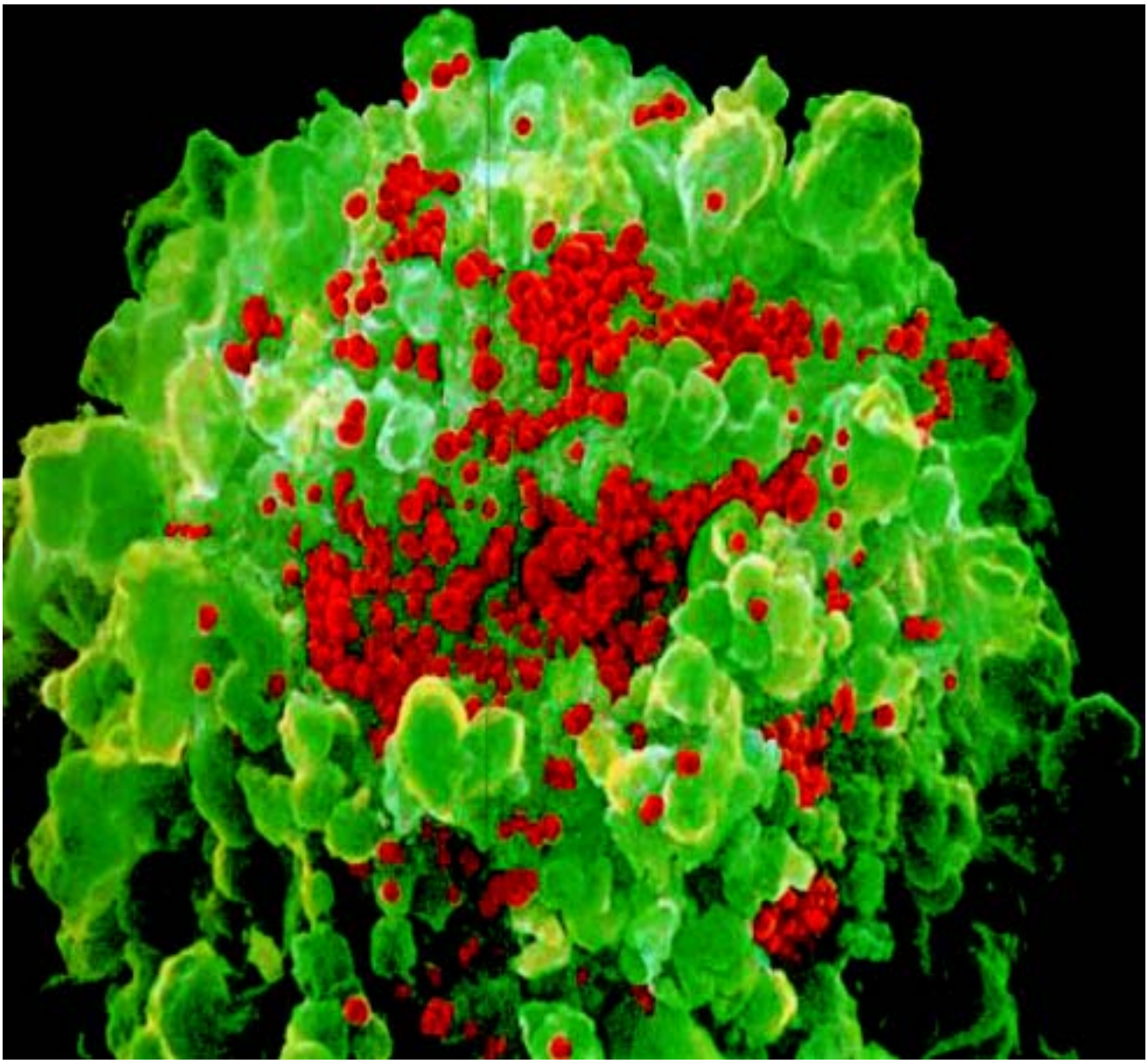
HUMIC ACID

INHIBITION OF HIV-1 REPLICATION

SOUTHERN RESEARCH INSTITUTE

Laub BioChemicals Corporation
1401 Quail St., Suite 121
Newport Beach, CA 92660

September 2000



FRONTISPIECE: AIDS virus (red dots) shown infecting a white blood cell.

Forward

This Report documents AIDS efficacy studies carried out on Laub BioChemicals Corp.'s natural-product and synthetic humate materials by Southern Research Institute, Frederick, MD. Natural-product humic acid is coded "*Hepsyl® HA*"; while synthetic humates are coded "*Hepsyl® xxx*", where *xxx* represents the starting material employed in the synthetic process–*CA*: caffeic acid; *CGA*: chlorogenic acid; *HGA*: homogentisic acid.

Natural-product and synthetic humates are protected by U.S. patents (5,946,445; 6,569,416; 6,524,566; 6,524,567; 6,534,049; 6,576,229) and other U.S. and international patents and patents pending. "*Hepsyl®*" is a Registered Trademark of Laub BioChemicals Corp. (U.S. 2,177,121).

Appendices are available upon request from Laub BioChemicals Corp.

CONFIDENTIAL REPORT FOR
LAUB BIOCHEMICAL CORPORATION

FINAL TECHNICAL REPORT

EVALUATION AND VERIFICATION OF ANTIVIRAL ACTIVITY OF SELECTED SYNTHETIC HUMATES

September 14, 2000

Prepared for:

Dr. Richard Laub
Laub Biochemical Corporation
1401 Quail Street, Suite 121
Newport Beach, CA 92660

Prepared by:

Jim A. Turpin Ph.D.
Manager Retrovirus Research Laboratory

Tracy Loftus
Supervisor Retrovirus Research Laboratory

Southern Research Institute
431 Aviation Way
Frederick, MD 21702
Phone: 301-694-3232 ext. 255
Fax 301-694-7223
Email turpin@serquest.com

Project SRI A085.01

September 15, 2000

Submitted By:

Tracy Loftus, M.S.
Supervisor Retrovirus Research Laboratory
Infectious Disease Research Department

Jim A. Turpin, Ph.D.
Manager Retrovirus Research Laboratory
Infectious Disease Research Department

REPORT SRF-00-A085.01

INTRODUCTION

This report outlines confirmatory and initial range and mechanism of action antiviral evaluations for four synthetic Humates for Laub Biochemical Corporation by Southern Research Institute.

The Humates have been previously evaluated for anti-HIV activity in laboratories outside Southern Research Institute. The objectives of the present studies were to confirm the antiviral activity of the Humates and extend previous observations. Based upon conversations with Dr. Laub we designed a series of experiments which would provide an initial assessment of antiviral range of action and mechanism of action by activity in specific HIV replication models and in the HIV attachment assay. We were able to confirm antiviral activity for the Humates and define their potential antiviral target to the pre-integration phase of HIV replication, providing preliminary data that a portion of the antiviral activity is due to inhibition of virus attachment.

MATERIAL AND METHODS

Test Material

Four Humates were hand delivered to Southern Research Institute by Dr. Laub on June 13, 2000. The compounds received are summarized in **Table 1**. The compounds were solubilized in DMSO and stored at –20°C until assay.

Table 1: Compounds Tested in This Study

Designation	Lot #
HEPSYL CA	010600
HEPSYL HA	011197
HEPSYL HGA	060800
HEPSYL CGA	050600

CEM-SS HIV-1_{RF} Cytoprotection Assay:

CEM-SS cells (obtained from the AIDS Research and Reference Reagent Repository, Bethesda, MD) are passaged in T-75 flasks in tissue culture media (RPMI 1640 medium (no

phenol red) with 10% Fetal Bovine Serum (heat inactivated), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin , and 10 μg/mL gentamycin). On the day preceding the assay, the cells are split 1:2 to assure they are in an exponential growth phase at time of infection. On the day of assay the cells are collected by centrifugation, washed twice with tissue culture medium and resuspended at 5 x 10^4 cells per mL and resuspended in fresh tissue culture medium. Total cell and viability counting is performed using a hemacytometer. Cell viability prior to the assay is determined by Trypan Blue dye exclusion and must exceed 95%. A pretitered aliquot of HIV-1_{Rf} (AIDS Research and Reference Reagent Repository, Bethesda, MD), 5 x 10^3 cells and compound where appropriate are placed into 0.2 cm round bottom microtiter plates (final volume 200 μL). Each plate contains cell control wells (cells only), virus control wells (cells plus virus), drug toxicity control wells (cells plus drug only), drug colorimetric control wells (drug only) as well as experimental wells (drug plus cells plus virus) (**Figure 1**). Cultures are incubated for 6 days at 37°C, 5% CO_2 and antiviral activity and compound toxicity determined by MTS staining. Activity is confirmed by both macroscopic and microscopic analysis of the assay. Please note this assay was originally identified as the XTT cytoprotection assay. The assays are identical except for the use of the MTS reagent in place of XTT for detection of cell viability.

BMC Isolation and Blasting:

Peripheral blood monocular cells (PBMCs) are obtained from normal hepatitis and HIV-1 negative donors by ficoll hypaque gradient separation. Briefly, anti-coagulated blood is diluted 1:1 with Dulbecco's phosphate buffered saline without Ca^{++} and Mg^{++} (PBS) and layered over 14 mL of Lymphocyte separation media in a 50 ml centrifuge tube. Tubes are then centrifuged for 30 minutes at 600 X g. Banded PBLs are gently aspirated from the resulting interface and subsequently washed 2X with PBS by low speed centrifugation. The mononuclear cells are counted, viability determined by Trypan Blue dye exclusion and resuspended in RPMI 1640 medium supplemented with 15 % FBS (heat inactivated), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 μg/mL gentamycin with 2 μg/mL phytohemagluttin (PHA) at 1 X 10^6 cells/mL. The cells are cultured for 48 to 72 h at 37°C, 5% CO_2. Following incubation, cells are collected by centrifugation, washed and resuspended in RPMI 1640 supplemented with 15 % FBS (heat inactivated), 2 mM L-glutamine, 100 U/mL penicillin, 100

μg/mL streptomycin, and 10 μg/mL gentamycin with 20 U/mL recombinant IL-2 (R & D Systems, Minneapolis, MN). IL-2 is included in the culture medium to maintain the cell division initiated by the PHA mitogenic stimulation. The cultures are then maintained until use by ½ culture volume change with fresh IL-2 containing medium every 3 days.

Figure 1: Plate Layout for the HIV Cytoprotection Assay

	1	2	3	4	5	6	7	8	9	10	11	12
A	Media	Media	Media	Media	Media	Media	Blank	Blank	Blank	Blank	Blank	Blank
B	Cells + Drug 1 0.32 μM	Cell Control	Cells + Virus + Drug 1 0.32 μM			Cells + Drug 1 0.32 μM	Cells + Drug 2 0.32 μM	Cells + Virus + Drug 2 0.32 μM			Cell Control	Cells + Drug 2 0.32 μM
C	Cells + Drug 1 1 μM	Cell Control	Cells + Virus + Drug 1 1 μM			Cells + Drug 1 1 μM	Cells + Drug 2 1 μM	Cells + Virus + Drug 2 1 μM			Cell Control	Cells + Drug 2 1 μM
D	Cells + Drug 1 3.2 μM	Cell Control	Cells + Virus + Drug 1 3.2 μM			Cells + Drug 1 3.2 μM	Cells + Drug 2 3.2 μM	Cells + Virus + Drug 2 3.2 μM			Cell Control	Cells + Drug 2 3.2 μM
E	Cells + Drug 1 10 μM	Virus Control	Cells + Virus + Drug 1 10 μM			Cells + Drug 1 10 μM	Cells + Drug 2 10 μM	Cells + Virus + Drug 2 10 μM			Virus Control	Cells + Drug 2 10 μM
F	Cells + Drug 1 32 μM	Virus Control	Cells + Virus + Drug 1 32 μM			Cells + Drug 1 32 μM	Cells + Drug 2 32 μM	Cells + Virus + Drug 2 32 μM			Virus Control	Cells + Drug 2 32 μM
G	Cells + Drug 1 100 μM	Virus Control	Cells + Virus + Drug 1 100 μM			Cells + Drug 1 100 μM	Cells + Drug 2 100 μM	Cells + Virus + Drug 2 100 μM			Virus Control	Cells + Drug 2 100 μM
H	Drug 1 100 μM + Media	Drug 1 32 μM + Media	Drug 1 10 μM + Media	Drug 1 3.2 μM + Media	Drug 1 1 μM + Media	Drug 1 0.32 μM + Media	Drug 2 100 μM + Media	Drug 2 32 μM + Media	Drug 2 10 μM + Media	Drug 2 3.2 μM + Media	Drug 2 1 μM + Media	Drug 2 0.32 μM + Media

PBMC Assay:

Human peripheral blood mononuclear cells from a minimum of 2 donors, that have been blasted with PHA and IL-2, are counted, viability determined by Trypan Blue dye exclusion and mixed in equal ratios. Pooled donors are used to minimize the variability observed between individual donors which results from quantitative and qualitative differences in HIV infection and overall response to the PHA and IL-2 of primary lymphocyte populations. The cells are resuspended at 1 x 10^6 cells /mL in RPMI 1640 without phenol red supplemented with 15% Fetal Bovine Serum (heat inactivated), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 μg/mL gentamycin and IL-2 (20 U/mL, R&D Systems, Minneapolis, MN). Fifty microliters of cells are then distributed to the inner 60 wells of a 96 well round bottom microtiter culture plate in a standard format developed by the Infectious Disease Research

department of Southern Research Institute. Each plate contains cell control wells (cells only), virus control wells (cells plus virus), and experimental wells (drug plus cells plus virus). Serially diluted compounds are added to the microtiter plate followed by the appropriate pre-titered strain of HIV-1. The studies presented here used the RoJo strain of HIV. RoJo is a low passage pediatric clinical isolate of HIV specifically isolated and developed in the laboratories of Southern Research Institute. All samples were assayed in triplicate with a replicate plate without virus for the determination of compound toxicity. The final volume per well was 200 μL. The assay was incubated for 6 days in a humidified atmosphere at 37°C, 5% CO_2, after which supernatants were collected, for analysis of RT activity and sister plates analyzed for cell viability by MTS dye reduction. Wells were also examined microscopically and any abnormalities noted.

Monocyte Isolation, Culture and Infection:

Peripheral blood monocytes were isolated from normal HIV-1 negative donors by plastic adherence following ficoll hypaque purification of the buffy coat, as described above for PBMCs. In many cases the same donor used to produce the PBMC populations will also be used to produce monocyte/macrophages, however unlike PBMC population monocyte/macrophage donors are never pooled. Following a 2h adherence in RPMI 1640 without phenol red supplemented with 10% human pooled AB serum (heat inactivated), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 μg/mL gentamycin, cultures were washed to remove non-adherent cells. The monocytes were released from the plastic by vigorous pipetting with Ca^{2+} and Mg^{2+} free PBS. Adherent cells were assessed for purity by nonspecific esterase staining (α-napthyl butyrate specific esterase, Sigma Chemical Co.), viability by Trypan Blue dye exclusion, counted and resuspended in RPMI 1640 supplemented with 10% Fetal Bovine Serum (heat inactivated), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 μg/mL gentamycin at 1 x 10^6 monocytes per ml. The monocytes (1 x 10^5 per 0.2 cm well) are then cultured for 6 days, allowing maturation of the cells to a macrophage-like phenotype. At day 6 the cultures were washed 3 times to remove any non-adherent cells and serially diluted test compounds added followed by the addition of a pre-titered amount of the Ba-L strain of HIV-1 otained from the NIAID AIDS Research and Reference Reagent Repository. Ba-L is a laboratory adapted HIV isolate with tropism for monocyte/macrophages. Cultures were washed a

final time by media removal 24 h post infection, fresh compound added and the cultures continued for an additional six days. The assays are preformed using a standardized microtiter plate format developed by the Infectious Disease Research department of Southern Research Institute, which uses only the inner 60 wells of a 96 well plate for assay purposes. The outer rows contain media and acts an a evaporation barrier. Each plate contains cell control wells (cells only), virus control wells (cells plus virus), and experimental wells (drug plus cells plus virus). HIV p24 antigen content to assess virus replication was measured at assay termination by a commercially available p24 ELISA assay (Coulter). Toxicity of test materials were measured on replicate plates; which do not receive virus, but are otherwise treated and setup identically to those receiving virus. AZT and/or ddC, HIV-1 reverse nucleoside transcriptase inhibitors, were used as positive control compounds and run in parallel with each determination. At termination of the assay culture plates were removed from the incubator and observed microscopically. Any unique findings were noted.

U1 and ACH-2 Assays

U1 and ACH-2 cells were obtained from the AIDS Research and Reference Reagent Program and maintained under standard culture conditions in RPMI 1640 supplemented with 10% fetal bovine serum (heat inactivated), supplemented with 2 mM L-glutamate, 100 U/mL penicillin and 100 μg/mL streptomycin. U1 cells are derived from the histocytic leukemia cell line U937, and contain a single integrated copy (HIV IIIB) of the cytokine and/or phorbol inducible provirus. ACH-2 cells are derived from the A3.01 T lymphoblastic cell line, and contain 2 copies of the HIV IIIB provirus, however only one is cytokine and/or phorbol inducible. Cultures are maintained in such a way as to ensure exponential growth of the populations. At the time of the assay cells are collected by centrifugation and counted by hemacytometer. If cell viability by Trypan Blue dye exclusion is less than 70% the assay is terminated. The cells are adjusted to 5x 10^4 cells/ mL and 100 μL placed in 96 well plates with 100 μL media containing a final concentration of 10 ng/mL TNFα and the test compound. Cultures are incubated for 3 days and supernatants harvested. Compound toxicity is determined by MTS dye reduction. Virus expression is measured by supernatant reverse transcriptase activity.

Virus Attachment Inhibition Assay:

The attachment inhibition assay is performed with the HeLa CD4 LTR β-gal cells available from the AIDS Research and Reference Reagent Repository. HeLa CD4 LTR β-gal cells are routinely cultured DMEM supplemented with 10% fetal bovine serum (heat Inactivated), 2 mM L-glutamate, 100 U/ml penicillin, 100 μg/mL streptomycin and the selection antibiotics hygromycin (100 μg/mL) and G418 (200 μg/mL). Twenty-four h prior to initiation of the assay the cells are trypsinized, counted and 1 x 10^4 cells placed in a 0.2 cm well in media without selection antibiotics. At 24h media is removed and compound in media placed on the cells and incubated for 15 to 30 min at 37°C. A known titer of virus is then added to the wells and the incubation continued for 1 h. At the end of the incubation the wells are washed 2 to 6 times with media and the culture continued for 48h. At 48h the media is removed and β–galactosidase enzyme expression determined by chemiluminescence per manufacturers instructions (Tropix Gal-screen™, Bedford Mass.). This chemiluminescent method uses a single solution containing cell lysis components and chemiluminescent substrates to detect activity in a single step. Compound toxicity is monitored on a sister plate using XTT dye reduction.

The interaction of gp120 and CD4 can also be assessed with this assay. Immediately following the 1 h virus adsorption the cells are washed 6 times and lysed. Cell–associated p24 antigen is then quantitated in the cell lysates by p24 antigen ELISA(Coulter).

MTS staining for cell viability:

At assay termination the assay plates were stained with the soluble tetrazolium-based dye MTS (CellTiter Reagent Promega) to determined cell viability and quantify compound toxicity. MTS is metabolized by the mitochondria enzymes of metabolically active cells to a soluble formazan product, allowing the rapid quantitative analysis cell viability and compound cytotoxicity. This reagent is a single stable solution that does not require preparation before use. At termination of the assay 20 μL of MTS reagent is added per well. The wells are incubated overnight for the HIV cytoprotection assay and for 4 h for monocyte/macrophages and PBMCs at 37°C. The incubation intervals were chosen based on empirically determined times for optimal dye reduction in each cell type. Adhesive plate sealers were used in place of the lids, the

sealed plate was inverted several times to mix the soluble formazan product and the plate was read spectrophotometrically at 490 nm with a Molecular Devices Vmax plate reader.

Reverse Transcriptase Assay:

Reverse transcriptase activity was measured in cell-free supernatants. Tritiated thymidine triphosphate (NEN) (TTP) was resuspended in distilled H_2O at 5 Ci/mL. Poly rA and oligo dT were prepared as a stock solution which was kept at -20°C. The RT reaction buffer was prepared fresh on a daily basis and consists of 125 µL 1.0 M EGTA, 125 µL dH_2O, 110 µL 10% SDS, 50 µL 1.0 M Tris (pH 7.4), 50 µL 1.0 M DTT, and 40 µL 1.0 M $MgCl_2$. These three solutions were mixed together in a ratio of 2 parts TTP, 1 part poly rA:oligo dT, and 1 part reaction buffer. Ten microliters of this reaction mixture was placed in a round bottom microtiter plate and 15 µL of virus containing supernatant was added and mixed. The plate was incubated at 37°C in a water bath with a solid support to prevent submersion of the plate and incubated for 60 minutes. Following reaction, the reaction volume was spotted onto pieces of DE81 paper, washed 5 times for 5 minutes each in a 5% sodium phosphate buffer, 2 times for 1 minute each in distilled water, 2 times for 1 minute each in 70% ethanol, and then dried. Opti-Fluor O was added to each sample and incorporated radioactivity was quantitated utilizing a Wallac 1450 Microbetaplus liquid scintillation counter.

P24 Antigen ELISA:

ELISA kits were purchased from Coulter Electronics. The assay is performed according to the manufacturer's instructions. Control curves are generated in each assay to accurately quantitate the amount of p24 antigen in each sample. Data are obtained by spectrophotometric analysis at 450 nm using a Molecular Devices Vmax plate reader. Final concentrations are calculated from the optical density values using the Molecular Devices Soft Max software package.

Data Analysis:

Using an in-house computer program, IC_{50} (50%, inhibition of virus replication), TC_{50} (50% reduction in cell viability) and a therapeutic index (TI, IC_{50}/TC_{50}) are provided. Raw data for both antiviral activity and toxicity with a graphic representation of the data are provided in a

printout summarizing the individual compound activity. We have provided AZT as a relevant positive control compounds for the individual assays. It should be noted that there are no available standardized control compounds for the U1 and ACH-2 assays.

RESULTS

The results of the antiviral evaluations for the compounds are summarized in the Appendices. The contents of the appendices are listed in **Table 2**. The antiviral data in the appendices for each test includes the relevant raw data values from the triplicate tests for virus replication (RT or p24) and cell viability (OD 490) for XTT dye reduction. The IC_{50} and TC_{50} values are calculated by linear regression using a program developed specifically for this purpose at Southern Research Institute. The TI represents the ratio of the TC_{50}/IC_{50}, and is used to determine relative potency between compounds. The graphical representation shows the relationship between antiviral efficacy (%VC) and compound toxicity (%CC) expressed as a percent of the control, virus no compound or cells no compound, respectively.

Table 2: Contents of the Appendices in this Report

Appendix	Contents
I	HIV Cytoprotection assay
II	PBMC assay
III	Monocyte/macrophage assay
IV	Chronically Infected CEM/SK-1 cells
V	U1 and ACH-2 assay
VI	Attachment assay
VII	gp120/ CD4 Association assay
VIII	Colorimetric assay

The assays performed meet our internal validation and standardization criteria. For successful antiviral assays (HIV cytoprotection, monocyte/macrophage and PBMC assays) AZT must demonstrate an IC_{50} between 1 and 10 nM without toxicity at the high test concentration of 4 μM. For U1 and ACH-2 assays since virus expression is dependent upon cytokine induction performance criteria identifying the level of virus production are used. For U1 cells this is

supernatant RT activity greater than 5000 counts per minute for 15 μL of supernate and greater than 20,000 for ACH-2 cells. In the HeLa CD4 LTR β-gal cells the virus must induce a minimum of 40,000 RFU for the assay to be considered valid. The assays used to evaluate the Humates meet the individual assay standards and other internal assay validation criteria including intra-triplicate variation and total virus replication. Thus, we consider the presented evaluations to be valid and representative of the antiviral activity of the tested compounds.

As part of our studies, we did identify an abnormal absorbance in MTS-based assays at very high concentrations of the Humates. A specific study was carried out to determine the contribution of this effect to our antiviral assays, and the results are contained in **Appendix VIII**. These studies show that the Humates are not interacting with the MTS reagent directly, but probably due to their brown color are causing abnormal absorbence. Additionally we identified a slight dependency upon the dilution scheme, suggesting with visual observations that this abnormal absorbence was due to compound precipitation at high concentrations. This colorimetric/absorbence problem could result in problems in interpretation of results from Humates experiments with concentrations higher than 100 μg/mL. However all MTS-based experiments include colorimetric controls (compound in medium) in the assay plate and the IC_{50} and TC_{50} calculation reflect correction for compound color. The IC_{50} for all antiviral assays where the Humates were active were below 5 μg/mL, suggesting that increased absorbance above 100 μg/mL will not affect the IC_{50} calculation, but may alter slightly the TC_{50} calculation. These results do point out that if experiments are to be performed with concentrations of the Humates higher than 100 μg/mL antiviral analysis and viability determinations should be performed using methods other than MTS-based absorbence.

The antiviral activity of the Humates in acute infection models of HIV replication are summarized in **Tables 3** and **4**. **Table 3** summarizes 2 independent HIV cytoprotection experiments where the IC_{50}s of the compounds were determined in an initial experiment and the compound high test concentration increased to 1 mg/mL in an attempt to identify the TC_{50}. The colorimetric effects identified above are shown by an increase in the absorbance of the color controls (compound in medium) used for each experiment. This data shows that the Hepsyl

Humates are in general non-cytotoxic. The CA and HA versions are more potent than the corresponding CGA and HGA versions, 3.3 and 4.4-fold respectively. The Hepsyl CA and HA were equipotent with IC_{50}s of 0.61 and 0.78 μg/mL, respectively. **Table 4** summarizes the antiviral effect of the compounds on HIV replication in primary lymphocytes and monocytes. In general, the Humates were more potent by 2 to 5–fold in PBMCs than monocyte/macrophages, with no cytotoxicity at 100 μg/mL for either. In general Hepsyl CA was the most active Humate in PBMCs (IC_{50} 0.28 μg/mL), while Hepsyl HGA was the most potent in monocyte macrophages (IC_{50} 0.99 μg/mL). Thus the Humates are potent inhibitors of acute infection in a transformed cell line (CEM-SS, T lymphoblastic) and primary peripheral blood cells.

Table 3: Activity of the Humates in the HIV Cytoprotection Assay

Compound[1]	IC_{50}	TC_{50}	TI
AZT	0.02	>1	>50
Hepsyl CA	0.61	605	991
Hepsyl HA	0.78	>1000	>1282
Hepsyl CGA	2.03	533	263
Hepsyl HGA	3.46	158	46[c]

[1] All concentrations in μg/mL, except AZT which is in μM units

Table 4: Activity of the Humates in PBMCs and Monocyte/Macrophage Assays

	PBMC			Monocyte/Macrophage		
Compound[1]	**IC_{50}**	**TC_{50}**	**TI**	**IC_{50}**	**TC_{50}**	**TI**
AZT	0.0007	>4	>5714	0.006	>4	>667
Hepsyl CA	0.28	>100	>357	1.70	>100	>59
Hepsyl HA	0.92	>100	>109	2.64	>100	>38
Hepsyl CGA	0.91	>100	>110	4.72	>100	>21
Hepsyl HGA	0.43	>100	>233	0.99	>100	>101

[1] All concentrations in μg/mL, except AZT which is in μM units.

In contrast, the Humates were marginally active to inactive in CEM-SS cells chronically infected with the SK-1 strain of HIV (**Table 5**). Since chronically infected populations of cells are a mixture of uninfected dividing cells, and long- and short-lived infected cells a low to marginal antiviral activity in this model would be expected if the Humates interact with an antiviral target early in the HIV replication cycle. In order to confirm or deny a potential target at later stages of HIV replication after virus integration we assessed the effects of the Humates on TNFα-induced U1 and ACH-2 cells. U1 cells derived from the monocyte-like histocytic lymphoma cell line U937 and ACH-2 cells derived from the T lymphoblastic cell line A3.01 each contain cytokine and phorbol inducible integrated copies of the HIV provirus (U1: 1 copy; ACH-2: 2 copies). Thus all virus production derives from an integrated virus via new transcription. **Table 6** shows that the Humates did not inhibit HIV replication in either ACH-2 or U1 cells. These results in comparison to the acute infection results in **Tables 3** and **4** strongly suggest that the antiviral target for the Humates is occurring during early phases of HIV replication before integration and new virus transcription occurs. The marginal activity seen in **Table 5** for Hepsyl Ha and Hepsyl CA potentially represents inhibition of new rounds of infection in the chronic population. Thus it is possible that increased antiviral activity would be identified if the assay periods were extended, allowing attrition of infected cells and reduction of the total infected cell number in the population via inhibition of new rounds of infection.

Table 5: Effect of Humates on Chronically Infected CEM-SS Cells

Compound[1]	IC_{50}	TC_{50}	TI
Hepsyl CA	193.7	383	2.0
Hepsyl HA	130.8	566	4.3
Hepsyl CGA	266.0	466	1.8
Hepsyl HGA	152.8	212	1.4

[1] All concentrations in μg/mL.

Table 6: Effect of the Humates on TNFα-Induced U1 and ACH-2 Cells

	U1 Assay			ACH-2 Assay		
Compound[1]	**IC_{50}**	**TC_{50}**	**TI**	**IC_{50}**	**TC_{50}**	**TI**
Hepsyl CA	155.1	129.5	--	77.9	50.1	--
Hepsyl HA	170.1	226.7	1.3	81.3	57.1	--
Hepsyl CGA	183.0	194.1	1.1	104.5	69.8	--
Hepsyl HGA	130.0	60.2	--	56.3	13.1	--

[1] All concentrations in μg/mL.

Based upon the chemical nature of the Humates we proposed, in addition to range of action testing to determine whether the antiviral target occurred pre- or post-integration, to assess their effect on attachment of the HIV virus to the cell. We proposed for these studies our standard HeLa CD4 LTR β-gal assay. Unmodified HeLa cells express the HIV coreceptor CXCR4, but not CD4. HeLa CD4 LTR β-gal cells are HeLa cells which express CD4 and contain a β-galactosidase enzyme under the transcriptional control of the HIV regulatory protein Tat. Thus upon infection and virus integration new Tat producion results in transactivation of the LTR and production of β-galactosidase, which is detected via chemiluminescence. In this assay the virus and test material are co-incubated for a short interval which allows interaction of the virus with the target cell, but limits metabolic modification such as the conversion of ATP to its active form AZP-TTP. Successful inhibition of virus attachment is then measured at 48 h by decreased induction of the β-galactosidase enzyme activity. This assay can be made more specific by measuring cell-associated p24 antigen. The binding of virus to cells via CD4 and the HIV coreceptor results in a complex that is stable to washing with isotonic solutions (tissue culture medium). Thus if the HeLa CD4 LTR β-gal cells are extensively washed immediately after virus adsorption, cell-associated p24 can be used as a measurement of virus cell interaction. This interaction is primarily mediated by the interaction of gp120 and CD4 interaction, since treatment with excess soluble CD4 and/or antibodies to the CD4/gp120 binding site or known inhibitors of the gp120/CD4 interaction significantly reduce cell bound p24 (JAT unpublished results and **Table 7**). **Table 7** shows the results of these studies. All four Humate congers were

potent inhibitors of virus cell attachment, with Hepsyl CGA between 40 to 80-fold more potent than the other Humates. The four Humates were also successful at blocking the association of virus to the HeLa CD4 LTR β-gal cells via gp120/CD4 interaction. However Hepsyl CGA was no more potent at blocking this interaction than the other Humates. The 69-fold differential in efficacy of the Hepsyl CGA for the general attachment assay versus measurement of the gp120 CD4 interaction suggests that this compound may have additional antiviral targets. The attachment fusion inhibitor Chicago Sky Blue was included as a positive control; with the Humates approximately 8-fold more efficacious at blocking the CD4:gp120 interaction than the positive control. The differential noted for Hepsyl CGA suggests that the Humates maybe able to mediate antiviral activity through mechanism other than prevention of the gp120:CD4 interaction.

Table 7: Effect of the Humates on Virus Attachment and CD4:gp120 Interaction

	Virus Attachment			gp120:CD4 Interaction		
Compound[1]	**IC_{50}**	**TC_{50}**	**TI**	**IC_{50}**	**TC_{50}**	**TI**
Chicago Sky Blue	0.53	>10	>18	2.06	>10	>5
Hepsyl CA	0.44	>1000	>2272	0.74	>1000	>1353
Hepsyl HA	0.48	>1000	>2083	0.23	>1000	>4347
Hepsyl CGA	0.01	>1000	>100000	0.69	>1000	>1449
Hepsyl HGA	0.88	>1000	>1136	0.68	>1000	>1471

[1] All concentrations in μg/mL.

DISCUSSION

We have assessed four synthetic Humates for antiviral activity in acute, chronic and latent HIV infection *in vitro* tissue culture models of HIV Infection. All four Humates are potent inhibitors of acute HIV infection in CEM-SS cells, PBMCs and monocyte/macrophages. Despite some trends in the different assays for antiviral potency, considering the error inherent in the *in vitro* assays employed, we must consider the 4 compounds to be of equal potency. In contrast the 4 synthetic Humates were inactive in latently infected cells and only marginally active

(Hepsyl CA and HA) in chronically infected cells. These results *in toto* are highly consistent with a pre-integration antiviral target for compound action. They are partially supported by the results of the virus attachment and gp120:CD4 interaction assay showing that the Humates are potent inhibitors of this interaction.

The recent discovery of the HIV coreceptor and the formation of novel conformational targets following the interactions of gp120:gp41:CD4: coreceptor(s) resulted in inhibitors of virus entry becoming more attractive clinical candidates. This has been helped by the failure of the highly active antiretroviral therapy (HAART) regimes to adequately control and/or eradicate HIV disease spurring the need for newer treatments and the recent successes of the coreceptor inhibitor AMD3100 and the HIV fusion inhibitor T-20 and T-1249 *in vivo*. In addition, the recent understanding that our current clinical candidates for inhibition of vaginal transmission of HIV (topical microbicides) are either ineffectual or potentially increase HIV transmission has again highlighted potential attachment inhibitors as possible lead compounds. Thus the development of inhibitors of the virus entry process is currently a viable and potentially efficacious approach to controlling HIV disease. The studies presented here identify the Humates as potent inhibitors of virus entry and the differential between the ability of Helsyl CGA to inhibit general virus entry and the gp120:CD4 interaction suggests mechanisms of antiviral action other than simple blocking of the gp120:CD4 interaction.

The anti-HIV data presented in these studies confirms and extends studies previously performed for Laub Biochemicals Corporation. Previous analysis of the antiviral activity of the Humates on HIV replication in Jurkat cells and PBMCs did not specifically identify IC_{50}'s for virus inhibition, but virus replication was suppressed greater than 90% at the low test concentration of 10 μg/mL. Interestingly in previous work, the Humates were able to suppress HIV replication in PBMCs derived from HIV infected individuals and prevent transmission to uninfected PBMCs. Although these later 2 studies have no direct correspondence to the studies performed for this report they are consistent with our observations. The treatment of the *in vivo* infected PBMCs suggests that treatment of chronically infected cells will also result in realized antiviral activity. The demonstration of a strong inhibition of RT activity but a more prolonged kinetics of inhibition for p24 in the previous data further supports this conjecture. This conjecture is possible if it is assumed that the chronically infected CEM cell line has a higher potential number of productively infected cells and a general longer survival of the chronically

infected cells than the *in vivo* infected PBMC (JAT unpublished observations). Thus, as identified above, extension of the chronically infected CEM assay for longer than 6 days may result in the identification of antiviral activity in the chronically infected cells employed in our assays. Finally, the observation the ability of the Humates to block virus transmission in PBMCs derived from HIV Infected individuals directly correlates with our observation of inhibition of virus attachment. Thus the efforts reported here compliment and extend previous observations of antiviral activity for the Humates.

The Humates are potent inhibitors of acute HIV infection in antiviral evaluation models. The present studies point to an antiviral target occurring before proviral integration, possibly involving virus attachment and entry.

Proposed Studies

Appendix IX contains an outline of proposed development of the Humates based on the data presented in this report. This outline has recently been submitted to Dr. Laub independently of this report. The outline in general is designed to evaluate the Humates in a manner consistent with the points to consider provided by the FDA for pre-clinical development of compounds, and taking into account the potential of an antiviral target involving HIV entry. The salient points of the proposal are:

- Evaluation of the mechanism of action of the Humates using current technology to identify and characterize potential inhibitors of the early phases of HIV replication and virus entry;
- Performing range of action studies to identify the Humates applicability to a number clinical isolates relevant to HIV disease, and show its ability to inhibit virus replication in chronically infected cells;
- Identify the potential of the Humates to inhibit viruses with known resistance profiles and engender resistance; with the caveat that attachment inhibitors are often very hard to engender resistance to;
- Assess the activity of the Humates in topical microbicide transmission models and their applicability to this area of HIV disease;

- Determine the *in vivo* efficacy of a lead Humate in treatment and topical microbicide models using non-human primates.

Thus the studies in **Appendix IX** provide a plan for the development of the Humates given current the information on their range and mechanism of antiviral action.