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BROAD-SPECTRUM ANTIVIRAL EFFICACY OF NATURAL-PRODUCT HUMATES NOW TRADEMARKED VIRACILLIN™

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For
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Picture of Ebola Virus Here.

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Preface

This Report presents the results of toxicology, cell proliferation, and efficacy testing work carried out on natural product Humates, Humic Acid (HA) in 2001-2002 by contract laboratories of the Virology Branch of the Antiviral Research and Antimicrobial Chemistry Program (Dr. Christopher Tseng, Program Officer), Division of Microbiology and Infectious Diseases *(DMID)* Screening and Testing Program for Antiviral, Immunomodulatory, Antitumor and/or Drug Delivery Activities, National Institutes of Allergy and Infectious Diseases *(NIAID),* under the auspices of the National Institutes of Health *(NIH,* Bethesda, Maryland). Samples of natural-product Humates Humic Acid (HA) were submitted directly to the contract laboratories for evaluation. Efficacy data are presented for five herpes viruses, three influenza viruses, and two hemorrhagic fever viruses.

Herpes simplex virus types 1 ***(HSV-1)*** and 2 ***(HSV-2)*** are responsible for orofacial and genital herpes, respectively. Approximately 45 million Americans are serum-positive for genital herpes (type 2), with 20 million routinely exhibiting symptoms. It is believed that 80% of Americans are serum-positive for orofacial herpes (type 1), virtually all of whom occasionally exhibit symptoms. Roughly half of herpes simplex viruses are today Acyclovir-resistant. Human cytomegalovirus ***(HCMV)*** is pervasive in the U.S.: 90% of the urban U.S. population is serum-positive for HCMV, although infection in immunocompetent individuals is generally asymptomatic. Varicella zoster virus ***(VZV)*** is the cause of chickenpox in children and shingles in adults. Epstein-Barr virus ***(EBV)*** is the cause of infectious mononucleosis in adolescents and young adults.

Influenza A ***(INFL-A)*** (6 strains) and B ***(INFL-B)*** (3 strains) are routinely responsible for 20,000-30,000 deaths annually in the U.S., and well over 100,000 hospitalizations. Pandemics occur when the virus mutates radically. The 1918 “Spanish flu” [Influenza A(H1N1)] caused approximately 500,000 deaths in the U.S. and 20,000,000 worldwide. The 1957-58 “Asian flu” [influenza A(H2N2)] resulted in 70,000 deaths in the U.S., while the 1968-69 “Hong Kong flu” [influenza A(H3N2)] brought about 34,000 U.S. dead.

Pichinde virus ***(PICV)*** is a "New-World" (South American) arenavirus whose members are generally associated with rodent-transmitted disease in humans. Punta Toro virus ***(PUTV)*** is a South American phlebovirus of the Bunyaviridae family of viruses, and is transmitted by sand flies. Crimean-Congo, Rift Valley, and Hanta viruses are also members of this family.

In order to be consistent with the nomenclature used in work carried out previously on Humates, the toxicology data are reported here as $_{TC50}$ values, that is, toxic concentrations of drug that result in 50% cell toxicity. Cell proliferation data are reported as $_{CP50}$ values, that is, concentrations of drug that produce a 50% decrease in cell proliferation. Drug efficacy data are given as $_{IC50}$ and $_{IC90}$ values, that is, inhibitory concentrations of drug that are efficacious in preventing infection of 50% or 90% of the cells treated. All concentrations are in units of μg/mL (corresponding to parts per million by weight/volume, *ppm)*.

TOXICITY ASSAYS

HFF Cells
MDCK Cells
LLC-MK_2 Cells

Toxicity Assays

Methodology

The Neutral Red method of assaying for drug toxicity was carried out in roughly the same manner for all cell lines tested; that employed for human foreskin fibroblast (HFF) cells utilized in the herpes work is provided below as a representative example.

Twenty-four hours prior to assay, HFF cells were plated into 96-well plates at a concentration of 2.5×10^4 cells per well. After 24 h, the medium was aspirated and 125 µL of drug was added to the first row of wells and then diluted serially 1:5 using the Cetus Liquid Handling System in a manner similar to that used in the CPE assay (see p. 7). After drug addition, the plates were incubated for seven days in a CO_2 incubator at 37°C. At this time the medium+drug was aspirated and 200 µL/well of 0.01% neutral red in PBS was added. This was incubated in the CO_2 incubator for 1 h. The dye was aspirated and the cells were washed using a Nunc Plate Washer. After removing the PBS, 200 µg/well of 50% EtOH/1% glacial acetic acid (in H_2O) was added. The plates were rotated for 15 min and the optical densities were read at 540 nm on a plate reader.

Visual observation was employed to confirm cell toxicity during the course of influenza and punta toro virus efficacy testing. Thus, during the cytopathic effect (CPE) inhibition tests, two additional wells of uninfected cells treated with each concentration of test compound were run in parallel with the infected, treated wells. At the time CPE was determined microscopically the toxicity control cells were also examined microscopically for any changes in cell appearance compared to normal control cells run in the same plate. These changes became manifest as enlargement, granularity, cells with ragged edges, a filmy appearance, rounding, detachment from the surface of the well, or other changes. The changes were given a designation of T (100% toxic), PVH (partially toxic-very heavy - 80%), PH (partially toxic-heavy - 60%), P (partially toxic - 40%), Ps (partially toxic-slight - 20%), or 0 (no toxicity - 0%), conforming to the degree of cytotoxicity seen. A 50% cytotoxic concentration (TC_{50}) was determined by regression analysis of these data.

Results

All drug evaluated were not cytotoxic at levels at least as high as 100 μg/mL, as shown below in **Table I.** Visual observation of Natural-Product Humate (Viracillin™), Humic Acid (HA) with uninfected MDCK cells in toxicity control wells appeared initially to indicate drug toxicity. However, the drugs were not in fact toxic as revealed by Neutral Red assays. Rather, the Humate, HA compounds were found to bind to cell surfaces, thereby changing their color and giving them an exanimate appearance. This discoloration was observed in a concentration-dependent manner at levels where antiviral activity was present.

Table I. Toxic Concentrations at 50% (TC_{50}) of Natural-Product Humates, Humic Acid (HA) Drugs with Indicated Cell Lines

	TC_{50}, μg/mL					
Natural Product Humate	BSC-1[a]	HFF[b]	MDCK[c]	LLC-ML_2[d] Trial 1	Trial 2a[f]	Trial 2b[g]
HA	>100	>100	>100	>100	>1000	>1000

[a]African green monkey kidney cells. [b] Human foreskin fibroblast cells. [c] Madin Darby canine kidney cells. [d]Adult rhesus monkey kidney cells. [e] Not evaluated. [f] Neutral Red assay. [g] Visual assay.

CELL PROLIFERATION VIABILITY) ASSAYS

HFF Cells
Daudi Cells

Cell Proliferation (Viability) Assays

Methodology

The counting method of assaying for cell proliferation (viability) was carried out in roughly the same manner for all cell lines tested; that employed for HFF cells utilized in the herpes work is provided below as a representative example.

Twenty-four hours prior to assay, HFF cells were seeded in 6-well plates at a concentration of 2.5 x 10^4 cells per well in minimum essential medium (MEM) containing 10% fetal bovine serum (FBS). On the day of the assay, drugs were diluted serially in MEM containing 10% FBS at increments of 1:5 covering a range from 100 µg/mL to 0.03 µg/mL. For drugs that were solubilized in DMSO, control wells received MEM containing 10% DMSO. The medium from the wells was then aspirated and 2 mL of each drug concentration was then added to each well. The cells were then incubated in a CO_2 incubator at 37°C for 72 h. At the end of this time, the medium+drug solution was removed and the cells washed. One milliliter of 0.25% trypsin was added to each well and incubated until the cells started to come off of the plate. The cell-medium mixture was then pipetted up and down vigorously to break up the cell suspension and 0.2 mL of the mixture was added to 9.8 mL of Isoton III and counted using a Coulter Counter. Each sample was counted three times with two replicate wells per sample.

Results

All natural product Humates did not inhibit cell proliferation at drug levels at least as high as 50 µg/mL, as shown below in **Table II.**

Table II. Cell Proliferation Inhibition Concentrations at 50% (CP_{50}) of Natural Product Humate, Humic Acid (HA) Drugs with Indicated Cell Lines

	CP_{50}, μg/mL	
Humate	HFF[a]	Daudi[b]
HA	88.4	>50

[a]Human foreskin fibroblast cells. [b] Burkitt's lymphoma derived cells.

HERPES VIRUSES

Herpes Simplex Virus Type 1 *(HSV-1)*

Herpes Simplex Virus Type 2 *(HSV-2)*

Epstein-Barr Virus *(EBV)*

Human Cytomegalovirus *(HCMV)*

Varicella Zoster Virus (VZV)

Herpes Viruses

Methodology

Preparation of Human Foreskin Fibroblast (HFF) Cells. Newborn human foreskins were obtained as soon as possible after circumcision and placed in minimal essential medium (MEM) containing vancomycin, fungizone, penicillin, and gentamicin, at the usual concentrations, for 4 h. The medium was then removed, the foreskin minced into small pieces and washed repeatedly with phosphate buffered saline (PBS) deficient in calcium and magnesium (PD) until red cells were no longer present. The tissue was then trypsinized using trypsin at 0.25% with continuous stirring for 15 min at 37°C in a CO_2 incubator. At the end of each 15-min period the tissue was allowed to settle to the bottom of the flask. The supernatant containing cells was poured through sterile cheesecloth into a flask containing MEM and 10% fetal bovine serum. The flask containing the medium was kept on ice throughout the trypsinizing procedure. After each addition of cells, the cheesecloth was washed with a small amount of MEM containing serum. Fresh trypsin was added each time to the foreskin pieces and the procedure was repeated until all the tissue was digested. The medium was then centrifuged at 1000 rpm at 4°C for 10 min. The supernatant liquid was discarded and the cells resuspended in a small amount of MEM with 10% FBS. The cells were then placed in an appropriate number of 25-mL tissue culture flasks. As cells became confluent and needed trypsinization, they were expanded into larger flasks. The cells were kept on vancomycin and fungizone to passage four, and maintained on penicillin and gentamicin.

Cytopathic Effect Inhibition Assay (CPE) for Herpes Simplex Viruses (HSV), Human Cytomegalovirus (HCMV), and Varicella Zoster Virus (VZV). Low-passage HFF cells were seeded into 96well tissue culture plates 24 h prior to use at a cell concentration of 2.5 x 10^5 cells per mL in 0.1 mL of MEM supplemented with 10% FBS. The cells were then incubated for 24 h at 37°C in a CO_2 incubator. After incubation, the medium was removed and 125 µL of experimental drug was added to the first row in triplicate wells, all other wells containing 100 µL of medium. The drug in the first row of wells was then diluted serially 1:5 throughout the remaining wells by transferring 25 µL using the Cetus Liquid Handling Machine. After dilution of drug, 100 µL of the appropriate virus concentration was added to each well excluding cell control wells, which received 100 µL of MEM. For HSV-1 and HSV-2 assays, the virus concentration utilized was 1000 PFU's per well. For CMV and VZV assays, the virus concentration added was 2500 PFU per well. The plates were then incubated at 37°C in a CO_2 incubator for 3 days for HSV-1 and HSV-2, 10 days for VZV, or 14 days for CMV. After the incubation period, the medium was aspirated and the cells stained with a 0.1% crystal violet solution for 4 h. The stain was then removed and the plates rinsed using tap water until all excess stain was removed. The plates were allowed to dry for 24 h and then read on a BioTek Plate Reader at 620 nm

Efficacy Assay for Epstein-Barr Virus (EBV).

There are two prototypes of infectious EBV. One is exemplified by the virus derived from supernatant fluids of the P3HR-1 cell line. This cell line produces nontransforming virus that induces the production of early antigen (EA) and viral capsid antigen (VCA) after primary infection or superinfection of B cell lines. The other prototype is exemplified by the B-95-8 virus. This virus immortalizes cord blood lymphocytes and induces tumors in marmosets. It does not, however, induce an abortive productive infection even in cell lines harboring EBV genome copies. The virus used in the assays of this work was P3HR-1.

Cell Lines. Daudi is a low level producer that contains 152 EBV genome copies/cell. These cells respond to superinfection by EBV by expressing EA and VCA. This cell line was maintained in RPMI-1640 medium supplemented by 10% FBS, L-glutamine and 100 μg/mL gentamicin. The cultures were fed twice weekly and the cell concentration adjusted to 3 x 10^5/mL. The cells were kept at 37°C in a humidified atmosphere with 5% CO_2.

ELISA Assay. Daudi cells were infected and treated with drug as described above. The cultures were incubated for 4 days at 37°C. The cells were counted, washed and brought to the desired final concentration. For each dilution of drug, cells were added to triplicate wells of a 96-well plate and air dried. The cells were then fixed for 20 min in an acetic acid/ethanol solution. A monoclonal antibody to EBV VCA was added and the cells were incubated for 1 h, followed by an incubation with horseradish peroxidase labeled goat antimouse IgG1 for 30 min. Plates were rinsed with PBS/Tween20 between incubations. Substrate containing Ophenylenediamine, citrate buffer and hydrogen peroxide was added to each well, and the plates were covered and gently shaken for 10 min. The reaction was stopped by adding 3N sulfuric acid, following which the plates were read on a microplate reader at 492 nm.

Reference Drugs. Acyclovir (Glaxo SmithKline) was the reference compound employed in the HSV-1, HSV-2, VZV, and EBV efficacy testing work. Ganciclovir (Roche) was the reference drug used with HCMV.

Results

The efficacy data for Natural-Product Humates Humic Acid (HA) with the five herpes viruses examined in this work are provided in the following tables. As shown, Natural Product HA was found to be effective against HSV-1 and HSV-2, and their efficacy approached that of Acyclovir. Natural Product HA was somewhat less effective against human cytomegalovirus, against varicella zoster virus. And also against Epstein-Barr virus.

Table III. Effective Inhibitory Concentrations at 50% (IC_{50}) and 90% (IC_{90}) of Natural-Product Humate, Humic Acid (HA) Drug and Acyclovir (ACV) Reference Compound with Herpes Simplex Virus Type 1 (HSV-1) (HFF Cells)

Drug	IC_{50}, μg/mL	IC_{90}, μg/mL
Natural Humate HA	4.7	13.1
Acyclovir	1.2–1.6	7.9

Table IV. Effective Inhibitory Concentrations at 50% (IC_{50}) and 90% (IC_{90}) of Natural-Product Humate, Humic Acid (HA) Drug and Acyclovir (ACV) Reference Compound with Herpes Simplex Virus Type 2 (HSV-2) (HFF Cells)

Drug	IC_{50}, µg/mL	IC_{90}, µg/mL
Natural Humate HA	2.5	6.7
Acyclovir	1.1–1.3	9.5

Table V. Effective Inhibitory Concentrations at 50% (IC_{50}) and 90% (IC_{90}) of Natural-Product Humate, Humic Acid (HA) Drug and Ganciclovir (GCV) Reference Compound with Human Cytomegalovirus (HCMV) (HFF Cells)

Drug	IC_{50}, µg/mL	IC_{90}, µg/mL
Natural Humate HA	32.3	47
Ganciclovir	0.3–0.76	0.6-1.3

Table VI. Effective Inhibitory Concentrations at 50% (IC_{50}) and 90% (IC_{90}) of Natural-Product Humate Drug and Acyclovir (ACV) Reference Compound with Varicella Zoster Virus (VZV) (HFF Cells)

Drug	IC_{50}, µg/mL	IC_{90}, µg/mL
Natural Humate HA	53.5	85.8
Acyclovir	0.23–0.38	16.3

Table VII. Effective Inhibitory Concentrations at 50% (IC_{50}) and 90% (IC_{90}) of Natural-Product Humate Drug and Acyclovir (ACV) Reference Compound with Epstein-Barr Virus (EBV) (Daudi Cells)

Drug	IC_{50}, µg/mL	IC_{90}, µg/mL
Natural Humate HA	>50	>50
Acyclovir	1.8–2.4	16.3

INFLUENZA VIRUSES

Influenza A/New Caledonia/20/99 (H1N1)

Influenza A/Panama/2007/99 (H3N2)

Influenza A/NWS/33 (H1N1)

Influenza A/PR/8/34 (H1N1)

Influenza A/Shangdong/09/93 (H3N2)

Influenza A/Sydney/05/97 (H3N2)

Influenza B/Beijing/184/93

Influenza B/Harbin/07/94

Influenza B/Hong Kong/5/72

Influenza Viruses

Methodology

Viruses and Cell Line Used in Primary Drug Screening. Influenza A and B were employed in this portion of the work. The virus strains were: A/New Caledonia/20/99(H1N1), A/Panama/2007/99(H3N2), A/NWS/33 (H1N1), A/PR/8/34 (H1N1), A/Shangdong/09/93(H3N2), and A/Sydney/05/97 (H3N2); and B/Beijing/184/93, B/Harbin/07/94, and B/Hong Kong/5/72. (All were tested in the presence of trypsin). The cell line was comprised of Madin Darby canine kidney (MDCK) cells.

Methods for Assay of Antiviral Activity.

Inhibition of Viral Cytopathic Effect (CPE). This test, run in 96-well flat-bottomed microplates, was used for the initial antiviral evaluation of all Natural-Product Humate, Humic Acid (HA) test compounds. In this CPE inhibition test, four $_{log10}$ dilutions of each drug (e.g. 1000, 100, 10, 1 mg/mL) were added to 3 cups, each containing a cell monolayer; within 5 min, the virus was then added and the plate sealed, incubated at 37°C, and the CPE read microscopically when untreated infected controls developed a 3 to 4+ CPE (approximately 72 to 120 h). A known positive control drug (Ribavirin; ICN Pharmaceuticals) was evaluated in parallel with the Natural-Product Humate in each test. Follow-up testing with compounds found active in initial screening tests were run in the same manner except 7 one-half log_{10} dilutions of each compound were used in 4 cups, each containing a cell monolayer per dilution.

Increase in Neutral Red (NR) Dye Uptake. This test was run to validate the CPE inhibition seen in the initial test, and utilized the same 96-well micro plates after the CPE had been read. Neutral red was added to the medium; cells not damaged by virus take up a greater amount of dye. Color intensity was read on a computerized micro plate autoreader. The method described by McManus *(Appl. Environ. Microbiol.* **1976,** *31,* 35-38) was employed. The $_{IC50}$ was determined from this dye uptake.

Decrease in Virus Yield (VY). Compounds considered active by CPE inhibition and by NR dye uptake were re-tested using both CPE inhibition and, using the same plate, effect on reduction of virus yield by assaying frozen and thawed eluates from each cup for virus titer by serial dilution onto monolayers of susceptible cells. Development of CPE in these cells was the indication of presence of infectious virus. As in the initial tests, a known active drug was run in parallel as a positive control. The 90% effective concentration (IC_{90}), i.e., a testdrug concentration that inhibits virus yield by 1 log_{10}, was determined from these data.

Secondary Test. Following confirmation of significant antiviral activity in initial testing and in virus yield assays an additional study was performed, consisting of determination of the effect of time of addition of test compounds to virus-infected cells.

Reference Drug. Ribavirin was the reference compound employed in the influenza efficacy testing work.

Results

The efficacy data for Natural Product Humates, Humic Acid (HA) with the influenza viruses examined in this work are provided in the following tables. As shown, Natural Product HA was found to be quite effective against all three influenza viruses.

In the time of addition studies **(Table XVII),** the most efficacious antiviral effect was observed when cells were pre-treated (at time 0) with Natural Product HA, that is, the drugs appeared to *prevent* infection. In addition, activity was also present with post-infection treatment regimens (Ribavirin lost all its antiviral activity by 24 h). For example, at 100 µg/mL concentration of Natural Product HA in infected cells, discrete virus foci were seen that appeared like small plaques (particularly when the drugs were added 24 h after virus exposure). These results suggest that the compounds also inhibited virus adsorption even after the infection process had begun. (Mature influenza virus buds out of the host cell, then goes on to infect new cells during its life cycle. Since the cells were continuously exposed to the Natural Product HA compounds, newly-formed virus exiting cells during the early rounds of virus replication would be blocked from attaching and entering uninfected cells to initiate new infections.)

Table VIII. Effective Inhibitory Concentrations at 50% (IC_{50}) and 90% (IC_{90}) of Natural Product HA Drugs and Ribavirin Reference Compound with Influenza Virus Type A (New Caledonia/20/99) (H1N1) (MDCK Cells)

	IC_{50}, µg/mL			
Drug	CPE Method	NR Method	VY Method	IC_{90}, µg/mL
Natural Product Humate HA	2.5	2.5	3.2	5
Ribavirin	0.55	0.38	0.32	1.4

Table IX. Effective Inhibitory Concentrations at 50% (IC_{50}) and 90% (IC_{90}) of Natural Product HA Drugs and Ribavirin Reference Compound with Influenza Virus Type A (Panama/2007/99) (H3N2) (MDCK Cells)

	IC_{50}, µg/mL			
Drug	CPE Method	NR Method	VY Method	IC_{90}, µg/mL
Natural Product Humate HA	<1	<1	0.22	0.4
Ribavirin	1.3	1.8	1.9	1.4

Table X. Effective Inhibitory Concentrations at 50% (IC_{50}) of Natural Product HA Drugs and Ribavirin Reference Compound with Influenza Virus Type A (NWS/33) (H1N1) (MDCK Cells)

	IC_{50}, µg/mL		
Drug	CPE Method	NR Method	VY Method
Natural Product			
Humate HA	1.3	1.3	–
Ribavirin	5-6.0	4.6-6.5	–

Table XI. Effective Inhibitory Concentrations at 50% (IC_{50}) of Natural Product HA Drugs and Ribavirin Reference Compound with Influenza Virus Type A (PR/8/34) (H1N1) (MDCK Cells)

	IC_{50}, µg/mL		
Drug	CPE Method	NR Method	VY Method
Natural Product			
Humate HA	14	18	–
Ribavirin	9	12	–

Table XII. Effective Inhibitory Concentrations at 50% (IC_{50}) of Natural Product HA Drugs and Ribavirin Reference Compound with Influenza Virus Type A (Shangdong/09/93) (H3N2) (MDCK Cells)

	IC_{50}, µg/mL		
Drug	CPE Method	NR Method	VY Method
Natural Product Humate HA	15	18	–
Ribavirin	1.5-3.2	1.7-3.2	–

Table XIII. Effective Inhibitory Concentrations at 50% (IC_{50}) of Natural Product HA Drugs and Ribavirin Reference Compound with Influenza Virus Type A (Sydney/05/97) (H3N2) (MDCK Cells)

	IC_{50}, µg/mL		
Drug	CPE Method	NR Method	VY Method
Natural Product Humate HA	0.35	0.55	–
Ribavirin	1	2	–

Table XIV. Effective Inhibitory Concentrations at 50% (IC_{50}) and 90% (IC_{90}) of Natural Product HA Drugs and Ribavirin Reference Compound with Influenza Virus Type B (Beijing/184/93) (MDCK Cells)

	IC_{50}, μg/mL			
Drug	CPE Method	NR Method	VY Method	IC_{90}, μg/mL
Natural Product				
Humate HA	<1	<1	0.5	2.5
Ribavirin	1	1.5	0.5	1

Table XV. Effective Inhibitory Concentrations at 50% (IC_{50}) of Natural Product HA Drugs and Ribavirin Reference Compound with Influenza Virus Type B (Harbin/07/94) (MDCK Cells)

	IC_{50}, μg/mL		
Drug	CPE Method	NR Method	VY Method
Natural Product			
Humate HA	0.7	0.65	–
Ribavirin	0.85	1.1	–

Table XVI. Effective Inhibitory Concentrations at 50% (IC_{50}) of Natural Product HA Drugs and Ribavirin Reference Compound with Influenza Virus Type B (Hong Kong/5/72) (MDCK Cells)

	IC_{50}, μg/mL		
Drug	CPE Method	NR Method	VY Method
Natural Product Humate HA	3.2	5	–
Ribavirin	1.2-1.8	1.8-1.8	–

Table XVII. Effect of Time of Addition on Efficacy of Natural Product HA Drugs and Ribavirin Reference Compound against Influenza Virus Type A (New Caledonia/20/99) (H1N1) (MDCK Cells)

	IC_{50}, μg/mL: Visual–Neutral Red Methods	
Time of Addition, h	Natural Product Humate HA	Ribavirin
0	5.5–5.5	7.5–6
1	14–15	6–5.5
2	16–17	7–8
4	10–10	7–7
8	14–14	9–12
24	48–55	>100–>100

HEMORRHAGIC FEVER VIRUSES

Pichinde Virus/An 4763

Punta Toro A Virus/Adames

Hemorrhagic Fever Viruses

Methodology

Viruses and Cell Lines Used in Primary Drug Screening. Pichinde and Punta Toro A viruses were employed in this portion of the work. The virus strains were An 4763 and Adames, respectively. The cell lines were African green monkey kidney cells (BSC-1; Pichinde virus) and adult Rhesus monkey kidney cells (LLC-MK_2; Punta Toro A virus).

Methods for Assay of Antiviral Activity. The methodologies employed with the hemorrhagic fever viruses (inhibition of viral cytopathic effect–*visual CPE;* increase in neutral red dye uptake–*NR;* time-ofaddition study) were identical to those used with influenza viruses described in the preceding Section. Values

of $_{IC100}$ were determined by virus titer.

Reference Drug. Ribavirin was again the reference compound employed in the efficacy testing work.

Results

The efficacy data for all Natural Product Humate, Humic Acid (HA) with the hemorrhagic fever viruses examined in this work are provided in the following tables. As shown, Humic Acid (HA) exhibited subtantial efficacy against both hemorrhagic fever viruses, Humic Acid (HA) particularly so against Punta Toro A virus. Virus titer experiments established that the $_{IC50}$ ^{and} $_{IC100}$ values for the former material were in fact 5-27 mg/mL and 270 mg/mL, respectively.

The addition of Humic Acid HA 1 h before virus exposure, at the time of virus exposure, and 1 h after virus exposure resulted in similar levels of inhibition of viral infection **(Table XXII).** When the Humic Acid HA compounds were added at 2 h after virus exposure or longer, HA was only weakly inhibitory. These data, reminiscent of the findings for influenza viruses **(Table XVII),** suggest that some early event in the virus replication cycle was inhibited; previous work has established that the operative mechanism is in fact the inhibition of viral fusion.

Table XX. Effective Inhibitory Concentrations at 50% (IC_{50}) of Humic Acid HA Drugs and Ribavirin Reference Compound with Pichinde Virus (BSC-1 Cells)

	IC_{50}, µg/mL	
Drug	CPE Method	NR Method
Natural Product		
Humate HA	<1	<1
Ribavirin	0.3	<1

Table XXI. Effective Inhibitory Concentrations at 50% (IC_{50}) and 100% (IC_{100}) of Humic Acid HA Drugs and Ribavirin Reference Compound with Punta Toro A Virus (LLC-MK_2 Cells)

	IC_{50}, µg/mL		IC_{100}, µg/mL
Drug	CPE Method	NR Method	Virus Titer
Natural Product			
Humate HA	5	15	378
Ribavirin	5	5	–

Table XXII. Effect of Time of Addition on Efficacy of Natural Product Humic Acid HA Drugs and Ribavirin Reference Compound against Punta Toro A Virus (LLC-MK$_2$ Cells)

	IC_{50}, µg/mL Visual–Neutral Red Methods	
Time of Addition, h	Natural Product HA	Ribavirin
-1	30–50	10–6
0	30–7	4–3
1	30–30	4–5
2	30–60	8–5
4	100–>100	4–9
6	100–50	10–8
8	>100–>100	8–10
12	>100–>100	8–12
24	>100–>100	20–35