Eur. J. Biochem. 245, 47–53 (1997)

Isolation and characterization of niphatevirin, a human-immunodeficiency-virus-inhibitory glycoprotein from the marine sponge *Niphates erecta*

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Anti-human immunodeficiency virus (HIV)-bioassay-guided fractionation of aqueous extracts of the Caribbean sponge *Niphates erecta* led to isolation of a novel anti-HIV protein, named niphatevirin. The protein was purified to homogeneity by ethanol precipitation, ammonium sulfate precipitation, gel-permeation chromatography and concanavalin-A–Sepharose affinity chromatography. Niphatevirin potently inhibited the cytopathic effects of HIV-1 infection in cultured human lymphoblastoid (CEM-SS) cells; the effective concentration of drug that results in 50% protection of the cells through inhibition of cell lethality, cell-cell fusion and syncytium formation was approximately 10 nM. Delay of addition of niphatevirin to infected cultures by two hours markedly decreased (≈50%) cytoprotection; delay of addition by eight hours resulted in no antiviral activity. Niphatevirin bound to CD4 in a manner that prevented the binding of gp120, but did not directly bind gp120. Niphatevirin (6.5 μM) was inactive in both hemagglutination and hemolysis assays. Niphatevirin had a molecular mass of about 19 kDa by matrix-assisted laser-desorption ionization-time of flight (MALDI-TOF) mass spectrometry, and a native molecular mass of approximately 18 kDa by gel-filtration chromatography. The protein had an acidic isoelectric point of 4.2–4.6, and was shown by periodate acid Schiff's staining to be glycosylated.

Keywords: anti-(human immunodeficiency virus); glycoprotein; sponge; CD4; *Niphates erecta*.

Marine organisms have been studied for several decades as potential sources of novel biologically active leads for drug development [2]. As part of the ongoing screening of natural products by the National Cancer Institute, aqueous and organic extracts of marine invertebrates are examined for their ability to protect human T-lymphoblastoid cells from human immunodeficiency virus (HIV-1)-induced cell killing [3, 4]. Aqueous extracts from the Caribbean sponge *Niphates erecta* have consistently shown potent activity in this screen.

Crude aqueous extracts from *Niphates digitalis* reportedly exhibited hematotoxicity in mice, although the responsible agent was not characterized [5]. Earlier studies of aqueous extracts of *N. digitalis* in our laboratory had identified HIV-inhibitory sulfated polysaccharides, which were isolated by ethanol precipitation [6]. In the case of *N. erecta*, however, ethanol precipitation did not completely eliminate the biological activity, raising the possibility that the antiviral activity was not due solely to the presence of sulfated polysaccharides; subsequent treatment with ammonium sulfate precipitated the biological activity, suggesting that the active component was proteinaceous. The present study was undertaken to isolate and characterize from aqueous extracts of *N. erecta* the anti-HIV constituent(s) not accountable to sulfated polysaccharides.

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Abbreviations. BCECF, biscarboxyethyl-5(6)-carboxyfluorescein acetoxymethyl ester; conA, concanavalin A; EC_{50}, effective concentration of drug that results in 50% protection of the cells; HIV, human immunodeficiency virus; MALDI-TOF, matrix-assisted laser-desorption ionization-time of flight; MOI, multiplicity of infection; MPA, *Maclura pomifera* agglutinin; PAS, periodate acid Schiff's; PVDF, poly(vinyldifloride); XTT, 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide inner salt.

MATERIALS AND METHODS

Sponge material. A sample of the sponge *N. erecta* (phylum Porifera, class Demospongiae, order Haplosclerida, family Niphatidae) [7] was collected by scuba at a depth of 36 m in March 1987, off Freeport-Lucaya, Grand Bahama Island, Bahamas. The sample collection and identification were made by Dr Shirley Pomponi (Harbor Branch Oceanographic Institution) under contract to the NCI. A taxonomic voucher specimen (sample number Q66B0331-V) is deposited at the Smithsonian Institution Sorting Center, Suitland, USA.

The sample is a cluster of erect branches, approximately 40 cm in length. The surface of the sponge is shaggy, particularly at the tips of the branches. Raised oscules, 5–8 mm in diameter, are irregularly distributed over the entire length of the branches. The consistency is firm and stiff, but compressible. The color in life is a dull pinkish purple; in ethanol the color fades to a light purple. Both the ectosome and choanosome are reticulate with multispicular spongin fiber tracts. The spicules

consist of oxeas, strongloxeas, strongyles and rare styles, approximately 170 μm×4 μm (based on microscopic measurements of 25 spicules). No microscleres were observed.

Protein determinations. The protein contents in various samples were determined by colorimetric assay using the Bio Rad protein assay system with bovine γ globulin as the standard according to Bradford [8].

Sulfated polysaccharide determinations. To determine the presence or absence of sulfated polysaccharides in aqueous fractions of *N. erecta*, samples were tested using the cationic dye, toluidine blue O, and analyzed for absorbance at 620 nm as described [6, 9].

Assays for anti-HIV activity. *Effect of niphatevirin on acute HIV-1 infection in CEM-SS cells.* CEM-SS cells [10] were maintained in RPMI 1640 media without phenol red and supplemented with 5% fetal bovine serum (BioWhittaker), 2 mM L-glutamine (BioWhittaker) and 50 mg · ml^{-1} gentamicin (BioWhittaker) (complete medium). Exponentially growing cells were washed, resuspended in complete medium, and a 50-μl aliquot containing 5000 cells was added to individual wells of a round-bottom, 96-well, microtiter plate containing serial dilutions of niphatevirin in a volume of 100 μl medium. Stock supernatants of HIV-1_{RF} were diluted in complete medium to yield a multiplicity of infection (MOI) of 0.8 (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 assayed for cellular viability, metabolic activity, DNA content, p24 antigen production, supernatant reverse-transcriptase activity and the synthesis of infectious virions as described [11].

Delayed addition of Niphatevirin to HIV-1_{RF} infected cells. CEM-SS cells were plated into individual wells of a 96-well microtiter plate at a density of 5000 cells/well in 50 μl medium. Diluted HIV-1_{RF} stock supernatants (50 μl) were added to appropriate wells to yield a MOI of 1.0. At various times after the addition of virus, a 100-μl aliquot of 4.4 μg · ml^{-1} niphatevirin was added to multiple wells. After a total of 6 days incubation, cellular viability was assessed using the 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) assay as described [11].

Treatment of HIV-1_{RF} with niphatevirin. Concentrated HIV-1_{RF} was treated for 60 min with 4.4 μg · ml^{-1} niphatevirin or medium alone. After incubation, the treated virus supernatant was diluted to yield a MOI of 0.8 and to dilute the niphatevirin beyond the effective concentration. The niphatevirin-treated virus (50 μl) was then added to individual wells of a 96-well microtiter plate containing 5000 CEM-SS cells (50 μl) and either 100 μl of medium alone or 4.4 μg · ml^{-1} niphatevirin. Plates were incubated for 6 days and cellular viability was assessed using the XTT assay.

Pretreatment of CEM-SS cells with niphatevirin. CEM-SS cells were incubated with 4.4 μg · ml^{-1} niphatevirin or complete medium for 60 min at 37°C. After incubation, CEM-SS cells were washed free of drug using two centrifugation steps. Treated cells were then resuspended in complete medium and added to individual wells of a 96-well microtiter plate (5000 cells · 50 μl^{-1}) containing 100 μl medium alone or 4.4 μg · ml^{-1} niphatevirin. A 50-μl aliquot of diluted HIV-1_{RF} was added to appropriate wells to yield a MOI of 0.8. Plates were incubated for 6 days at 37°C in an atmosphere containing 5% CO_2. After incubation, cellular viability was assessed using the XTT assay.

Effect of niphatevirin on cell-cell fusion. Uninfected CEM-SS cells (1×10^5 · 50 μl^{-1}) and CEM/HIV-1RF-infected cells (1×10^3 · 50 μl^{-1}) were incubated together in flat-bottomed, 96-well microtiter plates in the presence of various concentrations of niphatevirin (100 μl) or 100 μl of complete medium alone. The plates were incubated for 72 hours and the number of syncytia determined microscopically. Each experimental condition consisted of six replicate samples.

Stability tests. Crude protein samples from *N. erecta* were taken up in 50 mM sodium phosphate, pH 7.5, augmented with 0.02% NaN_3 and one of the following components: 10 mM EDTA, 5 mM dithiothreitol, and either 1% or 10% glycerol. The final concentration of the protein in solution was 500 μg · ml^{-1}. Samples treated with glycerol were separated into three aliquots and stored at either −20, 4 or 23°C for a period of 14 days prior to assay.

To determine pH stability, protein samples, purified through ethanolic precipitation and ammonium sulfate precipitation, were taken up in 50 mM sodium phosphate, pH 7.5 ($NaCl/P_i$) titrated to the following pH values: 4.0, 5.0, 6.0, 6.5, 7.0, 7.5, 8.0 and 9.0, and stored at 4°C for 24 hours prior to assay.

Protein purification. *Ethanol precipitation.* The removal of sulfated polysaccharides from crude aqueous extracts was performed essentially as described in Beutler et al. [6]. Briefly, an equal volume of absolute ethanol was added to aqueous extracts of *N. erecta* which had been dissolved in distilled, deionized water at a concentration of 50 mg · ml^{-1}. The ethanolic solutions were allowed to precipitate overnight at −20°C, then centrifuged at 3000 rpm for 60 min. The pellets were set aside and the supernatants were evaporated under vacuum to dryness prior to use in later purification steps.

Sephadex G-25 gel permeation. Freeze-dried ethanolic supernatants from *N. erecta* were taken up in 20 mM Hepes, pH 7.5, augmented with 0.02% NaN_3. The samples were placed on a Sephadex G-25 column (2.5 cm×50 cm, Sigma) and eluted with the same buffer at a flow rate of 2.5 ml · min^{-1}. The eluate was monitored at 280 nm and fractions were taken each minute.

Ammonium sulfate precipitation. Crystalline ammonium sulfate (molecular biology grade, Sigma) was added to the active fraction from the Sephadex G-25 column solution to bring the final concentration to 30% saturation. The mixture was allowed to precipitate on ice for 120 min and was then centrifuged at 3000 rpm for 60 min. The pellets were collected and the supernatant was then brought to 50% saturation with ammonium sulfate, followed again by precipitation and centrifugation. Finally, the second pellets were collected and the supernatant was brought to 75% saturation with ammonium sulfate; all other steps were repeated as above.

Concanavalin-A–sepharose affinity chromatography. Following ammonium sulfate precipitation, the 30–50% saturation protein pellet was brought up in 5 mM sodium acetate augmented with 0.1 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 0.02% NaN_3, pH 7.1, and placed on a 5-ml concanavalin-A–Sepharose 4B column (Sigma) previously equilibrated with the same buffer. Following application of the sample, the column was washed with 20 ml starting buffer, followed by 20 ml of the starting buffer augmented with 0.3 M methyl-α-D-mannopyranoside, and finally with 20 ml of the starting buffer augmented with 1.0 M sodium chloride. Buffer exchange and concentration of the resulting fractions was achieved by ultrafiltration on a YM-10 membrane.

G3000PW analytical gel permeation. Protein samples (100 μl) were injected onto a G3000PW gel-permeation column (21.5 mm×300 mm, Toso Haas) using a BioCad workstation (Perseptive Biosystems) and eluted with 25 mM sodium phosphate, pH 7.5, augmented with 0.2 M NaCl and 0.02% NaN_3, pH 7.5. The column was eluted at a flow rate of 5 ml · min^{-1} with fractions taken every 30 s. The native molecular mass was determined by calibrating standard proteins [bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome *c* (17 kDa), aprotinin (6.5 kDa)] by their retention time ($A_{280\,nm}$)

and comparing the resulting calibration curve to the retention time of the active protein.

Electrophoresis. *SDS/PAGE.* SDS/PAGE was carried out using 15% polyacrylamide resolving gels, 4% polyacrylamide stacking gels and standard discontinuous buffer systems according to Laemmeli [12] on a BioRad Mini Protean II apparatus. The gels (50×80×1 mm) were subjected to electrophoresis at a constant current of 25 mA · gel^{-1} at room temperature with chilled electrode buffer.

Isoelectric focusing. Isoelectric focusing was carried out using a non-urea 5% polyacrylamide tube gel system on a Bio-Rad Mini Protean II apparatus. Protein samples were focused at 4°C in a pH gradient 3.5−9.5 using BioRad ampholytes and the following voltage conditions: 500 V for 15 min followed by 750 V for 210 min. The isoelectric point of the protein was determined by comparison to a standard calibration using commercially available standard proteins (IEF Mix II, Sigma).

Periodate acid Schiff's (PAS) and silver staining. SDS/PAGE gels were stained for protein using either Coomassie brilliant blue or a modified Merrill silver stain procedure [13]. Gels were stained for glycoproteins by a periodic acid-Schiff-based stain (glycoprotein detection kit, Sigma).

Hemagglutination and hemolysis assays. Human type-O erythrocytes (1.0% in NaCl/P_i) were treated with various concentrations of niphatevirin and allowed to incubate for one hour at room temperature. Following incubation, the solution was centrifuged at 1500 rpm for 15 min, the supernatant was removed and mixed with an equal volume of NaCl/P_i in a cuvette, and hemolysis was quantified by measuring absorbance at 540 nm. Hemagglutination was determined by incubating a 2% solution of erythrocytes with various concentrations of niphatevirin, agglutination was determined microscopically at time points 1, 4 and 24 hours following administration. The agglutinin from *Lens culinaris* (Sigma) was used as a positive control for hemagglutination.

gp120 and CD-4 binding studies. To determine whether niphatevirin could bind directly to the HIV envelope protein gp120, dot-blot assays were conducted as follows: (a) 10 µl samples of either niphatevirin or standard proteins [bovine globulin and aprotinin as negative controls, soluble CD4 and horseradish peroxidase (HRP) as positive controls] were absorbed onto a poly(vinylidene difluoride) membrane by capillary action. (b) The protein-bound membrane was incubated overnight in a 1% solution of BSA in NaCl/P_i buffer. (c) The blocked membrane was washed with Tween/NaCl/P_i for 20 min (×3). (d) The washed membrane was incubated with a 1 µg · ml^{-1} solution of gp120-HRP conjugate for 60 min. (e) The membrane was again washed with Tween/NaCl/P_i for 20 min (×3). (f) The conjugate-bound membrane was then incubated in a color development solution consisting of the peroxidase substrate 3-amino-9-ethylcarbazole and 0.1% hydrogen peroxide in NaCl/P_i until spots became visible on the membrane.

To determine whether niphatevirin inhibited the binding of gp120 to CD4, dot-blot assays were carried out as described above except that 10-µg samples of soluble CD4 rather than niphatevirin were absorbed onto the poly(vinylidene difluoride) membranes and, following overnight incubation with 1% BSA and subsequent washes, membranes were incubated in solutions of either niphatevirin, standard proteins (bovine globulin, aprotinin) in NaCl/P_i or NaCl/P_i alone for 60 min. Following the niphatevirin wash, the membranes were washed with Tween/NaCl/P_i for 20 min (×3) and were then treated as in steps d−f above.

To confirm the binding of niphatevirin to sCD4, dot-blot assays were carried out in a similar manner to the initial gp120-binding assay. Briefly, poly(vinylidene difluoride) membranes were spotted with 10-µl aliquots of niphatevirin, blocked with BSA, washed with Tween/NaCl/P_i and then incubated for 60 min in a 1-µg/ml solution of sCD4. The membranes were then washed with Tween/NaCl/P_i and incubated with either anti-sCD4 polyclonal antibodies or gp120-binding site-specific anti-sCD4 monoclonal antibodies (OKT4a or Q4120). The membranes were again washed with Tween/NaCl/P_i and incubated with HRP-conjugated secondary antibodies, followed by a final washing in Tween/NaCl/P_i and incubation with the chromogenic substrate AEC.

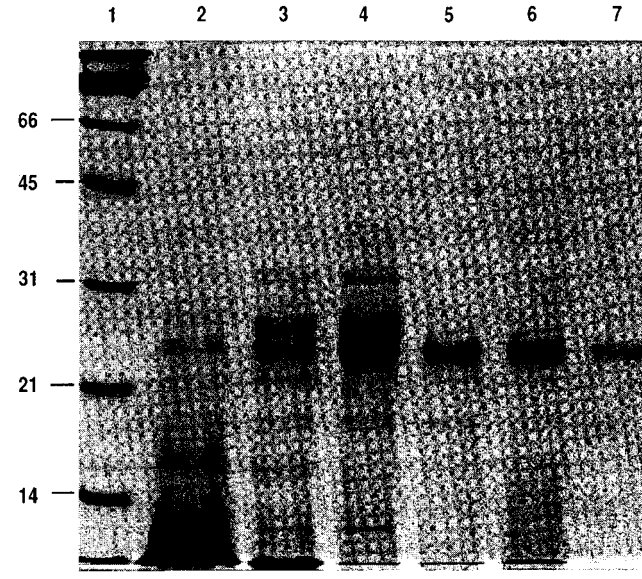

Fig. 1. SDS/PAGE analysis of various purification stages of niphatevirin. A 15% acrylamide gel (50×80×1 mm) was electrophoresed at 25 mA for 120 min and stained with Coomassie brilliant blue. Lane 1, molecular-mass standards; lane 2, crude aqueous extract of *N. erecta*; lane 3, ethanolic supernatant fraction; lane 4, Sephadex G-25 column fraction; lane 5, 50% ammonium sulfate precipitate; lane 6, concanavalin-A−Sepharose-purified fraction; lane 7, G3000PW gel-filtration protein fraction.

Mass spectroscopy. Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectroscopy was performed using a Kratos Kompact Maldi III 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 done using a Beckman model 6300 amino acid analyzer according to the protocols of the manufacturer. PROPSEARCH database analysis of the amino acid composition of niphatevirin was conducted as previously reported [14]. 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, version 8, September 1994, Genetics Computer Group, (Madison, USA).

RESULTS

pH and temperature stability, dithiothreitol, EDTA, glycerol. Analysis of partially purified protein extracts from *N. erecta* indicated that the anti-HIV activity was stable throughout the pH range tested (pH 5−9) and at temperatures from −20°C to room temperature for 2 weeks. Furthermore, the addition of 5 mM dithiothreitol did not significantly alter the activity, while the addition of 10 mM EDTA or 10% glycerol slightly reduced the activity.

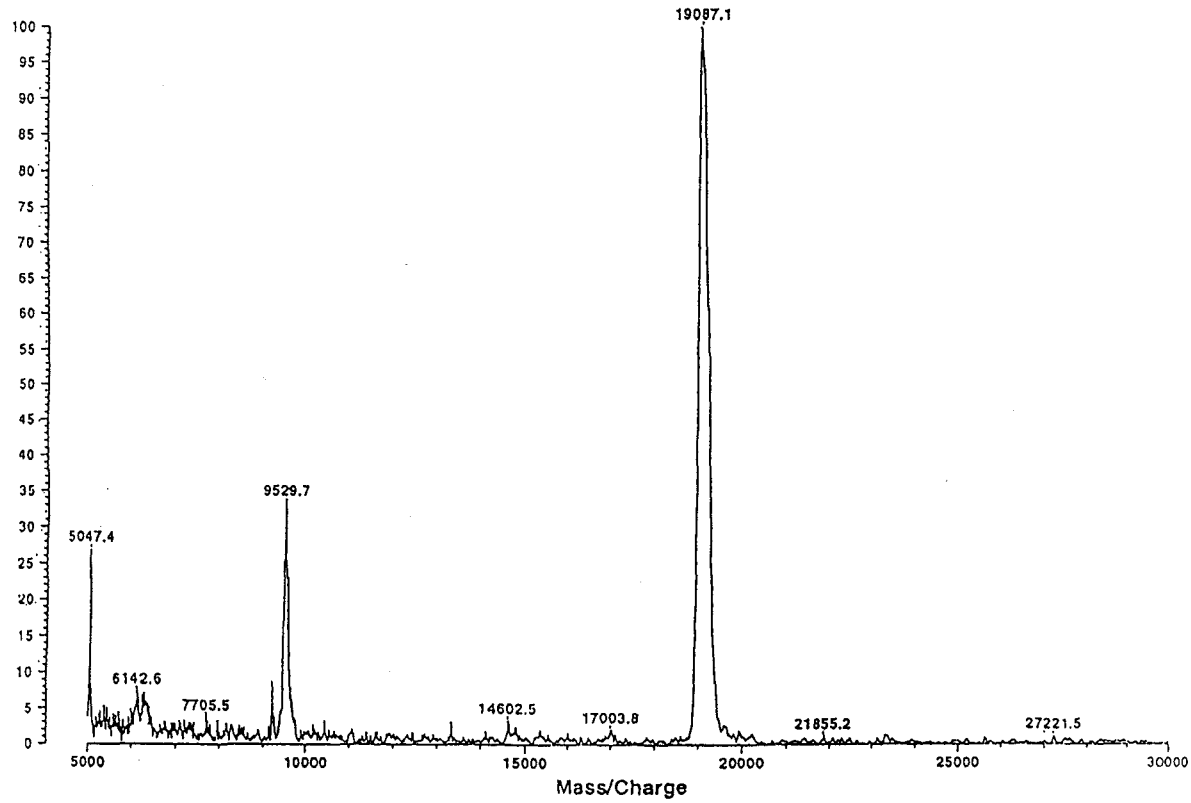

Fig. 2. MALDI-TOF analysis of purified niphatevirin. A sample of niphatevirin was analyzed by MALDI-TOF mass spectroscopy using sinapinic acid as a matrix and trypsin as an external standard, [M+H] = singly charged protein (*m/z* = 19087), [M+2H] = doubly charged protein (*m/z* = 9529).

Purification of niphatevirin. The active protein from *N. erecta* (niphatevirin) was purified to homogeneity by a combination of ethanol precipitation, ammonium sulfate precipitation, gel-permeation chromatography and concanavalin-A–Sepharose 4B affinity chromatography.

Ethanol precipitation was used to remove essentially all contaminating HIV-inhibitory sulfated polysaccharides present in the extract, as confirmed by negative toluidine blue O assay of the supernatant [11]. Fortunately, this procedure also removed the majority of inactive protein from the *N. erecta* extracts while leaving active protein in the supernatant. SDS/PAGE analysis of the supernatant indicated that the majority of residual contaminating proteins were of low molecular mass (<5 kDa; Fig. 1, lane 2). The subsequent Sephadex G-25 chromatography afforded a substantial purification of the active protein, which eluted in the void volume, followed by the 280 nm-absorbing contaminants later.

At this stage, the active material appeared to consist of two proteins of very similar molecular masses, as evidenced by SDS/PAGE (Fig. 1, lane 4). Neither ion-exchange chromatography nor hydrophobic-interaction chromatography was successful in separating the two bands. The two proteins were eventually separated by ammonium sulfate precipitation and concanavalin-A–Sepharose affinity chromatography (Fig. 1, lane 6). This procedure yielded an active fraction containing only one of the two candidate protein bands, which was purified to homogeneity by G3000 gel-permeation chromatography; this HIV-inhibitory protein was named niphatevirin. SDS/PAGE analysis of purified niphatevirin confirmed a single prominent band of protein at approximately 24 kDa (Fig. 1, lane 7).

Characterization of niphatevirin. SDS/PAGE analysis of purified niphatevirin consistently indicated a molecular mass of about 24 kDa when compared to standard proteins. However, analytical gel-filtration chromatography on a G3000PW column suggested a native molecular mass of 18 kDa for niphatevirin. Subsequent MALDI-TOF mass spectroscopic analysis of niphatevirin showed a single sharp peak corresponding to a molecular mass of 19087 Da (Fig. 2), confirming the analytical gel-permeation data.

Due to the fact that glycosylated lectins have previously been isolated from sponges, samples of niphatevirin were analyzed by SDS/PAGE followed by PAS staining for glycoproteins. By this method, it was determined that both of the candidate protein bands following ammonium sulfate precipitation were glycoproteins, although both were stained only lightly when compared to the horseradish peroxidase standard (possessing 16% sugar residues). The ammonium sulfate precipitate was then chromatographed on a concanavalin-A–Sepharose column. The biologically active protein, niphatevirin, did not bind to this column, though it was stained by the PAS stain. This indicated that niphatevirin was a glycoprotein and, further, that it did not bear the specific α-D-glucosyl residues or α-D-mannosyl residues necessary for binding to concanavalin A.

The isoelectric point of niphatevirin was determined to be in the range 4.2–4.6 by native isoelectric focusing of the protein in an acrylamide-based mini tube-gel system. The protein did not focus sharply, resulting in a range of pI values.

N-terminal amino acid sequencing of niphatevirin resulted in the following 37 residue sequence: H_2N-Ala-Val-Pro-Xaa-Pro-Gly-Val-Asn-Ile-Ala-Pro-Ala-Ala-Pro-Gln-Tyr-Met-Leu-Ala-Leu-Ser-His-Pro-Ala-Gly-Tyr-Xaa-Lys-Asn-Asn-Pro-Ala-Arg-Xaa-Pro-Val-Thr-.

Amino acid analysis of the blotted protein (Table 1) showed that the amino acid composition was unremarkable. A search of the PROPSEARCH data base [14] for identification of protein

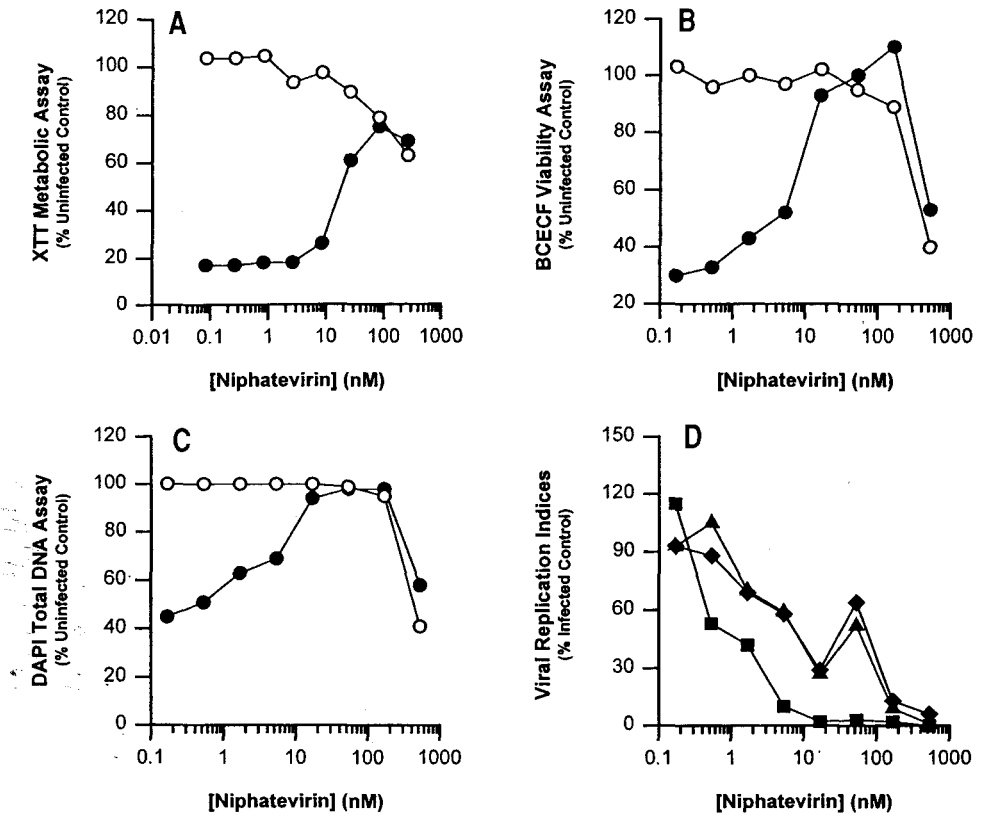

Fig. 3. Multiparameter analysis of the anti-HIV activity of niphatevirin. Effect of niphatevirin on uninfected (○) and HIV-infected (●) CEM-SS cells assessed after 6 days in culture using (A) cellular viability, (B) metabolic activity, (C) total DNA content, (D) supernatant reverse transcriptase activity (▲), p24 antigen production (◆) and synthesis of infectious virions (■). Values for infected cultures represent the mean of quadruplicate samples (SEM < 15%), while uninfected cell values represent the mean of duplicate samples. All points are graphically represented relative to the uninfected, non-drug-treated control values.

Table 1. Comparison of the amino acid composition of niphatevirin and Moraceae lectins. Amino acid analysis was performed as described in Materials and Methods.

Amino acid	Composition of		
	niphatevirin	jacalin	MPA
	mol/100 mol		
Ala	8.9	2.9	3.2
Asx	10.0	10.6	10.0
Glx	7.7	7.0	8.7
Phe	3.6	6.7	6.3
Gly	10.0	14.0	15.2
His	1.8	0.8	1.1
Ile	4.7	6.4	6.9
Lys	5.3	6.8	6.2
Leu	7.1	6.2	6.0
Met	1.2	0.9	0.5
Pro	5.3	4.5	3.4
Arg	3.6	1.3	3.1
Ser	7.7	9.3	7.9
Thr	8.3	7.0	8.4
Val	7.1	8.4	8.3
Tyr	4.7	7.1	4.6

similarity based on the amino acid composition was conducted using the molar amount of the amino acids in niphatevirin in comparison to those deposited in the SwissProt data base (> 30000 proteins). No significant match was found in the data base for the amino acid composition of niphatevirin.

Anti-HIV activity of niphatevirin. Purified niphatevirin was shown to be active in the primary anti-HIV assay (EC_{50} 0.24 μg · ml^{-1} [12 nM]). More extensive testing of the protein in a multiparameter assay (Fig. 3A and B) confirmed the cytoprotective activity in two independent cell-viability assay procedures (XTT, BCECF), DNA assay (Fig. 3C) and by supernatant levels of infectious virions, viral reverse transcriptase and p24 (Fig. 3D). In these assays, niphatevirin was cytotoxic to the host cells only at the highest tested concentration (500 nM).

To help ascertain the stage at which niphatevirin interfered with the viral life-cycle, delayed-addition studies were performed. Delay of addition of niphatevirin by 2 hours after HIV-1 infection of the CEM-SS host cells resulted in only 50% control anti-HIV activity; delay of addition by 8 hours resulted in essentially no anti-HIV activity (Fig. 4). These results suggested that niphatevirin acts early in the viral life cycle, possibly at the level of viral binding and/or entry into the cells.

The protein was then examined for its ability to bind to or inactivate virus particles and to bind to CEM-SS cells. Viral particles treated with niphatevirin, followed by dilution beyond effective antiviral concentrations of the protein, were fully infective, indicating that the protein was not directly virucidal. Similarly, when CEM-SS cells were treated with niphatevirin, then washed free of the protein, they retained full susceptibility to infection by the virus. Together with the time-course studies, these results indicated that niphatevirin acts reversibly to inhibit HIV infection of the host cells and that it must be continuously present in order to be maximally protective.

In additional experiments aimed at determining a possible mode of action for niphatevirin, the protein was tested for inhibition of cell-cell fusion and syncytia formation between chroni-

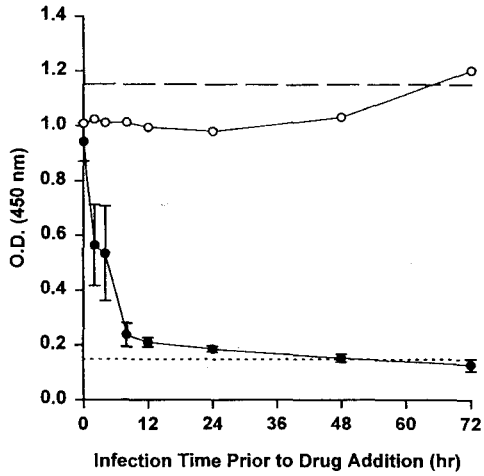

Fig. 4. Delayed-addition study. Effect of delayed addition of 0.23 μM concentrations of niphatevirin on HIV-1_{RF} infected (●) and uninfected (○) CEM-SS cells analyzed after 6 days in culture using the XTT assay. Absorbances ± SD are displayed and represent the mean of quadruplicate samples for infected cultures and duplicate samples for uninfected controls.

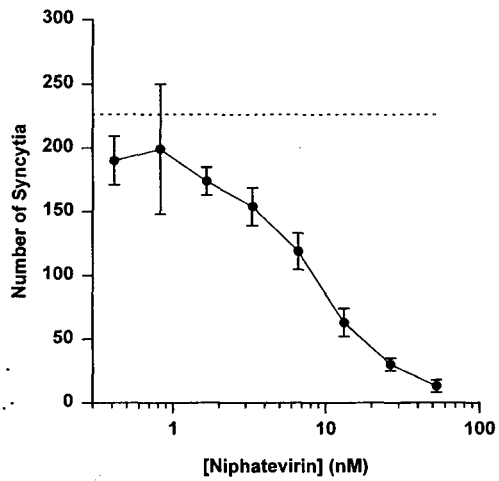

Fig. 5. Effect of niphatevirin on the inhibition of cell-cell fusion. Uninfected CEM-SS cells were co-cultured with CEM-SS cells chronically infected with HIV-1_{RF} in the presence of various concentrations of niphatevirin for 72 hours. After incubation, the number of syncytia (●) was determined microscopically and graphically represented as the mean of six samples ± SD.

cally infected and uninfected CEM-SS cells. Niphatevirin inhibited syncytium formation with an EC_{50} of 7 nM (Fig. 5), similar to the antiviral effective concentration found in the cell-viability assays.

Hemolysis and hemagglutination. In assays designed to determine the ability of niphatevirin either to agglutinate or to lyse erythrocytes, the protein was inactive, up to a concentration of 6.5 μM.

Binding studies. In an effort to determine potential molecular targets for niphatevirin, the protein was tested for its ability to bind either the viral protein gp120 or the cellular receptor CD4. Dot-blot assays, in which niphatevirin was bound to a poly(vinylidene difluoride) membrane, then washed with gp120, showed, as predicted by the virus treatment experiments, that the agent did not bind gp120. In similar assays in which soluble CD4 was first bound to the poly(vinylidene difluoride) membrane followed by washing with niphatevirin and then gp120, niphatevirin bound to CD4 in a manner that blocked the binding of gp120.

Finally, when niphatevirin was bound to the poly(vinylidene difluoride) membrane and washed with sCD4, visualization with anti-CD4 polyclonal antibodies showed clearly that niphatevirin bound sCD4. In contrast, when visualization of the niphatevirin-bound sCD4 was attempted using the gp120-binding site-specific monoclonal antibodies OKT4a and Q4120, neither monoclonal antibody was able to bind.

DISCUSSION

Previous studies on biologically active proteins from sponges have reported the isolation of either toxic polypeptides or agglutinating lectins (for general references, see [15, 16]). Lytic proteins of a size similar to that of niphatevirin have been isolated from the sponges *Tethya lycinurium* [17], *Iotrochota birotulata* [18] and *Suberites domuncula* [19]. In all of these studies, the purified proteins reportedly had hemolytic activity, which presumably contributed to their *in vivo* toxicity. Agglutinating proteins of a size similar to that of niphatevirin have been isolated from *Aaptos papillata* [20], *Axinella polypoides* [21, 22] and partially purified from *Haliclona* sp. and *Cinachyra tenuifolia* [5]. As with the toxic sponge proteins, isolation of the agglutinating sponge proteins was guided by assay of their interactions with red blood cells. In contrast, the novel protein niphatevirin, from *Niphates erecta*, neither agglutinates nor lyses erythrocytes, but instead was isolated based on its ability to protect cultured lymphocytes from the cytopathic effects of HIV-1.

N-terminal amino acid sequencing of niphatevirin resulted in a 37-amino-acid sequence that contained potential glycosylation sites but did not show significant (>30%) similarity to any known proteins following a GCG protein database search (Genetics Computer Group, Madison, USA). A PROP-SEARCH database analysis [14] of the molar amount of the amino acids in niphatevirin as compared to those deposited in the SwissProt database determined that no significant match was present in >30000 sequences searched. The amino acid composition of niphatevirin does, however, bear interesting similarities to the composition of the plant-derived lectins jacalin (*Artocarpus integrifolia* or *A. heterophyllus*) and *Maclura pommifera* agglutinin (MPA) [23] (Table 1). The lectin jacalin has previously been reported to inhibit the cytopathic effects of HIV-1 *in vitro* and this activity has been associated with its ability to bind to CD4 [24]. However, in contrast to our findings with niphatevirin, jacalin binding to CD4 was not sufficient to inhibit the subsequent binding of gp120 (data not shown); nor was jacalin able to inhibit syncytium formation between infected CEM cells and uninfected MT4 cells [24]. Furthermore, in side-by-side tests, jacalin and MPA, unlike niphatevirin, did not show >50% protection of the CEM-SS cells from the cytopathic effects of HIV-1 even at the highest tested dose of 10 μg/ml. Finally, both jacalin and MPA caused the dose-dependent proliferation of control lymphocytes, a cellular response not caused by niphatevirin (data not shown). Therefore, it is likely that niphatevirin binds to CD4 in a different manner or at a site different from jacalin.

Niphatevirin did not directly bind the viral protein gp120. This is in contrast to several plant-derived lectins (e.g., concanavalin A, *L. culinaris* agglutinin) which reportedly bind to gp120 through association with the various oligosaccharides attached to the glycoprotein [25, 26]. Niphatevirin did bind the lymphocyte membrane receptor CD4 in a manner that prevented CD4 association with gp120. This interaction is similar to that reported for

the lectin jacalin, except that jacalin binding to CD4 does not block the subsequent binding of gp120 [24]. The binding of niphatevirin to CD4 resembles more closely the binding of dextran sulfate to CD4 [27, 28], in that both bind to CD4, block the subsequent binding of gp120 and inhibit the infection and cytopathic effects of HIV-1 on $CD4^+$ host cells. Dextran sulfate differs from niphatevirin, however, in that it has also been reported to bind to gp120 at the major neutralizing V3 loop [29, 30].

To our knowledge, the isolation of the potent anti-HIV agent niphatevirin from protein extracts of the genus *Niphates* represents only the second report of a sponge protein with anti-HIV activity [31]. In contrast with the lectin from *Chondrilla nucula*, however, niphatevirin does not agglutinate human erythrocytes nor does it cause the proliferation of CEM cells.

We thank Kenneth Snader of NCI/NPB for the contract marine sample collections, Thomas G. McCloud for extractions, Donald G. Johnson for amino acid analysis and sequencing, and Perseptive Biosystems for loan of the BioCad workstation.

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