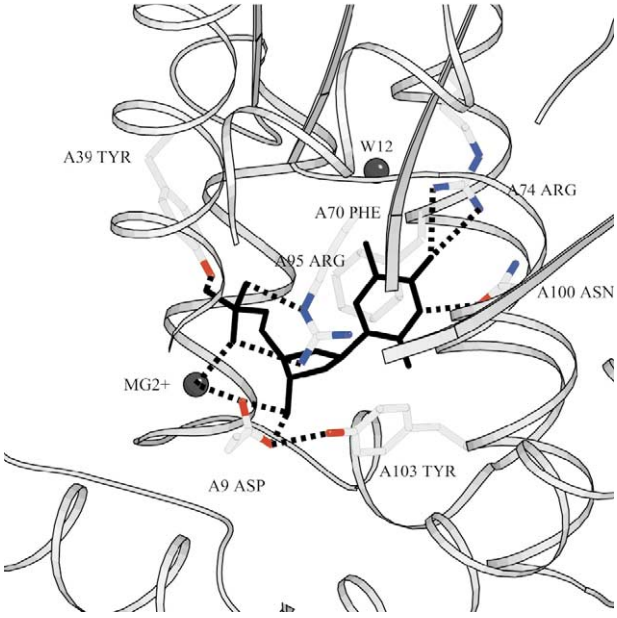

Figure 1. Schematic representation of the most important amino acid residues of TMPKmt interacting with TMP (in black).

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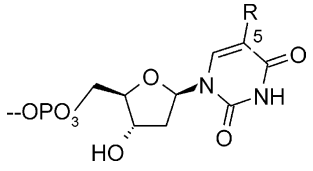
the interactions of the various dTMP substituents with the enzyme more straightforward. Next, alternative substitution patterns at the 5′-position would be explored and combined with optimal sugar and base modifications. These sugar- and base-modified nucleotides were examined on their affinity for the enzyme using a reported spectrophotometric assay,[7] unless mentioned otherwise.

First, systematic substitution of the 5-methyl group for various halogen atoms was explored. 5F-dUMP, 5Br-dUMP and 5I-dUMP turned out to be substrates for TMPKmt.[5] 5Br-dUMP ($K_m = 33\,\mu M$) showed the highest affinity, probably because a bromine occupies about the same steric space as a methyl group. The presence of the guanidinium group of Arg74 in the otherwise hydrophobic pocket around the 5-position could explain the measured affinity of the halogenated substrates. It is believed that the difference in the kinetic parameters of these compounds essentially reflects a size effect, with the halogen atoms serving as cavity filling atoms.[5] A co-crystal of 5I-dUMP with TMPKmt[6] showed that it, indeed, binds the phosphate acceptor binding site in a very similar fashion to dTMP.[2]

Since these findings confirmed the assumption that TMPKmt can accommodate sterically larger substituents at the 5-position, **1** was synthesised to accomplish a favourable interaction with a water molecule (W12), detected in the crystal structure. In contrast to the 5-halogeno-substituted substrates, this compound behaves as a (relatively weak) inhibitor of TMPKmt. Co-crystallisation with TMPKmt proved the predicted interaction with W12.[2] Other dTMP analogues with sterically more demanding substituents that maintain one polar atom (**2** and **3**) were found less active inhibitors than **1**, indicating that the volume of the cavity cannot be stretched too much. It is inferred that these three compounds are inhibitors because any modification, significantly perturbing the volume at the 5-position, changes the orientation of the sugar moiety as well as that of the α-phosphate.[2] Surprisingly, removal of the 5-methyl group of dTMP (dUMP) caused a drastic decrease in affinity ($K_m = 2100\,\mu M$ compared to 4.5 μM for dTMP) (Table 1).[5]

Table 1. Kinetic parameters of TMPKmt with base-modified nucleoside monophosphates



Compd	R	K_m (μM)	V_m (μmol/min mg of protein)	K_i (μM)
dTMP[5]	CH_3	4.5	10.6	
dUMP[5]	H	2100	3.5	
5F-dUMP[6]	F	420	4.7	
5Br-dUMP[6]	Br	33	9.8	
5I-dUMP[6]	I	140	7.5	
1[2]	CH_2OH			110[a]
2[2]	Furan-2-yl			140[a]
3[2]	Thien-2-yl			270[a]
4[2]	Benzyl			28

[a]The TMP kinase activity of these compounds was not determined using the spectrometric test described by Blondin et al.[7] but using HPLC chromatography. The K_m for dTMP obtained with this test is 40 μM.[2]

The observation that TMPKmt, as opposed to all TMPKs of other species examined so far,[5] is strongly inhibited by AZTMP ($K_i = 10\,\mu M$) offered good prospects for finding selective inhibitors of TMPKmt through altering the sugar part of the dTMP scaffold. At the 3′-position, the crystal structure reveals a hydrogen bonding network (denoted by the dotted lines, Fig. 1): the 3′-OH of dTMP interacts with the terminal carboxyl of Asp9, which, in its turn, is linked to the Mg^{2+}-ion that is responsible for positioning a phosphate oxygen of TMP. Through its interaction with Asp9, the 3′-azido function of AZTMP is believed to perturb the aforementioned hydrogen bonding interplay that is essential for catalysis, hence completely impeding the phosphoryl transfer.[6] Although an analogous interaction was expected upon reduction of the 3′-azido group of AZTMP to a 3′-amine (**5**), this drastically increased the K_i value to 235 μM. Replacing the 3′-OH of dTMP by a 3′-F (**8**), on the other hand, afforded an analogue that behaves as a substrate with a K_m of 30 μM.

The presence of Tyr103 close to the 2′-position is believed to render the enzyme catalytically selective for 2′-deoxynucleotides versus ribonucleotides. The ribo analogue (**6**) of **5**, however, unexpectedly showed a much higher affinity for the enzyme (45 μM vs 235 μM), this compound being the first example of a ribonucleotide with good affinity for TMPKmt. Modelling indicates that, in this case, Tyr103 stays somewhat further away from the 2′-position and is, therefore, unable to clash with the 2′-hydroxyl.[1] Introduction of halogens at the 2′-position (**9** and **10**) is better tolerated than a hydroxyl group at that position. Modelling showed that the halogen affects the relative position of the sugar ring. As a result, the 2′-chlorine occupies the pocket, in which the 3′-hydroxyl usually resides. As previously demonstrated with AZTMP, this particular domain can, indeed, accommodate more voluminous substituents.[1]

Replacing the five-membered sugar ring of dTMP by a 1,5-anhydrohexitol yielded a moderately active inhibitor. This effect is probably due to the inability of **11** to position both the phosphate and the 3′-hydroxyl groups in a favourable arrangement for catalysis.[2] With a K_i of 30 μM, the bisubstrate analogue Ap_5T, emerged as a good inhibitor of TMPKmt. The crystal structure of the complex of TMPKmt with Ap_5T, which has been established at 2.45 Å resolution,[2] revealed an unexpected binding mode for the adenine moiety of this compound. While the thymidine residue of this bisubstrate analogue occupies the binding pocket of dTMP, its ADP unit, surprisingly, fits a cavity on the surface of TMPKmt. This finding opens avenues to the design of branched molecules at the α-phosphate of Ap_5T, with

an additional group reaching out to this cavity present only in TMPKmt (Table 2).[2]

Obviously, monophosphate derivatives are unable to enter cells and, as a consequence, can not be delivered in such a way. Generally, nucleosides cross the cell–membrane barrier in a non-phosphorylated form and are then converted by intracellular kinases to the corresponding nucleotides. In this particular case where TMPK is the drug target, a thymidine analogue could be activated by thymidine kinase (TK), which converts the analogue into the corresponding monophosphorylated compound. However, a search of the *M. tuberculosis* genome[8] did not identify any gene coding for a TK. This result aligns with other biochemical studies indicating a lack of TK activity in mycobacteria.[9] At first sight, the absence of TK seems to be a severe limitation on the use of thymidine analogues or related compounds as anti-tuberculosis drugs. However, the finding that dT behaves as a competitive inhibitor of TMPKmt (with K_i in the same order of magnitude as K_m of dTMP[3]), raises the possibility of discovering non-phosphorylated nucleosidic inhibitors of TMPKmt. Enhancing the specific delivery of such inhibitors to bacteria within macrophages by modifying the 5′-position of dTMP is another option. Such modifications may also decrease toxicity by reducing interaction with the host cellular TK. Replacement of the 5′-hydroxyl by an azido or amino an group (**13** and **14**) yielded two high-affinity inhibitors of TMPKmt (K_i values of 7 and 12 μM, respectively).[3]

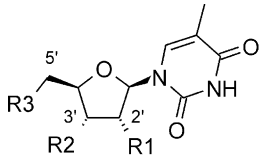
We investigated whether the negligible effect on TMPKmt affinity, observed upon deletion of the phosphate moiety of dTMP, is parallelled in other series. AZT, 5-BrdU and **22** exhibited similar affinities as their nucleotide congeners. In the case of **23**, the affinity decreased only 2.8 times upon removal of the 5′-*O*-phosphoryl. With regard to these observed similar affinities of nucleosides and nucleotides and in view of the drug delivery problems of phosphorylated compounds, nucleosides seem more useful leads for further drug design.

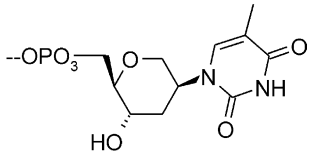
To further explore other substitution patterns at the 5′-position, the 5′-hydroxymethyl was substituted for a 5′-methyl. In **25**, this caused a 42-fold decrease in affinity compared to **6**. The affinities of **20** and **19**, on the contrary, were similar, making assumptions about the removal of the 5′-hydroxyl group unreliable. Compound **24**, with a 5′-iodo group, showed a low affinity ($K_i = 490$ μM), but it's not clear if the 5′-iodo group or the 2′-hydroxyl function accounts for this effect.

Because **6** conveyed the impression that ribo analogues are well tolerated by TMPKmt, two other ribonucleosides (**15** and **19**) were examined. The affinity of **15** was 26 times lower than the affinity of dT and **19** showed to be a poor inhibitor as well. These results indicate that the affinity improvement resulting from the introduction of a 2′-hydroxyl function in **5** (**6**) is exceptional. **16** even indicates that a 2′-β-OH is better tolerated.

Because AZTMP is one of the best known inhibitors, another nitrogen-containing substituent, a guanidine group (**26**), was introduced at the 3′-position. Although one would expect the delocalized positive charge of the guanidine moiety to strengthen the interaction with Asp9, the K_i value of 140 μM was not affirmative.

Table 2. Kinetic parameters of TMPKmt with sugar-modified nucleoside monophosphates



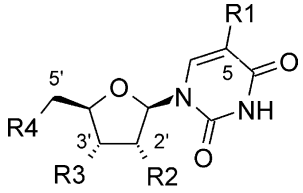


11

Compd	R1	R2	R3	K_i (μM)
dTMP	H	H	OPO_3^{2-}	4.5[a]
AZTMP[5]	H	N_3	OPO_3^{2-}	10
D4T-MP[5]	Dehydro	Dehydro	OPO_3^{2-}	140[a]
5[1]	H	NH_2	OPO_3^{2-}	235
6[1]	OH	NH_2	OPO_3^{2-}	45
7[1]	NH_2	OH	OPO_3^{2-}	120
8[1]	H	F	OPO_3^{2-}	30[a]
9[1]	Cl	OH	OPO_3^{2-}	19
10[1]	F	OH	OPO_3^{2-}	43
11[2]				150[b]
Ap_5T[2]				30[b]

[a] K_m value.
[b] The TMP kinase activity of these compounds was not determined using the spectrophotometric assay by Blondin et al.,[7] but using HPLC chromatography. K_m for dTMP is 40 μM.[2]

Table 3. Kinetic parameters of TMPKmt with sugar- and base-modified thymidine analogues



Compd	R1	R2	R3	R4	K_i (μM)
dTMP	CH_3	H	OH	OPO_3^-	4.5[a]
dT	CH_3	H	OH	OH	27
AZT	CH_3	H	N_3	OH	28
5Br-dU[3]	Br	H	H	H	5
12[3]	CH_3	H	OH	$NHCOCH_3$	90
13[3]	CH_3	H	OH	N_3	7
14[3]	CH_3	H	OH	NH_2	12
15	CH_3	OH	OH	OH	715
16	CH_3	OH[b]	OH	OH	238
17	CF_3	H	OH	OH	97
18	CH_2CH_3	H	OH	OH	1140
19	F	OH	OH	OH	521
20	F	OH	OH	H	560
21	CH_3	H	NH_2	OH	230
22	CH_3	H	F	OH	28
23	CH_3	F	OH	OH	122
24	CH_3	OH	N_3	I	490
25	CH_3	OH	NH_2	H	1900
26	CH_3	OH	$NHC(NH)NH_2$	OH	140

[a] K_m value.
[b] This hydroxyl lies at the β-side of the sugar ring.

Since the efficient affinity of **1** proved that the 5-position can accommodate intermediately sized substituents, **17** (which has a 5-CF_3 mimicking the size of the 5-methyl) and **18** were investigated as well. However, with K_i values of 97 and 1140 μM, they proved to be poor inhibitors of TMPKmt (Table 3).

In our search for high-affinity TMPKmt ligands as antituberculosis agents, most of the base- and sugar-modified dTMP-analogues (with the 5-halogeno dUMP derivatives, dUMP, 3′-F-dTMP and D4T-MP as exceptions) proved to be inhibitors of the enzyme. However, conversion to prodrugs is the only way to deliver phosphorylated compounds into cells. Since this recent work indicates that in many cases (AZT, 5-BrdU, **22** and **23**) nucleoside analogues show affinities that approach those of their phosphorylated counterparts, this problem could be easily circumvented by focusing on nucleosides instead of nucleotides. Concerning the 5′-modifications we can conclude that a 5′-amino and a 5′-azido group are good alternatives for a 5′-hydroxyl and that further study is recommended to probe the effect of deletion of the 5′-hydroxyl. At the 2′-position, a 2′-hydroxyl has proven to reduce the affinity for TMPKmt, with compound **6** as the only exception known so far.

Future research will be directed towards finding other high-affinity 5′-substituents that are unable to be phosphorylated by cellular kinases, thereby diminishing the risk for toxicity and further exploration of the 3′-position to improve the current inhibitors. Also, analogues exhibiting interesting inhibitory activity towards TMPKmt will be examined for selectivity vis-à-vis the human enzyme.

Acknowledgements

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