Structure, Physiological Role, and Specific Inhibitors of Human Thymidine Kinase 2 (TK2): Present and Future

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Abstract: Human mitochondrial thymidine kinase (TK2) is a pyrimidine deoxynucleoside kinase (dNK) that catalyzes the phosphorylation of pyrimidine deoxynucleosides to their corresponding deoxynucleoside 5′-monophosphates by γ-phosphoryl transfer from ATP. In resting cells, TK2 is suggested to play a key role in the mitochondrial salvage pathway to provide pyrimidine nucleotides for mitochondrial DNA (mtDNA) synthesis and maintenance. However, recently the physiological role of TK2 turned out to have direct clinical relevance as well. Point mutations in the gene encoding TK2 have been correlated to mtDNA disorders in a heterogeneous group of patients suffering from the so-called mtDNA depletion syndrome (MDS). TK2 activity could also be involved in mitochondrial toxicity associated to prolonged treatment with antiviral nucleoside analogues like AZT and FIAU. Therefore, TK2 inhibitors can be considered as valuable tools to unravel the role of TK2 in the maintenance and homeostasis of mitochondrial nucleotide pools and mtDNA, and to clarify the contribution of TK2 activity to mitochondrial toxicity of certain antivirals. Highly selective TK-2 inhibitors having an acyclic nucleoside structure and efficiently

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discriminating between TK-2 and the closely related TK-1 have already been reported. It is actually unclear whether these agents efficiently reach the inner mitochondrial compartment. In the present review article, structural features of TK2, MDS-related mutations observed in TK2 and their role in MDS will be discussed. Also, an update on novel and selective TK2 inhibitors will be provided.

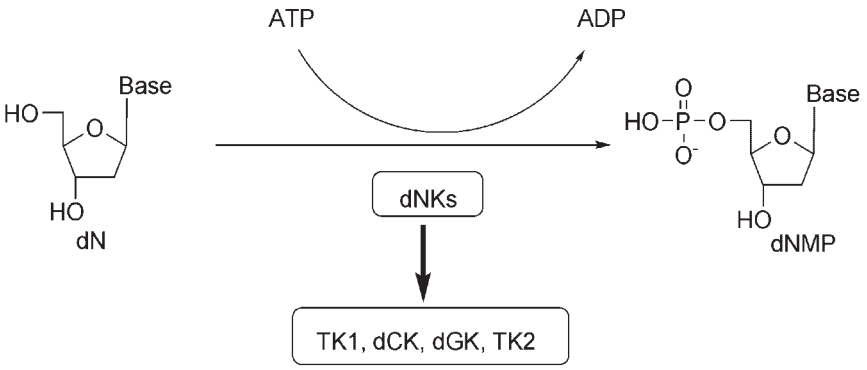
Key words: thymidine kinase 2 (TK2); deoxynucleoside kinases; inhibitors; mitochondrial depletion syndrome (MDS); acyclic nucleosides

1. INTRODUCTION

Human deoxynucleoside kinases (dNKs) are key enzymes in the salvage pathway of 2′-deoxynucleosides. dNKs catalyze the first phosphoryl transfer from a suitable donor (in general ATP) to the 5′-OH of a 2′-deoxynucleoside yielding the corresponding 2′-deoxynucleoside-5′-monophosphate (Scheme 1). In humans there are four different dNKs: thymidine kinase 1 (TK1) with a cytosolic localization; deoxycytidine kinase (dCK) with a cytosolic and/or nuclear localization; thymidine kinase 2 (TK2); and deoxyguanosine kinase (dGK) that represent mitochondrial nucleoside kinases. Besides their different localization, these enzymes have variable and even overlapping substrate specificities as well as a different pattern of expression according to the cell cycle and the nature or origin of the cell.[1,2]

Deoxynucleoside kinases are instrumental in the activation of nucleosides that are inhibitory against cancer cells and/or viruses.[3] They catalyze the initial, and often limiting, first phosphorylation step in the conversion of the nucleoside analogues to their corresponding triphosphates. Therefore, these enzymes have been extensively investigated in the chemotherapeutic field. Among the deoxynucleoside kinases, TK2 is probably the least studied enzyme. Its mitochondrial localization and its low levels of expression compared to the far more abundant cytosolic TK1 have hampered profound investigations. TK2 is the only dNK among the human enzymes whose three-dimensional structure has not been solved yet. Still there exists an increasing number of TK2 studies dealing with different issues such as mitochondrial toxicity of antiviral drugs; maintenance of balanced mitochondrial deoxyribonucleos(t)ide pools and mitochondrial DNA depletion syndromes; and mitochondrial homeostasis. In this respect, TK2 inhibitors can provide a useful tool to better understand the multiple roles of this mitochondrial nucleoside kinase.

In 2005, we reviewed in detail the compounds that had been described in the literature as TK2 inhibitors.[4] Readers are referred to that review for extensive structure–activity relationship studies.



Scheme 1. Phosphorylation reaction catalyzed by deoxynucleoside kinases (dNKs). TK1: thymidine kinase 1; dCK: deoxycytidine kinase; dGK: deoxyguanosine kinase; TK2: thymidine kinase 2.

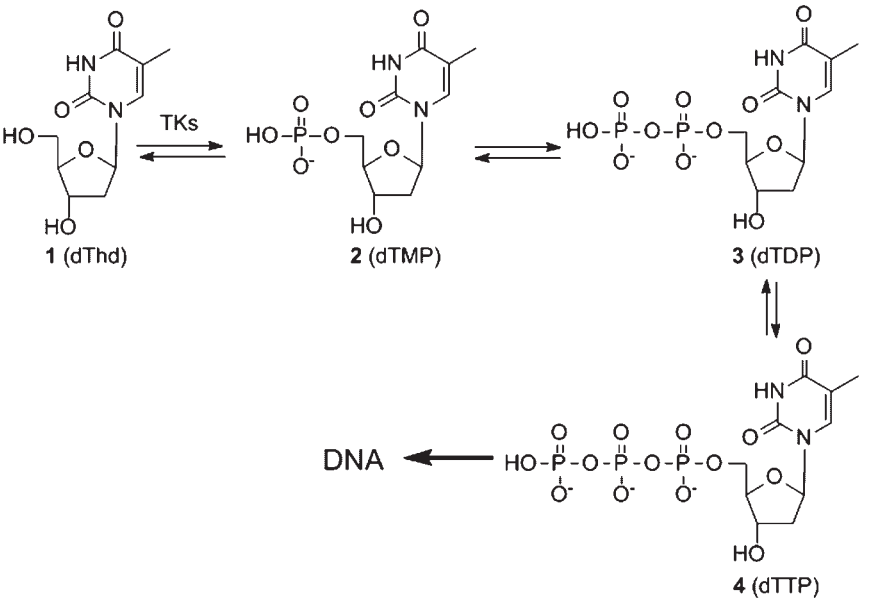
In the present review we focus on those compounds that have been reported in the last 2 years with a brief reference to the most potent inhibitors previously described. We also compiled the information related to TK2 that has become available through the use of TK2 inhibitors in different biological assays together with structural information on TK2 based on homology models and mutated enzymes.

2. TK2: DIFFERENCES BETWEEN TK1 AND TK2 AND THEIR SUBSTRATE SPECIFICITY

In mammals there are two enzymes able to phosphorylate thymidine (dThd, **1**): the cytosolic TK1 and the mitochondrial TK2, both affording thymidine monophosphate (dTMP, **2**) as shown in Scheme 2. Two additional phosphorylation steps are required to obtain thymidine triphosphate (dTTP, **4**) for incorporation into DNA.

Thymidine kinase 1 (TK1) and TK2 do not only differ in their intracellular localization, but they also contain a significantly different primary sequence, and are endowed with different substrate specificities and levels of expression according to the phase of the cell cycle.[2,5] Thus, in S phase cells, TK1 activity is much higher than that of TK2. However, in resting cells TK1 has very low, if any, measurable activity whereas the mitochondrial TK2 is constitutively expressed along the cell cycle and often is virtually the only thymidine kinase that is physiologically active in non-proliferating and resting cells.

Human TK2 was cloned in the late 1990s by two independent research groups.[6,7] Among natural substrates, TK2 phosphorylates thymidine (**1**), 2′-deoxyuridine (**5**) and 2′-deoxycytidine (**6**) (Fig. 1), although thymidine is the preferred substrate. Instead, TK1 seems to exclusively phosphorylate dThd and dUrd. Concerning nucleoside analogues used as antivirals, the anti-HIV nucleoside analogue 3′-azido-3′deoxythymidine (AZT, **7**) is a poor substrate for TK2, although its level of phosphorylation is significant in non-dividing tissues. Other drugs that are well-recognized and



Scheme 2. Successive phosphorylation steps of thymidine. dThd: thymidine; dTMP: thymidine monophosphate; dTDP: thymidine diphosphate; dTTP: thymidine triphosphate.

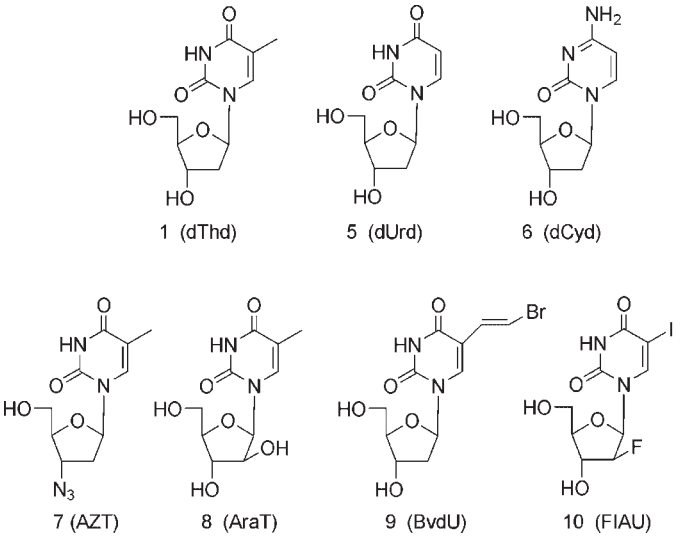


Figure 1. Nucleosides and nucleoside analogues that are substrates of TK2.

phosphorylated by TK2 include the antiherpetic drugs 1-β-D-(arabinofuranosyl)thymine (AraT, **8**), (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU, **9**) and its arabinosyl counterpart (*E*)-5-(2-bromovinyl)-1-β-D-(arabinofuranosyl)uracil (BVaraU). Another compound that is effectively recognized by TK2 is 2′-fluoro-5-iodo-(1-β-D-arabinofuranosyl)uracil (FIAU, **10**), a potent anti-hepatitis B nucleoside analogue. Additional information on TK2 substrates can be found in the literature.[2,3,8–10] It is interesting to note that mitochondrial toxicity has been observed in patients exposed to prolonged treatments with some of these antivirals (i.e., AZT, FIAU). The observed toxicity could be likely mediated by TK2 as will be discussed later.

In contrast to TK1, TK2 shows a relaxed enantioselectivity because it recognizes not only the natural D-nucleosides but also L-nucleosides like L-dThd, L-dCyd, L-BVDU, and L-IdU.[11] Other human deoxynucleoside kinases such as dCK or dGK are also not enantioselective either. A recent co-crystallization study of dCK with L-nucleosides concluded that the presence of a less restricted active site in enzymes such as dCK, dGK, or TK2, which accept several different substrates, may explain why these enzymes are also able to efficiently phosphorylate enantiomeric substrates.[12]

3. ROLE OF TK2 IN MITOCHONDRIAL DNA DEPLETION SYNDROME (MDS)

Mitochondrial DNA (mtDNA) synthesis occurs constitutively, throughout the whole cell cycle, therefore requiring a continuous supply of dNTPs. Thus, in resting cells, where cytosolic TK1 and ribonucleotide reductase are down-regulated, the mitochondrial salvage pathway may represent the only source of dNTPs for mtDNA. In the mitochondrial salvage pathway, the first (often limiting) phosphorylation step is carried out by TK2, which phosphorylates thymidine and deoxycytidine, and by dGK which phosphorylates deoxyguanosine and deoxyadenosine.[13] Mitochondrial thymidine metabolism is further regulated by 5′-deoxynucleotidase-2 (dNT-2) that is able to dephosphorylate dTMP.[14]

Mitochondrial DNA (mtDNA) depletion syndromes (MDS) are a heterogeneous group of mitochondrial disorders, that are characterized by multiple mutations in proteins that participate in mtDNA replication resulting in point mutations or deletions in mtDNA and/or a tissue-specific reduction of mtDNA levels. The best characterized disorders are associated with genes encoding

DNA polymerase γ, the nucleoside kinases TK2 and dGK, the Twinkle gene (a putative helicase), the adenine nucleotide translocator, the cytosolic thymidine phosphorylase (TPase) and the ADP-forming succinyl-CoA synthase, as reviewed by Elpeleg et al.[15] The number of mutations leading to mtDNA depletion continues to grow as exemplified by the recently reported mutations in RRM2B, encoding p-53 controlled ribonucleotide reductase.[16] This results in a compromised oxidative phosphorylation and mainly affect the muscle, liver and brain.[15] In non-replicating cells, where the cytosolic dNTP synthesis is down-regulated due to a lowering of the *de novo* nucleotide synthesis and S-phase-dependent salvage nucleoside kinases such as TK1, mtDNA synthesis highly depends on the mitochondrial salvage pathway enzymes such as TK2 and dGK.[17,18] Therefore, individuals with defects in TK2 show decreased intramitochondrial dTTP pools resulting in imbalance of dNTP ratios such as the dTTP/dCTP ratio.[19,20] It has been suggested that such mitochondrial nucleotide imbalances could affect the accuracy or rate of mtDNA synthesis and may be central to the observed disease mechanism. Indeed, deviations from the normal dNTP concentrations are known to be mutagenic which may account for the multiple mtDNA mutations found in MDS patients.

Since the first reports in 2001 assigning mutations in the TK2 gene to mitochondrial DNA depletion,[19] the number of clinical cases reported where TK2 mutations are associated with mtDNA depletion is clearly growing, depletion that often predominantly affects the muscles.[21–23] Saada et al.[19] initially identified two mutations in TK2 (i.e., His90Asn and Ile181Asn) in four individuals suffering from devastating myopathy and depletion of muscular mitochondrial DNA. Activity of TK2 in the muscle mitochondria was reduced by at least two- to sevenfold. Carrozzo et al.[24] also studied MDS in 16 patients and found TK2 mutations (Genbank mRNA XM_007855) in two patients: an Arg183Gly and an Arg254 stop codon (resulting in a truncated TK2) was observed in one patient, and a 142G insertion in TK2 was observed in another patient, resulting in a frameshift in the protein and early translation termination at amino acid position 149. Moreover, Mancuso et al.[25] also observed TK2 mutations in four patients with myopathy and MDS: the previously reported His90Asn mutation in TK2, and the novel mutations Thr77Met and Ile22Met. The pathogenicity of these mutations was confirmed by reduced TK2 activity in muscle (28–37% of controls). Interesting is also the report of Vilà et al.[26] on an Arg152Gly mutation and a Lys171 deletion in TK2 of a progressive myopathic 14-year-old patient. TK2 activity was reduced to only 1% of normal levels in isolated mitochondria from skin fibroblasts. The markedly reduced TK2 activity was most likely due to the Lys171 deletion-induced truncation of the enzyme and resulting disruption of the substrate-binding site of TK2. Interestingly, a patient has been recently described, suffering from a mitochondrial myopathy containing a homozygous His90Asn mutation in the TK2 gene but without showing mtDNA depletion, and exposing a relatively mild clinical course.[27] The biochemical basis for the lack of mtDNA depletion is still unclear, but compensatory mechanisms, relative higher muscle basal TK2 activity and/or a restricted mtDNA depletion to single fibers in the muscles (undetectable by the total muscle DNA extraction method) have been proposed. The authors conclude that all patients who present a mitochondrial myopathy, even with normal quantities of mtDNA, should be screened for TK2 mutations.[27]

It has been proposed that in replicating cells, cytosolic TK1, dCK and the *de novo* nucleotide synthesis pathway would compensate the defect of TK2 activity when TK2 activity is compromised upon mutations in the TK2 gene. However, in senescent cells where TK1 and ribonucleotide reductase activity are markedly down-regulated, the effect of TK2 mutations becomes more prominent and can have a devastating effect on mtDNA maintenance.[28] It is puzzling why TK2 deficiency preferentially affects muscle over other non-replicating tissues like liver, brain, heart or skin.[28] However a plausible explanation can be proposed when the TK2/cytochrome *c* oxidase (COX) and TK2/mtDNA ratio are compared. Thus, a low basal activity of TK2 together with a high requirement for mitochondrial encoded proteins, as happens in human muscle, has been proposed as the main determinant of the muscle involvement in TK2-deficient individuals.[17,28]

4. AZT TOXICITY

Long-term use of 3′-azido-3′-deoxythymidine (**7**) is associated with severe toxicity affecting different organs, mainly muscle, liver and heart.[29] It is reasonable to assume that while survival of HIV-1 infected individuals is prolonged by treatment with highly active anti-retroviral treatment (HAART), in which AZT continues to be an important drug cocktail ingredient, long-term side effects of AZT will also become more evident. This makes the understanding of the underlying mechanisms of AZT toxicity an important issue. It has been proposed that the AZT-related toxicities are the consequence of mitochondrial damage due to mitochondrial DNA depletion. Although different mechanisms of AZT-related mitochondrial damage may be involved,[30] the initial hypothesis postulated that inhibition of DNA polymerase γ by AZT-triphosphate (AZTTP)[31] leads to the depletion of mtDNA and consequently to depletion of mitochondrial RNA and polypeptides involved in oxidative phosphorylation.[30] However, recently, some investigators disagree with this explanation, and suggest that the concentration of AZTTP required for efficiently inhibiting DNA polymerase γ is very difficult to achieve under physiological conditions.[32,33] It was, therefore, proposed that the mechanism underlying mitochondrial toxicity may instead be related to the inhibition exerted by AZT on thymidine phosphorylation by TK2. In non-mitotic tissues like heart and liver, TK2 is virtually the only enzyme able to phosphorylate thymidine. Studies performed in isolated heart and liver mitochondria have indeed shown that AZT exerts a competitive inhibition of thymidine phosphorylation by TK2. Since TK2 is the only TK enzyme in these organs and thymidine phosphorylation is competitively inhibited in the presence of AZT, dTTP pools are depleted resulting in a decrease of mtDNA. These results have been confirmed in intact isolated perfused rat heart, where the presence of AZT in the perfusate inhibits the phosphorylation of [*methyl*-^{3}H]thymidine with an IC_{50} of 24 μM.[32] This mechanism of toxicity also provides a possible alternative to overcoming AZT toxicity, namely by raising thymidine levels in the AZT-treated patient through exogenous dThd administration.[33]

Different strategies are being described in an effort to better understand the mitochondrial toxicity associated to HAART regimens. Very recently transgenic mice with cardiac overexpression of TK2 have been described as a useful model to investigate AZT toxicity in the heart.[34] Also, computational methods are being applied to better understand mitochondrial AZT metabolism under different physiological conditions, exemplified by rapidly dividing, slowly dividing and post-mitotic cells.[35]

Certainly, unravelling the mechanism(s) underlying mitochondrial toxicity of antiviral nucleoside analogues turns out to be very complex, and more factors should probably also be taken into account. For example, the lethal mitochondrial toxicity of Fialuridine (FIAU, **10**) in the clinic was very recently associated to the presence of a nucleoside transporter (hENT1) that is functional in the mitochondrial membrane of human cells, but not in rodent cells, explaining why the mitochondrial toxicity of Fialuridine was not predicted by preclinical toxicity studies in mice.[36]

5. TK2: STRUCTURAL COMPARISON WITH OTHER dNKs

There are no crystal-based structures reported for TK2. Therefore, efforts have been made to build a reliable structural model of this enzyme. On the basis of its amino acid sequence, TK2 belongs to a large group of deoxynucleoside kinases that include other human enzymes like dCK and dGK, but similarities are even higher with the multi-functional deoxynucleoside kinase of the fruitfly *Drosophila melanogaster* (*Dm*-dNK).[2,37,38] The mGenTHREADER profile-based fold recognition method has been employed to compare these enzymes. This type of analysis indicates a common overall fold with other related enzymes despite a relatively low amino acid sequence identity: 29.3% with human deoxycytidine kinase (dCK), 17.7% with human dGK, and 47.7% with *Dm*-dNK,

covering approximately 220 amino acids.[39] A structure-based multiple sequence alignment of TK2, *Dm*-dNK and HSV-1 TK is shown in Figure 2. Interestingly, *Dm*-dNK has a broader substrate specificity than TK2 and phosphorylates all four natural deoxynucleosides, but still thymidine is the preferred substrate as is also the case for TK2.[40,41]

In 2001 the structure of *Dm*-dNK was solved in a complex with deoxycytidine.[37] Interestingly, the overall fold is very similar to that of the herpes virus thymidine kinase (HSV-1 TK), even though the amino acid sequence similarity is as low as 10% (Fig. 2). In 2003 additional complexes of *Dm*-dNK-dThd and *Dm*-dNK-dTTP were reported.[42]

Because of the markedly higher sequence identity with TK2, *Dm*-dNK has been used as the template for homology modeling of TK2.[2,38,39,43] Figure 3 represents the docking of thymidine and ATP in our reported TK2 model.[39] The thymine moiety of thymidine is sandwiched between the phenyl ring of Phe112 on one side, and Trp49 and Val82 on the other, while the carboxamide group in Gln79 establishes two hydrogen bonds with O4 and N3 of the thymine base (Fig. 3). As can be seen in Figure 2, these amino acid residues are highly conserved in *Dm*-dNK.

It has been suggested that a Glu-Arg pair close to the 5′-OH of the nucleoside in HSV-1 TK is crucial for catalysis since the Glu could act as a base in the deprotonation of the 5′-OH, assisted by the positively charged Arg.[44] A recent functional study on active site mutants of *Dm*-dNK[45] has confirmed that Glu52 in *Dm*-dNK could play this role in catalysis, while Arg105 should stabilize the transition state of the reaction. Therefore, a similar role can be proposed for the corresponding residues in TK2 (Glu50 and Arg103).

Gerth and Lutz[43] have reported a high degree of sequence identity of TK2, not only with the deoxynucleoside kinase of *Drosophila*, as already mentioned, but also with two other insect dNKs, derived from *Anopheles gambiae* (*Ag*-dNK) and *Bombyx mori* (*Bm*-dNK). These insect enzymes also have broad substrate specificity, as described for *Dm*-dNK. The data were used to engineer a mutant TK2 that could have broader substrate specificity. To pursue this aim Leu78 and Leu116 in TK2 were replaced with the corresponding residues in *Dm*-dNK (Phe and Met, respectively, as either single or

Figure 2. Structure-based multiple sequence alignment of TK2, *Dm*-dNK and *HSV*-1 TK computed by 3Dcoffee.[63] Numbering is according to TK2. Asterisks and dots denote amino acid identity or close similarity, respectively. Residues targeted for mutagenesis (L78, L116) are underlined and bold. Blue, red and green fonts denote residues involved in ATP, thymidine and Mg^{2+} binding, respectively.

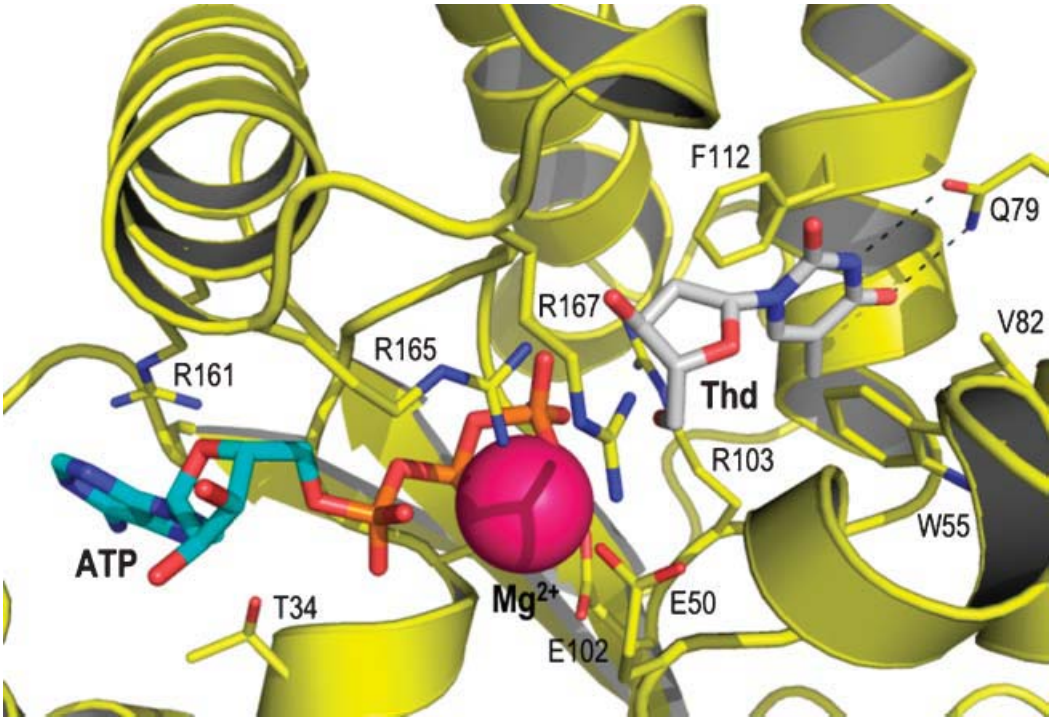


Figure 3. Docking of thymidine (C atoms in white) and ATP (C atoms in cyan) in the active site of a homology-based model of TK2.[39] Note the good stacking between the thymine base and the phenyl ring of Phe112, and the edge-to-face interaction of thymine with the indole ring of Trp55, which is held in position by the side chain of Val82, as well as the two hydrogen bonds between the carboxamide of Gln79 and N3 and O4 atoms of the thymine base. Mg^{2+} is represented as a magenta sphere.

double mutants). The result, however, was unexpected, leading to an enzyme (Leu78Phe/Leu116Met-TK2) that had an improved K_m for thymidine, but, instead, was endowed with narrower substrate specificity.[43]

6. STRUCTURES OF TK2 INHIBITORS

The availability of specific TK-2 inhibitors can be a useful tool to reveal the function of TK2 activity in mitochondrial homeostasis and to interfere with the phosphorylation of those antiviral/anticancer drugs that act as an efficient substrate for TK2. Since human recombinant TK2 became available, enzymatic assays have been performed to identify potential TK2 inhibitors. The similarities between TK2 and other dNKs, in particular *Dm*-dNK and HSV-1 TK have stimulated the testing of most of the synthesized inhibitors against these two other enzymes as well and this information is summarized in Tables I–III.

The first report on TK2 inhibitors originates from 1999, when different nucleosides modified at the sugar moiety were tested for their substrate/inhibitor properties against different human dNKs including dCK, TK1, and TK2.[46] It was found that 3′-hexanoylamino-3′-deoxythymidine (**11**) (Fig. 4), initially designed as a ligand for affinity chromatography for TK1,[47] exhibited a pronounced inhibition of thymidine phosphorylation by TK2 with a K_i value of 0.14 μM.[46]

In 2000, several ribofuranosylnucleosides were examined for TK2 inhibition.[48] In contrast to BVDU (**9**) which is an excellent substrate for TK2, its ribo analogue [5-(*E*)-(2-bromovinyl)uridine] (**12**) (Fig. 4) was found to be an inhibitor of thymidine phosphorylation. Another interesting

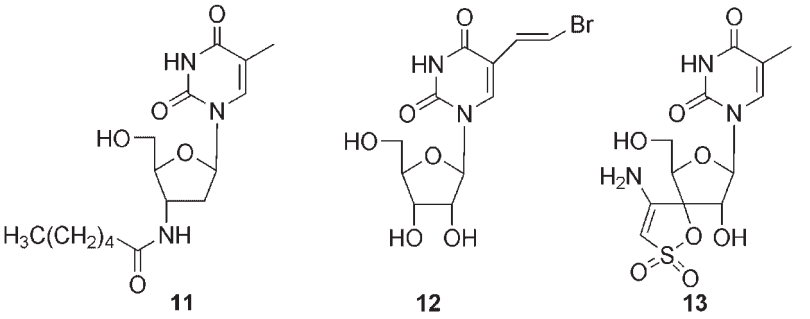


Figure 4. Initially reported TK2 inhibitors.

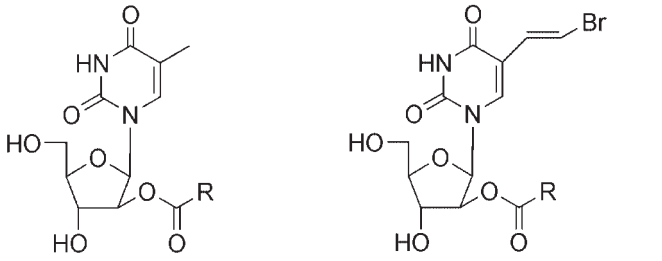
ribonucleoside that exerted significant TK2 inhibition was the 3′-spironucleoside **13** whose IC_{50} was 4.6 μM. No phosphoryl transfer from [γ-^{32}P]ATP to the 5′-position of these ribonucleosides was observed,[48] confirming a direct inhibitory activity rather than an alternative substrate behavior of these compounds.

Arabinofuranosyl nucleosides have also been explored as TK2 inhibitors. Manfredini et al.[49] communicated in 2001 the first compounds within this series, and a full report on these derivatives has recently been published.[50] It is interesting to note that while AraT (**8**) shows significant affinity for HSV-1 TK and a 10-fold lower affinity for TK2, introduction of long chain acyl substituents at the 2′-OH, as in the decanoyl and dodecanoyl esters (compounds **14** and **15**, respectively) (Fig. 5), enhanced the affinity for TK2 by 10-fold (Table I).[49,50] 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.[49,50] A similar approach has been applied to BVAraU derivatives. Thus, introduction of an octanoyl or decanoyl chain (compounds **16** and **17**) (Fig. 5) increased the inhibitory potency of the BVAraU derivatives against TK2-catalysed dThd phosphorylation by 7-fold

Table I. Inhibitory Effects of AraT and BvAraU Derivatives on [*methyl*-^{3}H]dThd Phosphorylation by Different Deoxyribonucleoside Kinases

	IC_{50}[a] (μM)		
Compound	TK2	HSV-1 TK	*Dm* dNK
AraT	285 ± 1	24 ± 3.1	65 ± 28
BvAraU	43 ± 5.8	3.6 ± 1.5	28 ± 10
14	27 ± 2.3	≥1000	872
15	28 ± 2	≥1000	>1000
16	6.3 ± 0.5	≥1000	178
17	6.8 ± 0.7	>1000	163
18	3.8 ± 0.2	>500	≥500
19	>500	322 ± 83	>500

[a]Concentration required to inhibit 1 μM [*methyl*-^{3}H]dThd phosphorylation by 50%. Data taken from Refs. 49,50 [in Ref. 50 it was erroneously indicated that the [methyl-^{3}H]dThd concentration was 2 μM (instead of 1 μM)].



14 R = $(CH_2)_8CH_3$
15 R = $(CH_2)_{10}CH_3$

16 R = $(CH_2)_6CH_3$
17 R = $(CH_2)_8CH_3$
18 R = $(CH_2)_{10}CH_2NHBoc$
19 R = $(CH_2)_5NH_2.HCl$

Figure 5. Selected TK2 inhibitors among 2′-O-acyl Ara-Tand Ara-BVU derivatives.

(IC_{50} values of ~6 μM) (Table I).[49,50] None of the araT and BVaraU derivatives showed appreciable inhibitory activity against cytosolic TK-1. Interestingly, when the acyl chain incorporates an NHBoc at the distal site as in compound **18**, TK2 inhibition is further slightly enhanced ($IC_{50} = 3.8 \pm 0.2$ μM).[50] However, when the pentanoyl chain is functionalized with a free NH_2 as in compound **19**, TK2 inhibition is lost. It should be mentioned that when the corresponding 2′-*O*-acyl-AraG and 2′-*O*-acyl-AraC derivatives were explored for TK2 inhibition, no inhibitory activity was observed.[50] Interestingly, the above described 2′-*O*-acylderivatives are highly selective in inhibiting TK2-catalyzed dThd-phosphorylation, while no inhibitory effect was detected against HSV-1 TK or *Dm*-dNK (Table I), despite the fact that the parent nucleosides (AraT and BVAraU) were inhibitory against the three enzymes.

The 2′-*O*-decanoyl BVAraU (**17**) was selected as the lead compound to perform additional biochemical studies. Detailed enzyme kinetic analyses performed with compound **17** showed purely competitive inhibition with respect to dThd as the natural substrate with a K_i value of 2.3 μM.[51] When tested for phosphoryl transfer from [γ-^{32}P]ATP to the 5′-OH of **17**, a very poor conversion to the 5′-monophosphate was observed. Thus, the introduction of these long acyl moieties at the 2′-OH of the arabinonucleosides AraT and BVAraU predominantly shifted the nucleosides from substrates to inhibitors and concomitantly increased the affinity and the selectivity for TK2.

Our research groups have been working on the identification of TK2 inhibitors since 2000. This line of research started with the observation that 5′-*O*-(4,4′-dimethoxytrityl)thymidine (**20**) (Fig. 6) showed a slight but measurable inhibitory activity against TK2-catalyzed dThd phosphorylation ($IC_{50} = 468$ μM) while the IC_{50} against HSV-1 TK was more than one order of magnitude lower (Table II). Replacement of the 4,4′-dimethoxytrityl group by a trityl as in 5′-*O*-tritylthymidine (**21**) markedly improved the inhibitory activity against both enzymes, affording a significant inhibition against TK2 ($IC_{50} = 33$ μM).[52] The presence of the trityl or dimethoxytrityl moiety at the 5′-position of the nucleoside converted the substrate (thymidine) into an inhibitor. Thus a synthetic program was initiated and designed to replace the deoxyribose moiety of the nucleoside by acyclic spacers preserving the thymine base and the trityl moiety at both ends of the spacer. These modifications were further accompanied by replacement of the thymine base by structurally related analogues and by exploration of the distal site with lipophilic groups different from the trityl present in the lead compounds **20** and **21**. It is not our intention to describe here in detail all the modifications performed because they were extensively reviewed in 2005,[4] but the most relevant inhibitors, collected in Figures 6 and 7 and in Table II, are briefly discussed. Replacement of the deoxyribose moiety of 5′-*O*-tritylthymidine (**21**) by a (*Z*)-butenyl spacer, as in compound **22**, allowed a significant increase in the

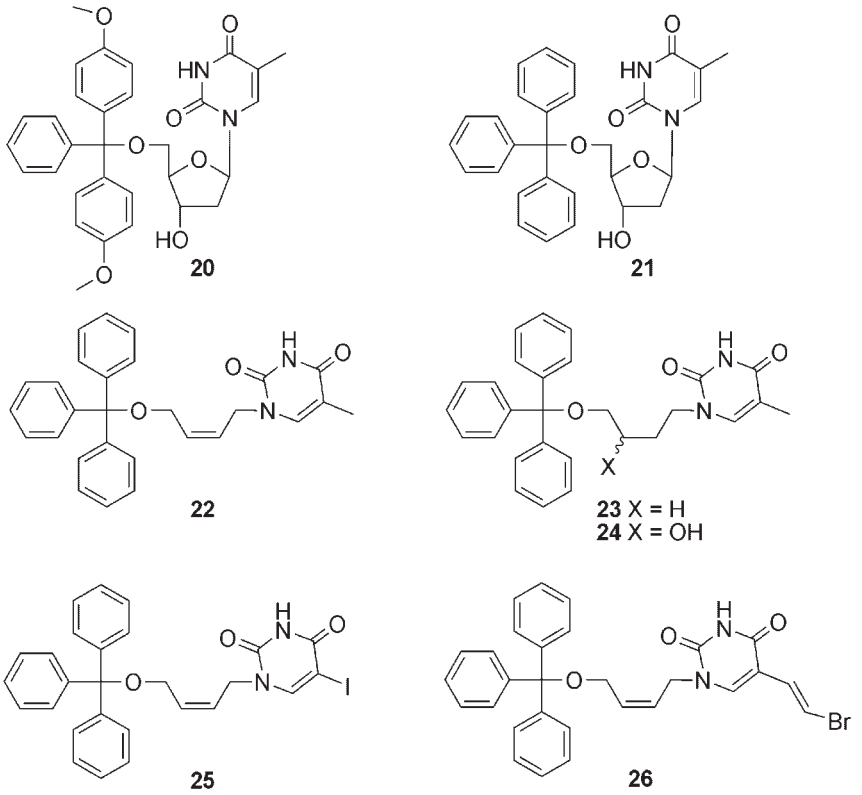


Figure 6. Selected TK2 inhibitors with an *O*-trityl substituent.

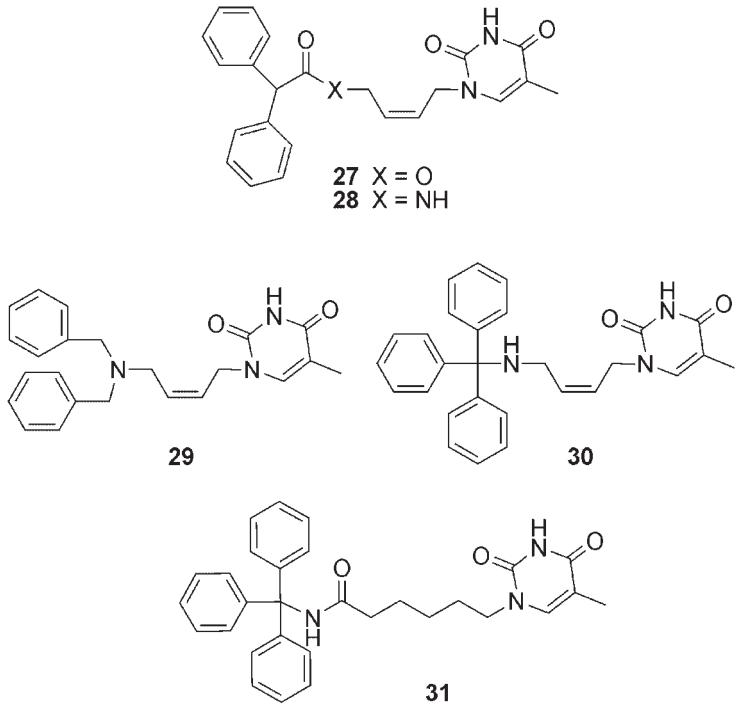


Figure 7. Selected TK2 inhibitors with a distal group different to *O*-trityl.

Table II. Inhibitory Effects of Acyclic Nucleosides **20**–**31** on [*methyl*-^{3}H]dThd Phosphorylation Catalyzed by Different Deoxyribonucleoside Kinases

	IC_{50}[a] (μM)		
Compound	TK-2	HSV-1 TK	*Dm* dNK
20	468 ± 45	14 ± 1	45 ± 9
21	33 ± 20	7.8 ± 0.3	12 ± 1
22	1.5 ± 0.2	45 ± 1	3.3 ± 0.9
23	3.3 ± 1.2	10 ± 1	19 ± 1
24	3.6 ± 0.4	1.2 ± 0.7	12 ± 4
25	4.6 ± 0.4	48 ± 14	1.6 ± 0.7
26	1.3 ± 1.1	>500	>500
27	4.6 ± 0.5	281 ± 48	16 ± 5
28	27 ± 1	115 ± 6	18 ± 2
29	3.5 ± 0.5	>500	16 ± 11
30	2.3 ± 0.4	26 ± 4	4.4 ± 0.4
31	19 ± 2	3.4 ± 0.4	19 ± 13

[a]Concentration required to inhibit 1 μM [*methyl*-^{3}H]dThd phosphorylation by 50%. Data taken from Refs. 52–55 [in Ref. 52 it was erroneously indicated that the [methyl-^{3}H]dThd concentration was 2 μM (instead of 1 μM)].

inhibitory activity against TK2 (Table II).[52] However other spacers like (*E*)-butenyl or butynyl led to considerably less active molecules against TK2. Compounds with a four-member spacer but with increased conformational freedom as in **23** or **24**, also showed potent TK2 inhibition but were less selective when compared to the other two enzymes (HSV-1 TK and *Dm*-dNK) (Table II). Therefore, the (*Z*)-butenyl spacer was kept present in most of the synthesized structures. It was observed that, concerning the base part, the thymine base can be replaced by structurally related analogues like 5-iodouracil (**25**) without compromising the inhibitory activity against any of the three enzymes.[52] However, it is interesting to note that the 5-(*E*)-(2-bromovinyl)uracil derivative **26** showed a potent and remarkable selective inhibition of TK2[53] (compare **22** with **26** in Table II). When considering the distal substituent, alternatives to the trityl group have been explored including acyl groups, amines and carboxamides. Among the acyl derivatives, the diphenylacetyl compound (**27**) (Fig. 7) was found as the most potent in this series with an IC_{50} of 4.6 μM.[52] However, the corresponding carboxamide, as shown for compound **28**, proved to be five- to sixfold less potent against TK2.[54] The synthesis and evaluation of amine derivatives at the distal site led to two new interesting compounds: the dibenzylamine derivative (**29**) with an IC_{50} against TK2 of 3.5 μM, lacking inhibition of HSV-1 TK; and the trityl amine **30** endowed with an inhibition profile very similar to that of the trityl ether **22**.[54] Compounds **21**, **24**, **25**, **28**–**30** did not inhibit cytosolic TK1 at 500 μM.

We have also performed the solution-phase parallel synthesis of a small library of thymine-derived carboxamides with cyano and/or phenyl groups at the distal site.[55] Compound **31** emerged as

the most potent TK2 inhibitor in this series of compounds, but interestingly, it is sixfold more active against the HSV-1 TK enzyme compared to TK2.

From the above-mentioned studies, the following conclusions were drawn: (i) the presence of the nucleic base is crucial for an efficient interaction of these inhibitors with TK2; (ii) the spacer connecting the nucleic base and the distal substituent has a major impact on the potency and selectivity of the inhibitors against TK2 and its related enzymes. The (*Z*)-2-butenyl linker has afforded the most selective compounds and increasing the conformational freedom of the spacer [as in compounds **23** and **24**] does not result in significant loss of the inhibitory potency against TK2; (iii) the substituents at the distal site of the molecules should be preferentially aromatic, for example, diphenylmethyl, biphenyl and dibenzyl substituents, but the triphenylmethyl (trityl) moiety provides the lowest IC_{50} values. Based on the above-mentioned premises, the most recent series of acyclic TK2 inhibitors keep the thymine base intact to allow interaction at the substrate binding site and an *O*-trityl moiety at the distal site, while more flexibility and different spacer lengths have been incorporated into the spacer (Fig. 8).[39] These modifications have been combined with the incorporation of small substituents at position 4 of one of the phenyl rings of the trityl moiety (Fig. 9).[39] The enzyme-inhibitory data are collected in Table III. It is interesting to note the strong relationship between the length of the spacer and the inhibitory activity against TK2. Derivatives with short linkers, for example, the propyl in **32** or long ones, for example, the octyl in **36**, afford TK2 inhibition at an IC_{50} around 45 μM, while those with intermediate length linkers (**33** and **35**) afforded at least 10-fold better inhibitory values (Table III). However, very noticeably, the hexamethylene linker (**34**) gave the highest inhibitory potency against TK2 with an IC_{50} of 0.50 μM. This compound is among the most potent inhibitors of the TK2-catalytic activity reported so far. Interestingly, replacement of the middle methylene group by oxygen in the five-methylene linker (compare **37** vs. **33** in Table III) slightly reduced the inhibitory potency against TK2.[39]

Concerning the substitutions replacing one of the phenyl rings of the trityl by other aryl moieties, it is interesting to note that the 4-pyridyl derivatives keep similar activity as the parent trityl against the three enzymes, both in the six-methylene (**38**) and the (*Z*)-butenyl series (**39**). However the corresponding *N*-methylpyridinium salts (**40** and **41**, respectively) were remarkably less active, probably indicating that the ionic nature of the salts provokes unfavorable interactions in the enzyme environment. Another substitution that is well tolerated by TK2 is the introduction of a methylcarboxamide moiety at position 4 of one of the phenyls of the trityl as shown in compound **42**. This compound emerged as the most potent inhibitor among the acyclic nucleosides against TK2, HSV-1 TK, and *Dm*-dNK at the same time.[39] It should be noticed that the most active compounds depicted in Table III were not found to be inhibitory to cytosolic TK1 at 500 μM pointing to a pronounced selectivity of these compounds for TK2.

Detailed kinetic analyses have been performed on native and recombinant human TK2 and with dThd and dCyd as the natural substrate[7,56] and with some of the most representative acyclic nucleoside analogues such as **22**, **26**, and **38**.[39,53] TK2 demonstrates negative cooperativity for dThd but not for dCyd.[7,56] However, for reasons of simplicity, the substrate affinities (represented by K_m) and the inhibition modes have been determined using the Michaelis–Menten equation. When examined against variable concentrations of thymidine (at saturating concentrations of ATP), **22**, **26**, and **38** showed a purely competitive inhibition with K_i values of 0.50, 0.78, and 0.29 μM, respectively. Since the K_m for dThd is estimated to be 1.2 μM, the resulting K_i/K_m values are lower than 1. When investigated against variable concentrations of ATP, the co-substrate of the TK2-catalyzed reaction, the three compounds behave as uncompetitive inhibitors with K_i values of 17, 24, and 10 μM, respectively. Also in these cases the K_i/K_m values of the compounds for TK2 against ATP were close to, or even lower than 1, based on an estimated K_m of 19 μM for ATP. These results led us to the conclusion that the acyclic nucleoside analogues, exemplified by **22**, **26**, and **38** bind at the thymidine binding site of TK2, but they only do so upon prior binding of ATP to the enzyme.

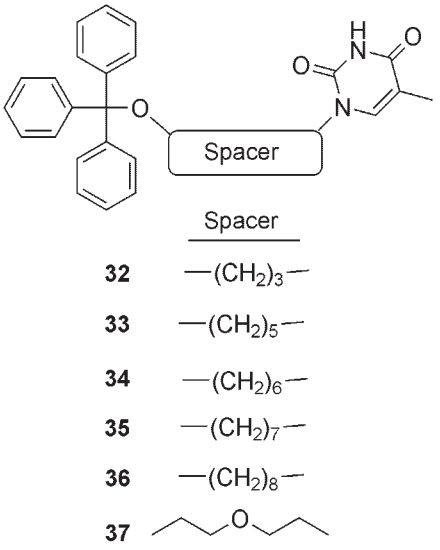


Figure 8. N1 substituted thymines tethered to a distal *O*-trityl through different spacers.

7. MOLECULAR MODEL OF THE BINDING OF INHIBITORS TO TK2

Since there are no crystal structures currently available for TK2, and to shed light on the experimentally obtained inhibition data for the latest series of acyclic nucleoside analogues, a reliable molecular model of the enzyme was built based on the already mentioned putative structurally closer nucleoside kinases detected by the mGenTHREADER profile-based fold recognition method, that is the human enzymes dCK and dGK, and the fruitfly enzyme, *D. melanogaster*

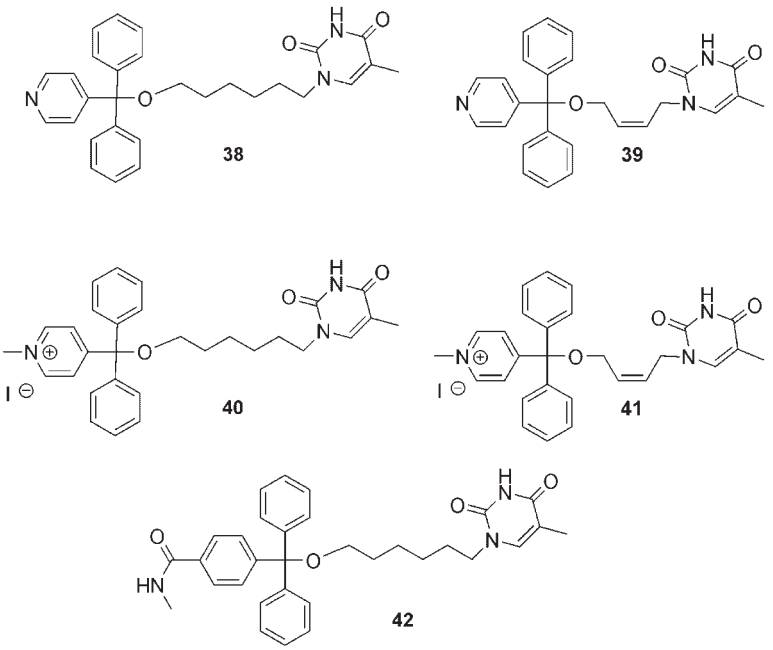


Figure 9. Selected TK2 inhibitors with triarylmetyl substituents structurally related to trityl.

Table III. Inhibitory Effect of the Acyclic Nucleoside Analogues **32**–**42** Against [methyl-^{3}H]dThd Phosphorylation by Different Deoxyribonucleoside Kinases

	IC_{50}[a] (μM)		
Compound	TK-2	HSV-1 TK	*Dm* dNK
22	1.5 ± 0.2	45 ± 1	3.3 ± 0.9
32	45 ± 2	46 ± 5	21 ± 6
33	2.5 ± 0.4	32 ± 1	15 ± 2
34	0.50 ± 0.01	3.7 ± 0.5	17 ± 10
35	4.7 ± 0.5	≥ 50	32 ± 8
36	45 ± 7	>500	30 ± 14
37	9.7 ± 0.1	17 ± 4	56 ± 21
38	0.47 ± 0.03	2.0 ± 0.4	2.7 ± 0.2
39	1.8 ± 0.1	25 ± 2	3.4 ± 0.1
40	23 ± 1	41 ± 6	29 ± 1
41	114 ± 71	>500	144 ± 58
42	0.39 ± 0.03	0.7 ± 0.4	3.5 ± 0.1

[a]Concentration required to inhibit 1 μM [*methyl*-^{3}H]dThd phosphorylation by 50%. Data taken from Ref. 39.

deoxyribonucleotide kinase (*Dm*-dNK). All of these enzymes belong to the same homologous superfamily of P-loop-containing nucleotide triphosphate hydrolases characterized by a three-layer (αβα) sandwich architecture and a Rossmann fold.

When addressing the problem of docking the acyclic nucleoside analogues, exemplified by **22** and **34**, into the enzyme, it is crucial to take into account the kinetic data showing that these compounds are competitive inhibitors against thymidine and uncompetitive inhibitors against ATP. The meaning of these observations is that the compounds bind in the thymidine binding site of the enzyme, presumably through the thymine moiety, but only in the presence of bound ATP. The first attempts to dock the (*Z*)-butenyl derivative **22** on a homology-built model of TK2 that also included ATP had to be performed in several steps and with some limitations.[54] In a more recent approach, it was reasoned that the application of normal mode analysis (NMA)[57] could afford a wider range of enzyme conformers for ligand docking purposes. Indeed, examination of the normal modes for TK2 revealed that closure of the P-loop in the ATP-binding site was closely coupled to the packing of

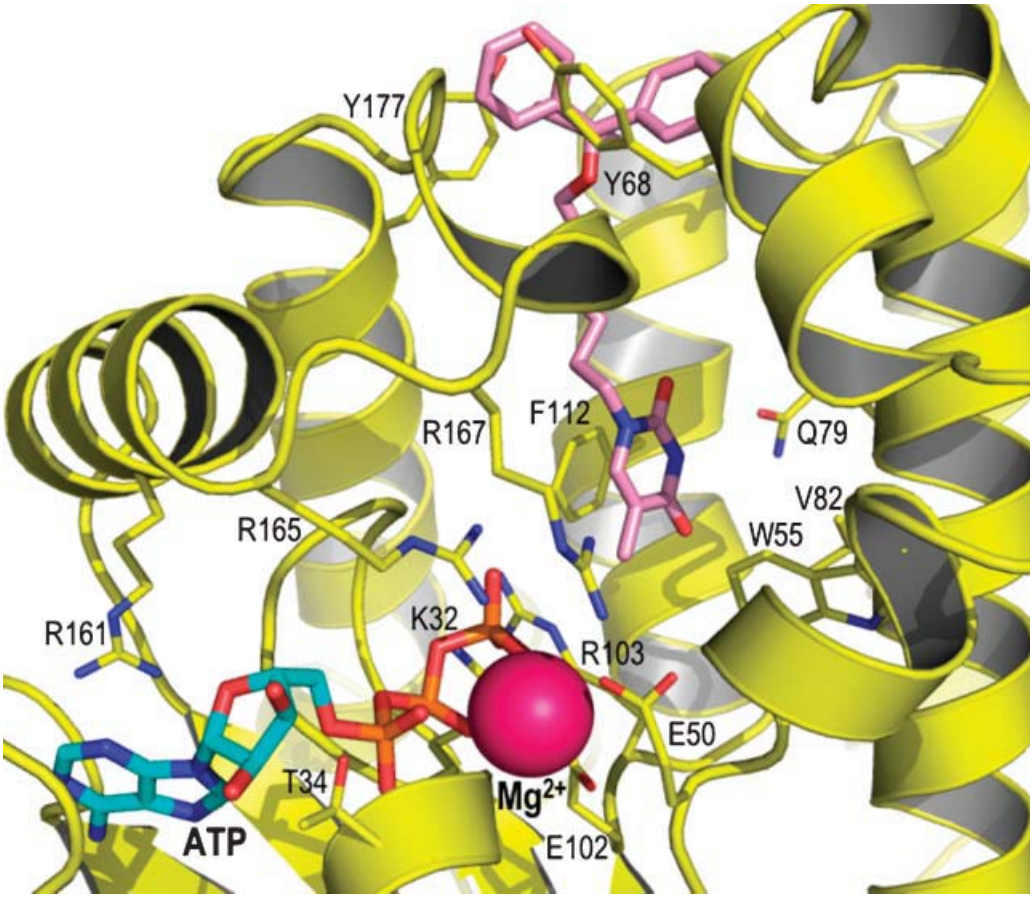


Figure 10. AutoDock best-scoring pose for inhibitor **34** (C atoms in pink) docked into the proposed binding site of TK2, which is displayed as a yellow ribbon. Note the almost perpendicular disposition of these inhibitors relative to ATP, and the interactions of the trityl group with Tyr68 and Tyr177 just above the plane delineated by these aromatic residues.

α-helices 3, 4, 6, and 7. Moreover, an essentially hydrophobic tunnel connecting the thymidine-binding pocket with the solvent was apparent at the interface between α-helices 3, 5, and 8, particularly when alternative locations for the side-chains of Tyr68 and Tyr177 were considered (Fig. 10). Furthermore, when this region was explored with the automated docking program AutoDock, the best-scoring solution for **22**, **34**, and **38** indeed placed the thymine moiety inside the dThd-binding cavity. In this orientation the O4 and N3 of thymine establish good hydrogen bonds with the carboxamide group of Gln79 whereas the planar heteroaromatic ring gets sandwiched between the side chains of Phe112 and Trp55 (Fig. 10). As a consequence, the spacer attached to the thymine exits this cavity at right angles from the ATP-binding site and traverses the hydrophobic tunnel, which is lined by the side-chains of Ile28, Leu64, Met67, Tyr68, Leu78, Leu116, Ile173, and Tyr177. Thus, depending on the length of the spacer, the distal triaryl moiety of **22**, **34**, and **38** will be positioned in a plane below ($n = 4$) or above ($n = 6$) the aromatic rings of Tyr68 and Tyr177 thus allowing these rings to establish favorable stacking and edge-to-face interactions. If the spacer is shorter, these interactions cannot take place and the inhibitor is strained in the interhelical region thus accounting for the decreased potency observed in going from **34** to **33** and **32**. Likewise, the detrimental effect on the inhibitory activity of increasing the length of the spacer, as in going from **34** to **35** and **36**, would result from the worsening of these interactions and an increased exposure to the solvent.

It is interesting to note that the substrate binding site of TK2 is able to accommodate such different inhibitors as 3′-hexanoyl derivatives (**11**), 2′-*O*-acylnucleosides, for example, 2′-*O*-decanoyl BVAraU (**17**), and acyclic nucleosides **22** or **38**, which points to a pronounced flexibility of the enzyme around the binding site. Such a flexibility must also be assumed to be present in other nucleoside kinases such as the varicella-zoster virus (VZV) TK which was shown to efficiently recognize bicyclic pyrimidine nucleoside analogues (BCNA) that contain, in addition to the bicyclic ring, a highly lipophilic and bulky alkylphenyl substituent.[58]

8. BIOLOGICAL ASSAYS PERFORMED WITH TK2 INHIBITORS

One of the main purposes of synthesizing and characterizing TK2 inhibitors is to better unravel and understand the multiple processes in which TK2 is involved, including mitochondrial homeostasis, mitochondrial toxicity of antiviral drugs and mtDNA depletion syndromes associated with TK2 mutations. However, direct evaluation of the effect of TK2 inhibitors in cell culture has proven to be extremely difficult due to the mitochondrial localization of the enzyme and its low levels of expression. Besides this, the expression of the recombinant enzyme is accompanied by important limitations. For example, there have been reported difficulties in heterologously expressing TK2 due to aggregation under standard overexpression conditions.[38] Recently an optimized protocol for the expression and purification of TK2 has been reported.[43] Also transgenic overexpression of TK2 was lacking until 2007, when the construction of the first transgenic mice has been reported.[34] This will allow a better understanding of the impact of different mtDNA contents and the effect of anti-retrovirals.[34] Moreover, a very recent article describes the expression of the human TK2 reporter gene truncated at the *N* terminus alone or fused with green fluorescent protein (GPF) in the cytoplasm of transduced cells for PET imaging with radiolabeled antivirals, such as ^{124}I-FIAU.[59]

Thus, at the time of writing of this review article, the biological assays where TK2 inhibitors can be efficiently tested are rather limited and in some cases the role of TK2 could only be deduced in an indirect way. The availability of novel cell lines, transgenic mice and assay protocols will certainly contribute to a better understanding of the physiological role of TK2.

As mentioned earlier, the expression of TK2 in the cytosol of tumor cell lines by a TK2 gene construct has been unsuccessful for a long time. Based on the existing similarities between TK2 and HSV-1 TK, and since some TK2 inhibitors also markedly inhibit HSV-1 TK, a number of TK2 inhibitors have been evaluated in OST-TK$^-$/HSV-1 TK$^+$ (cells that lack TK1 and express HSV-1 TK in the cytosol)[60] in combination with HSV-1 TK substrates such as the nucleoside analogues Ara-T or ganciclovir.[39] As a representative example data are shown for compound **38** in Table IV. It is well-established that when OST-TK$^-$/HSV-1 TK$^+$ cell cultures are exposed to HSV-1 TK substrates like Ara-T or ganciclovir, a marked cytotoxicity is observed in the lower nanomolar range (CC_{50} or 50% cytotoxic concentration ranging from 0.0019 to 0.0062 μM). Addition of the HSV-1 TK inhibitor **38** at a concentration of 10 μM resulted in a 60-fold reduction of the cytotoxicity of these drugs compared to the untreated cells. At lower inhibitor concentrations, that is, 5 and 2.5 μM, there is a dose-dependent reduction of the cytostatic activity by 40- and 20-fold, respectively.[39] This reduction of the cytostatic activity of AraT and ganciclovir is exerted at non-toxic concentrations of the inhibitor (IC_{50} = 21 μM). These data indicate that TK2 inhibitors like **38** are able to efficiently penetrate into intact tumor cells and are able to reach the target enzyme when expressed in the cytosol. However, it is not clear yet whether these compounds are also able to cross the mitochondrial double membrane in the intact cells. Thus, selective inhibitors of TK2 may be useful to decrease the toxic side-effects of some nucleoside analogues without affecting their biological (i.e., antiviral) activity, and may increase the therapeutic potential and selectivity of those nucleoside analogues that cause mitochondrial toxicity due to interaction with TK2. Obviously, to obtain such an effect, efficient mitochondrial penetration of the TK2 inhibitors is of paramount importance. Also, since selective

Table IV. Effect of Compound **38** on the Cytostatic Activity of Ara-T and GCV in OST-TK$^-$/HSV-1 TK$^+$ Cell Lines

	IC_{50}[a] (μM)	
Inhibitor (conc.)	Ara-T	GCV
None	0.0062 ± 0.0015	0.0019 ± 0.000
38 (10 μM)	0.37 ± 0.00	0.11 ± 0.01
38 (5 μM)	0.24 ± 0.03	0.081 ± 0.013
38 (2.5 μM)	0.12 ± 0.02	0.050 ± 0.005

[a]Concentration required to inhibit cell proliferation by 50%. Data are the mean (± SD) of at least two to three independent experiments. Data taken from Ref. 39.

TK2 inhibition may intrinsically lead to MDS-like side effects, TK2 inhibitors should likely be applied for a relatively short time period to counteract or ameliorate the toxic side effects of antiviral/anticancer nucleoside analogues that act as an efficient substrate for TK2, and to avoid potential eventual long-term side effects due to TK2 inhibition.

Another interesting application of TK2 inhibitors is the reliable discrimination and/or determination of TK1 and TK2 activity in different cell lines or during different phases of the cell cycle. In a recent report, radiolabeled [^{3}H]BVDU, an excellent TK2 substrate with negligible affinity for TK1, has been employed to determine TK1 and TK2 levels in combination with the TK2 inhibitor KIN109 (**38**).[61] In cell extracts obtained from human osteosarcoma cell lines that lack TK1 (OST TK1$^-$), but where TK2 is the only dThd phosphorylating enzyme, 25 μM of KIN109 completely blocked 0.2 or 1 μM dThd phosphorylation; also, at the same concentration KIN109 blocked the phosphorylation of 0.2 μM BVDU by $\geq$90%. In HOS TK1$^+$ cells, where TK1 activity largely exceeds that of TK2, administration of the TK-2 inhibitor KIN109 at even higher concentrations (100 μM) showed only negligible effects on dThd phosphorylation indicating that TK1 is the predominant dThd-phosphorylating enzyme in these cells. However, in the presence of [^{3}H]BVDU, KIN109 had a marked inhibitory effect on BVDU phosphorylation, confirming that BVDU conversion to its 5′-monophosphate is largely carried out by TK2.

Assays were also performed in extracts from proliferating (cycling) and quiescent (non-cycling) cultures of human lung fibroblasts (CCD cells). In extracts from cycling cells, KIN109 (**38**) did not inhibit dThd phosphorylation, demonstrating that TK1 is the predominant enzyme. In these extracts, BVDU phosphorylation is affected by the presence of the inhibitor KIN109 at 25 μM (87% of phosphorylation of inhibition at 0.20 μM [^{3}H]BVDU) indicating that there is TK2 activity present in proliferating CCD. Instead, in quiescent cells, KIN109 efficiently inhibits both thymidine and BVDU phosphorylation confirming that TK2 is the predominant thymidine phosphorylating enzyme during quiescence. Thus, [^{3}H]BVDU has been proposed as a suitable substrate to measure TK2 activity in cell extracts and the TK2 inhibitor KIN109 proved very helpful to discriminate between TK1 and TK2 enzyme activity levels.[61]

Among the arabinonucleosides, 2′-*O*-decanoyl-BVAraU (**17**) was also used for additional biological studies.[50] Compound **17** was tested on isolated human fibroblast mitochondria and the 50% inhibitory concentrations (IC_{50}) against dThd and dCyd phoshorylation were determined as 40 and 97 μM, respectively. These higher IC_{50} values with respect to the value previously described in a (purified recombinant) enzymatic assay (6.8 μM) may be explained by a possible partial hydrolysis of the inhibitor by esterases (and thus, by partial inactivation of the inhibitor). Still other explanations can be proposed including an incomplete uptake of the compound by the intact mitochondria; a reduction in the inhibitory activity due to relatively high endogenous thymidine concentrations present in the mitochondria as compared to the enzymatic assays; and/or a potential effect of the decanonyl compound at the level of uptake of thymidine by the isolated intact mitochondria. Interestingly, additional experiments indicate that the 2′-*O*-decanoyl-BVAraU (**17**) has an effect on mitochondrial function and thus, is able to penetrate into the mitochondria. For example, compound **17** was tested in a tissue culture of primary fibroblasts under two different experimental conditions: a permissive medium and a restrictive medium that lacks glucose but contains enough galactose to sustain the pentose phosphate pathway but not ATP production by glycolysis. After 1 week of treatment, and at concentrations up to 60 μM, compound **17** behaved similarly under both experimental conditions and had no measurable impact on the mitochondrial function regardless of the medium. However, at concentrations between 60 and 100 μM, cell viability was impaired only in the restrictive medium, but not under the permissive conditions, indicating that at these concentrations compound **17** inhibits the mitochondrial respiratory chain (MRC) function in the cells grown in the restrictive medium. Unfortunately at concentrations higher than 100 μM, a general toxic effect was observed under both experimental conditions so that the window to perform relevant additional experiments is quite narrow. Also, in the permissive medium, the effect of compound **17** on MRC enzymes was measured. After 1 week and at concentrations between 60 and 100 μM, COX activity was decreased while the effect was much less significant on succinate dehydrogenase (SDH). Since COX contains subunits encoded exclusively by mtDNA while SDH is encoded by the nucleus, the effect of compound **17** on COX without affecting SDH could be ascribed to a selective effect on mtDNA. Finally, the mtDNA content was determined by real time PCR in fibroblasts that had been treated with 100 μM of 2′-*O*-decanoyl-BVAraU (**17**) for 1 week. The mtDNA content relative to nuclear DNA was measured and found to be decreased by 50% compared to untreated cells. According to the authors, this confirms that the mitochondrial disfunction observed in the previous experiments is indeed the consequence of mtDNA depletion due to TK2 inhibition by compound **17**. However, it seems currently unclear whether the inhibitor **17** is a substrate for TK2 (being inhibitory after incorporation into mitochondrial DNA) or behaves as a pure TK2 inhibitor. Moreover, since compound **17** can be hydrolyzed by esterases thus providing BVAraU, which is a good TK2 substrate, it is unclear whether the reported effects can be ascribed to a TK2 inhibitor and/or to the action of a TK2 substrate. More research is needed to reveal the exact mode of action of compound **17** and the proposed impact of TK2 inhibition on mitochondrial function in intact cells/mitochondria.

9. TK2: PROGRESS, PERSPECTIVES, AND CHALLENGES

It becomes increasingly clear that mitochondrial TK2 activity plays an important role in mitochondrial homeostasis (i.e., maintenance of mitochondrial DNA and proper dNTP pool balances) and mitochondrial toxicity of some nucleoside analogues. Tissue-specific alteration of TK2 activity upon mutations in the TK2 gene results in mtDNA disorders including the mtDNA depletion syndrome (MDS) due to dTTP pool depletion affecting predominantly muscle, and to a lesser extent liver and brain. It is expected that an increasing number of individuals suffering from a defect in TK2 will be identified now that the MDS have been recognized to be potentially caused by TK2 mutations and that the tools to search for mutations in the TK2 gene are now available. The better

understanding of the mitochondrial toxicity caused by a number of nucleoside analogues will allow for a more efficient anticipation on such side-effects potentially linked to novel nucleoside analogues that are under clinical development.

There are now available TK2 inhibitors active in the submicromolar range, such as compounds **38** and **42**. These and other TK2 inhibitors collected in this review article do not inhibit the cytosolic TK1, indicating that selectivity among human TKs is achievable. In fact, compound **38** has already been proven to discriminate between TK2 and TK1 activity in different cell lines and phases of the cell cycle.[61] Moreover, it is unlikely that these inhibitors should be recognized by any of the other cellular nucleoside kinases (i.e., dCK, dGK) given the nature of the base part in the TK2 inhibitors.

However, there are a number of challenges that should be addressed in the near future. Resolving the tertiary structure of the TK2 enzyme and its molecular interaction with the dThd substrate and inhibitors should be helpful to allow the rational design of novel inhibitors with improved potency (i.e., at lower nanomolar inhibitory concentrations) while keeping the specificity for TK2 inhibition. At this moment it is also unclear whether the current TK2 inhibitors are able to cross the double membrane of the mitochondria, and efficiently reach the mitochondrial compartment. Therefore, more research should be done to design TK2 inhibitors that efficiently reach the interior of the mitochondria. In parallel attempts should be made to reveal the intracellular compartment where the compounds accumulate. The construction of knock-out mice would open exciting perspectives to study the physiological role of TK2 in an animal model, and to develop potential strategies to overcome TK2 activity defects as observed in MDS patients. Transgenic mice with cardiac over-expression of TK2 have been recently reported[34] and open new possibilities to investigate the effect of different mtDNA contents and the effects of anti-retrovirals on mitochondrial toxicity. Also non-homologous recombination of TK2 and *Dm*-dNK has led to enzyme chimeras[62] with higher catalytic activity than the parental enzymes, opening the field to biotechnological applications such as combined gene/chemotherapy of cancer or non-invasive imaging of the efficiency of gene transfection and expression in a broad variety of diseased individuals.

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