Backbone Cyclic Peptide, Which Mimics the Nuclear Localization Signal of Human Immunodeficiency Virus Type 1 Matrix Protein, Inhibits Nuclear Import and Virus Production in Nondividing Cells†

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ABSTRACT: Here, we describe an application of the backbone cyclic (BC) proteinomimetic approach to the design and the synthesis of a BC peptide which functionally mimics the nuclear localization signal (NLS) region of the human immunodeficiency virus type 1 matrix protein (HIV-1 MA). On the basis of the NMR structure of HIV-1 MA, a library of BC peptides was designed and screened for the ability to inhibit nuclear import of NLS−BSA in digitonin-permeabilized HeLa and Colo-205 cultured cells. The screening yielded a lead compound (IC_{50} = 3 μM) which was used for the design of a second library. This library led to the discovery of a highly potent BC peptide, designated BCvir, with an IC_{50} value of 35 nM. This inhibitory potency is compared to a value of 12 μM exhibited by the linear parent HIV-1 MA NLS peptide. BCvir also reduced HIV-1 production by 75% in infected nondividing cultured human T-cells and was relatively resistant to tryptic digestion. These properties make BCvir a potential candidate for the development of a novel class of antiviral drugs which will be based on blocking nuclear import of viral genomes.

Proteinomimetics are small molecules that mimic the structure and/or the activity of a large parent protein. The availability of such small molecules can be useful for the detailed study of the biological function, molecular structure, and folding of proteins. Moreover, proteinomimetics are excellent candidates for becoming a new type of drugs, since they overcome some of the limitations that currently hamper the therapeutic use of proteins and polypeptides such as antigenicity, metabolic instability, and poor bioavailability. Many structural proteinomimetics have already been described; most of them were deprived of the biological function which characterizes the parent protein (*1*−*7*). Also, attempts to obtain small peptides which mimic catalytic sites of enzymes and preserve their enzymatic activity have so far failed (*8*). Only few examples of structural proteinomimetics which retain the biological activity and resemble the structure of the corresponding proteins have so far been reported, such as the zinc finger (*9*), metal-binding proteinomimetics (*10*), and RGD peptidomimetics (*11*). We have recently developed a general approach for the design and synthesis of small BC[1] peptides which mimic the structure and function of active regions in proteins. This approach is based on the concepts of backbone cyclization (*12*) and cycloscan (*13*) and was designated the "BC proteinomimetic" approach (*14*).

Backbone cyclization is a general method by which a conformational constraint is imposed on peptides through the connection of the N^{α} or C^{α} atoms in the peptide backbone either to each other or to side chains or to the carboxyl and amino termini (*12*). Backbone cyclization has been previously shown to convert peptides into selective and metabolically stable peptidomimetics having an enhanced biological activity as compared to that of the linear parent peptides (*15*, *16*). Cycloscan is a selection method based on conforma-

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[1] Abbreviations: BC, backbone cyclic; NLS, nuclear localization signal; HIV-1, human immunodeficiency virus type 1; MA, matrix protein; NMR, nuclear magnetic resonance; BSA, bovine serum albumin; IC_{50}, inhibitory concentration at 50%; BPTI, bovine pancreatic trypsin inhibitor; SPPS, solid phase peptide synthesis; MBHA, 4-methylbenzhydrylamine; DTT, dithiothreitol; SMPS, simultaneous multiple-peptide synthesis; Fmoc, 9-fluorenylmethyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; Z, benzyloxycarbonyl; Bzl, benzyl; pMeOBzl, *p*-methoxybenzyl; PyBroP, bromotris(pyrrollidino)phosphonium hexafluorophosphate; DIEA, ethyldiisopropylamine; NMP, *N*-methylpyrrollidinone; HOBt, *N*-hydroxybenzotriazole; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; TFA, trifluoroacetic acid; DCM, dichloromethane; TBTU, 2-(1-hydroxybenzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TOF-MS, time-of-flight mass spectrometry; TDW, triply distilled water; AAA, amino acid analysis; FCS, fetal calf serum; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethanesulfonyl flouride; Vpr, viral protein r.

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tionally constrained BC peptide libraries that allows a rapid detection of the most active BC peptide derived from a given sequence (*13*). The diversity of cycloscan, which includes modes of backbone cyclization, ring position, ring size, and ring chemistry, allows us to generate a large number of sequentially biased peptides that differ solely in their conformation in a gradual discrete manner. The principles of the BC proteinomimetic approach are based on the following steps: (i) elucidation of the active residues in the target protein, (ii) design and modeling of an ensemble of prototypic BC peptides that encompass the active residues and their conformation resembling that of the parent protein, (iii) cycloscan of each BC prototype until a lead(s) is discovered, (iv) structural analysis of the best lead, and (v) optimization through iteration. This approach was demonstrated using BPTI as a model protein. A backbone bicyclic nonapeptidomimetic of BPTI was identified and was found to inhibit trypsin with an IC_{50} value in the low micromolar range (*14*).

The aim of this study is to examine the feasibility of the BC proteinomimetics approach for the design and synthesis of BC peptides which mimic NLS regions in proteins.

The life cycle of eukaryotic cells depends on translocation of functional proteins into the cell nucleus, in which DNA replication and RNA biosynthesis occur. Also, nucleocytoplasmic transport of viral genomes is essential for the replication and assembly of many animal viruses. For example, the nuclear import of HIV-1, which is mediated by the viral MA protein, is crucial for the productive infection of nondividing cells (*17*).

NLS is a transport signal within proteins which mediates their nuclear uptake by a complex mechanism (*18*, *19*). It has a semiconsensus sequence mostly composed of 5−15 amino acid residues, a significant proportion of which are positively charged (lysine and arginine) (*20*). Linear peptides derived from NLS sequences function as an active NLS since their covalent conjugation to BSA causes nuclear import of the resulting conjugate (*21*, *22*). Such peptides also inhibit nuclear import as was demonstrated by inhibition of the HIV-1 MA protein and its preintegration complex (*23*). However, due to their structural flexibility and metabolic instability, the therapeutic use of linear NLS peptides as antiviral drugs is impractical.

Here, we present the application of the BC proteinomimetic approach for the discovery of a peptide which inhibits nuclear import of NLS−BSA in an in vitro assay system and was found to reduce HIV-1 production in nondividing infected cultured cells, apparently by blocking its MA-mediated nuclear uptake. On the basis of these results, we suggest that this peptide mimics the NLS region of the HIV-1 MA protein.

MATERIALS AND METHODS

Chemicals. Protected amino acids, MBHA resin, and coupling reagents were purchased from NOVA Biochem (Laufelfingen, Switzerland). Other chemicals were purchased from Sigma (St. Louis, MO) or Merck (Darmstadt, Germany). Solvents for peptide synthesis were purchased from Baker (Phillipsburg, NJ) and were of anhydroscan quality.

Transport buffer was comprised of 20 mM Hepes (pH 7.3), 110 mM potassium acetate, 5 mM sodium acetate, 0.5 mM EGTA, 2 mM DTT, 1 mg/mL leupeptin, 1 mg/mL pepstatin, 1 mg/mL aprotinin, and 0.1 mM PMSF.

Cultured Cells. (a) Colo-205 [human colon adenocarcinoma cells (ATCC CCL 222)] and Hut 78 cells (human T-cell line) were maintained in RPMI 1640 medium, supplemented with 10% FCS, 0.3 g/L L-glutamine, 100 units/mL penicillin, and 100 units/mL streptomycin (Beit Haemek, Israel).

(b) The monolayer culture of HeLa cells was grown in DMEM growth medium supplemented with 10% FCS as previously described (*24*).

Virus. The HIV-1_{IIIB} strain was kindly provided by M. Wainberg (Lady Davis Institute, Montreal, Canada).

BC Peptide Synthesis. BC peptides were prepared as described before (*15*, *16*). Briefly, peptides were synthesized on MBHA resin (loading 0.56 mequiv/g). Peptide libraries were synthesized by the SMPS "tea bags" methodology (*25*), with 200 mg resin portions in each bag in the first library and 300 mg resin portions in the second library. The resin was sealed in 4 × 5 cm polypropylene fabric bags which were placed in polypropylene boxes and shaken with a Labotron shaker (INFORS HT, Bottmingen, Germany). The synthesis of peptide 22 was performed in a manual SPPS vessel. The vessel was shaken with a MilliGen 504 shaker. All amino acids were Fmoc-protected on the N^{α} atoms, except for Gly and Val which were Boc-protected. The side chain protecting groups were Lys(Z), Ser(Bzl), and Cys(pMeOBzl). PyBroP (3 equiv) was used as a coupling agent, with a 3-fold excess of protected amino acid and a 7-fold excess of DIEA in each coupling. All protected amino acids were preactivated for 10 min prior to coupling. The coupling reactions were performed for 2 h. The coupling of the first amino acid in each peptide was repeated twice. Capping was performed with 0.5 M acetic anhydride, 0.125 M DIEA, and 0.015 M HOBT in DMF for 1 h. Fmoc deprotection was performed with 20% piperidine in DMF for 30 min and was repeated twice each time. Fmoc-N^{α}-[ω-(Boc-amino)-alkyl]Gly [alkyl = ethyl (n = 2), n-propyl (n = 3), n-butyl (n = 4), and n-hexyl (n = 6)] building units for the synthesis of the library were prepared in situ on the resin as described (*26*), except that a 10-fold excess of bromoacetyl bromide and a 10-fold excess DIEA were used instead of bromoacetic acid and the reaction took place for 1 h. Nucleophilic substitution with mono Boc-alkylenediamine in the same step was performed for 24 h with the reagent at 1.5 M in DMSO. The coupling of Lys after the building unit was repeated three times. Boc deprotection was performed with 55% TFA in DCM for 2 min and then for an additional 30 min. Lactam cyclization was performed three times using TBTU (3 equiv) with 4 equiv of DIEA, after Boc deprotection. HF cleavage was performed in the Kel F system (Peptide Institute, Osaka, Japan). Thioanisole was used as a scavenger in the first library, and anisole was used for the same purpose in the cleavage of the other peptides. The reaction took place at −5 °C for 2 h. After HF evaporation, the cleaved peptides and the resin were treated with TFA, and the resin was filtered off and washed three times with 2 mL of TFA. The combined TFA washings were evaporated under nitrogen, and the crude peptide was precipitated from cold ethyl ether. The crude peptide was washed several times with cold ether, dried, dissolved in 30% acetic acid, and lyophilized. The crude peptides were analyzed by TOF-MS and had the

Table 1: Analytical Data for the Active BC Peptides[a,b]

peptide no.	AAA data	MW calcd	MW found
19.1	no AAA performed	941.2	941
21	Leu 1, Lys 3.9	1013.2	1012.98
22	Leu 1, Val 0.8, Lys 4	1009.2	1008.9
mix 4	no AAA performed	942, 956, 971	943, 955, 971
19	Gly 1, Lys 3.95	956	955
19.2	Gly 1, Leu 1.08, Lys 3.42	967	967.2
19.4	Gly 1, Lys 4.3	854	853.8

[a] For experimental details of peptide synthesis and peptide structure, see Materials and Methods and Figures 1 and 2. [b] There are no AAA data available for the building unit and for the cysteine.

expected molecular weights. The first library was analyzed as mixtures. All other peptides were characterized separately. The peptides were analyzed by HPLC (Merck Hitachi 655A instrument equipped with a L-6200A gradient pump and a UV−vis detector with the tunable wavelength set to 215 nm). The flow was fixed at 1 mL/min, and the gradient was 5 to 60% acetonitrile/TDW (containing 0.1 and 0.085% TFA, respectively) over the course of 35 min. The column was the RP-3 type from Shandon. Peptides 17−20, 19.1−19.4, 21, and 22 were also characterized by AAA. Peptides 19.1−19.4, 21, and 22 were purified by semipreparative HPLC. The separations were performed on a RP-8 250 × 10 mm inside diameter column (Merck) with a constant flow of 4.5 mL/min. The gradient was 5 to 60% acetonitrile/TDW (containing 0.1 and 0.085% TFA, respectively) over the course of 45 min. The purified peptides were also characterized by analytical HPLC, AAA, and TOF-MS. Characterization data for the active peptides are shown in Table 1.

Synthesis of Linear Peptides. Linear NLS peptides were synthesized on Rink amide resin (loading 0.5 mmol/g) using the model 433A Applied Biosystems peptide synthesizer with the FastMoc chemistry. The peptides were cleaved from the resin using TFA with 5% anisole as a scavenger, precipitated from cold ether, dissolved in 30% acetic acid, and lyophilized. Crude peptides were analyzed by reverse-phase HPLC (C3 column, gradient of 5 to 60% acetonitrile/TDW containing 0.1% TFA over the course of 35 min) and characterized by TOF-MS and amino acid analysis.

Digestion of the Peptides by Trypsin. A mixture containing 0.05 mL of trypsin solution (8% w/v in 0.001 M HCl), 0.5 mL of TEA buffer at pH 7.8 (*27*), and 0.2 mL of peptide 22 (BCvir, see the Results) solution in water (initial concentration of 1.25 mg/mL) was subjected to HPLC (C3 column, gradient of 5 to 60% acetonitrile/TDW containing 0.1% TFA over the course of 35 min) immediately after addition of the peptide ($t = 0$). The mixture was incubated at room temperature, and samples were injected every 70 min. The same experiment was performed with peptide 19.4 (initial concentration of 3 mg/mL) and with the HIV MA NLS linear peptide (initial concentration of 20 mg/mL). The peak area of the peptide at each time interval was compared to the peak area of the peptide when $t = 0$, as a measure of the relative peptide concentration.

Quantitative Analysis of Nuclear Import in an in Vitro System. Nuclear import was quantitatively determined essentially as described before (*28*), except that a suspension of Colo-205 cells was used instead of a suspension of HeLa cells and biotinylated BSA-SV40 T-antigen NLS (500 nM) was used as a transport substrate [SV40 NLS has been used throughout this work to promote nuclear import since it is considered the prototypic NLS (*22*)]. The immunomodule stripes were coated with rabbit anti-BSA serum instead of using the IgG fraction. The peptides were preincubated with the permeabilized cells for 30 min at room temperature, and then reticulocyte extract was added at 4 °C. Transport was initiated by the addition of the substrate (biotynilated BSA−NLS), and then the reaction mixture was kept at 30 °C for 30 min. All other experimental conditions were as described before (*28*). The results given are an average of triplicate ELISA determinations whose standard deviation never exceeded 20%.

Estimation of Nuclear Import by Fluorescence Microscopy Observations. HeLa cells were cultivated on 10 mm coverslips to subconfluent density and then permeabilized with digitonin as described before (*24*). Linear peptides bearing the NLS of the SV40 large T-antigen were covalently attached to fluorescently labeled (FITC-labeled) BSA molecules (*22*), and translocation of the resulting fluorescently labeled NLS−BSA molecules (FL−NLS−BSA) into nuclei of digitonin-permeabilized HeLa cells was followed by fluorescent microscopy observations, as previously described (*24*).

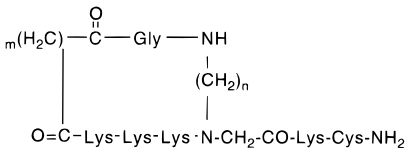
Determination of BCvir Cytotoxicity. Hut 78 cells (0.5×10^6/mL) were cultured in the absence or presence of 0.1, 1, 10, and 100 μg/mL BCvir. The number of living cells was determined by staining with tryphan blue 1, 2, 4, and 7 days after seeding.

Inhibition of HIV-1 Growth by BCvir. Hut 78 cells (0.5×10^6/mL) were incubated for 2 h at 37 °C in the absence or presence of 5 μg/mL aphidicolin (Sigma). Cells were then infected with HIV-1_{IIIB} (at a multiplicity of infection of 0.1) in the presence or absence of BCvir (100 μg/mL). Following a 1 h absorption, cells were incubated in 1.5 mL of the same media and samples of 120 μL were taken 3, 5, 7, and 9 days after infection. Virus propagation was determined according to the amounts of p24-CA antigen released into the media, using a Vironostika HIV-1 Antigen Microelisa system (Organon Teknika).

RESULTS

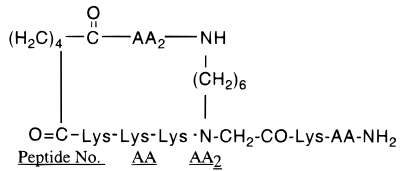
Design, Synthesis, and Screening of the First Backbone Cyclic NLS Mimetic Library. The design of the backbone cyclic peptide library was based on the structure of HIV-1 MA as determined by NMR (*29*, *30*). The sequence of the NLS region in this protein is KKQYK [or KKKYK in other HIV-1 strains (*29*)], and it is located within the outer strand of a β-sheet. Residues K26, K27, Q28, and K30 are exposed to the solvent, while the side chain of Y29 faces the interior of the protein, forming a hydrogen bond with T97. Replacement of Y29 by a Phe residue significantly reduced nuclear uptake of the HIV-1 MA (*17*). Since Y29 is not exposed to the surface of the protein, we assumed that its role is to stabilize the bioactive conformation of the NLS region. Conceivably, this conformation renders the basic residues accessible for binding to the putative NLS receptor (*31*). This assumption led us to design the first peptide library in such a way that the BC building unit would replace Y29. The role of the building unit is to mimic that of Y29, namely, to impose a conformational constraint (by cyclization) on the NLS. A ring size cycloscan (*13*) was also performed in the first library to determine the optimal ring size required for an inhibitory effect.

(a)



peptide No.	atoms in ring	n	m
1	18	0	2
2	19	0	3
3	20	0	4
4	20	2	2
5	21	0	5
6	21	2	3
7	21	3	2
8	22	2	4
9	22	3	3
10	22	4	2
11	23	2	5
12	23	3	4
13	23	4	3
14	24	3	5
15	24	4	4
16	24	6	2
17	25	4	5
18	25	6	3
19	26	6	4
20	27	6	5

(b)



Peptide No.	AA	AA_2
19.1	Ser	Gly
19.2	Leu	Gly
19.3	Met	Gly
19.4	----	Gly
21*	Leu	Cys
22*	Leu	Val

*These two peptides were synthesized after screening of the second library, to improve its inhibitory activity

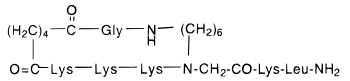
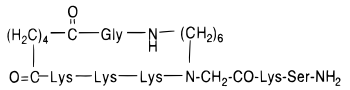
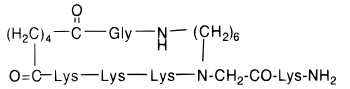
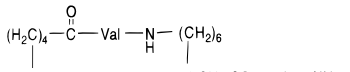
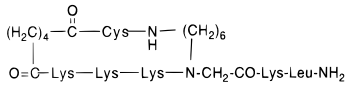
FIGURE 1: General structure of the backbone cyclic NLS mimetic peptides: (a) the first library and (b) the second library.

On the basis of the information described above, we designed an N-backbone to end (*12*) cyclic library of 20 peptides having the general structure shown in Figure 1a. The BC building unit used had a free amine at the end of the N-alkyl chain, and the lactam ring cyclization was formed by an amide bond to a dicarboxylic acid spacer attached to the N terminus of the peptide.

Initial screening of the first library was performed by estimating the ability of its peptides to competitively inhibit nuclear translocation of FL−NLS−BSA in the in vitro system (see Materials and Methods). The library was divided into four crude peptide mixtures which were screened separately. Of these mixtures, only one, designated mixture 4 (containing peptides 17−20, see Figure 1a), was found to possess an inhibitory activity. The activity of each individual crude peptide present in mixture 4 was then determined by the same method, from which peptide 19 (ring size of 26 atoms) was found to be the most potent inhibitor and served as a lead compound for a second library.

Design, Synthesis, and Screening of the Second Backbone Cyclic NLS Mimetic Library. To optimize the inhibitory activity of the NLS mimetic lead compound (peptide 19), we designed and synthesized a second library having the general structure shown in Figure 1b. In this library, the ring size and ring position were maintained as in peptide 19 and an amino acid scan was performed both in the ring and in the parent sequence. The cysteine residue, which is not an integral part of the NLS sequence, was replaced by various amino acids (see Figure 1b). The activity of the crude peptides (designated 19.1−19.4, see Figure 1b) was determined by fluorescent microscopy, out of which two peptides, 19.2 and 19.4, were found to be inhibitory. The IC_{50} values of the purified peptides from this library, in comparison to those of the corresponding linear parent NLS peptides, are shown in Table 2. Peptide 19.2 was found to be the most active backbone cyclic peptide (Table 2), having an IC_{50} value of 3 μM. To improve the inhibitory activity, this peptide was used as a lead compound for further iterations of the cycloscan procedure.

Table 2: Peptide Structures and Their Corresponding in Vitro IC_{50} Values[a]

Peptide structure	Peptide name	IC_{50} (μM)
Gly-Lys-Lys-Lys-Tyr-Lys-Leu-Lys-His-Cys-NH_2	HIV-MA NLS	12
Pro-Lys-Lys-Lys-Arg-Lys-Val-Cys-NH_2	SV40 large T-antigen NLS	0.001
	19.2	3
	19.1	>1000
	19.4	150
	22 (BCvir)	0.035
	21	no inhibition observed

[a] Nuclear import was determined by the quantitative method as described in Materials and Methods; IC_{50} values were determined by using peptides and transport substrates in molar ratios between 1000:1 and 1:1000, respectively.

The results in Table 2 show that lead peptide 19.2 is much less potent than the prototypic SV40 NLS, but is already more potent than the HIV MA NLS. We assumed that the relatively low activity of peptide 19.2 is due to the fact that the Gly residue in the ring is indifferent to the expected β-structure, resulting in a peptide having a less stable β-structure. To further stabilize the β-structure and thereby improve the inhibitory activity of peptide 19.2, the Gly residue was replaced by a Val residue which is known to be the best β-sheet former (*32*). The structure of the resulting new peptide, which was designated by us BCvir (peptide 22), is shown in Figure 2. As anticipated, the purified BCvir was found to be highly active and its IC_{50} value reached 35 nM, 2 orders of magnitude higher than that of the lead compound, 19.2. For comparison, the IC_{50} value of the linear parent peptide, namely, that of the HIV-1 MA NLS, was 12 μM. Cuurent experiments are in progress to further improve the inhibitory activity as well as the stability of BCvir.

The above results were confirmed by using a nonquantitative experimental system, namely, nuclear import of

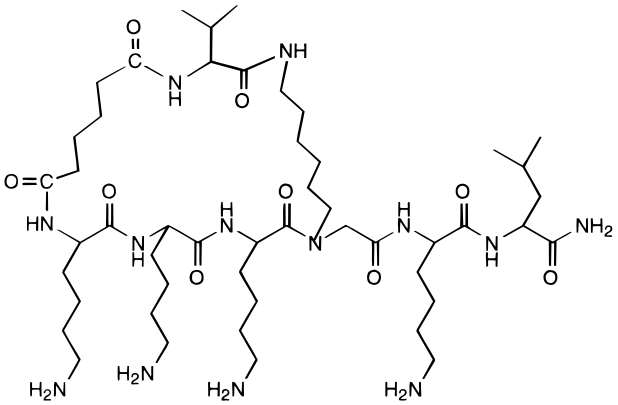


FIGURE 2: Structure of BCvir.

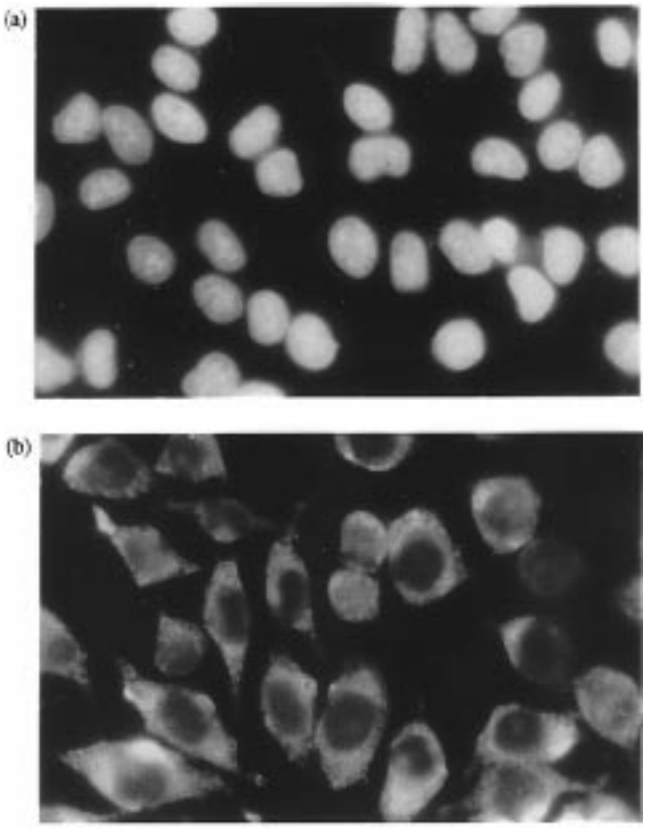


FIGURE 3: BCvir inhibits the translocation of FL−NLS−BSA into nuclei of permeabilized HeLa cells. Permeabilized HeLa cells were incubated with FL−NLS−BSA as described in Materials and Methods, in the absence (a) or in the presence (b) of BCvir. For inhibition of nuclear import, a mixture of BCvir and the transport substrate were added in a ratio of 2:1 (w/w).

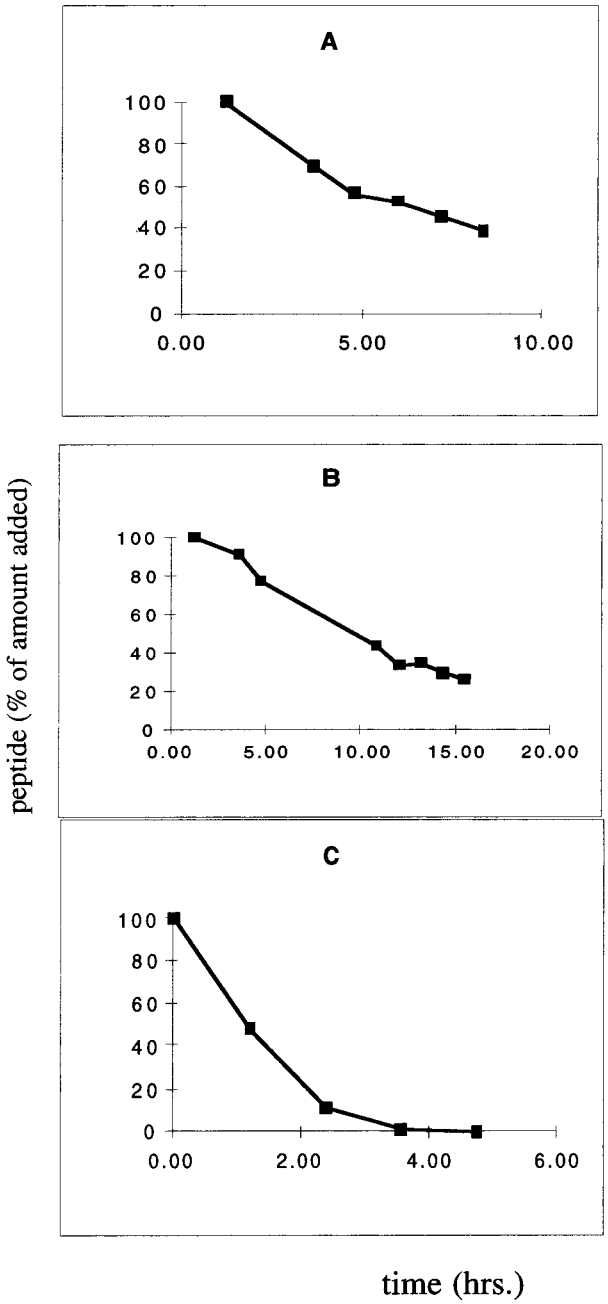


FIGURE 4: Determination of the susceptibility of BCvir, peptide 19.4, and HIV MA NLS to tryptic digestion. Treatment with trypsin was performed as described in Materials and Methods: (A) BCvir, (B) peptide 19.4, and (C) HIV MA NLS linear peptide. The half-life ($t_{1/2}$) values are 7, 9, and 1.3 h, respectively.

fluorescently labeled NLS−BSA and fluorescent microscopy observations (ref *22* and Materials and Methods). As can be seen in Figure 3, BCvir completely abolished nuclear import.

Stability of BCvir to Proteolytic Cleavage by Trypsin. BCvir contains four lysine residues, and therefore, it was important to study its susceptibility to proteolytic cleavage by trypsin (*27*). As mentioned above, BC peptides are expected to be relatively resistant to proteolytic digestion. The results given in Figure 4 show that the $t_{1/2}$ value of BCvir digestion by trypsin is 7 h, that of peptide 19.4 is 9 h, and that of the linear HIV MA NLS parent peptide is only about 1.3 h. Evidently, BCvir possesses a relatively high resistance to cleavage by trypsin, as reflected by its higher $t_{1/2}$ value. On the basis of the HPLC chromatogram, in which the area of the peak corresponding to BCvir decreased and the area of another peak with a shorter retention time increased, we assume that the exocyclic lysine−leucine bond in BCvir was slowly cleaved. Proteolysis within the ring would result in multiple product peaks. This assumption is supported by the result showing that peptide 19.4, which lacks an exocyclic peptide bond, has a higher $t_{1/2}$ value.

Inhibition of HIV-1 Production by BCvir. Since BCvir inhibited nuclear import of NLS−BSA in permeabilized culture cells, we tested its effect on HIV-1 production in virus-infected cells. Since the MA NLS is essential for HIV-1 replication only in nondividing cells (*17*, *21*), the effect of BCvir was studied in aphidicolin cell cycle-arrested

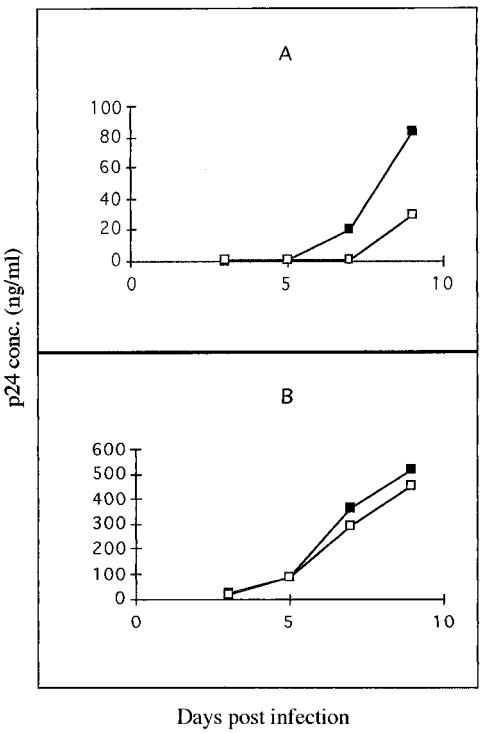


FIGURE 5: Inhibition of HIV-1 production by BCvir. BCvir was added to human Hut 78 cells infected by HIV-1 2 h prior to infection. (A) Inhibition of HIV-1 growth by BCvir in cell cycle-arrested human Hut 78 cells. The number of Hut 78 cells cultivated in the presence of BCvir and aphidicolin was not altered during the course of the experiment (dark boxes, without BCvir; and light boxes, with BCvir). (B) No inhibition by the same peptide in dividing cells (dark boxes, without BCvir; and light boxes, with BCvir). Please note that virus production in cells grown in the absence of aphidicolin is more efficient than in cells grown in its presence.

cells. Figure 5A shows that 9 days after infection BCvir (100 μg/mL) reduced virus production by 75% in nondividing Hut 78 cells, compared to that in untreated cells. BCvir did not affect virus production in aphidicolin-untreated dividing cells (Figure 5B).

It should be mentioned that BCvir did not affect the growth rate of uninfected cultured Hut 78 cells (data not shown).

DISCUSSION

This work demonstrates a successful application of our novel approach of BC proteinomimetics in obtaining a functional mimetic of an active region that bears a defined secondary structure within a viral protein. This was demonstrated by the discovery of BC peptides, whose amino acid sequence corresponds to the NLS of the HIV MA, which were able to inhibit nuclear import in in vitro assay systems as well as HIV-1 replication in infected cultured cells.

Linear NLS peptides, as opposed to the BC peptides, are flexible and exist in multiple conformations. It is not surprising, therefore, that most of the linear NLS peptides studied so far were found to inhibit nuclear import in various systems (for example, see ref *23*). Upon interaction with the cellular NLS receptor, these linear peptides, due to their flexibility, may adapt the appropriate bioactive conformation which allows their attachment to the receptor binding site, thus inhibiting nuclear uptake by competing with cellullar karyophilic proteins.

Despite the fact that all the peptides within each library bear the same parent sequence, only a few of the BC peptides described herein were found to be inhibitory. It is conceivable that due to their conformational constraint only some of the BC peptides are able to adapt the appropriate bioactive conformation. Conformational restriction renders the BC NLS peptides less flexible and probably more selective than the linear peptides. Our finding that the libraries described above also contained nonactive peptides further strengthens this assumption. In addition, the BC peptides are resistant to proteolysis, a fact that should potentiate their metabolic stability. Being metabolically stable makes such BC peptides attractive candidates for therapeutic applications. Evidently, such an approach may be essential in cases that the NLS region is composed of discontiguous amino acid residues, e.g. bipartite NLS sequences (*33*).

The lead compound discovered in the second library, designated 19.2, inhibits nuclear uptake of NLS−BSA with an IC_{50} value in the micromolar range. Other BC peptides bearing the same parent sequence were found to be inactive. Peptide 19.2 possesses an inhibitory activity higher than that of the linear parent peptide (HIV MA NLS) but lower than that of the peptide carrying the prototypic SV40 large T-antigen NLS. BCvir, in which Gly was replaced by Val, had an improved inhibitory activity. These results may indicate that we have succeeded in mimicking the β-sheet structure of the NLS region. The dramatic increase obtained in the inhibitory activity may be attributed to an additional stabilization of the β-sheet in BCvir due to the presence of the Val residue, which is the best β-sheet former (*32*), as mentioned above. NMR experiments are currently being conducted to prove this assumption.

Important conclusions regarding the role of the amino acid residues comprising the NLS region in HIV-1 MA can also be drawn from the observed IC_{50} values. Our hypothesis that Y29 has mainly a structural role, namely, to stabilize the conformation of the NLS, was supported by the result showing that BCvir, in which Y29 was replaced by a BC building unit, was found to be a potent inhibitor. This confirmed our view that amino acid residues that display structural roles can be replaced by BC building units.

Peptide 19.2 and BCvir contain Leu at the C terminus. These peptides were the most active peptides in the second library. They possess identical NLS sequences, the same as that of the HIV MA. Peptide 19.4, which also bears the same NLS sequence, but lacks the leucine residue, shows an activity 2 orders of magnitude lower than that of 19.2. On the other hand, peptide 19.1, in which a Ser residue replaces the Leu residue, exhibits a negligible inhibitory activity (Table 2). These results emphasize the important role of the Leu residue for the inhibitory activity of the above peptides.

Preliminary results have also demonstrated that, in addition to its inhibitory effect on nuclear import in vitro, BCvir also inhibits HIV-1 production in nondividing human T-cells but not in dividing cells. The decrease in virus production may be attributed to an effect on the general nuclear import machinery in vivo. However, it is noteworthy that under the conditions used, BCvir was not toxic to the cells.

A possible explanation for our observation that inhibition of virus production by BCvir was not complete, and plateaued at 75%, is the following: it was recently reported

that, in addition to the MA protein, the viral Vpr protein can support nuclear import of the viral preintegration complex. BCvir, which bears the MA NLS sequence, should not block the Vpr-mediated translocation since Vpr probably uses a different and independent cellular pathway (*34*). Alternatively, the incomplete inhibition of HIV-1 production can be attributed to a low concentration of BCvir in its site of activity due to insufficient permeability into the cells. Current attempts are being made in our laboratories to discover BC Vpr mimetics and to study their effect on nuclear import and HIV infection.

Blocking of HIV nuclear import as a therapeutic objective was already suggested, and the use of small nonpeptidic molecules for that purpose was demonstrated (*35*). Due to their inherent properties and their ability to block nuclear import, BC peptides from the type described in this work can be considered potential candidates for the development of a novel class of antiviral drugs in general, and anti-HIV drugs in particular.

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