ELSEVIER

Biochemical Pharmacology

Biochemical Pharmacology 61 (2001) 727–732
Short communication

2′-*O*-Acyl/alkyl-substituted arabinosyl nucleosides as inhibitors of human mitochondrial thymidine kinase

Jan Balzarini[a,*], Bart Degrève[a], Chaoyong Zhu[b], Elisa Durini[c], Luca Porcu[c], Erik De Clercq[a], Anna Karlsson[b], Stefano Manfredini[c]

[a]*Rega Institute for Medical Research, K.U. Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium*
[b]*Division of Clinical Virology, Huddinge University School, S-141 86 Huddinge/Stockholm, Sweden*
[c]*Dipartimento di Scienze Farmaceutiche, Universita di Ferrara, I-44100 Ferrara, Italy*

Received 19 June 2000; accepted 28 August 2000

Abstract

Introduction of a bulky lipophilic acyl entity at the 2′-OH position of both 1-β-D-arabinofuranosylthymine (araT) and (*E*)-5-(2-bromovinyl)-1-β-D-arabinofuranosyluracil (BVaraU), consistently resulted in a marked (~10-fold) increase in the inhibitory activity of these new arabinosyl nucleoside analogues for the mitochondrial thymidine kinase (TK-2)-catalysed conversion of 2 μM [*methyl*-^{3}H]dThd to [*methyl*-^{3}H]dTMP. The most potent derivatives were inhibitory to [*methyl*-^{3}H]dThd phosphorylation by TK-2 within the lower micromolar concentration range. Substitution of the arabinosyl nucleoside derivatives with the acyl groups also dramatically increased the selectivity of these compounds. The inhibitory activity of araT and BVaraU to dThd phosphorylation by other related nucleoside kinases, including herpes simplex virus type 1 TK, varicella–zoster virus TK, and cytosolic TK-1, was completely annihilated upon 2′-*O*-acyl substitution ($IC_{50} \geq 1000$ μM). Kinetic analysis revealed purely competitive inhibition of 2′-*O*-acyl-BVaraU against TK-2-catalysed thymidine phosphorylation (K_i/K_m: 2.3). However, 2′-*O*-acyl-BVaraU was extremely poorly converted to the corresponding arabinosyl nucleoside 5′-monophosphate by TK-2 as revealed by [γ-^{32}P]phosphate transfer studies from [γ-^{32}P]ATP. Thus, the 2′-*O*-acyl derivatives of BVaraU did not behave as substrates, but rather as potent and highly selective inhibitors of TK-2. This is the first report on such a highly selective arabinosyl nucleoside inhibitor of mitochondrial TK-2, and opens perspectives for the rational design of selective mitochondrial TK-2 inhibitors.

Keywords: Thymidine kinase-2; Mitochondria; Antivirals; Anticancer; BVaraU; AraT

1. Introduction

There exist two different 2′-deoxynucleoside kinases in the cells that phosphorylate thymidine to its 5′-monophosphate derivative dTMP [1]. One enzyme is a cytosolic dThd kinase, designated TK-1, and the other is a mitochondrial dThd kinase, designated TK-2. Whereas TK-1 solely recognises the natural pyrimidine nucleosides dThd and dUrd, TK-2 phosphorylates, in addition to dThd and dUrd, 2′-deoxycytidine (dCyd) as well [2–4]. Interestingly, herpes simplex virus-specified 2′-deoxynucleoside kinase has a similar substrate specificity as TK-2, and it has been shown that mitochondrial TK-2 is sequence-related to HSV-1 TK [5], whereas TK-1 is not sequence-related to any of the mammalian or herpes virus nucleoside kinases. Thymidine kinases are important in view of their capacity to phosphorylate (activate) a number of anticancer and antiviral nucleoside analogues. It has indeed been demonstrated that the anticancer agent 5-fluoro-dUrd is a fairly good substrate for TK-1. Also, 3′-azido-2′,3′-dideoxythymidine (zidovudine), 2′,3′-didehydro-2′,3′-dideoxythymidine (d4T, stavudine), and FIAU are well recognised by cytosolic TK-1. In contrast, several antiviral nucleoside analogues such as (*E*)-5-(2-bromovinyl)-dUrd, (*E*)-5-(2-bromovinyl)-araU, and acyclovir are exclusively recognised by herpetic thymidine

* Corresponding author. Tel.: +32-16-33.73.41; fax: +32-16-33.73.40.
E-mail address: jan.balzarini@rega.kuleuven.ac.be (J. Balzarini).

Abbreviations: dThd, 2′-deoxythymidine; TK-1, cytosolic thymidine kinase; TK-2, mitochondrial thymidine kinase; dUrd, 2′-deoxyuridine; AZT, 3′-azido-2′,3′-dideoxythymidine; FIAU, 2′-fluoro-5-iodo-1-β-D-arabinofuranosyluracil; BVDU, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; BVaraU, (*E*)-5-(2-bromovinyl)-1-β-D-arabinofuranosyluracil; HSV, herpes simplex virus; VZV, varicella–zoster virus; and araT, 1-β-D-arabinofuranosylthymine.

0006-2952/01/$ – see front matter
PII: S0006-2952(01)00529-9

kinases (BVDU, BVaraU, ACV) and by mitochondrial TK-2 (BVDU, BVaraU) [5–9].

Several highly specific and potent inhibitors of HSV-1 and HSV-2 TK have been described [10–12]. These inhibitors are nucleoside analogues generally containing a (substituted) guanine or thymine base and an acyclic or intact (deoxy)ribose sugar part, substituted at the 5′-position with a bulky lipophilic entity. HSV TK inhibitors have been shown to play a role in preventing HSV reactivation from the ganglia [10,12]. In contrast, no potent or specific inhibitors have been described for mammalian thymidine kinases TK-1 and TK-2. However, the availability of specific TK-2 inhibitors may be useful for several reasons. They can be helpful in delineating the physiological role of mitochondrial TK-2 in maintaining the integrity of the mitochondria and its contribution to specific mitochondrial or cellular events (e.g. mitochondrial DNA damage repair). Mitochondrial TK-2 has been envisaged as a determinant of mitochondrial toxicity of the anti-hepatitis B virus (HBV) drug FIAU [13], as well as the anti-human immunodeficiency virus (HIV) drug AZT [14]. Thus, inhibitors of TK-2 could be helpful in protecting liver and muscle, which mainly consist of resting and terminally differentiated cells that almost solely express TK-2 (and not TK-1), from the toxic effects of the antimetabolites (i.e. FIAU and AZT) that are recognized as substrates by TK-2. In this study, we identified a new class of compounds that inhibit TK-2 without affecting the closely related HSV-1 TK, VZV TK, or cytosolic TK-1.

2. Materials and methods

2.1. Test compounds

2′-*O*-Substituted BVaraU and araT derivatives were prepared and characterised as will be described in detail in a forthcoming work. Briefly, the 5′,3′-protected araT and BVaraU derivatives were treated with the appropriate acyl or alkyl chloride to obtain the expected 2′*O*-acyl or 2′-*O*-alkyl derivatives and in turn de-protected at the 5′- and 3′-positions to give the 2′-*O*-substituted arabinosyl nucleosides [15]. The chemical structures and nature of the 2′-*O*-substituents of these compounds are shown in Table 1.

2.2. Enzyme

Human TK-2 cDNA was inserted into the *pGEX-5X-1* vector, expressed as fusion protein to glutathione *S*-transferase, and purified as previously described [4]. HSV-1 TK and VZV TK were prepared and characterised as described previously [16,17].

2.3. Radiochemicals

The radiolabelled substrate [*methyl*-^{3}H]dThd (70 Ci/mmol) was obtained from Moravek Biochemicals. [γ-^{32}P]ATP (3000 Ci/mmol) was from Amersham Pharmacia Biotech.

*2.4. Thymidine kinase assay using [*methyl-^{3}*H]dThd as substrate*

The activity of purified TK-1 and recombinant TK-2 was assayed in a 50-μL reaction mixture containing 50 mM Tris–HCl, pH 8.0, 2.5 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS), 3 mg/mL of BSA, 2.5 mM ATP, the indicated concentrations of [*methyl*-^{3}H]dThd and enzyme. The samples were incubated at 37° for 30 min in the presence or absence of different concentrations of the test compounds. Aliquots of 45 μL of the reaction mixtures were spotted on Whatman DE-81 filter paper disks. The filters were washed 3 times for 5 min in 1 mM ammonium formate, once for 1 min in H_2O, and once for 5 min in ethanol. Radioactivity was determined by scintillation counting, and K_m, K_i, and V_{max} values were derived from Lineweaver–Burk plots. IC_{50} values were derived from enzyme assays in which 2 μM [*methyl*-^{3}H]dThd was used as the radiolabelled substrate.

2.5. Phosphoribosyl transfer assay using [γ-^{32}P]ATP as phosphate donor

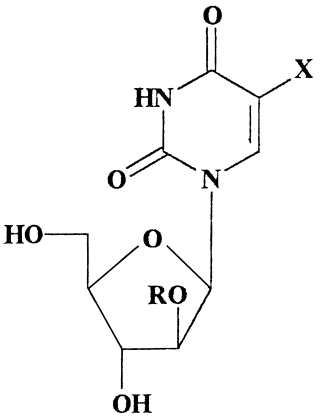
The [^{32}P]phosphoryl transfer assay was performed using [γ-^{32}P]ATP (3000 Ci/mmol, Amersham Pharmacia Biotech). dThd and the arabinosyl nucleosides were added at a final concentration of 5 and 100 μM in a 10-μL reaction mixture containing 50 mM Tris, pH 8.5, 5 mM $MgCl_2$, 1 mM unlabelled ATP, 100 μCi [γ-^{32}P]ATP, and 1 μg of recombinant TK-2. The samples were incubated for 30 min at 37°. Two microlitres of the reaction mixtures was spotted on polyethyleneimine-cellulose F TLC sheets (Merck), and the ribonucleotides were separated in a buffer containing NH_4OH:isobutyric acid:distilled H_2O (1:66:33). The sheets were autoradiographed using phosphorimaging plates (BAS 1000, Fujix).

3. Results

*3.1. Inhibitory activity and selectivity of new 2′-O-*acyl derivatives of araT and BVaraU against [methyl-^{3}*H]dThd phosphorylation by thymidine kinase from different origins*

AraT is recognised as a good substrate by the nucleoside kinases encoded by HSV-1 and VZV (Table 1). The phosphorylation of 2 μM [*methyl*-^{3}H]dThd catalysed by these viral enzymes was inhibited by this drug at an IC_{50} between 17 and 24 μM. Mitochondrial TK-2 showed a 10-fold lower sensitivity to the inhibitory activity of araT against dThd phosphorylation (IC_{50}: 285 μM), whilst no sign of inhibition

Table 1
Inhibitory effects of araT and BVaraU derivatives on 2 μM [*methyl*-^{3}H]dThd phosphorylation by 2′-deoxynucleoside kinases from different origin



	R	IC_{50}[a] (μM)			
		TK-1	TK-2	HSV-1 TK	VZV TK
AraT derivatives					
X					
—CH_3	—H	>1000	285 ± 94	24 ± 3.1	17 ± 8.7
—CH_3	—C(=O)—$(CH_2)_8CH_3$	>1000	27 ± 2.3	≥1000	>1000
—CH_3	—C(=O)—$(CH_2)_{10}CH_3$	≥1000	28 ± 2	≥1000	>1000
—CH_3	—C(=O)—$(CH_2)_3$—CH_3	>1000	>1000	>1000	>1000
—CH_3	—$(CH_2)_7CH_3$	>1000	120 ± 14	>1000	>1000
—CH_3	—$CH_2(C_5H_6)$	>1000	801 ± 71	>1000	>1000
BVaraU derivatives					
X					
—CH=CHBr	—H	>500	43 ± 5.8	4.3 ± 0.36	3.3 ± 1.1
—CH=CHBr	—C(=O)—$(CH_2)_6CH_3$	>1000	6.3 ± 0.5	≥1000	718 ± 59
—CH=CHBr	—C(=O)—$(CH_2)_8CH_3$	>1000	6.8 ± 0.7	>1000	845 ± 30
—CH=CHBr	—C(=O)—CH_2OCH_3	>1000	402 ± 234	84 ± 1.8	35 ± 1.5

[a] 50% inhibitory concentration, or compound concentration required to inhibit the enzyme-catalysed phosphorylation of 2 μM [*methyl*-^{3}H]dThd by 50%.

of TK-1-catalysed dThd phosphorylation was noted at 1 mM. Introduction of bulky acyl groups (i.e. decanoyl and dodecanoyl ester) at the 2′-OH ("up") position of araT increased the inhibitory effect of the araT derivatives on dThd phosphorylation by mitochondrial TK-2 10-fold, whereas the inhibition of dThd phosphorylation by HSV-1 and VZV TK by these compounds was decreased >50-fold. Thymidine phosphorylation by TK-1 was insensitive to araT and the 2′-*O*-acyl-araT derivatives. Thus, introduction of a bulky acyl group at the 2′-position of araT increased its selectivity more than 500-fold for TK-2 versus the related viral thymidine kinases. Shortening the 2′-*O*-acyl chain to a pentanoyl resulted in a complete loss of its inhibitory activity against TK-2-catalysed dThd phosphorylation. In order to overcome the possible hydrolysis of the 2′-*O*-acyl-araT derivatives by esterases, the 2′-octyl- and 2′-*O*-benzyl ethers were also synthesised. However, in both cases, the inhibitory activity of the 2′-*O*-alkyl-araT derivatives against dThd phosphorylation was inferior to that found for the 2′-*O*-acyl ester derivatives (Table 1).

Since BVaraU has a 7-fold greater affinity for TK-2, HSV-1 TK, and VZV TK than araT, and encouraged by the marked increase in inhibitory activity of the 2′-*O*-acyl-araT derivatives against dThd phosphorylation, 2′-*O*-acyl-substituted BVaraU derivatives were synthesised. Substitution at the 2′-OH ("up") position of BVaraU by octanoyl and

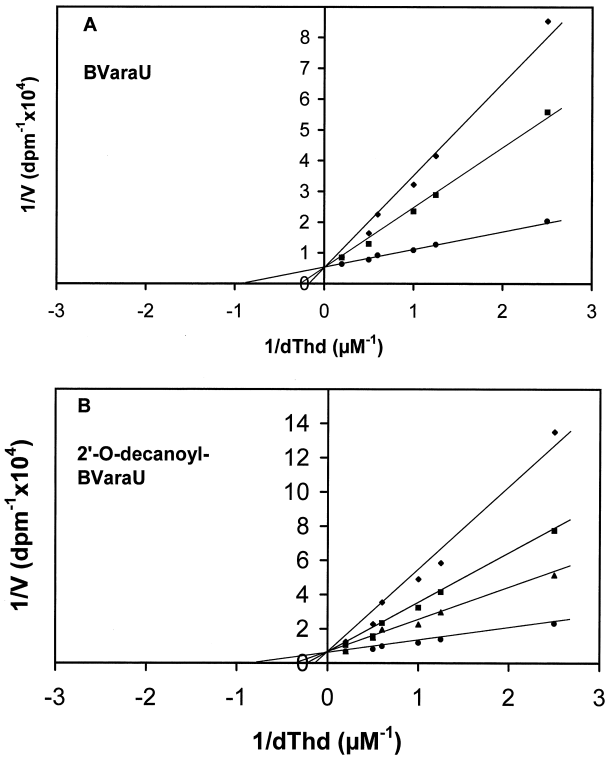


Fig. 1. Lineweaver–Burk plots for mitochondrial TK-2 in the presence of 50 μM (◆), 25 μM (■), or 0 μM BVaraU (panel A) and 25 μM (◆), 10 μM (■), 5 μM (▲), and 0 μM 2′-*O*-acyl-BVaraU (i.e. decanoyl ester of BVaraU) (panel B), with [*methyl*-^{3}H]dThd as the natural (competing) substrate.

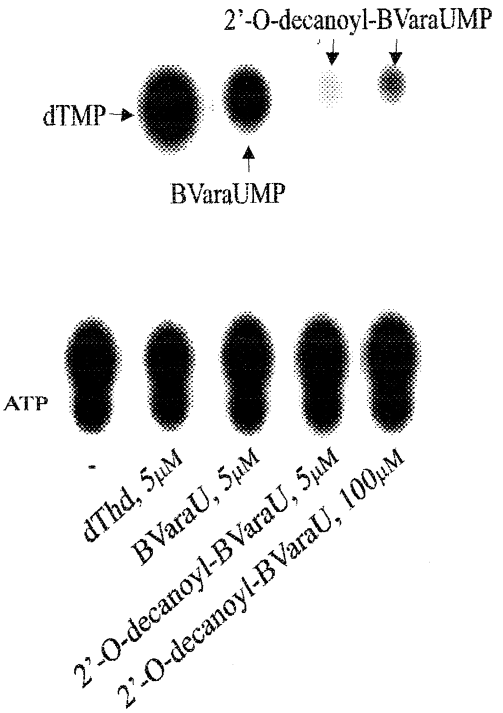


Fig. 2. Phosphorylation of dThd, BVaraU, and 2′-*O*-acyl-BVaraU (i.e. decanoyl ester of BVaraU) by mitochondrial TK-2 in a [^{32}P]phosphate transfer assay from [γ-^{32}P]ATP. The compounds were exposed to TK-2 at 5 and 100 μM. The upper spots represent the formation of [^{32}P]monophosphate products. The spots at the bottom (start) of the thin-layer chromatogram represent [^{32}P]ATP. The radiolabelled spots just above the [^{32}P]ATP spots most likely represent a radiolabelled (i.e. ATP) adduct to the enzyme, because control experiments lacking test compound contain an identical spot, whereas control experiments lacking TK-2 have a markedly decreased radiolabelled spot at this location (data not shown) (a slight radiolabelled spot can still be observed, most likely due to a radiolabelled adduct to BSA, present in the reaction mixture).

decanoyl moieties further markedly increased the inhibitory effect of BVaraU on TK-2-catalysed dThd phosphorylation by 7-fold (Table 1). IC_{50} values against TK-2 as low as 6–7 μM were obtained. As observed for the araT derivatives, the 2′-*O*-acyl-BVaraU derivatives lost their inhibitory activity against dThd phosphorylation by HSV-1 TK and VZV TK (IC_{50}: 3–4 μM for BVaraU vs 845–>1000 μM for the 2′-*O*-acyl-BVaraU derivatives). The less bulky 2′-*O*-methoxyacetyl derivative of BVaraU lost 10- to 20-fold inhibitory activity against TK-2, HSV-1 TK, and VZV TK. Again, TK-1 remained insensitive to all substituted BVaraU derivatives (Table 1).

3.2. Enzyme kinetic analysis of BVaraU and 2′-O-acyl-BVaraU

Both the parental BVaraU and the 2′-*O*-decanoyl-BVaraU derivative were subjected to detailed kinetic analysis. Two concentrations of BVaraU (50 and 25 μM) and 2′-*O*-decanoyl-BVaraU (10 and 5 μM) were added to six different [*methyl*-^{3}H]dThd concentrations (ranging from 0.4 to 5 μM) and incubated in the presence of mitochondrial TK-2. In both cases, the TK-2-catalysed dThd phosphorylation was competitively inhibited by the compounds (Fig. 1). The K_m value for dThd was 1.2 μM, while the K_i values were 9.2 for BVaraU versus 2.3 for 2′-*O*-decanoyl-BVaraU. Thus, the 2′-*O*-acyl derivative of BVaraU acquired a marked affinity for TK-2 and showed purely competitive inhibition with respect to dThd as the natural substrate.

3.3. Phosphoryl transfer from [γ-^{32}P]ATP to 2′-O-acyl-BVaraU

Experiments were designed to reveal whether the 2′-*O*-acyl-BVaraU derivative acted as either an efficient substrate or a specific inhibitor of mitochondrial TK-2 (Fig. 2). TK-2-catalysed [^{32}P]phosphate transfer from γ-^{32}P-labelled ATP was measured in the presence of 5 and 100 μM 2′-*O*-decanoyl-BVaraU. In parallel experiments, the natural substrate dThd and BVaraU acted as control substrates of the reaction. Whereas both dThd and BVaraU proved to act as very efficient substrates of TK-2, the 2′-*O*-decanoyl-BVaraU was very poorly converted to its 5′-monophosphate derivative under similar experimental conditions.

4. Discussion

In contrast with cytosolic TK-1, mitochondrial TK-2 has long been a poorly studied enzyme. Moreover, its physiological role in mitochondrial functioning and integrity is not well understood. Nowadays, there is increased interest in mitochondrial function and the role of mitochondrial nucleoside salvage enzymes in the phosphorylation and antiviral/cytostatic activity of antiviral, antimetabolic, and anticancer compounds. TK-2 was recently expressed in *Escherichia coli* and the recombinant enzyme became available for structural and biochemical studies [4]. It has been shown that TK-2 has sequence homology with the herpes virus nucleoside kinases (i.e. HSV TK and VZV TK), but not with TK-1, and mimics more closely the properties of HSV TK and VZV TK with regard to substrate recognition than does cellular TK-1 [5]. Besides its recognition of dThd, dUrd, and 2′-deoxycytidine (dCyd) as natural substrates, TK-2 also efficiently recognises pyrimidine nucleoside analogues such as BVDU and BVaraU, two antiherpetic compounds that are efficient substrates for HSV-1 and VZV TK but are not recognised as substrate by TK-1 [5,18].

In the present study, we have demonstrated that substitution of pyrimidine arabinosyl nucleoside derivatives at the 2′-OH position of the sugar moiety by a bulky lipophilic acyl moiety markedly enhances their affinity for the mitochondrial enzyme and their inhibition of dThd phosphorylation catalysed by TK-2. In addition, it dramatically increases the selectivity of these compounds for TK-2 and shifts the capacity of these novel compounds from an efficient substrate (i.e. BVaraU) to an effective (competitive) inhibitor of the enzymatic reaction (i.e. 2′-*O*-acyl-BVaraU). Introduction of such acyl groups in two different arabinosyl nucleoside analogues (araT and BVaraU) consistently increased affinity and selectivity for TK-2. Recently, Kierdaszuk *et al*. [19] reported the sugar-modified 3′-hexanoylamino-2′,3′-dideoxythymidine as a discriminator between TK-1 and TK-2, with a K_i of ~600 μM for TK-1 and 0.1 μM for TK-2. However, ^{32}P transfer experiments have not been carried out to reveal whether this compound acted as a substrate or as an inhibitor of TK-2. From our studies, we could conclude that, in contrast to dThd and BVaraU, 2′-*O*-acyl-substituted BVaraU derivatives predominantly act as potent and selective inhibitors of the TK-2 enzyme. Studies are currently ongoing to optimise the TK-2 inhibitor leads (i.e. by introducing a 5′-substitution in the molecules as is present in selective inhibitors of the herpes simplex virus TKs [11] and by increasing the stability of the lead structures). Indeed, the 2′-*O*-acyl derivatives of BVaraU and araT are unstable in cell culture. We obtained strong evidence that the 2′-*P*-acyl derivatives of BVaraU were readily converted to the parent compound in cell culture, displaying a similar potent anti-herpes simplex virus type 1 activity to that of the parent BVaraU, but completely losing their anti-herpes activity against TK-deficient HSV-1 strains.[1] Improvement of the stability of the compounds against esterases is a prerequisite for enabling the drugs to eventually reach, in an intact (inhibitory) form, the TK-2 enzyme located in the mitochondrial compartment.

The availability of specific TK-2 inhibitors will be useful for a number of applications. Selective TK-2 inhibitors can be used in studies on the role of mitochondrial TK-2 in the phosphorylation of nucleoside analogues and in investigations into mitochondrial DNA repair and DNA damage caused by antiviral and anticancer nucleoside analogues [14]. Furthermore, the unexpected delayed adverse effects, allegedly due to mitochondrial toxicity, caused by nucleoside analogues such as the anti-HBV drug FIAU, could possibly be avoided in the presence of potent and specific TK-2 inhibitors. This report describes new lead inhibitors of mitochondrial TK-2 that should permit such studies after these compounds have been further optimised in terms of inhibitory potential against TK-2 and stability (resistance) against esterases.

Acknowledgments

This work was supported by grants from the Medical Faculty of the Karolinska Institute, the Biomedical Research Programme of the European Commission, the “Geconcerteerde Onderzoeksacties (GOA)” (Krediet no. 00/12), the “Belgische Federatie tegen Kanker”, and Ferrara University. We thank Mrs. Lizette van Berckelaer for excellent technical assistance and Mrs. Christiane Callebaut for dedicated editorial help. B.D. is the recipient of a fellowship from the “Belgische Federatie tegen Kanker”.

References

[1] Arnér ES, Eriksson S. Mammalian deoxyribonucleoside kinases. Pharm Ther 1995;67:155–86.
[2] Munch-Petersen B, Cloos L, Tyrsted G, Eriksson S. Diverging substrate specificity of pure human thymidine kinases 1 and 2 against antiviral dideoxynucleosides. J Biol Chem 1991;266:9032–8.
[3] Eriksson S, Kierdaszuk B, Munch-Petersen B, Öberg B, Johansson NG. Comparison of the substrate specificities of human thymidine kinase 1 and 2 and deoxycytidine kinase toward antiviral and cytostatic nucleoside analogs. Biochem Biophys Res Commun 1991;176:586–92.
[4] Johansson M, Karlsson A. Cloning of the cDNA and chromosome localization of the gene for human thymidine kinase 2. J Biol Chem 1997;272:8454–8.
[5] Johansson M, van Rompay AR, Degrève B, Balzarini J, Karlsson A. Cloning and characterization of the multisubstrate deoxyribonucleoside kinase of *Drosophila melanogaster*. J Biol Chem 1999;274:23814–9.

[1] Andrei G, Snoeck R and De Clercq E, unpublished observations.

[6] Elion GB, Furman PA, Fyfe JA, de Miranda P, Beauchamp L, Schaeffer HJ. Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl)guanine. Proc Natl Acad Sci USA 1977;74: 5716–20.
[7] Balzarini J, Baba M, Pauwels R, Herdewijn P, De Clercq E. Anti-retrovirus activity of 3′-fluoro- and 3′-azido-substituted pyrimidine 2′,3′-dideoxynucleoside analogues. Biochem Pharmacol 1988;37:2847–56.
[8] Balzarini J, Herdewijn P, De Clercq E. Differential patterns of intracellular metabolism of 2′,3′-didehydro-2′,3′-dideoxythymidine (D4T) and 3′-azido-2′,3′-dideoxythymidine (AZT), two potent anti-HIV compounds. J Biol Chem 1989;264:6127–33.
[9] Wang J, Eriksson S. Phosphorylation of the anti-hepatitis B nucleoside analog 1-(2′-deoxy-2′-fluoro-1-β-D-arabinofuranosyl)-5-iodouracil (FIAU) by human cytosolic and mitochondrial thymidine kinase and implications for cytotoxicity. Antimicrob Agents Chemother 1996;40:1555–7.
[10] Gebhardt BM, Wright GE, Xu H, Focher F, Spadari S, Kaufman HE. 9-(4-Hydroxybutyl)-N2-phenylguanine (HBPG), a thymidine kinase inhibitor, suppresses herpes virus reactivation in mice. Antiviral Res 1996;30:87–94.
[11] Martin JA, Thomas GJ, Merrett JH, Lambert RW, Bushnell DJ, Dunsdon SJ, Freeman AC, Hopkins RA, Johns IR, Keech E, Simmonite H, Wong Kai-In P, Holland M. The design, synthesis and properties of highly potent and selective inhibitors of herpes simplex virus types 1 and 2 thymidine kinase. Antivir Chem Chemother 1998;9: 1–8.
[12] Watkins AM, Dunford PJ, Moffatt AM, Wong-Kai-In P, Holland MJ, Pole DS, Thomas GM, Martin J, Roberts NA, Mulqueen MJ. Inhibition of virus-encoded thymidine kinase suppresses herpes simplex virus replication *in vitro* and *in vivo*. Antivir Chem Chemother 1998; 9:9–18.
[13] Colacino JM. Mechanisms for the anti-hepatitis B virus activity and mitochondrial toxicity of fialuridine (FIAU). Antiviral Res 1996;29: 125–39.
[14] Lewis W, Dalakas MC. Mitochondrial toxicity of antiviral drugs. Nat Med 1995;1:417–22.
[15] Manfredini S, Baraldi PG, Bazzanini R, Bortolotti F, Vertuani S, Ashida N, Machida H. Enzymatic synthesis of 2′-*O*-acyl prodrugs of 1-(β-D-arabinofuranosyl)-5(*E*)-(2-bromovinyl)uracil and of 2′-*O*-acyl-araU, -araC and -araA. Antivir Chem Chemother 1998;9:25–31.
[16] Balzarini J, Bohman C, De Clercq E. Differential mechanism of cytostatic effect of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine, 9-(2,3-dihydroxy-2-propoxymethyl)guanine, and other antiherpetic drugs on tumor cells transfected by the thymidine kinase gene of herpes simplex virus type 1 or type 2. J Biol Chem 1993;268:6332–7.
[17] Degrève B, Andrei G, Izquierdo M, Piette J, Morin K, Knaus EE, Wiebe LI, Basrah I, Walker RT, De Clercq E, Balzarini J. Varicella–zoster virus thymidine kinase gene and antiherpetic pyrimidine nucleoside analogues in a combined gene/chemotherapy treatment for cancer. Gene Ther 1997;4:1107–14.
[18] Balzarini J, De Clercq E, Mertes MP, Shugar D, Torrence PF. 5-Substituted 2′-deoxyuridines: correlation between inhibition of tumor cell growth and inhibition of thymidine kinase and thymidylate synthetase. Biochem Pharmacol 1982;22:3673–82.
[19] Kierdaszuk B, Krawiec K, Kazimierczuk Z, Jacobsson U, Johansson NG, Munch-Petersen B, Eriksson S, Shugar D. Substrate/inhibitor properties of human deoxycytidine kinase (dCK) and thymidine kinases (TK1 and TK2) towards the sugar moiety of nucleosides, including *O*′-alkyl analogues. Nucleosides Nucleotides 1999;18:1883–903.