Modulated Nucleoside Kinases as Tools to Improve the Activation of Therapeutic Nucleoside Analogues

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Dedicated to Professor Dieter Gallwitz on the occasion of his 65th birthday

The use of nucleoside analogues in anticancer and antiviral treatments is often impaired by the slow intracellular activation of these drugs. This problem can be addressed by the modulation of rate-limiting enzymes in the activation pathways of the nucleoside analogues. Therapeutic strategies based on the combination of optimized activating enzymes and established nucleoside drugs promise significant improvements to traditional chemotherapy.

KEYWORDS:

cancer · human immunodeficiency virus · nucleoside analogues · protein design · thymidylate kinase

1. Introduction

The synthesis of DNA in virus-infected or malignant cells represents an attractive target for the development of antagonistic drugs. In particular, nucleoside analogues which disturb DNA replication in target cells have emerged as a widely used class of drugs in anticancer and antivirus therapy.[1–3]

Nucleoside analogues generally enter cells in a nonphosphorylated state (the "prodrug") and need to become phosphorylated by several cellular or viral nucleoside and nucleotide kinases before they can interact with their target enzyme, for example, a DNA polymerase (Figure 1). This dependency on the interaction with a series of enzymes is thought to be the major source of the side effects and low efficiency that often accompany the therapeutic use of nucleoside prodrugs. In addition, it limits the chances to find new potent substances by the traditional screening of chemical libraries. Limiting nucleoside kinases can be bypassed in some cases by the use of nucleoside monophosphate (NMP) analogues whose phosphate group is chemically masked; these lipophilic NMP precursors (also called pronucleotides) are capable of entering the cells and efficiently releasing the NMP analogue.[4] Furthermore, concepts that aim to enhance the activation rate of an established prodrug by introducing catalytically optimized variants of rate-limiting enzymes of the drug's phosphorylation pathway into cells may help to overcome the limitations.

2. Cancer Treatment with Antiherpes Nucleoside Drugs and Herpes Thymidine Kinase

The human herpes simplex virus type 1 encodes its own thymidine kinase (HSV1-TK) which ensures the production of dTMP independently of the cell-cycle-regulated cellular thymidine kinase 1. The HSV1-TK has a low substrate selectivity, which allows it to phosphorylate the acyclic nucleoside prodrugs acyclovir (ACV) and ganciclovir (GCV) that are not accepted by cellular nucleoside kinases (Figure 1). Thus, the formation of ACV-MP and GCV-MP is restricted to virus-infected cells.[5] The monophosphates become further activated to their triphosphate forms by cellular kinases. Once incorporated into growing DNA, both analogues inhibit further DNA elongation and cause the rapid death of HSV-infected cells.[6, 7]

The success of ACV in the control of HSV infections has initiated a strategy to kill tumors by the selective introduction of the HSV1-*tk* gene into malignant cells, thereby rendering them sensitive for ACV or GCV. This so called "suicide gene therapy"[8] should overcome the limited specificity of common drugs in cancer chemotherapy. Several suicide gene/prodrug combinations have been tested in preclinical and clinical trials, with HSV1-*tk* and GCV being the most widely used system.[9] Nevertheless, a usually low HSV1-*tk* transfection rate and a low GCV phosphorylation rate of HSV1-TK, in part due to competition by cellular thymidine,[10] reduce the efficiency of this system. Thus, an enzyme variant, which allows the selective and efficient phosphorylation of GCV, would significantly enhance this therapeutic strategy.

In order to find such a variant, certain amino acid positions in the nucleoside-binding site of HSV1-TK were randomized. An *Escherichia coli* expression library was screened for mutants with an increased sensitivity for GCV or ACV.[11] One variant with several amino acid changes (L159I/I160F/F161L/A168F/L169M;

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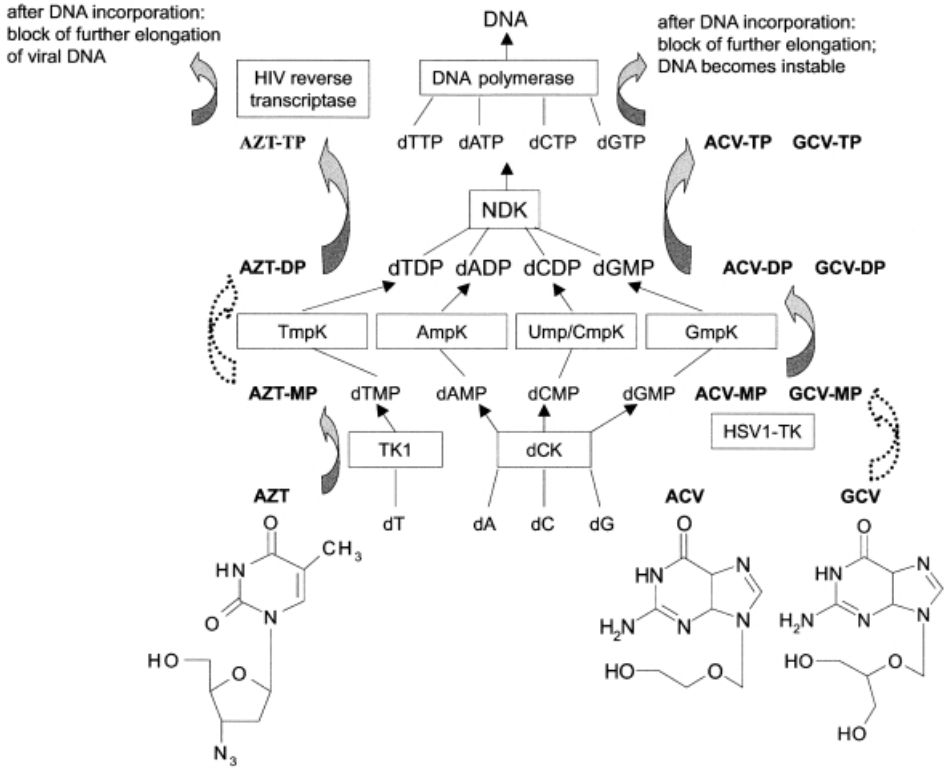

Figure 1. *Intracellular activation pathways of the antiviral nucleoside prodrugs 3′-azido-3′-deoxythymidine (AZT), acyclovir (ACV), and ganciclovir (GCV). The successive phosphorylation of these nucleoside prodrugs depends mainly on cytosolic nucleoside and nucleotide kinases. Target selectivity is mediated by the viral enzymes HIV reverse transcriptase (specific drug: AZT) or HSV1-TK (specific drugs: ACV, GCV). Bottleneck steps are indicated by dotted arrows. TK1: thymidine kinase 1, dCK: deoxycytidine kinase, HSV1-TK: herpes simplex virus type 1 thymidine kinase, TmpK: thymidylate kinase, AmpK: adenylate kinase, Ump/CmpK: uridine monophosphate/cytidine monophosphate kinase, GmpK: guanylate kinase, NDK: nucleoside diphosphate kinase, dT: deoxythymidine, dA: deoxyadenosine, dC: deoxycytidine, dG: deoxyguanosine, MP: monophosphate, DP: diphosphate, TP: triphosphate.*

Figure 2) was selected, that proved to be superior to wildtype HSV1-TK in a gene therapy approach for a xenograft tumor model in nude mice.[11]

A second approach was driven by the idea of a rational modulation of HSV1-TK based on its 3D structure in order to selectively destroy the phosphorylation activity for dT and dTMP, but not for GCV.[12–14] Residue Gln125, which mediates the preferred binding of thymidine bases, was changed to Asn, Asp, or Glu.[7] Ala167, which is located close to the thymine base of dT (Figure 2), was changed to the bulkier Tyr residue.[15] These mutants did not significantly alter the sensitivity of transfected mammalian cells to GCV compared with cells expressing wild-type HSV1-TK.[10, 15] One possible reason may be that these mutations not only impair the phosphorylation capacity of HSV1-TK for pyrimidines but also for the nucleoside analogues ACV and GCV (Table 1).

In further attempts to identify more efficient kinases, thymidine kinases of other herpes viruses were evaluated for their competence as suicide enzymes. The equine herpes virus type 4 thymidine kinase (EHV4-TK) rendered mammalian cells up to 12 times more sensitive to GCV than HSV1-TK.[19] Furthermore, an Ala143Tyr mutant of EHV4-TK (equivalent to Ala167Tyr in HSV1-TK) does not bind thymidine any more, but still efficiently phosphorylates GCV;[16] this recommends it as a promising alternative to HSV1-TK.

3. AIDS Treatment with a Modified Human Thymidylate Kinase Showing Improved Activation of the Anti-HIV Prodrug AZT

The human immunodeficiency virus (HIV) transcribes its RNA-coded genome into DNA by a virus-encoded reverse transcriptase (HIV-RT) before it integrates into the cellular genome. The dTTP analogue 3′-azido-3′-deoxythymidine triphosphate (AZT-TP) is accepted by HIV-RT almost as well as dTTP, but is a bad substrate for cellular DNA polymerases.[20] After incorporation predominantly into viral DNA, the 3′-azido group of AZT prevents further elongation of the viral DNA.

The bottleneck of the intracellular AZT activation is the conversion of AZT-MP into AZT-DP, catalyzed by the cellular thymidylate kinase (TmpK; Figure 1). As a consequence, cytotoxic AZT-MP accumulates[21] and the overall low level of AZT-TP is supposed to favor the selection of AZT-resistant virus strains.

While human and yeast TmpK have a phosphorylation rate for AZT-MP that is 200-fold slower than that for dTMP, the *E. coli* enzyme phosphorylates AZT-MP only 2-fold slower than dTMP. In order to derive the structural features of the *E. coli* TmpK that allow this efficient phosphorylation of AZT-MP and to transfer it to the human enzyme, the structures of all three proteins were solved.[22–27]

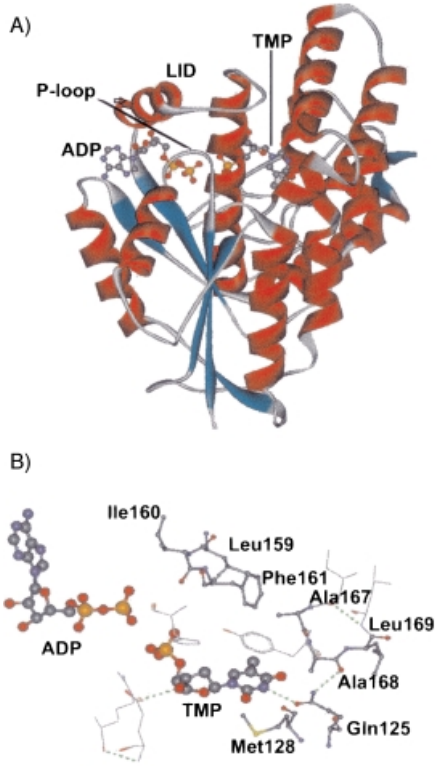

Figure 2. *Sites of modification in HSV1-TK. A) Ribbon diagram of HSV1-TK in complex with ADP and dTMP. The thymine base is embedded in the binding pocket. B) Focus on the nucleoside binding region around dTMP. Amino acid residues of the binding pocket which were analyzed by mutational studies to improve the GCV prodrug phosphorylation capacity are highlighted. Hydrogen bonds are indicated by green dots. The figure was generated from the PDB database entry 1VTK[13] with the ViewerLite 4.2 software.*

Amino acids from the flexible LID region and the phosphate binding loop (P-loop) in all three TmpK homologues are involved in the fixation of the phosphoryl donor (ATP) and acceptor (dTMP), and they also provide positive charges important for catalysis of phosphoryl transfer. The LID region of *E. coli* TmpK contains three arginine residues, which are conserved in other nucleoside monophosphate kinases (Figure 3). The middle arginine is missing in the LID motifs of human and yeast TmpK;

Table 1. *Maximum catalytic activities, k_{cat} [s^{-1}], of HSV1-TK variants and EHV4-TK for the physiological thymidine substrate and the guanosine analogue prodrugs ACV and GCV.[a]*

	dThd	ACV	GCV	Reference
HSV1-TK (wt)	0.43	0.13	0.17	[16]
HSV1-TK (Gln125Asn)	0.44	0.064	0.023	[17]
HSV1-TK (Gln125Glu)	0.003	no data	0.04	[10]
HSV1-TK (Gln125Asp)	0.1	n.d.	n.d.	[10]
HSV1-TK (Ala167Tyr)	n.d.	0.0048	0.013	[18]
EHV4-TK (wt)	0.56	0.17	0.44	[16]

[a] n.d.: not detectable.

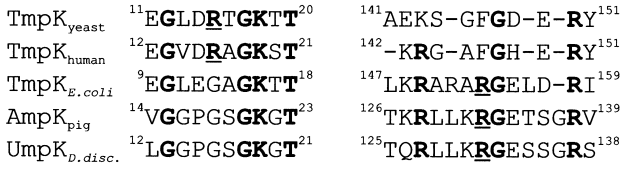

Figure 3. *Sequence alignment of the P-loop and LID motifs of different nucleoside monophosphate kinases. Conserved amino acids are marked in bold. Structurally conserved arginines that were shown to participate in phosphoryl transfer are bold and underlined.*

instead, both enzymes have an arginine in their P-loop (Arg16 in human, Arg15 in yeast) which is placed in the same spatial position in the active site as is the LID-based Arg153 in *E. coli* TmpK (Figure 4). This arginine residue is supposed to be essential for stabilization of the transition state in all TmpK enzymes.[25, 28]

The bulky azido-group of AZT-MP interferes with the aspartate next to the arginine located in the P-loop of yeast and human TmpK and induces a misplacement of the whole P-loop. Since the LID region cannot correctly close over the catalytic center, important hydrogen bonds are lost, especially the contacts of the P-loop arginine residue to the γ-phosphate group of ATP (Figure 4 A/B). This is thought to be the reason for the low phosphorylation rate of AZT-MP. In *E. coli* TmpK, however, AZT-MP is much better tolerated, such that the LID-based Arg153 still forms hydrogen bonds to the ATP γ-phosphate group and thus fulfills a catalytic role analogous to that of Arg16 in the human enzyme (Figure 4 C).

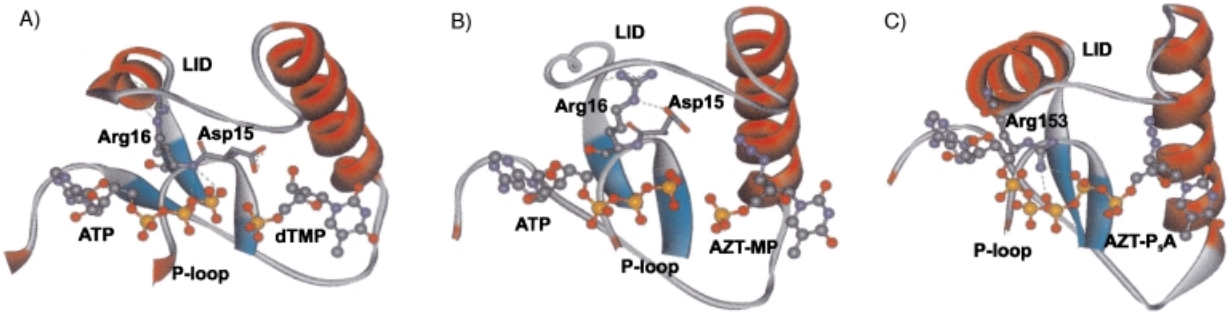

Figure 4. *Substrate interactions with the LID and P-loop motifs in human and* E. coli *TmpK. A) Focus of the LID region and P-loop of human TmpK (PDB entry 1E2Q[26]). The P-loop Arg16 interacts with the γ-phosphate of ATP through hydrogen bonds (green dots). Asp15 is located close to the 3′-OH group of the dTMP ribose. B) LID and P-loop of human TmpK with bound AZT-MP (PDB entry 1E9A[27]). The bulky azido group of AZT-MP interferes with Asp15 and induces a 0.5 Å movement of the P-loop away from the substrates; the LID region is also misplaced. C) LID and P-loop of* E. coli *TmpK with bound AZT-P_3A (PDB entry 5TMP[25]). In this protein, neither the P-loop nor the LID-region is impaired by the azido group (blue balls). The LID-based Arg153 still interacts with the ATP γ-phosphate equivalent of AZT-P_3A.*

These structural data were used for rational mutagenesis of human TmpK. The P-loop arginine residue was changed to a small and uncharged glycine residue. In addition, the LID region was replaced by that of *E. coli* TmpK in order to adapt the human TmpK to the *E. coli* homologue. Most dramatically, this mutant enzyme shows an approximately 200-fold higher activity with AZT-MP as compared to the wild-type enzyme, and, moreover, phosphorylates AZT-MP better than dTMP (Table 2). Furthermore, the levels of AZT-DP and AZT-TP in mammalian melanoma cells transfected with this mutant were increased eightfold.[29]

Table 2. *Maximum catalytic activities, k_{cat} [s^{-1}], of yeast,* E. coli, *human wild-type, and engineered human thymidylate kinases.*[28]

	dTMP	AZT-MP
yeast TmpK	35	0.175
E. coli TmpK	15	6
human TmpK	0.73	0.01
human TmpK R16G+*E. coli* LID	1.5	2.14

4. Outlook: Improved Drug-Activating Enzymes as Valuable Tools for Therapeutic Application

Traditional screening for new drugs, even in its modern form of high-throughput screening technologies, is confronted with the rising problem of declining output.[30] On the other hand, protein engineering offers techniques to understand and modulate proteins that interact with drugs. As a consequence, "protein tools" can be designed which significantly increase the efficiency of established drugs, with nucleoside prodrugs as one prominent example. Nevertheless, the task to transfer such proteins to their site of action is not yet sufficiently solved. Suicide gene therapy with HSV1-TK and GCV is in clinical phase III trials[31] but is still far away from clinical routine. Gene delivery systems need further improvements in transfection efficiency as well as target specificity. Most importantly, the recent setbacks in human gene therapy led again to questions about the safety of commonly used adenoviral[32] and retroviral vectors.[33] In some cases, protein transduction domains, which mediate the fast delivery of covalently fused proteins into a wide range of mammalian cells and tissues,[34] may serve as an alternative to DNA-based vector systems.

Taken together, the directed design and intracellular application of enzymes as tools to enhance prodrug activation is emerging as an additional concept in chemotherapy. In the long run, the combined introduction of, for example, a nucleoside prodrug and a kinase efficiently activating this prodrug may hold enormous promise in the treatment of cancer and viral infections.

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[1] C. M. Galmarini, J. R. Mackey, C. Dumontet, *Lancet Oncol.* **2002**, *3*, 415 – 424.
[2] L. Naesens, E. De Clercq, *HERPES* **2001**, *8*, 12 – 16.
[3] K. E. Squires, *Antiviral Ther.* **2001**, *6*, Suppl. 3, 1 – 14.
[4] C. Meier, *Mini Rev. Med. Chem.* **2002**, *2*, 219 – 234.
[5] P. A. Furman, M. H. St Clair, J. A. Fyfe, J. L. Rideout, P. M. Keller, G. B. Elion, *J. Virol.* **1979**, *32*, 72 – 77.
[6] S. M. Freeman, C. N. Abboud, K. A. Whartenby, C. H. Packman, D. S. Koeplin, F. L. Moolten, G. N. Abraham, *Cancer Res.* **1993**, *53*, 5274 – 5283.
[7] R. R. Drake, T. N. Wilbert, T. A. Hinds, K. M. Gibert, *J. Biol. Chem.* **1999**, *274*, 37 186 – 37 192.
[8] F. L. Moolten, *Cancer Res.* **1986**, *46*, 5276 – 5281.
[9] M. Aghi, F. Hochberg, X. O. Breakefield, *J. Gene Med.* **2000**, *2*, 148 – 164.
[10] T. A. Hinds, C. Compadre, B. K. Hurlburt, R. R. Drake, *Biochemistry* **2000**, *39*, 4105 – 4111.
[11] M. E. Black, M. S. Kokoris, P. Sabo, *Cancer Res.* **2001**, *61*, 3022 – 3026.
[12] D. G. Brown, R. Visse, G. Sandhu, A. Davies, P. J. Rizkallah, C. Melitz, W. C. Summers, M. R. Sanderson, *Nat. Struct. Biol.* **1995**, *2*, 876 – 881.
[13] K. Wild, T. Bohner, G. Folkers, G. E. Schulz, *Protein Sci.* **1997**, *6*, 2097 – 2106.
[14] J. N. Champness, M. S. Bennett, F. Wien, R. Visse, W. C. Summers, P. Herdewijn, E. De Clercq, T. Ostrowski, R. L. Jarvest, M. R. Sanderson, *Proteins* **1998**, *32*, 350 – 361.
[15] B. Degreve, R. Esnouf, E. De Clercq, J. Balzarini, *Mol. Pharmacol.* **2000**, *58*, 1326 – 1332.
[16] C. Monnerjahn, unpublished data.
[17] S. Kussmann-Gerber, O. Kuonen, G. Folkers, B. D. Pilger, L. Scapozza, *Eur. J. Biochem.* **1998**, *255*, 472 – 481.
[18] J. Balzarini, S. Liekens, R. Esnouf, E. De Clercq, *Biochemistry* **2002**, *41*, 6517 – 6524.
[19] L. Loubiere, M. Tiraby, C. Cazaux, E. Brisson, M. Grisoni, J. C. Zao-Emonet, G. Tiraby, D. Klatzmann, *Gene Ther.* **1999**, *6*, 1638 – 1642.
[20] P. A. Furman, J. A. Fyfe, M. H. St Clair, K. Weinhold, J. L. Rideout, G. A. Freeman, S. N. Lehrman, D. P. Bolognesi, S. Broder, H. Mitsuya, *Proc. Natl. Acad. Sci. USA* **1986**, *83*, 8333 – 8337.
[21] Y. Tornevik, B. Ullman, J. Balzarini, B. Wahren, S. Eriksson, *Biochem. Pharmacol.* **1995**, *49*, 829 – 837.
[22] A. Lavie, M. Konrad, R. Brundiers, R. S. Goody, I. Schlichting, J. Reinstein, *Biochemistry* **1998**, *37*, 3677 – 3686.
[23] A. Lavie, I. R. Vetter, M. Konrad, R. S. Goody, J. Reinstein, I. Schlichting, *Nat. Struct. Biol.* **1997**, *4*, 601 – 604.
[24] A. Lavie, I. Schlichting, I. R. Vetter, M. Konrad, J. Reinstein, R. S. Goody, *Nature Med.* **1997**, *3*, 922 – 924.
[25] A. Lavie, N. Ostermann, R. Brundiers, R. S. Goody, J. Reinstein, M. Konrad, I. Schlichting, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 14 045 – 14 050.
[26] N. Ostermann, I. Schlichting, R. Brundiers, M. Konrad, J. Reinstein, T. Veit, R. S. Goody, A. Lavie, *Structure* **2000**, *15*, 629 – 642.
[27] N. Ostermann, A. Lavie, S. Padiyar, R. Brundiers, T. Veit, J. Reinstein, R. S. Goody, M. Konrad, I. Schlichting, *J. Mol. Biol.* **2000**, *304*, 43 – 53.
[28] R. Brundiers, A. Lavie, T. Veit, J. Reinstein, I. Schlichting, N. Ostermann, R. S. Goody, M. Konrad, *J. Biol. Chem.* **1999**, *274*, 35 289 – 35 292.
[29] L. Loubière, personal communication.
[30] D. F. Horrobin, *Nat. Biotechnol.* **2001**, *19*, 1099 – 1100.
[31] K. Yazawa, W. E. Fisher, F. C. Brunicardi, *World J. Surg.* **2002**, *26*, 783 – 789.
[32] N. Somia, I. M. Verma, *Nat. Rev. Genet.* **2000**, *1*, 91 – 99.
[33] E. Check, *Nature* **2002**, 420, 116 – 118.
[34] J. Wadia, S. F. Dowdy, *Curr. Opin. Biotechnol.* **2002**, *13*, 52 – 56.

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