Recent Advances in Thymidine Kinase 2 (TK2) Inhibitors and New Perspectives for Potential Applications

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Abstract: Thymidine kinase 2 (TK2), encoded on chromosome 16q22 of the human genome, is a deoxynucleoside kinase (dNK) that catalyzes the phosphorylation of the pyrimidine deoxynucleosides 2'-deoxythymidine (dThd), 2'-deoxyuridine (dUrd) and 2'-deoxycytidine (dCyd) to the corresponding deoxynucleoside 5'-monophosphate derivatives. In contrast to the S-phase-specific thymidine kinase 1 (TK1), TK2 is constitutively expressed in the mitochondria and plays an important role in providing dNTPs for the replication and maintenance of mitochondrial DNA (mtDNA). The severe mitochondrial DNA depletion syndrome (MDS) has been associated with mutations in TK2, resulting in mtDNA depletion, isolated skeletal myopathy, and death of the individual at an early stage of life. Some antiviral nucleoside analogs, such as 3'-azido-dThd (AZT) that is targeting the human immunodeficiency virus (HIV)-encoded reverse transcriptase, are substrates for TK2 and it has been proposed that the mitochondrial toxicity observed after prolonged treatment with such drugs could be due to their interaction with TK2. Therefore, the design of specific TK2 inhibitors may be useful to investigate the role of TK2 in the maintenance and homeostasis of mitochondrial dNTP pools and its contribution to the mitochondrial toxicity of several antiviral and anticancer drugs. Since 2000, several potent and selective TK2 inhibitors have been described. Besides bringing together previously reported inhibitors, special attention will be paid in this review to the new families of TK2 inhibitors more recently described, together with modeling studies and biological assays. Moreover, the last section will be focused on several recent investigations that suggest that depletion of mtDNA can take place both in tumorigenesis and during cancer treatment with certain nucleoside analogues.

Keywords: Mitochondrial thymidine kinase 2 (TK2), nucleoside metabolism, enzymatic inhibitors, mitochondrial depletion syndrome (MDS), tumorigenesis.

INTRODUCTION

In mammalian cells, the pyrimidine nucleoside thymidine (dThd) (**1**, Fig. 1) can be phosphorylated to its corresponding 5'-monophosphate (dTMP, **2**) by two different enzymes: thymidine kinase 1 or TK1, and thymidine kinase 2, also referred to as mitochondrial thymidine kinase or TK2 [1, 2]. The phosphate group that is transferred to the 5'-OH of thymidine is derived, in general, from ATP, although other nucleoside triphosphates can also act as phosphate donors. The reaction is performed in the presence of Mg²⁺. Both enzymes belong to the family of deoxynucleoside kinases (dNKs) and are involved in the pyrimidine nucleoside salvage pathway. dNKs are instrumental in the activation of a variety of nucleoside analogues [3, 4]. These agents represent a drug family extensively studied for therapeutic applications mostly against the proliferation of cancer cells and virus replication. Their chemotherapeutic properties have, more recently, also been evaluated against bacterial, parasitic and protozoal infections.

Despite their similar role in thymidine phosphorylation, the differences between TK1 and TK2 are very pronounced in view of their cellular localization, encoding genes, primary sequence, levels of expression according to the cell type and/or phase of the cell cycle, capacity to recognize nucleoside analogues besides their natural substrate, etc. Even their complex regulation shows significant differences [5].

Human TK1 (hTK1) was cloned and characterized in the eighties and the encoding gene is located at chromosome 17q25 [6, 7]. TK1 has a cytosolic localization and its expression is strongly cell-cycle dependent. High enzyme levels are detected during the G₁/S phase of the cell cycle while they decrease during the G₂/M phase

and are absent in non-proliferating (resting) cells. Interestingly, TK1 levels are high in several proliferating and malignant cells even up to 15-fold compared to normal cells and therefore are considered as a marker with prognostic value for certain tumors [8-10]. From an amino acid sequence point of view, hTK1 has no close structural congeners. Concerning substrate recognition (Fig. 2), TK1 phosphorylates dThd (**1**) and dUrd (**3**) (but not dCyd (**4**)) among the natural substrates and some nucleoside analogues such as 3'-azido-dThd (AZT) (**5a**) and 3'-fluoro-dThd (FLT) (**5b**). Both drugs are highly active against HIV [11] (Fig. 2), and AZT and FLT are excellent substrates for TK1 [12].

Both hTK1 and TK2, the latter gene being cloned in the late nineties [13, 14], are expressed from a nuclear gene located at chromosome 16q22. The enzyme contains an N-terminal mitochondrial targeting sequence and is imported into the mitochondria, its final localization. The levels of TK2 are not cell-cycle regulated. It is a constitutively-expressed enzyme regardless of the cell cycle. In proliferating cells, although TK2 is expressed, the levels of hTK1 are much higher (>10- to 20-fold) than those of TK2. Therefore the contribution of TK2 to cellular deoxynucleoside phosphorylation is rather marginal in such cells. However, in resting (post-mitotic) cells, TK2 is the predominant, if not the exclusive nucleoside kinase able to phosphorylate all natural pyrimidine deoxynucleosides (i.e. thymidine (**1**), deoxyuridine (**3**) and deoxycytidine (**4**)). Interestingly, TK2 is also able to recognize as substrates a variety of nucleoside analogues with antiviral and/or anticancer properties such as 1-β-D-arabinofuranosylthymine (AraT) (**6**), 1-β-D-(2'-fluoroarabinofuranosyl)-5-iodouracil (FIAU) (**7**) and, particularly (E)-5-(2-bromovinyl)-2'-dUrd (BvdU) (**8**) (Fig. 2) [14]. The anti-HIV drug AZT (**5a**) is quite a poor substrate for TK2, but in non-dividing cells TK2-catalysed phosphorylation of AZT becomes relevant [15, 16].

On the basis of its amino acid sequence, the enzyme that most closely resembles TK2 is the multisubstrate deoxynucleoside kinase

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Fig. (1). Thymidine phosphorylation reaction catalyzed by TK1 and TK2.

Fig. (2). Representative substrates of TK2.

of the fruitfly, *Drosophila melanogaster* (*Dm*-dNK), with a sequence identity of ~48 %, and to a lesser extent, the thymidine kinase encoded by herpes simplex virus type 1 (HSV-1 TK). Interestingly, these two enzymes, which are more divergent in their sequence but have a similar 3D-structure [17], recognize a broad range of substrates, a characteristic that is also present in TK2.

Due to their differences in expression (depending the cell cycle) and localization (cytosol/mitochondria), TK1 and TK2 differently contribute to the phosphorylation of dThd and its analogues. In proliferating cells, the contributions of TK1 and ribonucleotide reductase (which converts ribonucleotides to 2'-deoxyribo-nucleotides) and thymidylate synthase (that converts 2'-dUMP to 2'-dTTP) are sufficient to provide the required dThd-5'-triphosphate (dTTP) pools [18]. But in resting cells the picture is significantly different. It is interesting to mention that mitochondrial DNA (mtDNA) undergoes turnover in post-mitotic cells while nuclear DNA does not [19]. This turnover requires a continuous dNTP demand and balanced dNTP pools. Mitochondrial TK2 and deoxyguanosine kinase (dGK) are the two sole nucleoside kinases that catalyze the first activation step for all natural nucleosides, and eventually control the mtDNA synthesis rate [20]. The importance of TK2 activity for mtDNA maintenance has indeed been demonstrated in a mouse model deficient in TK2 [21], as will be discussed later.

TK2 is also involved in mitochondrial DNA depletion syndrome (MDS), a clinically heterogeneous group of mitochondrial disorders characterized by a reduction of the mtDNA copy number in affected tissues without mutations or rearrangements in the

mtDNA [22]. Among the causes directly involved in the appearance of MDS are mutations in the gene encoding TK2. The first reported cases of patients with a TK2 deficiency suffered from severe myopathy [23, 24]. Since then, more than 20 different pathologies associated to TK2 mutations have been described. While weakness and hypotonia are common symptoms, hepatopathy and/or encephalopathy have also been reported [25, 26]. Interestingly, similar symptoms have been described in patients exposed to prolonged treatment with nucleoside analogues [27].

With the information provided so far it is likely that TK2 plays a role in mtDNA synthesis particularly in non-proliferating cells and therefore TK2 inhibitors can be considered as valuable tools to study the role of TK2 in the maintenance and homeostasis of mitochondrial nucleotide pools and mtDNA. Moreover, specific TK2 inhibitors may also be useful to determine the involvement of TK2 in the mitochondrial toxicity of a number of antiviral and anticancer drugs, or to investigate the contribution of TK1 and TK2 to thymidine phosphorylation in different cell types.

Since 2000, several research groups have been working on the design, synthesis and evaluation of specific TK2 inhibitors, as reviewed in 2005 [28] and later on in 2008 [29]. In the present review article attention has been paid to the most relevant compounds described up to 2008 and to the novel compound families described since then. Also, computer-assisted molecular modeling studies to explain the interactions between the inhibitors and their target enzyme TK2, will be addressed here. In the last section, the importance of TK2 in mtDNA maintenance will be further illustrated,

including very recent findings that suggest an important role of mtDNA and TK2 in tumorigenesis.

Chemical Structures of TK2 Inhibitors: from Nucleosides to Acyclic Nucleoside Analogues and Back to Nucleosides

There is no doubt that the availability of human recombinant TK2 to perform enzymatic assays has allowed the identification of a variety of compounds capable of inhibiting TK2 activity. Besides, the similarities between TK2 and other related enzymes such as *Dm*-dNK and HSV-1 TK have driven the design, synthesis and evaluation of new chemical entities against these enzymes in parallel, trying to rationally explore similarities and differences among them.

In 1999, different nucleosides with modifications on the sugar moiety were tested as substrates or inhibitors of different human deoxynucleoside kinases including dCK, TK1 and TK2 [30]. Among them, 3'-hexanoylamino-3'-deoxythymidine (**9**, Fig. 3) was identified as an inhibitor of dThd phosphorylation by TK2 with a K_i of 0.15 μ M.

Later on, in 2000, different ribofuranosyl nucleoside analogues were reported as TK2 inhibitors (derivatives **10** and **11**, (Fig. 3)), with IC_{50} values ranging from 4 to 19 μ M (Table 1) [31]. The most surprising finding was that, although 2'-deoxynucleoside BvdU (**8**) is an excellent substrate for TK2, its ribo analogue (**10**) is a rather poor substrate for TK2 but mainly behaved as a competitive inhibitor of thymidine phosphorylation by TK2.

One year later, in 2001, Manfredini *et al.* described the first family of arabinofuranosyl nucleosides as selective and effective TK2 inhibitors [32-34]. Within this series of compounds, the introduction of different lipophilic chains at the 2'-OH of AraT (**6**) and BvAraU (**13a** in Fig. 3) led to purely competitive inhibitors of TK2 with IC_{50} values in the micromolar range without a measurable effect on cytosolic TK1 (compounds **12**, **13b** and **14** in (Fig. 3) and Table 1). Again, by means of a simple chemical modification, good substrates of TK2 were converted into efficient TK2 inhibitors. These compounds were also tested against the related enzymes

HSV-1 TK and *Dm*-dNK. In contrast to the parent nucleosides AraT (**6**) and BvAraU (**13a**), compounds **12**, **13b** and **14** showed no particular effect on these enzymes. Further studies carried out with **12**, the most active compound in these series, revealed that **12** was able to inhibit the TK2 enzyme "in organello" [34]. Still these 2'-*O*-acyl nucleosides are not ideal tools for studies in intact cells due to the esterase-sensitive acyl group, whose presence in the molecule is crucial for efficient TK2 inhibition [32].

It was in 2002 when our research groups described a unique family of acyclic nucleoside analogs as specific TK2 inhibitors. The starting point was the enzymatic evaluation of dThd phosphorylation by different dNKs in the presence of different nucleoside analogues synthesized in our laboratories as well as others derived from commercial sources. In this way 5'-*O*-tritylthymidine (**15**, Fig. 4) was identified as a compound able to inhibit TK2 and HSV-1 TK activities with IC_{50} values of 33 and 7.8 μ M, respectively [35]. The sole presence of the trityl group at position 5' of the deoxyribose was able to convert the natural substrate thymidine into a TK2 inhibitor. It is not the aim of this review to detail all the synthesized compounds and the SAR studies that have been carried out within this family of analogs including their acyclic derivatives, and readers are referred to previous review articles [28, 29]. A brief summary containing the most relevant derivatives is here included. Their structures are collected in Figs. 4 and 5, and their enzymatic activities are displayed in Table 2. The working hypothesis consisted on the replacement of the sugar moiety of 5'-*O*-tritylthymidine **15** by conformationally constrained acyclic spacers keeping the thymine base and the trityl group at each side of the spacer. Thus, introduction of a (*Z*)-butenyl spacer led to derivative **16** (Fig. 4), which showed a significant increase of the inhibitory activity against TK2 with an IC_{50} value of 1.5 μ M, and this compound was considered as the lead for further optimizations [35].

The importance of the nucleic base was then explored by replacement of thymine with related pyrimidines such as 5-iodouracil or 5-(*E*)-(2-bromovinyl)uracil (BVU) as in compound **17** (Fig. 4) [36]. The latter compound showed similar activity against TK2 (IC_{50} = 1.3 μ M) compared to **16** (IC_{50} = 1.5 μ M) but emerged as a

Fig. (3). Selected nucleoside derivatives reported as TK2 inhibitors.

Table 1. Inhibitory Activity of Selected Nucleoside Derivatives on [*methyl*-³H]dTHd Phosphorylation by Different Deoxyribonucleoside Kinases

Compound	IC ₅₀ ^a (μ M)			
	TK1	TK2	HSV-1 TK	Dm-dNK
10^b	>1000	19 $\pm$ 1.1	-	-
11^b	>1000	4.6 $\pm$ 0.35	-	-
AraT(7)^c	>1000	285 $\pm$ 1	24 $\pm$ 3.1	64 $\pm$ 28
BvAraU (13a)^c	>500	43 $\pm$ 5.8	3.6 $\pm$ 1.5	28 $\pm$ 10
12^c	$\geq$ 1000	28 $\pm$ 2	$\geq$ 1000	>1000
13^c	>1000	6.8 $\pm$ 0.7	>1000	163
14^c	ND	3.8 $\pm$ 0.2	>500	$\geq$ 500

^a50% Inhibitory concentration required to inhibit phosphorylation of ^b2 μ M or ^c1 μ M [*methyl*-³H]dTHd by 50%. Data taken from references 31 and 34

Fig. (4). Selected TK2 inhibitors from the acyclic nucleoside derivative family.

highly selective inhibitor of TK2 because it completely lost inhibitory potency against HSV-1 TK and Dm-dNK (Table 4).

Next, replacement of the trityl ether moiety by different amines and carboxamides was explored while maintaining the thymine base and the (*Z*)-2-butenyl spacer [37]. Most of these compounds showed moderate inhibitory activity against TK2. The most relevant compounds among this series were the trityl amine derivative **19**, which showed a profile similar to that of the *O*-trityl analogue **16**, and the dibenzylamine derivative **18**, which displayed a remarkable selectivity *versus* HSV-1 TK [37].

Alternatively, the trityl group and the thymine base were linked by a variety of polymethylene [38] or amide containing spacers [39]. The data obtained with polymethylene chains revealed a clear dependence between the length of the alkyl chain and the inhibitory potency against TK2, as exemplified by derivative **20** (Fig. 5), with a hexamethylene spacer, that was the most potent TK2 inhibitor with an IC₅₀ of 0.5 μ M. Keeping the thymine base and the hex-

amethylene spacer, subtle modifications were performed at the trityl substituent. In particular, replacement of one of the phenyl rings of the trityl by a 4-pyridyl afforded a similarly potent TK2 inhibitor, designated as KIN109 (compound **21**, Fig. 5), that has proved useful in further biological studies, as will be illustrated later. However, the corresponding *N*-methylpyridinium salt (**22**) was significantly less active against TK2. Another interesting example is compound **23** with a methyl carboxamide at position 4 of one of the phenyl rings of the trityl group. This compound has been shown to be the most potent inhibitor of TK2 within the acyclic nucleoside compound series. It should be stressed that all of these compounds were not inhibitory towards TK1 at concentrations up to 500 μ M and thus are endowed with an unprecedented selectivity against TK2.

Since our last report in 2008 [29], the research on TK2 inhibitors has been headed mainly by Van Calenbergh and collaborators who reported in 2009 a series of 3'-substituted thymidine derivatives as potent and selective inhibitors of human TK2 (Fig. 6) [40].

Table 2. Inhibitory Activity of Selected Acyclic Tritylated Nucleoside Derivatives on [methyl-³H]dThd Phosphorylation by Different Deoxyribonucleoside Kinases

Compound	IC ₅₀ ^a (μM)			
	TK1	TK2	HSV-1 TK	Dm-dNK
15	> 500	33 ± 20	7.8 ± 0.3	12 ± 1
16	> 500	1.5 ± 0.2	45 ± 1	3.3 ± 0.9
17	> 500	1.3 ± 1.1	> 500	> 500
18	> 500	3.5 ± 0.5	> 500	16 ± 11
19	> 500	2.3 ± 0.4	26 ± 4	4.4 ± 0.4
20	> 500	0.50 ± 0.01	3.7 ± 0.5	17 ± 10
21	> 500	0.47 ± 0.03	2.0 ± 0.4	2.7 ± 0.2
22	> 500	23 ± 1	41 ± 6	29 ± 1
23	> 500	0.39 ± 0.03	0.7 ± 0.4	3.5 ± 0.1

^a50% Inhibitory concentration required to inhibit phosphorylation of 1μM [methyl-³H]dThd by 50%. Data taken from references 35, 37 and 38.

Fig. (5). Structures of acyclic nucleoside derivatives bearing a hexamethylene spacer and distal modified groups.

Originally designed and evaluated as inhibitors of *Mycobacterium tuberculosis* thymidylate kinase (TMPK_{mt}) [41], the 3'-thiourea-dThd derivatives **24-31** (Fig. 6) showed moderate inhibition against this enzyme (K_i : 5-67 μM), and the authors decided to evaluate them against TK2, HSV-1 TK and TK1. As shown in Table 3, all thiourea derivatives potently inhibited TK2 with IC₅₀ values ranging from 0.43 to 3.1 μM. It was also observed that the inhibitory activity was influenced by the nature of the aryl group present on the thiourea moiety. The 4-benzyloxyphenyl (**27**), 3,4-dichlorophenyl (**29**) or 4-chloro-3-trifluoromethylphenyl (**28**) derivatives afforded the best inhibitory activity, with significant selectivity for TK2 *versus* TK1 (selectivity index ≥1000) and poor, if any, inhibitory activity against HSV-1 TK (IC₅₀ 46-500 μM). Moreover, compound **30** in which the thiourea substituent is connected directly to

the sugar ring, was even more active against TK2 than the branched-chain analogue **28**, while the replacement of the sulfur atom by an oxygen, as in derivative **31**, led to a slightly less inhibitory activity against TK2 when compared to compound **30**. It should be noticed that α-nucleosides, as exemplified by the 5'-thiourea derivative **32** (Fig. 6, Table 3) [41], were 10-100 fold less potent than the 3'-thiourea-β-dThd derivatives against TK2.

In order to increase the potency of the newly discovered 3'-thiourea-thymidine derivatives, and based on the results of the molecular docking of compound **30** in the active substrate-binding site of TK2 (*vide infra*), the thiourea moiety was replaced by the bioisosteric 1,2,3-triazole group [42]. Therefore, several 1,4- and 1,5-disubstituted-1,2,3-triazole derivatives were synthesized and evaluated (Fig. 7, Table 4). As in the thiourea series, the inhibitory activ-

Fig. (6). Structures of 3'-thiourea-dThd and 5'-thiourea- α -dThd derivatives.

Table 3. Inhibitory Activity of 3'- and 5'- thiourea Derivatives on [methyl-³H]dThd Phosphorylation by Different Deoxyribonucleoside Kinases

Compound	IC ₅₀ ^a (μM)		
	TK1	TK2	HSV-1 TK
24	>500	1.2 ± 0.1	444 ± 18
25	>500	3.1 ± 0.3	≥500
26	>500	1.2 ± 1.1	400 ± 54
27	≥500	0.43 ± 0.04	232 ± 39
28	≥500	0.47 ± 0.04	46 ± 4.0
29	≥500	0.64 ± 0.31	87 ± 36
30	316 ± 1.2	0.15 ± 0.01	195 ± 54
31	416 ± 6.0	0.42 ± 0.0	335 ± 25
32	>500	38 ± 1.0	472 ± 40

^a50% Inhibitory concentration required to inhibit phosphorylation of 1μM [methyl-³H]dThd by 50%. Data taken from reference 40.

ity was influenced by the nature of the substituent at C-4 of the triazole ring. Thus, compounds with an alkyl (**34**), a phenyl (**35**), a benzyl (**36**) or a cyclopentylmethyl (**37**) substituent afforded sub-micromolar IC₅₀ values. Interestingly, the introduction of an electron-withdrawing Cl atom at the *para* position of the phenyl substituent, as in **38**, significantly improved the anti-TK2 activity up to the nanomolar range. However, compound **40** (bearing a 2-pyridinyl group) or the 1,5-triazoles **41** and **42** (regioisomers of **35** and **38**, respectively) were much less potent. When thymine in derivatives **35** and **38** was replaced by BVU (well-known as a privileged base for the binding to TK2), the potency against TK2 was at least as good, if not slightly improved (IC₅₀ values 0.32 μM for **35** vs. 0.25 μM for **43** or 0.046 μM for **38** vs. 0.036 for **44**), whereas the selectivity against TK1 was significantly increased.

The most recent 3'-substituted nucleoside derivatives described by Van Calenbergh and colleagues involve the incorporation of benzylamines, guanidine and (amino)tetrazole groups at the 3' position (Figs. **8** and **9**) [43]. In this limited series only benzyl, 4-chlorophenyl and 4-chloro-3-trifluoromethylphenyl derivatives

were synthesized on the basis of results obtained in the 1,2,3-triazole series. In the case of (amino)tetrazole derivatives (Fig. **9**), both thymine and BVU were used as the nucleic acid base. As shown in Table **5**, the benzylamine (**45-47**) and guanidine (**48-50**) derivatives displayed inhibition of TK2 with IC₅₀ values ranging from 0.33 to 4.6 μM. In all cases, the presence of a 3-CF₃-4-Cl substituted phenyl group (compounds **47** and **50**) improved the TK2 inhibitory activity. The most potent inhibition was exhibited by the 1,5-aminotetrazole derivatives in which the tetrazole ring is attached to C3' via an NH bridge, as in compounds **51** and **53**. The inhibitory potential of compounds **54-56** were clearly inferior to **51** and **53**. To the best of our knowledge, compound **53**, with an IC₅₀ of 0.014 μM, emerged as the most potent and selective TK2 inhibitor reported to date [43].

Kinetic Analysis of TK2 Inhibitors and Biological Studies

In order to obtain additional information about the mode of TK2 inhibition by the compounds described, detailed kinetic analyses have been carried out with different series of compounds.

Table 4. Inhibitory Activity of 3'-triazol-1-yl Derivatives of Thymidine and BVDU on [*methyl*-³H]dThd Phosphorylation by Different Deoxyribonucleoside Kinases

Compound	IC ₅₀ ^a (μM)			
	TK1	TK2	HSV-1 TK	Dm-dNK
33	>500	4.7 ± 2.1	212 ± 17	40 ± 1
34	366 ± 4	0.23 ± 0.05	95 ± 1	4.1 ± 0.8
35	54 ± 16	0.32 ± 0.01	28 ± 6	2.2 ± 0.8
36	318 ± 110	0.30 ± 0.12	158 ± 42	3.9 ± 0.6
37	177 ± 182	0.15 ± 0.02	48 ± 2	3.0 ± 0.4
38	71 ± 0	0.046 ± 0.002	27 ± 5	1.8 ± 0.9
39	75 ± 49	0.042 ± 0.008	30 ± 2	0.99 ± 0.53
40	316 ± 17	1.3 ± 0.4	46 ± 1	3.8 ± 0.1
41	≥500	0.6	445 ± 78	17 ± 0
42	109 ± 79	4.0 ± 0.0	392 ± 18	30 ± 4
43	>500	0.25 ± 0.09	36 ± 6	2.9 ± 0.8
44	>500	0.036 ± 0.003	4.5 ± 0.6	0.46 ± 0.06

^a50% Inhibitory concentration required to inhibit phosphorylation of 1μM [*methyl*-³H]dThd by 50%. Data taken from ref. 42.

Fig. (7). General structure of the 3'-[4-Aryl-(1,2,3-triazol-1-yl)] nucleoside analogue family.

Among the acyclic nucleoside derivatives, compounds **16**, **17** [36] and **20** [38] were included in these assays. When evaluated against variable concentrations of thymidine (at saturating concentrations of ATP), these compounds behaved as competitive inhibitors of TK2 (*K_i* values of 0.50, 0.78 and 0.29 μM, respectively). On the other hand, if the variable substrate was ATP, uncompetitive inhibition was observed (*K_i* values of 17, 24 and 10 μM, respectively). Thus it may be concluded that the acyclic nucleoside inhibitors occupy the thymidine binding site of TK2 only when the cosubstrate ATP is already bound to the enzyme.

As regards the 3'-substituted nucleosides, the kinetic properties of compounds **30** [40] and **38** [42] have also been investigated against TK2. When tested against variable concentrations of dThd, these inhibitors inhibited TK2 in a purely competitive fashion with considerable low *K_i* values of 0.054 and 0.012 μM, respectively. In the presence of variable concentrations of ATP, the inhibitors displayed an uncompetitive behavior (as assessed by the parallel lines in the Lineweaver-Burk kinetic plots) with *K_i* values of 2 and 0.41 μM, respectively. Therefore, also in this case, it seems that the compounds need the pre-formation of the TK2-ATP complex in

order to interact with TK2 and exert their inhibition by occupying the thymidine-binding site.

Direct evaluation of TK2 inhibitors in cell culture is challenging. The mitochondrial localization of the enzyme and its relatively low expression levels, particularly in proliferating tissues where TK1 is far more abundant, really complicates the testing of TK2 inhibitors. Alternatively, on the basis of (i) the similarities between TK2 and other dNKs such as HSV-1 TK and *Dm*-dNK, and (ii) the fact that the most potent TK2 inhibitors also significantly inhibit *Dm*-dNK and/or HSV-1 TK, as illustrated in Tables 2-5, cell culture assays have been performed with cell lines transfected with these dNKs in the cytosol, but lacking the cytosolic TK1. In this respect, compounds **16**, **20** and **21** were tested in OST-TK/HSV-1 TK⁺ cell cultures (cells that lack TK1 and express HSV-1 TK in the cytosol) in the presence of efficient HSV-1 TK substrates such as Ara-T. The results obtained indicate that the acyclic nucleosides efficiently penetrate into the transfected cells and are able to reach their target enzyme in the intracellular environment when present in the cytosol [35, 38].

Fig. (8). Structure of the 3'-benzylamine and 3'-guanidine dThd and BvDU derivatives.

Fig. (9). Structure of the 1,5-aminotetrazole series of dThd and BvDU derivatives.

Similar experiments have been carried out with the 3'-substituted nucleosides, exemplified by compound **44**. In this case human osteosarcoma (OST TK⁻) cells transfected with the *Dm-dNK* gene (OST TK⁻/*Dm-dNK*⁺) were used. Cell cultures were exposed to these excellent *Dm-dNK* substrates like BvdU in the presence or absence of **44**. While BvdU was cytostatic against the *Dm-dNK*⁺-transfected cells at an IC₅₀ of 0.76 μM, its antiproliferative effect was markedly decreased in the presence of **44** [~ 80% at 10 μM (IC₅₀ 2.95 μM) and ~ 50% at 4 μM (IC₅₀ 1.64 μM)]. Thus, under these experimental conditions, in the presence of the *Dm-dNK*/TK2 inhibitor **44** the conversion of BvdU mediated by *Dm-dNK* (expressed in the cytosol) was inhibited in a dose-dependent manner [42].

There are also reported examples where a specific TK2 inhibitor, KIN109 (**21**) in combination with radiolabeled [³H]BvdU, has been used as a tool to determine the TK1 and TK2 contribution to thymidine phosphorylation in different cell lines or during different phases of the cell cycle [44, 45]. In extracts from cycling cells KIN109 (**21**) did not inhibit dThd phosphorylation, demonstrating that TK1 is the predominant enzyme. In these same extracts, BvdU phosphorylation is affected by the presence of KIN109 (**21**) indicating that there is TK2 activity present in the proliferating cell cultures. However, in quiescent cells, KIN109 (**21**) efficiently inhibits both thymidine and BvdU phosphorylation confirming that TK2 is the predominant thymidine-phosphorylating enzyme under quiescence conditions [44]. Thus, [³H]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. In fact, [³H]BvdU and

KIN109 (**21**) have recently been successfully applied to measure TK2 activity in fibroblasts from two TK2-deficient patients with a slow progressing syndrome [45].

Despite the progress made in the synthesis and characterization of TK2 inhibitors, it is still unclear and even doubtful whether the available TK2 inhibitors are able to cross the double membrane of the mitochondria and reach the target enzyme TK2 in intact cells.

3D Homology Model for TK2 and Docking Studies

Since the experimental resolution of the three-dimensional structure of TK2 remains elusive, construction of homology models still represents the most suitable tool to gain insight into the binding mode of the different families of enzyme inhibitors. Thus, the protein fold recognition method mGenTHREADER [46] was used to identify TK2's structural neighbours, which turned out to be the human enzymes deoxycytidine kinase (dCK), dGK and *Dm-dNK*, with sequence identities ranging from 17.7 to 47.7%. More recent work using the Phyre2 server [47] has extended this structural similarity to HSV1 TK and equine herpesvirus type 4 TK. Sequence and structural alignment, as performed by Dali [48], are shown in Figs.

10 and **11**, respectively. In common with these structural templates, the overall fold of TK2 is proposed to contain a three-layer (αβα) sandwich architecture and a Rossmann fold. ATP and Mg²⁺ were also incorporated into the model since the reported enzyme kinetics for the described inhibitors indicated uncompetitive inhibition *versus* ATP, thus suggesting that the compounds bind to the enzyme-ATP complex. Additionally, the normal mode analysis of the initial constructed model of TK2 [37] revealed some interesting confor-

Table 5. Inhibitory Activity of 3'-substituted Thymidine and BVDU Derivatives on [*methyl*-³H]dThd Phosphorylation by Different Deoxyribonucleoside Kinases

Compound	IC ₅₀ ^a (μ M)			
	TK1	TK2	HSV-1 TK	Dm-dNK
45	>500	4.6 $\pm$ 0.3	>500	48 $\pm$ 3
46	$\geq$ 500	3.3 $\pm$ 0.2	>500	41 $\pm$ 4
47	442 $\pm$ 37	0.33 $\pm$ 0.04	492 $\pm$ 12	4.8 $\pm$ 0.2
48	355 $\pm$ 69	0.43 $\pm$ 0.02	>500	11 $\pm$ 6
49	468 $\pm$ 12	1.3 $\pm$ 0.5	372 $\pm$ 39	7.7 $\pm$ 4.1
50	$\geq$ 500	0.58 $\pm$ 0.29	>500	12 $\pm$ 4
51	382 $\pm$ 52	0.035 $\pm$ 0.007	316 $\pm$ 39	1.2 $\pm$ 0.0
52	>500	0.90 $\pm$ 0.01	>500	10 $\pm$ 6
53	>500	0.014 $\pm$ 0.001	34 $\pm$ 3	0.41 $\pm$ 0.04
54	29 $\pm$ 1	0.59 $\pm$ 0.19	40 $\pm$ 0	3.6 $\pm$ 0.7
55	222 $\pm$ 112	2.6 $\pm$ 1.2	39 $\pm$ 1	31 $\pm$ 4
56	>500	0.40 $\pm$ 0.04	26 $\pm$ 4	3.6 $\pm$ 0.2

^a50% Inhibitory concentration required to inhibit phosphorylation of 1 μ M [*methyl*-³H]dThd by 50%. Data taken from reference 43.

1	-----MLLWPLRGWAARALRCFGPGSRGSPASGPGPRRVQRRAWPPDKEQEKEKKSVCV	55	KITM_HUMAN
1	-----MAEAASCARKGTKYAEGTQP-----FTVLI	25	DNK_DROME
1	MAAGRLFLSRLRAPFSSMAKSPLEGVSSSRGLHAG-----RGPRLSI	43	DGUOK_HUMAN
56	EGN IASGKTTCLEFFSN-ATDVEVLTEPVSKWRNVRGH-----NPLGLMYHDA	102	KITM_HUMAN
26	EGN I GSGKTTYLNHFKEYKNDICLLTEPVEKWRNVNGV-----NLLELMYKDP	73	DNK_DROME
44	EGN IAVGKSTFVKLLTKTYPPEWHVATEPVATWQNIQAAGTQKACTAQSLGNLLDMMYREP	103	DGUOK_HUMAN
103	SRWGLTLQTYVQLTMLDRHTRP-----QVSSVRLMERSIHSARYIFVENLYRSGKMPE	155	KITM_HUMAN
74	KKWAMPFQSYVTLTMLQSHTAP-----TNKKLKIMERSIFSARYCFVENMRRNGSLEQ	126	DNK_DROME
104	ARWSYTFQTFSLSRLKVQLEPFPEKLLQARKPVQIFERSVYSDRYIFAKNLFENGSLSD	163	DGUOK_HUMAN
156	VDYVVLSEWFDWILRNMD--VSVDLIVYLRTNPETCYQLKKRCREEEKVIPLEYLEAIH	213	KITM_HUMAN
127	GMYNTLEEWYKFIEESIH--VQADLIYLRTSPEVAYERIRQARSEESCVPLKYLQELH	184	DNK_DROME
164	IEWHIYQDWSFLLWEFASRITLHGFIYLAQSPQVCLKRLYQARAREEEKGIELAYLEQLH	223	DGUOK_HUMAN
214	HLHEEWLIK-----SLFPMAAPVLVIEADHMERMLELFEQNRDRILTPENRKHCP---	265	KITM_HUMAN
185	ELHEDWLIH-----QRRPQSCVKLVLDADLNLENIGTEYQRSESSIFDAISSNQQPSPV	238	DNK_DROME
224	GQHEAWLIHKTTKLHFEALMNIPVLVLDVNDDFSEEVTQQEDLMREVNTFVKNL-----	277	DGUOK_HUMAN

Fig. (10). Sequence alignment of TK2 and related enzymes.

mational changes involving the closure of the P-loop in the ATP binding site concomitant to the packing of α -helices 3, 4, 6 and 7. These changes provoked the appearance of a rather hydrophobic tunnel connecting the thymidine-binding pocket with the solvent at the interface between α -helices 3, 5 and 8 and alternative side-chain orientations for Tyr99 and Tyr208 [38].

With these observations in hand, docking of the acyclic nucleoside derivative **20** was carried out (Fig. 12). The putative binding mode of **20** revealed that the thymine moiety occupied the thymidine-binding cavity with the heteroaromatic ring sandwiched between Phe143, Trp86 and Val115. This packing allowed O4 and N3 atoms of the pyrimidine ring to establish hydrogen bonds with the carboxamide group of Gln110. It should be noted that this inter-

Fig. (11). Structural alignment, as performed by Dali⁴⁸, of gemcitabine-bound Dm dNK (PDB id 2VPP, green), varicella zoster thymidine kinase in complex with BVDU-monophosphate and ADP (PDB id 1OSN, cyan), human dCK in complex with 2'-dC and ADP (PDB id 1P61, magenta), HSV6 TK in complex with *N*-methyl-dihydroxy-isobutyl-thymine (PDB id 3F0T, yellow), sulphate-bound HSV1 TK (PDB id 1E2H, salmon), and HSV1 TK in complex with dihydroxy-isobutyl-thymine (PDB id 1E2P, white).

Fig. (12). (a) Schematic representation of the docking solution of acyclic nucleoside analogue **20** together with the tunnel (semitransparent surface), ATP and Mg^{2+} ion. (b) Closer look at the putative binding mode of **20**.

action pattern is proposed to be conserved for all thymine-containing inhibitors studied (see below). The hexamethylene spacer of **20** was placed along the hydrophobic tunnel and the trityl moiety was positioned so as to interact with Tyr99 and Tyr208. This model accounts for the SAR studies within these series of acyclic nucleoside inhibitors in so far as there is a critical spacer length below or above which interactions of the distal trityl group with the enzyme cannot take place and much lower TK2 affinities are observed.

Docking studies of some of the newest 3'-substituted-dThd analogues have also been carried out. Based again on the experimental data, inhibitor **30** was docked inside TK2 in the presence of bound ATP, and molecular dynamics simulations were carried out for the enzyme-inhibitor complex [40]. The final structure (Fig. 13) revealed the same features concerning the anchoring of the thymine base as observed for inhibitor **20**, that is, stacking of the base with

Phe143, Trp86 and Val115 and two hydrogen bonds between O4 and N3 and the carboxamide group of Gln110. Besides, the orientation of the sugar is such that the 5'-OH is fixed by the guanidinium nitrogen of Arg134. The substituted phenyl ring stacks against Ile59 and its orientation makes the trifluoromethyl group pointing to Arg196. More importantly, both thiourea nitrogens are hydrogen-bonded to the γ -phosphate of ATP.

These new interactions between **30** and the cosubstrate ATP brought the attention to two major issues in the current model when addressing the docking of triazole derivative **38** [42], (i) the conformation of the C-terminal low-homology His-rich loop that stabilizes the adenine ring of ATP, and (ii) the precise side-chain conformation of the residues making up the "lid loop" that closes the entrance to the active site and stabilizes ATP and inhibitor binding. Through extrapolation from the available data from *Dm*-dNK in

Fig. (13). Docking of the 3'-thiourea derivative **30**. ATP and a Mg^{2+} ion are also shown.

complex with drugs, substrates and “feedback inhibiting” deoxyribonucleoside triphosphates, some conformational features were derived for TK2: (i) in the presence of bound ATP- Mg^{2+} the lid region must be found in a closed conformation with strong interactions between Glu200, Arg187 and Asn93, as well as between Glu201, Arg196 and Arg198. The latter Arg residues are also responsible for the anchoring of the α and β phosphate groups of ATP, respectively; (ii) the 3'-OH of the thymidine substrate can be held in position by two direct hydrogen bonds with Glu201 and Tyr99, whose OH group is further anchored to a water molecule that bridges to Tyr208 and O2 of the thymine base; (iii) the presence of Mg^{2+} bound to the ATP phosphates promotes a small displacement of the side-chains of Glu81 and Glu133 so that, together with Thr64, these residues become part of the coordination sphere of the cation; and (iv) a free 5'-OH of a nucleosidic ligand can be hydrogen-bonded by the guanidinium nitrogen of Arg134 and the carboxamide group of Glu81, whose equivalent residues in *Dm*-dNK have been shown to be crucial for the phosphoryl transfer reaction [49]. Taking all of these features into account, the docking solution obtained for derivative **38** is shown in (Fig. 14). As in previous studies, the thymidine moiety of **38** fills the substrate-binding site thus giving rise to the same interactions as described for deoxythymidine. In this location, the C2'-endo puckering of the sugar ring places the 3'-triazole ring with its nitrogens facing Glu200 and the phenyl ring forces the side-chain of Tyr99 to flip and also displaces the aforementioned water molecule. Interestingly, interaction with the carboxylate of Glu200 is mediated by a bridging water molecule that is also bonded to Arg198. As these two residues are part of the “lid loop”, this arrangement can account for the fact that the loop is closed over the active site and therefore the ATP molecule can get trapped. Besides, the binding orientation of the triazole ring is fully compatible with the modulation of the inhibitory activity upon introduction of substituents at position 4. Thus, the phenyl ring in **38** makes van der Waals interactions with Val203 and Ile204, and therefore occupation of this hydrophobic cavity leads to an increase in binding affinity.

Using the same methodology, the tetrazole derivatives were also docked in the model of ATP-bound TK2 [43]. It seemed clear that the closed loop conformation of the enzyme was better stabilized by the inhibitor having the amino group bridging the tetrazole ring to the sugar moiety. In the case of **51** (Fig. 15), the tetrazole moiety could interact with the guanidinium of Arg167 while the amino group could establish a hydrogen bond with the hydroxyl group of Tyr177. The structural equivalent of this Tyr177 in *Dm*-dNK is also a tyrosine (Tyr179) but in HSV-1 TK it is a methionine

(Met231), which cannot establish the mentioned hydrogen bond. This amino acid replacement may account for the fact that these compounds are much better inhibitors of TK2 and *Dm*-dNK than of HSV-1 TK.

Important Role of TK2 in mtDNA Maintenance and Potential Implications in Tumorigenesis

As mentioned in the introduction, TK2 is a nuclear encoded mitochondrial enzyme that phosphorylates dThd and dCyd for mitochondrial DNA synthesis. Together with one other mitochondrial enzyme, dGK, TK2 catalyses the formation of deoxyribonucleotides required for DNA synthesis [20]. The importance of TK2 for mitochondrial DNA maintenance has been demonstrated in a mouse model deficient in TK2 [21]. The TK2-deficient mice develop normally during their first week of life but the following days they show growth retardation and progressive loss of mtDNA in multiple organs that is most pronounced in skeletal muscle, heart, brain and liver. In actively dividing cells several enzymes contribute to the synthesis of dNTPs that are used for both nuclear and mitochondrial DNA biogenesis and a specific deficiency of mitochondrial nucleoside kinases is therefore not symptomatic in S-phase cells. In actively dividing cells ribonucleotide reductase, thymidylate synthase and cytosolic TK1 play a major role in dNTP synthesis for both nuclear and mitochondrial DNA. However, when nuclear DNA replication is completed, one of the subunits of ribonucleotide reductase, the smaller R2 subunit, is rapidly degraded and also TK1 activity gets downregulated and is absent in quiescent cells [50]. The enzymes left to provide dNTPs for mitochondrial DNA replication in non-proliferating cells are TK2, dGK and an enzyme named p53R2, which replaces the ribonucleotide reductase R2 subunit but is present in much lower amounts compared to the R2 subunit. The compensatory enzymes present in quiescent cells are sufficient to provide dNTPs for mitochondrial DNA synthesis in certain cells like fibroblasts, with low levels of mtDNA, but not in cells from tissues with a high demand on mtDNA, like brain and muscle [51].

Deficient mtDNA synthesis is found in a group of severe genetic diseases collectively called mitochondrial DNA depletion syndromes (MDS). MDS entail multi-organ failure but can vary greatly in symptoms, age of onset and tissue-specificity [26]. In addition to MDS, alterations in mtDNA are important also in acquired human pathologies such as mitochondrial toxicity induced by HIV therapy that can manifest itself as severe clinical phenotypes [52]. It was indeed recently shown that patients treated with anti-retroviral nucleoside analogues accumulated mtDNA mutations caused by accelerated mtDNA turnover and a clonal expansion of

Fig. (14). Docking of the 3-triazole derivative **38**. ATP and Mg^{2+} ion are shown on the left side of the enzyme, and the inhibitor on the right side.

Fig. (15). Docking of the tetrazole derivative **51**. ATP and Mg^{2+} ion are shown on the left side of the enzyme, and the inhibitor on the upper middle side.

preexisting age-related mtDNA mutations [53]. This was suggested to cause the premature aging phenotype frequently observed in drug-treated HIV-infected patients. If TK2 plays a role in the activation of these nucleoside analogues, it may be assumed that specific TK2 inhibitors could protect mtDNA from side-effects caused by those drugs, that inhibit the function of the mitochondrial DNA polymerase (pol γ), without affecting the antiviral efficiency of the compounds in the extra-mitochondrial compartments.

It would also be of interest to investigate TK2 inhibition in those diseases that are characterized by unwanted cell proliferation. The role of mitochondria, specifically mtDNA, in tumorigenesis remains largely unclear [54]. Decreased levels of mtDNA content have been detected in a variety of cancers such as breast cancer, renal carcinoma, hepatocellular carcinoma, and gastric tumors.

Furthermore, mtDNA is also depleted as a response to certain nucleoside analogues used in the treatment of malignant diseases. Thus, several studies suggest that depletion of mtDNA can be involved in tumorigenesis and may also occur in the treatment of cancer by certain nucleoside analogues. Previous studies have shown that certain organs, such as brain, skeletal muscle and liver, are completely dependent on TK2 for cell proliferation and tissue development [55]. Indeed, although the level of TK2 activity in brain tumors, sarcomas or liver tumors is not known, the TK2 dependence of the tissues from which these tumors originate make them prime candidates to study the effects of TK2 inhibition. With the altered ATP production in malignant cells they may be more vulnerable for TK2 inhibitors. In fact, malignant cells from tissues with high dependence on TK2 may indeed be even more sensitive

to TK2 inhibition than the non-malignant cells, because the mitochondrial function is already turned down in malignant cells and thus the TK2 level for function is very close to the threshold level in the malignant cells, as compared to the non-malignant cells. Thus, the specificity of such TK inhibitors would rely on differences between adult differentiated cells as compared to transformed malignant cells. Available mouse models will be of interest to study the mtDNA synthesis in tumors and how tumors respond to mtDNA damage. In tissues less affected by TK2 deficiency, such as skin and hematopoietic cells, TK1 or ribonucleotide reductase activity can compensate for TK2 deficiency [56]. Whether these compensatory mechanisms are also valid for cancer cells originated from these tissues still remains to be proven. Cancer cells have a defective oxidative phosphorylation and usually prefer oxidation of fatty acids to produce ATP [57]. This property has been suggested to promote chemoresistance. Inhibition of mitochondrial function by TK2 inhibitors may deprive the cancer cells of ATP and induce tumor cell death. It still has to be investigated whether mtDNA-deficient cells are more susceptible to chemotherapy and other anti-cancer strategies. It is possible that the increased vulnerability that may be caused by TK2 inhibitors would facilitate other therapeutic approaches in cancer treatment.

CONCLUSIONS

TK2 turns out to be an important enzyme in several aspects. Since it provides phosphorylated pyrimidine nucleotides important for mitochondrial DNA synthesis, in particular in non-proliferating (resting) cells, it is therefore instrumental for the homeostasis of the mitochondrial nucleotide pools and mtDNA. TK2 deficiency results in the mitochondrial DNA depletion syndrome characterized by a reduced mtDNA copy number in several cell types. TK2 can also activate a variety of mitochondria-related antiviral and anti-cancer drugs, resulting in side-effects that could be potentially avoided if efficient TK2 inhibitors were available. Although highly potent and selective TK2 inhibitors have been designed and synthesized (Ki in the nanomolar range; specific TK2 inhibition and no measurable TK1 inhibition), efficient uptake into the mitochondrial compartment of intact cells is still questionable and a challenge for future research and development of novel efficient TK2 inhibitors.

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ABBREVIATIONS

TK	=	Thymidine kinase
<i>hTK</i>	=	Human TK gene
dNTP	=	2'-deoxynucleoside-5'-triphosphate
dThd	=	(2'-deoxy)thymidine
dUrd	=	2'-deoxyuridine
dCyd	=	2'-deoxycytidine
dGuo	=	2'-deoxyguanosine
AZT	=	3'-azido-dThd
dTMP	=	dThd-5'-monophosphate
dUMP	=	dUrd-5'-monophosphate
FLT	=	3'-fluoro-dThd
AraT	=	1-β-D-arabinofuranosylthymine

FIAU	=	1-β-D-(2'-fluoroarabinofuranosyl)-5-iodouracil
BvdU	=	(<i>E</i>)-5-(2-bromovinyl)-2'-dUrd
HIV	=	Human immunodeficiency virus
HSV	=	Herpes simplex virus
Dm-dNK	=	<i>Drosophila melanogaster</i> -deoxynucleoside kinase
dTTP	=	dThd-5'-triphosphate
MDS	=	Mitochondrial DNA depletion syndrome
ATP	=	Adenosine-5'-triphosphate
K _i	=	Inhibitory constant
dCK	=	dCyd kinase
dGK	=	dGuo kinase
dNK	=	2'-deoxynucleoside kinase

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