Isolation and characterization of adociavirin, a novel HIV-inhibitory protein from the sponge *Adocia* sp.

Barry R. O'Keefe[a], Tolga Erim[1,a], John A. Beutler[a], John H. Cardellina II[a], Robert J. Gulakowski[a], Benjamin L. Krepps[a], James B. McMahon[a], Raymond C. Sowder II[b], Donald G. Johnson[b], Robert W. Buckheit Jr.[c], Susan Halliday[c], Michael R. Boyd[a,*]

[a] *Laboratory of Drug Discovery Research and Development, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, Frederick Cancer Research and Development Center, Bldg 1052, Rm. 121, Frederick, MD 21702-1201, USA*
[b] *AIDS Vaccine Program, S.A.I.C.-Frederick, NCI-Frederick Cancer Research and Development Center, Frederick, MD 21702-1201, USA*
[c] *Southern Research Institute, Frederick Research Center, Frederick, MD 21701, USA*

Received 16 April 1998; revised version received 9 June 1998

Abstract Aqueous extracts of the New Zealand sponge *Adocia* sp. (Haplosclerida) displayed potent anticytopathic activity in CEM-SS cells infected with HIV-1. Protein fractions of the extract bound both to the viral coat protein gp120 and to the cellular receptor CD4, but not to other tested proteins. The purified active protein, named adociavirin, was characterized by isoelectric focusing, amino acid analysis, MALDI-TOF mass spectrometry and N-terminal sequencing. Adociavirin, a disulfide-linked homodimer with a native molecular weight of 37 kDa, was active against diverse strains and isolates of HIV-1, as well as HIV-2, with EC_{50} values ranging from 0.4 nM to >400 nM. The anti-HIV potency of adociavirin appears dependent on host cell type, with macrophage cultures being the most sensitive and peripheral blood lymphocytes the most resistant.

Key words: Human immunodeficiency virus; Macrophage; Anti-HIV protein; Sponge protein; gp120

1. Introduction

As part of the search for new drug candidates for the treatment of AIDS, the National Cancer Institute has carried out a screening program to test natural product extracts for their ability to protect T-lymphoblastoid cells from human immunodeficiency virus (HIV-1)-induced cell killing [1,2]. In addition to this cell-based assay system, secondary assays developed for specific binding to the viral coat protein gp120 and to the cellular receptor CD4 were instituted within the Laboratory of Drug Discovery Research and Development to prioritize further the protein fractions of marine extracts. Using these two prioritization methods, as well as previously described dereplication procedures for antiviral extracts [3,4], aqueous extracts of the New Zealand sponge *Adocia* sp. (Haplosclerida) were selected for bioassay-guided fractionation of their HIV-inhibitory constituents.

Earlier studies on organic extracts of the sponge genus *Adocia* have resulted in the identification of several classes of biologically active compounds, including diterpene isocyanides [5,6] and xestoquinone/halenaquinone derivatives [7]. Previous work in our laboratory has shown that the aqueous extracts of a variety of organisms are a productive source of novel proteins and peptides with anti-HIV activity [8,9]. Here we report the isolation and characterization of a novel protein from an aqueous extract of the sponge *Adocia* sp. with potent inhibitory activity against HIV-induced cytopathicity.

2. Materials and methods

2.1. Sponge material

A sample of the sponge *Adocia* sp. (670 g) was collected at a depth of 14 m on a rocky reef in Ocean Bay, 30 m offshore of Chatham Island, New Zealand. A taxonomic voucher specimen (sample number Q66D293) has been deposited at the Smithsonian Institution Sorting Center, Suitland, MD, USA.

2.2. Protein determinations

Protein content in various samples was determined by colorimetric assay using the Bio-Rad protein assay system with bovine γ-globulin as the standard according to the procedures of Bradford [10].

2.3. Sulfated polysaccharide determinations

To determine the presence or absence of sulfated polysaccharides in aqueous fractions of *Adocia* sp., samples were tested using the cationic dye toluidine blue O and analyzed for absorbance at 620 nm as described previously [3,11].

2.4. Bioassay for monitoring the purification of adociavirin

CEM-SS cells [12] were maintained in RPMI 1640 medium without phenol red and supplemented with 5% fetal bovine serum (BioWhittaker, Walkersville, MD), 2 mM L-glutamine (BioWhittaker) and 50 mg/ml 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 adociavirin in a volume of 100 μl of medium. Stock supernatants of

*Corresponding author. Fax: (1) (301) 846-6177.
E-mail: Boyd@dtpax2.ncifcrf.gov;

[1] National Institutes of Health, Student Research Training Program, 1995.

Abbreviations: BCECF, biscarboxyethyl-5(6)-carboxyfluorescein acetoxymethyl ester; BSA, bovine serum albumin; CV, column volume; DTT, dithiothreitol; EC_{50}, effective concentration of drug that results in protection of 50% of the host cells from the cytopathic effects of HIV; HIV, human immunodeficiency virus; IEF, isoelectric focusing; MALDI-TOF, matrix assisted laser desorption ionization-time of flight; MOI, multiplicity of infection; PAS, periodate acid Schiff's; PBS, phosphate buffered saline; PVDF, polyvinylidenedifluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TPBS, Tween phosphate buffered saline; XTT, 2,3-*bis*[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt

Part 51 in the series 'Anti-HIV Natural Products'; for part 50, see Fuller, R.W., Westergaard, C.K., Collins, J.W., Cardellina, J.H. II and Boyd, M.R. (1998) J. Nat. Prod. (submitted).

PII: S0014-5793(98)00736-4

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 and then assayed for cellular viability, metabolic activity, DNA content, p24 antigen production, supernatant reverse transcriptase activity and the synthesis of infectious virions as previously described [13].

2.5. Delayed addition of adociavirin 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 of medium. Diluted HIV-1_{RF} stock supernatants (50 μl) were added to appropriate wells to yield an MOI of 1.0. At various times after the addition of virus, a 100 μl aliquot of 2.5 μg/ml adociavirin was added to multiple wells. After a total of 6 days incubation, cellular viability was assessed using the XTT assay as previously described [13].

2.6. Effect of adociavirin on cell-cell fusion

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

2.7. Assays for anti-HIV activity in fresh human cells

Descriptions and sources of the T-tropic virus strains, primary isolates and cell lines have been previously published [14,15]. The HIV-1 dual-tropic isolate 89.6 [16] and the M-tropic isolates Ba-L [17] and Ada-M [18] were obtained from the National Institute of Allergy and Infectious Diseases (NIAID) AIDS Research and Reference Program. The M-tropic isolate SKLA was obtained from a pediatric patient of the University of Alabama (Birmingham) pediatric AIDS clinic by cocultivation of the patient's peripheral blood mononuclear cells with uninfected normal donor cells; the virus pool was expanded and frozen in liquid nitrogen after one passage.

For antiviral assays, peripheral blood lymphocytes (PBL) and macrophages (MAC) were isolated as necessary following Ficoll-Hypaque centrifugation as described elsewhere [19].

2.8. Stability tests

Crude protein samples from *Adocia* sp. were taken up in 50 mM sodium phosphate buffer (pH 7.5) augmented with 0.02% NaN_3 and one of the following components: 10 mM EDTA, 5 mM DTT, and either 1% or 10% glycerol. The final concentration of the protein in solution was 50 μg/ml. Samples treated with glycerol were separated into three aliquots and stored at −20°C, 4°C or room temperature 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 buffer titrated to the following pH values: 4.0, 5.0, 6.0, 6.5, 7.0, 7.5, 8.0 and 9.0, and were stored at 4°C for 24 h prior to assay.

2.9. Protein purification

2.9.1. Ethanol precipitation. The removal of sulfated polysaccharides from crude aqueous extracts was performed essentially as reported previously [3]. Briefly, an equal volume of absolute ethanol was added to *Adocia* sp. aqueous extract that had been dissolved in distilled deionized water at a concentration of 50 mg/ml. The ethanolic suspension was allowed to precipitate overnight at −20°C and then centrifuged at 3000 rpm for 60 min. The pellet was set aside and the supernatant was evaporated under vacuum to dryness prior to use in later purification steps.

2.9.2. Ammonium sulfate precipitation. Freeze-dried ethanolic supernatants from *Adocia* sp. were taken up in 25 mM sodium phosphate buffer (pH 7.5) at a concentration of 50 mg/ml and maintained on ice. Crystalline ammonium sulfate (Sigma, molecular biology grade) was added to the 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 set aside and the supernatant was then brought to 55% saturation with ammonium sulfate followed again by precipitation and centrifugation. Finally, the second pellets were saved and the supernatant was brought to 75% saturation with ammonium sulfate; all other steps were repeated as above.

2.9.3. Sephadex G-100 gel permeation. Freeze-dried ethanolic supernatants from *Adocia* sp. were taken up in 50 mM sodium phosphate buffer (pH 7.5) augmented with 0.4 M NaCl and 0.02% NaN_3. The samples were placed on a Sephadex G-100 column (2.5×50 cm, Sigma) and eluted with the same buffer at a flow rate of 2.5 ml/min. The eluant was monitored at 280 nm and fractions were taken each minute. Active fractions were concentrated by ammonium sulfate precipitation.

2.9.4. Anion exchange chromatography. Active fractions from the Sephadex G-100 column were first concentrated and desalted by ultrafiltration on a YM-10 membrane (10 kDa molecular weight limit, Amicon) into the starting buffer (25 mM sodium phosphate, pH 7.5). The desalted fractions were then injected onto a Poros HQ column (5×100 mm, Perseptive Biosystems) pre-equilibrated with the same starting buffer. The column was eluted at a flow rate of 2.0 ml/min using the following gradient: (1) 5 column volumes (CV, equal to 1.66 ml) of the starting buffer; (2) 6 CV of 0.1 M NaCl in the starting buffer; (3) 0.1–0.4 M NaCl over 20 CV; (4) 0.4 M NaCl for 4 CV; (5) 0.4–1.0 M NaCl over 4 CV; (6) 1.0 M NaCl for 5 CV. The eluate was monitored for both conductivity and absorbance (210 and 280 nm). Fractions were collected every 3 ml.

2.9.5. Hydrophobic interaction chromatography. Active fractions from the Poros HQ column were combined and concentrated on YM-10 membranes and then brought up to a concentration of 1.5 M ammonium sulfate. This protein solution was then injected onto a Poros PE column (5×100 mm, Perseptive Biosystems) pre-equilibrated with a starting buffer of 50 mM sodium phosphate, 1.5 M $(NH_4)_2SO_4$, pH 7.5. The column was then eluted at a flow rate of 5 ml/min over the following gradient: (1) 12 CV of the starting buffer; (2) 1.5–0 M $(NH_4)_2SO_4$ over 75 CV; (3) 0 M $(NH_4)_2SO_4$ for 15 CV. The eluate was monitored for both conductivity and absorbance (210 and 280 nm). Fractions were collected every 5 ml.

2.9.6. G3000PW analytical gel permeation. Protein samples (100 μl) were injected onto a G3000PW gel permeation column (21.5×300 mm, Toso Haas) using a BioCad workstation (Perseptive Biosystems) and eluted with 25 mM sodium phosphate buffer augmented with 0.4 M NaCl and 0.02% NaN_3 (pH 7.5). The column was eluted at a flow rate of 5 ml/min with fractions taken every 30 s. Native molecular weight 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 (absorbance 210 and 280 nm) and comparing the resulting calibration curve to the retention time of the active protein.

2.9.7. C-18 reverse phase chromatography. To prepare purified adociavirin samples for N-terminal sequencing, the protein was injected onto a Dynamax C-18 column (4.6×300 mm, 5 μm, 300 Å, Rainin) and eluted with a linear gradient of 0–80% acetonitrile in H_2O (augmented with 0.1% TFA) over 5 CV (∼25 ml) at a flow rate of 0.8 ml/min. The eluate was monitored for absorbance at 210 nm and fractions were collected by hand.

2.10. Electrophoresis

2.10.1. SDS-PAGE. SDS-PAGE was carried out using 18% polyacrylamide resolving gels, 4% polyacrylamide stacking gels and standard discontinuous buffer systems according to Laemmli [20] on a Bio-Rad Mini Protean II apparatus. The gels (50×80×1 mm) were subjected to electrophoresis at a constant current of 30 mA/gel at room temperature with chilled electrode buffer.

2.10.2. Isoelectric focusing. soelectric 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 of 3.5–9.5 using Bio-Rad 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).

2.10.3. PAS and silver staining. SDS-PAGE gels were stained for protein using either Coomassie brilliant blue or a modified Merril silver stain [21]. Gels were stained for glycoproteins by a periodic acid Schiff (PAS)-based stain (glycoprotein detection kit, Sigma).

2.11. gp120 and CD-4 binding studies

To determine the affinity of adociavirin for the HIV envelope pro-

tein gp120, dot-blot assays were conducted as follows: (1) 10 μl samples of either adociavirin or standard proteins (bovine globulin and aprotinin as negative controls, horseradish peroxidase as a positive control) were absorbed onto a PVDF membrane by capillary action. (2) The protein-bound membrane was incubated overnight in a 1% solution of BSA in PBS buffer. (3) The blocked membrane was washed with TPBS for 20 min (×3). (4) The washed membrane was incubated with a 1 μg/ml solution of gp120-horseradish peroxidase conjugate for 30 min. (5) The membrane was again washed with TPBS for 20 min (×3). (6) The conjugate-bound membrane was then incubated in a color development solution consisting of the peroxidase substrate 3-amino-9-ethylcarbazole (AEC) and 0.1% hydrogen peroxide in TPBS until spots became visible on the membrane.

To determine the affinity of adociavirin for the cellular receptor CD4, dot-blot assays were conducted as follows: (1) 10 μl samples of either adociavirin or standard proteins (bovine globulin and aprotinin as negative controls, horseradish peroxidase as a positive control) were absorbed onto a PVDF membranes by capillary action. (2) The protein-bound membranes were incubated overnight in a 1% solution of BSA in PBS buffer. (3) The blocked membranes were washed with TPBS for 20 min (×3). (4) The washed membranes were then incubated with solution of 1 μg/ml soluble CD4 (sCD4, Advanced Biotechnologies) in PBS for 45 min. (5) The membranes were again washed with TPBS for 20 min (×3). (6) The membranes were then incubated with either rabbit anti-CD4 polyclonal antibodies or a monoclonal antibody specific to the gp120 binding site of CD4 (OKT4a or Q4120). (7) The membranes were again washed with TPBS for 20 min (×3) and incubated with HRP-conjugated secondary antibodies, followed by a final washing in TPBS and incubation with the chromogenic substrate AEC.

2.12. Mass spectroscopy

Matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectroscopy was done using a Kratos Kompact Maldi II instrument (Shimadzu) using sinapinic acid as a matrix and trypsin and BSA as internal standards. For the analysis of reduced adociavirin, β-mercaptoethanol (β-MCE) (2%) was added to the sample prior to the addition of the matrix.

2.13. Amino acid analysis and sequencing

Adociavirin (500 μg) was taken up in 8 M guanidine hydrochloride and reduced by the addition of 10 μl β-MCE. The protein was allowed to react with the β-MCE for 1 h. Following reaction with β-MCE, a 10-fold molar excess of 4-vinylpyridine was added to the reaction vessel and the mixture was blanketed with N_2 and allowed to react. After 2 h the reaction mixture was evaporated under N_2 to remove volatile β-MCE from the mixture. The mixture was then injected onto a C-18 reverse phase column and eluted as described previously.

Pyridinylated adociavirin was digested by endoprotease Lys-C using a 50:1 ratio of adociavirin to Lys-C (Boehringer Mannheim) in Tris-HCl buffer (pH 8.5). The mixture was allowed to react for 18 h followed by C-18 reverse phase chromatography to purify the peptide fragments.

Amino acid analysis was performed using a Beckman Model 6300 amino acid analyzer according to manufacturer protocols. N-terminal amino acid sequencing was performed on an Applied Biosystems Model 477A sequencer according to manufacturer protocols.

3. Results and discussion

The bioassay-guided fractionation of aqueous extracts of the marine sponge *Adocia* sp. resulted in the isolation of a novel HIV-inhibitory protein named adociavirin. The protein was purified to homogeneity by the sequential use of ethanolic and ammonium sulfate precipitation followed by gel filtration, anion exchange and hydrophobic interaction chromatography. The protein, homogenous by SDS-PAGE, had a molecular weight of approximately 19 kDa (Fig. 1).

Analytical gel filtration studies of adociavirin showed that the protein was a homodimer in its native state with a molecular radius equivalent to 37 kDa. In subsequent SDS-PAGE and MALDI-TOF mass spectroscopy studies, the adociavirin monomers (18.4 kDa) were shown to be linked through disulfide bonds which were cleaved by β-MCE (Fig. 2A,B,C, respectively). This disulfide linkage between monomers was also confirmed by MALDI-TOF analysis of adociavirin following reduction and modification of Cys residues with 4-vinylpyridine (data not shown).

Adociavirin had an isoelectric point of 4.0 by native isoelectric focusing and was not stained in the PAS staining system for glycoproteins, thereby indicating that the protein was not glycosylated.

Following pyridinylation of the reduced cysteine residues with 4-vinylpyridine and C-18 reverse phase purification of adociavirin, the protein was characterized by both amino acid analysis (Table 1) and by N-terminal Edman degradation sequence analysis. Protease cleavage of the 4-vinylpyridine modified protein with Lys-C endoprotease, and isolation and sequencing of the resulting peptide fragments, was also attempted but yielded little additional sequence information. Adociavirin was a very hydrophobic protein, failing to elute from C-18 until a level of 75% acetonitrile was reached. Certain peptide fragments of the pyridinylated protein, however, could only be eluted from the column by the use of hot isopropanol. Despite this problem, enough peptide fragments were recovered and sequenced to confirm the 41 amino acid N-terminal sequence as follows: H_2N-Ser-Asn-Gly-Glu-Ile-Phe-Val-Asn-Ser-Ile-Gly-Asn-Leu-Glu-Lys-Ile-Phe-Ile-Ala-Ser-Met-Asp-Asp-Cys-Pro-Asn-Asp-Lys-Arg-Arg-Leu-Thr-Leu-Glu-Glu-Ala-Thr-Ala-Val-Gln-Asp-.

Database searches using this 41 amino acid sequence as a seed for the GCG search program determined that no significant homology (>20%) existed between adociavirin and any of the amino acid or translated DNA sequences in the database, thereby indicating that it is a novel protein.

Examination of the stability of partially purified adociavirin indicated that the HIV-inhibitory activity of the protein was stable for periods of one month when stored at 4°C, −20°C or −70°C but was reduced by approximately 50% when stored at room temperature for a month. In addition, the anti-HIV activity of adociavirin was stable in the pH range 6–8. However, the addition of either the chelating agent EDTA or the

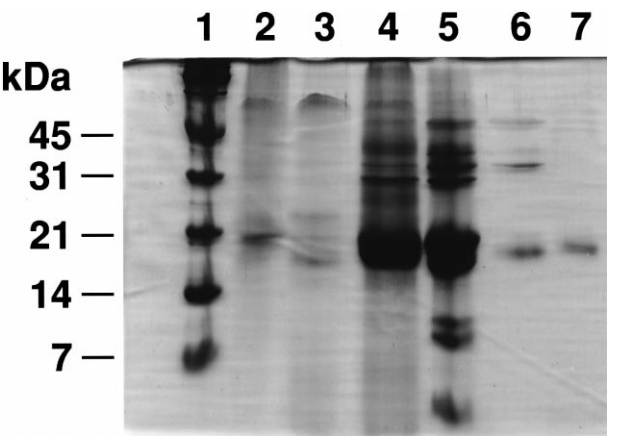

Fig. 1. SDS-PAGE analysis of various purification stage of adociavirin. A 15% acrylamide gel (50×80×1 mm) was electrophoresed at 25 mA and stained with Coomassie brilliant blue. Lane 1, molecular mass standards; lane 2, crude aqueous extract of *Adocia* sp.; lane 3, ethanolic supernatant fraction; lane 4, ammonium sulfate precipitate; lane 5, Sephadex G-100 column fraction; lane 6, anion exchange column fraction; lane 7, phenyl-ether column fraction.

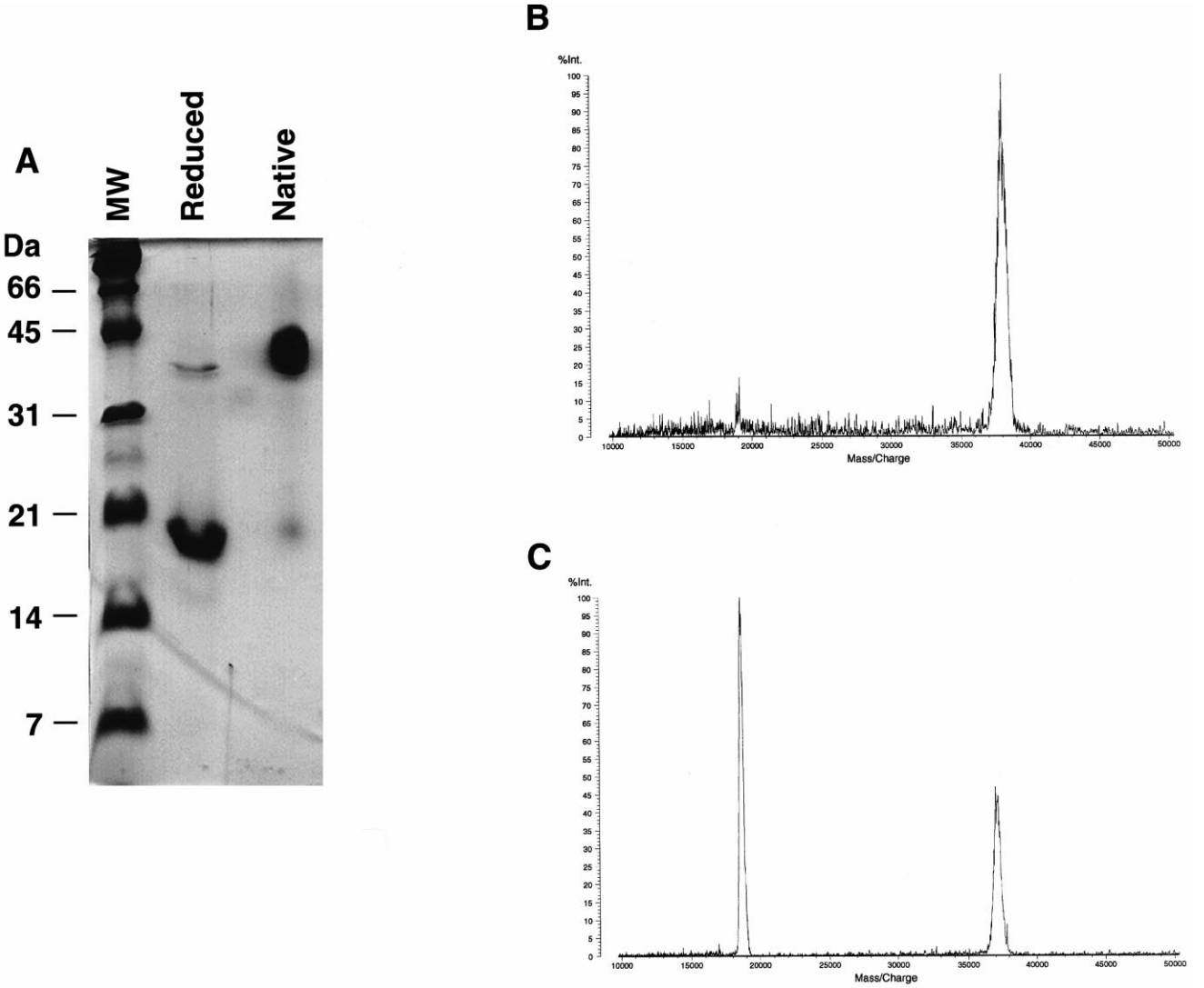

Fig. 2. Analysis of the dimeric nature of adociavirin. A: SDS-PAGE analysis of reduced and native adociavirin. Native adociavirin and adociavirin reduced by the addition of β-mercaptoethanol were electrophoresed on a 15% acrylamide gel (50×80×1 mm) at 25 mA and stained with Coomassie brilliant blue. B: MALDI-TOF mass spectroscopic analysis of native adociavirin using siapinic acid as a matrix and trypsin and BSA as external standards. C: MALDI-TOF mass spectroscopic analysis of adociavirin reduced by the addition of 2% β-mercaptoethanol, matrix and standards the same as above.

reducing agent dithiothreitol to the storage buffer (50 mM sodium phosphate, pH 7.5) decreased the activity of adociavirin by 75%.

Adociavirin bound both to the cellular receptor CD4 and the viral envelope glycoprotein gp120 but not to control proteins (horseradish peroxidase and BSA) in dot-blot assays (data provided to the reviewers). Studies on a similarly 'dual-binding' anti-HIV peptide named T-22 ([$Tyr^{5,12}$,Lys^{7}]-polyphemusin II), have also been reported, but this arginine-rich 2 kDa peptide has no homology to adociavirin [22]. The specific interactions between adociavirin and CD4 and gp120 will require further molecular studies.

Conventional anti-HIV-induced cytopathicity assays using CEM-SS lymphoblastoid cells [12] were used to monitor the progress of the purification of adociavirin. Adociavirin containing samples consistently displayed activity in the 10–100 nM range in this assay system. Furthermore, a multiparameter assay [13] demonstrated that adociavirin not only inhibited the cell killing associated with HIV infection but also decreased the production of infectious virions and the accumulation of viral reverse transcriptase activity in the cell culture supernatants (Fig. 3).

Additional studies of the time course of adociavirin activity against incipient HIV-1 infection showed a 50% reduction of activity if addition of the protein was delayed 2 h after virus addition. The decrease in cytoprotection was mirrored by an increase in supernatant accumulation of reverse transcriptase activity (Fig. 4). These studies provided further confirmation that the protein interacts very early in the infection process, most likely at the level of viral binding or fusion. In subsequent cell-cell fusion assays, wherein cells chronically infected with HIV-1 were allowed to fuse with uninfected cells, adociavirin inhibited cell-cell fusion (as determined by the number of syncytia) with an EC_{50} of 10 nM (Fig. 5). Taken together, these two studies strongly suggest that adociavirin is most likely a surface-active HIV-1 inhibitory protein which binds to either viral and/or cell surface molecules.

Adociavirin displayed potent activity against a broad range

Table 1
Amino acid analysis of adociavirin

Amino acid	Residues	Molar %
Asx	17	10.5
Thr	8	5.0
Ser	8	5.0
Glx	13	8.0
Gly	18	11.2
Ala	14	8.7
Cys[a]	2	1.2
Val	9	5.6
Met	2	1.2
Ile	11	6.8
Leu	11	6.8
Tyr	5	3.1
Phe	8	5.0
His	1	0.6
Lys	9	5.6
Arg	7	4.3
Pro	5	3.1

[a]Determined as pyridylethylcysteine.

of laboratory-adapted strains and primary isolates of HIV-1, as well as HIV-2 (Table 2). Adociavirin appeared most potent in protecting macrophage cell cultures from the cytopathic effects of the virus with EC_{50} values in the low nanomolar range (0.4–3.0 nM). The protein appeared less active in T-cell cultures, with EC_{50} values in the range of 30–60 nM and was least active in protecting PBLs from the virus with EC_{50}s two to three orders of magnitude weaker than in macrophage cultures (97 to >400 nM). Of particular interest were the simultaneous assays performed using the dual-tropic HIV-1

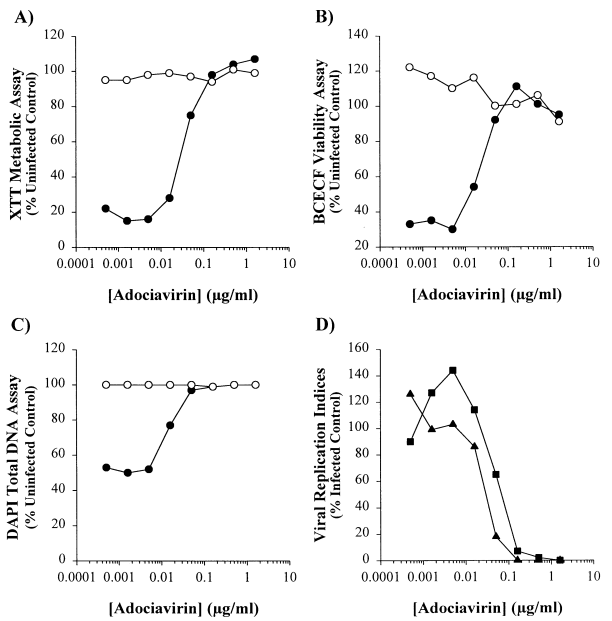

Fig. 3. Multiparameter analysis of the anti-HIV activity of adociavirin. Effect of adociavirin on uninfected (○) and HIV-infected (●) CEM-SS cells assessed after 6 days in culture using (A) cellular viability, (B) metabolic activity, (C) total DNA content, (D) supernatant reverse transcriptase activity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

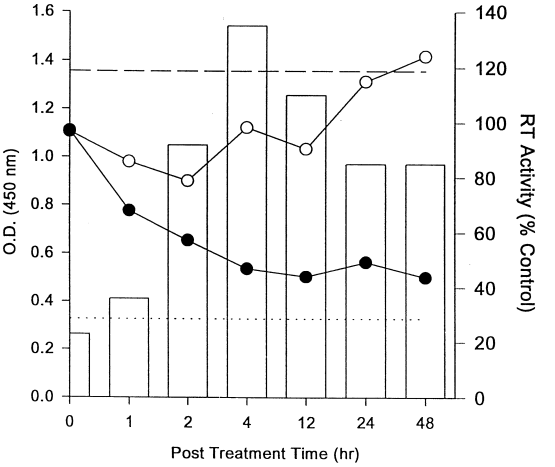

Fig. 4. Time course of the protective effects of adociavirin. Effect of delayed addition of 1.3 μg/ml adociavirin on HIV-1_{RF} infected (●) and uninfected (○) CEM-SS cells analyzed after 6 days in culture using the XTT assay. Reverse transcriptase activity (open bars) was also measured at each time point. The dotted line indicates untreated virus-infected cell controls and the dashed line indicates untreated uninfected cell controls.

strains 89.6 and ADA. In this assay, PBL and macrophage cells isolated from the same leukopack were infected with the dual-tropic viruses and simultaneously treated with various dilutions of adociavirin. The results confirmed earlier assays in which adociavirin exhibited significantly better efficacy in macrophage cultures; in this case approximately 250-fold better.

The apparent cell-line tropism displayed by adociavirin may be indicative of some form of preferential interaction with the surface of macrophage cells or with the viral surface constituents that preferentially interact with macrophage cells. Recent reports have indicated that certain seven-transmembrane domain, G-coupled cell surface receptors (i.e. CKR-5, CXCR4) in the chemokine receptor family are involved with the specific tropisms displayed by HIV-1 subtypes (for recent

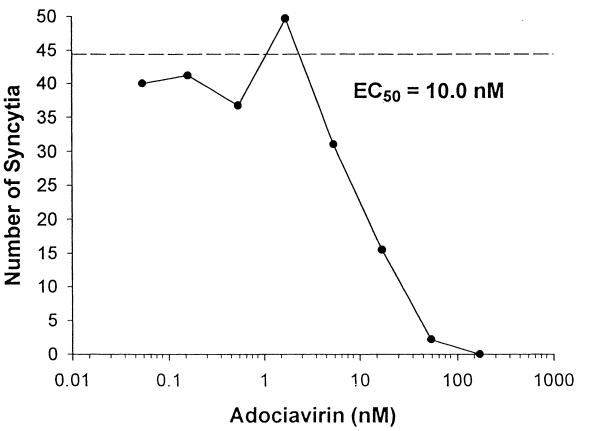

Fig. 5. Effect of adociavirin on the inhibition of cell-cell fusion. Uninfected CEM-SS cells were co-cultured with CEM-SS cells chronically infected with HIV-1_{RF} in the presence of various concentrations of adociavirin for 72 h. After incubation the number of syncytia (●) was determined microscopically. The results represent the mean of quadruplicate samples, graphically represented as the percent of the non-drug-treated controls.

Table 2
ANti-HIV activity of adociavirin

Cell type	Virus strain	EC_{50} (nM)	IC_{50} (nM)
Macrophage[a]	ADA	0.4	>405
Macrophage[a]	89.6	0.4	>405
Macrophage	Ba-L	3.0	319
Macrophage	ADA	2.0	319
PBMC[a]	ADA	97	>405
PBMC[a]	89.6	111	>405
PBMC	WEJO	278	>405
PBMC	TEKI	119	>405
PBMC	SLKA	122	>405
CEM-SS	HIV-2 ROD	100	270
CEM-SS	IIIB	59	>405
CEM-SS	RF	35	>405
MT2	IIIB	n.p.	205
MT2	RF	32	221
H9	IIIB	>405	>405

[a]Assays run simultaneously from the same leukopack.

reviews, see [23,24]). Whether or not adociavirin's activity is related to specific interactions with any of these receptors is a question that will be addressed in further studies.

Acknowledgements: We thank M.H.G. Munro and J.W. Blunt of the University of Canterbury, New Zealand for sponge collections, C.N. Battershill of the National Institute of Water and Atmospheric Research, New Zealand for both sponge collection and identification, T.G. McCloud for extractions and K. Snader of NCI/NPB for arranging the contract marine sample collections.

References

[1] Boyd, M.R. (1988) in: AIDS: Etiology, Diagnosis, Treatment, and Prevention (DeVita, V.T., Hellman, S. and Rosenberg, S.A., Eds.), J.B. Lippincott, Philadelphia, PA.
[2] Weislow, O.S., Kiser, R., Fine, D.L., Bader, J., Shoemaker, R.H. and Boyd, M.R. (1989) J. Natl. Cancer Inst. 81, 577–586.
[3] Beutler, J.A., McKee, T.C., Fuller, R.W., Tischler, M., Cardellina II, J.H., Snader, K.M., McCloud, T.G. and Boyd, M.R. (1993) Antiviral Chem. Chemother. 4, 167–172.
[4] Cardellina II, J.H., Munro, M.H.G., Fuller, R.W., Manfredi, K.P., McKee, T.C., Tischler, M., Bokesh, H.R., Gustafson, K.R., Beutler, J.A. and Boyd, M.R. (1993) J. Nat. Prod. 56, 1123–1129.
[5] Baker, J.T., Wells, R.J., Oberhänsli, W.E. and Hawes, G.B. (1976) J. Am. Chem. Soc. 98, 4010–4012.
[6] Kazlauskas, R., Murphy, P.T., Wells, R.J. and Blount, J.F. (1980) Tetrahedron Lett. 21, 315–318.
[7] Schmitz, F.J. and Bloor, S.J. (1988) J. Org. Chem. 53, 3922–3925.
[8] Boyd, M.R., Gustafson, K.R., McMahon, J.B., Shoemaker, R.H., O'Keefe, B.R., Mori, T., Gulakowski, R.J., Wu, L., Rivera, M.I., Laurencot, C.M., Currens, M.J., Cardellina II, J.H., Buckheit Jr., R.W., Nara, P.R., Pannell, L.K., Sowder II, R.C. and Henderson, L.E. (1997) Antimicrob. Agents Chemother. 41, 1521–1530.
[9] O'Keefe, B.R., Beutler, J.A., Cardellina II, J.H., Gulakowski, R.J., Krepps, B.L., McMahon, J.B., Sowder II, R.C., Henderson, L.E., Pannell, L.K., Pomponi, S.A. and Boyd, M.R. (1997) Eur. J. Biochem. 245, 47–53.
[10] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[11] Albano, R.M. and Mourão, P.A.S. (1983) Biochim. Biophys. Acta 760, 192–196.
[12] Nara, P.L. and Fischinger, P.J. (1988) Nature 332, 469–470.
[13] Gulakowski, R.J., McMahon, J.B., Staley, P.G., Moran, R.A. and Boyd, M.R. (1991) J. Virol. Methods 33, 87–100.
[14] Buckheit Jr., R.W., Fliakas-Boltz, V., Decker, W.D., Roberson, J.L., Pyle, C.A., White, E.L., Bowden, B.J., McMahon, J.B., Boyd, M.R., Bader, J.P., Nickell, D.G., Barth, H. and Antonucci, T.K. (1994) Antiviral Res. 25, 43–56.
[15] Buckheit Jr., R.W., Roberson, J.L., Lackman-Smith, C., Wyatt, J.R., Vickers, T.A. and Ecker, D.J. (1994) Hum. Retroviruses 10, 1497–1506.
[16] Collman, R., Balliet, J.W., Gregory, S.A., Friedman, H., Kolson, D.L., Nathanson, N. and Srinivasan, A. (1992) J. Virol. 66, 7517–7521.
[17] Gartner, S., Markovits, P., Markovitz, D.M., Kaplan, M.H., Gallor, R.C. and popovic, M. (1986) Science 233, 215–219.
[18] Gendelman, H.E., Orenstein, J.M., baca, L.M., Weiser, B., Burger, H., kalter, D.C. and Meltzer, M.S. (1989) AIDS 3, 475–495.
[19] Gartner, S. and Popovic, M. (1990) in: Techniques in HIV Research (Aldovini, A. and Walker, B., Eds.), pp. 59-63, Stockton Press, New York.
[20] Laemmli, U.K. (1970) Nature 227, 680–685.
[21] Merrill, C.R., Goldman, D., Sedman, S.A. and Ebert, M.H. (1981) Science 211, 1437–1438.
[22] Tamamura, H., Otaka, A., Murakami, T., Ishihara, T., Ibuka, T., Waki, M., Matsumoto, A., Yamamoto, N. and Fujii, N. (1996) Biochem. Biophys. Res. Commun. 219, 555–559.
[23] Bates, P. (1996) Cell 86, 1–3.
[24] Cohen, J. (1997) Science 275, 1261–1264.