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Substrate specificity of feline and canine herpesvirus thymidine kinase

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ABSTRACT

The thymidine kinases from feline herpesvirus (FHV TK) and canine herpesvirus (CHV TK) were cloned and characterized. The two proteins are closely sequence-related to each other and also to the herpes simplex virus type 1 thymidine kinase (HSV-1 TK). Although FHV TK and CHV TK have a level of identity of 31 and 35%, respectively, with HSV-1 TK, and a general amino acid similarity of ≈54% with HSV-1 TK, they do not recognize the same broad range of substrates as HSV-1 TK does. Instead the substrate recognition is restricted to dThd and pyrimidine analogs such as 1-β-D-arabinofuranosylthymine (araT), 3′-azido-2′,3′-dideoxythymidine (AZT) and (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU). FHV TK and CHV TK differ in substrate recognition from mammalian cytosolic thymidine kinase 1 (TK1) in that TK1 does not phosphorylate BVDU and they also differ from mammalian mitochondrial thymidine kinase 2 (TK2), which, in addition to thymidine and thymidine analogs also phosphorylates dCyd. Although the nucleoside analog BVDU was a good substrate for FHV and CHV TK, the compound was poorly inhibitory to virus-induced cytopathic effect in FHV- and CHV-infected cells. The reason is likely the poor, if any, thymidylate kinase activity of FHV and CHV TK, which in HSV-1 TK-expressing cells convert BVDU-MP to its 5′-diphosphate derivative.

1. Introduction

Nucleoside kinases are important enzymes for the activation of pharmacologically active nucleoside analogs. The triphosphate product of the nucleoside analogs competes with the natural nucleotides for incorporation into the elongating DNA at the level of the cellular polymerases, which can lead to DNA chain termination. Cellular nucleoside kinases phosphorylate several important anticancer and antiviral nucleoside analogs but nucleoside kinases are also exploited as suicide genes in gene therapy trials (Floeth et al., 2001; Voges et al., 2003). Herpes simplex virus type 1-encoded thymidine kinase (HSV-1 TK) provides unique properties to cells that express it, and makes the cells susceptible to several nucleoside pro-drugs. The most studied nucleoside kinase for suicide gene application is the expression of HSV-1 TK in tumor cells and subsequent systemic chemotherapy with ganciclovir (GCV) (Wei et al., 1998; Beltinger et al., 2000). This combination has been used in a wide variety of animal tumor models, and also in several clinical trials for human cancer. The trials have indicated the need for optimization of the strategy of combined gene/chemotherapy of cancer (Fillat et al., 2003).

In search of nucleoside kinases with properties that can be useful in therapeutic approaches we have cloned and characterized thymidine kinases from two other herpes viruses; feline herpesvirus TK (FHV TK) and canine herpesvirus TK (CHV TK) (Nunberg et al., 1989; Rémond et al., 1995). These two proteins have a general identity of 47% and a homology of 66%, but considering only the amino acids involved in the binding of the substrate or in the enzyme active site, the identity reaches 80%. In comparison with HSV-1 TK both enzymes have an identity of 31–35% and a general amino acid similarity of about 54% to the HSV-1 TK sequence. Taking into account the amino acids involved in the substrate binding and the active site residues, the percentage of identity of FHV TK/CHV TK and HSV-1 TK is approximately 60%.

Despite the high sequence similarity with HSV-1 TK, our study demonstrates that the substrate specificity of FHV TK and CHV TK is much narrower than for HSV-1 TK and limited to thymidine and pyrimidine nucleoside analogs. The present study also shows that the kinetic properties regarding dThd, AZT and BVDU of FHV TK and CHV TK are different as compared to HSV-1 TK.

2. Materials and methods

2.1. Test compounds

Following nucleoside analogues were used in the study: araT (Sigma, St. Louis, MO); araG (R.I. Chemical Inc., Orange, CA); AZT

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(Sigma); GCV (N.V. Roche, Brussels, Belgium); dFdC (Gemcitabine) (J. Colacino, at that time at Eli Lilly); dFdG (J. Colacino, at that time at Eli Lilly); BVDU (Searle, United Kingdom); BVaraU (provided by H. Machida, Yamasu Shoyu Co., Choshi, Japan); FIAU (J. Colacino, at that time at Eli Lilly). The nucleotides dTMP, dUMP, AMP, UMP and ATP were purchased from Sigma.

2.2. Cloning and sequencing

We used PCR to amplify the fragments encoding FHV TK and CHV TK from the viral DNA. The FHV TK forward primer was: 5′-TCGTCATATGCTCGAAATGGCGAGTGGAACCATCCCCG-3′, and the FHV TK reverse primer was: 5′-CAGCCGGATCCTCGATTTAATGGTATATCGTCAAGGC-3′. The CHV TK forward primer was: 5′-TCGTCATATGCTCGAAATGGAAGTAAAAATTTGCCG-3′ and the CHV TK reverse primer was: 5′-CAGCCGGATCCTCGATTTAATCACAATACATTTCTT-3′. The fragments were cloned into linearized pET 16b (Novagen) using the In-fusion system (Clontech) protocol. The FHV TK and CHV TK sequencer were verified by sequence determinations of both strands using an ABI 310 sequencer (Applied Biosystems) and the BigDye cycle sequencing kit.

2.3. Protein expression and purification

The plasmids were transformed into *Escherichia coli* BL21(DE3)pLysS (Novagen) and single colonies were inoculated into LB-medium supplemented with 100 μg/ml ampicillin. The bacteria were grown at 37 °C and protein expression was induced at an OD_{600} of 0.8 with 1 mM isopropyl-1-thio-β-D-galacto-pyranoside for 12 h at 27 °C. The expressed protein was purified as described by the supplier. The purity of the enzymes were verified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Phast system, Amersham Pharmacia Biotech) and the protein concentration was determined with Bradford Protein Assay (Bio-Rad) using BSA as the concentration standard.

2.4. Enzyme assays

The activity of the purified recombinant enzymes was assayed in a 50-μl reaction mixture containing: 50 mM Tris–HCl pH 7.6, 0.1 mg/ml BSA, 2.5 mM ATP, 5 mM $MgCl_2$, 5 mM dithiothreitol and 2.5 μM [methyl-^{3}H]-thymidine (MT6032, Moravek Biochemicals) or 1 μM [methyl-^{3}H]-3′-azido-3′-deoxythymidine (MT 817, Moravek Biochemicals), and thymidine with a concentration ranging from 20 μM to 3 mM or AZT with concentration ranging from 5 to 250 μM. The samples were incubated for 30 min at 37 °C and every 10 min 10 μl aliquots were spotted on Whatman DE-81 filter paper disks. The filters were dried 1 h, washed 3 × 5 min in 5 mM ammonium formate and once in sterile water. The filter-bound nucleoside monophosphates were eluted in 500 μl of 0.1 M HCl and 0.1 M KCl and the radioactivity quantified by scintillation counting. The Michaelis–Menten constants were calculated using the GraphPad Prism software.

The substrate specificity of the purified enzymes was assayed by thin layer chromatography. Briefly, the assay was performed in 50 mM Tris–HCl pH 7.6, 0.5 mg/ml BSA, 5 mM $MgCl_2$, 5 mM DTT, 10 mM ATP, 15 μCi [γ-^{32}P]ATP (Amersham Pharmacia Biotech), 1 mM of each substrate and 5–10 μg of recombinant FHV TK or CHV TK. The samples were incubated for 30 min at 37 °C. Two microlitres of the reaction mixtures were spotted on polyethyleneimine-cellulose F thin layer chromatography sheets (Merck). The nucleosides monophosphate were separated in a buffer containing NH_4OH:isobutyric acid:dH_2O (1:66:33) and the nucleoside diphosphates were separated in 0.5 M ammonium formate (pH 3.5). The sheets were autoradiographed using phosphoimaging plates (BAS-1000, Fujix).

2.5. Antiviral activity assays

Anti-FHV and -CHV experiments were performed in Crandell-Rees Feline Kidney (CrFK) and Madin-Darby Canine Kidney (MDCK) cells. CrFK cells were seeded in wells of 96-well plates at 30,000 cells per well and incubated for 1 day at 37 °C until confluency was reached. Medium was aspirated and replaced by serial dilutions of the test compounds (100 μl per well). 100 μl of FHV, diluted in medium to obtain a virus input of 100 $CCID_{50}$ (1 $CCID_{50}$ being the virus dose that is able to infect 50% of the cell cultures), was added to each well. Mock-treated cell cultures receiving solely the test compounds were included in each plate, to determine the cytotoxicity. After 4 days of incubation at 37 °C, microscopy was performed to score the virus-induced cytopathic effect. MDCK cells were seeded in wells of 96-well plates at 3500 cells per well and incubated for 1 day at 35 °C. Serial dilutions of the test compounds (50 μl per well) were added, followed by 50 μl of CHV, diluted in medium to obtain a virus input of 100 $CCID_{50}$. Mock-treated cell cultures receiving solely the test compounds were included in each plate, to determine the cytotoxicity. After 7 days of incubation at 35 °C,

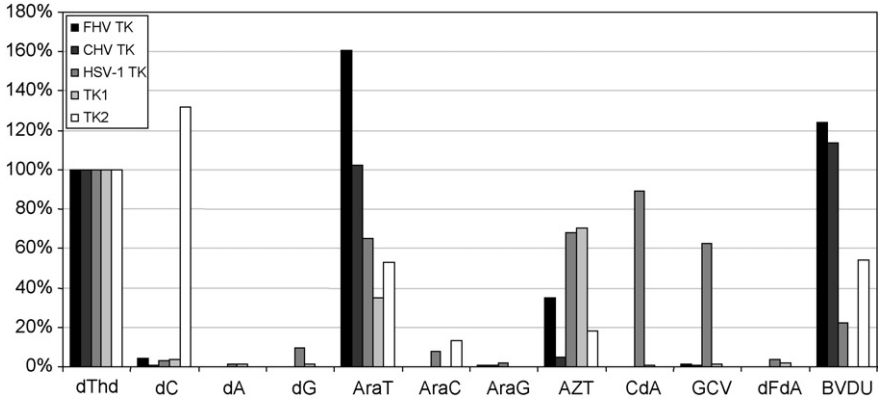

Fig. 1. Nucleoside and nucleoside analog phosphorylation by FHV TK, CHV TK, HSV-1 TK, human TK1 and human TK2. Relative levels of phosphorylation expressed in relation to % dThd phosphorylation, of one representative out of three independent experiments. (AraT: 1-β-D-arabinofuranosylthymine; AraC: 1-β-D-arabinofuranosylcytosine; AraG: 9-β-D-arabinofuranosylguanine; AZT: 3′-azido-2′,3′-dideoxythymidine; CdA: 2-chloro-2′-deoxyadenosine; GCV: ganciclovir; dFdA: 2′,2′-difluorodeoxyadenosine BVDU: (*E*)-5-(2-bromovinyl)-2′-deoxyuridine.)

Table 1
Kinetic properties of FHV TK and CHV TK

Substrate	FHV TK				CHV TK			
	K_m (μM)	V_{max} (μmol/mg/min)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})	K_m (μM)	V_{max} (μmol/mg/min)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
dThd	41 ± 15	33 ± 2.5	23	560×10^3	16 ± 7	9.5 ± 0.8	6.5	405×10^3
BVDU	10 ± 2.5	1.23 ± 0.06	0.86	86×10^3	15 ± 5.1	0.58 ± 0.04	0.40	26×10^3
AZT	15 ± 2.4	1.4 ± 0.05	0.97	65×10^3	50 ± 5.2	0.78 ± 0.03	0.53	10×10^3

k_{cat} was calculated using a molecular mass of 41.8 and 40.9 kDa, respectively, for FHV TK and CHV TK.

microscopy was performed to score the virus-induced cytopathic effect.

3. Results

3.1. Kinetic properties of feline and canine herpes virus TK

From the natural deoxyribonucleosides, CHV TK and FHV TK preferentially phosphorylated dThd (Fig. 1). The enzymes also efficiently phosphorylate the pyrimidine nucleoside analogs 1-β-D-arabinofuranosylthymine (araT), (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU) and 3′-azido-2′,3′-dideoxythymidine (AZT). Very low levels of dCyd phosphorylation and virtually no phosphorylation of purines or purine analogs tested was detected (Fig. 1).

We determined the kinetic parameters of FHV TK and CHV TK for the substrates dThd, BVDU and AZT. With dThd, CHV TK showed the lowest K_m (16 μM) while FHV TK showed the highest V_{max} (33 μmol/mg/min). With BVDU as substrate, FHV TK showed a K_m of 10 μM and CHV TK a K_m of 15 μM, both with a low V_{max}. AZT as substrate was similar to BVDU with a K_m of 15 μM for FHV and 50 μM for CHV TK and both with a low V_{max} (Table 1). Thus, the phosphorylating capacity (K_{cat}/K_m) of both enzymes was most efficient for the natural substrate dThd, and about 10- to 40-fold less efficient for BVDU and AZT (Table 1).

3.2. Monophosphate kinase assay

In addition to nucleosides, we also studied the phosphorylation of nucleoside monophosphates to the corresponding diphosphates by FHV and CHV TK. Both enzymes had the ability to phosphorylate AMP, UMP, dUMP and dTMP (Fig. 2). FHV TK showed a slightly higher efficiency to phosphorylate nucleoside monophosphates at the assay conditions used, but in general both kinases had the same phosphorylation pattern (dTMP > dUMP > AMP > UMP).

3.3. In vivo cytopathic effect assay

In the antiviral activity assays, the analogs 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil (FIAU) and araT showed the best inhibition of the virus-induced cytopathic effect, both in FHV- and CHV-infected cells (Table 2). AZT, BVDU and 1-β-D-arabinofuranosyl-5-(*E*)-(2-bromovinyl)uracil (BVaraU) needed higher concentrations to achieve significant inhibition (Table 2).

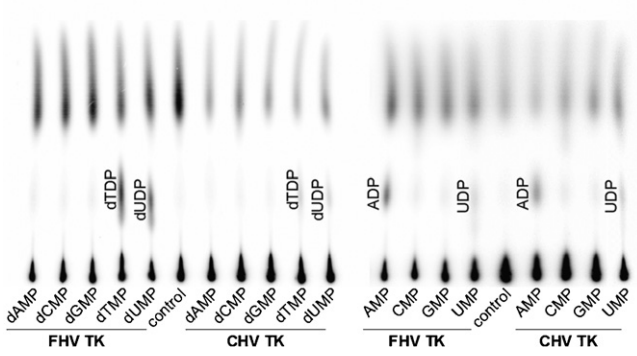

Fig. 2. TLC analysis of FHV TK and CHV TK for monophosphates phosphorylation in [γ-^{32}P]ATP phosphotransferase assay. (The control reactions are without addition of substrate.)

Table 2
Antiviral activity of nucleoside analogues against FHV TK and CHV TK in cell culture

Substrate	EC_{50}[a] (μM)		MCC[b] (μM)	
	FHV	CHV	CrFK	MDCK
araT	15	4.8	>125	160
araG	75	>100	125	≥100
AZT	>125	>125	>125	>125
GCV	30	6.7	>50	>50
dFdC	>0.8	>0.1	4.0	≥0.3
dFdG	>20	≥3.0	80	30
BVDU	30	>200	>200	>200
BVaraU	>125	>200	>125	>200
FIAU	0.09	0.02	≥20	≥8

[a] 50% effective concentration, or compound concentration, required to inhibit virus-induced cytopathic effect in cell culture by 50%.

[b] Minimal inhibitory concentration, or compound concentration required to afford a microscopically visible alteration of cell morphology. CrFK, Crandell-Rees Feline Kidney. MDCK, Madin-Darby Canine Kidney. dFdC, 2′,2′-difluorodeoxycytidine (gemcitabine), dFdG, 2′,2′-difluorodeoxyguanosine.

3.4. Analysis of the amino acids involved in the substrate binding

The alignment of the FHV TK and CHV TK (Fig. 3A) showed that the two TK sequences are closely related (identity of 47% and homology of 66%) and, in particular, the amino acids involved in the active site showed 87% of identity (14 residues out of 16, as shown in Fig. 3A). The few amino acids that differ between the enzymes are not directly involved in the substrate binding, and this may explain the differences in the K_m and V_{max}, while they do not affect the substrate specificity of the enzymes. A comparison with the HSV-1 TK sequence showed an identity of 31–35% and a general amino acid similarity of about 54%. The amino acids defining the active pocket in HSV-1 TK and FHV TK and CHV TK showed a similarity of 62% (Fig. 3A). The presence of a tyrosine in the ATP binding site (p-loop) has not previously been reported for other nucleoside kinases (Fig. 3B), while the phenylalanine is extremely well conserved in all human nucleoside kinases as well as in nucleoside kinases of other species. This different residue may partially explain the poor capacity of FHV TK and CHV TK to recognize purines and purine nucleoside analogs.

HSV-1 TK has two alanines at positions 167–168, while both FHV TK and CHV TK have an alanine and a serine at the corresponding positions (Fig. 3C). These two amino acids also differ in other nucleoside kinases (Fig. 3C). Deoxyguanosine kinase and deoxycytidine kinase have serine and aspartic acid at these positions, while thymidine kinase 2 and *Dm*-dNK have corresponding serine and alanine residues (Fig. 3B). The observed differences suggest an important role of these amino acids for the eventual substrate recognition of the enzymes.

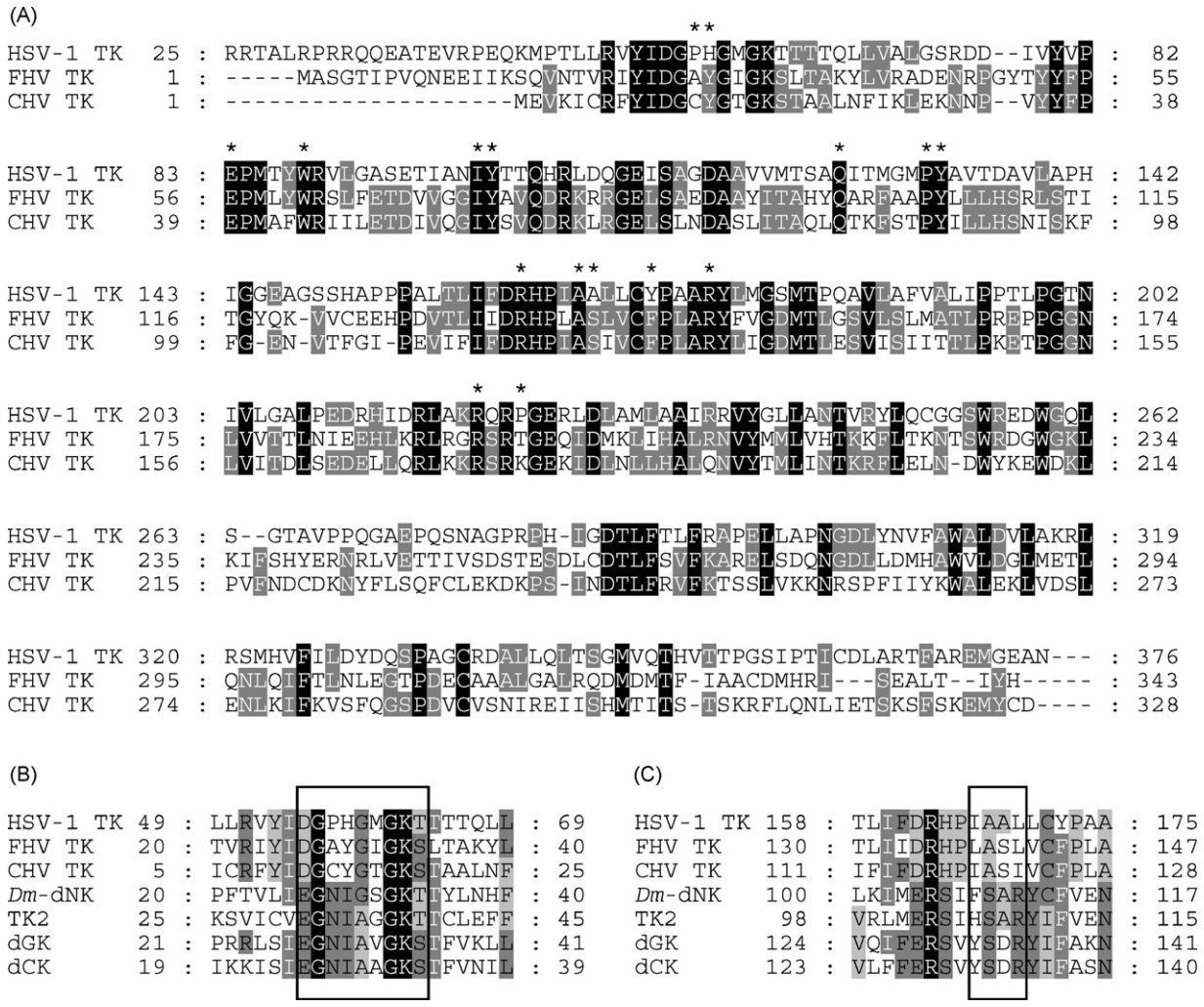

Fig. 3. (A) Alignment of HSV-1 TK, FHV TK and CHV TK. (B) Alignment of the p-loop in FHV TK, CHV TK, *Dm*-dNK and the human nucleoside kinases: TK2, dGK and dCK. (C) Alignment of the amino acids 158–175 of HSV-1 TK with the corresponding amino acids in FHV TK, CHV TK, *Dm*-dNK and the human nucleoside kinases: TK2, dGK and dCK. (Black boxes indicate completely conserved amino acid residues and different shades of grey boxes indicate different levels of conserved residues. Symbol (*) indicates amino acids that are involved in the substrate binding or in the active site pocket definition.)

4. Discussion

Deoxyribonucleoside kinases constitute a family of enzymes with high sequence similarity between different species. Despite the high degree of conservation, a thorough characterization reveals differences in substrate specificity and unique properties for several of the nucleoside kinases. The multisubstrate nucleoside kinase from *Drosophila melanogaster* (*Dm*-dNK) and the herpes simplex virus thymidine kinases all show a broad substrate specificity and are therefore investigated as suicide genes in gene/chemotherapy protocols (Fillat et al., 2003). In search of even more efficient suicide gene candidates we have studied two viral nucleoside kinases, FHV TK and CHV TK. FHV TK has been already subject investigated with regard to its capacity to phosphorylate antiviral compounds such as penciclovir and acyclovir (Maggs and Clarke, 2004; Hussein et al., 2008).

The kinetic determinations of FHV TK and CHV TK showed a K_m for dThd of 41 and 16 μM respectively, which is relatively high as compared to HSV-1 TK (0.23 μM) or the highly catalytically active *Dm*-dNK (1.6 μM) (Johansson et al., 1999, Balzarini et al., 2006). Similarly to HSV-1 TK and *Dm*-dNK, both FHV TK and CHV TK recognized AZT and BVDU as substrates. FHV TK and CHV TK showed a limited substrate recognition that differed from HSV-1 TK, which phosphorylates both purine and pyrimidine nucleoside analogs. FHV TK and CHV TK substrate recognition was exclusively restricted to dThd and pyrimidine analogs. Also the human thymidine kinases 1 and 2 (TK1 and TK2) were included in the comparison of substrate specificity of the thymidine kinases. Interestingly, except for the similarity between FHV TK and CHV TK, all of the studied thymidine kinases had a unique individual pattern of substrate recognition. They all recognized dThd and the thymidine analogs AZT and araT. However, BVDU was not recognized by TK1; TK2 was unique in its dCyd recognition; and HSV-1 TK was the only enzyme among the tested thymidine kinases that recognized GCV as substrate. The phosphorylation capacity of the anti-HSV-1 drug BVDU was more pronounced for FHV TK and CHV TK than for HSV-1 TK and TK2. Both enzymes showed a weak capacity (FHV TK > CHV TK) to phosphorylate nucleoside monophosphates, although they could recognize and phosphorylate dTMP, dUMP, AMP and UMP to the corresponding diphosphate.

BVDU, and its closely related analog BVaraU, were not markedly inhibitory to the FHV- and CHV-induced cytopathic effect in cell culture (30 and >200 μM). This is very different compared to the well-documented inhibitory effect of BVDU to HSV-1 (but not HSV-2) infection. It is likely due to the fact that the FHV- and CHV-encoded TKs have poor dTMP kinase activity, which is required

to further convert BVDU-MP to its 5′-diphosphate derivative as HSV-1 (but not HSV-2) TK is doing. In this respect, FHV TK and CHV TK rather mimic HSV-2 TK that has a high affinity for BVDU but also lacks dTMP kinase activity to further convert BVDU-MP (and BVaraU-MP) to their 5′-diphosphate derivative. In contrast to BVDU-MP and BVaraU-MP, araT- and FIAU-5′-monophosphates can be further converted to their 5′-diphosphate derivatives by cellular enzymes and thus become toxic derivatives. Our findings suggest that it would be of interest to transduce tumor cells with the FHV TK or CHV TK genes. Such cells would be expected to become extremely sensitive to the cytostatic activity of BVDU due to accumulation of BVDU-MP and subsequent inhibition of thymidylate synthase in tumor cells. A similar phenomenon has been previously observed for HSV-2 TK-gene transduced murine mammary carcinoma FM3A cells in which accumulation of BVDU-MP and selective thymidylate synthase inhibition was demonstrated (Balzarini et al., 1987).

The comparison of the sequences of FHV- CHV- and HSV-1 TKs reveals interesting amino acid substitutions that may explain the differences in substrate recognition observed, despite the overall large similarity among these enzymes. Especially interesting is the area corresponding to HSV-1 TK positions 167 and 168 that in HSV-1 TK are two alanines, while both FHV TK and CHV TK have an alanine and a serine at the corresponding positions. This may be important for the differences in substrate recognition between the enzymes since substitutions of these residues has markedly altered the dThd and GCV recognition of HSV-1 TK (Balzarini et al., 2006). Other amino acids suggested as being important for HSV-1 TK GCV recognition are Pro57, His58 and Tyr172 (Munir et al., 1992; Solaroli et al., 2003). These residues are absent in FHV TK and CHV TK and the lack of conservation of this structure may contribute to explain the poor capacity of FHV TK and CHV TK to recognize purines and purine nucleoside analogs.

We conclude that FHV TK and CHV TK are not endowed with a broad substrate specificity, as seen with HSV-1 TK, but instead solely recognize thymidine and pyrimidine nucleoside analogs, including BVDU. The efficiency of FHV and CHV TK to convert BVDU to its 5′-monophosphate is higher than observed for HSV-1 TK and TK2 and suggests that these genes could be candidates for possible further investigations as suicide genes.

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