The Role of Thymidine Kinases in the Activation of Pyrimidine Nucleoside Analogues

Ashraf S. Al-Madhoun[1], Werner Tjarks[2] and Staffan Eriksson*[1]

[1]*Department of Molecular Biosciences, Division of Veterinary Medical Bio-Chemistry, Swedish University of Agricultural Sciences, The Biomedical Center, Box 575, S-751 23 Uppsala, Sweden*

[2]*College of Pharmacy, The Ohio State University, Columbus, Ohio 43210, USA*

Abstract: Deoxynucleoside analogues need activation by deoxynucleoside kinases to serve as antiviral or anticancer agents. Here we review the properties of cellular cytoplasmic thymidine kinase 1, mitochondrial thymidine kinase 2, the multisubstrate deoxynucleoside kinase from Drosophila melanogaster and Herpes virus 1 thymidine kinase. Important substrate activity relationships will be discussed.

Keywords: TK1, TK2, Dm-dNK, HSV1-TK, nucleoside analogues, chemotherapy, BNCT.

INTRODUCTION

During the last four decades, deoxynucleoside analogues have been used in the treatment of viral and cancer diseases and their importance is increasing. Nucleoside analogues are prodrugs, requiring 5′-phosphorylation to form active nucleotides that can function as inhibitors of viral or cellular replication processes. The initial and usually rate limiting reaction is performed by deoxynucleoside kinases. This mini-review attempts to summarize the biochemistry of the pyrimidine specific thymidine kinase enzymes with emphasis on structure activity relationships. Several reviews relevant to this topic have been published previously [1-9].

The major route for the synthesis of DNA precursors is the reduction of ribonucleotide diphosphates to deoxyribonucleotides by the ribonucleotide reductase enzyme system [10]. This enzyme is strictly controlled at transcriptional, post-transcriptional, and allosteric levels. Recently, it has also been reported that a special form of this enzyme is induced in response to DNA damage, possibly as part of the DNA repair mechanism [10, 11]. However, it is widely accepted that this *de novo* synthesis pathway is restricted to S-phase cells or damaged cells. A complementing salvage pathway is found in most cells and it is responsible for the direct phosphorylation of deoxynucleosides imported from extracellular compartments or derived from dephosphorylation of deoxynucleotides. Several deoxynucleoside kinases are involved in this pathway, forming nucleoside monophosphates, which are subsequently phosphorylated to deoxynucleoside triphosphates by nucleoside monophosphate kinases [12-15] and nucleoside diphosphate kinases [16, 17], respectively. The initial phosphorylation reaction leads to the trapping of charged nucleotides inside cells.

In mammalian cells, there are four cellular deoxynucleoside kinases including two cytosolic enzymes, thymidine kinase (TK1) and deoxycytidine kinase (dCK), as well as two mitochondrial enzymes, thymidine kinase 2 (TK2), and deoxyguanosine kinase (dGK) [3, 4, 6, 9]. Here, we will try to summarize the properties of the pyrimidine specific cellular deoxynucleoside kinases (TK1 and TK2), as well as the multisubstrate deoxynucleoside kinase from *Drosophila melanogaster* (Dm-dNK) and one member of the viral TK family i.e. HSV1-TK. We will try to illustrate the basic pattern of substrate recognition of these enzymes.

CYTOSOLIC THYMIDINE KINASE (TK1)

TK1 (EC 2.7.1.21) is found in almost all organisms with a few exceptions such as yeast and some insects [1, 7-9]. Most bacteria [17-19] and many DNA viruses (such as bacteriophages, vaccinia viruses, poxviruses and herpes viruses) code for proteins with thymidine kinase activity [1, 2, 9]. Interestingly, the Pox-virus TKs have similar characteristics as TK1, whereas the Herpes virus TKs have very broad specificity and they belong to the same enzyme family as dCK and TK2, as will be described below.

The expression of TK1 is cell cycle regulated and the active enzyme is found in S-phase cells. TK1 regulation is complex and the mRNA level peaks in proliferating cells. Splicing and translation of TK1 mRNA also varies in cells at different growth stages [7, 20]. TK1 transcripts encode a protein of 25.5 kDa with highly conserved regions typical for other nucleoside kinases. However, the crystal structures of this enzyme family have not yet been determined. TK1 levels are mainly regulated by post-translational mechanisms, in particular by differential degradation due to a highly active protease expressed in mitotic cells [21]. The biological role of TK1 is still unclear, since several cell lines and, more recently, transgenic mice have been engineered lacking TK1 activity. These mice developed kidney sclerosis and died before one year of age, but apparently showed only minimal alterations due to cell proliferation [22].

With the exception of CTP, ATP and other nucleoside- and deoxynucleoside triphosphates serve as phosphate donors for TK1. ATP also induces the formation of a highly active TK1 tetramer, starting from the dimeric TK1 with reduced activity [23]. The end product, TTP, is a feed back inhibitor, most likely acting as a bi-substrate analogue that

*Address correspondence to this author at the Department of Molecular Biosciences, Division of Veterinary Medical Bio-Chemistry, Swedish University of Agricultural Sciences, The Biomedical Center, Box 575, S-751 23 Uppsala, Sweden; Tel.: 46 18 471 41 87; Fax: 46 18 55 07 62; E-mail: Staffan.Eriksson@vmk.slu.se or Ashraf.Al-Madhoun@bmc.uu.se

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binds to the active site of TK1. The substrate specificity of TK1 with regard to nucleosides will be described below.

MITOCHONDRIAL THYMIDINE KINASE (TK2)

Similarly to TK1, TK2 transfers the γ-phosphate group of ATP to the 5'-hydroxyl group of Thd and dUrd. In addition, TK2 also phosphorylates dCyd and related nucleosides. Although the level of TK2 is very low compared to TK1 in proliferating cells, TK2 is the only pyrimidine-phosphorylating enzyme in resting cells. TK2 is localized in the mitochondrial matrix and different levels of expression were detected in various tissues, presumably in correlation to the mitochondrial content [1, 3, 9].

The open reading frame of human TK2 cDNA encodes a 265 aminoacids protein with an extra N-terminal 33 aminoacids mitochondrial leader peptide. The processed TK2 has a calculated molecular weight of 28 kDa and the active enzyme is a dimer. Recombinant truncated TK2 showed similar enzyme activity as the native enzyme [24-26]. The amino acid sequence of TK2 share approximately 40% identity with Dm-dNK. The key aminoacid residues in substrate recognition in the Dm-dNK sequence are also conserved in the TK2 sequence, explaining the overlapping substrate specificity of both enzymes. The structure determination of Dm-dNK has provided a model for the structure of TK2 [9,27].

Human TK2 utilizes Thd, dCyd, and dUrd as substrates with different efficiency and kinetic mechanisms. TK2 phosphorylates Thd with negative cooperativity, which means that the apparent affinity for the substrate decreased with increasing substrate concentrations. On the other hand, the phosphorylation of dCyd and dUrd showed normal Michaelis-Menten kinetics. Both TTP and dCTP act as TK2 feed back inhibitors, and the enzyme utilizes ATP and CTP as phosphate donors [3, 9, 26]. The specificity of TK2 for nucleoside analogues is described below.

The role of TK2 in providing precursors for mtDNA synthesis has recently been clarified. Saada *et al.* [28] reported a case study involving children, who died before two years of age, suffering from a genetic disorder characterized by severe muscle myopathy and mtDNA depletion. Molecular analysis demonstrated that their TK2 gene was harboring critical point mutations leading to inactivation of the enzyme [25, 28]. These results clearly demonstrated that TK2 plays an essential role in mtDNA precursors synthesis.

DROSOPHILA MELANOGASTER DEOXYNUCLEOSIDE KINASE (Dm-dNK)

Dm-dNK (EC 2.7.1.145) has the capacity to phosphorylate all the four natural deoxynucleosides with high turn over rates, thus it is considered a deoxynucleoside kinase [29, 30]. However, pyrimidines are at least two orders of magnitude more efficient as substrates compared to the purines and it is therefore appropriate to include the enzyme in this mini-review. Recently, several other deoxynucleoside kinases with broad specificity from insects such as *Bombyx mori* [31] and the mosquito *Anopheles gambiae* [32], and from the amphibian *Xenopus laevis* [31] have been cloned and characterized. Although these enzymes are closely related to the human TK2, enzymes from insects are characterized by broad substrate specificities including all four natural deoxynucleosides [29-32].

Dm-dNK is encoded by a gene at chromosome 3 with an open reading frame of 750 bp. The polypeptide contains 250 aminoacids with a molecular mass of 29 kDa and the active enzyme is a dimer. The enzyme is most likely essential for normal larva development but its exact role and cellular localization remains to be clarified. The kinetic mechanism of Dm-dNK is in accordance with the Michaelis-Menten equation, following an ordered reaction mechanism with the formation of a ternary complex. The recombinant enzyme, which in most cases is a C-terminal truncated version of the wild type enzyme, has kinetic properties similar to the purified protein from insect cells.

The broad specificity of Dm-dNK is also demonstrated by its capacity to phosphorylate nucleoside analogues with an overall pattern similar to that of TK2 (Table 1). In extended structure activity studies, Dm-dNK also phosphorylated many cytosine- and purine nucleosides [29, 31, 32]. However there are some differences in substrate specificity compared to TK2, including a 10-100 fold higher catalytic rate of the insect enzyme, as described below. This fact in addition to the broad specificity has encouraged attempts to produce new Dm-dNK variants with expanded capacity to phosphorylate analogues that could be used in gene therapy or dNTPs synthetic procedures [29]. A detailed structure function study of Dm-dNK is now possible due to the determination of its 3D crystal structure as well as the isolation and characterization of a number of mutants [9, 27, 33].

HERPES SIMPLEX 1 - THYMIDINE KINASE (HSV1-TK)

Herpes simplex virus type 1 (HSV1) belongs to the alpha group of the *Herpesviridae* family of animal viruses while the beta- and gamma groups include the cytomegalo-viruses and the Epstein-Barr virus, respectively. Virus specific thymidine kinases have been found in all members of the alpha- and gamma herpes viruses. The biological role of these kinases is not fully understood, since viruses lacking TK were detected in patients treated with antiviral nucleoside analogues. It appears that HSV1-TK is not necessary for the establishment of latent infection or virus multiplication in rapidly dividing cells but it is essential for the reactivation of the virus from latency or for replication in non-dividing e. g. neuronal cells [1, 2 and references therein].

HSV1-TK is a dimer of two identical 41 kDa subunits. Despite the fact that the viral TK is larger than the corresponding eukaryotic enzymes with ~ 100 additional aminoacids and a low sequence identity to TK2 and Dm-dNK (about 10-15 %), there are several key sequence motifs found to be conserved within this enzyme family [34-37]. Analysis of the protein crystal structure revealed that the core structure of HSV1-TK has a similar fold to that of Dm-dNK [27].

HSV1-TK differs from the mammalian deoxynucleoside kinase TK1 and TK2 concerning the substrate specificity. In addition to Thd and dUrd, the viral enzyme phosphorylates

dCyd and a variety of purine nucleosides. Pyrimidine and purine analogues, such as BVDU, acyclovir and ganciclovir, are very good substrates for HSV1-TK but they are, if at all, only poorly phosphorylated by cellular deoxynucleoside kinases. This is the basis for the excellent antiviral properties of these nucleoside analogues [1, 2 and references therein].

Both HSV1-TK and Dm-dNK are characterised by their broad substrate specificity; however, the capacity to phosphorylate acyclic nucleosides is a unique property of the viral enzyme. Another unique feature of HSV1-TK is their capability to convert the 5′-monophosphates of Thd and several of its analogues to the corresponding 5′-diphosphates via its thymidylate kinase activity [1, 2].

Nowadays, there is a large interest in using HSV1-TK in gene therapy of cancer as a suicide gene that confers sensitivity of transfected tumor cells to nucleoside analogues [38]. Mutants of HSV1-TK have been engineered with improved specificity for nucleoside analogues e.g. BVDU, AZT, ganciclovir and acyclovir [39 and references therein]. More recently, mutants of Dm-dNK have also been generated for potential use in suicide gene therapy [33].

THE STRUCTURES OF DM-DNK AND HSV1-TK

During the last eight years, several groups have determined the three-dimensional structure of HSV1-TK/substrate complexes [34-37]. The structure of C-terminal truncated active Dm-dNK, lacking the last 20 amino acids, was determined by Johansson *et al.* [27]. Both Dm-dNK and HSV1-TK are dimeric proteins and their folding is remarkably similar although there are several extra domains in the viral enzyme, including an extra C-terminal anti-parallel beta strand and an alpha helix. Moreover, some of the helixes are considerably longer (Fig. **1**).

There are several important aminoacid sequence motifs in the deoxyribonucleoside kinases. For instance, there is a conserved sequence; -GXXGXGKS/TT- that corresponds to a glycine-rich loop found among ATP/GTP binding proteins. This motif forms a turn without hydrogen bonds, also known as the P-loop, which accommodates the γ-phosphate of ATP. There is a lysine-arginine rich region that functions as a lid for the active site cleft. The consensus sequence -RXXXRXRXXE- forms a turn with several hydrogen bonds and an LID loop between the α7 and the α8 helix (residues 167-176 in Dm-dNK and 220-231 in HSV1-TK, Fig. **1**).

The substrate clefts in Dm-dNK and HSV1-TK have several conserved features, e.g. P/TEPV/M, D/ERS, and A/DRY motifs. These regions are involved in the interaction with nucleoside substrates. The triplet -DRH- is conserved in all the herpes viral TKs, which most likely corresponds to the -ERS- triplet of the mammalian deoxynucleoside kinases. In addition, there are glutamine residues (Gln 81 in Dm-dNK and Gln 125 in HSV1-TK) interacting with the nucleoside base via hydrogen bonds and a conserved Tyr-Glu couple (70 and 172 in Dm-dNK and 101 and 225 in HSV1-TK) that is involved in hydrogen donor interaction with the 3′-OH of the deoxyribose moiety [27]. Several studies with HSV1-TK mutants, e.g. Gln125 to Asn [39 and references therein], have demonstrated surprising functional effects both on specificity and the capacity to phosphorylate nucleoside monophosphates.

SUBSTRATE ACTIVITY RELATIONSHIPS OF TK1, TK2, Dm-dNK AND HSV1-TK

In addition to endogenous Thd and dUrd, TK1 phosphorylates many 3′-analogues of Thd/dUrd e.g. 2′-3′-dideoxythymidine (ddT, **5**) and the anti-HIV compounds 3′-azido-2′,3′-dideoxythymidine (AZT, **10**), 3′-fluoro-2′,3′-

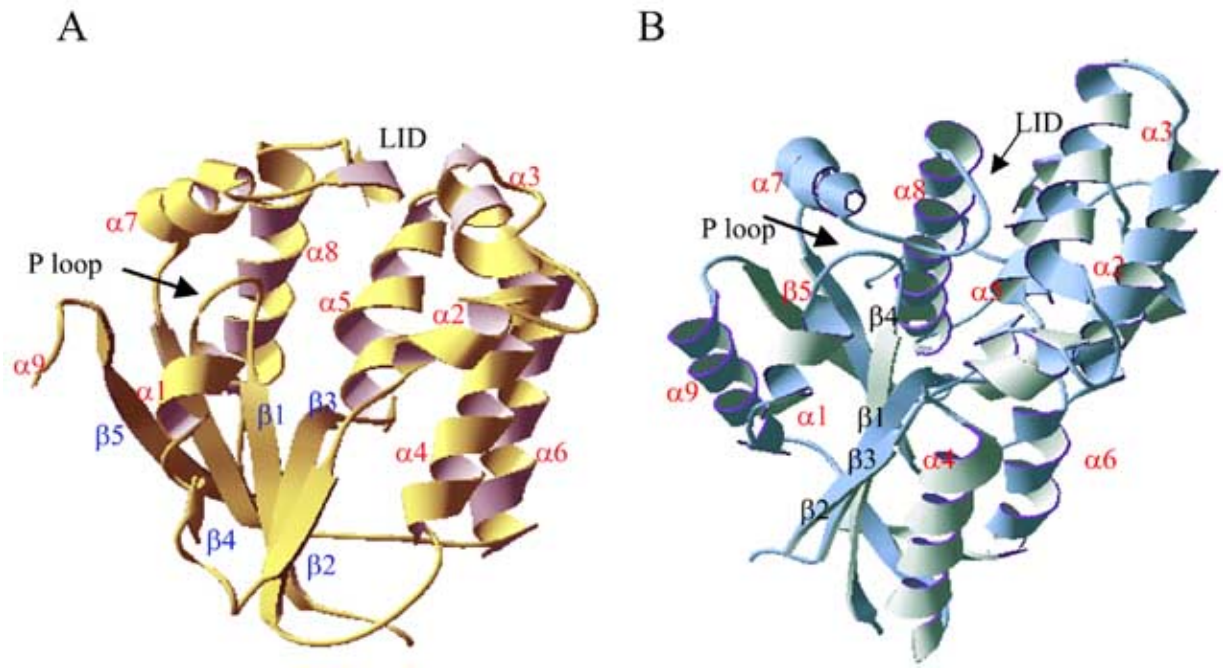


Fig. (1). The structures of A. Dm-dNK [27] and B. HSV1-TK [67].

Table 1. The Phosphorylation of Thymidine, Deoxyuridine, and some of their Derivatives. The values are in percentage relative to Thd = 100%. Compound concentration: 100 μM

	Compound	TK1	TK2	Dm-dNK	Reference
1	β-D-Thd	100	100	100	
2	β-L-Thd	2 ± 1	88 ± 21		51
3	α-D-Thd	5 ± 3	21 ± 3		51
4	α-L-Thd	<0.1	24 ± 3		51
5	2′,3′-di-Thd (ddT)	40	4	4.4 ± 0.4	4,70,29,33
6	2′-methylene-2′-Thd	<0.1	4		4
7	2'-hydroxy-Thd	2	3		4
8	1-β-D-arabinofuranosyl-thymine (AraT)	<0.1	60 ± 10	54 ± 5	4,70,71,29,73,33
9	2′,3′-didehydro-2′,3′-di-Thd (D4T)	7	1 ± 0.5	0.5	71,72,73
10	3′-azido-2′,3′-di-Thd (AZT)	52	4	1.1 ± 0.3	71,72,29
11	3-methyl-Thd	43	5		75
12	3-ethyl-Thd	43	<1		75
13	3-isopropyl-Thd	17	<0.1		75
14	dUrd	77 ± 2	140	113 ± 2	73,72,29,33
15	Urd	<0.1	4		74
16	2′,3′-di-dUrd (ddU)	10	2		4
17	1-β-D-arabinofuranosyl-uracil (AraU)	≤1	20 ± 1		6,4,73
18	3′-azido-2′,3′-di-Urd	70	5		74
19	3′-methyl-dUrd	<0.1	3		74
20	3′-ethyl-dUrd	<0.1	1		74
21	2′,2′-difluoro-2′-dUrd (dFdU)	<0.1	60	0.3	71,70,74
22	5-fluoro-2′-dUrd (5-FdUrd)	95	230	92 ± 11	71,74,70,29
23	5-amino-dUrd	3	50		3
24	5-ethyl-dUrd	80	100		3
25	5-(2-bromovinyl)-2′-dUrd (BVDU)	≤1	25 ± 2	54 ± 4	6,70,73,33
26	1-(2′-deoxy-2′-fluoro-1-β-L-arabinofuranosyl)-5-iodouracil (FIAU)	76	130	93.6 ± 0.1	71,73
27	1-(2′-deoxy-2′-fluoro-β-L-arabinofuranosyl)-5-methyluracil (FMAU)	48	170	86.6 ± 1.2	71

dideoxythymidine, and 2′,3′-dideoxy-2′,3′-didehydro-thymidine (d4T, **9**) [40-42] (Table 1). On the other hand, all 3′-analogues of Thd/dUrd are poor substrates for TK2 (Table 1). 2′-Hydroxy-Thd (**7**) shows equally low activity both with TK1 and TK2 whereas arabinosyl-Thd (**8**) as well as 3′-methyl- and 3′-ethyl- dUrd (**19, 20**) are fairly good substrates for TK2 but not TK1 (Table 1). As expected, 3′-azido-Urd (**18**) is a good substrate for TK1 but is only poorly phosphorylated by TK2. Overall, dUrd nucleosides follow the same activity pattern with TK1 and TK2 as the Thd nucleosides although to a reduced extent. A limited analysis of the Dm-dNK substrate specificity revealed strong similarities to TK2 (Table 1).

Many 5-substituted dUrds, such as 5-fluoro-, 5-ethyl, and 5-bromo-dUrd (**22**, **24**, **25**), are good substrates for TK1, TK2, and Dm-dNK while 5-amino-dUrd (**23**) is primarily phosphorylated by TK2. Analogues of dUrd with bulkier substitutions, such as 5-propenyl-, 5-(2-chloroethyl)- and 5-(2-bromovinyl)-, 5-(2-thienyl)-, 5-(2-furanyl)-dUrd, are good substrates both for TK2 and Dm-dNK [43] while only the latter two analogues are also efficient substrates for HSV1-TK [44]. The anti-hepatitis B virus analogues 2′-deoxy-2′-fluoro-5-methyl-β-D-arabinofuranosyluracil (FMAU) and 2′-deoxy-2′-fluoro-5-iodo-β-D-arabinofuranosyluracil (FIAU) are efficient substrates for TK2 but showed only minimal activity with TK1 [45, 46]. The high capacity of TK2 to

activate FIAU may be a contributing factor for the severe mitochondrial toxicity observed following treatment with this analogue [47]. Unexpectedly, the L-enantiomer of FMAU was found to be a relatively good substrate both for TK1 and TK2 (Table 1) [48], thus being the only reported L-nucleoside with TK1 substrate characteristics [49]. In contrast to TK1, both TK2 and HSV1-TK show relaxed enantioselectivity since they efficiently phosphorylate L-Thd as well as other L- nucleosides, such as L-BVDU, L-FMAU, and L-5-iodo-dUrd (**26**, **27**, Table 1) [48-51 and references therein].

The elevated expression levels of TK1 in rapidly proliferating tumors, in contrast to quiescent cells, encouraged the synthesis of boronated nucleoside for boron neutron capture therapy (BNCT) [52]. The aim was to generate boron containing nucleoside analogues that are specifically phosphorylated and, consequently, entrapped in tumor cells but not in non-proliferating cells. Following radiation with low-energy neutrons, an activated form of boron undergoes fission generating cytotoxic particles that specifically kill tumor cells primarily by causing DNA double strand breaks [53]. Thd analogues with methyl, ethyl, isopropyl, as well as various bulky *o*-carboranylalkyl substituents at the N3 position were found to be surprisingly good substrates for TK1 [54, 55], while dUrd analogues with similar bulky substituents at the 5-position were not accepted as substrates by TK1. The distances between opposite hydrogen atoms within the *o*-carborane cage average ~ 5.5 Å [54], thus the volume of this highly lipophilic cluster is about 50% larger than the space occupied by the three-dimensional sweep of a phenyl group [54]. The distances between the nitrogen atom at the 3-position of Thd and the terminal hydrogen atoms of the *o*-carborane cluster in compounds **28-33** (Table 2) range from ~ 6.8 Å (compound **28**) to ~ 10.3 Å (compound **33**), as determinant by molecular mechanisms (MM+) calculations [Jayaseharan Johnsamuel, personal communication]. Although in particular those *o*-carboranyl Thd analogues with ethylene and pentylene spacers between boron cluster and the Thd scaffold showed the highest phosphorylation rates, all compounds of this series (compounds **28-39**, Table 2) were substantially phosphorylated. This indicates that TK1 can tolerate bulky groups at N3 of Thd, implying the existence of a large cavity in the active site of TK1, which is probably extending as a funnel-type opening to the surface of the enzyme.

The TK1 substrate characteristics of *o*-carboranyl Thd analogues **34-39** (Table 2), containing dihydroxypropyl groups at the *o*-carborane cage, were somewhat better than those of their non-dihydroxypropylated counterparts (**28-33**, Table 2), indicating a possible role of hydrogen bonds with amino acid residues and/or water molecules. Neither of the carboranyl-Thd analogues (**28-39**) was a substrates of TK2 and Dm-dNK.

Another series of compounds with carboranyl cage substitution at the 3′-position of Thd showed low but

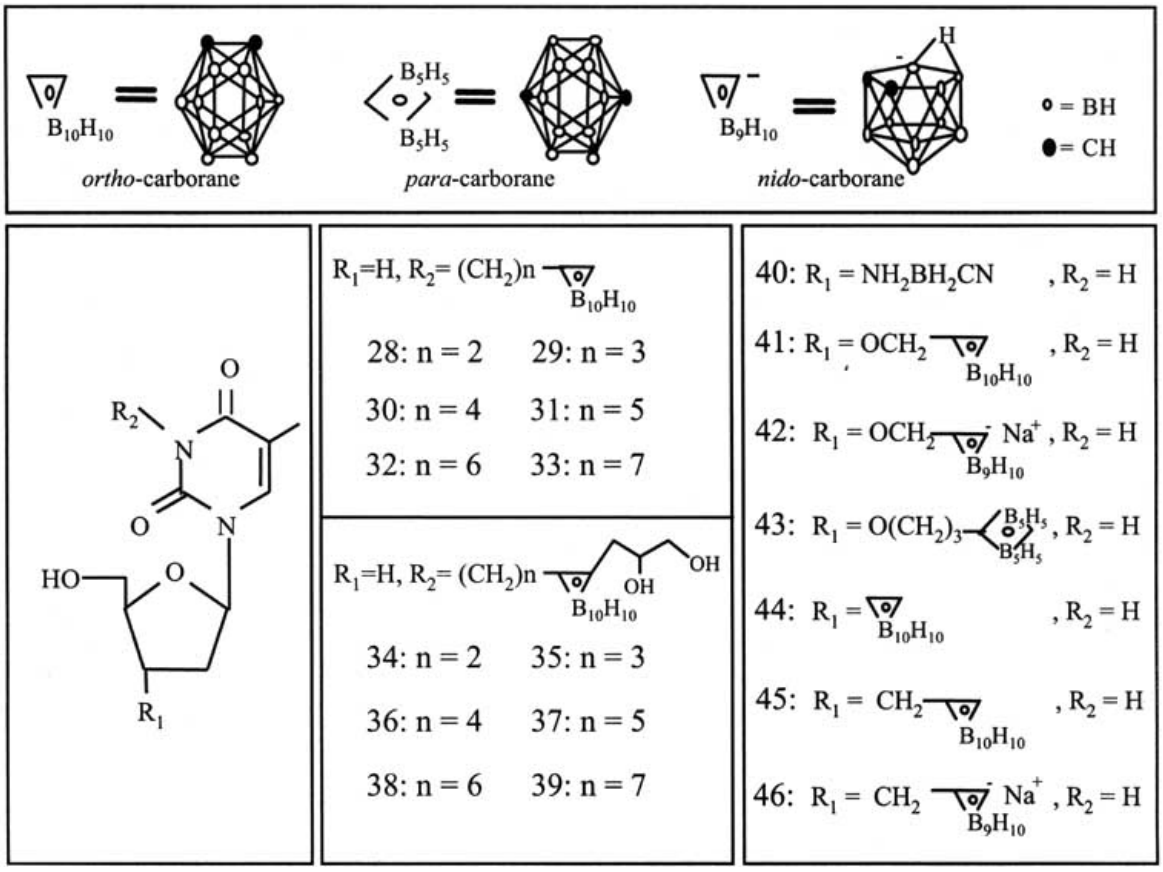


Fig. (2). The structures of compounds described in Table (2).

Table 2. The Phosphorylation of Boronated Thymidine Analogues in Relation to Thymidine. The values for thymidine (at 10 or 100 μM) were set to 100. Substrate concentrations were 100 μM for 3′-boronated thymidines, and 10 μM for 3-carboranyl-thymidines using TK1. nd: not detected

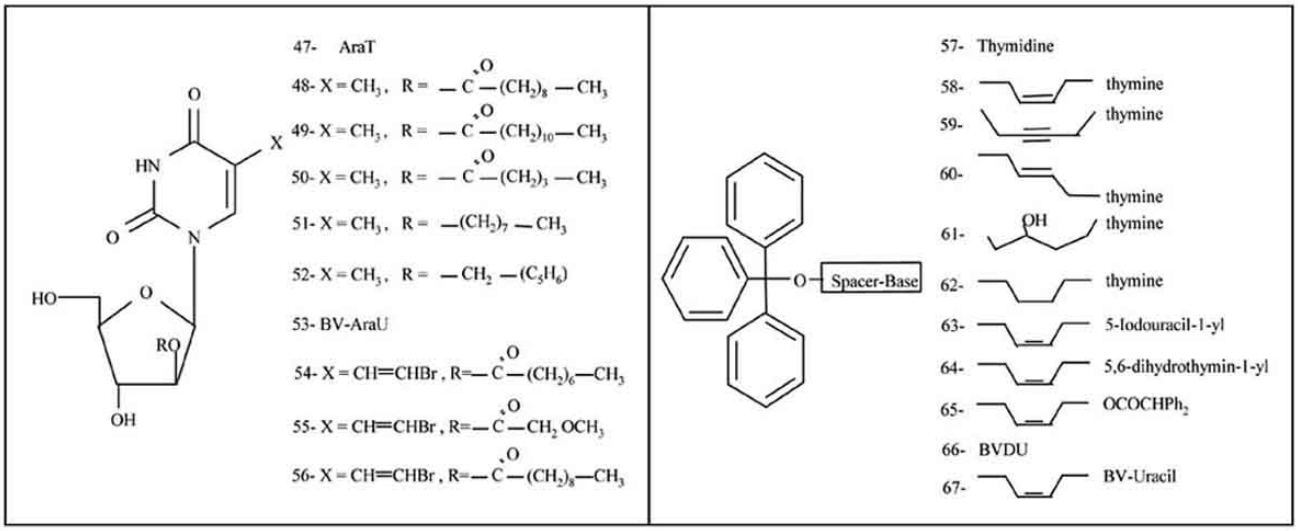
	Compound	TK1	TK2	Dm-dNK	Reference
	Thd	100	100		
28	3-[2-(*o* -carboran-1-yl)ethyl] -Thd	39 ± 3	0.2	<0.1	55
29	3-[3-(*o* -carboran-1-yl)propyl]-Thd	30 ± 5	0.2	<0.1	55
30	3-[4-(*o* -carboran-1-yl)butyl] -Thd	13 ± 4	0.3	<0.1	55
31	3-[5-(*o* -carboran-1-yl)pentyl]-Thd	41 ± 6	0.1	<0.1	55
32	3-[6-(*o* -carboran-1-yl)hexyl] -Thd	28 ± 5	<0.1	<0.1	55
33	3-[7-(*o* -carboran-1-yl)heptyl] -Thd	11 ± 3	<0.1	<0.1	55
34	3-[2-(2-(2,3-dihydroxyprop-1-yl)-*o* -carboran-1-yl)ethan-1-yl] -Thd	45 ± 3	0.2	<0.1	55
35	3-[3-(2-(2,3-dihydroxyprop-1-yl)-*o* -carboran-1-yl)propan-1-yl] -Thd	40 ± 4	0.4	<0.1	55
36	3-[4-(2-(2,3-dihydroxyprop-1-yl)-*o* -carboran-1-yl)butan-1-yl] -Thd	21 ± 1	0.3	<0.1	55
37	3-[5-(2-(2,3-dihydroxyprop-1-yl)-*o* -carboran-1-yl)pentan-1-yl] -Thd	41 ± 5	0.2	<0.1	55
38	3-[6-(2-(2,3-dihydroxyprop-1-yl)-*o* -carboran-1-yl)hexan-1-yl] -Thd	32 ± 8	0.2	<0.1	55
39	3-[7-(2-(2,3-dihydroxyprop-1-yl)-*o* -carboran-1-yl)heptan-1-yl] -Thd	13 ± 7	<0.1	<0.1	55
40	3′-cyanoborane-Thd	5.6 ± 0.3	<0.1		56
41	3′-*ortho* -carboranylmethylether-Thd	+	nd		56
42	3′-*nido* -carboranylmethylether-Thd	<0.1	nd		56
43	3′-*para* -carboranylpropylether-Thd	<0.1	nd		56
44	3′-*ortho* -carboranyl-Thd	8.1 ± 0.3	1.2 ± 0.3		56
45	3′-*ortho* -carboranylmethyl-Thd	0.8 ± 0.3	0.5 ± 0.2		56
46	3′-*nido* -carboranylmethyl-Thd	0.1 ± 0.02	<0.1		56

significant phosphorylation by TK1. Direct attachment of the carborane cage to the 3′-position of the sugar moiety without a tether gave the highest phosphorylation rate (compound **44**). Insertion of an ether group or a single methylene linkage between the carboranyl group and the sugar, as well as degradation of the *o*-carborane cage to the corresponding negatively charged *nido*-carborane, resulted in a drastic reduction of phosphorylation by TK1 (**41-43**, **45**, **46**, Table 2) [55, 56]. This indicates that TK1 is able to accommodate fairly bulky and lipophilic moieties in immediate proximity to the 3′-position of Thd. It has been suggested that the TK1 affinity of 3′-substituted Thds may be highly influenced by their electrostatic properties [57], in particular the atomic charge of the atom in the 3′-substituent adjacent to the C3′ carbon of the Thd scaffold, which may be more important than the steric properties of the 3′-substituent [58]. This appears to be the case for the 3′-azido functions in AZT (**10**) and its nitrogen adjacent to C3′ and it could also apply to the carboranyl substituent in compound **44** and the carbon atom within this cluster that is situated next to C3′ (Table 2, Fig. **2**) [59].

A series of publications has described the synthesis and evaluation of nucleoside analogues that selectively inhibit TK2 [60-64]. The IC_{50} values of a representative set of these analogues evaluated in inhibitory assays using TK1, TK2, HSV1-TK, and Dm-dNK are shown in Table 3.

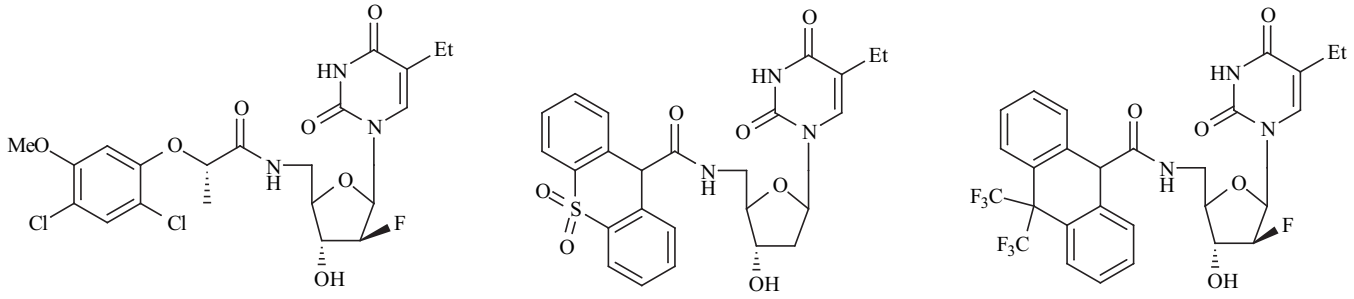
One set of non-nucleoside TK2 inhibitors is based on 5′-O-trityl derivatives of Thd and BVDU [63, 64]. Cyclic 5′-O-trityl-Thd (**57**, Table 3) and BVDU (**66**, Table 3) were found to be ~ 3-fold more efficient as inhibitors of HSV1-TK and Dm-dNK than TK2 while TK1 function was not inhibited by these agents. Compounds **58-65**, **67** (Table 3) are acyclic derivatives of Thd and BVDU that possess relative flexible, lipophilic, and very bulky side chains attached to N1 of thymine/BV-uracil that may occupy a large hydrophobic cavity common to TK2, HSV1-TK, and Dm-dNK. This finding is supported by results from extensive inhibitory studies with HSV1-TK and HSV2-TK demonstrating that in particular 2′-fluoro-2'-deoxy-5-ethyl-ara-Urd derivatives possessing bulky three-membered ring systems at the 5′-postion were highly effective inhibitors of both herpes virus enzymes as will be described below [65]. These new acyclic 5′-O-trityl analogues **58-65**, **67** were apparently stable under physiological conditions. In kinetic experiments they performed as reversible, non-competitive TK2 inhibitors, thus making them potentially useful *in vivo* TK2 inhibitors. Another type of TK2 inhibitors are 2′-O-acyl/alkyl arabinosyl nucleosides [61, 62]. Significant differences in

Table 3. Inhibitory Activity of Pyrimidine Analogues on Nucleoside Kinases Activities. The IC_{50} value is the inhibitory concentration (μM) that is required to inhibit 1 μM radio- labeled thymidine phosphorylation by 50%. NEA: not estimated accurately



	Compound	TK1	TK2	HSV-1 TK	Dm-dNK	Reference
47	1-β-D-arabinofuranosyl-thymine (AraT)	>1000	285 ± 94	24 ± 3.1	65 ± 28	61,62
48	2′-*O*-decanoyl-AraT	>1000	27 ± 2.3	≥1000	872	61,62
49	2′-*O*-dodecanoyl-AraT	>1000	28 ± 2	≥1000	>1000	61,62
50	2′-*O*-pentanoyl-AraT	>1000	>1000	≥1000	>1000	61,62
51	2′-octyl-ether-AraT	>1000	120 ± 14	>1000	≥1000	61,62
52	2′-bentyl-ether-AraT	>1000	801 ± 71	>1000	>1000	61,62
53	(*E*)-5-(2-bromovinyl)-1-β-D-arabinofuranosyl-uracil (BV-AraU)	>500	43 ± 5.8	4.3 ± 3.6	31.6 ± 10	61,62
54	2′-*O*-octanoyl-BV-AraU	>1000	6.3 ± 0.5	≥1000	178	61,62
55	2′-*O*-methyoxyacetyl-BV-AraU	>1000	402 ± 234	84 ± 1.8		61,62
56	2′-*O*-decanoyl-BV-AraU	>1000	6.8 ± 0.7	>1000	163	61,62
57	5′-*O*-trityl-thd	>100	33 ± 20	7.8 ± 0.3	12 ± 1.0	63,64
58	1-[(*Z*)-4-(triphenylmethoxy)-2-butenyl]-thymine	>100	1.5 ± 0.2	45 ± 1	3.3 ± 1.0	63,64
59	1-[4-(triphenylmethoxy)-2-butenyl]-thymine	>100	NEA	3.1 ± 0.2		63
60	1-[(*E*)-4-(triphenylmethoxy)-2-butenyl]-thymine	>100	25 ± 13	3.0 ± 0.0		63
61	(*RS*)-1-[3-hydroxy-4-(triphenylmethoxy)-butyl]-thymine	>100	3.6 ± 0.4	1.2 ± 0.7	12 ± 4.0	63,64
62	1-[4-(triphenylmethoxy)-butenyl]-thymine	>100	3.3 ± 1.2	10 ± 1		63
63	5-iodo-1-[(*Z*)-4-(triphenylmethoxy)-2-butenyl]-uracil	>100	4.6 ± 0.4	48 ± 14		63
64	1-[(*Z*)-4-(triphenylmethoxy)-2-butenyl]-5,6-dihydro-thymine	>100	9.8 ± 1.9	NEA		63
65	4-(thymin-1-yl)but-2-enyl-diphenylacetate	>100	4.6 ± 0.5	NEA		63
66	5′-*O*-trityl-BVDU	>100	64 ± 21	5.6 ± 2.7	13 ± 2.0	63,64
67	(*E*)-5-(2-bromovinyl)-1-[(Z)-4-(triphenylmethoxy)-2-butenyl]-uracil	>100	1.3 ± 1.1	>100	>100	63,64
68	5-(2-bromovinyl)-2′-dUrd (BVDU)	>100	0.3 ± 0.1	2.8 ± 1.5	2.5 ± 0.63	63,64
69	3′-spiro-(4′′-amino-1′′-2′′-oxathiole-2′′,2′′-deoxide)-5-methyl-Urd	>1000	4.6 ± 0.4			60

inhibitory effects on TK2, HSV1-TK, and Dm-dNK were observed for 2′-ara-Urd/Thd nucleosides. The unsubstituted compounds were effective inhibitors of HSV1-TK and Dm-dNK but not TK2, while derivatives 2′-substituted with long and lipophilic carbon chains were effective only in case of TK2 and but not HSV1-TK and Dm-dNK. Interestingly, this unusual inhibitory effect in case of TK2 could only be observed for 2′-substituents significantly larger than the pentanoyl-ester (compound **50**, Table 3) with an approximate effective length of 6 Å between the C=O carbon and the terminal hydrogen. It could be speculated that in case of TK2, but not HSV1-TK and Dm-dNK, the long, flexible, and



70 71 72

Fig. (3). The structure of sub-nanomolar inhibitors of HSV1-TK.

lipophilic carbon chains in compounds **48**, **49**, **54**, and **56** occupy the same hydrophobic cavity that has been suggested to be involved in the inhibitory activity of compounds **57-68** (Table 3). These 2′-ara-Urd/dThd analogues apparently did not serve as substrates for TK2 and they were subject to degradation by esterases. The latter renders these agents possibly unsuitable as *in vivo* inhibitors of TK2.

Another unusual uridine derivative, 3′-spiro-(4′′-amino-1′′-2′′-oxathiole-2′′,2′′-dioxide)-5-methyluridine (**69**) is apparently also a relatively efficient and selective inhibitor of TK2 (about 30-fold more effective than 5-methyl-Urd (Table 3) [60]

A series of very efficient inhibitors of HSV-TK has been developed based on isosteric and isoelectric analogues of Thd monophosphate e.g. phosphonate- and sulphonate-esters as well as amide and sulphonamide derivatives of 5′-amino-5′-Thd [65 and references therein]. Examples of these HSV-TK inhibitors are depicted in Fig. **3**. Tolerance of the viral enzyme for relatively bulky groups at the 5-position in the pyrimidine base, as discussed earlier and shown in Table 1, was the basis for the design of 5-ethyluridine analogue **70** (Fig. **3**). Introduction of a fluorine atom in the 2-arabinose position of this analogue further increased potency, acid stability and bioavailability [66]. This compound, as well as the other analogues presented in Fig. **3**, did not show any activity with partially purified TK1 at 10 μM concentrations. Due to very low water solubility of compound **70**, its 3′-L-valyl-ester was synthesized, which effectively suppressed HSV replication *in vitro*, as well as in murine models of recurrent herpes virus infections [67]. The antiviral effects of the more recently synthesized thioxanthene (**71**) and dihydroanthracene carboxylic acid (**72**) derivatives have not yet been published [65].

Information regarding the structure of the active site of HSV1-TK has apparently not been used in the design of these sub-nanomolar HSV-TK inhibitors (compounds **70-72**). Nevertheless, it can be concluded from these studies that HSV1-TK possesses a lipophilic region in the vicinity of the 5′-position of the nucleoside substrates, similar but not identical to that described for TK2 and Dm-dNK. Extensive knowledge of the structure of the active site of HSV1-TK [34-37] and the recent determination of the crystal structure of the highly homologous Varicella Zoster TK (VZV-TK/HSV3-TK) [68] will hopefully enable rational drug design projects, which will lead to inhibitors of viral enzymes with high selectivity and potency. An interesting recent example of such design strategies for HSV-TK based suicide gene therapy is N-methanocarba-Thd [69 and references therein] with a fixed northern type sugar conformation. This nucleoside showed anti-tumor activity in murine colon cancer cells expressing HSV1-TK [69].

CONCLUSIONS

The substrate activity profiles of the four thymidine kinases can be summarized as follows: TK1, TK2, Dm-dNK, and HSV1-TK all accept substituents at the 5-position of dUrd that are comparable in size and physicochemical properties to the methyl group (van der Walls radius: 2.00 Å). In case of TK2, Dm-dNK, and HSV1-TK, medium sized 5-substiuents of dUrd are still phosphorylated to a limited extent, while the introduction of a bulky carboranyl group results in poor substrate characteristics. It appears that in case of TK2, Dm-dNK, and HSV1-TK, but not TK1, relatively small groups at the 5-position at dUrd with the potential to interact via charge or hydrogen bonds with the enzyme may also be effective substrates. TK1 displays dramatically different substrate specificity compared to TK2, Dm-dNK, and HSV1-TK, tolerating even bulky substituents at the 3-position and, to a limited extent, also at the 3′-position. Exceptional is the tolerance of HSV1-TK for acyclic carbohydrate moieties as in the cases of acyclovir, ganciclovir and penciclovir.

The inhibitor activity profiles of the four kinases discussed in this review are less well understood, in particular that of TK1. For TK2, Dm-dNK, and HSV1-TK, 5′- and 2′-ara- modifications at various types of nucleosides, partially with bulky and highly lipophilic substituents, appear to be important factors.

Overall, research efforts during the last eight years have resulted in significant advances in the use both of substrates and inhibitors of TK1, TK2, Dm-dNK, and HSV1-TK for antiviral therapy, conventional cancer chemotherapy, suicide gene therapy, and experimental BNCT. Nevertheless, more knowledge of structural features of viral and cellular thymidine kinases is necessary to complement results from existing structure activity studies and to provide essential additional information for modeling studies and rational drug design that will guide the synthesis of new compounds with improved efficiency and minimal side effect.

Due to pivotal role of the TK1 family of enzymes in various therapeutic areas, future research efforts should

include in particular the development of selective TK1 inhibitors to be used as antiviral, anticancer and antibiotic drugs.

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