Isolation and Characterization of *Myrianthus holstii* Lectin, a Potent HIV-1 Inhibitory Protein from the Plant *Myrianthus holstii*[1]

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Aqueous extracts from the African plant *Myrianthus holstii* potently inhibited the infection of the T-lymphoblastoid cell line, CEM-SS, by human immunodeficiency virus-1_{RF} (HIV-1_{RF}). The active constituent, *M. holstii* lectin (MHL), was purified by LH-20 column chromatography and reversed phase HPLC. MHL, a 9284-Da cysteine-rich protein, was characterized by amino acid analysis, *N*-terminal sequencing, ESIMS, and matrix-assisted laser-desorption ionization−time-of-flight mass spectrometry. Pure MHL had anti-HIV activity, with an EC_{50} value of 150 nM. Delaying the addition of MHL for up to 8 h after initial exposure of CEM-SS cells to virus did not result in loss of the antiviral activity; however, if addition of the compound was delayed for 16 h or more, there was a marked decrease in the antiviral activity. MHL bound to a virus-free, soluble form of the viral envelope protein gp120 but did not inhibit the subsequent binding to a cell-free, soluble form of the cellular receptor CD4.

Certain proteins and peptides isolated from plants inhibit human immunodeficiency virus (HIV). Examples include *Urtica dioica* agglutinin (UDA);[2−5] lectins from *Artocarpus heterophyllus*,[6,7] *Galanthus nivalis*,[8,9] *Narcissus pseudonarcissus*;[9−11] circulin, a cyclic peptide from *Chassalia parvifolia*;[12] and concanavalin A.[13] The present study was initiated based upon the detection of anti-HIV activity in the aqueous extracts of the tropical plant *Myrianthus holstii* Pal. (Urticaceae) when tested in the XTT−tetrazolium-based anti-HIV screen.[14]

Although no previous phytochemical investigations of *Myrianthas holstii* have been reported to date, the related species *M. arboreus* contains peptide alkaloids,[15] triterpene acids,[16−18] pentacyclic triterpenoids,[19,20] and a pentacyclic diacid,[21] none of which was shown to have anti-HIV activity. Herein we present the bioassay-guided fractionation of the aqueous extract from the roots of *M. holstii*, leading to the isolation and characterization of a protein, *M. holstii* lectin (MHL), which has potent anti-HIV activity.

The active protein from *M. holstii* was purified from the aqueous extract of the wood of roots by LH-20 column chromatography and reversed phase HPLC, yielding a white solid with a molecular mass of approximately 9 kDa that behaved as a monomeric protein by SDS/PAGE analysis when compared with standard proteins (Figure 1). HPLC, ESIMS, and matrix-assisted laser-desorption ionization−time-of-flight (MALDI−TOF) analysis, however, indicated that MHL consists of two major constituents having molecular mass (M_r) of 9284 and 9300 Da (Figure 2). In addition, smaller signals 16 and 32 Da higher were also noted. These are most likely oxidation products of sulfur-containing amino acids in the native protein. The two major components were separated by reversed phase HPLC. The protein having the M_r of 9284 was carried through the subsequent analytical steps.

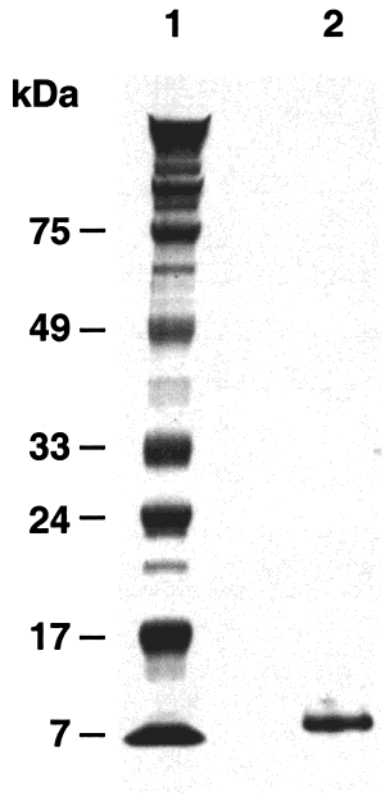

Figure 1. SDS/PAGE analysis of MHL against standard proteins. A 10−20% gradient polyacrylamide gel (50 × 80 × 1 mm) was electrophoresed at 30 mA for 60 min and stained with Coomassie brilliant blue. Lane 1, molecular-mass standards; Lane 2, MHL.

Amino acid analysis of the native protein suggested 16 cysteine residues (data not shown). Attempts to obtain the *N*-terminal sequence of MHL directly via Edman degradation were unsuccessful, indicating that the *N*-terminus was blocked. The protein was then reduced with β-mercapto-

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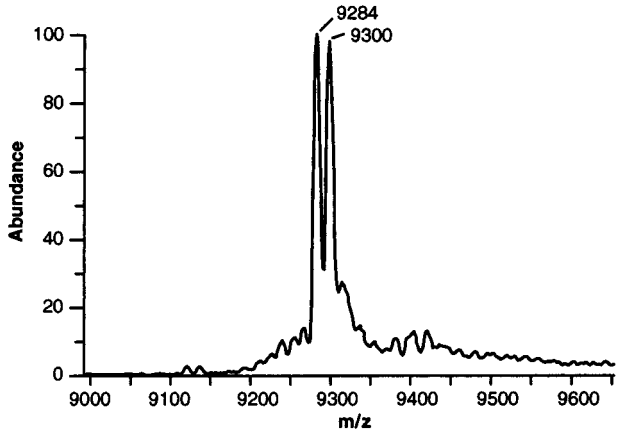

Figure 2. ESIMS analysis of purified MHL. A sample of MHL was analyzed by ESIMS using a lysozyme standard (M_r = 14 305.2). 9284: MHL; 9300: oxidized MHL.

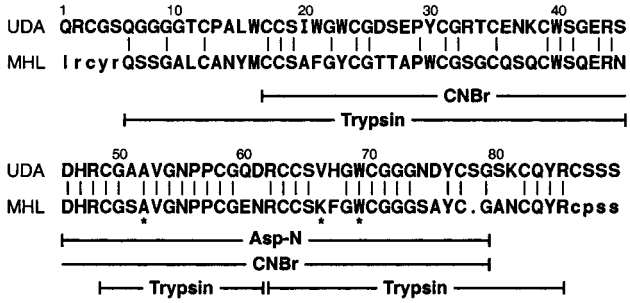

Figure 3. Amino acid sequence of MHL. The protein was sequenced by Edman degradation of a series of overlapping fragments generated by enzymatic and chemical cleavages. Selected peptides isolated by reversed-phase HPLC from digests with endoproteinase Asp-N, trypsin, and CNBr are shown. The deduced sequence indicates that there are multiple isoforms differing in their primary sequences at the three positions indicated by *. Comparison of the amino acid sequence of domain 1−89 of the UDA (P51613) with MHL is also shown. The homologous amino acids found in both MHL and UDA are indicated by a connector |. Residues indicated by lower case letters are placed by prediction, based on the difference between the amino acid composition of the whole protein minus the composition from the known sequence segment plus homology alignments with other chitin binding domains from other species.

ethanol and subsequently alkylated using 4-vinyl pyridine to yield the 4-pyridylethyl cysteine (PEC) derivative. SDS/PAGE analysis of the alkylated protein indicated a molecular mass of about 11 kDa. Further, ESIMS analysis of the PEC derivative gave a M_r of 10.980 kDa, supporting the presence of 16 disulfide-linked cysteines in the nonreduced starting material.

The PEC derivative was cleaved using both the enzyme endoproteinase Asp-N and cyanogen bromide (CNBr). The PEC derivative was also succinylated to increase solubility, and the resulting product cleaved with trypsin. The peptide fragments resulting from each digest were purified and sequenced. The sequence data obtained from Asp-N, CNBr, and trypsin digests resulted in 79 contiguous amino acid assignments of the expected 88 amino acids as shown in Figure 3. The sequence data suggested that there are multiple isoforms of MHL differing in their primary sequences at positions 52, 66, and 69 (marked as *). A search using the Wisconsin package 9.1 enabled alignment of the sequence of MHL with that of UDA, a previously reported lectin (8500 Da) isolated from the stinging-nettle plant,[2,4,5] showing that 48 out of 79 amino acids from the sequence were homologous to residues in the UDA sequence. This indicated a 61% homology between MHL and UDA (Figure 3). The amino acid composition of native MHL is typified by high contents of cysteine (18.2%), a result

Table 1. Anti-HIV Assay Results of MHL vs. *S. griseus* chitinase, *N. pseudonarcissus* lectin, and *P. tetragonolobus* lectin

sample	EC_{50} (μg/mL)	IC_{50} (μg/mL)
MHL	1.4	>50
S. griseus chitinase	no protection	>50
N. pseudonarcissus lectin	2.5	>50
P. tetragonolobus lectin	1.5	>50

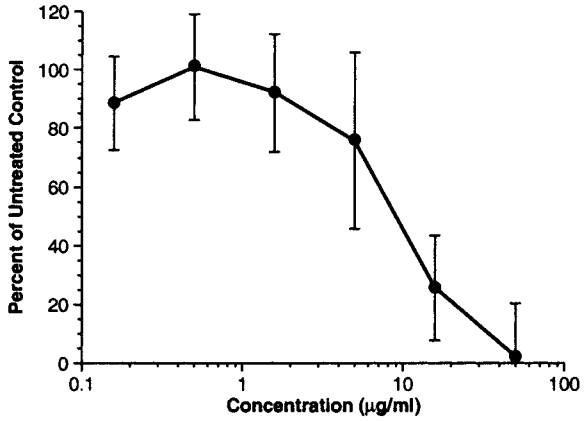

Figure 4. Effect of MHL on the inhibition of cell−cell fusion. Uninfected CEM-SS cells were co-cultured with CEM-SS cells chronically infected with HIV-1_{RF} in the presence of various concentrations of MHL for 24 h. After incubation, the number of syncytia (●) was determined microscopically and is graphically represented as the mean of four samples ± SD.

similar to that obtained for UDA (15.6%). In contrast, however, methionine and phenylalanine form 3.4% of the total amino acid composition in MHL although UDA does not contain these two amino acids. Using the same Wisconsin 9.1 database search package, the sequence results of MHL also showed significant homology with other plant lectins such as wheat germ agglutinin[22] and rice lectin,[4] as well as chitinases such as those isolated from maize[23] and tobacco.[24]

MHL protected CEM-SS cells from the cytopathic effects of HIV-1_{RF}, with an EC_{50} value of 1.4 μg/mL (150 nM), a result similar to that reported for UDA (EC_{50} = 105 nM).[3] In this assay, MHL was not toxic to the target cells, even at the highest tested concentration (250 μg/mL). Reductive cleavage of the disulfide bonds in MHL, however, resulted in loss of anti-HIV activity (data not shown), implying that the disulfide bonds are required for anti-HIV activity. MHL was assayed for anti-HIV activity simultaneously with *Streptomyces griseus* chitinase, *Narcissus pseudonarcissus* lectin, and *Psophocarpus tetragonolobus* lectin. The chitinase was not active against HIV-1 although the lectins were active in the same concentration range as MHL (Table 1).

MHL was examined for its ability to bind to or inactivate virus particles and to bind to CEM-SS cells. Virus particles treated with MHL (10 μg/mL), followed by dilution beyond effective concentrations of the protein, were fully infective, indicating that the protein was not directly virucidal. Likewise, when CEM-SS target cells were treated with 4 μg/mL of MHL, then washed free of the protein, they retained full susceptibility to infection by the virus (data not shown). MHL was additionally tested for inhibition of cell−cell fusion and syncytium formation between chronically infected CEM-SS cells and uninfected CEM-SS cells.[25] MHL inhibited syncytium formation, with an EC_{50} value of 9.8 μg/mL (Figure 4). Results from these studies indicate that MHL acts reversibly to inhibit HIV infection of the

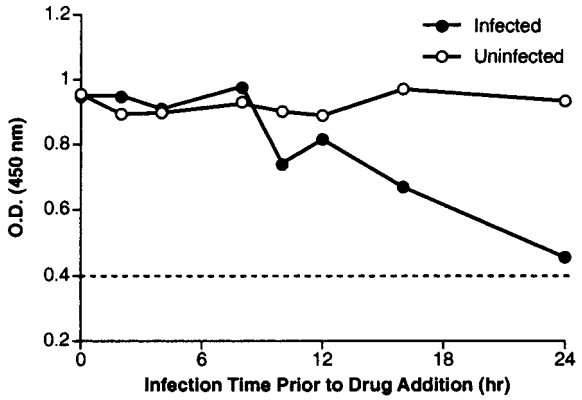

Figure 5. Delayed-addition study. Effect of delayed addition of 10 μg/mL of MHL on HIV-1_{RF} infected (●) and uninfected (○) CEM-SS cells analyzed after 6 days in culture using the XTT assay. Absorbances ± SD are displayed and represent the mean of quadruplicate samples for infected cultures and duplicate samples for infected controls.

host cells and that it has to be continuously present to be fully active.

To help ascertain the stage at which MHL interfered with the viral life-cycle, delayed-addition studies were performed. MHL was still fully active when addition was delayed by 8 h after HIV-1 addition to the CEM-SS host cells (Figure 5); delay of addition by 12 h resulted in partial activity, while after 16 h or more there was no appreciable anti-HIV-1 activity. The results of these studies also suggest that MHL does not act on the cell surface.

In an initial effort to screen potential molecular targets of MHL, the protein was tested for its ability to bind to a soluble form of the viral protein gp120 in fluorescence-polarization experiments. Addition of MHL to fluorescein-5-isothiocyanate-labeled gp120 (FITC-gp120) resulted in an increase in polarization (17 mP), implying that the protein bound to gp120 (Figure 6A). Further, as indicated by Figure 6B, both MHL and sCD4 could bind to gp120 simultaneously, causing an increase in fluorescence polarization by 10 and 12 mPa, respectively. This result suggests, that unlike other plant proteins such as jacalin,[4,5] MHL did not inhibit sCD4 from binding to gp120. From this result, it appears that MHL binds to the viral protein gp120 at a different site (or in a different manner) from sCD4. Further binding experiments in the presence of monomeric sugars showed that myrianthin was not inhibited from binding to gp120 by any of the sugars tested except for *N*-acetyl-D-glucosamine (GlcNAc). When MHL was added to FITC-gp120 in the presence of 0.5 M GlcNAc no change in polarization was noted, implying that the protein was inhibited from binding to gp120 (Figure 6C). Similar results were obtained when 0.7 and 1.0 M GlcNAc were used (data not shown). These results suggest that MHL binds to GlcNAc and, hence, is subsequently unable to bind to gp120, thereby implying that MHL interacts with sugar moieties present on gp120. Our results on MHL binding to GlcNAc compares very well with that reported for UDA, which specifically binds to GlcNAc.[2,3,5]

MHL displays certain lectin-like properties (*e.g.*, sugar-specific binding) similar to those reported for UDA.[2−4] However, unlike many other plant lectins,[6−10,13] MHL does not agglutinate human erythrocytes (high test concentration 27 μM). Furthermore, in the chitinase assay MHL did not show activity itself, but did inhibit the activity of *S. griseus* chitinase (data not shown).

Proteins and lectins with therapeutic potential isolated from natural product extracts such as MHL have previously been reported. For example, extracts from the

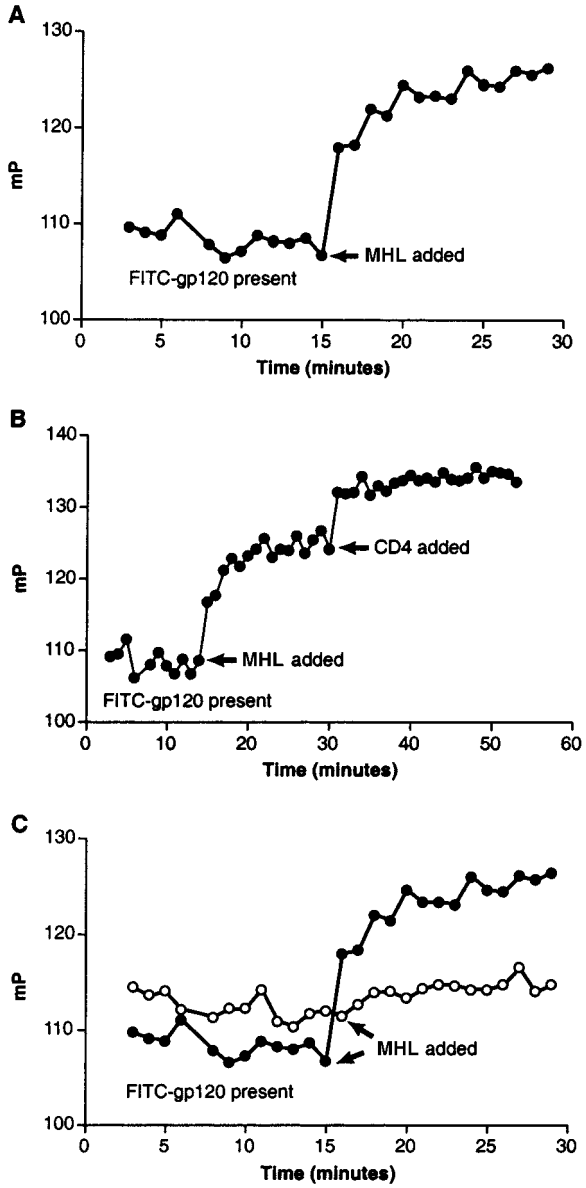

Figure 6. (A) Fluorescence polarization of FITC-gp120 upon addition of MHL. (B) Fluorescence polarization of FITC-gp120 upon addition of MHL and sCD4. (C) Fluorescence polarization of FITC-gp120 (●) upon addition of MHL, (○) FITC-gp120 in the presence of 0.5 M GlcNAc upon addition of MHL.

rhizomes of *U. diocia* are used in Europe for the treatment of benign prostatic hyperplasia,[26,27] while leaf extracts are used as antiinflammatory remedies in rheumatoid arthritis.[28] Furthermore, the anti-HIV natural product protein cyanovirin-N, isolated from the cyanobacterium *Nostoc ellipsosporum*, is currently being investigated for clinical use as a topical microbicide against HIV. The limiting factor for MHL, as with all lectins as therapeutic agents, is their inherent lack of specificity. For MHL, because it readily interacts with GlcNAc, any structure bearing this terminal monosaccharide would potentially bind the protein. Should one find lectins with greater specificity for higher-order oligosaccharide structures, they could prove more interesting and also provide a new class of potential therapeutic agents.

Experimental Section

Plant Material. The wood of roots of *M. holstii* was collected under an NCI contract in 1991, from a riverine forest in Tanzania, Africa, by the Missouri Botanical Garden.

Voucher specimens (Q66R786) from this collection are maintained at the Missouri Botanical Garden and the Smithsonian Institution. The plant was identified by C. C. Berg (Herbarium, Botanical Institute, University of Bergen, Norway).

Extraction and Purification. The freeze-dried plant material was ground to a coarse powder (241.0 g) and sequentially extracted with 1:1 MeOH−CH_2Cl_2 followed by water. The aqueous extract was frozen and lyophilized to give a dark brown powder (6.32 g). A 1.15-g portion of the extract was first chromatographed by size-exclusion chromatography on a Sephadex LH-20 column (MeOH−H_2O, 7:3). The active fractions were pooled and further purified by reversed phase HPLC (2.14 × 25 cm; C_4; 300 Å, Dynamax) using a gradient of 0−30% (by volume) of acetonitrile in water. The elution of active components was monitored by UV absorption with a photodiode array detector (Waters 990). The active fraction was further purified by reversed phase HPLC (1.0 × 25 cm; C_{18}; 300 Å, Dynamax) using a gradient of 5−30% (by volume) of acetonitrile in water. All the mobile phases for HPLC contained 0.05% TFA. The two major active fractions were collected, pooled, and lyophilized (overall yields: MHL M_r 9284 3.1%; MHL M_r 9300 2.5% of extract).

Mass Spectrometry. EIMS was performed with a JEOL SX102 equipped with an analytical electrospray source. The spectrometer was calibrated using an external lysozyme standard (M_r 14 305.2) prior to each analysis. Samples were injected into the source in a 1:1 solution of hexafluoro-2-propanol and 2% acetic acid. MALDI-TOF mass spectrometry was performed using a Kratos Kompact Maldi II instrument (Shimadzu) operated in a linear mode with sinapinic acid as a matrix and trypsin as an external standard.

Amino Acid Analysis and Sequencing. Amino acid analysis was performed using a Beckman model 6300 amino acid analyzer, according to the protocols of the manufacturer. *N*-Terminal amino acid sequencing was performed on an Applied Biosystems model 477A sequencer, according to the protocols of the manufacturer. *N*-Terminal amino acid sequence similarity was searched using the program manual for the Wisconsin package 9.1 Unix, September 1997, Genetics Computer Group, (GCG, Madison, WI).

Reduction and Alkylation of the Disulfide Bonds. MHL (10.4 mg) was dissolved in 1 mL of 8 M guanidine hydrochloride. Then, 10 μL of 3 M Tris HCl (pH 8.5) and 10 μL of β-mercaptoethanol were added and the reaction incubated in the dark at 37 °C under an atmosphere of nitrogen. After 1 h, 30 μL of 4-vinyl pyridine (Sigma) was added to the reaction mixture, which was incubated as above, for 3 h, dried under nitrogen, and then maintained at −20 °C overnight. The 4-pyridylethylated product was purified by reversed phase HPLC (1.0 × 25 cm; C_{18}; 300 Å, Dynamax) using a gradient of 5−30% (by volume) acetonitrile in water (plus 0.05% TFA).

Cleavage of the 4-Pyridylethylated Product with Asp-N. The 4-pyridylethylated MHL (138 μg) was dissolved in 20 mM Tris HCl (pH 7.5), and 2 μg of endoproteinase Asp-N (Boehringer, Mannheim) was added and the reaction maintained at room temperature for 18 h. To complete the reaction, additional Asp-N (2 μg) was added to the mixture to afford a 1:35 enzyme−substrate ratio (w/w), and the reaction was incubated at 37 °C for an additional 2 h. The Asp-N-cleaved pyridylethylated-peptide products were purified by reversed phase HPLC (0.39 × 30 cm; C_{18}; Waters μbondapak, 10 μm) using a gradient of 0−60% (by volume) of acetonitrile in water (plus 0.1% TFA).

Cleavage of the 4-Pyridylethylated Product with Cyanogen Bromide. The 4-pyridylethylated product (138 μg) was dissolved in 500 μL of 70% TFA, and 1 mg of crystalline CNBr was added. The reaction mixture was incubated at room temperature for 18 h under nitrogen. The volume of the reaction mixture was reduced to 150 μL under nitrogen, 500 μL of water was added and the mixture lyophilized. The resulting solid was dissolved in 500 μL of 50% CH_3CN−H_2O containing 0.1% TFA and lyophilized. The sample was purified by reversed phase HPLC (0.39 × 30 cm; C_{18}; Waters μbondapak, 10 μm) using a gradient of 0−30% (by volume) of acetonitrile in water (plus 0.1% TFA).

Succinylation of the 4-Pyridylethylated Product. The 4-pyridylethylated product (138 μg) was dissolved in 138 μL of water, and 7 μL of 0.2 M Na_2CO_3 was added to give a final pH of 10. Succinic anhydride (2 mg) was added with magnetic stirring, and the pH was continually adjusted by adding 1 M NaOH and 0.2 M Na_2CO_3 to pH 9. After 30 min, more succinic anhydride (2 mg) was added and the pH adjusted to 9 as before. At 1 h, the solution was acidified to pH 2 with concentrated HCl. The product was purified by reversed phase HPLC (0.39 × 30 cm; C_{18}; Waters μbondapak, 10 μm) using a gradient of 0−60% (by volume) of acetonitrile in water (plus 0.1% TFA), and the succinylated product was lyophilized.

Cleavage of the 4-Pyridylethylated Succinylated Product with Trypsin. The purified succinylated protein (138 μg) was dissolved in 600 μL of 40 mM Tris HCl (pH 8.5), and trypsin (2.8 μg) was added to give a 1:50 enzyme−substrate ratio (w/w). The reaction was allowed to proceed at room temperature for 3 days. The reaction mixture was adjusted to pH 2 with hydrochloric acid, and the cleaved product was purified by reversed phase HPLC (0.39 × 30 cm; C_{18}; Waters μbondapak, 10 μm) as above.

Electrophoresis. SDS/PAGE was carried out using 10−20% gradient polyacrylamide resolving gels (precast, Novex) and buffer systems according to Laemmeli.[29] The gels (50 × 80 × 1 mm) were subjected to electrophoresis at a constant current of 30 mA/gel for 60 min at room temperature with chilled electrode buffer.

Assays for Anti-HIV Activity: Effect of MHL on Acute HIV-1_{RF} Infection in CEM-SS Cells. CEM-SS cells were maintained in RPMI 1640 medium without phenol red and supplemented with 10% fetal bovine serum (BioWhittaker), 2 mM L-glutamine (BioWhittaker), and 50 μg/mL gentamicin (BioWhittaker) (complete medium). Exponentially growing cells were washed, resuspended in complete medium, and a 50-μL aliquot containing 5 × 10^3 cells was added to individual wells of a 96-well round-bottom microtiter plate containing serial dilutions of MHL in 100-μL medium. Stock supernatants of HIV-1_{RF} were diluted in complete medium to yield sufficient cytopathicity (80−90% cell kill in 6 days), and a 50-μL aliquot was added to appropriate wells. Plates were incubated for 6 days at 37 °C, then stained for cellular viability using 2,3-bis-[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) as described.[12] This standard procedure was used for other samples tested in this study.

Treatment of HIV-1_{RF} with MHL. Concentrated HIV-1_{RF} was treated for 1 h with 1 mL of culture medium containing 4 μg/mL of MHL or control medium. After incubation, the treated and control virus samples were diluted to yield a multiplicity of infection (MOI) of 0.2, in a total volume of 50 μL, which diluted the compound beyond the antiviral protective range. The pretreated virus (50 μL) was then added to individual wells of a 96-well microtiter plate containing 5 × 10^3 CEM-SS cells and either medium alone or 4 μg/mL of MHL. The cultures were then incubated for 6 days, and cellular viability was determined using the XTT assay.

Treatment of CEM-SS Cells with MHL. CEM-SS cells were incubated with medium containing 4 μg/mL of MHL or with control medium for 1 h at 37 °C. After incubation, the CEM-SS cells were washed free of MHL using two centrifugation steps. Pretreated cells were then resuspended in culture medium and added to individual wells of a 96-well microtiter plate containing 5 × 10^3 CEM-SS cells and either 50 μL of compound-free medium or 4 μg/mL of MHL. A 50-μL aliquot of diluted HIV-1_{RF} was added to appropriate wells to yield an MOI of 0.2. The plates were incubated for 6 days at 37 °C in an atmosphere containing 5% CO_2, and cellular viability was assessed using the XTT assay.[12]

Effect of MHL on Cell−Cell Fusion.[25] Uninfected CEM-SS cells (1 × 10^5 cells/50 μL) were co-cultured with chronically infected CEM/HIV-1_{RF} cells (1 × 10^3 cells/50 μL) in flat-bottomed 96-well microtiter plates in the presence of various concentrations (50, 15.8, 5, 1.58, 0.5, 0.16 μg/mL) of MHL or in control medium. After 1 and 3 days of incubation, the

cultures were evaluated microscopically for the presence and number of syncytia (quadruplicate samples).

Delayed Addition of MHL to HIV-1$_{RF}$ Infected Cells. The standard anti-HIV assay was performed as previously described in this text, except that MHL (10 μg/mL) was added to quadruplicate wells at 0, 2, 4, 6, 8, 12, 16, and 24 h after the addition of the virus. After a total of 6 days of incubation, the cultures were observed microscopically for the presence of cytopathic effects (syncytia) and then stained by XTT.[12]

gp120 and CD4 Binding Studies. To investigate the physical interaction of MHL with a soluble form of the HIV envelope protein, gp120, and with the soluble form of the cellular receptor CD4 (sCD4), fluorescence-polarization experiments were carried out using an FPM-1 fluorescence-polarization analyzer (Jolley Consulting and Research, Inc.). A stock solution of aqueous MHL (0.1 mM) was prepared and 10-μL aliquots were used in all experiments. Fluorescein-5-isothiocyanate-labeled gp120 (FITC-gp120, Intracel) was the molecule detected in all experiments. FITC-gp120 (0.42 μM) was added to 990 μL of BGG phosphate buffer (pH 7.4, PanVera, Quality Biological Inc.) to give a 4.19 nM solution of FITC-gp120, and 50-μL aliquots were used in all experiments. The sCD4 (40 μg, 18.2 μM) was obtained from the AIDS Research and Reagent Program. Monomeric sugars (D-fructose, D-fucose, D-galactose, D-glucose, D-mannose, *N*-acetyl-D-glucosamine, *N*-acetyl-neuraminic acid, *N*-acetyl-nitrosamine, and D-xylose) were purchased from Sigma and tested at concentrations up to 1 M. Cyanovirin-N (CV-N), which is known to bind to gp120, was used as a positive control in this study.[30] Fluorescence readings were taken at 30-s intervals.

Fluorescence polarization was measured with FITC-gp120 alone, and change in fluorescence polarization was measured upon addition of MHL (Figure 6A) and upon addition of MHL followed by sCD4 (18.2 mM) (Figure 6B). The experiment was also performed with FITC-gp120 in the presence of 0.5 M GlcNAc (Figure 6C) and 0.7 M GlcNAc (data not shown), followed by addition of MHL in each experiment. The experiment was repeated in the presence of 0.7 M GlcNAc and MHL as before, followed by addition of CV-N (2.8 μM) to the assay mixture, and the change in fluorescence polarization was measured (data not shown).

In separate experiments, fluorescence polarization was measured with FITC-gp120 alone and then with the addition of 9 different sugars (D-fructose, D-fucose, D-galactose, D-glucose, D-mannose, *N*-acetyl-D-glucosamine, *N*-acetyl-neuraminic acid, *N*-acetyl-nitrosamine, and D-xylose) at concentrations up to 1 M. MHL was added, and the change in fluorescence polarization was measured in each case.

Chitinase assay. The colorimetric chitinase assay was performed using the manufacturer's protocol (Sigma Chemical Company, MO). Absorbance was measured at l540 nm on a UV spectrophotometer (Beckman).

Hemagglutination assay. Hemagglutination was determined by incubating a 2% solution of human type-O erythrocytes with various concentrations of MHL in a 24-well plate. Agglutination was determined by visual examination 2 h after administration. The lectin from *Lens culinaris* (Sigma) was used as a positive control.

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