

SUBSTRATE SPECIFICITY OF THREE VIRAL THYMIDINE KINASES (TK): VACCINIA VIRUS TK, FELINE HERPESVIRUS TK, AND CANINE HERPESVIRUS TK

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□ *In search of novel suicide gene candidates we have cloned and characterized thymidine kinases from three viruses; vaccinia virus TK (VVTk), feline herpesvirus TK (FHV-TK), and canine herpesvirus TK (CHV-TK). Our studies showed that VVTk primarily is a thymidine kinase, with a substrate specificity mainly restricted to dThd and only minor affinity for dCyd. VVTk also is related closely to mammalian thymidine kinase 1 (TK1), with 66% identity and 75% general homology. Although CHV-TK and FHV-TK are sequence related to herpes simplex virus types 1 thymidine kinase (HSV1-TK), with 31% and 35% identity and a general similarity of 54%, the substrate specificity of these enzymes was restricted to dThd and thymidine analogs.*

Keywords Nucleoside analog; Gene therapy; Suicide gene; Nucleoside kinases

INTRODUCTION

The prototype for combined gene/chemotherapy of malignant tumors is the expression of herpes simplex virus thymidine kinase (HSV-TK) in tumor cells and subsequent systemic chemotherapy with the nucleoside analog ganciclovir. In search of novel suicide gene candidates we have cloned and characterized thymidine kinases from three other viruses; vaccinia virus TK (VVTk), feline herpesvirus TK (FHV-TK), and canine herpesvirus TK (CHV-TK).

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MATERIAL AND METHODS

Protein expression and purification. We expressed the three kinases in *E. coli* as poly-histidine fusion proteins in the pET-16b vector (Clontech, Mountain View, CA, USA). The expressed proteins were purified with TALON metal affinity resin (Clontech, Mountain View, CA, USA).

Enzyme assays. The activity of the purified recombinant enzymes was assayed in a 50 μ l reaction mixture containing: 50 mM Tris-HCl pH 7.6, 0.1 mg/ml BSA, 2.5 mM ATP, 5 mM MgCl₂, 5 mM dithiothreitol, and [³H] analogs (Amersham Biosciences, Uppsala, Sweden). The samples were incubated 30 minutes at 37°C and every 10 minutes 10 μ l aliquots were spotted on Whatman DE-81 filter paper disks. The filter bound nucleoside monophosphates were eluted in 500 μ l of 0.1 M HCl and 0.1 M KCl and the radioactivity quantified by scintillation counting. The substrate specificity of the purified enzymes was assayed by thin layer chromatography.

RESULTS

VVTK had the ability to recognize both dCyd and dThd among the natural substrates although the efficiency of dCyd phosphorylation was much less compared with dThd phosphorylation (Table 1). VVTK also efficiently converted araT, AZT and BVDU (data not shown) to their corresponding monophosphates (Figure 1). The substrate specificity of both FHV-TK and CHV-TK was limited to dThd and thymidine analogs, with poor affinity for dCyd. Neither the purines dAdo and dGuo nor several purine- and deoxycytidine analogs tested were recognized by FHV-TK or CHV-TK (Table 2, Figure 2).

DISCUSSION

Deoxyribonucleoside kinases are a family of enzymes with high sequence similarity between different species. Although this high degree of conservation a thorough characterization often reveal differences in substrate specificity and unique properties for several of the nucleoside kinases. The multisubstrate nucleoside kinase from *Drosophila melanogaster* and the

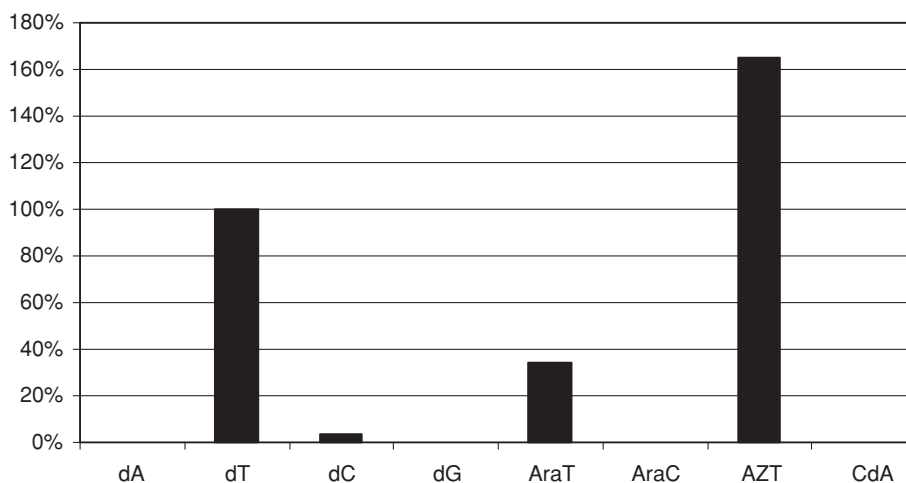
TABLE 1 Kinetic Properties of VVTK (k_{cat} was Calculated Using a Molecular Mass of 22.5 kDa)

Substrate	K_m (μ M)	V_{max} (μ mol/mg/min)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1} \text{M}^{-1}$)
dThd	330	585	220	665×10^3
dCyd	>10,000			
AZT	600	1,100	412	687×10^3

TABLE 2 Kinetic Properties of FHV-TK and CHV-TK (k_{cat} was Calculated Using a Molecular Mass of 41.8 kDa for FHV-TK and 40.9 kDa for CHV-TK)

Substrate	FHV-TK				CHV-TK			
	K_m (μM)	V_{max} ($\mu\text{mol}/\text{mg}/\text{min}$)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1} \text{M}^{-1}$)	K_m (μM)	V_{max} ($\mu\text{mol}/\text{mg}/\text{min}$)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1} \text{M}^{-1}$)
dThd	41	33	23	560×10^3	16	9.5	6.47	405×10^3
AZT	15	1.4	0.97	65×10^3	50	0.78	0.53	10×10^3

herpes viral thymidine kinases all show a broad substrate specificity and are therefore used as suicide genes in gene/chemotherapy protocols. In search of even more efficient suicide gene candidates, we have investigated three viral nucleoside kinases with regard to their substrates specificities and kinetic properties. All of the enzymes studied in this report were strictly pyrimidine nucleoside kinases that most efficiently phosphorylated dThd among the natural substrates. The VVTK also recognized dCyd with low efficiency. VVTK also efficiently convert the pyrimidine analogs araT, AZT, and BVDU to their corresponding mono-phosphates. The feline and canine herpes virus TKs showed a sequence similarity of approximately 54% with the herpes simplex virus TK. However, the substrate specificity of FHV-TK and CHV-TK was limited to dThd and thymidine analogs.

**FIGURE 1** Nucleoside and nucleoside analog phosphorylation by VVTK. Relative levels of phosphorylation expressed in relation to percentage dThd phosphorylation. (AraT = 1- β -D-arabinofuranosylthymine; AraC = 1- β -D-arabinofuranosylcytosine; AZT = 3'-azido-2',3'-dideoxythymidine; CdA = 2-chloro-2'-deoxyadenosine).

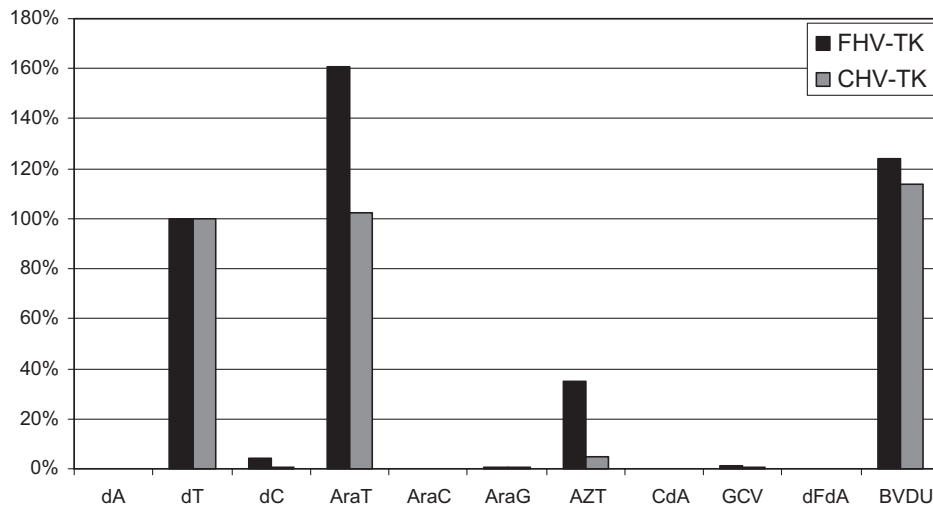


FIGURE 2 Nucleoside and nucleoside analog phosphorylation by FHV-TK and CHV-TK. Relative levels of phosphorylation expressed in relation to percentage dThd phosphorylation. (AraG = 9- β -D-arabinofuranosylguanine; GCV = Ganciclovir; dFdA = 2',2'-difluoro deoxyadenosine BVDU = (E)-5-(2-bromovinyl)-2'-deoxyuridine).

We conclude that the substrate specificity of VVTK, FHV-TK and CHV-TK is limited to thymidine and thymidine analogs, although VVTK also recognized dCyd with low efficiency. None of the three investigated thymidine kinases in our study demonstrate the broad substrate specificity characteristic for HSV-TK.

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