Mitochondrial Thymidine Kinase Inhibitors

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Abstract: Mitochondrial thymidine kinase or TK-2 belongs to the family of mammalian deoxynucleoside kinases (dNKs) that catalyze the phosphorylation of deoxynucleosides to their corresponding deoxynucleoside monophosphates by γ-phosphoryl transfer of ATP. These enzymes are instrumental in the activation of deoxynucleoside analogues with biological and therapeutic properties. Moreover, dNKs are fundamental to maintain dNTPs pools for DNA synthesis and repair. TK-2 has a mitochondrial localization and is the only thymidine kinase that is physiologically active in non-proliferating and resting cells. Several recent investigations point to an important role of TK-2 in the maintenance of mitochondrial dNTPs pools. Indeed, mutations in the gene encoding TK-2 have been associated with mitochondrial DNA (mtDNA) depletion that mostly affects skeletal muscle. Moreover, TK-2 has been suggested to be implicated in mitochondrial toxicity associated to prolonged treatments with nucleoside analogues (i.e AZT for the treatment of AIDS patients). In this scenario, TK-2 inhibitors could be a useful tool to further clarify both the physiological role of TK-2 in the maintenance of mitochondrial dNTP pools, and the possible contribution of TK-2 to the mitochondrial toxicity of pyrimidine nucleoside analogues. In the present article we review the most recent literature covering different aspects of TK-2 as well as published TK-2 inhibitors, with special emphasis on acyclic nucleoside analogues that have been described by our research groups and whose prototype compound is 1-[(*Z*)-4-(triphenylmethoxy)-2-butenyl]thymine.

Keywords: Thymidine kinase, TK-2, nucleoside metabolism, anticancer drugs, antiviral drugs, mitochondrial toxicity, acyclic nucleoside analogues.

INTRODUCTION

Deoxynucleoside analogues are extensively used in the treatment of viral and cancer diseases. Most nucleoside analogues are prodrugs that require phosphorylation at the 5´-OH to generate the active nucleotide drugs, generally the triphosphates. In this activation, the rate-limiting step usually is the first phosphorylation event that is catalyzed by deoxynucleoside kinases (dNKs). Deoxynucleoside kinases catalyze the phosphorylation of deoxynucleosides to their corresponding deoxynucleoside monophosphates (dNMPs) by γ-phosphoryl transfer of ATP (or other deoxynucleoside triphosphates), and are, in general, a crucial step in the salvage of deoxynucleosides.

In mammalian cells, there are four different deoxynucleoside kinases: thymidine kinase 1 (TK-1), which is located in the cytosol; deoxycytidine kinase (dCK), which is located in the cytosol and/or the nucleus; and deoxyguanosine kinase (dGK) and thymidine kinase 2 (TK-2), both of which have a mitochondrial localization [1,2]. These four dNKs have different but overlapping substrate specificities. To illustrate how important these enzymes are in the chemotherapy of nucleoside analogues, it should be mentioned that clinically established anticancer drugs such as Cytarabine, Fludarabine and Cladribine, as well as the more recently developed Gemcitabine and Troxacitabine, require activation by dCK to be pharmacologically useful [3]. Similarly, Zalcitabine (ddC), Lamivudine (3TC) and Emtricitabine (FTC), which are used for the treatment of HIV infection, are phosphorylated by dCK [3]. The role of this enzyme is so critical that cells lacking dCK activity are highly resistant to most of these drugs. Similarly, TK-1 is crucial for the activation of AZT and d4T, which are extensively used in HIV-infected patients [3].

Besides the activation of nucleoside analogues with pharmacological properties, dNKs have a fundamental role in the salvage pathway of deoxynucleotide synthesis. This is not only important in cell proliferation, but also possibly in DNA repair. Moreover, mitochondrial DNA synthesis, which is independent of nuclear DNA synthesis, occurs during the whole cell cycle.

PHOSPHORYLATION OF THYMIDINE AND ANALOGUES: TK-1 AND TK-2

Among the above-mentioned dNKs, in mammals there are two enzymes that phosphorylate thymidine, TK-1 and TK-2. Human TK-1 was cloned in 1984 [4] and further characterized in 1987 [5]. TK-2 was not cloned till the late nineties [6,7]. Both thymidine kinases show important differences in their primary amino acid sequences, substrate specificities and levels of expression in the different phases of the cell cycle [2,8]. TK-1 has the highest level of expression in S phase cells, with very low or no activity in resting cells. By contrast, TK-2 is constitutively expressed throughout the cell cycle, and therefore it is virtually the only thymidine kinase that is physiologically active in non-proliferating and resting cells. Among natural deoxynucleosides, TK-1 phosphorylates thymidine (dThd, **1**)

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and 2´-deoxyuridine (dUrd, **2**), while TK-2 additionally phosphorylates 2´-deoxycytidine (dCyd, **3**) (Fig. **1**). Yet, the preferred substrate for both TKs is dThd. The ribonucleosides uridine (**5**) and cytidine (**6**) are not substrates for TK-1 and TK-2, and 1-(β-D-ribofuranosyl)thymine (**4**) has a very poor substrate activity for both enzymes. Substrate specificities of TK-1 and TK-2 against antiviral biologically active nucleoside analogues have been extensively studied and reviewed. [2,3,9-11]. A few examples are included herein to illustrate that there are striking differences in substrate affinities. Thus, AZT (3´-azido-3´-deoxythymidine, **7**) is an excellent substrate for TK-1 and is a poor substrate for TK-2, but in nondividing tissues TK-2 phosphorylation becomes significant. On the other hand, 1-(β-D-arabinofuranosyl)thymine (Ara-T, **8**) proved to be a much better substrate for TK-2 than for TK-1. Another interesting example is the antiherpetic agent 5-(*E*)-(2-bromovinyl)-2´-deoxyuridine (BVDU, **9**) that has no measurable affinity for TK-1 but is an excellent substrate for TK-2. Also 2´-fluoro-5-iodo-(1-β-D-arabinofuranosyl)uracil (FIAU, **10**), a nucleoside analogue active against Hepatitis B virus replication, is efficiently recognized by TK-2. Very recently, thymidine mimics incorporating isocarbostyril- or difluorophenyl residues as alternatives to the thymine base have been tested as substrates of TK-1 and TK-2 [12]. Interestingly, compounds JW1 (**11**) and JW2 (**12**) were found to be much better substrates for TK-2 than for TK-1, in particular compound JW2, which expanded the range of nucleoside analogues that can be activated by these dNKs.

Besides the activation of nucleoside analogues with pharmacological properties, TK-1 and TK-2 have a fundamental functional role in the salvage pathway of deoxynucleotide synthesis. Although the level of TK-2 is very low compared to that of TK-1 in proliferating cells (≤ 5%), in resting cells TK-2 is the predominant, if not the exclusive dThd phosphorylating enzyme. Interestingly, critical point mutations in the gene encoding TK-2 have been correlated to mitochondrial DNA (mtDNA) disorders [13], suggesting an essential role of TK-2 in mtDNA synthesis.

THYMIDINE KINASE 2 AND MITOCHONDRIAL DNA DISORDERS

Mitochondrial DNA replication occurs throughout the whole cell cycle. This means that dNTPs are constantly required for mtDNA synthesis. It has been assumed for a long time that the mitochondrial and cytosolic dNTP pools belonged to different compartments since the inner mitochondrial membrane represents an impermeable barrier to the negatively charged nucleotides [14]. However, a very recent study on the origin of the mitochondrial thymidine-5´-triphosphate (dTTP) has suggested extensive communication and a dynamic equilibrium with the cytosolic pools [15].

In non-replicative tissues, cytosolic TK-1 activity is poor, if present at all, and the mitochondrial TK-2, involved in the mitochondrial salvage pathway, will provide nucleotides for mtDNA synthesis. In this pathway the first, and often, limiting step in the phosphorylation of the pyrimidine nucleosides dThd, dCyd and dUrd, is carried out by TK-2, while the other mitochondrial dNK, that is, dGK, phosphorylates 2´-deoxyguanosine (dGuo) and 2´-deoxyadenosine (dAdo). The dNMPs resulting from this phosphorylation can be further phosphorylated to the triphosphates, or can be dephosphorylated to the corresponding nucleosides by 5´-nucleotidases. In particular,

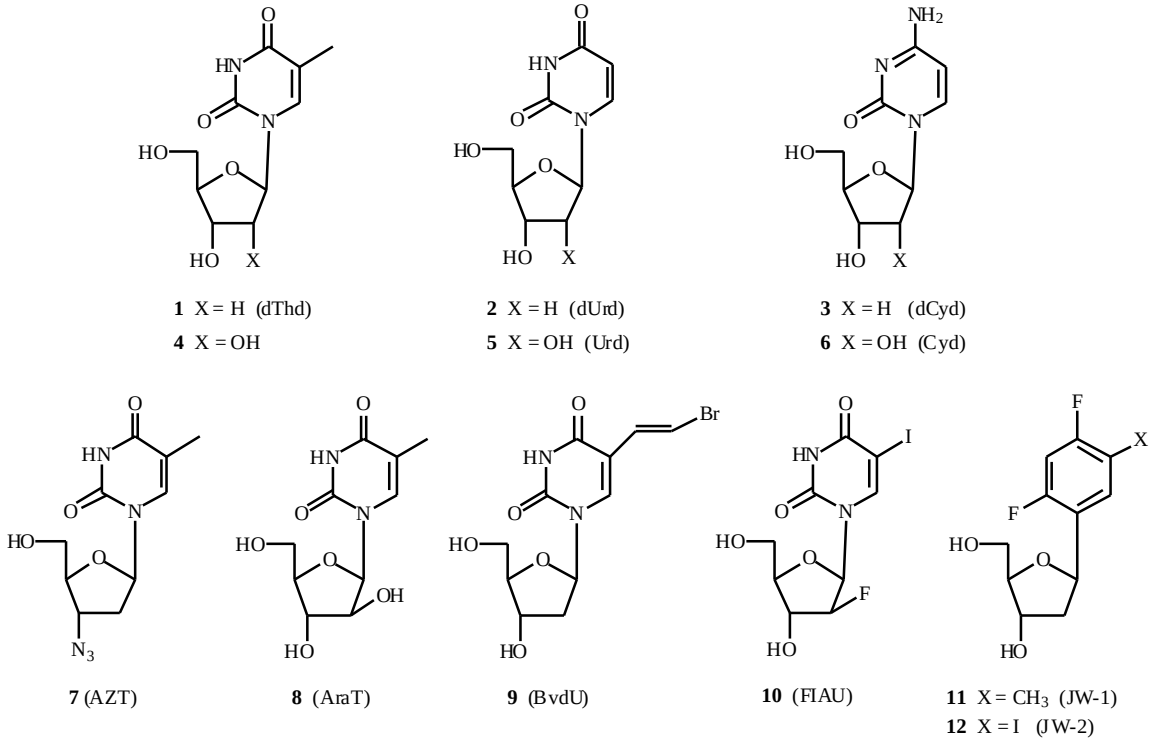


Fig. (1). Substrates that are recognized by TK-1 and/or TK-2

deoxynucleotidase-2 (dNT-2) localizes in the mitochondria and can efficiently use dTMP as a substrate, thus playing an important role in regulation of dTTP metabolism [16].

In the same way that cytosolic dNTP pools have to be highly controlled and balanced to properly replicate and repair cellular DNA [17], it is very likely that imbalances in mitochondrial dNTP pools would lead to mitochondrial disorders. Indeed, there are now a group of disorders recognised and designated as mtDNA depletion syndromes, some of which have been associated with mutations in mitochondrial dNKs (TK-2 and dGK) [18,19]. In particular, certain mutations in the gene encoding TK-2 have been associated with mtDNA depletion that mostly affects skeletal muscle [13]. Some very recent papers have tried to explain how this TK-2 deficiency has an impact on mtdNTP pools [20], and particularly affect the muscle tissues [21]. Also, site-directed mutagenesis has been used to introduce the reported mutations in the TK-2 cDNA and to measure the catalytic activity of the mutant enzymes, in an effort to get further insights on how these mutations affect dNTP pools [22,23].

Long-term treatment with antiviral nucleoside analogues such as AZT or FIAU has been associated with severe mitochondrial toxicity [24,25]. Since these nucleoside analogues are phosphorylated by TK-2, it can be assumed that their corresponding triphosphates may accumulate inside the mitochondria and may produce mtDNA depletion. Interestingly, there are analogies between the genetic mtDNA depletion syndromes and mtDNA depletion caused by nucleoside analogues [19]. Still, the mechanism(s) by which these nucleoside analogues exert their mitochondrial toxicity is not fully understood [25]. Because of its clinical impact in AIDS patients, many efforts have been directed to elucidating the mitochondrial toxicity reported in patients receiving prolonged treatment with AZT. Although AZT is not an excellent substrate for TK-2, in nonreplicating cells where TK-1 is virtually not expressed, AZT phosphorylation by TK-2 is significant. It has been suggested that the accumulation of AZT-TP could affect DNA polymerase-γ resulting in mtDNA depletion. This has been designated as the "DNA pol-γ hypothesis" [24,25]. However, more recent studies in heart mitochondria indicate that the toxicity may be due not to AZT-TP but to AZT-MP, which inhibits TK-2 and therefore disrupts the correct supply of dTTP for mtDNA replication [26]. It is also interesting to note that AZT-MP is poorly dephosphorylated by dNT-2 [27], and this could contribute to the accumulation of AZT-MP and/or AZT-TP. Moreover, it has been proposed that the deoxynucleotide carrier (DNC), which can exchange deoxynucleoside di- and triphosphates between the cytoplasm and the mitochondria, could also be involved in the mitochondrial toxicity of antiviral nucleosides [28]. In this case, phosphorylation by TK-2 should not necessarily be relevant to mitochondrial toxicity. However, a very recent study employing DNC-overexpressing cells as well as cells in which DNC expression was down-regulated by small interfering RNA, concluded that DNC does not play an important role in the mitochondrial DNA depletion associated with anti-HIV dideoxynucleoside analogues [29]. More efforts are required to clarify the contribution of these different observations to the final outcome seen in patients. The different expression of the nucleoside-metabolising enzymes and/or nucleotide transporters may eventually determine the susceptibility of patients and tissues to the mitochondrial toxicity associated with nucleoside analogues. In this regard, a very recent study has determined the activities and mRNA levels of dNKs, including TK-2, and 5´-nucleotidases, in different cell types such as adipocytes, muscle tissue and peripheral blood cells, in an attempt to correlate the activity of these enzymes with the mitochondrial toxicity associated with nucleoside analogues [30].

DIFFERENCES AND SIMILARITIES WITH OTHER DNKS

As mentioned above, there are important differences between human kinases TK-1 and TK-2. Based on their amino acid sequences, TK-1 forms a group on its own, while TK-2 belongs to a larger group of nucleoside kinases with strong similarities to other human dNKs like dCK and dGK, and particularly to the multisubstrate deoxynucleoside kinase (dNK) of the fruitfly *Drosophila melanogaster* (Dm dNK) [2]. Dm dNK was cloned by two independent groups [31,32]. It has a broad specificity and phosphorylates all four natural deoxynucleosides, although with different efficiencies, as well as a large number of deoxynucleoside analogues. Still, the preferred substrate is dThd. This broad nucleoside specificity together with its high catalytic rate makes Dm dNK a particularly attractive enzyme for biotechnological applications as well as an alternative to HSV-1 TK which is employed in suicide cancer gene therapy [33,34].

The amino acid sequence identity between human TK-2 and Dm dNK is about 40% and there are no long insertions or deletions. In 2001, the structure of Dm dNK was solved in complex with dCyd [35]. It is interesting to note that Dm dNK only shares a 10% amino acid sequence identity with HSV-1 TK, but the core structure of these two enzymes has a similar overall fold. The main differences are an extra amino acid domain in HSV-1 TK (between residues 197 and 198 in Dm dNK) and an extra C-terminal antiparallel β-strand and one α-helix compared to Dm dNK [35]. There are three major regions in the three-dimensional structures common to all these dNKs: the P-loop, the lid region and the substrate cleft. The P-loop is a Gly-rich loop that accommodates the γ-phosphate of ATP. The lid region is a Lys-Arg-rich region that functions as a cover for the active site cleft. Finally, the substrate cleft has several conserved amino acids, even in the larger cleft of HSV-1 TK. In this cleft there is a highly conserved Gln residue that makes two hydrogen bonds to the nucleosidic base. In the Dm dNK/dCyd complex, the amino group of Gln81 forms a hydrogen bond to N3 of cytosine, and the carbonyl of Gln81 forms an hydrogen bond to the 4-NH_2 of cytosine [35]. In a more recent complex between Dm dNK and dThd [36], Gln81 is differently oriented so that the carbonyl of Gln81 forms a hydrogen bond to NH-3 of thymine and the amino group forms hydrogen bond to 4-CO of thymine, as represented in (Fig. **2**). In this second complex, the methyl group of thymine has displaced two water molecules from the binding site compared to the dCyd complex, so that there are now hydrophobic interactions with Arg105, Val84, Met88 and Ala110 [36]. A π-π interaction is

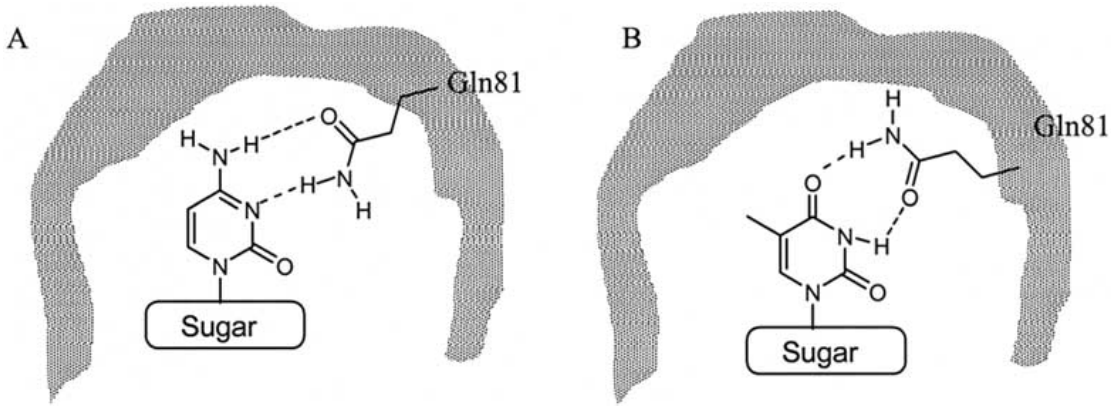


Fig. (2). Representation of the interaction of the highly conserved Gln81at the substrate binding site of Dm dNK with cytosine (A) or thymine (B). Hydrogen bonds are shown as dotted lines.

also observed between the base and the phenyl ring of Phe114. A detailed view of the dThd binding site in Dm dNK is shown in (Fig. **3a**). Concerning the deoxyribose moiety, the 3´-OH forms hydrogen bonds to Tyr70 and Glu172. Another important interaction is between the 5´-OH of the nucleoside and Glu52 and Arg169. According to previous studies on HSV-1 TK [37], the Glu52 carboxylate plays a key role in the mechanism of phosphorylation acting as the base that deprotonates the 5´-OH, a step that is possibly facilitated by the close Arg residues.

Among dNKs, feedback inhibition by the end-product deoxynucleoside triphosphates is quite common, the most potent inhibitors being the triphosphates of the preferred substrates. This fine regulation of the salvage pathway has significant physiological importance for the maintenance of balanced dNTP pools. For Dm dNK, dTTP is a strong competitive inhibitor with respect to ATP. The structure of the Dm dNK-dTTP complex has recently been solved [36], and the most relevant interactions of dTTP with Dm dNK are shown in (Fig. **3b**). dTTP was found to be a bisubstrate inhibitor with its nucleoside part interacting in the nucleoside binding site, while the phosphate groups partially occupy the phosphate donor site, so that the γ-phosphate is bound to the P-loop main chain and Arg167, the β-phosphate to Arg167 and Arg169, and the α-phosphate to Lys33 and Arg105. Oxygens of the γ- and β-phosphates coordinate a magnesium ion. When compared to the Dm dNK-dThd complex the differences in the P-loop are generally less than 1Å. When both complexes are compared, the major conformational changes are seen in the lid region, as shown in (Fig. **3a** and Fig. **3b**), and also affect Glu52 and Arg105, two residues that could be involved in the catalysis mechanism. In the Dm dNK-dTTP complex (Fig. **3b**), the carboxylate of Glu52 is part of the coordination sphere of the magnesium ion whereas the guanidinium of Arg169 fixates the β-phosphate of dTTP. It has been proposed that this binding mode as a bisubstrate inhibitor is in accordance with the specific and effective feedback inhibition that dTTP exerts on Dm dNK.

So far, no crystallographic determination of TK-2 has been reported. However, the 40% identity in amino acid sequence between this enzyme and Dm dNK has allowed the construction of three-dimensional models of TK-2 using the Dm dNK structure as a template [2,35,38,39]. In the substrate cleft of these models there are only two differences with respect to Dm dNK: (i) the position occupied by Met118 in Dm dNK, which is ~8Å from the base of the

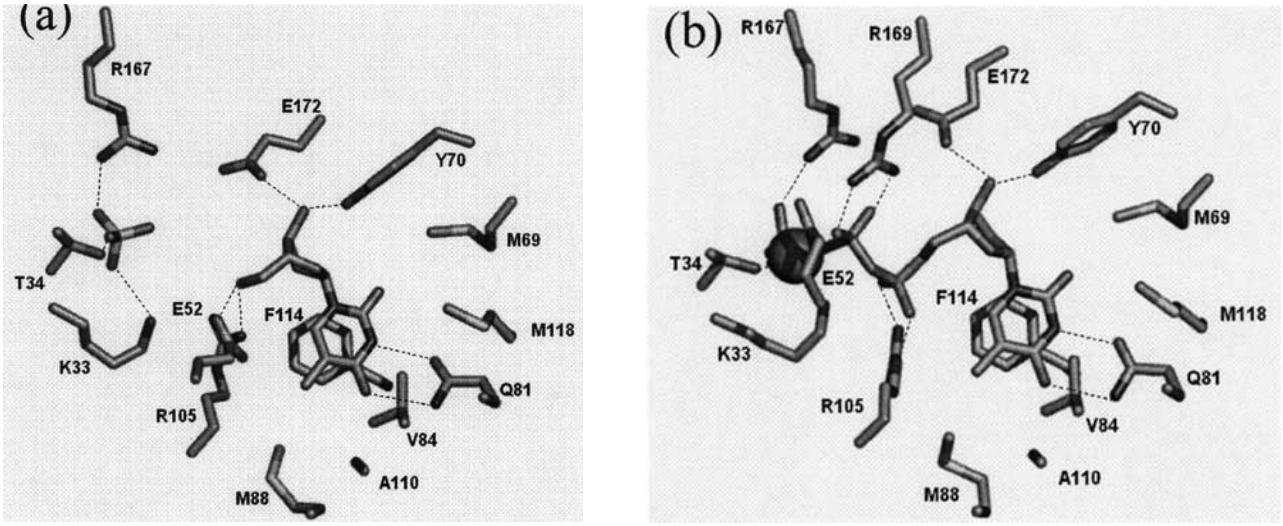


Fig. (3). Details of the binding site interactions between Dm dNK and either dThd (a) or dTTP (b), as found in Protein Data Bank entries 1ot3 and 1oe0 [36]. Hydrogen bonds are shown as broken lines. The sphere in (b) represents the magnesium ion.

alternative substrate dCyd, is Leu116 in TK-2, and (ii) the residue positionally equivalent to Phe80, which is at van der Waals contact distance with the nucleoside base in the Dm dNK complex, is a Leu in TK-2. Both substitutions result in a slightly enlarged substrate cleft in TK-2 relative to Dm dNK.

Barroso *et al*. [39] have reported a three-dimensional model of TK-2 that was obtained using the protein structure homology-modeling server SWISS-MODEL. The substrates dThd and dCyd, the phosphate donor ATP and the feedback inhibitors dTTP and dCTP were docked in the active site of the modelled TK-2 using DOCK 4.0.1. All the ligands were located at the expected binding site by analogy with the available complexes Dm dNK-dThd, Dm dNK-dCyd and Dm dNK-dTTP. The modelled binding mode of dTTP and dCTP as bisubstrate inhibitors helped to explain the high affinity and specificity of these feedback inhibitors, as previously described for the Dm dNK-dTTP complex. Interestingly, these authors reported that in the purification of recombinant human TK-2 obtained after expression in *E. coli*, deoxynucleoside triphosphates like dTTP, dCTP or dATP remained tightly bound to the enzyme. The partial occupancy of the substrate binding site by these feedback inhibitors could affect enzyme kinetics performed with these recombinant enzymes [39]. Indeed, in a very recent study, these authors reported on a dNTP-free TK-2 system obtained by incubation of the enzyme with high concentrations of dThd during its purification [40]. The catalytic activity of the dNTP-free dimeric and tetrameric TK-2 increased 16-18 fold with dThd compared to previously characterized enzyme [39]. Also, by employing different techniques such as intrinsic trytophan fluorescence, far-UV circular dichroism and limited proteolysis, it was suggested that TK-2 has a remarkable ability to adopt different conformations on binding different ligands, be they substrates or inhibitors.

A model of TK-2 based on the available Dm dNK structure has also been constructed to explain the binding mode of a series of TK-2 inhibitors [38]. The ATP-binding region that is quite disordered in all the available dNKs complexes was also incorporated into this model by using the structure of human thymidylate kinase as a template [38]. More details of this particular model will be given in a later section.

TK-2 INHIBITORS

From the above described features, we may conclude that TK-2 is implicated in the phosphorylation of pyrimidine nucleosides needed for mtDNA synthesis and could also be involved in the mitochondrial toxicity associated to antiviral nucleoside analogues. However, there are still many open questions related to the real contribution of TK-2 to these issues. TK-2 inhibitors could be valuable tools to unravel the role of TK-2 in mitochondrial dNTP pools and homeostasis, and may also help to clarify the contribution of TK-2-catalyzed phosphorylation of antiviral drugs to the mitochondrial toxicity of these agents. Such inhibitors could also be helpful to investigate the activity of TK-1 and TK-2 in different cell types.

The direct testing of TK-2 inhibitors in cell culture is quite difficult. The mitochondrial localization of TK-2, its low level of expression and the presence of the more active and abundant TK-1 in the cytosol, preclude the direct testing in cell culture. Attempts have been made to construct a cell system where TK-2 is expressed and this has been achieved in *Escherichia coli* [41]. However, the differences in nucleoside metabolism between *E. coli* and mammalian cells, as well as the different transmembrane transport in bacteria, limit the conclusions that can be derived from this system. Therefore, for the time being, the most reliable data come from the direct evaluation of the ligands against the recombinant enzyme in a cell-free assay. Based on the sequence similarity between TK-2 and Dm dNK, and to a lesser extent, HSV-1 TK, the testing of compounds has been performed in many cases against the three enzymes. The information gained from these experiments can also be relevant to further explore similarities and differences among this group of enzymes. For the most active compounds, testing has also been performed against cytosolic TK-1 to ensure the selectivity among human thymidine kinases.

Ara-, Ribo- and 2´-Deoxyribo- Nucleoside Analogues

In 1999, several nucleoside analogues modified at the sugar moiety, including *O*-alkyl analogues, were tested as substrates or inhibitors against TK-1 and TK-2 [42]. It was found that 3´-*O*-methyl- and 3´-*O*-ethyl-2´-deoxyuridine (**13** and **14**) (Fig. **4**) were between 20 and 100-fold more inhibitory to TK-2 than to TK-1, with K_i values of 15 and 10 μM, respectively. Even more pronounced was the inhibitory effect of 3´-hexanoylamino-3´-deoxythymidine (**15**), with a more than 1000-fold higher affinity for TK-2 and a K_i value of 0.15 μM.

Another interesting finding concerning TK-2 inhibition by nucleoside analogues was reported in 2000, where different ribofuranosylnucleosides were found to be potent and selective TK-2 inhibitors [43]. 5-(*E*)-(2-bromovinyl)uridine (**16**) markedly inhibited dThd phosphorylation by TK-2, while the 2´-deoxy analogue (BvdU, **9**) is an excellent alternative substrate for this enzyme. Also, the 3´-substituted nucleoside 3´-spiro-(4´´-amino-1´´,2´´-oxathiole-2´´,2´´-dioxide)-5-methyluridine (**17**) (Fig. **4**) inhibited dThd phosphorylation by TK-2 with an IC_{50} of 4.6 μM. Despite the presence of a free 5´-OH, both compounds were proven not to be substrates for TK-2 as revealed by phosphate transfer from [γ-^{32}P]ATP experiments, and therefore could be considered as rather specific TK-2 enzyme inhibitors.

As mentioned above, Ara-T is a good substrate of TK-2. However, introduction of long chain acyl substituents at the 2´-OH, as in the decanoyl and dodecanoyl esters (compounds **18** and **19**, respectively) (Fig. **4**), enhanced the affinity for TK-2 by 10-fold [44]. By shortening the 2´-*O*-acyl chain to a pentanoyl moiety, or by replacing the acyl chain by an alkyl moiety, an important decrease in the inhibitory activity was observed. Since BvAraU is also a substrate of TK-2, acyl chains were also incorporated at the 2´-OH of BvAraU. Introduction of a octanoyl or decanoyl chain (compounds **20** and **21**) increased the inhibitory potency against TK-2-catalysed dThd phosphorylation with IC_{50} values of ~ 6 μM [44]. Detailed enzyme kinetics analyses performed with compound **21** showed purely competitive inhibition with respect to dThd as the natural substrate (K_m value for dThd

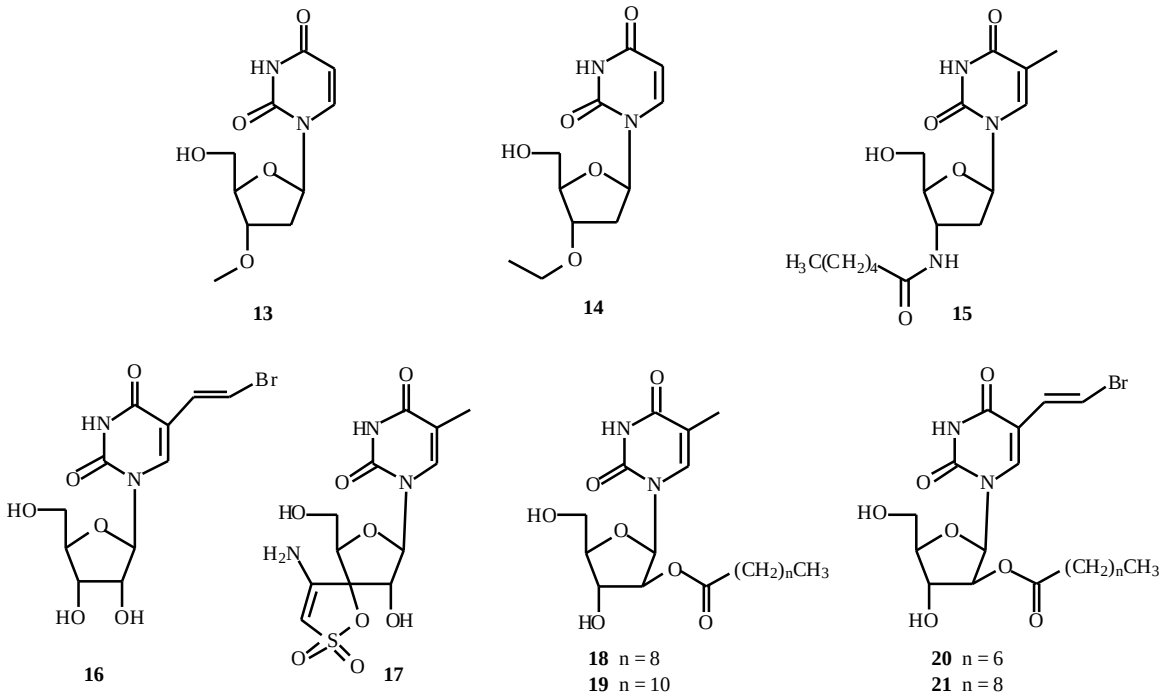
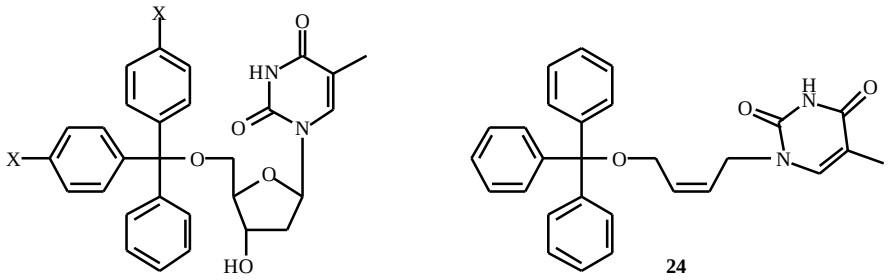


Fig. (4). Ara-, Ribo- and 2-deoxyribo-nucleoside analogues as TK-2 inhibitors.

was 1.2 μM; K_i value for compound **21** was 2.3 μM) [45]. Compound **21** was only poorly converted to the corresponding 5´-monophosphate. Therefore, it is interesting to note how introduction of these acyl moieties shifted the nucleosides from substrates to inhibitors, and furthermore increased the affinity for the target enzyme TK-2. Unfortunately, these 2´-*O*-acyl derivatives are unstable in cell culture and are readily converted to the parent nucleoside [45]. Therefore, they do not qualify as tools to study TK-2 in intact cells.

Acyclic Nucleoside Analogues

Our common project on the identification of TK-2 inhibitors started with the testing of nucleoside analogues previously synthesized in our laboratories as well as commercial samples. Among the latter, it was found that 5´-*O*-(4,4´-dimethoxytrityl)thymidine (**22**) (Fig. **5**) showed inhibitory activity against HSV-1 TK-catalyzed dThd phosphorylation with an IC_{50} = 14 μM, while inhibition of TK-2 could only be achieved at a higher concentration (IC_{50} = 468 μM). Replacement of the strongly acid labile dimethoxytrityl group by the more stable trityl analogue resulted in 5´-*O*-Tr-thymidine (**23**) that was a markedly more potent inhibitor against both enzymes, particularly against TK-2 (IC_{50} = 33 μM). In both compounds, the presence of the (dimethoxy)trityl group at the 5´-position of thymidine converted the thymidine substrate into an inhibitor since both compounds lack a free available 5´-OH group susceptible of being phosphorylated by TK-2. On the basis of this observation, we hypothesized that the role of the 2´-deoxyribose moiety in compounds **22** and **23** was to locate the thymine base and the trityl substituents in the right way to interact with the enzyme, which suggested to us that the sugar moiety could be replaced by acyclic spacers. Acyclic nucleoside analogues offer several advantages compared to natural nucleosides such as ease of synthesis, higher chemical and metabolic stability, and major discrimination *versus* other nucleoside processing enzymes (i.e. TK-1). Therefore, we envisioned the synthesis and evaluation of a series of acyclic nucleoside analogues of general formula [Thy]-spacer-[trityl] [46]. The initially synthesized compounds and their inhibitory activity against dThd phosphorylation catalyzed by TK-2, HSV-1 TK and Dm dNK are summarized in Table **1**. The results obtained with 5´-*O*-Tr-dThd (**23**) are also included in Table **1** for comparative purposes. Interestingly, replacement of the 2-deoxyribose moiety by acyclic spacers did not only retain the inhibitory ability but also resulted in enhanced inhibitory potency against TK-2. In particular, compound **24** (Fig. **5**) possessing a (*Z*)-butenyl spacer displayed an increase of 20-fold in inhibitory activity against TK-2 while showing a 6-fold decrease in inhibitory potency against HSV-1 TK. Other compounds with rigid spacers such as those present in compounds **25** and **26** were less inhibitory to TK-2, although they demonstrated good inhibition of HSV-1 TK. By increasing the conformational freedom of the spacer (compounds **28**-**30**), the inhibitory potency against TK-2 was maintained (around 3 μM), but the compounds also became more active against HSV-1 TK. As expected on the basis of the sequence homology between these enzymes, the inhibitory values against TK-2 and Dm DNK are more similar than those comparing the inhibitory potency against HSV-1 TK. Compounds **24** or **30** were not inhibitory to TK-1-catalyzed phosphorylation of dThd, as desired. From these data, it was concluded that the (*Z*)-butenyl spacer affords the appropriate core to further explore substitutions at both the thymine base and the trityl moiety of the parent molecule.

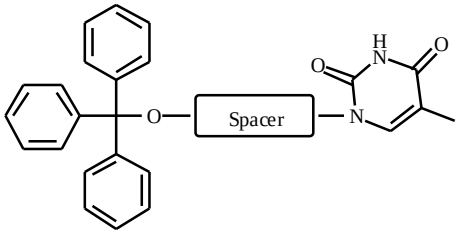


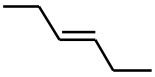
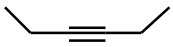
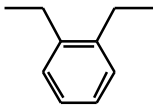
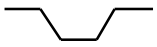
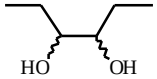
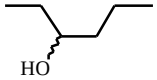
22 X = OCH_3 (DMTr-dThd)
23 X = H (Tr-dThd)

24

Fig. (5). DMTr-dThd, Tr-dThd and 1-[(*Z*)-4-(triphenylmethoxy)-2-butenyl]thymine.

Table 1. Inhibitory Effect of Acyclic Nucleoside Analogues on the Phosphorylation of [*methyl*-^{3}H]dThd by TK-2, HSV-1 TK, Dm dNK and TK-1



Compound	Spacer	IC_{50} (μM)[a]			
		TK-2	HSV-1 TK	Dm dNK	TK-1
23	5´-O-Tr-dThd	33 ± 20	7.8 ± 0.3	12 ± 1	>100
24		1.5 ± 0.2	45 ± 1	3.3 ± 0.9	>100
25		25 ± 13	3.0 ± 0.0	81 ± 36	>100
26		404 ± 136	3.1 ± 0.2	47 ± 2	n.d.[b]
27		>500	>500	>500	n.d.
28		3.3 ± 1.2	10 ± 1	19 ± 1	n.d.
29		17 ± 1	13 ± 1	22 ± 12	n.d.
30		3.6 ± 0.4	1.2 ± 0.7	12 ± 4	>100

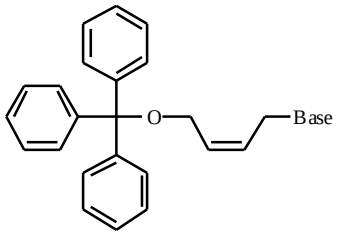
[a] 50% Inhibitory concentration or compound concentration (expressed in μM) required to inhibit dThd phosphorylation by 50%. [b] n.d.: not determined

Modifications at the base level [46] (Table **2**) including replacement of the thymine base by guanine (**31**) or 5-methylcytosine (**32**) abolished the inhibitory ability. Introduction of a methyl group at N-3 of thymine (**33**) also led to an inactive compound. Substitution of the thymine base by the close analogue 5-iodouracil (**34**) gave an IC_{50} value against TK-2 similar to that of the thymine compound (**24**), and showed a 10-fold lower activity against HSV-1 TK. Instead, the 5-ethyluracil derivative (**35**) was one order of magnitude less active against TK-2 and completely lost its inhibitory potential against HSV-1 TK. Similarly, the 5,6-dihydrothymine derivative (**36**) showed an IC_{50} of 10 μM against TK-2 and no activity against HSV-1 TK. Interestingly, the 5-(*E*)-(2-bromovinyl)uracil derivative (**37**) was equipotent with the thymine analogue against TK-2 (IC_{50} = 1.3 ± 1.1 μM), but showed no inhibition of HSV-1 TK or Dm dNK, therefore becoming the most selective compound against TK-2 in this series [47]. Moreover, compound **37** showed no inhibition of TK-1.

Different alternatives to the *O*-trityl group have been explored at the terminal site. Initially, we focused on the acyl derivatives (**38**-**43**) that are represented in Table **3** [46]. These compounds were, in general, inactive against HSV-1 TK and one order of magnitude less active against TK-2 than the *O*-trityl prototype (**24**). The most potent inhibition was seen with the diphenylacetyl derivative (**42**) with an IC_{50} against TK-2 of 4.6 μM. It should be pointed out that the unsubstituted compound (**44**) was almost inactive against the three enzymes, stressing the importance of a lipophilic moiety at the distal site.

A further step consisted in synthesizing a series of compounds analogous to **24** in which the *O*-trityl moiety was replaced by aromatic carboxamides [38]. Examples of such molecules are shown in Table **4**. In the carboxamide series, moderate inhibition of TK-2 was observed with IC_{50} values around 20-30 μM, with the exception of the diphenyl derivative **50** that was only 3-fold less potent than the *O*-trityl derivative (**24**) against TK-2. Inhibition of Dm dNK was similar to that observed for TK-2 while the compounds were less inhibitory to HSV-1 TK. Interestingly, it has been previously reported that carboxamide derivatives of 5´-amino-2´,3´-dideoxy-5-ethyluridine, and in particular, derivatives of xanthene carboxylic acid and phenylacetic acid, are extremely potent HSV-1 TK inhibitors [48]. In this acyclic series both the xanthene amide **47** and the phenylacetamide derivative **49** were moderately active against TK-2, but still 3- to 8-fold more potent against TK-2 than against HSV-1 TK. These observations may indicate

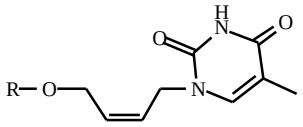
Table 2. Inhibitory Effect of Base-Modified Acyclic Nucleosides Analogues on the Phosphorylation of [*methyl*-^{3}H]dThd by TK-2, HSV-1 TK, Dm dNK and TK-1



Compound	Base	IC_{50} (μM)[a]			
		TK-2	HSV-1 TK	Dm dNK	TK-1
24	Thymin-1-yl	1.5 ± 0.2	45 ± 1	3.3 ± 0.9	>100
31	Guanin-9-yl	>500	>500	>500	n.d.[b]
32	5-Methylcytosin-1-yl	484 ± 11	>500	75 ± 36	n.d.
33	3-*N*-Methylthymin-1-yl	>500	>500	>500	n.d.
34	5-Iodouracil-1-yl	4.6 ± 0.4	48 ± 14	1.6 ± 0.7	n.d.
35	5-Ethyluracil-1-yl	20 ± 7	>500	5.3 ± 1.6	n.d.
36	5,6-Dihydrothymin-1-yl	10 ± 2	>500	15 ± 4	n.d.
37	5-(*E*)-(2-Bromovinyl) uracil-1-yl	1.3 ± 1.1	>500	>500	>100

[a] 50% Inhibitory concentration or compound concentration (expressed in μM) required to inhibit dThd phosphorylation by 50%. [b] n.d.: not determined

Table 3. Inhibitory Effect of Acyl-Derivative Analogues of 24 on the Phosphorylation of [*methyl*-^{3}H]dThd by TK-2, HSV-1 TK, Dm dNK and TK-1



Compound	R	IC_{50} (μM)[a]			
		TK-2	HSV-1 TK	Dm dNK	TK-1
38	CO—*t*Bu	40 ± 11	>500	37 ± 7	n.d [b]
39	CO—CH_2—*t*Bu	28 ± 5	>500	33 ± 3	n.d
40	CO—$(CH_2)_5$—CH_3	11 ± 7	420 ± 8	30 ± 8	n.d
41	CO—$(CH_2)_{10}$—CH_3	19 ± 12	>500	275 ± 120	n.d
42	CO—$CHPh_2$	4.6 ± 0.5	281 ± 48	16 ± 5	n.d.
43	CO—Ph	30 ± 4	>500	33 ± 3	n.d.
44	H	173 ± 68	224 ± 20	306 ± 85	n.d.
45	CH_2—Ph	22 ± 4	>500	24 ± 1	n.d.

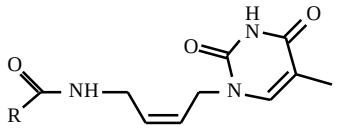
[a] 50% Inhibitory concentration or compound concentration (expressed in μM) required to inhibit dThd phosphorylation by 50%. [b] n.d.: not determined

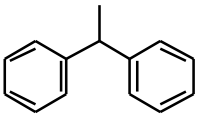
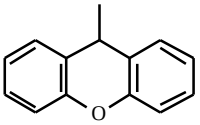
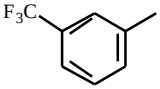
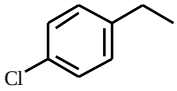
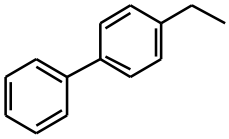
that the *Z*-butenyl spacer present in compounds **47** and **49** could be responsible for the selectivity observed against TK-2.

Another series of compounds include the replacement of the *O*-trityl moiety of the prototype compound **24** by several secondary and tertiary amines [38], whose formulae are included in Table **5**. Two compounds in this series deserve a special comment. The tritylamine derivative (**53**) shows an inhibitory potency against the three enzymes which was very similar to that of the *O*-trityl derivative (**24**). Secondly, the dibenzylamine derivative (**52**) is only 3-fold less potent against TK-2 than the *O*-trityl analogue, but showed no inhibition of HSV-1 TK at 500 μM. All these data stress the importance of an aromatic moiety, preferentially diphenylmethyl, triphenylmethyl or dibenzyl at the distal part of these acyclic nucleoside analogues for efficient TK-2 inhibition.

Most of the substitutions described up to this point have been performed with a (*Z*)-butenyl spacer connecting the thymine base and the distal substituent, mainly because incorporation of this bridging entity has resulted in certain selectivity against TK-2 and much decreased activity against HSV-1 TK, as will be explained in a later section. However, it is important to remember that the butyl analogue (**28**) or the 3-hydroxybutyl analogue (**30**) were only slightly less potent than the (*Z*)-butenyl derivative (**24**) in their inhibition of TK-2 (Table **1**). On the other hand, further evaluation of our own collection of thymine derivatives against TK-2 has shown that 1-(ω-cyanoalkyl)thymines, and, in particular, 1-(8-cyanooctyl)thymine showed significant inhibition of TK-2 (IC_{50}= 5.9 μM). SAR studies with the different series of acyclic nucleoside analogues that we have just described allowed us to conclude that the *O*-trityl moiety of the prototype compound **24** could be replaced by a dibenzylamine (**52**) or a triphenylmethylamine (**53**) without significantly affecting TK-2 inhibition. On the basis of these observations, we have performed the solution-phase parallel synthesis of a small library of thymine-derived carboxamides functionalized at the distal part with cyano and/or phenyl rings [49]. The structures of these compounds and their evaluation against TK-2 and related enzymes are included in Table **6**. To our surprise, compounds that carry a cyano group, (compounds **56**-**58**, **61**-**63**, **66**-**68**), independent of the length and/or the nature of the spacer, were virtually inactive, which is in contrast with the significant TK-2 inhibition of the parent 1-(8-cyanooctyl)thymine. When analyzing the compounds that carry one or more phenyl rings in the tail (compounds **58**-**60**, **63**-**65**, **68**-**70**), it is clear that increasing the number of phenyl rings also increased the potency of inhibition. In particular, compound **70** is the most inhibitory against TK-2 among this series of thymine-derived carboxamides, but it is approximately one order of magnitude less active than the parent compounds (**24**, **52** or **53**). However, it is interesting to mention that compound **70** is 6-fold more active against the HSV-1 TK enzyme than against TK-2.

Table 4. Inhibitory Effect of Carboxamide Derivative Analogues of 24 on the Phosphorylation of [*methyl*-^{3}H]dThd by TK-2, HSV-1 TK, Dm dNK and TK-1



Compound	R	IC_{50} (μM)[a]			
		TK-2	HSV-1 TK	Dm dNK	TK-1
46		27 ± 1	115 ± 6	18 ± 2	n.d.[b]
47		33 ± 7	131 ± 70	22 ± 2	n.d.
48		28 ± 1	72 ± 37	29 ± 7	n.d.
49		23 ± 9	198 ± 29	41 ± 9	n.d.
50		4.1 ± 0.5	119 ± 37	7 ± 3	n.d.

[a] 50% Inhibitory concentration or compound concentration (expressed in μM) required to inhibit dThd phosphorylation by 50%. [b] n.d.: not determined

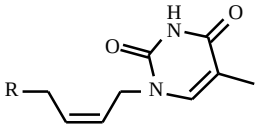
Enzyme Kinetic Analysis of Acyclic Nucleoside Analogues Against TK-2

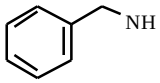
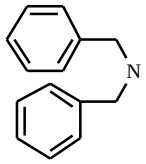
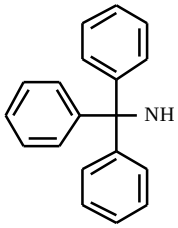
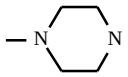
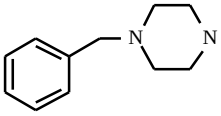
Detailed kinetic analyses have been performed with the prototype compound **24** and with the highly selective TK-2 inhibitor (*E*)-5-(2-bromovinyl)uracil derivative (**37**) [47]. Both compounds gave rise to reversible inhibition of TK-2. When tested against dThd as a variable substrate, compounds **24** and **37** showed a purely competitive inhibition of TK-2, with K_i values of 0.50 and 0.78 μM, respectively, resulting in K_i/K_m ratios lower than 1. This means that the affinity of the TK-2 inhibitors for the enzyme is at least as good as, if not higher than, that of the natural substrate dThd. If the concentrations of inhibitor that can be attained in the mitochondria of intact cells are higher than the substrate concentrations, inhibition of TK-2 may become physiologically very relevant. Interestingly, when both compounds were tested against variable concentrations of ATP, the co-substrate of the TK-2-catalyzed reaction, they behaved as uncompetitive inhibitors, with K_i values of 17 and 24 μM. Since the K_m value for ATP is 23 μM, the K_i/K_m ratios were close to 1 (0.81 and 1.1 for **24** and **37**, respectively). The uncompetitive inhibition against ATP indicates that compounds such as **24** and **37** do not bind to the free enzyme; instead, they would only bind to TK-2 after the binding of ATP as a co-substrate [47]. On the basis of their competitive inhibition with dThd, it can be concluded that compounds **24** and **37** bind at the thymidine-binding site of the enzyme, but they can only do so once ATP is already bound to the enzyme. This suggests that ATP binding may afford a conformational change at the substrate binding-site that allows binding of the inhibitor.

Molecular Modelling of TK-2 in Complex with Inhibitor 24

As previously mentioned, although there are no direct structural determinations of TK-2 currently available, the relatively high sequence homology between TK-2 and *Dm* dNK [2] has been used to create models of TK-2 [35,38,39]

Table 5. Inhibitory Effect of Amine-Derivative Analogues of 24 on the Phosphorylation of [*methyl*-^{3}H]dThd by TK-2, HSV-1 TK, Dm dNK and TK-1

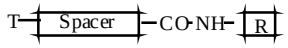


Compound	R	IC_{50} (μM)[a]			
		TK-2	HSV-1 TK	Dm dNK	TK-1
51		57 ± 22	>500	41 ± 4	n.d[b]
52		3.5 ± 0.5	>500	16 ± 11	>500
53		2.3 ± 0.4	26 ± 4	4.4 ± 0.4	>500
54		491 ± 12	>500	>500	n.d.
55		180 ± 110	>500	34 ± 2	n.d.

[a] 50% Inhibitory concentration or compound concentration (expressed in μM) required to inhibit dThd phosphorylation by 50%. [b] n.d.: not determined

Fig. (6). Sequence alignment of selected parts (P-loop, and helical and lid regions) of the deoxyribonucleoside kinases that are discussed in the text. *D. melanogaster* dNK and HSV-1 TK are structurally aligned using molecular graphics. Residue numbers refer to the primary sequence of each protein.

Table 6. Inhibitory Effect of Thymine-Derived Carboxamides on the Phosphorylation of [*methyl*-^{3}H]dThd by TK-2, HSV-1 TK, Dm dNK and TK-1



Comp	Spacer	R	IC_{50} (μM)[a]		
			TK-2	HSV-1 TK	Dm dNK
56	$(CH_2)_3$	CH_2CN	>500	>500	>500
57	$(CH_2)_3$	CH_2CH_2CN	460 ± 56	>500	>500
58	$(CH_2)_3$	CH(Ph)CN	208 ± 32	495 ± 7	218 ± 91
59	$(CH_2)_3$	$CHPh_2$	238 ± 90	>500	346 ± 41
60	$(CH_2)_3$	CPh_3	44 ± 7	175 ± 81	21 ± 0
61	$(CH_2)_3CONHCH_2$	CH_2CN	>500	>500	>500
62	$(CH_2)_3CONHCH_2$	CH_2CH_2CN	>500	>500	>500
63	$(CH_2)_3CONHCH_2$	CH(Ph)CN	318 ± 18	>500	375 ± 14
64	$(CH_2)_3CONHCH_2$	$CHPh_2$	287 ± 35	>500	292 ± 112
65	$(CH_2)_3CONHCH_2$	CPh_3	194 ± 48	191 ± 132	84 ± 33
66	$(CH_2)_5$	CH_2CN	268 ± 46	>500	475 ± 35
67	$(CH_2)_5$	CH_2CH_2CN	353 ± 56	>500	>500
68	$(CH_2)_5$	CH(Ph)CN	75 ± 36	275 ± 8	141 ± 110
69	$(CH_2)_5$	$CHPh_2$	38 ± 3	43 ± 2	103 ± 59
70	$(CH_2)_5$	CPh_3	19 ± 2	3.4 ± 0.4	19 ± 13
71	(*E*)-CH_2-CH=CH-CH_2CH_2	CH(Ph)CN	371 ± 36	>500	276 ± 25
72	(*E*)-CH_2-CH=CH-CH_2CH_2	$CHPh_2$	265 ± 44	≥500	232 ± 44

[a] 50% Inhibitory concentration or compound concentration (expressed in μM) required to inhibit dThd phosphorylation by 50%

based on the three dimensional structure reported for Dm dNK [35,36] and the Swiss-Model protein modeller server [50]. A primary sequence alignment of selected key regions of HSV-1 TK, Dm dNK, and TK-2 based on a structural alignment of the former two proteins is shown in (Fig. **6**).

Kinetic analyses of the inhibition of the acyclic nucleosides **24** and **37** have shown that they must interact at the thymidine binding site (competitive inhibition with dThd) but only when ATP is already bound (uncompetitive inhibition with ATP). Therefore, in order to construct a reliable model of the interaction of **24** and analogues with TK-2, it was mandatory to include ATP in the TK-2 structure prior to performing the docking experiments [38]. Since the ATP binding region in the crystal structure of *Dm* dNK (PDB code = 1j90) is highly disordered [35], the structure of human thymidylate kinase (TMPK, PDB code = 1e2q) was used to provide the template for the highly homologous C-terminal region of TK-2 which interacts with N6 of ATP. The conformation and docking orientation that were used for ATP in this model were based on a best-fit superimposition of the C_a 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 [51]. Due to the volume of the trityl substituent, the docking of **24** 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 (Fig. **7**). Binding of **24** 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 in agreement with our experimental findings that compound **24** is a competitive inhibitor with respect to thymidine [47]. In this orientation, the apolar *Z*-butenyl spacer faces the hydrophobic Ile26 (Fig. **7**), whose equivalent residue in HSV-1 TK is the more polar His58 (Fig. **6**). The different nature of this amino acid in TK-2 and HSV-1 TK may account, at least in part, for the differences in inhibition potencies against the two enzymes observed with compound **24**, and in general, with the inhibitors that contain a (*Z*)-butenyl spacer. To accommodate the triphenylmethoxy substituent, it was 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'. For compound **24**

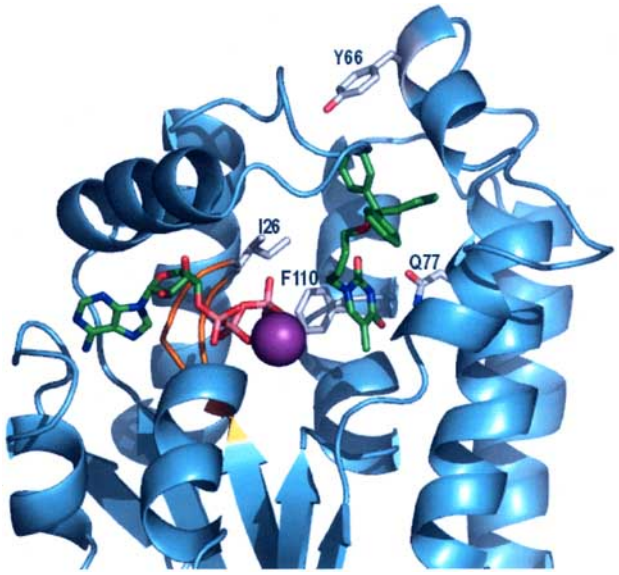


Fig. (7). Detail of the modelled human TK-2 in complex with inhibitor **24**. The protein Cα trace is shown as a ribbon coloured in cyan except for the P-loop region which is coloured in orange. Carbon atoms in ATP (left) and inhibitor **24** (right) are coloured in green whereas carbon atoms in Gln77, Tyr66, Phe110 and Ile26 are coloured in grey. The magenta sphere represents the magnesium ion.

and analogues there is no possibility for such interaction since they lack the corresponding 3´-OH. Therefore we searched for alternative preferred rotamers of this residue. It was 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 *O*-trityl moiety. Similar conformational changes appear to be precluded in the case of the herpes TK enzyme. The final complex depicted in (Fig. **7**) suggests that the selectivity of compound **24** and related inhibitors for TK-2 in preference to HSV-1 TK, in addition to the Ile26 vs. 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 longer and straight in HSV-1 TK (residues 97-128) (Fig. **6**).

Cell Culture Assays Performed with Acyclic Nucleoside Analogues

Unfortunately, direct evaluation of TK-2 inhibitors in cell culture has proven to be difficult, and alternative ways to express TK-2 in tumour cells and to evaluate the inhibitors in an intact cell system have been proposed. However, a tumour cell line expressing TK-2 in the cytosol is not available yet.

Based on the existing similarities between TK-2 and HSV-1 TK, and since some of our most potent TK-2 inhibitors showed also significant activity against HSV-1 TK, we have investigated two of these inhibitors (compounds **24** and **25**) in OST-TK$^-$/HSV-1 TK$^+$ cell lines in combination with HSV-1 TK substrates, such as ganciclovir (GCV) and (*E*)-5-(2-bromovinyl)-1-β-D-arabinofuranosyl-uracil (BVaraU) (Table **7**) [46]. The OST-TK$^-$/HSV-1 TK$^+$ cells represent human osteosarcoma cells that are deficient in cytosolic TK-1 but express HSV-1 TK in the cytosol [52]. When GCV and BVaraU were exposed to OST-TK$^-$/HSV-1 TK$^+$ cell cultures, a marked cytotoxicity was observed with IC_{50} values in the low nanomolar range (0.004-0.008 μM). Addition of HSV-1 TK inhibitors such as compounds **24** or **25** at a concentration of 20 μM to these cell cultures resulted in a marked reduction (2 – 3 orders of magnitude) in the cytotoxic effect of both GCV and BVaraU. This "detoxifying" effect was more pronounced and efficient by the addition of these inhibitors than by the addition of the natural substrate thymidine (last column in Table **7**). These results support the potential interest of inhibitors such as **24** or **25** to be combined in cell culture with TK-2-dependent nucleoside analogues to avoid or prevent potential toxic side effects. Finally, the results obtained with compounds **24** and **25** in the cell culture (OST-TK$^-$/HSV-1 TK$^+$) model demonstrate that these acyclic nucleoside inhibitors are efficiently taken up by intact cells to reach the cytosolic compartment, and are sufficiently stable and potent to exert their inhibitory activity in intact cells. One of the major challenges now would be the development of modalities to direct the TK-2 inhibitors to the mitochondrial compartment.

SUMMARY

Since the cloning of TK-2 in 1999 by two independent research groups, much progress has been achieved in kinetic studies and inhibitor development against this enzyme and much has been learnt regarding its role in mitochondrial DNA synthesis and homeostasis. Deficiency in TK-2, due to genetic alterations, has been shown to cause severe myopathy, and these findings have spurred an interest in other TK-2-related research areas such as efficiency of the mutated enzymes to phosphorylate dThd, impact of such mutated or deficient enzymes on mitochondrial dNTPs pools, elucidation of tissue specificity for TK-2 deficiency, etc. Significant progress has also been made in advancing different hypotheses that correlate TK-2 and mitochondrial toxicity associated to prolonged treatments with nucleoside analogues, particularly AZT. However, as we have tried to summarize in this review article, still much work is needed to clarify the contribution of the different proposed mechanisms to the final outcome seen in patients. It has been suggested that availability of efficient TK-2 inhibitors could be a valuable tool to better clarify these issues.

Our research groups have been involved in the identification of novel TK-2 inhibitors since 2000. We have presented here the most relevant structure-activity relationship studies with a variety of acyclic nucleoside analogues with respect to TK-2, HSV-1 TK and Dm dNK. The results obtained can be summarized in three major points: (1) The nucleic base is crucial in the interaction of these inhibitors with the target enzyme(s); (2) the spacer connecting the nucleic base and the distal substituent has a major impact on the potency and selectivity of the inhibitors against TK-2 and related enzymes. Most of the synthesized structures contain a (*Z*)-butenyl spacer since it combines a potent inhibition and certain selectivity against TK-2, and (3) the effect of substituents attached at the distal site indicates

Table 7. Effect of the Acyclic Nucleoside Analogues 24 and 25, as well as dThd, on the Cytostatic Activity of GCV and BvAraU in OST-TK$^-$/HSV-1 TK$^+$ Cell Lines

	IC_{50} (μM)[a]			
	As such	+ **24** (20 μM)	+ **25** (20 μM)	+ dThd (50 μM)
GCV	0.004 ± 0.002	0.18 ± 0.17	0.23 ± 0.19	0.026± 0.002
BvAraU	0.008 ± 0.006	2.2 ± 1.6	3.1 ± 0.85	0.016± 0.009

[a] 50% Inhibitory concentration or compound concentration (expressed in μM) required to inhibit cell proliferation by 50%

that aromatic substituents such as diphenylmethyl, biphenyl and dibenzyl, and particularly triphenylmethyl (trityl), afford the best inhibitory values. Enzyme kinetic studies have clarified the mode of interaction of these compounds with TK-2 and related enzymes, and the results have been rationalized using a model-built complex between the prototype compound (**24**) and a homology-modelled TK-2. Moreover, these compounds have been shown to exert their inhibitory action in an intact cell culture system using OST-TK$^-$/HSV-1 TK$^+$ cells that express the HSV-1 TK in the cytosolic compartment. However, no direct evaluation on TK-2 in cell culture has been assayed so far. Still, at this stage of research, it is unclear whether these compounds can enter the mitochondrial compartment. New assays are needed to evaluate the impact of these inhibitors on TK-2 function. If efficient targeting of TK-2 inhibitors to the mitochondria becomes an achievable goal, it will become easier to investigate the role of TK-2 in mitochondrial toxicity of antiviral and anticancer compounds, as well as other important issues such as mitochondrial homeostasis and integrity, the dynamics of mitochondrial dNTP pools, mitochondrial DNA repair, the possible existence of a substrate cycle between TK-2 and mitochondrial nucleotidase(s), and the communication between mitochondria and the cytosol/nucleus.

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