Viracillin™

ACTIVATED HUMIC ACID™

THE CHEMICALLY INDUCED INHIBITION OF HSV INFECTION

Viral Therapeutic Technologies
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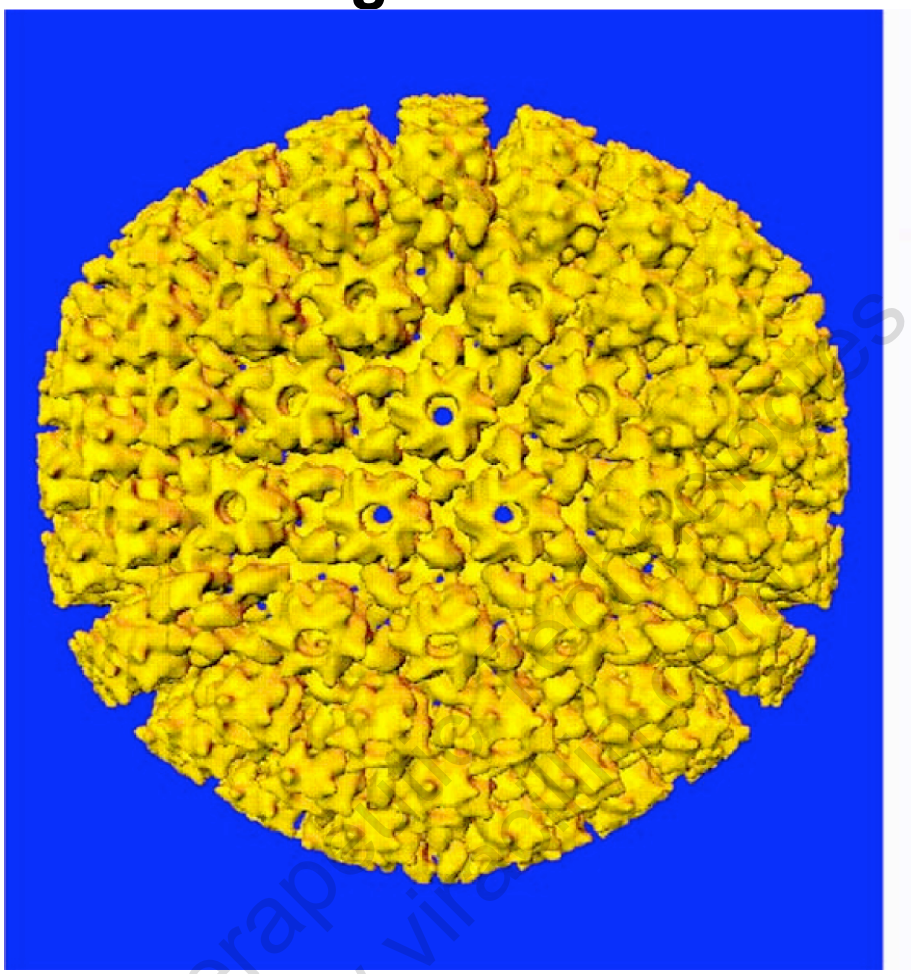
August 1998



Structural representation of a herpesvirus.

SPECIALTY LABORATORIES

Final Report

Antiviral Effects of Humates on HSV-1 and HS-2

***Specialty* Project Number CT80659**

August 14, 1998

Performing Laboratory

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Final Report

Antiviral Effects of Humates on HSV-1 and

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Final Report

Antiviral Effects of Humates on HSV-1 and HSV-2

Introduction

Soil extract materials, particularly the class of substances known collectively as "Humus," "humics," "humic Acid(s)," or "humates," have been widely used in a number of applications for many years. Both natural soil extracts and synthetic humates have been employed extensively in horticultural and related industries, particularly for soil enhancement and as soil remediation agents. In addition, both natural and synthetic materials have been employed as additives for use in organic gardening and landscaping, and in fresh-water aquaria.

Medicinal benefits have also been claimed for both synthetic and naturally occurring humates. For example, humic materials have long been used as "Folk remedies" for a wide variety of illnesses; their anti-inflammatory properties are also well documented. In addition, humic substances have been shown to exhibit anti-microbial as well as anti-viral efficacy, and are particularly active against coxsackie virus A9, herpes simplex virus (HSV) types 1 and 2, human immunodeficiency virus (HIV), and influenza virus types A and B.

Laub BioChemicals Corporation contracted with Specialty Laboratories (SL) to investigate the antiviral activities of proprietary synthetic humic acid as well as natural-product humic acid on HSV-1 (ATCC Strain VR-260) and HSV-2 (ATCC Strain VR-734), respectively. SL agreed to test both naturally-occurring humic acid and synthetic products at different concentrations to determine their respective IC_{50} and IC_{99}.

Three series of experiments were designed and performed to verify the antiviral activities natural-product humic acid:

(1) **Experiment Series I:** Cell Protection against Viral Infection (a). Fresh monkey kidney cells (target cells) were incubated with virus and testing compounds for one hour, then cultured without the testing compounds.

(2) **Experiment Series II:** Cell protection against viral infection (b). Target cells were first exposed to drugs, then to virus, and then were cultured without drugs.

(3) **Experiment Series III:** Infected-cells recovery from viral infection. Target cells were first infected with virus first and then cultured with drugs.

Materials and Methods

Materials

Synthetic and natural-product humate materials provided by Laub BioChemicals Corporations were tested and compared. HSV-1 (ATCC strain VR-260) and HSV-2 (ATCC Strain VR-734) were purchased from ATCC (Rocvkville, MD) for testing. Hybriwix^{tm}Probe Sytems: Herpes (HSV) Antiviral Susceptibility Test Kit (Catalogue # 100-1-072 or 200-1-072) was purchased from Diagnostic Hybrids, Inc. (Athens, OH).

Assay Principle

Cultured monkey kidney cells are used to test for the degree of sensitivity of Herpes simplex virus (Type I and II) to anti-viral chemotherapeutic agents by a radiometric DNA Hybridization assay. The general procedure consists of obtaining a tube culture which contains virus-infected cells showing 50-100% cytopathic effect (CPE), inoculating diluted samples of this cultured virus into cell wells containing cultured cells and a various dilutions of the anti-viral drugs, and incubating the cultures at 35–37–C in a CO_2-humidifed incubator until "no-drug" control wells demonstrate 50-100% CPE (routinely 24-36 hours, can be up to 48 hours). Multiple wells are inoculated with virus for each concentration of drug to be tested, including multiple wells inoculated for use as a "no-drug-added" control.

After the culture-amplification period, the supernatant fluid is removed from cell wells, the cell monolayer is lysed and the DNA denatured and captured on Hybriwix^{tm} filter membrane supports. All Hybriwix^{tm} are then batch-hybridized with an ^{125}I radiolabeled DNA probe which is specific to HSV-1 and -2. The processed Hybriwix^{tm} are counted, and the mean reactivity for each concentration of drug is determined. The amount of raidioactivity measured is proportional to the amount of virus produced. There is an inverse relationship between the measured count per minute (CPM) and the potency of the drug tested. The concentration of drug resulting in a 50% reduction in DNA hybridization compared to the no-drug control is used to establish the Inhibitory Concentration 50 (IC_{50}). The concentration of drug resulting in a 99% reduction in DNA hybridization compared to the no-drug control establishes the Inhibitory Concentration 99 (IC_{99}).

Drug-related Cytotoxicity

Drug-related cytotoxicities were examined with six drug concentrations and one on drug

(0 ug/mL). All four compounds were tested in African Green Monkey Kidney Cells (CV-1) in triplicate. The cells were purchased from Diagnostic Hybrids, Inc. (Athens, Oh) and were provided in flat dishes containing multiple cell wells. The cells were cultured in the presence of different concentrations of drugs for 24-36 hours and 35-37–C in a CO_2 humidified incubator. The morphology of the cultured cells was examined to determine any cytotoxic effects.

Anti-viral Effects of Testing Compounds

Drug-susceptibilities of the HSV-1 and HSV-2 were done in African Green Monkey Kidney Cells (CV-1) in triplicate. Both HSV-1 (ATCC strain VR-260) and HSV-2 (ATCC Strain VR-734) purchased from ATCC were used in these experiments.

Both synthetic and natural-product humate materials were tested for their anti-HSV-1 and HSV-2 effects at seven concentrations (0,2, 6, 19, 56, 167, and 500ug/mL) in triplicate to determine the IC_{50} and IC_{99} values.

Three mechanisms of drug action were examined, as described below:

(1) Experiment Series I: Cell Protection against Viral Infection (a). Fresh monkey kidney cells (target cells) are incubated with virus and testing compounds for one hour, then cultured without the testing compounds.

(2) Experiment Series II: Cell protection against viral infection (b). Target cells were first exposed to drugs, then to virus, and then were cultured without drugs.

(3) Experiment Series III: Infected-cells recovery from viral infection. Target cells were first infected with a virus and then are cultured with drugs.

Results

1. Sterility Test of Natural Humic Acid

The natural humate material was tested for sterility under both aerobic and anaerobic conditions before it was used in the experiment (Specialty Labs Test Codes 5774 and 5708). No bacterial growth was observed in either environment, suggesting that the natural compound does not carry life microorganism that may interfere with the subsequent assays. The natural compound was also tested for its cytotoxicity (next Section). No bacterial growth was observed during the study as well. Based on these observations, it is concluded that under these conditions, the natural compound is suitable for this study posing no concern of live microorganism contamination.

2. Drug-related cytotoxicity

The cytotoxicity was determined by visual observation. No abnormal cell morphology was observed in cultures with no drug nor containing drug concentrations up to 500

ug/mL. Furthermore, no apparent CV-1 cell death (ie, cell detachment from the bottom of the wells) was observed in any concentration tested. These results suggested that no cytotoxicity to CV-1 cells with drug concentrations of up to 500ug/mL of both humic acid compounds. It is therefore concluded that it is possible to test the anti-viral effects of all four compounds up to 500ug/mL without any drug-related cytotoxicity.

3. Anti-viral Effects

Three mechanisms of drug action were examined, as described below.

3.1. Experiment Series I. Cell Protection Against Viral Infection (a)

Virus culture supernatant was mixed with different concentrations of drug (final concentrations of 0, 2, 6, 19, 56, 167, and 500ug/mL). 200uL of the mixture was incubated with cells at 37⁻ for one hour. The supernatant was removed, supplemented with fresh culture medium without drugs, and incubated for an additional 36-48 hours at 35-37⁻C. At the end of the culturing, the cells were lysed and their DNA was captured on the filter paper. Radioisotope-labeled HSV-specific probe was hybridized with the DNA collected on the filter paper. After hybridization, the paper was washed, and the remaining radioactivity on the filter paper was measured to determine IC_{50} and IC_{99}. An inverse relationship exists between the measured CPM and the drug potency.

The anti-viral effects (as defined with IC_{50} and IC_{99}) of each compound on HSV-1 and HSV-2 are shown in the Appendix and summarized in Table 1 below.

Table 1. Experiment Series I. Cell Protection Against Viral Infection (a)

Compound	HSV-1		HSV-2	
	IC_{50}(ug/mL)	IC_{99}(ug/mL)	IC_{50}(ug/mL)	IC_{99}(ug/mL)
Natural-product humate	16	74	11	31

3.2 Experiment Series II: Cell Protection Against Viral Infection(b)

Different concentrations of drug (final concentrations of 0, 2, 6, 19, 56, 167, and 500ug/mL) were first incubated with cells for one hour at 35-37⁻C. After washing off the drug, the virus culture supernatant (200uL) was incubated with cells at 35-37⁻C for another hour. The supernatant was removed, supplemented with culture medium without any drugs and incubated for an additional 36-48 hours at 35-37⁻C. At the end of the culturing, the cells were lysed and their DNA was captured on the filter paper. Radioisotope labeled HSV-specific probe was hybridized with the DNA collected on the filter paper. After hybridization, the paper was washed and the remaining radioactivity

on the filter paper was measured to determine IC_{50} and IC_{99}. The amount of radioactivity measured is proportional to the amount of virus produced. An inverse relationship exists between the measured CPM and the drug potency.

The anti-viral effects (as defined by IC_{50} and IC_{99}) of each compound on HSV1 and HSV-2 are shown in the Appendix and summarized in Table 2, below.

Table 2. Experiment series II: Cell Protection Against Viral Infection (b)

Compound	HSV-1		HSV-2	
	IC_{50}(ug/mL)	IC_{99}(ug/mL)	IC_{50}(ug/mL)	IC_{99}(ug/mL)
Natural-product humate	19	95	27	52

3.3 Experiment Series III: Infected-cell Recovery from Viral Infection.

Virus culture supernatant was incubated with cells for one hour. After incubation, the unbound viruses were washed off, fresh media containing different concentrations (0, 2, 6, 19, 56, 167, and 500ug/mL) of testing compounds was added, and the cells were incubated at 35-37⁻C for 36-48 hours. The cells were lysed and their DNA was captured on the filter paper. Radioisotope_labeled HSV-specific probe was hybridized with the DNA collected radioactivity on the filter paper was measured to determine IC_{50} and IC_{99}. The amount of radioactivity measured is proportional to the amount of virus produced. An inverse relationship exists between the measured CPM and the drug potency.

The anti-viral effects (as defined by IC_{50} and IC_{99}) of each compound on HSV1 and HSV-2 are shown in the Appendix and summarized in Table 2, below.

Table 3. Experiment series III: Infected-cells Recovery from Viral Infection

Compound	HSV-1		HSV-2	
	IC_{50}(ug/mL)	IC_{99}(ug/mL)	IC_{50}(ug/mL)	IC_{99}(ug/mL)
Natural-product humnate	6.0	19	3.4	19

Conclusions

No cytotoxicities were observed in any of the compounds tested up to 500 ug/mL, suggesting that neither the synthetic humic acid compounds nor the natural-product humate material are toxic to the cells tested

The anti-HSV-1 and anti-HSV-2 effects were examined. Furthermore, three series of experiments were designed to explore the potential mechanisms of action. The first series of experiment demonstrated that both compounds can inactivate both HSV-1 and HSV-2 infections with different anti-viral activities. The second series of experiments demonstrated that the compounds can protect cells against HSV-1 and HSV-2 infection. The third series of experiments demonstrated that the compounds can interfere the proliferation of HSV-1 and HSV-2 after the virus enters the cells.

The primary goal of these experiments was to provide "proof of the principle." It is concluded that synthetic as well as natural-product humates have anti-viral effects on well-characterized HSV-1 and HSV-2 testing strains. Different potencies were observed against HSV-1 and HSV-2 under different experimental conditions. Further evaluation of the anti-viral effects on drug-resistant HSV-1 or HSV-2 strains is warranted.

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