Published in final edited form as:

J Med Chem. 2010 August 12; 53(15): 5792–5800. doi:10.1021/jm1005379.

The sugar ring of the nucleoside is required for productive substrate positioning in the active site of human deoxycytidine kinase (dCK): implications for the development of dCK-activated acyclic guanine analogs

Saugata Hazra¹, Manfred Konrad², and Arnon Lavie^{1,*}

¹ Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago, 900 S. Ashland, Chicago, IL 60607, USA

² Max Planck Institute for Biophysical Chemistry, Am Fassberg 11, D-37077 Göttingen, Germany

Abstract

The low toxicity of acyclovir (ACV) is mainly due to the fact that human nucleoside kinases have undetectable phosphorylation rates with this acyclic guanine analog. In contrast, herpes virus thymidine kinase (HSV1-TK) readily activates ACV. We wanted to understand why human deoxycytidine kinase (dCK), which is related to HSV1-TK and phosphorylates deoxyguanosine, does not accept acyclic guanine analogs as substrates. Therefore, we crystallized dCK in complex with ACV at the nucleoside phosphoryl acceptor site, and UDP at the phosphoryl donor site. The structure reveals that while ACV does bind at the dCK active site, it does so adopting a non-productive conformation. Despite binding ACV, the enzyme remains in the open, inactive state. In comparison to ACV binding to HSV1-TK, in dCK the nucleoside base adopts a different orientation related by about a 60 degree rotation. Our analysis suggests that dCK would phosphorylate acyclic guanine analogs if they can induce a similar rotation.

Introduction

Human deoxycytidine kinase (dCK) demonstrates intrinsically high substrate promiscuity. Its role in the nucleotide salvage pathway is to phosphorylate the pyrimidine nucleoside deoxycytidine (dC) as well as the purine nucleosides deoxyadenosine (dA) and deoxyguanosine (dG).¹ Modifications of these physiological substrates in both the base and sugar moieties are largely tolerated by the dCK active site^{2, 3}. This has made dCK an essential enzyme for the activation of myriad nucleoside analogs used to treat both cancers^{4, 5} and viral infections.^{6, 7} Examples of tolerated substitutions include the addition of a halogen at the base (e.g. cladribine⁸), sugar (e.g. gemcitabine⁹), or at both locations (e.g. clofarabine¹⁰), and of hydroxyl group in the sugar part (e.g. AraC and fludarabine^{11, 12}). Nucleosides of the non-physiological L-form¹³ are also efficiently phosphorylated by dCK (e.g. L-dC, L-dA^{3, 14}). Remarkably, analogs of deoxycytidine in its L-form are tolerated even when the ribose moiety is replaced by a dioxolane (as in the case of troxacitabine) or oxathiolane (as in lamivudine) ring.³

*corresponding author; ¹Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago, 900 S. Ashland, Chicago, IL 60607, USA; Lavie@uic.edu; 1-312-355-5029.

Supplementary Data: A table with the crystallographic statistics (Table S1) and three figures (Figures S1, S2, and S3) are available as supplemental information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

In contrast, the guanosine analogs acyclovir (ACV; 2-amino-9-((2-hydroxyethoxy)methyl)-1H-purin-6(9H)-one) and ganciclovir (GCV; 2-amino-9-[[[(1,3-dihydroxypropan-2-yl)oxy]methyl]-6,9-dihydro-3H-purin-6-one]¹⁵ are not phosphorylated by dCK. ACV and GCV (for a schematic of these compounds see Figure 1A) are used to treat herpes infections. These compounds owe much of their lack of toxicity to the fact that human nucleoside kinases do not convert them into their monophosphate form. Thus, only in cells that possess a viral kinase such as the herpes virus thymidine kinase type 1 (HSV1-TK) do these compounds become activated. Since human dCK phosphorylates dG¹⁶ and possesses an active site that tolerates diverse modifications of the nucleoside, it is surprising that it does not activate ACV or GCV. In comparison to the parent compound dG, these antiviral compounds simply lack atoms in the sugar moiety, so steric exclusion cannot be the reason for their lack of acceptance by dCK. To understand the factors that prevent ACV or GCV from being phosphorylated by dCK we solved the crystal structure of the enzyme in complex with ACV. The crystallographic asymmetric unit contains four monomers of dCK, and we observe different binding modes of ACV in the dCK active sites. Notably, all the binding modes represent a non-productive binding conformation. Comparison of the dCK+ACV complex structure with the dCK complex structures in which dG or dA are bound^{16, 17} provide an explanation for the lack of phosphorylation activity with ACV. We extend this analysis to rationalize the ability of HSV1-TK to phosphorylate ACV/GCV, while only marginally accepting dG as a substrate.¹⁸ This molecular-level understanding can guide the design of novel acyclic nucleoside analogs that are accepted by human dCK, or alternatively, the design of dCK variants that have been endowed with the ability to phosphorylate acyclic nucleoside analogs such as ACV. Such engineered enzymes could play a role in suicide gene therapy application.

Results

Crystallization, data collection, and structure solution of the dCK+ACV+UDP complex

Human dCK catalyzes phosphoryl transfer to the nucleoside acceptor using either ATP or UTP as phosphoryl donors.^{19, 20} We obtained crystals of dCK in the presence of the nucleoside ACV at the acceptor site and the nucleotide UDP at the donor site. This complex mimics a dead-end complex, and allows us to observe a state very similar to the one expected for the substrates complex. Data to 2.1 Å resolution were collected using a home X-ray source. The structure was solved using molecular replacement with PDB entry 2ZI6 (dCK in complex with dA and UDP) as the search model. Inspection of the data revealed that the crystals were twinned. The structure was solved in the P₄₁ space group with four molecules in the asymmetric unit. Data collection and refinement statistics are presented in Table S1.

Binding of ACV to human dCK

Inspection of the nucleoside-binding sites (one in each of the four monomers in the asymmetric unit) revealed electron density consistent with a purine base. The electron density for the guanine portion of ACV was clearest in monomers A (monA) and B (monB), of lower quality in monomer D (monD), and too weak for confident modeling in monomer C (monC). In the monomers for which we modeled ACV, the guanine moiety of the nucleoside occupied similar positions. In contrast, the acyclic sugar moiety took alternate conformations between the four monomers (Figure 1, panels B, C, & D). The presence of multiple conformations for the acyclic portion of ACV was also observed in the crystal structure of HSV1-TK²¹ (Figure 1, panels E & F). This suggests that flexibility of the acyclic sugar moiety of ACV is an inherent feature of this class of nucleoside analogs, even when bound to the active site of enzymes. In our dCK structure in complex with ACV, the most easily interpretable electron density was observed for ACV bound to monB, and

consequently we will focus the analysis to this mode of ACV binding to dCK. In contrast to the diversity in binding mode observed for ACV at the nucleoside binding site, all four monomers had identical electron density for UDP at the nucleotide binding site. The structure was refined to a final crystallographic R-factors of 24.7%^{work}/28.9%^{free}.

Glutamine 97 is a critical residue in the dCK active site that is observed to recapitulate Watson-Crick type interactions with the nucleoside base.²² The ability of the glutamine side chain to rotate and appropriately place its hydrogen bond donor and acceptor moieties correctly explains the ability of dCK to bind nucleosides with contrasting hydrogen bond patterns (e.g. dA versus dG).¹⁶ In all previous structures of dCK in complex with various nucleosides, Gln97 makes use of both the hydrogen bonding donor (-NH₂ group) and acceptor (-C=O group) moieties to bind the base of the nucleoside. In contrast, the ACV complex structure reveals that the guanine base presents two hydrogen accepting moieties to the side chain of Gln97: the carbonyl group at position 6, and the nitrogen atom at position 7 (Figure 2, panels A & B). We modeled the side chain of Gln97 such that its amino group interacts with the base carbonyl group. However, this constellation places the side chain's carbonyl group in proximity (2.5 Å) to the base's N7 atom, which are both hydrogen bond acceptors. The alternative Gln97 conformation, achieved by rotating the side chain by 180 degrees, would also result in only a single favorable hydrogen bond interaction, with the other being an unfavorable interaction. The fact that ACV fails to build dual favorable hydrogen bond interactions with Gln97 may explain the inability of this nucleoside analog to inhibit the dG phosphorylation rate of dCK (data not shown).

We asked what promotes ACV's guanine moiety to bind in this seemingly unfavorable state. The reason lies in the nature of the interaction between ACV and the side chain of Glu53. In our structure we observe that the Glu53 side chain interacts favorably (distance of 2.1 Å) with the base 2-amino group (Figure 2B). Thus, it seems that this positive interaction with Glu53 compensates for the non-optimal interactions between the base and Gln97. Additional polar interactions between ACV and dCK involve the terminal hydroxyl group of the acyclic moiety and the side chain of Arg128. In terms of hydrophobic interactions between the nucleoside and enzyme, the guanine base is sandwiched by phenyl residues 96 and 137.

In summary, ACV is observed to bind in a mode not seen before with complexes of dCK and nucleosides, in which only one of the Gln97 side chain functional groups can build a favorable hydrogen bonding interaction with the nucleoside. In terms of function, the conformation of ACV adopted in the dCK active site is non-productive, since the terminal hydroxyl group of the acyclic moiety is too far (>4.4 Å) from the side chain of Glu53 (Figure 2B, red squiggly line). For productive binding to occur, the carboxylic group of Glu53 must be able to activate the nucleoside's hydroxyl group for a nucleophilic attack on the gamma-phosphate of the phosphoryl group donor (in the case of dCK, this being either ATP or UTP).

Discussion

The goal of this work is to decipher the reasons that prevent the guanine analog ACV (and its homolog GCV) from being phosphorylated by human dCK. The crystallographic studies clearly demonstrate that ACV does bind at the nucleoside-binding site of the enzyme. So why does this dG-analog not become phosphorylated, whereas dG is a relatively good dCK substrate?¹⁶ To address this question we compared the ACV binding modes observed in the dCK+ACV+UDP complex to that earlier dCK complexes observed with the purines dG and dA, which were also solved in the presence of UDP at the phosphoryl donor site^{16, 17}.

ACV binds to dCK with non-productive conformations and fails to elicit a transition into the closed and active state of the enzyme

Our previous studies of dCK in complex with the purines dG and dA revealed that the purine must bind in the *anti* conformation for correct positioning.^{16, 17} This also applies to pyrimidine binding to dCK.^{3, 14, 22, 23} Binding in the *anti* conformation places the sugar's 5'-hydroxyl group in proximity of Glu53. The putative role of this conserved carboxylic acid is to supply the catalytic base that activates the nucleoside 5'-hydroxyl group for nucleophilic attack on ATP/UTP. Such a role for Glu53 is supported by the fact that mutation of Glu53 abrogates activity (data not shown), as also seen when the analogous glutamic acid of dNK (Glu52) is mutated.²⁴ For a sequence alignment between dCK and the other human nucleoside kinases, the *Drosophila* nucleoside kinase (dNK), and HSV1-TK, see Figure 3. Thus, for productive nucleoside binding to occur, the substrate must be so positioned that its 5'-hydroxyl group can directly interact with the side-chain of Glu53.

In the case of the dCK+dG+UDP ternary complex structure (PDB ID 2ZI7),¹⁶ the distance between the dG 5'-hydroxyl group and Glu53 is 2.2 Å. Therefore, this corresponds to a productive binding mode. In contrast, in the case of ACV binding to dCK, the corresponding distance is 4.4 Å at the conformation seen in monB (Figure 2), and larger for the other monomers (ACV does not possess a ribose sugar; hence the distance is measured from the ACV terminal hydroxyl group that corresponds to a nucleoside's 5'-hydroxyl group). Based on this criterion, it is clear that ACV binds in a non-productive fashion.

In addition to mispositioning of its hydroxyl group, ACV binding fails to elicit a transition in dCK to its closed state. In most known ligand-associated structures of dCK, the enzyme adopts a so-called "closed" conformation. An example for the closed state would be the complex with dG at the nucleoside site, and UDP at the acceptor site.¹⁶ Recently, in the structure of dCK in complex with the purine dA at the acceptor site and UDP at the donor site, we observed a more open enzyme conformation (PDB ID 2ZI6).¹⁷ This "open" conformation is not consistent with catalysis, requiring an additional enzyme conformation step to bring the acceptor nucleoside and donor nucleotide within interaction distance. The superpositions of monB of the ACV+UDP complex structure on that of the dA+UDP complex (representing the open state) and on that of the dG+UDP complex (representing the closed state) are shown in Figure 4. Whereas the superposition with the open dA-containing complex structure shows a very good fit (see arrows Figure 4A), clear differences are seen with the open dG-containing complex structure (arrows, Figure 4B). The conformation adopted by dCK when bound to ACV is similar within the four monomers in the asymmetric unit (Figure S1 shows an overlay of the three monomers for which ACV was modeled). The conclusion is that ACV fails to induce the closed and active enzyme state.

Note that despite the fact that the dA+UDP complex was observed in the open inactive state, dA can be phosphorylated by dCK using UTP as phosphoryl donor. Thus, the mere fact that we observed the enzyme in its inactive state does not denote that the particular nucleoside in the complex is not a dCK substrate. It does, however, imply that a further conformational change must occur before dCK can phosphorylate the nucleoside. The fact that there is no measurable phosphorylation activity with ACV (data not shown) implies that this nucleoside gets trapped in an inactive dCK state, which is likely to be very similar to the one we observe in our structure.

The positions of Trp58 and Tyr86 act as differentiators between the open and closed enzyme states (Figure 4C). Trp58 is situated just prior, and Tyr86 just following the insert region (Figure 3). In the closed state, Trp58 is adjacent to the base of the nucleoside, but it swings away in the open state. The change of Tyr86 position is also significant between the open and closed state. Notably, Tyr86 maintains the interaction to the nucleoside 3'-OH group, as

the change in nucleoside position tracks the change in the position of Tyr86 between the two states. Interestingly, Trp58 and Tyr86 are conserved among the human nucleoside kinases family, that includes dCK, dGK, and TK2 (TK1 forms its own distinct family), in the drosophila nucleoside kinase (dNK), and in HSV1-TK (Figure 3). Thus, it seems likely that this family of nucleoside kinases uses these conserved residues to facilitate the transition to the closed and active state in response to nucleoside binding.

Insight into the ability of HSV1-TK to phosphorylate ACV

What structural feature of the nucleoside ACV prevents it from binding in a productive conformation to dCK and in inducing the closed enzyme conformation? It cannot simply be the lack of a 3'-hydroxyl group, since dideoxycytidine and the cytidine analogs lamivudine and troxacitabine are phosphorylated by dCK despite lacking a 3'-hydroxyl.^{3, 14} Toward this understanding, we first analyzed the productive binding of ACV to HSV1-TK.

The Achilles heel of the herpes virus is its kinase HSV1-TK. This nucleoside kinase is highly promiscuous being able to activate ACV and other acyclic nucleoside analogs such as GCV. This fact is exploited for achieving selective ACV activation in virally infected cells, which leads to chain termination during viral replication. Why does this kinase, a homolog of dCK (Figure 3 and S2), have the ability to phosphorylate ACV whereas dCK does not? The crystal structures of HSV1-TK in complex with thymidine (dT; PDB ID 1KIM) and with ACV have been reported (PDB ID 2KI5).²¹ The overlay of HSV1-TK active site residues from the dT and ACV complex structures is depicted in Figure 5. Analogous to our observation with dCK, the acyclic portion of ACV binds to HSV1-TK in multiple conformations. Critically, in one of the conformations (labeled ACV conf 1 in Figure 5), the hydroxyl group in ACV that would act to attack ATP is only 3.3 Å away from the side chain of Glu83. This carboxylic acid corresponds to Glu53 in dCK (Figure 3). Thus, in the HSV1-TK complex, an active conformation is attained that brings the nucleoside hydroxyl group to the correct position for being activated by the carboxylic acid. This explains why ACV is a substrate for HSV1-TK. Remember that in the case of dCK, the corresponding distance from the ACV hydroxyl group to the side chain of Glu53 is 4.4 Å or greater depending on the ACV binding mode. These distances preclude activation of ACV by Glu53. An additional point made apparent by this superposition is that ACV adopts a quasi-*syn* conformation when binding to HSV1-TK. In contrast, dT binds in the *anti* conformation.

We next wanted to understand the reasons that make dG a very poor HSV1-TK substrate (5% of the thymidine phosphorylation rate¹⁸), whereas it is a good dCK substrate.¹⁶ Since there is no crystal structure of HSV1-TK in complex with dG, we modeled its binding. One option to model dG binding to HSV1-TK assumes that the common guanine base present in ACV and dG will occupy the same position. As the nucleoside conformation for dG we used the one observed in the dCK+dG complex structure, and overlaid the guanine moiety between ACV and dG (Figure 6A). This superposition reveals that ACV in fact binds to HSV1-TK upside-down in comparison to the way dG binds to dCK. This overlay recapitulates the previous observation that ACV is in the quasi-*syn* conformation, in contrast to the *anti* conformation of dG. From this modeling study it follows that, were dG to bind to HSV1-TK and keep the same base position for the guanine moiety as seen with ACV, its sugar group would point the 5'-hydroxyl group away from the catalytic Glu83, and would in fact clash with Tyr101 (Figure 6A, red arrow). Thus, this mode of binding of dG to HSV1-TK does not occur. In other words, the residual activity observed with dG for the enzyme HSV1-TK means that dG must bind in a different mode than the dG-analog ACV.

An alternative approach to model dG binding to HSV1-TK is to assume that it would bind in an analogous fashion to dT binding to HSV1-TK, or for that matter, in a similar way as dG binds to dCK. To address this possibility we overlaid the HSV1-TK structure in complex

with dT with that of dCK in complex with dG (Figure 6B). This overlay reveals the factors that disfavor dG from binding in this manner. Most important would be the presence of a repulsive interaction between the side chain of Arg176 and the amino group present in the guanine base (Figure 6B, red arrow). An additional factor may be the lack of a positive interaction that takes place in dCK, where the side chain of Arg104 interacts with the guanine carbonyl group (Figure 6B, green arrow). HSV1-TK lacks this arginine residue and has a tyrosine in its place. The favorable role of an arginine at this position for dG phosphorylation is supported by the fact that in dGK, which phosphorylates both dG and dA, does indeed contain an arginine at this position, whereas TK2, which does not phosphorylate dG, has a methionine at this position (Figure 3). However, the lack in HSV1-TK of an arginine residue at this position may be a secondary reason for dG not being a good substrate, since dNK, which also lacks an arginine at the analogous position, can still phosphorylate dG.²⁵

In conclusion to this section, HSV1-TK binds the purine ACV in a productive fashion, but this is achieved by turning the base upside-down as compared to a physiological nucleoside. Moreover, the acyclic nucleoside analog adopts a quasi-*syn* conformation, in contrast to the *anti* conformation adopted by deoxyribose-containing nucleosides when bound to nucleoside kinases. The same explanation would apply to the similar guanine analog GCV. In fact, the two conformations seen for ACV in the HSV1-TK complex structure mimic the structure of GCV. Note that HSV1-TK binds dT in the right-side-up mode (Figure 5), and hence it is a substrate. It is the acyclic nature of the ACV/GCV sugar moiety that permits the upside-down mode of binding to HSV1-TK. Lacking this conformational flexibility in the sugar moiety, dG cannot bind in an analogous manner as ACV. Thus, the only way dG can bind to HSV1-TK is to adopt a binding mode similar to that seen with dT. Such binding has to overcome several disfavorable interactions, and this clarifies the very low rate of dG phosphorylation by HSV1-TK.

Insight into the inability of dCK to phosphorylate ACV

In complete reversal with HSV1-TK, dCK can phosphorylate dG well, but ACV not at all. Yet, it is a trivial undertaking to fit ACV in place of dG at the dCK active site, as the only difference between the two nucleosides is the lack of C2' and C3' in ACV (see Figure 1). So, why does ACV not adopt such a conformation when bound to dCK? We suggest that the presence of a rigid sugar ring is required to place dG correctly in the dCK active site, and its absence in ACV results in the mispositioned binding we observed. Supporting this hypothesis is the result of modeling dG binding to dCK in the same position as ACV (Figure S3). This analysis reveals that steric hindrance between the deoxyribose ring of dG and Met85 and Tyr86 would prevent dG binding in the same position as we observe ACV binding to dCK.

What are the unique features in HSV1-TK compared to dCK that allow the viral enzyme to phosphorylate ACV? To address this question, we overlaid the ACV complex structures of HSV1-TK (representing the active state) and dCK (representing the inactive state). Since ACV binds to HSV1-TK and dCK with multiple conformations, we limited the analysis to the ACV conformation that brought its 5'-hydroxyl-equivalent nearest to the catalytic glutamic acid (Figure 7). This comparison reveals that the guanine moiety of ACV binds at approximately the same plane (Figure 7, inset), but at a different angle between the two enzymes. Despite high conservation of active site residues between dCK and HSV1-TK, the ACV in dCK is ~60 degrees rotated versus that in HSV1-TK (Figure 7). The consequence is a longer distance – 4.4 versus 3.3 Å – between the terminal hydroxyl group and the activating carboxylic acid (Glu53 in dCK, Glu83 in HSV1-TK). This difference in orientation seems to be due to the interaction between ACV and Arg176 in HSV1-TK. Arg176 makes a bidentate interaction with the guanine base. As a result of the Arg176-

guanine base interaction, the nucleoside adopts a different orientation relative to that adopted when bound to dCK. In dCK, the residue at the equivalent position to Arg176 is a leucine, so this interaction cannot be recapitulated. Note that the difference in position of Tyr86 (dCK) and Tyr101 (HSV1-TK) is due to the fact that dCK is in the open conformation. In the closed dCK enzyme state, Tyr86 overlays perfectly on Tyr101.

Conclusion

Nucleoside analogs with an acyclic sugar moiety such as ACV and GCV have become important antiviral agents. The pharmacological action of this class of compounds is dependent on their initial activation by a viral kinase such as the HSV1-TK. In contrast, no human nucleoside kinase activates these prodrugs to a significant extent, and, in particular, human dCK, while a highly promiscuous enzyme, does not phosphorylate these guanine analogs. The structure of dCK in complex with ACV presented here, and the comparison with the dCK structure in complex with dG, exposed the unexpected finding that guanine-containing nucleosides have two potential modes of binding. If a cyclic sugar moiety is present, such as in dG, only one of these modes of binding is an option. In this case, the nucleoside is in the *anti* conformation. However, if an acyclic sugar moiety is present, as in ACV, an alternative mode of binding, where the nucleoside is in a quasi-*syn* conformation, is preferred. This analysis pertains to both dCK and HSV1-TK. The difference though is that only in HSV1-TK, does this alternative mode of binding result in ACV positioning that is consistent with catalysis. Comparison of our structure to that of HSV1-TK with ACV revealed the difference in active sites that enables HSV1-TK to phosphorylate ACV. Residue Arg176 of HSV1-TK positions ACV so that its terminal hydroxyl group is at interaction distance to the catalytic Glu83. Since dCK has a leucine residue at the analogous position, such positioning of the nucleoside does not take place. This understanding can guide the development of novel acyclic nucleosides that are phosphorylated by human dCK. The criteria for such compounds would be the inclusion of functional groups that promote an orientation as seen in HSV1-TK.

Experimental Section

All laboratory reagents were purchased from Fisher and Sigma. Human dCK was expressed and purified as described earlier.³ We used the C₄S variant (a mutant in which cysteines 9, 45, 59, and 146 were exchanged with serines) of dCK since this variant is more amenable for crystallization, and has been shown to be a good mimic of wild-type dCK.³ Crystals of dCK (at 20 mg/ml) in complex with ACV and UDP (both at 5 mM) were obtained by vapor diffusion from hanging drops (1 μ l of protein/nucleoside/nucleotide mix plus 1 μ l reservoir) using a reservoir solution that contained 0.90–1.5 M trisodium citrate dihydrate and 100 mM HEPES, pH 7.5. The crystals were grown at 12 °C. X-ray data on a frozen crystal cryoprotected with mineral oil were collected at UIC using a RAXIS-IV⁺⁺ detector mount on a Rigaku RH-200 rotating anode x-ray generator. Data were processed using XDS.²⁶ The crystal structure was solved by molecular replacement, using the program MOLREP²⁷ and dCK structure 2ZI6 as search model. Refinement of the structure was done by using REFMAC²⁸ and model rebuilding accomplished with the graphics program O.²⁹ Figures were prepared using MOLSCRIPT³⁰ and RASTER3D.³¹ The coordinates and structure factors were deposited at the RCSB under accession code 3MJR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by a National Institutes of Health grant (S.H., and A.L.) and the Max-Planck-Society (M.K.). We thank Victor Marques for helpful discussions.

Abbreviations

dCK	human deoxycytidine kinase
dGK	human deoxyguanosine kinase
dNK	Drosophila deoxynucleoside kinase
HSV1-TK	herpes simplex virus thymidine kinase type 1
ACV	acyclovir
GCV	ganciclovir

References

1. Eriksson S, Munch-Petersen B, Johansson K, Eklund H. Structure and function of cellular deoxyribonucleoside kinases. *Cell Mol Life Sci.* 2002; 59:1327–1346. [PubMed: 12363036]
2. Arner ES, Eriksson S. Mammalian deoxyribonucleoside kinases. *Pharmacol Ther.* 1995; 67:155–186. [PubMed: 7494863]
3. Sabini E, Hazra S, Konrad M, Burley SK, Lavie A. Structural basis for activation of the therapeutic L-nucleoside analogs 3TC and troxacitabine by human deoxycytidine kinase. *Nucleic Acids Res.* 2007; 35:186–192. [PubMed: 17158155]
4. Ewald B, Sampath D, Plunkett W. Nucleoside analogs: molecular mechanisms signaling cell death. *Oncogene.* 2008; 27:6522–6537. [PubMed: 18955977]
5. Galmarini CM, Mackey JR, Dumontet C. Nucleoside analogues: mechanisms of drug resistance and reversal strategies. *Leukemia.* 2001; 15:875–890. [PubMed: 11417472]
6. Cihlar T, Ray AS. Nucleoside and nucleotide HIV reverse transcriptase inhibitors: 25 years after zidovudine. *Antiviral Res.* 2010; 85:39–58. [PubMed: 19887088]
7. De Clercq E. The design of drugs for HIV and HCV. *Nat Rev Drug Discov.* 2007; 6:1001–1018. [PubMed: 18049474]
8. Lamanna N, Weiss M. Purine analogs in leukemia. *Adv Pharmacol.* 2004; 51:107–125. [PubMed: 15464907]
9. Huang P, Chubb S, Hertel LW, Grindey GB, Plunkett W. Action of 2',2'-difluorodeoxycytidine on DNA synthesis. *Cancer Res.* 1991; 51:6110–6117. [PubMed: 1718594]
10. Zhenchuk A, Lotfi K, Juliusson G, Albertioni F. Mechanisms of anti-cancer action and pharmacology of clofarabine. *Biochem Pharmacol.* 2009; 78:1351–1359. [PubMed: 19576186]
11. Cai J, Damaraju VL, Groulx N, Mowles D, Peng Y, Robins MJ, Cass CE, Gros P. Two distinct molecular mechanisms underlying cytarabine resistance in human leukemic cells. *Cancer Res.* 2008; 68:2349–2357. [PubMed: 18381442]
12. Mansson E, Spasokoukotskaja T, Sallstrom J, Eriksson S, Albertioni F. Molecular and biochemical mechanisms of fludarabine and cladribine resistance in a human promyelocytic cell line. *Cancer Res.* 1999; 59:5956–5963. [PubMed: 10606241]
13. Mathe C, Gosselin G. L-nucleoside enantiomers as antiviral drugs: a mini-review. *Antiviral Res.* 2006; 71:276–281. [PubMed: 16797735]
14. Sabini E, Hazra S, Konrad M, Lavie A. Nonenantioselectivity property of human deoxycytidine kinase explained by structures of the enzyme in complex with L- and D-nucleosides. *J Med Chem.* 2007; 50:3004–3014. [PubMed: 17530837]
15. Elion GB. Mechanism of action and selectivity of acyclovir. *Amer J of Med.* 1982; 73(1A):7–13. [PubMed: 6285736]

16. Sabini E, Hazra S, Konrad M, Lavie A. Elucidation of different binding modes of purine nucleosides to human deoxycytidine kinase. *J Med Chem.* 2008; 51:4219–4225. [PubMed: 18570408]
17. Sabini E, Hazra S, Ort S, Konrad M, Lavie A. Structural basis for substrate promiscuity of dCK. *J Mol Biol.* 2008; 378:607–621. [PubMed: 18377927]
18. Fyfe JA, Keller PM, Furman PA, Miller RL, Elion GB. Thymidine kinase from herpes simplex virus phosphorylates the new antiviral compound, 9-(2-hydroxyethoxymethyl)guanine. *J Biol Chem.* 1978; 253:8721–8727. [PubMed: 214430]
19. White JC, Capizzi RL. A critical role for uridine nucleotides in the regulation of deoxycytidine kinase and the concentration dependence of 1-beta-D-arabinofuranosylcytosine phosphorylation in human leukemia cells. *Cancer Res.* 1991; 51:2559–2565. [PubMed: 2021937]
20. Shewach DS, Reynolds KK, Hertel L. Nucleotide specificity of human deoxycytidine kinase. *Mol Pharmacol.* 1992; 42:518–524. [PubMed: 1406603]
21. Champness JN, Bennett MS, Wien F, Visse R, Summers WC, Herdewijn P, de Clerq E, Ostrowski T, Jarvest RL, Sanderson MR. Exploring the active site of herpes simplex virus type-1 thymidine kinase by X-ray crystallography of complexes with aciclovir and other ligands. *Proteins.* 1998; 32:350–361. [PubMed: 9715911]
22. Sabini E, Ort S, Monnerjahn C, Konrad M, Lavie A. Structure of human dCK suggests strategies to improve anticancer and antiviral therapy. *Nat Struct Biol.* 2003; 10:513–519. [PubMed: 12808445]
23. Hazra S, Sabini E, Ort S, Konrad M, Lavie A. Extending thymidine kinase activity to the catalytic repertoire of human deoxycytidine kinase. *Biochemistry.* 2009; 48:1256–1263. [PubMed: 19159229]
24. Egeblad-Welin L, Sonntag Y, Eklund H, Munch-Petersen B. Functional studies of active-site mutants from *Drosophila melanogaster* deoxyribonucleoside kinase. Investigations of the putative catalytic glutamate-arginine pair and of residues responsible for substrate specificity. *FEBS J.* 2007; 274:1542–1551. [PubMed: 17302737]
25. Knecht W, Sandrini MP, Johansson K, Eklund H, Munch-Petersen B, Piskur J. A few amino acid substitutions can convert deoxyribonucleoside kinase specificity from pyrimidines to purines. *Embo J.* 2002; 21:1873–1880. [PubMed: 11927571]
26. Kabsch W, Gast WH, Schulz GE, Leberman R. Low resolution structure of partially trypsin-degraded polypeptide elongation factor, EF-TU, from *Escherichia coli*. *J Mol Biol.* 1977; 117:999–1012. [PubMed: 342708]
27. Vagin A, Teplyakov A. MOLREP: an automated program for molecular replacement. *J Appl Cryst.* 1997; 30:1022–1025.
28. Murshudov GN, Vagin AA, Dodson EJ. Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr D Biol Crystallogr.* 1997; 53:240–255. [PubMed: 15299926]
29. Jones TA, Zhou JY, Cowan SW, Kjeldgaard M. Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crystallogr A.* 1991; 47:110–119. [PubMed: 2025413]
30. Kraulis PJ. MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. *J Appl Crystallogr.* 1991; 24:946–950.
31. Merritt EA, Bacon DJ. Raster3D: photorealistic molecular graphics. *Methods in Enzymology.* 1997; 277:505–524. [PubMed: 18488322]

Figure 1.

Binding of ACV to human dCK. **A.** Chemical structure of dG and its acyclic analogs GCV and ACV. **B.** Parallel and perpendicular views of ACV as bound in monA of the dCK+ACV+UDP structure. The terminal hydroxyl group in ACV that corresponds to the ribose 5'-hydroxyl is labeled. **C.** Analogous views of ACV from monB. **D.** Analogous views of ACV from monD. **E.** Similar to the situation observed in the dCK+ACV complex structure, in the HSV1-TK+ACV complex structure, ACV adopts multiple conformations. Shown are the parallel and perpendicular views of ACV in conformation 1. **F.** Analogous views of ACV in conformation 2. **G.** For reference, shown are the analogous views of dG from the dCK+dG+UDP structure.

Figure 2.

A ball-and-stick representation (**A**) and a schematic representation (**B**) of ACV binding to monB. Note the 4.4 Å distance between the side chain of Glu53 and the terminal hydroxyl group of ACV. Such a long distance precludes the activation of the nucleoside to attack the phosphoryl donor.

Figure 3.

Structure-based sequence alignment of human nucleoside kinases of the same family (deoxycytidine kinase (hdCK); deoxyguanosine kinase (hdGK); mitochondrial thymidine kinase 2 (hTK2)), drosophila nucleoside kinase (dNK) that is able to phosphorylate all four nucleosides, and thymidine kinase from herpes virus, type I (HSV1- TK). Color scheme: orange for residues conserved in all 5 sequences; yellow when at least 3 of the 5 residues are identical; the non-identical residues in such cases are also colored yellow if homologous. The P-loop and insert regions are indicated, as are select critical residues based on the dCK numbering. E53 – acts to activate the 5'-hydroxyl group; W58 and Y86 – indicators of the open and closed enzyme states; Q97 – binds the base of the nucleoside; R104 and D133 are residues we have identified to be responsible for the lack of thymidine kinase activity by dCK; R128, a part of the ERS motif, activates E53; R188 – involved in binding the nucleotide donor; R192/R194 – in the Lid region, play a role in stabilizing the transition state; E197 – seems to play a role in stabilizing the closed state by directly interacting with the 3'-hydroxyl group of the nucleoside.

Figure 4. ACV binds to dCK but does not elicit the transition to the closed enzyme state. **A.** Overlay of dCK in complex with ACV mon B (light green) and dA (blue; PDB ID 2ZI6). The nucleosides are at the center of the image, UDP at the right. The good fit between the structures shows that ACV binds to the same open enzyme state as observed previously in the complex structure of dCK with dA+UDP. For visual clarity only a single monomer of the dimeric dCK is shown. **B.** Overlay of dCK in complex with ACV mon B (light green), and dG (light blue; PDB ID 2ZI7). In the dCK complex with dG+UDP, the enzyme adopts the closed state. Arrows point to regions where the differences in enzyme conformation are most apparent. **C.** A close-up of the overlay of ACV monB (light green) and dG (light blue). Note the different conformation of Trp58 and Tyr86, which are indicators of the enzyme conformation ‘open’ in the case of the ACV complex, ‘closed’ in the dG complex. Zoom on the nucleosides (right side) demonstrates the difference of guanine base orientation between the two complexes. To aid in seeing this difference, a gray sphere highlights the guanine amino group at the 2-position. Note how the bases are related by a relative rotation of ~180 degrees.

Figure 5.

Upon binding to HSV1-TK, ACV achieves the critical close contact between the terminal hydroxyl group and the activating glutamic acid. Shown is an overlay of HSV1-TK in complex with thymidine (red; PDB ID 1KIM) and ACV (yellow; PDB ID 2KI5). ACV binds in two conformations, labeled conf1 (orange) and conf2 (yellow). Gray spheres indicate the positions of the hydroxyls that would become phosphorylated. Note that, both in the case of dT and ACV-conf1 binding, a close contact is achieved between the terminal hydroxyl group and the side chain of Glu83. Therefore, the carboxylic acid can activate either nucleoside to attack the γ -phosphate of the phosphoryl donor. Inset: side view parallel to the base of the nucleosides. While the position of the guanine base is similar to that of the thymine base, they are not totally parallel.

Figure 6.

Modeling dG binding to HSV1-TK. **A.** We superimposed a dG molecule (light blue) on the ACV molecule based on the common guanine base, using the HSV1-TK ACV structure (yellow). ACV makes positive interactions with Glu83 and Arg178 – green arrows. Were dG to bind in the same position, the ribose ring would be too close to Tyr101 (red arrow). Hence, dG cannot bind to HSV1-TK in a similar fashion as ACV. **B.** Overlay of the HSV1-TK structure in complex with dT (red) on the dCK structure in complex with dG (light blue). Arg176 would act to repel dG due to the unfavorable interactions with the guanine amino group (red arrow), and would also lack the positive interaction present in dCK between Arg104 and the guanine carbonyl group (green arrow). These factors suggest the reasons that make dG a very poor HSV1-TK substrate.

Figure 7.

Contrasting ACV binding to dCK (light green) and HSV1-TK (yellow). For clarity, only the ACV conformation that brings the terminal hydroxyl group closer to the catalytic glutamic acid is shown. In both enzymes, the guanine base is in the same location, but different by a relative rotation of ~60 degrees. The bidentate interaction between Arg176 of HSV1-TK and ACV cannot be made by dCK, where the homologous residue is Leu141. The relative rotation of the nucleoside in HSV1-TK positions the terminal hydroxyl group within 3.3 Å to Glu83. Lacking this interaction in dCK, the terminal hydroxyl is 4.4 Å from Glu53. Thus, Glu53 is too far to be able to activate the hydroxyl group for attack on the phosphoryl donor, revealing why ACV is not phosphorylated by dCK. Inset: The plane of the guanine base is slightly off-set between its position in dCK and in HSV1-TK.