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Establishment and use of a cell line expressing HSV-1 thymidine kinase to characterize viral thymidine kinase-dependent drug-resistance

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Abstract

To understand the mechanisms of antiviral drug resistance and to have a system to examine the cytotoxicity of herpes simplex virus type 1 (HSV-1) inhibitors that are thymidine kinase (TK)-dependent, we have constructed a plasmid pFTK1 by inserting a DNA fragment containing the TK gene of HSV-1 strain F into the eukaryotic expression vector pcDNA3.1/His A. TK-deficient 143B cells were transfected with this vector and neomycin-resistant cells were selected. Cell survival in HAT medium and TK activity of the cell lysates were examined to ascertain HSV-1 TK expression. A cell line expressing the viral TK gene, FTK143B (FTK), was established and used for characterization of two laboratory-derived TK-deficient drug-resistant HSV-1 mutants of strain F. The antiviral activities of several drugs, mostly nucleoside analogues, were compared in the Vero, 143B and FTK cell culture systems. We showed that both mutant viruses lost their resistance to acyclovir and to other HSV-1 TK-dependent compounds in FTK cells but not in Vero and 143B cells. Significantly increased cytotoxicity of ganciclovir and (*E*)-5-(2-bromovinyl)-2′-deoxyuridine was also observed in the FTK cells. This HSV-1 TK gene-transfected cell model is a useful tool to rapidly determine HSV-1 drug resistance at the viral TK level.

Keywords: Herpes simplex virus type 1; Thymidine kinase; Drug-resistance; Acyclovir; Antivirals; Thymidine kinase expressing cells

1. Introduction

Thymidine kinase (TK) is a pyrimidine salvage enzyme responsible for the synthesis of deoxythymidine monophosphate (dTMP) from deoxythymidine and ATP. The TKs encoded by the *Herpesviridae* are unique in that they possess low substrate specificities and additional enzymatic activities, i.e. deoxycytidine kinase and dTMP kinase (TMPK). HSV-1 TK (vTK) is composed of two identical subunits each containing 376 amino acids (43 kDa), and the native protein has binding sites for both the natural nucleoside substrates and also for the phosphate donor (Wagner et al.,

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1981; Black and Loeb, 1993; Brown et al., 1995). The vTK is an important pharmacological target of most herpesvirus treatments, because it catalyzes the initial activation (phosphorylation) of antiviral nucleoside analogues such as acyclovir (ACV), penciclovir (PCV), ganciclovir (GCV) and many five-substituted deoxyuridine compounds (Elion et al., 1977; Fyfe, 1982). After transfer of the purified vTK gene to cell cultures (attempted by Wigler et al., 1977), establishment of various vTK-expressing cell lines upon transfection of (usually) cellular TK-negative cells with the vTK DNA have been reported (Reyes et al., 1982; Ayusawa et al., 1985; Shimizu et al., 1986; Caruso and Klatzmann, 1992; Drake et al., 1997), to improve the antiviral activity of nucleoside analogues by increasing intracellular concentrations of the active forms (Guettari et al., 1997), but also, and more importantly, for genetic chemomodulation (Balzarini et al., 1985a,b, 1993; Caruso et al., 1993; Caruso and Klatzmann, 1994; Danthinne et al., 1998; Degrève et al., 1999) to selectively kill genetically modified cancer cells by antiherpetic drugs.

Most of the reported antiviral resistance to vTK-dependent antiherpetic compounds are due to TK deficiency and very rarely due to alteration of vTK or viral DNA polymerase activity (Field et al., 1980; Darby et al., 1981, 1986; Crumpacker et al., 1982; Larder et al., 1983; Matthews et al., 1989; Chatis and Crumpacker, 1992; Field and Biron, 1994; Sasadeusz et al., 1997). According to the level of vTK production, TK deficiency can be divided into two classes, namely TK-negative HSV-1 and TK-altered HSV-1 mutants. Studying drug-resistance is important for elucidation of the mode of drug action and for understanding virus replication mechanisms and pathogenesis. It is also clinically important to anticipate the potential emergence of drug resistance and to try using alternative drugs with a different mode of action. It is useful to have a simple test system to confirm that antiviral resistance is due to TK-deficiency. If cells produce vTK by themselves, the TK-deficient virus will loose its drug resistance caused by failure of phosphorylation (activation) of the drugs.

Here we report that we established a cell line producing vTK by transfection of cellular TK-deficient 143B cells with the wild-type HSV-1 TK gene and confirmed that two TK-negative and TK-altered HSV-1 strains lost their drug-resistance to vTK-dependent antivirals in this cell line, while in the parental TK-competent 143B cells and in other cell lines (i.e. Vero), these mutant viruses kept their drug-resistant phenotype. In addition, we demonstrated that several compounds had significantly increased cytotoxicity in the 143B cells expressing the HSV-1 TK gene.

2. Materials and methods

2.1. Cells and viruses

The following cell lines were obtained from the American Type Culture Collection (ATCC) (Rockville, MD): Vero (African green monkey kidney), 143B (human osteosarcoma, TK-negative) and 143B PML BK TK (143TK) (143B cells transfected with an HSV TK-expression plasmid) cells. HSV-1/AR1 and HSV-1/AR2 were laboratory-derived TK-deficient ACV-resistant mutants of HSV-1 strain F obtained from ATCC. Vero cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Gaitherburg, MD) supplemented with 5% fetal bovine serum (FBS) (Life Technologies) and 40 μg/ml gentamycin sulfate (Sigma Chemicals, St. Louis, MO). For maintenance of the 143B cells, 15 μg/ml 5-bromo-2′-deoxyuridine (BDU) (Sigma) was added to DMEM with 10% FBS (DMEM/10% FBS) to suppress TK-positive cells. For 143TK, HAT (100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine) (Sigma) was added to DMEM/10% FBS to maintain vTK production. To maintain the FTK phenotype, HAT medium and 600 μg/ml G418 (Sigma) were added. For antiviral evaluation, all the cells were prepared in the absence of BDU, HAT or gentamycin G418 for two passages prior to the experiment. Vero cells were used for virus amplification.

2.2. Compounds

ACV [9-(2-hydroxyethoxymethyl)guanine] was kindly provided by Samchully Pharmaceuticals, Seoul, Korea and PCV [9-(4-hydroxy-3-hydroxymethyl-but-1-yl)guanine] by SmithKline Beecham Pharmaceuticals, Harlow, Essex, UK. GCV [9-(1,3-dihydroxy-2-propoxymethyl)guanine] was obtained from Hoffmann La Roche, Basel, Switzerland in the form of the sodium salt. (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU) and phosphonoformic acid trisodium salt (PFA, foscarnet) were purchased from Sigma. GCV and PFA were dissolved in DMEM and the other compounds in dimethylsulfoxide (DMSO) at the concentration of 20 mg/ml and stored at −20 °C as stock solutions. Working dilutions were prepared in assay medium just before use.

2.3. Plasmid construction

The isolation and modification of DNAs, transformation of *Escherichia coli* (*E. coli*) and other techniques used for this work were performed as described by Maniatis et al., 1982. The general strategy of the construction of the vTK expression plasmid was as described in Fig. 1. Viral DNAs were isolated from virus particles collected from Vero cells infected with HSV-1 strain F and digested with *Bam*HI. The *Bam*HI Q fragment (3.4 kb) was purified from agarose gel and inserted into *Bam*HI sites of a cloning vector, BS/KS(−) (Stratagene, La Jolla. CA). *E. coli* strain DH5α was transformed with the ligated construct. Ampicillin-resistant colonies were amplified and screened for plasmids containing the appropriate fragment of 3.4 kb, BamHIQ/BS-KS(−) (pBamHIQ). *Bam*HI and *Not*I sites were introduced by using the polymerase chain reaction method with synthetic oligonucleotides at the 5′-end and 3′-end of the vTK open reading frame (ORF) region of pBamHIQ, respectively. DNA fragments of the TK ORF with artificial restriction enzyme sites were isolated and inserted into an eukaryotic expression vector, pcDNA3.1/His A (Invitrogen, Groningen, the Netherlands), digested with *Bam*HI and *Not*I. The plasmid DNA, pFTK1/pcDNA3.1 (pFTK1) was amplified and isolated from the *E. coli* strain DH5α for further DNA transfection.

2.4. DNA Transfection and selection of cells expressing vTK

143B cells were transfected with pFTK1 or vector plasmid by the calcium phosphate–DNA coprecipitation method (Graham and van der Eb, 1973), incorporating the glycerol shock procedure (Parker and Stark, 1979). They were diluted in order to transfer one or a few cells into 96-well plate wells and cultivated in the presence of 600 μg/ml of G418 for 14 days. Cell colonies were trypsinized and suspended in the presence of G418 and HAT.

2.5. Thymidine kinase activity assay

Confluently grown cells or HSV-1-infected 143B cells showing 50% cytopathic effect (CPE) were harvested, washed twice with magnesium- and calcium-free phosphate buffered saline [PBS(−)] and resuspended in PBS(−)/3 mM dithiothreitol (DTT). The cells were treated with 0.1% of Nonidet P-40 (NP-40) and then incubated on ice for 5 min before extraction. Following centrifugation for 2 min, aliquots of the supernatant was stored at −70 °C until the enzyme assay. The 50 μl enzyme reaction mixture contained 20 mM phosphate buffer, pH 7.5, 10 mM $MgCl_2$, 10 mM ATP, 10 mM DTT, 10 μM thymidine, 5 μM [^{3}H]-thymidine (specific activity 83 mCi/mmol, Amersham Pharmacia Biotech, Piscataway, NJ) and various volumes of crude lysates. The reaction was performed at 37 °C for 30 min. The mixture was filtered through Whatman DE 81 filters and washed twice with ice-cold 4 mM ammonium formic acid and twice with ethanol (Chatis et al., 1989). The radioactivity of the dried filters was measured by using a liquid scintillation counter (LS 6000TA, Beckman Instruments, Palo Alto, CA).

2.6. Protein staining and Western blot analysis

Identification of the vTK protein was performed by western blotting probed with rabbit

polyclonal antibody against HSV-1 TK (anti-vTK) prepared by us with purified recombinant vTK proteins of HSV-1 strain F from the *E. coli* expression system. Cells in culture dishes were inoculated with virus at a multiplicity of infection (M.O.I.) of 10, adsorbed for 1 h, and incubated with DMEM/2% FBS at 37 °C. When 50% CPE was observed, the infected cells were harvested, and washed with ice-cold isotonic buffer (10 mM Tris–HCl, pH 7.5, 0.14 M NaCl, 1.5 mM $MgCl_2$). After centrifugation, isotonic buffer with 0.5% NP-40 and 10% glycerol solution was added to the cell pellets to afford cell lysis on ice for 10–60 min. The supernatant was denatured at 100 °C for 5 min, electrophoresed at 10 mA for 1 h, 30 mA for 1 h through a set of 12.5% polyacry-

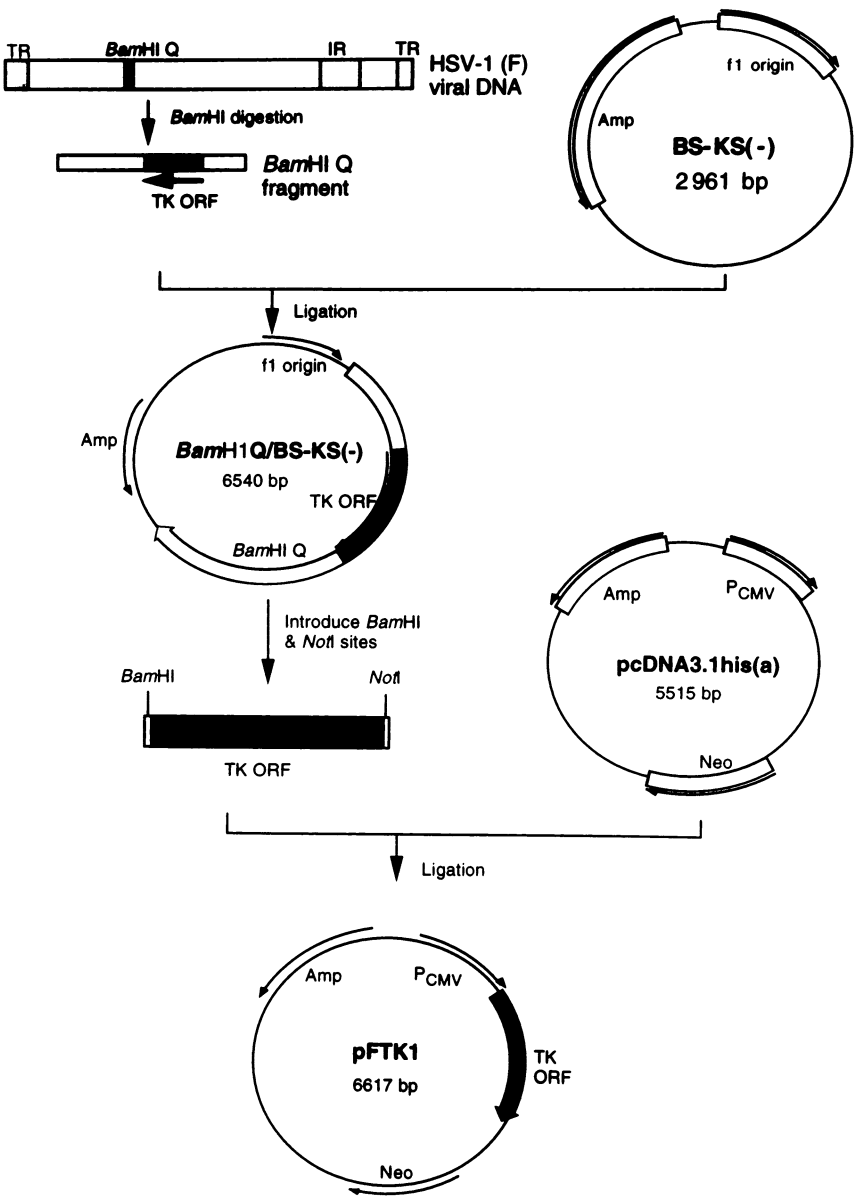

Fig. 1. General outline of the construction of an eukaryotic expression plasmid encoded TK gene of HSV-1.

lamide/SDS gel. One of the gels was silver-stained according to the Manufacturer's description and the other was transferred onto a nitrocellulose membrane. The membrane was blocked with 3% bovine serum albumin (BSA) in TBS buffer (10 mM Tris–HCl, pH 8.0, 0.15 N NaCl) for 1 h at room temperature and washed twice with 1 × TBST buffer (0.05% Tween-20 in TBS). Polyclonal rabbit anti-vTK antibody 1/1,000 diluted with 1X TBST including 1% BSA, was applied to the membrane. It was incubated for 1 h at room temperature and washed twice with 1 × TBST. The secondary antibody (anti-rabbit IgG, alkaline phosphatase conjugate) (Sigma) at a 1/10,000 dilution in 1 × TBST including 1% BSA was added, incubated for 1 h at room temperature and washed twice with 1 × TBST and once with buffer 3 (0.1 M Tris–HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5). A chemiluminescent substrate (5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium) (Sigma) in buffer 3 was then applied to the membrane and kept in a dark room. When the bends were detected, stop solution (1 M Tris–HCl, pH 8.0, 0.5 M EDTA) was added to the membrane to stop further reaction.

2.7. Antiviral evaluation

Each virus strain was titrated and subsequently tested for their in vitro susceptibilities to a broad range of antiviral compounds by a CPE inhibition assay in Vero, 143B and FTK cells. Confluent cells grown in 96-well microplates (at ~3–4 days after seeding) without BDU, HAT or G418 for two passages were infected with the different virus strains at a M.O.I. of 100 $CCID_{50}$ per well. Inoculum sizes were verified by a simultaneous CPE assay. After 1 h incubation, the residual virus was removed and 100 μl of the drugs diluted with DMEM/2% FBS was applied to the infected cells in duplicate. The cells were incubated at 37 °C for 3 days. Antiviral activity in the virus-infected cell cultures and the cytocidal effects of the drugs in the mock-infected cells were measured by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Pauwels et al., 1988) and also by microscopic observation. For the MTT assay, the culture medium was removed and 50 μl of an MTT solution at the concentration of 2.5 mg/ml in PBS(−) was added to each well and incubated in a humidified CO_2 incubator at 37 °C for 1.5 h. Then, 100 μl of acidified isopropanol/10% Triton X-100 solution was added and the plates were shaken to dissolve the formazan products. The absorbances at dual wavelength (540 nm as main and 690 nm as reference) were measured with a microplate reader (Thermomix, Molecular Devices, Sunnyvale, CA). The cell survival in the control wells of the mock-infected cell cultures without drug was considered as 100% cell survival. The 50% cytotoxic (cytocidal) concentration (CC_{50}) was defined as the concentration of compound that reduced the absorbance of the mock-infected control samples by 50%. Antiviral activity of the drugs at the dose achieving 50% cell survival (i.e. protection) of the cell cultures against the CPE of the virus strains, was defined as the 50% effective concentration (EC_{50}). The assays for the cytostatic effects of the compounds were performed as follows. The cells were seeded in microtiter plate wells and incubated at 37 °C. One day later, the compounds diluted with DMEM/5% FBS were added to the growing cell cultures. The cells were further incubated for 3 days at 37 °C and their survival was compared to those of the cell controls without drug by using the above described MTT assay.

3. Results

3.1. Construction of the TK-expression plasmid

For transfection of cellular TK-deficient 143B cells, the eukaryotic expression plasmid pFTK1 containing the vTK gene was cloned by transformation of *E. coli* with the ligated construct, amplified and purified as described in Section 2 and in Fig. 1. By DNA sequence analysis of pBamHIQ and pFTK1, it was confirmed that there was no base difference in the TK ORF region (data not shown).

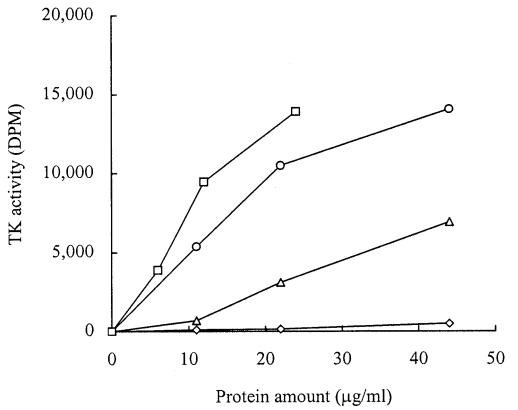

Fig. 2. Thymidine kinase activity of crude cell lysates: ◇, mock-infected 143B; ○, HSV-1 (F)-infected 143B; △, mock-infected FTK; □, mock-infected 143TK cells.

3.2. Cloning of the vTK-expressing cell line, FTK143B

Selection markers for cloning of the cell line expressing the vTK gene were firstly neomycin-resistance and secondly survival in HAT medium. 143B cells were transfected with pFTK1 or vector DNA (pcDNA3.1/His A) as a control. Then, the cells were cultivated in the presence of G418 for 14 days. Cell colonies from the pFTK1-transfected cell culture were split with trypsin and cultivated in the presence of 600 μg/ml G418 and HAT medium and from the vector-transfected cell culture in the absence of HAT medium. Several cell lines survived in HAT medium and were verified for their vTK activity and gene expression by Western blot analysis (data not shown). Most of the clones were HSV-1 TK-positive. The cell clone that showed the highest TK activity was selected for further characterization and designated FTK143B (FTK). The TK activities of the crude lysates of the various cell cultures are shown in Fig. 2. The TK enzyme activity proportionally increased with higher protein extract concentrations. However, the enzyme activity was lower than that of 143B cells infected with HSV-1 or 143TK obtained from ATCC. The 143B cells and 143B cells transfected with the vector plasmid (lacking the viral TK gene) showed no significant TK activity (data not shown). Expression of the vTK gene was confirmed by Western blot analysis probed with polyclonal rabbit anti-vTK antibody (Fig. 3). 43 kDa protein products were detected in both FTK and 143TK cells, while not in 143B cells. An additional smaller but stronger band, about 37 kDa, observed in HSV-1 (F)-infected cell lysates was also found in FTK. It was very stable when it was kept in the medium containing G418 and HAT. Therefore, we concluded that FTK was a stable cell line properly expressing the vTK gene.

3.3. Antiviral evaluation

To find out whether FTK cells can be used for the confirmation of the role of the vTK in antiviral drug-resistant HSV-1 strains, the antiviral activity of several compounds against TK-deficient HSV-1/AR1 and HSV-1/AR2, and their parental strain HSV-1/F in 143B cells, HSV-1 TK gene-transfected FTK cells and also Vero cells were compared. A summary of the antiviral activity and the cytotoxicity of the compounds are shown in Table 1 and resulted from at least three separate experiments. AR1 and AR2 showed a comparable 20- to 50-fold resistance to ACV in Vero and 143B cells. However, their sensitivity to GCV, PCV, BVDU and BDU were markedly different. In general, the AR1 mutant strain was markedly more resistant to these nucleoside drugs than the AR2 mutant strain (Table 1). PFA showed a similar inhibitory activity in Vero and in 143B cells against both isolates. ACV, GCV, PCV and BVDU known as vTK-dependent antiherpes agents regained their antiviral activity against AR1 and AR2 in the HSV-1 TK gene-transfected FTK cells. Plaque reduction assays clearly showed that strain AR1 became indeed completely sensitive to BVDU in FTK cells (data not shown). The cytotoxicity of GCV and BVDU in FTK cells was markedly increased. The CC_{50} of the other compounds including ACV, PCV, BDU and PFA, remained at >100 μg/ml. Detailed dose response curves of ACV, GCV, PCV, BVDU and BDU against the different virus strains in 143B and FTK cell cultures are shown in Fig. 4. There was no significant difference between the cytocidal and cytostatic effect of all the tested compounds mea-

Table 1
Summary of thea anti-HSV-1 activity of various compounds in Vero, 143B and FTK cells

Compounds	Mock-infected (CC_{50}: μg/ml)			HSV-1-infected (EC_{50}: μg/ml)								
				F			AR1			AR2		
	Vero	143B	FTK	Vero	143B	FTK	Vero	143B	FTK	Vero	143B	FTK
ACV	>100	>100	>100	1.2	4.2	0.8	44.4	70.3	2.4	57.5	86.7	13.9
GCV	>100	>100	2.6	0.5	0.8	0.1	33.8	>100	0.1	5.2	100.0	0.8
PCV	>100	>100	>100	1.4	3.5	0.6	>100	>100	3.7	9.5	51.7	1.0
BVDU	>100	>100	17.3	1.3	0.7	0.4	>100	>100	0.8	50.9	14.9	0.4
BDU	>100	>100	>100	5.7	2.4	2.1	>100	>100	>100	3.8	11.7	5.4
PFA	>100	>100	>100	88.6	61.4	50.3	84.2	57.5	57.4	85.0	57.4	56.1

Average data from at least three separate experiments.

sured for 3 days in 143B and FTK cells, as shown in Fig. 4.

4. Discussion

We have characterized several TK-deficient drug-resistant HSV-1 mutant strains isolated after selecting for drug resistance with high doses of ACV in cell culture. To create a cell culture model to confirm easily whether their drug resistance was due to dysfunction of vTK and to reveal whether the nucleoside analogues were vTK-dependent or not, a stable cell line expressing the vTK gene of HSV-1 strain F, designated FTK cells, was constructed and cloned. The cells showed neomycin-resistance and survived in the presence of HAT. Expression of the vTK gene in FTK cells was confirmed by TK activity measurements of crude cell lysates and by Western blot analysis. The cell line was stable and grew well and dense enough to afford MTT assays. Further experiments were performed with the parental HSV-1 strain F, and two of our TK-deficient mutants, HSV-1/AR1 and HSV-1/AR2. The mutants showed almost no TK-activity when virus-infected Vero cell lysates were used as enzyme source and [^{3}H]-thymidine as the radiolabeled substrate, AR1 was considered to be TK-negative and AR2 TK-partial. In a plaque autoradiograph performed with [^{14}C]-thymidine as the radiolabeled substrate AR1 showed no radioactive signal, and showed no virulence and no ex vivo reactivation from latency in the mouse intranasal infection model and in the 'zoster'-like model (data not shown). The purified and concentrated recombinant AR1 TK proteins expressed in *E. coli* system did not exhibit TK activity (data not shown). AR2 showed a radioactive signal in plaque autoradiography, and comparable virulence to that of the F strain and *ex vivo* reactivation in both mouse infection models occurred.

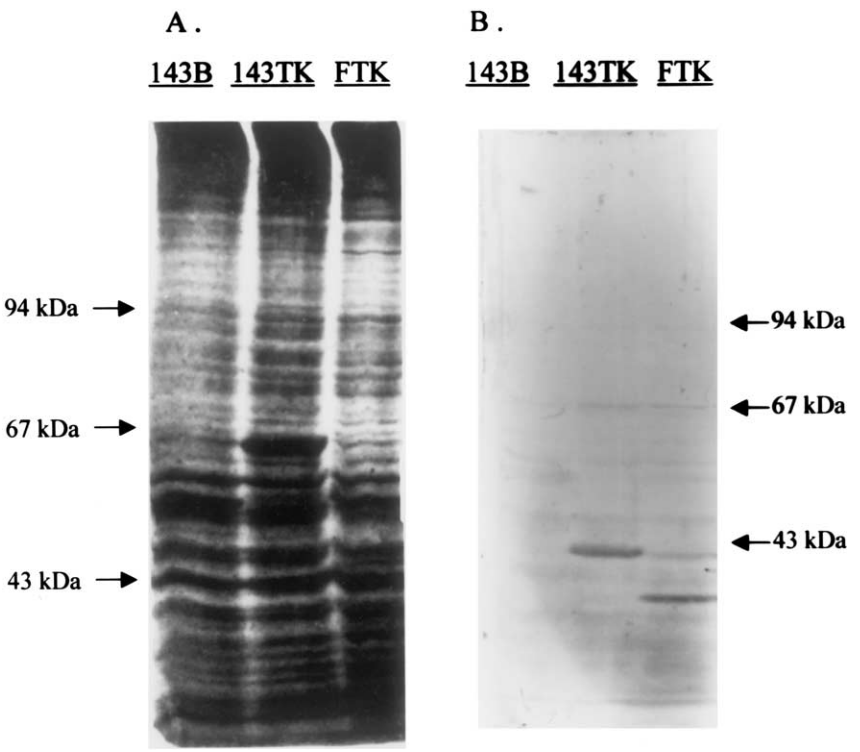

Fig. 3. Western blot analysis of crude cell lysates probed with polyclonal rabbit anti-HSV-1 TK antibody. (A) Silver stained; (B) Western blot.

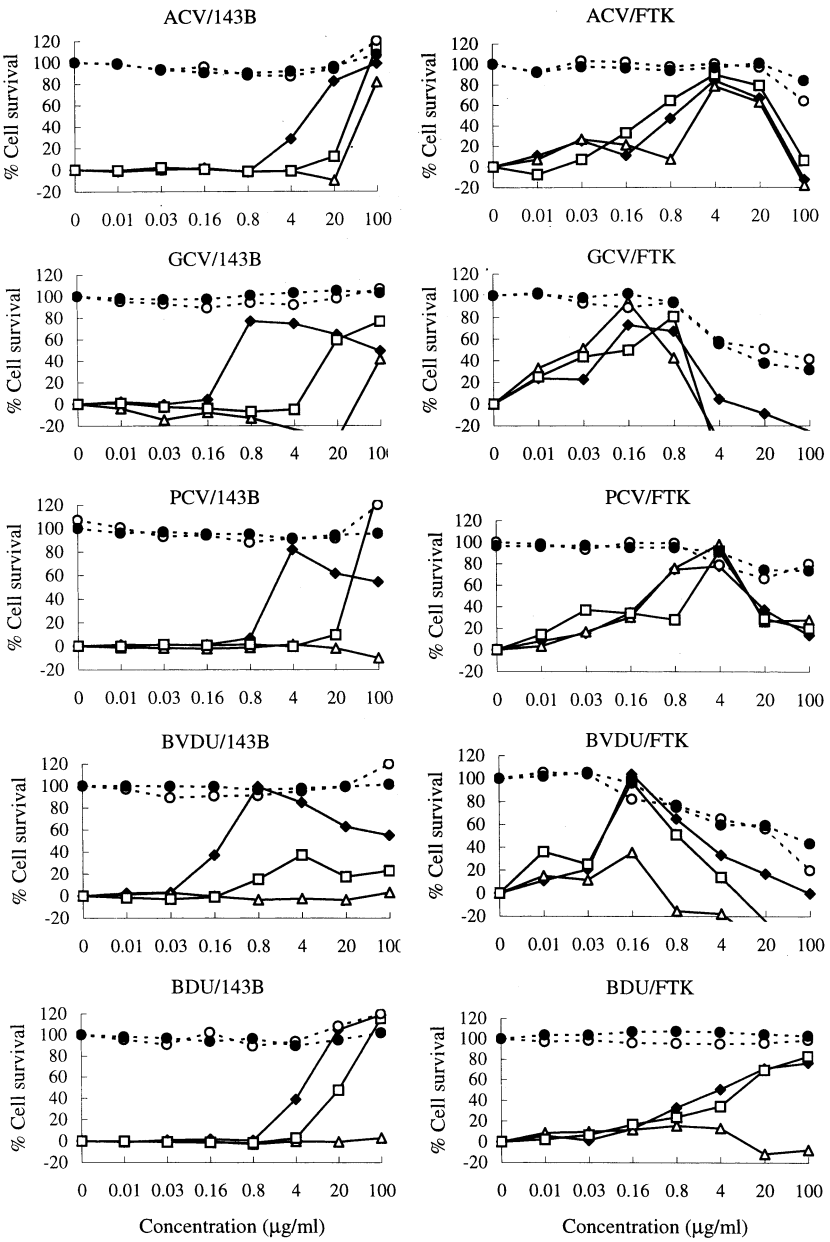

Fig. 4. Dose–response curves for various compounds against HSV-1 in 143B and FTK cell cultures. Antiviral activity against HSV-1 strain F (–◆–), mutant strain AR1 (–△–) and mutant strain AR2 (–□–). Cytocidal effects (--○--) and cytostatic effects (--●--).

AR1 has a substitution of the methionine residue by lysine at amino acid position 322 of the vTK protein, and AR2 showed the substitution of an alanine residue by leucine at amino acid position 189. The recovery of the antiviral activity of the vTK-dependent compounds in FTK cells against both types of mutated viruses indicated that their antiviral resistance was indeed due to vTK-deficiency and that FTK cells could be a useful tool in confirmation of the mechanism of drug-resis-

tance caused by vTK-deficiency, since ACV, GCV, PCV and BVDU regained their antiviral activity in FTK cells. A partial decrease of resistance to ACV was observed in one of the other TK-deficient mutants that we generated and that was shown to have an additional DNA polymerase mutation causing resistance to PFA and much higher resistance to ACV than the other virus mutants with only TK deficiency in Vero or 143B cells—the EC_{50} values of ACV in the Vero, 143B and FTK cells being >100, >100 and 20 μg/ml, respectively. Therefore, we could conclude that the additional drug resistance in the FTK cells was due to a DNA polymerase mutation.

In some cases, however, BVDU and GCV became more cytotoxic to the FTK cells than in mock-infected cells. This again indicates and confirms that the FTK cells produce the vTK protein with functional activity. This phenomenon has been well reported in literature and forms the rational basis of combined gene/chemotherapy of cancer cells (Balzarini et al., 1985a,b; Balzarini and De Clercq, 1989). Cytostatic drug-resistance of mammary carcinoma cells transfected with the HSV-1 TK gene has also been reported (Moolten et al., 1990; Golumbek et al., 1992). Unlike antiviral-resistance mainly caused by change(s) in the DNA sequences (i.e. mutations or deletions), resistance at the TK level was due to partial or complete loss of HSV-1 TK activity (Moolten et al., 1992; Yang et al., 1998), or inhibition of transcription caused by methylation of the vTK gene (Tasseron-de Jong et al., 1989; Degrève et al., 2000).

The biological functions and the regulation of the vTK gene is not clearly understood yet. Expression of the vTK gene in FTK cells showed a cytostatic as well as a cytocidal effect. Dramatic morphological changes were often observed in some cell clones we had selected for screening, and this may have been influenced by a different integration site of the gene construct into the chromosomes. Too high an expression of the vTK gene in cells may also be toxic. FTK seemed to express less vTK than 143TK obtained from ATCC and to grow better than 143TK. The vTK-dependent compounds and other nucleosides showed much less toxicity and more pronounced antiviral activity in FTK cells than in 143TK cells (data not shown). GCV and BVDU became significantly toxic in FTK, whereas ACV and PCV did not. However, all of them showed much higher toxicity in 143TK cells (data not shown). It is not clear if this is due to the different expression vector system or to the His-tagged vTK protein, or to a higher enzyme expression in the 143TK cells. Retarded viral growth was observed in FTK cells compared to 143B cells even though they were kept in the absence of G418, HAT or BDU for two passages to remove the possible harmful effects of these supplements on virus amplification. Although there are still several issues about FTK cells to be solved, we have shown in this study that there may be a useful tool to quickly elucidate the role of viral TK in drug-resistant mutant HSV-1 strains.

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