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BRIEF COMMUNICATION

The equine herpes virus 4 thymidine kinase is a better suicide gene than the human herpes virus 1 thymidine kinase

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The herpes simplex virus type 1 thymidine kinase suicide gene (HSV1tk) together with ganciclovir (GCV) have been successfully used for in vivo treatment of various experimental tumors, and many clinical trials using this system have been launched. With the aim to improve this therapeutic system, we compared the potential efficacy of different herpes virus derived thymidine kinases (HSV1, varicella-zoster virus, equine herpes virus type-4 and Epstein–Barr virus) as suicide genes in association with the nucleoside analogs acyclovir, ganciclovir and bromovinyldeoxyuridine. Using various murine and human cell lines expressing these viral tk, we show that HSV1- and EHV4tk are the more efficient suicide genes for the different nucleoside analogs tested. Moreover, EHV4tk expressing murine and human cells were three- to 12-fold more sensitive to GCV than HSV1tk expressing cells. This was correlated with the presence of five-fold higher amounts of the toxic triphosphated-GCV in EHV4- versus HSV1tk expressing cells. Altogether, these experiments underline the potential advantages of the EHV4tk as a suicide gene.

Keywords: *nucleoside analogs; cancer; fusion proteins*

The herpes simplex virus type 1 thymidine kinase gene (HSV1*tk*) is the most widely used suicide gene, both in experimental settings and clinical trials.[1] Expression of the HSV1*tk* gene renders tumor cells sensitive to antiviral agents like acyclovir (ACV), ganciclovir (GCV) and bromovinyl deoxyuridine (BVDU). These nucleoside analogs are efficiently converted to their monophosphate form by HSV1*tk*, and are then converted to triphosphate compounds by host cellular kinases. Incorporation of these metabolites into elongating DNA blocks elongation leading to cell death.[2–4] The HSV1*tk*/GCV system has proved efficient for inducing the regression of transplanted tumors in various animal models,[5] as well as of carcinogen-induced tumors.[6] Based on these favourable results, several clinical gene therapy trials are in progress aimed at assessing safety and efficacy of this treatment for treating malignancies, and preliminary results have now been reported.[7,8]

Despite the effectiveness of the HSV1*tk*/GCV system for killing tumor cells, it remains important to try to improve it, with the aim to maximize therapeutic efficacy and/or to facilitate treatment modalities. We thus looked for new suicide gene/prodrug combinations. Biochemical and *in vivo* studies have demonstrated different substrate specificity of herpes *tk* towards antiviral and cytostatic nucleoside analogs.[9–11] We thus compared *tk* from HSV1, varicella-zoster virus (VZV), equine herpes virus type-4 (EHV4) and Epstein–Barr virus (EBV) for their capacity to sensitize tumor cells to ACV, GCV and BVDU.

We generated expression vectors with each of these *tk* genes fused in frame with the *Sh Ble* gene conferring resistance to zeocin.[12] This should facilitate the selection of transduced cells and the detection of the chimeric proteins by immunodetection with anti-SH polyclonal antibodies. The dual functionality of such chimeric proteins expressed by these vectors has been previously demonstrated in bacteria lacking endogenous *tk* activity.[12] From endogenous *tk*-deficient murine fibroblasts (*tk*(–) 3T3), we generated cells expressing the different viral *tk* based on zeocin selection. These cells could survive when grown in selective HAT medium and could incorporate tritiated thymidine (^{3}HT) (data not shown) demonstrating the dual functionality of *tk*/SH fusion proteins. These results are in agreement with previous experiments showing that HSV1*tk* retain its functionality after amino- or carboxy-terminal fusion to various proteins.[13–16]

We next analyzed these selected bulk populations for their *in vitro* sensitivity to nucleoside analogs. We measured inhibition of cell proliferation in comparison with parental cells and determined 50% inhibitory concentrations (IC_{50}) (Table 1). Incorporation of ^{3}HT in cells in the absence of drug was in the same range for the four bulks (data not shown). ACV IC_{50} values were approximately 15-fold lower in HSV1-, EHV4- and VZV*tk*

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Table 1 Cytotoxic activity (IC_{50}) of ACV, GCV and BVDU against murine *tk*(–) 3T3 transfected and non-transfected cells

	ACV				GCV				BVDU			
	IC_{50} (μm)	s.d.	n	Selectivity index	IC_{50} (μm)	s.d.	n	Selectivity index	IC_{50} (μm)	s.d.	n	Selectivity index
NIH-3T3	165	49.5	2		70	14.1	2		55	7.1	2	
HSV1-TK	8.7	5.1	3	19	0.2	0.11	3	350	0.015	0.007	2	5500
EHV4-TK	11.1	16.4	3	15	0.1	0.02	3	700	0.02	0.03	2	2750
VZV-TK	11	5.7	3	15	8	1.4	2	9	0.016	0.02	2	2750
EBV-TK	43.3	5.8	3	4	6	3.6	3	12	0.04	0.05	3	1375

s.d., standard deviation to the mean; n, number of experiments.
Tk(–) 3T3 cells were grown in DMEM medium supplemented with 1% l-glutamine (GibcoBRL), 1% penicillin, streptomycin and neomycin mixture (GibcoBRL, Paisley, UK) and 10% new born calf serum (GibcoBRL). Cells were transfected using a calcium phosphate precipitation kit (GibcoBRL) and 48 h after cells were grown in selective medium with 25 μg/ml of zeocin (Cayla, Toulouse, France). Drug toxicity was assessed by measuring inhibition of cell proliferation. Cells were seeded at 2.5×10^3 per well in 24-well plates. 24 h later, cells were grown in drug-containing medium (from 0.001 to 100 μm, in triplicate) for 7 days. Methyl-^{3}H-thymidine (Amersham, UK) was added at day 6 (1.5 μCi per well) and incorporated radioactivity was measured with a Micro-Beta Plus counter (EG&G Instruments and Wallac, Turku, Finland). Cytotoxic doses were expressed as means of IC_{50} values (the drug concentration required to reduce the methyl-^{3}H-thymidine incorporation by 50%) of two or three experiments, and selectivity indexes were calculated as the IC_{50} ratios of parental to transfected cells. ACV (9-(2-hydroxyethoxymethyl)guanine), GCV (9-(1,3-dihydroxy-2-propoxymethyl)guanine), and BVDU ((E)-5-(2-bromovinyl)-2′-deoxyuridine), were purchased from Wellcome (Issy-les-Moulineaux, France), Syntex (Palo Alto, CA, USA) and Sigma Chemical (St Louis, MO, USA), respectively.

Table 2 Selectivity indexes and cytotoxic activity (IC_{50}) of ACV, GCV and BVDU against murine *tk*(–) 3T3 HSV1- and EHV4*tk* expressing clones

	ACV			GCV			BVDU		
	IC_{50} (μm)	n	Selectivity index	IC_{50} (μm)	n	Selectivity index	IC_{50} (μm)	n	Selectivity index
NIH-3T3	53.3 ± 4.4	3		36.7 ± 15.6	3		43.3 ± 12.5	3	
HSV1*tk*									
1	11.5 ± 1.5	3		0.17 ± 0.04	2		0.03 ± 0.01	2	
2	7.3 ± 2.2	3		0.1 ± 0.02	3		0.02 ± 0.010	2	
3	20 ± 10	3		0.13 ± 0.08	2		0.01 ± 0.005	2	
4	22.5 ± 7.5	3		0.18 ± 0.02	2		0.004 ± 0.001	2	
5	20	2		0.3	1		0.004 ± 0.004	2	
mean ± s.d.	16.3 ± 6.5		3	0.18 ± 0.08		210	0.013 ± 0.011		3300
EHV4*tk*									
1	5	1		0.08	1		0.023 ± 0.02	2	
2	12 ± 3	2		0.05 ± 0.01	2		0.057 ± 0.004	2	
3	8 ± 2	3		0.06 ± 0.03	3		0.032 ± 0.008	2	
4	8	1		0.1	1				
5	3.5 ± 1.5	2		0.06 ± 0.05	2		0.067 ± 0.06	2	
6	5	1		0.023	1		0.02 ±	2	
mean ± s.d.	6.9 ± 3.1[a]		8	0.06 ± 0.03[a]		600	0.04 ± 0.021[a]		1100

[a]Different from HSV1*tk* IC_{50} value.
n, number of experiments.
Independent clones were picked up after transfection and selection in medium with 25 μg/ml of zeocin and individually expanded. Drug sensitivity of each *tk*-expressing clones was tested as described in Table 1. Individual IC_{50} means for each five HSV1*tk* and six EHV4*tk* clones were determined and means for each *tk* were reported ± s.d. with selectivity index. The amount of the *tk*/ZEO fusion proteins detected in HSV1-*tk*(+) clones 1, 2, 3 and 4 was similar to the amount detected in EHV4-*tk*(+) clones 1, 2, 3 and 4 (data not shown).

expressing cells compared with the parental NIH-3T3 cells, and only four-fold lower for EBV*tk* expressing cells. HSV1- and EHV4*tk* expressing cells were 350- and 700-fold more sensitive to GCV than NIH-3T3 cells, compared with only seven-fold for VZV- or EBV*tk* expressing cells. Unlike ACV and GCV, IC_{50} values for BVDU were in the same range for all herpes virus-*tk* expressing cell bulks, about 1300- to 3600-fold lower than for NIH-3T3 cells. Similar results were obtained by assessing directly the drug's toxicity in a semi-quantitative assay evaluating the cell survival in the presence of increased drug concentrations (data not shown).[17] These observations were extended using the murine pancreatic tumor PANC-O2 cell line. Again, it was found that IC_{50} values were three-fold lower for EHV4*tk* than for HSV1*tk* expressing cell bulk, 0.55 ± 0.07 and 1.7 ± 0.5 μm, respectively (means of two independent experiments).

Among the four herpes viruses *tk* tested for their

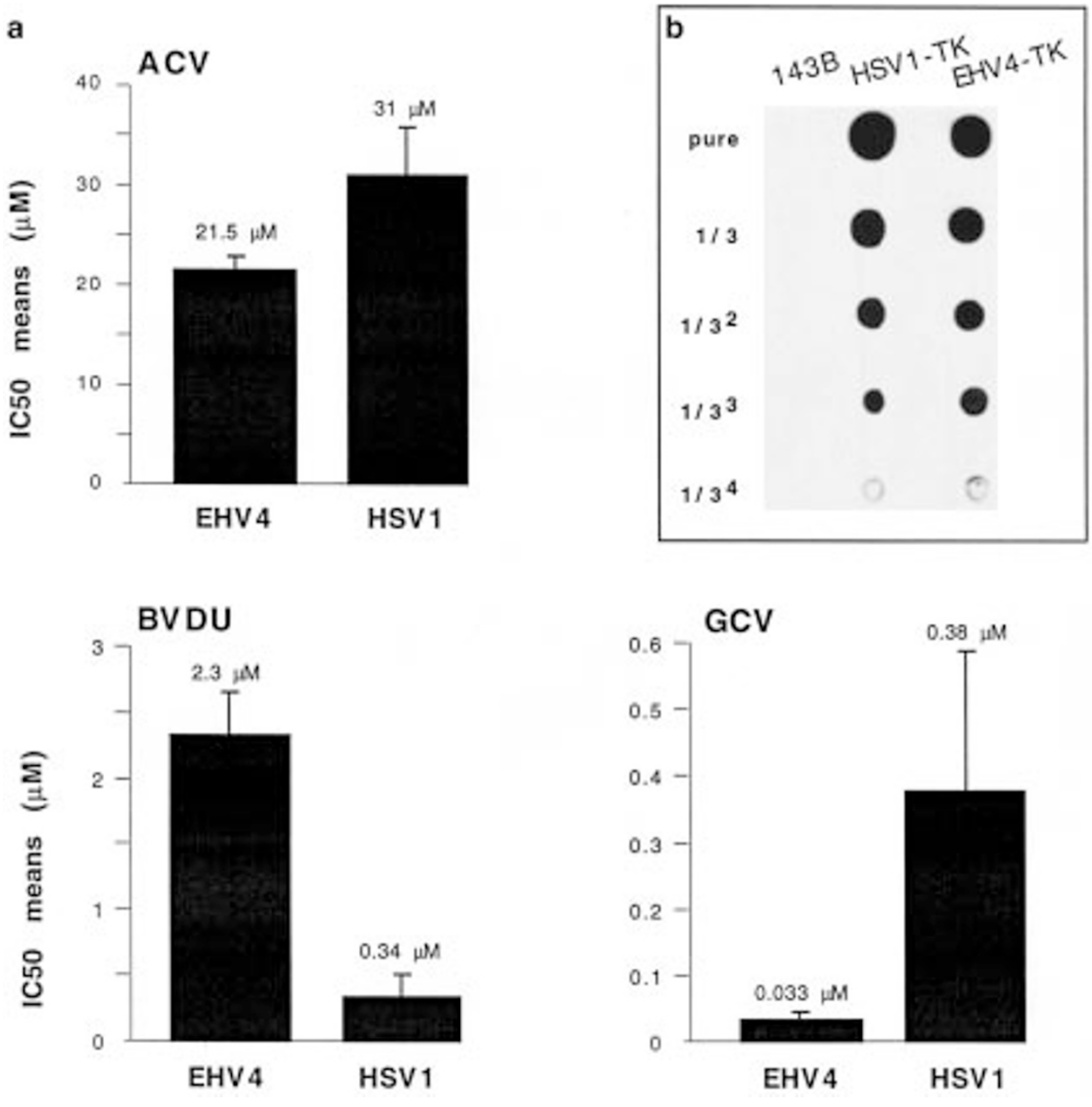

Figure 1 *Cytotoxic activity of ACV, GCV and BVDU against human tk(–) 143B cells transfected with HSV1- or EHV4tk. tk(–) 143B cells were grown in DMEM medium supplemented with 1% l-glutamine, 1% penicillin, streptomycin and neomycin mixture and 10% fetal calf serum (GibcoBRL). Cells were transfected using a calcium phosphate precipitation kit and cell bulks were selected with 50 μg/ml zeocin in grown medium. (a) Drug toxicity was assessed by measuring inhibition of cell proliferation as described in Table 1. Results were presented as mean of IC_{50} values ± s.d. The means, determined on three independent experiments, are indicated on the top of each histogram. (b) Dot blot analysis of tk expression in transfected and non-transfected tk(–) 143B cells. Cells were incubated in lysis buffer at the final cellular concentration of 20×10^6 cell/ml. 5 μl of successive dilutions of cellular extracts were dropped on nitrocellulose membrane. Zeo-fusion proteins were revealed as described in Cazaux et al.*[12] *There was no detectable band corresponding to fusion protein in tk(–) 143B cell extracts.*

capacity to sensitize cells to nucleoside analogs toxicity, HSV1- and EHV4*tk* demonstrated the more interesting selectivity indexes. Because cell bulks represent a mixture of clones with different sensitivities, we next analyzed the drug cytotoxicity on individual clones expressing either of these two *tk* (Table 2). For ACV and GCV, the sensitivity of EHV4*tk* expressing cells was statistically more important than the one of HSV1*tk* expressing cells ($P < 0.02$ and 0.008, respectively). However, HSV1*tk*-expressing clones were three times more sensitive to BVDU than the EHV4*tk* expressing clones (Table 2). These differences in sensitivity to nucleoside analogs were not due to differences in *tk* expression levels. Indeed, expression of EHV4*tk*/ZEO and HSV1*tk*/ZEO proteins detected by Western blot using polyclonal antibodies against SH protein was similar in the four EHV4*tk* (+) and four HSV1*tk* (+) clones tested (number 1 to 4 on Table 2 for both fusion proteins) (data not shown).

Since nucleoside analog's metabolism might be different in cells of different species we thus analyzed the efficiency of the system for killing human cells. Human *tk*(–) osteosarcoma 143B cell bulks expressing HSV1- or EHV4*tk* were thus generated. Herpes virus *tk* protein expression levels were similar for each cell bulk as assessed by dot-blot using polyclonal antibodies against SH protein (Figure 1b). We then, studies cell sensitivity to ACV, GCV and BVDU (Figure 1a). The toxicity of ACV was similar in HSV1- and EHV4*tk*-expressing cells. It is worth noting that EHV4*tk* expressing cells were approximately 12-fold more sensitive to GCV than HSV1*tk*

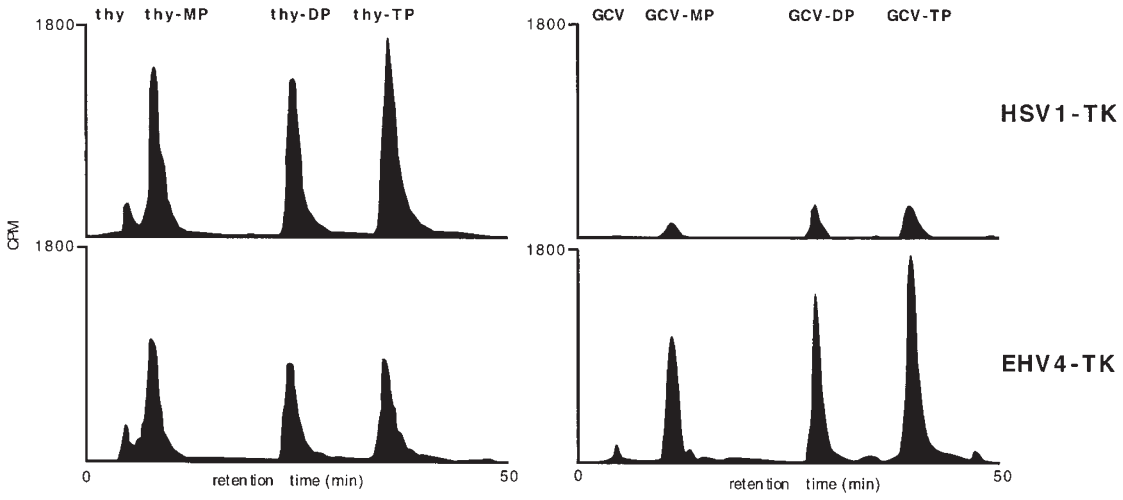

Figure 2 *Thymidine and GCV phosphorylation in transfected and non-transfected tk(–) 143B cells. 3×10^6 cells were incubated for 7 h with 1 μm of 8-^{3}H-GCV (3 Ci/mmol, Isotopchim, France) or methyl-^{3}H-thymidine (5 Ci/mmol, Amersham, UK) in a final volume of 5 ml of medium. After trypsin treatment and washes with PBS, the nucleotides were extracted with 1 ml of ice cold methanol 60% over the night at –20°C. The samples were analyzed by Fast Protein Liquid Chromatography (FPLC, Pharmacia-LKB Instruments, Uppsala, Sweden) on an anion-exchange column. Radioactivity was counted at the column exit with a radiamatic Flo-one Beta A-500 apparatus (Packard, Meriden, CT, USA), and peaks were analyzed with Flo-one/Data software.*[25] *The percentages of thy-MP, thy-DP and thy-TP were respectively 29.3, 27.5 and 39.2% in HSV1-tk expressing cells and 36.8, 27.5 and 28.8% in EHV4-tk expressing cells. The percentages of GCV-MP, GCV-DP and GCV-TP were respectively 15.5, 36.5 and 45% in HSV1-tk expressing cells and 23.9, 29.3 and 45% in EHV4-tk.*

expressing cells. On the contrary the toxicity of BVDU was greater in HSV1*tk* than in EHV4*tk* expressing cells (Figure 1a). These observations were extended to an additional tumor cell line, human colonic carcinoma HCT116 cells. EHV4- and HSV1*tk* expressing cells were 485- and 170-fold more sensitive to GCV than parental HCT116 cells (mean of IC_{50} values for GCV was 53.4 ± 15 μm). IC_{50} values for GCV were three-fold lower for EHV4*tk* than for HSV1*tk* expressing cells, 0.11 ± 0.02 and 0.32 ± 0.08 μm, respectively (means ± s.d. for three independent experiments).

In order to understand why GCV is more toxic in EHV4*tk* compared with HSV1*tk* expressing cells, we analyzed the GCV phosphorylation in *tk*(–) 143B and HSV1- or EHV4*tk* expressing cells. While there was no detectable phosphorylation of thymidine in *tk*(–) 143B cells (data not shown), thymidine was similarly phosphorylated in both TK expressing cells (Figure 2). For GCV, the proportions of the different phosphorylated forms were the same in both *tk* cells, while the total amount of intracellular GCV was five-fold higher for EHV4- than for HSV1*tk* expressing cells. Such a difference might be explained by the uptake of nucleoside and their analogs that has been reported to be correlated with *tk* expression. Indeed, the uptake of thymidine into *tk*(–) *Escherichia coli* is proportional to the amount of *tk* activity expressed from the heterologous HSV1*tk* gene.[18] Furthermore, mammalian HSV1*tk* expressing cells showed higher GCV uptake and phosphorylation than control cells.[19] Since *tk* expression was similar in both *tk*(+) cells, the greater amount of intracellular GCV in EHV4*tk* expressing cells could indicate a greater EHV4*tk* activity toward GCV compared with HSV1*tk* activity. Detailed kinetic studies of EHV4*tk* and HSV1*tk* enzymatic activities should confirm these observations.

In conclusion, significant differences in the activity of the different herpes *tk* were observed. EBV- and VZV*tk* did not demonstrate any advantages over HSV1*tk* whatever the nucleoside analog used. Two recent studies investigated VZV*tk* as a suicide gene in human breast cancer and osteosarcoma cells.[20,21] In these articles IC_{50} values for BVDU ranged from 0.06 to 0.6 μm, quite similar to our values, but no comparison between VZV- and HSV1*tk* was shown. It is worth nothing that EHV4*tk* expressing cells were always more sensitive to GCV than HSV1*tk* expressing ones whether as bulks that provide an estimate of the overall enzyme activity in a polyclonal population, or as clones. This enhanced toxicity was not due to a difference in *tk* expression levels as assessed by Western blot. This is also further indicated by the observation of an opposite effect with BVDU that is more toxic for HSV1*tk* expressing cells, demonstrating a qualitative rather than a quantitative difference between the activity of these enzymes. Depending on the cell lines, EHV4*tk* was three- to 12-fold more potent than HSV1*tk*. Such a difference appears significant when contemplating the clinical use of suicide genes. Indeed the treatment of experimental tumors in mice or rats demonstrate that the GCV dosage is often critical for obtaining efficacy, notably for brain tumors. When administered at 10 mg/kg twice a day, the usual dosage in humans, the GCV plasmatic concentration range between 0.44 to 2.2 μg/ml, and the cerebral spinal fluid (CSF) concentrations are approximately three-fold lower.[22,23] It is therefore possible that CSF GCV concentrations are suboptimal for the treatment of brain tumors transduced with HSV1*tk*. In any case, a better efficiency of the enzyme should lead to a better treatment efficiency and/or a decrease of the GCV dose used. This latter point is also significant since GCV toxicity in human appears cumulative depending on the overall amounts administered.[24] Altogether, our results underline the potential advantages of the EHV4*tk* as a suicide gene.

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