OUKA
Osaka University Knowledge Archive

Title	Isolation of Drug Resistant Mutants of Varicella-Zoster Virus : Cross Resistance of Acyclovir Resistant Mutants with Phosphonoacetic Acid and Bromodeoxyuridine
Author(s)	Shiraki, Kimiyasu; Ogino, Takao; Yamanishi, Koichi et al.
Citation	Biken journal : journal of Research Institute for Microbial Diseases. 1983, 26(1), p. 17-23
Version Type	VoR
URL	https://doi.org/10.18910/82467
rights	
Note	

Osaka University Knowledge Archive : OUKA

https://ir.library.osaka-u.ac.jp/

Osaka University

ISOLATION OF DRUG RESISTANT MUTANTS OF VARICELLA-ZOSTER VIRUS: CROSS RESISTANCE OF ACYCLOVIR RESISTANT MUTANTS WITH PHOSPHONOACETIC ACID AND BROMODEOXYURIDINE

KIMIYASU SHIRAKI, TAKEO OGINO[1]
KOICHI YAMANISHI and MICHIAKI TAKAHASHI

Department of Virology, Research Institute for Microbial Diseases, Osaka University, Yamadaoka, Suita, Osaka 565, Japan

(Received December 1, 1982)

SUMMARY Mutants of Varicella-Zoster Virus (VZV) which are resistant to phosphonoacetic acid (PAA), bromodeoxyuridine (BuDR), and acyclovir (ACV) were obtained by serial passages of VZV with increasing concentrations of these drugs. A PAA-resistant mutant and a BuDR-resistant mutant were found also to be resistant to ACV. Five of 8 ACV-resistant mutants acquired resistance to PAA, but none acquired resistance to BuDR. The BuDR-resistant mutant did not induce viral thymidine kinase (TK) activity, but all the ACV-resistant mutants selected in ACV showed viral TK activity which was suppressed with anti-VZV serum and had almost the same electrophoretic mobility as that of the parent strain on polyacrylamide gel electrophoresis in non-denaturing conditions. However, in competitive TK assay with ACV, 2 of 8 ACV-resistant mutants showed no change of phosphorylation of radioactive thymidine, while the other 6 showed decreased phosphorylation of radioactive thymidine. It was suggested that TK induced by the former 2 ACV-resistant mutants had lost affinity to ACV, and so the mutants could grow in the presence of ACV. Thus of the 8 ACV-resistant mutants selected in ACV, 2 were sensitive to PAA with altered TK activity, 5 were resistant to PAA with unaltered TK activity, and 1 was sensitive to PAA with unaltered TK activity, and may have altered DNA polymerase activity to ACV, retaining sensitivity to PAA.

These results suggest that resistance of VZV to ACV results from alterations in the virus-specified TK or DNA polymerase, as demonstrated in HSV resistant to ACV.

1 Paesent address: Hiroshima City Institute of Public Health, Shoko-Center, Nishi-ku, Hiroshima 733, Japan

INTRODUCTION

Acyclovir strongly inhibits growth of herpes simplex virus (HSV) (Elion et al. 1977). In HSV, ACV is phosphorylated by viral TK and the resulting triphosphorylated ACV becomes a substrate for viral DNA polymerase (Fyfe et al. 1978; Coen and Schaffer, 1980; Schnipper and Crumpacker, 1980) thus blocking DNA synthesis at its 3′ terminal incorporation site (Furman et al. 1980).

VZV is also inhibited by PAA, BuDR and ACV (May et al. 1977; Dobersen et al. 1976; Crumpacker et al. 1979; Biron and Elion. 1980). In this study we isolated PAA, BuDR and ACV resistant mutants of VZV and studied their properties with regard to the roles of TK and DNA polymerase by investigating their cross-resistance to other drugs.

MATERIALS AND METHODS

1. *Cells*

Human Embryonic lung (HEL) cells were propagated in a mixture of equal amounts of Eagle's minimal essential medium (MEM) and medium 199 supplemented with 10% calf serum, and were maintained in the same medium with 3% calf serum.

2. *Virus*

The Kawaguchi strain of VZV (Takahashi et al., 1975) was propagated in HEL cells and cell-free virus was plaque-purified 6 times and stocked as cell-free virus in SPGC medium (Asano and Takahashi, 1978) at -70 C.

3. *Isolation of drug resistant mutants*

Cell-free virus was inoculated onto HEL cells and after appearance of the 50% cytopathic effect (CPE), infected cells were inoculated onto uninfected HEL cells at a ratio of 1: 5 and maintained in the presence of drug. A PAA-resistant mutant (PAA-R) was isolated by passage in increasing concentrations of the drug, (10 μg/ml, 20 μg/ml, 50 μg/ml, 75 μg/ml) with 3 passages in each concentration until the appearance of CPE. When 50% of the monolayer showed CPE, the infected cells were washed once with ice-cold PBS, treated with 0.1% of EDTA in PBS and sonicated in SPGC medium. The sonicate was centrifuged at 2,000 *g* for 10 min at 4 C and the supernatant was used for plaque purification. After plaque purification twice, the virus was used as PAA-R.

VZV was passaged three times each with BuDR at 5 μg/ml, and 10 μg/ml until the appearance of CPE, and after plaque purification twice in the presence of 10 μg/ml of BuDR, it was used as BuDR-R.

ACV-resistant mutants were isolated in the presence of increasing concentrations of the drug (20 μM, 50 μM, 100 μM) by 3 passages at each concentration until the appearance of CPE. After plaque purification twice in the presence of 100 μM of ACV they were used as ACV-resistant mutants (A1-A8).

4. *Measurement of sensitivity of virus to drugs*

Monolayers of HEL cells in 60 mm plastic Petri dishes were inoculated with 100 plaque forming units (PFU) / dish of virus in 0.2 ml SPGC medium. After incubation for 1 h to permit adsorption, 5 ml of maintenance medium and the required concentration of drug were added. After the appearance of CPE, the cells were fixed and stained, and plaques were counted. The effective dose for 50% plaque reduction (ED_{50}) was determined by plotting the reduction in plaque number against the log of the drug concentration in μg/ml and reading the dose required for 50% plaque reduction from the graph.

5. *Assay of thymidine kinase activity*

The TK assay used was based on the method of Ogino et al. (1977). When more than 70% of the monolayer showed CPE in the presence of ACV, BuDR, PAA or without drug, the cells were treated with 0.1% EDTA in PBS and washed 3 times with PBS by brief centrifugation. The resulting pellet was suspended in 150 mM KC1, 3 mM 2-mercaptoethanol, 50 mM Tris buffer (pH 8.0) at a cell concentration of 5×10^6 cells/ml, and sonicated for 1 min in an ice bath. The sonicated samples were centrifuged at 3,000 rpm for 10 min at 4 C, dialysed against 150 mM KC1, 3 mM 2-mercaptoethanol, 50 mM Tris buffer (pH 8.0) and centrifuged at 20,000 rpm in a Hitachi RP 40 rotor at 4 C. The supernatant was used for enzyme assay. The enzyme assay mixture, in a final volume of 0.25 ml,

contained 0.2 μCi of ^{14}C thymidine (54 mCi/mmol, Amersham), 5 m*M* ATP, 5 m*M* $MgCl_2$, and 50 m*M* Tris buffer (pH 8.0). The reaction was conducted at 38 C for 15 min and was stopped by immersing the mixture in a boiling water bath for 2 min. The amount of phosphorylated thymidine was determined by the DEAE cellulose disc method. TK activity in the linear phase of the reaction was measured.

6. *Neutralization of thymidine kinase activity by anti-VZV serum*

Antiserum to VZV was obtained by repeated immunization of green monkeys with VZV-infected green monkey cells. The enzyme extract was incubated with an equal volume of twice-diluted preimmunization serum or post-immunization serum for 1 h at 4 C and then TK activity was assayed.

7. *Polyacrylamide gel electrophoresis*

Non-denaturing polyacrylamide gel electrophoresis (PAGE) was carried out by the procedure of Larder and Darby (1982). The separating gel contained 5% acrylamide, 0.2% bisacrylamide, 0.38 *M* Tris base (pH 8.0) 1.25 m*M* $MgCl_2$, 0.2 m*M* thymidine, 0.025% ammonium persulfate and 0.025% N,N,N',N'-tetramethylethylenediamine (TEMED), and the stacking gel contained 2% acrylamide 0.08% bisacrylamide, 0.12 *M* Tris base (pH 6.8), 1.25 m*M* $MgCl_2$, 0.2 m*M* thymidine, 0.025% ammonium persulfate and 0.025% TEMED. The running buffer contained 25 m*M* Tris base, 0.19 *M* glycine, 1 m*M* $MgCl_2$, 0.2 m*M* thymidine and 10 m*M* 2-mercaptoethanol. The buffer in the upper chamber (cathode) was supplemented with 2.5 m*M* ATP. Samples were applied in a volume of 30–50 μl containing 10% glycerol. Electrophoresis was carried out for approximately 4 h at 4 C at a constant current of 60 mA using slab gel. Bromophenol blue was used as a tracking dye. After electrophoresis, TK was located as follows. A sheet of DE-81 paper was layered over the gel and soaked in 5 ml of TK assay mixture (50 m*M* Tris base, pH 8.0 5 m*M*, ATP, 1 μCi/ml of ^{14}C thymidine) and the paper and gel were sandwiched between glass plates, sealed into a plastic bag, and incubated at 38 C for 1 h. The paper was then removed and washed with 1 m*M* ammonium formate, and the reaction products were located by autoradiography.

8. *Competitive assay of thymidine kinase activity with ACV*

For examination of quantitative or qualitative alterations in drug-resistant mutants, the TK activities of cells infected with the drug-resistant mutants were assayed. TK activity was assayed in the presence of the indicated concentration of ACV. The activity with each concentration of ACV was plotted on a graph, expressing TK activity without ACV as 100%.

9. *Antiviral drugs*

Phosphonoacetic acid was obtained from ICN Pharmaceuticals Inc. (Plainview N.Y.), bromodeoxyuridine from Sigma Chemical Co. (St. Louis U.S.A.), and acyclovir was a gift from the Japan Wellcome Co.

RESULT

1. *Isolation of drug resistant mutants*

VZV was passaged in the presence of drugs, and drug resistant VZV mutants were isolated. The mutant clones isolated were one PAA-resistant mutant clone (PAA-R), one BuDR-resistant mutant clone (BuDR-R), and 8 ACV-resistant mutant clones (A1-A8) (Table 1).

2. *Sensitivity of viruses to drugs*

Drug-resistant mutants were examined for cross-sensitivities to PAA, BuDR, and ACV, and the results are also summarized in Table 1. The parent strain is sensitive to PAA, BuDR, and ACV. PAA-R and BuDR-R are resistant to the selecting drug and ACV. The ACV resistant mutants selected in ACV are all sensitive to BuDR but 5 mutants (A1, A2, A3, A4, and A5) have acquired resistance to PAA and 3 mutants (A6, A7, and A8) remain sensitive to PAA.

3. *Induction of viral thymidine kinase activity*

Table 2 shows the TK activity of infected cells and the neutralization of its activity by preimmunization serum and post-immunization serum to VZV. The parent strain and mutants other than BuDR-R had ability to induce TK activity in infected cells. The in-

TABLE 1. *Drug susceptibilities (ED_{50}'s) of the parent strain and drug resistant mutants*

Strain	ACV (μM)	BuDR (μg/ml)	PAA (μg/ml)
Parent	4.6	2.0	5.2
A1[a]	>100	1.9	24
A2	>100	2.1	>50
A3	>100	1.05	>50
A4	>100	1.45	44
A5	>100	1.6	34
A6	>100	1.05	<5.0
A7	>110	1.7	<5.0
A8	>100	1.6	<5.0
PAA-R[b]	>100	<1.0	>75
BuDR-R[c]	>100	>10.0	<5.0

[a] A1-A8: Acyclovir resistant mutants selected in ACV.
[b] Phosphonoacetic acid resistant mutant selected in PAA.
[c] Bromodeoxyuridine resistant mutant selected in BuDR. Mutants were plaque purified twice in the presence to the following concentrations of drugs: ACV; 100 μM, PAA; 75 μg/ml, BuDR; 10 μg/ml.

TABLE 2. *Neutralization of thymidine kinase activity by anti-VZV serum*

	Pre-serum	Post-serum
Original	30,245(100%)	2,156(7.1%)
A1	8,560(100%)	833(9.7%)
A2	20,312(100%)	2,778(13.7%)
A3	6,635(100%)	2,279(34.3%)
A4	3,868(100%)	395(10.2%)
A5	5,645(100%)	1,675(29.7)%
A6	8,106(100%)	684(8.4%)
A7	23,849(100%)	4,249(17.8%)
A8	8,825(100%)	778(8.8%)
PAA-R	29,119(100%)	3,330(11.4%)
BuDR-R	1,494(100%)	1,208(80.9%)
HEL	1,938(100%)	1,306(67.4%)

TK activity was measured after incubation with preimmunization serum and postimmunization serum to VZV. The activity with postimmunization serum was calculated as a percentage of that with preimmunization serum.

duced TK activity was neutralized with postimmunization serum but not with preimmunization serum, suggesting that the TK activity expressed is induced by virus, and that all mutants except BuDR-R possess the capacity to induce viral TK. The degrees of activity induced by each mutant are difficult to compare from these data, because the mode of infection is not uniform.

4. *Polyacrylamide gel electrophoresis of TK induced by the mutants*

The location of TK in PAGE is shown in Fig. 1. No TK activity was detectable in enzyme extracts from uninfected HEL cells, HEL cells infected with BuDR-R or cells infected with the parent strain which had been preincubated with VZV antiserum. In contrast, enzyme extract of cells infected with the parent VZV strain which had been preincubated with preimmunization serum, those of cells infected with PAA-R, A2 and A7 showed TK activity with almost the same electrophoretic mobility (Fig. 1). The TK induced by all the ACV-resistant mutants (A1-A8) had almost the same electrophoretic mobility as that of the parent strain (data not shown).

5. *Competitive assay of TK activity with ACV*

All mutants except BuDR-R induced viral TK activity. To determine whether some of them induce TK activity with different substrate specificity from that of the parent strain, we assayed the TK activity of the mutants in the presence of ACV. In the presence of ACV, the parent strain, PAA-R, and the ACV-resistant mutants A2, A3, A4, A5, A7 showed greatly decreased TK activity, while mutant A1 showed slightly decreased TK activity and mutants A6 and A8 showed no decrease in TK activity (Fig. 2). ACV inhibits virus growth because the triphosphorylated form of ACV, which is produced by viral TK, inhibits DNA polymerase activity. Thus in the former group ACV is phosphorylated and so the uptake of radioactive thymidine is suppressed, whereas in the latter group ACV is presumably not

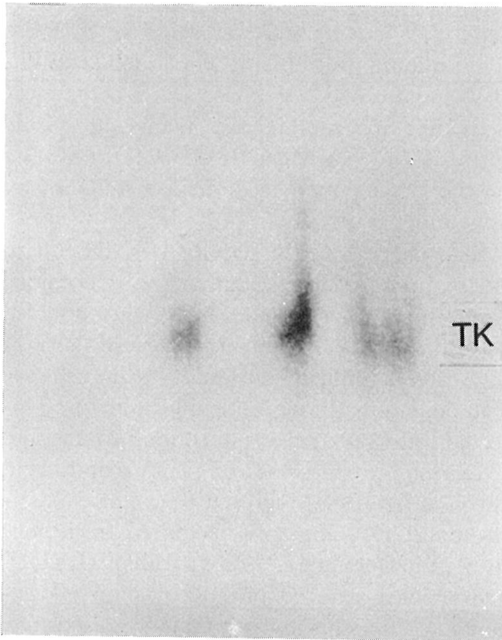

FIGURE 1. The location in nondenaturing polyacrylamide gel of TK induced by infection with various strains of VZV. High speed supernatants of cell extracts were subjected to nondenaturing polyacrylamide gel electrophoresis, and TK was located by autoradiography as described in the text,

1. Extract of HEL cells.
2. Extract of parent strain preincubated with postimmunization serum to VZV.
3. Extract of parent strain preincubated with preimmunization serum to VZV.
4. Extract of BuDR-R infected HEL cells.
5. Extract of PAA-R infected HEL cells.
6. Extract of A2 infected HEL cells.
7. Extract of A7 infected HEL cells.

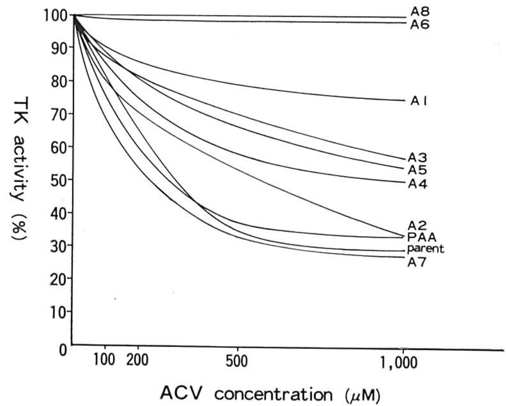

FIGURE 2. TK activity induced by infection with drug resistant mutants was assayed in the presence of ACV. The TK activity at various concentrations of ACV is plotted.

phosphorylated and so the virus is resistant to ACV. Therefore, the TK induced by A6 and A8 probably has altered substrate specificity to ACV.

DISCUSSION

ACV resistancy in HSV has been explained by mutation in the TK gene and DNA polymerase gene. ACV is phosphorylated by viral TK, not host TK, thus making it possible to inhibit virus growth selectively (Elion et al., 1977; Biron and Elion 1980; Field et al. 1980). The TK locus involves the absence of TK induction upon HSV infection (Schnipper and Crumpacker 1980; Crumpacker et al. 1980) and altered substrate specificity of viral TK which phosphorylates thymidine but not acyclovir. (Darby et al. 1981; Larder and Darby 1982). The DNA polymerase locus shares resistance to PAA and ACV (Schnipper and Crumpacker 1980; Crumpacker et al. 1980; Furman et al. 1981), but a BW^r mutant was isolated that is sensitive to PAA but resistant to ACV (Furman et al. 1981). This means that the triphosphorylated form of ACV cannot be a substrate for viral DNA polymerase in this mutant and that mutation on DNA polymerase conferred resistance to ACV but not to PAA.

Furman et al. (1980) determined the mechanism of inhibition of DNA synthesis using biochemically transformed cells. ACV is phosphorylated and then incorporated terminally at the 3′-end of growing DNA chains stopping DNA synthesis at this point.

In this study we isolated PAA, BuDR, ACV-

TABLE 3. *Biological Characters of ACV Mutants*

Strain	Altered TK activity	PAA resistance
Parent	−	−
A1	±	+
A2	−	++
A3	−	++
A4	−	+
A5	−	+
A6	+	−
A7	−	−
A8	+	−

resistant mutants, and observed cross-resistance of the PAA and BuDR resistant mutants to ACV. Five of 8 ACV-resistant mutants (A1, A2, A3, A4, A5) acquired resistance to PAA. PAA-R may be a mutant of the DNA polymerase locus, like the PAA-resistant mutant of HSV. BuDR-R did not induce viral TK activity (Table 2, Fig. 1). All the ACV-resistant mutants selected in ACV induced viral TK activity upon infection, but 2 of 8 mutants (A6, A8) showed altered substrate specificity to ACV. The TK of Al seemed to have slightly altered substrate specificity to ACV. As A1 was resistant to PAA, it may be a double mutant of the TK locus and DNA polymerase locus. A7 showed wild type TK activity and was sensitive to PAA. This mutant presumably corresponds to the BW^r mutant of HSV whose DNA polymerase does not incorporate the triphosphorylated form of ACV (Furman et al. 1981). Studies on the DNA polymerase of these mutants are now under way.

The degree of TK activity was difficult to assess, because enzyme extracts were obtained from infected cells inoculated with infected cells in this work. This was because, VZV of high titer is difficult to obtain in the cell-free state and thus a low input multiplicity of infected cells was used as inoculum for infection.

During preparation of this manuscript, Biron et al. (1982) reported that 3 of 5 ACV-resistant VZV mutants acquired resistance to 5-(2-bromovinyl)-2′-deoxyuridine (BVDU) and showed reduced phosphorylation of acyclovir. The other two mutants were sensitive to BVDU, and all 5 mutants were sensitive to PAA. The former mutants probably correspond to A6 and A8 and the latter mutants to A7. They did not report a PAA resistant ACV-resistant mutant.

PEFERENCES

Asano Y., Takahashi M. 1978. Studies on neutralization of varicella-zoster virus and serological follow-up of cases of varicella and zoster. Biken J. 21: 15–23.

Biron K. K., Elion G. B. 1980. In vitro susceptibility of varicella-zoster virus to acyclovir. Antimicrob. Agents Chemother. 18: 443–447.

Biron K., Fyfe J. A., Noblin J. E., Elion G. B. 1982. Selection and preliminary characterization of acyclovir-resistant mutants of varicella zoster virus. Proceedings of a Symposium on Acyclovir. Am. J. Med. July 20, 1982: 383–386.

Coen D. M., Schaffer P. A. 1980. Two distinct loci confer resistance to acycloguanosine in herpes simplex virus type 1. Proc. Natl. Acad. Sci. U.S.A. 77: 2265–2269.

Crumpacker, C. S., Schnipper, L. E., Zaia J. A., Levin M. J. 1979. Growth inhibition by acycloguanosine of herpesviruses isolated from human infections. Antimicrob. Agents Chemother. 15: 642–645.

Crumpacker, C. S., Chartrand, P., Subak-Sharpe, J. H., Wilkie, N. M. 1980. Resistance of herpes simplex virus to acycloguanosine-Genetic and physical analysis. Virology 105: 171–184.

Darby, G., Field, H. J., Salisbury, S. A. 1981. Altered substrate specificity of herpes simplex virus thymidine kinase confers acyclovir-resistance. Nature 289: 81–83.

Dobersen, M. J., Jerkofsky, M., Greer, S. 1976. Enzymatic basis for the selective inhibition of varicella-zoster virus by 5-halogenated analogues of deoxycytidine. J. Virol. 20: 478–486.

Elion, G. B., Furman, P. A., Fyfe, J. A., Miranda,

P. D., Beauchamp, L., Schaeffer, H. J. 1977. Selectivity of action of an antiherpetic agent, 9-(2-hydoxyethoxymethyl) guanine. Proc. Natl. Acad. Sci. U.S.A. 74: 5716–5720.

Field H. J., Darby G., Wildy P. 1980. Isolation and characterization of acyclovir-resistant mutants of herpes simplex virus. J. Gen. Virol. 49: 115–124.

Furman, P. A., McGuirt, P. V., Keller, P. M., Fyfe, J. A., Elion, G. B. 1980. Inhibition by acyclovir of cell growth and DNA synthesis of cells biochemically transformed with herpesvirus genetic information. Virology 102: 420–430.

Furman, P. A., Coen, D. M., Clair, M.H.S., Schaffer, P. A. 1981. Acylovir-resistant mutants of herpes simplex virus type 1 express altered DNA polymerase or reduced acyclovir phosphorylating activities. J. Virol. 40: 936–941.

Fyfe, J. A., Keller, P. M., Furman, P. A., Miller, R. L., Elion, G. B. 1978. Thymidine kinase from herpes simplex virus phosphorylates the new antiviral compound, 9-(2-hydoxyethoxymethyl) guanine. J. Biol. Chem. 253: 8721–8727.

Larder, B. A., Darby, G. 1982. Properties of a novel thymidine kinase induced by an acyclovir resistant herpes simplex virus type 1 mutant. J. Virol. 42: 649–658.

May, D. C., Miller, R. L., Rapp, F. 1977. The effect of phosphonoacetic acid on the in vitro replication of varicella-zoster virus. Intervirology 8: 83–91.

Ogino, T., Otsuka, T., Takahashi M. 1977. Induction of deoxypyrimidine kinase activity in human embryonic lung cells infected with varicella-zoster virus. J. Virol. 21: 1232–1235.

Schnipper, L. E., Crumpacker, C. S. 1980. Resistance of herpes simplex virus to acycloguanosine: Role of viral thymidine kinase and DNA polymerase loci. Proc. Natl. Acad. Sci. U.S.A. 77: 2270–2273.

Smith, K. O., Kennell, W. L., Poirier, R. H., Lynd, F. L. 1980. In vitro and in vivo resistance of herpes simplex virus to 9-(2-hydroxyethoxymethyl) guanine. (acycloguanosine) Antimicrob. Agents Chemother. 17: 144–150.

Takahashi, M., Okuno, Y., Otsuka, T., Osame, J., Takamizawa, A., Sasada, T., Kubo, T. 1975. Development of a live attenuated varicella vaccine. Biken J. 18: 25–33.