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Antiviral activity of Viracea® against acyclovir susceptible and acyclovir resistant strains of herpes simplex virus

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Abstract

Viracea, a topical microbicide, is a blend of benzalkonium chloride and phytochemicals derived from *Echinacea purpurea* and is a proprietary formula from Destiny BioMediX Corp. Viracea was tested against 40 strains of herpes simplex virus (HSV): 15 strains (five HSV-1 and ten HSV-2) were resistant to acyclovir (ACV-R) and 25 strains (13 HSV-1 and 12 HSV-2) were susceptible to ACV (ACV-S). The median ED_{50} of Viracea for the five ACV-R strains of HSV-1 was a 1:100 dilution of the drug with a range of 1:50–1:400. The median ED_{50} of Viracea for the ten ACV-R strains of HSV-2 was 1:200 with a range of 1:50–1:3200. For the ACV-S strains of HSV-1 and HSV-2, the median ED_{50} of Viracea was 1:100 and 1:200, respectively. The cytotoxicity of Viracea was evaluated in a standard neutral red dye uptake assay in human foreskin fibroblasts. The cytotoxicity of Viracea approached only 50% at the highest concentration of the drug tested, a 1:2 dilution, indicating that Viracea is non-toxic in this cell cytotoxicity assay. Although the active component(s) in Viracea that has anti-HSV activity is not known, it appears that this extract has good antiviral activity against both ACV resistant and ACV susceptible strains of HSV-1 and HSV-2.

Keywords: Acyclovir resistant HSV; Anti-herpes compound; *Echinacea purpurea*; Phytochemical

1. Introduction

Herpes simplex virus type 1 (HSV-1) infections are common in the US population and oropharyngeal infections are the most frequent infections caused by this virus (Corey and Spear, 1986a,b). Genital herpes is an important sexually transmitted disease that is usually caused by HSV-2, but HSV-1 can also cause these infections (Johnson et al., 1989). In addition, one report suggests that HSV-2 may be a risk factor for the transmission of human immunodeficiency virus (Holmberg et al., 1988).

Recently, Fleming et al. (1997) reported that the seroprevalence of HSV-2 in persons 12 years

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of age or older in the USA is 21.9%, a 30% increase over the previous survey for all groups. The initial infection with HSV can be quite severe but is often mild or subclinical. Less than 10% of those who were seropositive reported a history of genital herpes (Fleming et al., 1997) and recurrences with HSV can vary from none to frequent. In immunocompromised patients and neonates, however, HSV infections can be severe and may cause systemic illnesses.

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 (Beuscher et al., 1994, Taylor et al., 1996). Ferrea et al. (1993) found that methanolic extracts of dried *Combretum micranthum* leaves contained antiviral activity against HSV-1 and HSV-2. Meyer et al. (1996) described the inhibition of the cytopathic effect of HSV-1 in human lung fibroblasts using an aqueous extract of *Helichrysum aureonitens* (Asteraceae), a southern African medicinal plant. Of the 142 traditional medicines used in China, Indonesia, and Japan and evaluated by Kurokawa et al. (1993), 32 were found to have activity against HSV-1. The Indian medicinal plant, *Pongamia pinnata*, also has been shown to have in vitro activity against HSV (Elanchezhiyan et al., 1993). These materials were all prepared as extracts and the modes of action of most of these extracts are not known; however, an extract of the culture medium of mycelia from the edible mushroom *Lentinus edodes* appears to block the late stage of replication of HSV-1 (Sarkar et al., 1993). Yip et al. (1995) isolated the compound fluoranthene from the leaves and twigs of *Elsholtzia ciliata*, a Chinese medicinal plant, that has activity against Sindbis and murine cytomegalovirus.

HSV infections continue to increase, and ACV resistance has become a serious problem in certain patients; therefore, new anti-HSV drugs continue to be developed (Cassady and Whitley, 1997). Various phytochemicals have been shown to have in vitro antiviral activity and may be a good source of new antiviral agents. These agents have the potential to be modified and become more effective analogs than the original compounds. Viracea, a proprietary formula from Destiny BioMediX Corp., is a blend of benzalkonium chloride and phytochemicals derived from the aerial parts of *Echinacea purpurea* (L.) Moench. *E. purpurea*, the purple coneflower, is a native American plant and a member of the Asteraceae family. This study was designed to determine the in vitro antiviral activity of Viracea against ACV susceptible and resistant strains of HSV.

2. Materials and methods

2.1. Antiviral compounds

A stock solution of ACV (Glaxo-Wellcome) was prepared in water at a concentration of 10 mM and frozen in aliquots at −20°C. The stock ACV was diluted in Eagle's minimal essential medium (MEM; BioWhittaker, Walkersville, MD) for use in susceptibility assays.

Echinacea purpurea powder, provided by Destiny BioMediX, Chicago, IL, was prepared from the aerial parts of mature *E. purpurea* plants. The plants were dried, powdered and sterilized with ethylene oxide. Viracea was made into an aqueous solution by adding 100 mg *E. purpurea* powder per ml of water containing 5.0 μg of benzalkonium chloride per ml.

To determine the effect of temperature on the extraction of Viracea from the *E. purpurea* powder, three separate preparations were made by placing the mixture at either 37, 60, or 100°C for 1 h. These stock extracts were centrifuged to remove the particulate material and the supernatants were filtered through a 0.45 μm filter. Aliquots of each preparation were frozen at −70°C. To prepare the Viracea for antiviral testing, an aliquot was diluted in an equal volume of 2 × MEM. From this initial dilution, eight serial twofold dilutions of the drug were prepared in 1 × MEM beginning at a 1:25 dilution of the stock Viracea.

2.2. Cell cultures and viruses

Human foreskin fibroblasts (HFF) were obtained from ViroMed Laboratories, Minneapolis,

MN, at low passage and used throughout this study. The cells were grown in MEM supplemented with glutamine, antibiotics, and 10% fetal bovine serum (FBS). Maintenance medium was the same MEM with 1% FBS.

A total of 40 HSV strains were tested in this study: 18 HSV-1 strains and 22 HSV-2 strains. Ten HSV-1 and ten HSV-2 strains were isolated in the Clinical Virology Laboratory at The University of Chicago. Each strain was passaged ≤ 2 times before testing. A stock of each virus was grown in cultured fibroblasts and aliquots were frozen at -70°C. The titer of each virus was determined using HFF in 24-well plates with an agarose overlay. These 20 strains were determined to be susceptible to acyclovir (ACV-S) (Rabalais et al., 1987). An additional five ACV-S strains obtained from Dr Karen Biron, Glaxo-Wellcome, Research Triangle Park, NC, were also tested.

Fifteen acyclovir resistant (ACV-R) strains of HSV were tested in this study. Eight ACV-R strains were isolated in our laboratory, one HSV-1 and seven HSV-2. These eight strains were passed and frozen as described above. In addition, we obtained seven ACV-R strains from Dr Karen Biron, four HSV-1 and three HSV-2 strains. The strains received from Glaxo-Wellcome have been characterized and the mutations associated with ACV resistance are known.

A control strain, either HSV-1 strain F or HSV-2 strain G, was included with each set of assays. These control strains are susceptible to ACV.

2.3. Susceptibility testing by ELISA

HFF cells were inoculated into 96-well microtiter trays at a density of 7×10^3 cells per well. The plates were incubated at 37°C in 5% CO_2 until the cells were confluent, usually 3 days. Sixty wells of each plate were used: six uninfected cell control wells, six virus-infected control wells without drug, and six replicates of eight dilutions of the drug. Dilutions of each virus were prepared in MEM. The growth medium was removed from all wells and 50 μl of MEM was added to the cell control wells and 50 μl of virus inoculum (multiplicity of infection (MOI) of 0.05) was added to the remaining wells. The virus was allowed to adsorb for 2 h at 37°C. The inoculum was removed and 100 μl of MEM was added to the cell control wells and the virus control wells. Eight serial two-fold dilutions of Viracea were prepared in MEM and 100 μl of the diluted drug was added to the remaining wells beginning at a 1:25 dilution through a 1:3200 dilution of the drug. All plates were incubated at 37°C in 5% CO_2.

After incubating for 48 h, the plates were examined using an inverted phase contrast microscope to insure that viral cytopathic effect (CPE) was present in the virus control wells and to score the CPE in all wells of the plate including the virus control wells and all drug dilution wells. Each row of wells was scored from 0 to 4+, 4+ indicating that all cells showed CPE. This was done to insure that the inhibition of CPE correlated with the quantitative ELISA results. The medium was then removed from all microtiter wells and 100 μl of a blocking solution consisting of 0.5% bovine serum albumin (BSA) in phosphate buffered saline, pH 7.2, was added to each well for 30 min at room temperature. The blocking solution was removed, the cells were fixed by adding 100 μl of ethanol–acetone (95:5, v/v) to each well and the plates were placed at -20°C for 30 min. Each well was washed four times with 200 μl of wash solution (PBS containing 0.5% BSA and 0.05% Tween 20).

The antibodies used in the ELISA were obtained from Dako, Carpinteria, CA, and were prepared by immunizing rabbits with an antigen prepared by sonication and extraction of HSV-1 or HSV-2 infected rabbit cornea cells. All the virion proteins were present in the antigen preparation used to produce the antibody. To determine the effective dose (ED_{50}), the rabbit polyclonal antibody to HSV-1 or HSV-2 conjugated to horseradish peroxidase was diluted in PBS containing 10% normal rabbit serum. A volume of 100 μl of the antibody was added to each well and the plates were incubated at 37°C for 2 h. The antibody was removed and the wells were washed four times as before. The enzyme substrate, 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma, St Louis, MO) was added to each well and the plates were incubated at room temperature for 3–4 min. The optical density (OD) of each well

was read in a dual wavelength ELISA plate reader at 630–490 nm. The average OD was determined for the uninfected cell control wells, the virus control wells, and each drug dilution. The percent change in OD was calculated as follows: [(average drug sample OD) − (average cell control OD)]/[(average virus control OD) − (average cell control OD)] × 100. The ED_{50} is defined as the dilution of antiviral compound that produces a 50% or greater reduction in the OD of the colored substrate product.

The susceptibility of the HSV strains to ACV was determined by using the same in situ ELISA procedure as above. The concentrations of ACV tested were from 0.1 to 50 μM.

The susceptibility of HSV to benzalkonium chloride alone was also tested beginning with a 5.0 μg/ml solution, the same concentration that was used to make the Viracea extracts. The stock 5.0 μg/ml solution was diluted using serial two-fold dilutions identical to the Viracea extract dilution scheme.

2.4. Cytotoxicity assay

The cytotoxicity of Viracea was determined by using a standard in vitro cytotoxicity procedure with minor modifications (Neyndorff et al., 1990). Briefly, eight serial two-fold dilutions of Viracea were prepared in MEM beginning at a 1:2 dilution and 100 μl of the diluted drug was added to HFF cells in a 96-well microtiter plate. The plates were incubated for 48 h. The MEM was removed from the microtiter plates, replaced with 100 μl of MEM containing 50 μg/ml neutral red dye (Sigma, St Louis, MO) and incubated for 3 h at 37°C. The dye solution was removed and replaced with 200 μl of neutral buffered 4% formaldehyde for 2 min to fix the cells. The fixative was removed and replaced with 200 μl of a solution containing 1% acetic acid and 50% methanol to extract the dye and the plates were incubated for 20 min at room temperature. The OD of the neutral red dye was read in a dual wavelength spectrophotometer at 490–630 nm. The cytotoxic dose (Tox_{50}) was determined as the percentage change in OD and was calculated as follows: [(average drug sample OD) − (average cell control OD)]/[(average dye control OD) − (average cell control OD)] × 100. The Tox_{50} is defined as the dilution of drug that produces a 50% or greater reduction in the OD of the neutral red dye.

3. Results

3.1. The effect of temperature on the extraction of Viracea

The two control strains of HSV, HSV-1 strain F and HSV-2 strain G, were tested with the three extracts prepared at 37, 60 and 100°C. Each preparation was tested ten times. No differences were seen in the activity of Viracea prepared by extraction at either 37 or 60°C. The median ED_{50} for the preparations that were extracted at 37 or 60°C was the 1:200 dilution of Viracea. The median ED_{50} for the preparation extracted at 100°C was a 1:25 dilution. Since extracts prepared at either 37 or 60°C gave identical results, we chose to use the extract prepared at 60°C for the remainder of this study.

3.2. The anti-HSV activity of Viracea

Five characterized ACV-S strains of HSV were received from Glaxo-Wellcome and tested in our assay in addition to the 20 ACV-S clinical strains of HSV collected at the University of Chicago. These 25 HSV strains were tested in an in situ ELISA to determine the inhibitory activity of Viracea. For the 13 ACV-S strains of HSV-1, the median ED_{50} of Viracea was a 1:100 dilution of the drug with a range of 1:25–1:400 (Table 1). The median ED_{50} of Viracea for the 12 ACV-S strains of HSV-2 was 1:200 with a range of 1:50–1:1600 (Table 2).

The inhibitory activity of Viracea was also determined for the 15 ACV-R strains of HSV. For the five ACV-R strains of HSV-1, the median ED_{50} was a 1:100 dilution of the drug with a range of 1:50–1:400 (Table 3). The median ED_{50} of the ten ACV-R strains of HSV-2 was a 1:200 dilution of the drug with a range of 1:50–1:3200 (Table 3). The 40 strains used in this study were tested two or more times in the in situ ELISA to

Table 1
ACV susceptible strains of HSV-1

Strain	Susceptibility to:	
	ACV (μM)	Viracea (dilution)
1. V61665	0.5	1:50
2. M35290	0.25	1:200
3. T18327-1	0.5	1:50
4. V70843	1.0	1:200
5. H30716	0.5	1:50
6. V74140	0.5	1:100
7. X17626	1.0	1:200
8. F21664	0.5	1:400
9. S6434	0.5	1:400
10. H31191	1.0	1:100
11. 15616 SC16 [a]	0.5	1:100
12. 15990 BW-S [a]	0.5	1:25
13. 13231WL KOS [a]	1.0	1:50
Median ED_{50}		1:100
HSV-1 control strain F	0.5	1:50

[a] Virus obtained from Glaxo-Wellcome, Research Triangle Park, NC.

determine the activity of Viracea. The data presented are derived from a representative assay.

Control strains were also tested with each run of the ELISA. The median ED_{50} of seven assays with HSV-1 strain F was a 1:50 dilution of Viracea (Table 1). The median ED_{50} of eight assays with HSV-2 strain G was a 1:100 dilution of the drug (Table 2).

Table 2
ACV susceptible strains of HSV-2

Strain	Susceptibility to:	
	ACV (μM)	Viracea (dilution)
1. T7788	2.5	1:100
2. T43648	2.5	1:200
3. V72019	0.5	1:800
4. F51695	2.5	1:200
5. M23277	2.5	1:200
6. R-326	1.0	1:50
7. T33301	0.5	1:200
8. V72833	0.5	1:200
9. V69730	0.5	1:200
10. V74269	1.0	1:200
11. 14875 MS2 [a]	1.0	1:1600
12. 16053 8702 [a]	1.0	1:800
Median ED_{50}		1:200
HSV-2 control strain G	0.5	1:100

[a] Virus obtained from Glaxo-Wellcome, Research Triangle Park, NC.

Table 3
Acyclovir resistant strains of HSV

	Susceptibility to:	
	ACV (μM)	Viracea (dilution)
HSV-1 strains		
1. T28387	>50	1:200
2. 11573 PAAr5 [a]	5.0	1:400
3. 12959 DM21 [a]	>50	1:100
4. 15985 SC16-S1 [a]	>50	1:100
5. 15991 BW-R [a]	>50	1:50
Median ED_{50}		1:100
HSV-2 strains		
1. V60780	>50	1:3200
2. V68697	>50	1:800
3. V74262	>50	1:100
4. F66556	>50	1:200
5. F23226	>50	1:1600
6. R-324	>50	1:200
7. R-325	>50	1:50
8. 14893 Kost [a]	20	1:100
9. 16052 8708 [a]	10	1:800
10. 16064 8713 [a]	>50	1:1600
Median ED_{50}		1:200

[a] Virus obtained from Glaxo-Wellcome, Research Triangle Park, NC.

To control for the possibility that Viracea was inhibiting the horseradish peroxidase in the ELISA in a dose-dependent manner rather than inhibiting HSV, an ELISA was performed in which one-half the plate (three rows of wells) was treated with dilutions of Viracea in the usual manner and the other half of the plate was fed with MEM without any drug. After 48 h of incubation, the MEM was removed from the second half of the plates and replaced with the same dilutions of Viracea and incubated for an additional 4 h. The ELISA results for the half of the plate that received Viracea for 48 h showed the typical inhibition for both HSV-1 and HSV-2 with ED_{50} values for both viruses of 1:100. The half of the plates treated with Viracea after 48 h of incubation showed no inhibition of either virus in the ELISA. We concluded that Viracea had no

inhibitory effect on the horseradish peroxidase in the ELISA.

3.3. The anti-HSV activity of benzalkonium chloride

To determine if the benzalkonium chloride used in the preparation of Viracea has an inhibitory effect on HSV, susceptibility assays were performed using benzalkonium chloride alone. Benzalkonium chloride was prepared at a concentration 5.0 μg/ml and diluted in MEM using the same dilution scheme used to test Viracea, 1:25–1:3200. The benzalkonium chloride was tested against the control strains of HSV-1 and HSV-2 and the assays were repeated two times with each virus. The ED_{50} of benzalkonium chloride was $<$1:25 dilution in all four assays. These results indicate that benzalkonium chloride by itself has no inhibitory effect on HSV at the concentrations used in the Viracea preparation.

3.4. Cytotoxicity of Viracea

The toxicity of Viracea to HFF was determined using the neutral red dye uptake cytotoxicity assay. After 48 h of incubation, the toxicity of Viracea approached 50% only at the highest concentration of the drug tested, the 1:2 dilution. This was the highest possible concentration that could be tested since the aqueous extract must first be diluted 1:2 in 2 $\times$ MEM. Of the ten cytotoxicity assays performed, the Tox_{50} of Viracea was equal to 1:2 in three assays and less than 1:2 in seven assays, i.e. no toxicity was evident.

4. Discussion

We examined the anti-HSV activity of Viracea against ACV susceptible and resistant strains of HSV-1 and HSV-2. The clinical strains were passed a limited number of times before the isolates were tested with ACV or Viracea. These isolates were characterized only as to their susceptibility to ACV and the mutations associated with ACV resistance in these clinical strains are not known.

Viracea was shown in this study to have good in vitro activity against clinical strains of HSV. No difference was found in the antiviral activity of Viracea against the ACV-S and the ACV-R strains of HSV. The inhibition of HSV by Viracea appears to function after the adsorption and penetration steps because in these studies, the drug was added after 2 h of incubation of the virus with the cells. The active component(s) in Viracea is not known at present. It may be that there is more than one active component in this aqueous extract or that the active component is a metabolite of a compound found in the extract. Knowing the nature of the active component is important so that the mode of action can be determined. Phytochemicals may be a good source of new antiviral agents that have the potential to be modified and become more effective analogs than the original compounds.

The role of benzalkonium chloride in the Viracea preparation is not known. Benzalkonium chloride alone at the concentration used in the preparation of Viracea has no inhibitory effect on HSV in these assays. It is possible that benzalkonium chloride forms a complex with a compound in the *E. purpurea* powder. It is also possible that the benzalkonium chloride acts to stabilize the active component in the preparation.

The effect of temperature on the extraction of the active component indicated that only the high temperature of 100°C reduced the anti-HSV activity of Viracea; however, the drug retained significant activity after boiling for 1 h, indicating that the active component(s) is relatively stable at high temperature.

Experiments to assess the toxicity of Viracea indicate that this preparation of the drug is non-toxic in cell cultures. The therapeutic index, the inhibitory dilution divided by the toxic dilution, is in the range of 50–100.

Phytochemicals are beginning to be investigated as therapeutic agents for a variety of infectious agents. These materials, like Viracea, may be relatively non-toxic and some may have a long history of use in traditional herbal medicines. The use of a non-toxic topical microbicide for the treatment of recurrent HSV would be ideal, especially for those patients who experience frequent recurrences.

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References

Beuscher, N., Bodinet, C., Neumann-Haefelin, D., Marston, A., Hostettmann, K., 1994. Antiviral activity of African medicinal plants. J. Ethnopharmacol. 42, 101–109.

Cassady, K.A., Whitley, R.J., 1997. New therapeutic approaches to the alphaherpesvirus infections. J. Antimicrob. Chemother. 39, 119–128.

Corey, L., Spear, P.G., 1986a. Infections with herpes simplex virus. New Engl. J. Med. 314, 686–691.

Corey, L., Spear, P.G., 1986b. Infections with herpes simplex virus. New Engl. J. Med. 314, 749–755.

Elanchezhiyan, M., Rajarajan, S., Rajendran, P., Subramanian, S., Thyagarajan, S.P., 1993. Antiviral properties of the seed extract of an Indian medicinal plant, *Pongamia pinnata*, Linn., against herpes simplex viruses: in-vitro studies on Vero cells. J. Med. Microbiol. 38, 262–264.

Ferrea, G., Canessa, A., Sampietro, F., Cruciani, M., Romussi, G., Bassetti, D., 1993. In vitro activity of a *Combretum micranthum* extract against herpes simplex virus types 1 and 2. Antiviral Res. 21, 317–325.

Fleming, D.T., McQuillan, G.M., Johnson, R.E., Nahmias, A.J., Aral, S.O., Lee, F.K., St. Louis, M.E., 1997. Herpes simplex virus type 2 in the United States, 1976 to 1994. New Engl. J. Med. 337, 1105–1111.

Holmberg, S.D., Stewart, J.A., Gerber, A.R., Byers, R.H., Lee, F.K., O'Malley, P.M., Nahmias, A.J., 1988. Prior herpes simplex virus type 2 infection as a risk factor for HIV infection. J. Am. Med. Assoc. 259, 1048–1050.

Johnson, R.E., Nahmias, A.J., Magder, L.S., Lee, F.K., Brooks, C.A., Snowden, C.B., 1989. A seroepidemiologic survey of the prevalence of herpes simplex virus type 2 infection in the United States. New Engl. J. Med. 321, 7–12.

Kurokawa, M., Ochiai, H., Nagasaka, K., Neki, M., Xu, H., Kadota, S., Sutardjo, S., Matsumoto, T., Namba, T., Shiraki, K., 1993. Antiviral traditional medicines against herpes simplex virus (HSV-1), poliovirus, and measles virus in vitro and their therapeutic efficacies for HSV-1 infection in mice. Antiviral Res. 22, 175–188.

Meyer, J.J.M., Afolayan, A.J., Taylor, M.B., Engelbrecht, L., 1996. Inhibition of herpes simplex virus type 1 by aqueous extracts from shoots of *Helichrysum aureonitens* (Asteraceae). J. Ethnopharmacol. 52, 41–43.

Neyndorff, H.C., Bartel, D.L., Tufaro, F., Levy, J.G., 1990. Development of a model to demonstrate photosensitizer-mediated viral inactivation in blood. Transfusion 30, 485–490.

Rabalais, G.P., Levin, M.J., Berkowitz, F.E., 1987. Rapid herpes simplex virus susceptibility testing using an enzyme-linked immunosorbent assay performed in situ on fixed virus-infected monolayers. Antimicrob. Agents Chemother. 31, 946–948.

Sarkar, S., Koga, J., Whitley, R.J., Chatterjee, S., 1993. Antiviral effect of the extract of culture medium of *Lentinus edodes* mycelia on the replication of herpes simplex virus type 1. Antiviral Res. 20, 293–303.

Taylor, R.S.L., Manandhar, N.P., Hudson, J.B., Towers, G.H.N., 1996. Antiviral activities of Nepalese medicinal plants. J. Ethnopharmacol. 52, 157–163.

Yip, L., Hudson, J.B., Towers, G.H.N., 1995. Isolation of the anthropogenic compound fluoranthene in a screening of Chinese medicinal plants for antiviral compounds. Planta Med. 61, 187–188.