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Improving the Selectivity of Acyclic Nucleoside Analogues as Inhibitors of Human Mitochondrial Thymidine Kinase: Replacement of a Triphenylmethoxy Moiety with Substituted Amines and Carboxamides

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Abstract—Two series of analogues of the novel human mitochondrial thymidine kinase inhibitor 1-[(*Z*)-4-(triphenylmethoxy)-2-butenyl]thymine were synthesized by replacing the triphenylmethoxy moiety by a variety of substituted amines and carboxamides. In all the cases, the selectivity against the mitochondrial enzyme was either maintained or improved, and several derivatives were almost as potent as the parent compound. A molecular model was built that can account for the observed selectivities.

Deoxyribonucleoside kinases (dNK) catalyze the phosphorylation of deoxyribonucleosides to the corresponding deoxyribonucleoside monophosphates. In mammalian cells, there are four different dNKs with partially overlapping substrate specificities: TK-1 and dCK, located in the cytosol and/or the nucleus, and dGK and TK-2 with a mitochondrial localization.[1] There is a high sequence homology between TK-2, dCK and dGK, but also with herpes simplex virus type 1 thymidine kinase (HSV-1 TK).[2] Both TK-2 and HSV-1 TK recognize thymidine, 2′-deoxyuridine and 2′-deoxycytidine as substrates for phosphorylation.

As enzymes involved in the activation of nucleoside and nucleoside analogues, dNKs are an important and active area of research both in the anticancer and antiviral fields.[1] They have also been used in the gene-directed enzyme/prodrug therapy of cancer, where transduction of tumor cells with the corresponding dNK will activate a relative nontoxic nucleoside analogue (prodrug) to kill tumor cells.[3] Interest in TK-2 is quite recent. Its role in mitochondrial DNA synthesis as well as in mitochondrial toxicity observed under prolonged treatment with antiviral drugs such as AZT or FIAU is under debate.[4–6] Moreover, TK-2 is constitutively expressed throughout the cell cycle, and, in nonproliferating and resting cells, TK-2 is virtually the only pyrimidine nucleoside kinase that is physiologically active.[7] Therefore, the use of TK-2 inhibitors may help to resolve fundamental questions such as the physiological role of TK-2 and may also help clarify the contribution of TK-2 catalyzed phosphorylation of antiviral drugs and their mitochondrial toxicity.

Reports on TK-2 inhibitors are scarce and include 2′-*O*-alkylether orester derivatives of arabinofuranosyl nucleosides,[8,9] 5-substituted ribonucleosides, as well as 3′-highly functionalized nucleosides.[10] Also, the substrate/inhibitor properties of a series of nucleoside derivatives have been described.[11] Very recently, we have reported on the first acyclic nucleoside analogues as TK-2 inhibitors, the lead compound being 1-[(*Z*)-4-(triphenylmethoxy)-2-butenyl]thymine (**1**).[12] Further studies with this compound and related analogues have

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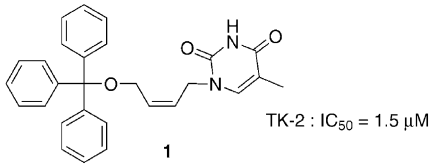
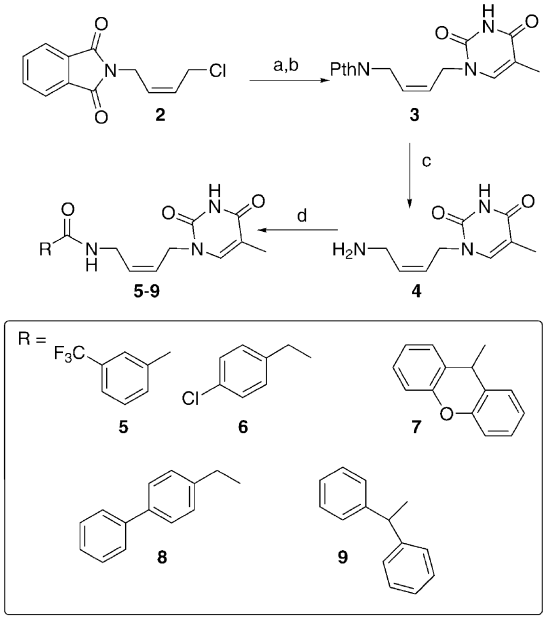
revealed that these compounds constitute the first examples of reversible non-nucleoside, non-substrate inhibitors of TK-2 that are competitive with respect to thymidine and uncompetitive with respect to ATP.[13] Here we describe further structural modifications on our lead compound that examine replacement of the trityl ether moiety by different amines and carboxamides, and the effect of these substitutions on the inhibitory activity against TK-2 and the closely related HSV-1 TK enzyme.



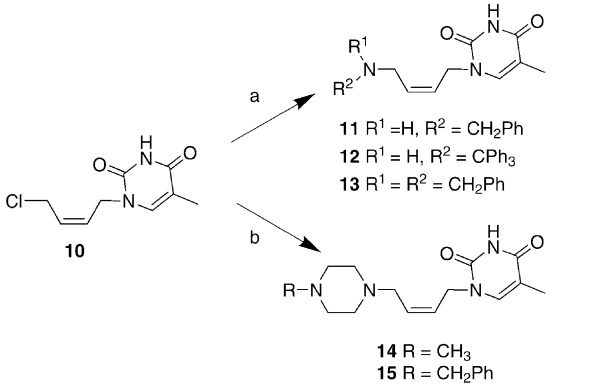
Two sets of analogues of 1-[(*Z*)-4-(triphenylmethoxy)-2-butenyl]thymine (**1**) have been prepared that conserve both the thymine base and the (*Z*)-2-butenyl spacer. In the first set, the triphenylmethoxy moiety was replaced by different amides. The synthetic approach, represented in Scheme 1, involved the coupling of (*Z*)-*N*-(4-chloro-2-butenyl)phthalimide (**2**)[14] with silylated thymine in the presence of NaI, to afford the N-1 derivative (**3**) in 76% yield. Deprotection of the phthalimide moiety with hydrazine hydrate gave the primary amine (**4**) (83% yield) that was subjected to condensation with a variety of carboxylic acids in the presence of BOP and Et_3N. This procedure afforded the carboxamides **5–9** in 75–98% yield.[15]

In the second set of compounds, the *O*-triphenylmethyl moiety in compound **1** was replaced with amines by reaction of 1-[(*Z*)-4-chloro-2-butenyl]thymine (**10**)[16] with either primary or secondary amines (Scheme 2). The chloro derivative **10** was obtained in 84% yield by reaction of (*Z*)-1,4-chloro-2-butene with in situ silylated thymine, which improved the yield with respect to the previously reported procedure.[16] This chloro derivative **10** was reacted with benzyl, trityl or dibenzyl amine as well as *N*-methyl and *N*-benzyl piperazine, in the presence of Et_3N (Scheme 2). The benzyl and trityl amine derivatives (**11** and **12**) were isolated in modest yields (34 and 33% yield, respectively) by ion-exchange chromatography (Dowex 50 WX4, H^+ form) followed by a reversed phase SPE cartridge. When a secondary amine was used as reactant, such as dibenzylamine or *N*-methyl or *N*-benzylpiperazine, the best results in the isolation of the reaction products were obtained with the aid of phthalic anhydride and ion-exchange resins. As has been described for other reaction mixtures containing non-volatile secondary amines, treatment with phthalic anhydride transforms the excess secondary amine into the corresponding 2-amidobenzoic acids, which are then captured by a basic ion exchange resin (IRA 400, OH^- form).[17] In this way, the reaction product (**13–15**) is left in solution and is easily obtained (75–85% yield) after filtration and evaporation.



Scheme 1. (a) *N,O*-Bis(trimethylsilyl)acetamide, thymine, CH_3CN, 80 °C; (b) NaI, CH_3CN, 80 °C; (76%); (c) $H_2NNH_2{\cdot}H_2O$, EtOH, rt, (83%); (d) RCO_2H, Et_3N, BOP, rt: **5** (96%); **6** (98%); **7** (76%); **8** (85%); **9** (75%).

Compounds **5–9** and **11–15** were evaluated for their inhibitory activity against phosphorylation of dThd by recombinant TK-2 and the very closely related HSV-1 TK, following described procedures.[12,18] The results are shown in Table 1. Our parent compound, the *O*-triphenylmethyl derivative **1** is also included in the Table for comparision. In the carboxamide series (**5–9**), moderate inhibition of TK-2 and HSV-1 TK was observed although all compounds showed selectivity against human TK-2. It has been previously reported that several carboxamide derivatives of 5′-amino-2′,3′-dideoxy-5-ethyluridine, in particular derivatives of xanthene carboxylic acid and phenyl acetic acid, are extremely potent HSV-1 TK inhibitors.[19] However, in our case, both the phenylacetamide **6** and the xanthene amide **7** are 3- to 8-fold more potent against TK-2 than against HSV-1 TK, which may indicate that the *Z*-butenyl spacer may be responsible for the selectivity observed here against TK-2. The diphenyl derivative **8** in the carboxamide series deserves special mention as it is equally selective and only 3-fold less potent than the parent *O*-trityl compound **1** against TK-2.



Scheme 2. (a) R^1R^2NH, Et_3N, CH_3CN, Δ; **11** (34%); **12** (33%); **13** (85%); (b) *N*-methyl or *N*-benzylpiperazine, Et_3N, CH_3CN, Δ: **14** (75%); **15** (78%).

Table 1. Inhibitory effect of **5–9** and **11–15** on the phosphorylation of [*methyl*-^{3}H]dThd by TK-2, HSV-1 TK and TK-1

Compd	IC_{50} (μM)[a]		
	TK-2	HSV-1 TK	TK-1
1	1.5±0.2	45±1	>500
5	28±1	72±37	n.d.[b]
6	23±9	198±29	n.d.
7	33±7	131±70	n.d.
8	4.1±0.5	119±37	>500
9	27±1	115±6	n.d.
11	57±22	>500	n.d.
12	2.3±0.4	26±4	>500
13	3.5±0.5	>500	>500
14	491±12	>500	n.d.
15	180±110	>500	n.d.

[a]50% Inhibitory concentration or compound concentration (expressed in μM) required to inhibit dThd phosphorylation by 50%. Data are mean value (±SD) of at least two or three independent experiments.
[b]n.d., not determined.

In the amine series, the methyl and benzyl piperazine derivatives **14** and **15** were almost devoid of inhibitory activity against both enzymes. The benzyl amine derivative **11** showed modest inhibition against TK-2 and no inhibition on the herpes enzyme whereas the inhibitory profile of the triphenylmethyl (trityl) amine **12** was very similar to that of the *O*-trityl analogue **1**. Finally, the dibenzylamine derivative **13** was a highly selective TK-2 inhibitor with an IC_{50} of 3.5±0.5 μM. To date, this compound is one of the most selective TK-2 inhibitors in our hands. For the most potent compounds, cytosolic TK-1-catalyzed phosphorylation of dThd was measured, but no inhibition was detected at 500 μM.

In an attempt to account for the observed selectivity of the *Z*-butenyl thymine derivatives against TK-2, a model of human TK-2 was built using the X-ray crystal structure of *Drosophila melanogaster* dNK (*Dm*dNK; PDB code = 1j90)[20] as a template. Since *Dm*dNK and TK-2 share 44% of sequence identity and display very similar substrate binding and inhibition preferences,[13] a common overall fold was assumed. Indeed, in our model the substrate binding clefts of both enzymes are lined with the side chains of identical residues, except for positions 76 and 114 of TK-2, which are both Leu in TK-2 and correspond to Phe80 and Met118 in *Dm*dNK. The structure of human thymidylate kinase (TMPK, PDB code = 1e2q)[21] provided the template for the highly homologous C-terminal region of TK-2 which interacts with N6 of ATP but is highly disordered in the ATP-free crystal structure of *Dm*dNK. ATP was also incorporated into our model as the reported inhibition mechanism for **1**[13] implies that ATP is bound to the enzyme–inhibitor complex. The conformation and docking orientation for ATP in our TK-2 model was based on a best-fit superimposition of the C_α traces of *Dm*dNK, HSV-1 TK (PDB code = 2vtk) and human TMPK, as this latter enzyme has been co-crystallized with a non-hydrolyzable analogue of ATP. AMBER-compatible RESP charges[22] for ATP^{4-} were calculated for the Mg^{2+}-bound conformer using Gaussian 98.[23] Standard AMBER[24] parameters were used and a charge of +2 was assigned to the magnesium ion. Docking of **1**

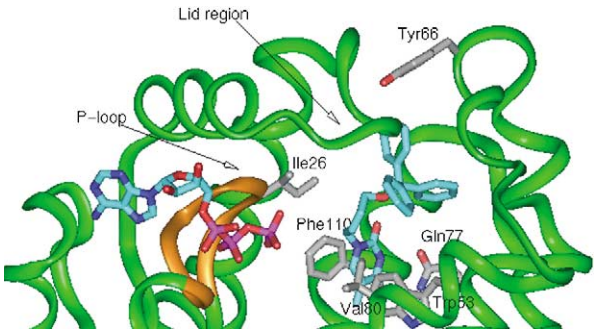


Figure 1. Detail of the modeled TK-2 in complex with inhibitor **1**. The protein Cα trace is shown as a ribbon colored in green except for the P-loop region which is colored in orange. Carbon atoms in ATP (left) and 1 (right) are colored in cyan. Carbon atoms of Gln77 and side chains of residues lining the cavity where the nucleobase binds are colored in grey.

was accomplished in several steps. First, the pyrimidine ring of the nucleobase present in the inhibitor was sandwiched between the phenyl ring of Phe110 (Phe114 in *Dm*dNK) on one side and the side chains of both Trp53 and Val80 (Trp57 and Val 84 in *Dm*dNK) on the other side. Binding of **1** was further stabilized by direct hydrogen bonds between N3 and O4 of the nucleobase and the carboxamide group of the highly conserved Gln77 (Gln81 in *Dm*dNK). This location for the thymine base is further supported by our experimental finding that **1** is a competitive inhibitor with respect to thymidine, so both compounds must share a common binding site. In this orientation, the apolar *Z*-butenyl spacer faces the hydrophobic Ile26 (Fig. 1), whose equivalent residue in HSV-1 TK is the more polar His58,[19] which may account, at least in part, for the differences in inhibition potencies against these two enzymes (Table 1). To accommodate the triphenylmethoxy substituent we found it necessary to displace the side chain of Tyr66, whose equivalent residue in other crystal structures has been shown to establish a hydrogen bond with the nucleosidic O3′. Since no such possibility exists in the present case, we searched for alternative preferred rotamers of this residue and found that the hydroxyphenyl ring of Tyr66 could be stabilized in an alternative location by a hydrogen bonding interaction with the carbonyl oxygen of Glu167. In this orientation a hydrophobic cavity lined by Ile26, Leu62, Met65, Met66, Leu76, Ile171, and Tyr175 became apparent that was further expanded using molecular dynamics simulations to lodge the trityl moiety. Similar conformational changes appear to be precluded in the case of the herpes enzyme (manuscript in preparation).

The final complex (Fig. 1) suggests that the selectivity of **1** and related inhibitors for TK-2 in preference to HSV-1 TK, in addition to the Ile26 versus His58 replacement discussed above, could be related to a motion involving a mostly helical domain (residues 62–80) that is probably kinked in TK-2 (by analogy to the *Dm*dNK structure) but is straight in HSV-1 TK (residues 97–128). That these imposed conformational changes can be made even more detrimental for inhibitor binding to HSV-1 TK relative to TK-2 is exemplified here by

compound **13**, whose dibenzylamino substituent appears to be more discriminatory against HSV-1 TK than the trityl group present in **1**. Further work is in progress to test this hypothesis.

In conclusion, two series of 1-[(*Z*)-butenyl]thymine derivatives have been prepared by replacing the triphenylmethoxy group of our lead compound by a variety of amides and amines. The synthesized compounds showed marked selectivity against TK-2 when compared to HSV-1 TK. The structural similarities between both enzymes allow them to accommodate a variety of lipophilic substituents. Due to the lack of a crystallographic structure of TK-2, at the present time we can only speculate about how these compounds may interact with their target enzyme, although it seems that the *Z*-butenyl spacer is largely responsible for the TK-2 selectivity.

Acknowledgements

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