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Characterization of herpes simplex virus type 1 selected in culture for resistance to acyclovir or foscarnet

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Abstract:

To determine the characteristics of herpes simplex virus type 1 (HSV-1) resistant to either acyclovir (ACV) or foscarnet (FOS), HSV-1 KOS (wild-type) strain was used for selection of drug-resistant mutants in Vero cells. In one-step growth curve, all mutants showed their abilities to multiply in different manners from the wild type. Based on plaque reduction assay, there was no cross resistance between ACV-resistant mutants (AR1, AR2, AR3) and FOS-resistant mutants (FR1, FR2, FR3). Moreover, AR2 and AR3 exhibited higher sensitivity to FOS. All the mutants demonstrated higher sensitivity to at least 1 of the 3 plant extracts of Barleria lupulina, Clinacanthus siamensis and Nephelium lappaceum. By neutralization test, FR1 was more sensitive to neutralizing antibodies. The results suggested that the mechanisms of ACV and FOS resistances of mutants studied were different. One mechanism of FOS resistance of mutant may involve in changing its antigenicity to the neutralizing antibodies. The medicinal plants studied showing better activities on the drug-resistant HSV than ACV and FOS would be useful for the alternative therapy in the future.

Keywords: ACV-resistant HSV-1; Barleria lupulina; Clinacanthus siamensis; Cross resistance; FOS-resistant HSV-1; Nephelium lappaceum; Neutralization test; One-step growth curve; Plaque reduction assay
Introduction

Herpes simplex virus (HSV) infections are very common in the general population and may be responsible for serious complication among immunocompromised patients [1]. HSVs whose genomes consist of a single large double-stranded DNA molecule, are members of the family Herpesviridae. HSV type 1 (HSV-1) is more frequently associated with nongenital (above the belt) infection whereas HSV type 2 (HSV-2) is associated with genital (below the belt) disease. Today, the principle of type specificity and location is not as predictable as it was. Nevertheless, this observation was pivotal for many of the early clinical, serologic, immunologic, and epidemiologic studies. Primary HSV-1 infections usually occur in the young child or young adult via mouth or lip. Recurrent herpes labialis is the largest reservoir of HSV-1 infection [2] and probably is the most recurrent HSV-1 disease.

Acyclovir (ACV) has been used as the treatment of choices for HSV infection. This widespread use of ACV has lead to the emergence of HSV strains resistant to ACV. Resistant HSV isolates have been reported [3-6] mainly in immunocompromised patients (prevalence around 5-7%) and particularly in allogeneic bone marrow transplants patients (prevalence reaching 30%) [1, 7-10].

Patients with ACV-resistant HSV infections usually are treated with foscarnet (FOS). Nevertheless, initial descriptions of in vitro resistance to FOS in HSV isolated from immunocompromised patients [11] and a child under bone marrow transplantation [12] have raised the possibility that FOS resistance may become a clinically relevant problem.

A large number of plants have been used by various societies for the treatment of diseases. Some traditional medicines consisting of these natural products have been shown by in vitro techniques to possess antiviral activity [13]. Of the 142 traditional medicines used in China, Indonesia, and Japan evaluated by Kurokawa et al. [14], 32 were found to have activity against HSV-1. Some Thai medicinal plants, Aglaia edulis, Barleria lupulina, Centella asiatica, Clinacanthus nutans, Glyptopetalum sclerocarpum, Macura cochinchinensis, have been proven for in vitro anti-HSV [15-19]. Previously, we screened 80 extracts from 16 plants for anti-HSV-2 activity and found that 19 extracts of 9 plants demonstrated good efficacy [20]. So, we selected 3 plants including Barleria lupulina, Nephelium lappaceum and Clinacanthus siamensis with the best efficacy to check their activity on our drug-resistant HSV-1 mutants in this study.

HSV-1 infections continue to increase, and drug resistance has become a serious problem in certain patients. Understanding the nature of drug-resistant HSV-1 would be the basic knowledge for help to dissolve the public problem. Little is known about the characteristics of ACV- and FOS-resistant HSV-1 isolates.

The advantage of the in vitro system is that it is a well-defined entity which contains just the viruses and the cells, while other factors, such as immunological ones, taking part in the defense mechanism of the animal are not involved. On the other hand, the in vitro system has its own limitations concerning what is actually happening with the virus in the animal [21].

Here, we reported an in vitro characteristics of ACV- and FOS-resistant HSV-1, in ability of replication, drug-cross resistance, sensitivity to medicinal plants possessing good anti-HSV activities and antigenicity to anti-HSV-1 polyclonal antibodies. The drug-resistant HSV-1 mutants demonstrated different characteristics from their wild type in several ways especially in abilities of replication. Some mutants retained the same characteristics as the wild type in sensitivities to drug/plant or specific neutralizing antibodies. Other(s) changed to be more sensitive to drug/plant or to the neutralizing antibodies.

Materials and Methods

Cell line and virus strains

Vero [American Type Culture Collection (ATCC)], an African green monkey kidney cell line was grown in
the growth medium [Minimum Essential Medium with Earle's balance salts and 290 mg/L L-glutamine, 2.2 g/L sodium bicarbonate (MEM), 10% fetal bovine serum and antibiotic-antimycotic agents which contained 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate and 0.25 μg/ml fungizone] and incubated at 37°C and 5% CO₂.

HSV-1 KOS strain was used as a wild type. ACV-resistant mutants included AR1, AR2 and AR3. FOS-resistant mutants were consisted of FR1, FR2 and FR3. All virus strains were plaque-purified for 3 cycles.

Plaque purification of viruses

Serial dilutions of the wild type virus stock were prepared and then inoculated onto confluent Vero cell monolayers of each well of a 6-well plate. After 1 hr adsorption, extracellular virus was removed. The infected cells were washed twice with MEM and incubated on ice for several minutes. Agarose medium (1.6% low melting agarose [Seaplaque agarose, BMA, Rockland, ME, USA] and 2 × growth medium without antibiotics [2 × GM] in a ratio 1:1) containing ACV or FOS trisodium salt was added onto the cold Vero cell plate. Following solidification of the agarose overlay, the culture plate was incubated at 37°C and 5% CO₂ until the plaques became visible. Virus from a single plaque was amplified in Vero cells. Following 3 cycles of plaque purification to ensure homogeneity, an eventually last passage was done by drug-free medium. Virus stocks were prepared in Vero cells that were infected at multiplicity of infection (MOI) of 0.01 and in drug-free medium.

Total virus was harvested by freezing-thawing the infected culture for 3 times, then was centrifuged (1,500 x g for 5-10 min) to get the virus in the supernatant. The supernatant virus was aliquoted and kept at -80°C until used. The viral stocks were titrated by plaque assay as previously described [20].

Compounds and plant extracts

ACV and FOS trisodium salt, hexahydrate were purchased from Sigma (USA) and prepared by dissolving the powder in sterile phosphate buffer saline (PBS, pH 7.4) aseptically in concentration of 400 μg/ml and 3 mg/ml. The stock solution was aliquoted and stored at -20°C until used.

Plants were collected during August 2001 and prepared as plant extracts. All plants were authenticated by Dr. Rutt Suttisri, Department of Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University. Each plant was chopped into small pieces and extracted with 95% ethanol for 3-7 days. Then, the extract was filtered and the filtrate was evaporated under reduced pressure at 40°C. Each dry extract was prepared at a concentration of 50 mg/ml in 100% dimethyl sulfoxide and sit at room temperature for 30 minutes. Then the extracts were kept at -20°C until used.

Selection of drug-resistant mutants

This procedure was performed by using a method modified from Andrei et al. [22] and Sarisky et al. [23]. Confluent Vero cell monolayers (seeded with 7 x 10⁵ cells/well) prepared in 6-well plates were infected with MOI of 0.1 for 1 hr at 37°C. The inoculum was then removed and 2 ml of growth medium containing ACV (1 μg/ml) or FOS (40 μg/ml) was added per well. When a culture demonstrated complete cytopathic effect, it was freeze-thawed for 3 times. Next, 0.5 ml of the resulting virus pool was passaged in the presence of increasing concentrations of ACV or FOS. Drug-resistant HSV-1 mutants were obtained by serial passage of the wild type virus in Vero cells in the presence of increasing concentrations of ACV or FOS. After the highest possible concentrations (80 μg/ml of ACV or 180 μg/ml of FOS) had been reached (which allowed mutant to survive), each drug mutant was selected [22].

Plaque reduction Assay

This procedure was performed by using a modification of Sarisky et al. [23]. Fifty microliters of each virus isolate (50 PFU) were added onto monolayer of Vero cells in each well of 96-well plate, triplicately and incubated at 37°C for 1 hr. One hundred microliters of the overlay medium (2 x GM and 1% methylcellulose
solution in a ratio 1:1 with 2-fold dilutions of plant extracts or ACV or FOS were overlayed onto the infected cultures, triplicately. Final concentrations of plant extracts or FOS were 250, 125, 62.5, 31.25 to 1.9 μg/ml and of ACV were 40, 20, 10, 5 to 0.06 μg/ml. The cultures were incubated in the CO2 incubator at 37°C until the plaques became visible. The plaques were enumerated and 50% inhibitory concentration (IC50) was determined as previously described [20]. Briefly, the relationship between the dose (or concentration) of drug (or extract) (x) and the plaque numbers (y) was plotted in a graph. The linear regression equation, y = a + bx or y = a + b log x, was obtained and 50% of the virus control (no drug and extract) plaque numbers on y axis were drawn to the regression line to find the 50% of the drug (extract) concentration on x axis.

One-step growth

The virus was inoculated onto Vero cells at MOI of 10. After 1 h at 37°C, the cells were washed twice with MEM in order to remove the unabsorbed virus and then the growth medium was added. Twelve hour, 24, 36 and 48 h of incubation, medium containing extracellular progeny viruses was collected, clarified by centrifugation (1,500 x g for 5 min) and frozen. The viruses were quantified by plaque assay [20]. In each viral isolate, the viral titers at different times (0, 12, 24, 36 and 48 h were plotted as a one-step growth curve. It was noted that the virus yield at time zero was counted after 1 h adsorption and twice washing, so no extracellular progeny virus was observed.

Neutralization test

Serial 2-fold dilutions (started at 1:50) of rabbit anti-HSV-1 neutralizing polyclonal antibodies (Dako code number B 0114) were prepared in MEM. Two hundred microliters of each dilution were incubated with 200 μl of each virus (200 PFU) at 37°C for 1 hr. The mixture were inoculated onto Vero cell monolayers in each well (100 μl/well, 4 wells/dilution) (antibody titers were started from 1:100, 1:200, 1:400 to 1:6,400) of a 96-well plate and reincubated at 37°C for 1 hr. One hundred microliters of overlay medium were added onto each infected well of the 96-well plate. The infected culture plate was incubated for 3 days. The plaque numbers in each well were counted and the antibody titer in virus isolate was calculated in term of 50% neutralization dose (ND50). The differences in the ND50 titer were considered significant if the differences were more than or equal to four-fold [24].

Results and Discussion

In this study, drug-resistant HSV-1 mutants were obtained by 7-10 serial passages of the wild type HSV-1 KOS strain in Vero cells in the presence of increasing concentrations of ACV or FOS. The highest possible concentration of ACV that allowed the mutants to survive was 80 μg/ml. However, IC50 of ACV against AR1-3 isolates were 10, 20 and 20 μg/ml, respectively (IC50 of ACV against the wild type was 0.15 μg/ml) (Table 1). Our results were different from the studies of Sarisky et al. [23], Saijo et al. [25], and Andrei et al. [22]. ACV-resistant HSV-1 isolates (14 mutants) selected in MRC-5 cells by Sarisky et al. [23] replicated in 10 μg/ml ACV and IC50 values of ACV against them were 17.3, 19.4, 20.4, 22.4, 25.8, 26.3, 36, 52.5, 56, 60.4 and more than 100 (4 isolates) (μg/ml (IC50 of ACV against their wild type, SC16 strain, was 0.2 μg/ml). ACV-resistant HSV-1 isolates (15 mutants) selected in Vero cells by Saijo et al. [25] survived in 3 μg/ml ACV and IC50 values of ACV against them were 4.8, 10, 16, 40, 70, 72, 80, and more than 100 (8 isolates) μg/ml (IC50 of ACV against their wild type, TAS strain, was 0.6 μg/ml). Andrei et al. [22] observed that ACV-resistant HSV-1 strains selected in human embryonic lung cells and 100 μg/ml ACV demonstrated IC50 of ACV was 22.5 ± 21.1 μg/ml (IC50 of ACV against their wild type, KOS strain was 0.014 ± 0.01 μg/ml). The reason why they were different from ours may be because the mutations occurred randomly; the cell lines [22, 23] and the wild type strains [23, 25] were different.
The highest possible concentrations of FOS which allowed FR1-3 mutants to grow was 180 μg/ml and IC_{50} values of FOS against FR1-3 were 40, 51.25 and 125 μg/ml, respectively (IC_{50} of FOS against the wild type was 23.44 μg/ml) (Table 1). The results were different from those of Andrei et al. [22] who reported that for FOS-resistant HSV-1 strains selected in 40 μg/ml FOS, IC_{50} of FOS was 127 ± 40 μg/ml (IC_{50} of FOS against the wild type was 29.6 ± 13.7 μg/ml). Andrei et al. [22] used fixed concentration of FOS to select HSV-1 mutants while we increased FOS concentrations to select the mutants. In addition, the wild type strain used in Andrei et al. [22] was different from the wild type strain in our study. Consequently, FOS-resistant HSV-1 mutants of Andrei et al. [22] and FR1-3 demonstrated different IC_{50}.

To study drug-cross resistance, plaque reduction assay was performed. Based on IC_{50}, there was no cross resistance between the ACV-resistant and FOS-resistant mutants (Table 1). Stranska et al. [10, 26], Andrei et al. [22] and Gaudreau et al. [27] also found that ACV-resistant HSV-1 mutants were susceptible to FOS. Moreover, our AR2 and AR3 were more sensitive to FOS (Table 1). Contrarily, Saijo et al. [12] and Stranska et al. [28] found that ACV-resistant HSV-1 strains isolated from immunocompromised children demonstrated cross resistance to FOS. One of these strains was never exposed to FOS before [28].

All FOS-resistant HSV-1 mutants were still susceptible to ACV (Table 1). Our results supported some of Safrin et al study [11]. Safrin et al reported that 7 of 10 FOS-resistant isolates from 6 patients, developing under the selective pressure of FOS usage, were ACV-susceptible. However, one isolate was of borderline susceptibility and 2 strains were ACV-resistant. Moreover, Andrei et al. [22] reported that FOS-resistant HSV-1 mutants were ACV-resistant.

ACV is nucleoside analogue of guanosine. The first phosphorylation is completed by the viral encoded thymidine kinase (TK) protein that allows ACV to become active only in virus infected cells. The second and third phosphorylations are achieved by cellular TK. ACV triphosphate acts by competitive

### Table 1. Susceptibilities to anti-HSV drugs and Thai plant extracts of ACV-resistant and FOS-resistant HSV-1 mutants and wild type (HSV-1 KOS strain) in Vero cells

<table>
<thead>
<tr>
<th>Virus isolate</th>
<th>IC_{50} (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ACV</td>
</tr>
<tr>
<td>KOS</td>
<td>0.15</td>
</tr>
<tr>
<td>AR1</td>
<td>10.00</td>
</tr>
<tr>
<td>AR2</td>
<td>20.00</td>
</tr>
<tr>
<td>AR3</td>
<td>20.00</td>
</tr>
<tr>
<td>FR1</td>
<td>0.15</td>
</tr>
<tr>
<td>FR2</td>
<td>0.15</td>
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<tr>
<td>FR3</td>
<td>0.15</td>
</tr>
</tbody>
</table>

KOS is HSV-1 KOS strain. AR1, AR2 and AR3 are ACV-resistant HSV-1 mutants. FR1, FR2 and FR3 are FOS-resistant HSV-1 mutants. BL = Barleria lupulina; CS = Clinacanthus siamensis; NL = Nephelium lappaceum
inhibition of viral DNA polymerase and it is a DNA chain terminator. Mechanism of ACV resistance in HSV has been involved in mutation of TK or DNA polymerase gene [25, 26, 28].

FOS presents a very different structure, analogous to a pyrophosphate and acts on viral DNA polymerase without previous activation by viral TK [1]. Mechanism of FOS resistance in HSV has been localized to mutation in the DNA polymerase gene [29, 30]. Consequently, FOS is active on ACV-resistant HSV that is altered in its TK protein but not DNA polymerase. Our AR1-3 mutants probably mutated their TK genes resulting in susceptibility to FOS. However, polymerase mutations may span a broad area of about 386 amino acids within 5 proposed domains [29, 30] resulting in a variety of potential permutations in antiviral susceptibility patterns [11].

Previously, plant extracts from 16 plants were screened for anti-HSV-2 activity [20]. We found that 3 plant extracts of B. lupulina (leaves), C. siamensis (leaves) and N. lappaceum (pericarp) exhibited the highest anti-HSV-2 activities. In this study, we checked anti-HSV-1 activity of these 3 plants on the drug-resistant mutants. Based on the IC50, all the mutants did not resist to the 3 plant extracts. Moreover, they were more sensitive to at least 1 of the 3 plant extracts compared with the wild type (Table 1). AR1, FR1 and FR2 demonstrated higher sensitivities to all the 3 extracts (IC50 < 31.25 μg/ml for B. lupulina, < 62.5 μg/ml for C. siamensis (leaves) and < 31.25 μg/ml for N. lappaceum). AR2 was more sensitive to extract of B. lupulina whereas FR3 was more susceptible to extracts of N. lappaceum. AR3 showed more sensitivities to both extracts of B. lupulina and C. siamensis. These Thai plants showing better activities on the ACV or FOS-resistant HSV-1 would be useful for the alternative therapy in the future.

One-step growth analysis can be used to study the single-cell life cycle of virus. The key to the one-step growth cycle is accomplished by infecting cells with a sufficient number of virus particles to ensure that most of the cells are infected rapidly. Most one-step growth experiments are conducted at a MOI of 5-10 PFU/cell to ensure that almost of the cells receive at least one infectious unit [31]. When the results of a one-step growth experiment are plotted graphically, a number of important features about virus replication are revealed. The number of viral progeny can be determined in either extracellular or intracellular viral particles per ml [31-32]. The pattern of one-step growth curve of each specific virus is characteristic. Moreover, the one-step growth curves of extracellular progeny viruses and intracellular progeny ones of the same virus strain are similar in pattern but the viral titers at each time may be slightly different [31-32]. In this study, we chose to measure the extracellular progeny viruses of the wild type virus and its mutants. All the mutants showed their abilities to multiply in different manner from the wild type (Figure 1). A titer of the wild type reached a plateau within 36 hours after infection whereas most of mutants did not. AR2, AR3 and FR1 did not show the plateau within 48 hours after the infection while FR2 replicated at the highest level within 24 hours of inoculation. Rate of replication of AR3 was very low whereas multiplication of AR2 became higher at 48 hours. Only FR3 replicated without exhibiting the plateau. Although the titer of AR1 reached the plateau within 36 hours, it was higher than that of the wild type.

To study antigenicity of the mutants, we performed the neutralization test and used ND50 to determine the titer of neutralizing anti-HSV-1 polyclonal antibodies. The antibodies react with HSV-1 specific antigens and with antigens common for HSV-1 and HSV-2. The finally highest concentration of sodium azide (antibody reagent preservative) used in this study was 0.000975% that was not toxic to Vero cells [33] as there was no cytopathic effect observed in our study (data not shown). We found that FR1 was more sensitive to the neutralizing antibodies than the wild type (Figure 2) (antibody concentration used was inverse to the antibody titer) suggesting that one mechanism of FOS-resistance may involve in changing antigenicity to the neutralizing antibodies. Nevertheless, AR1-3 and FR2-3 did not change the antigenicity significantly to the specific antibodies.
In summary, we determined drug cross-resistance and cross-resistance to some Thai plants, the characteristics of one-step growth and antigenicity expressed by ACV-and FOS-resistant HSV-1 mutants. The results suggested that B. lupulina, C. siamensis and N. lappaceum showing better activities on the drug-resistant HSV would be useful for the alternative therapy in the future. The mechanisms of ACV and FOS resistances of mutants studied were different. One mechanism of FOS resistance of mutant may involve in changing its antigenicity to the neutralizing antibodies. The patterns of one-step growth curve of ACV-resistant and FOS-resistant HSV-1 mutants were different from their parent wild type strain.

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