Development of a Functional Backbone Cyclic Mimetic of the HIV-1 Tat Arginine-rich Motif

We have used the backbone cyclic proteinomimetics approach to develop peptides that functionally mimic the arginine-rich motif (ARM) of the HIV-1 Tat protein. This consensus sequence serves both as a nuclear localization signal (NLS) and as an RNA binding domain. Based on the NMR structure of Tat, we have designed and synthesized a backbone cyclic ARM mimetic peptide library. The peptides were screened for their ability to mediate nuclear import of the corresponding BSA conjugates in permeabilized cells. One peptide, designated “Tat11,” displayed active NLS properties. Nuclear import of Tat11-BSA was found to proceed by the same distinct pathway used by the Tat-NLS and not by the common importin β pathway, which is used by the SV40-NLS. Most of the Tat-derived backbone cyclic peptides display selective inhibitory activity as demonstrated by the inhibition of the nuclear import mediated by the Tat-NLS and not by the SV40-NLS. The Tat-ARM-derived peptides, including Tat-11, also inhibited binding of the HIV-1 Rev-ARM to its corresponding RNA element (Rev response element) with inhibition constants of 5 nM. Here we have shown for the first time (a) a functional mimic of a protein sequence, which activates a nuclear import receptor and (b) a mimic of a protein sequence with a dual functionality. Tat11 is a lead compound which can potentially inhibit the HIV-1 life cycle by a dual mechanism: inhibition of nuclear import and of RNA binding.

Proteinomimetics are small molecules that can mimic the structure and/or function of active sites within proteins (1). They are useful for detailed study of protein folding, structure, and function. The major potential application of proteinomimetics is, however, therapeutic; such molecules can be used to block protein-protein and/or protein-nucleic acid interactions, thus interfering with undesired biological processes (2–4). Because relatively small, proteinomimetics often solve acute problems associated with the use of proteins as drugs, such as antigenicity, low bioavailability, high cost, and rapid enzymatic degradation.

Two properties of the parent protein must be retained when designing proteinomimetics: (i) the bioactive conformation of the desired active site and (ii) a certain degree of conformational flexibility to allow induced fit. Linear peptides are not optimal candidates to mimic proteins, because they equilibrate between multiple conformations and thus adaptation of the bioactive conformation is at an entropic cost. Introduction of conformational constraints into the peptide is thus needed to generate a proteinomimetic. Being relatively small and conformationally constrained, cyclic peptides are excellent candidates to serve as proteinomimetics. Backbone cyclization of peptides is the method of cyclization developed and used in our lab. It results in peptides with improved selectivity, enhanced metabolic stability, and high bioavailability (5–9). To select the most active backbone cyclic (BC)1 peptide based on a given sequence, we have developed the “cycloscan” technology (10), which involves the structure-based design, synthesis, and screening of BC peptide libraries. All peptides in a BC library are based on the same primary sequence and differ only in parameters that influence their conformation. These parameters include ring size, position, and chemistry. The feasibility of these methodologies has been demonstrated with several naturally occurring peptides, including substance P (6, 7, 10, 11), somatostatin (9), and pheromone biosynthesis activating neuropeptide (5), resulting in the development of receptor-selective and metabolically stable BC peptides.

We have recently extended the use of backbone cyclization from peptides to proteins and coined this novel methodology “the backbone cyclic proteinomimetics approach.” We have demonstrated its utility with the parent proteins bovine pancreatic trypsin inhibitor (1, 11, 12) and HIV-1 matrix protein nuclear localization signal (NLS) (13).

Nuclear import of proteins is normally mediated by a specific signal, termed the nuclear localization signal (NLS). The prototypic NLS is a short sequence of mostly basic amino acids, as in the SV40 large T-antigen. Nuclear import in the common pathway is initiated by binding of the NLS to the cellular receptor importin α. The complex formed is then attached to the receptor importin β, which anchors it to the nuclear pore complex (NPC), and is transported into the nucleus via the NPC by an energy-dependent process (15). It has recently been
shown that several other nuclear import pathways also exist. For example, nuclear import of the HIV-1 Tat protein is mediated by the binding of the Tat-NLS directly to importin β at the same site through which it binds importin α (16).

HIV-1 Tat protein is a viral transcriptional activator that is actively imported into the nucleus. It binds the viral RNA in a specific region within the LTR promoter called TAR, thereby increasing the processivity of RNA polymerase II (reviewed in Refs. 17 and 18). The Tat-NLS is a semiconsensus arginine-rich motif (ARM) (reviewed in Refs. 19–21) and is found in several proteins, including the HIV-1 Rev protein (19–21). Interestingly, the ARM also functions as the specific TAR RNA binding domain. The three-dimensional structure of the HIV-1 Tat protein and the TAR RNA have been separately determined by NMR (22, 23), but a structure of the complex is not yet available.

As mentioned above, previous research in our group resulted in the development of matrix protein-NLS mimetics. The most potent mimetic, termed BCvir by us, inhibited nuclear import via the importin α pathway in vitro with an IC$_{50}$ value of 35 nM (13). However, BCvir was not able to mediate nuclear import of the appropriate conjugate. It inhibited the function of the corresponding receptor but was not able to activate it, showing an antagonistic activity (13, 24). BCvir is not an optimal NLS mimic because it does not display the properties of an active NLS (agonistic activity).

In the present study we describe the development of the functional mimetics of the HIV-1 Tat NLS. A BC library based on the Tat-NLS sequence was designed and synthesized. Several peptides from the library specifically inhibited in vitro nuclear import via the importin β pathway. One of the peptides, Tat11, is an active NLS, as demonstrated by its ability to mediate nuclear import of a BSA conjugate in an in vitro assay system. Our results show, for the first time, the use of the BC-P approach to develop a functional proteinomimetic capable of undergoing receptor-based nuclear import.

### EXPERIMENTAL PROCEDURES

**Chemicals**—Protected amino acids, methyl benzhydryl amine resin, and reagents were purchased from Sigma or Merck. Solvents for peptide synthesis were purchased from Baker, Phillipsburg, NJ.

**BC Peptide Synthesis**—BC peptides were prepared as described previously (13) with the following modifications: peptides were synthesized on methyl benzhydryl amine resin (loading 0.56 meq/g). Peptide libraries were synthesized by the simultaneous multiple peptide synthesis “tea bags” methodology (35), with 300-ng resin portions in each bag. The protected amino acid derivatives used were: Fmoc-Arg(Mts)-OH, Fmoc-Lys(Z)-OH and Fmoc-Cys(pMeOBzl)-OH. The coupling reactions were performed for 2 h following a 10-min preactivation in NMP with PyBroP (3 eq) as a coupling agent. 3 eq of protected amino acid and 7 eq of diisopropylphosphoramidite were used in each coupling. The coupling of Fmoc-Arg(Mts) was performed after the building unit was repeated three times. The coupling of all the amino acids following this arginine were repeated twice. Succinic and glutaric acids were coupled as their anhydrides (10 eq) with 10 eq diisopropylphosphoramidite and 1 eq dimethylaminopyridine in NMP for 3 h. Couplings of adipic and pimelic acid were performed with 10 eq acid and 10 eq DIC, which were preactivated in DMF for 30 min and then added to the resin with 1 eq dimethylaminopyridine and shaken for 3 h. HP cleavage and work up were performed as described (13). The crude peptides were analyzed by time of flight-mass spectroscopy and had the expected molecular weights. The peptides were analyzed by AAA and HPLC (Merck Hitachi, wavelength 215 nm, RP-4 column (Vydac)). The flow was fixed at 1 ml/min, and the gradient was 5–60% acetonitrile: triple distilled water (containing 0.1% trifluoroacetic acid) in 35 min. The gradient was 5–60% acetonitrile: triple distilled water (containing 0.1% trifluoroacetic acid) in 45 min. The purified peptides were also characterized by analytical HPLC, AAA, and time of flight-mass spectroscopy. All molecular masses found were exactly as the calculated values. The purified peptides were all >95% clean, as determined by analytical HPLC.

**Synthesis of Linear Peptides**—Linear Tat-ARM and SV40-NLS peptides were synthesized on Rink amide resin (loading 0.5 mmol/g) using the Applied Biosystems peptide synthesizer model 431A as described in Ref. 13. Ac-GRKKRRQRRRAHQN-NH$_2$ was used for the linear Tat-ARM. This sequence has been shown by Siomi et al. (26) to act as an NLS. The peptides were cleaved from the resin using a mixture of 95% trifluoroacetic acid, 2.5% anisole, and 2.5% disopropylamine and were further treated, purified by HPLC, and characterized as described for the BC peptides. Modified Rev34–50 peptides of the sequence succ-TQRARRRRRRWREWQRAAAAC-NH$_2$ have been utilized for all Rev-RRE experiments. Synthesis of Rev peptides was carried out using standard Fmoc/HBTU chemistry on a ABI Applied Biosystems 431A peptide synthesizer. The crude peptide was purified on a C-18 semipreparative HPLC column with an isocratic mixture of 14% acetonitrile (0.1% trifluoroacetic acid) in water (0.1% trifluoroacetic acid), lyophilized, and reacted with 100 equivalents of 5-Iodomethylfluorescein in 100 mM sodium phosphate (pH 8.0), 2 mM EDTA, and 30% (v/v) Me$_2$SO at room temperature in the dark for 2 h. The resulting fluorescently labeled peptide and the unlabeled Rev-ARM peptide were purified on a C-18 semipreparative HPLC column with a mixture of acetonitrile (0.1% trifluoroacetic acid) and water (0.1% trifluoroacetic acid); isocratic conditions were 19% acetonitrile for Rev-FI and 14% acetonitrile for the unlabeled Rev ARM peptide.

**Cultured Cells**—Colo-205 (human colon adenocarcinoma cells (ATCC CCL-225)) were maintained in RPMI 1640 medium, supplemented with 10% fetal calf serum, 0.3 g/l L-glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin (Beit hemeek, Israel).

### Quantitative Analysis of Nuclear Import in an in Vitro System

Nuclear import was quantitatively determined by the ELISA-based assay system using biotinylated BSA conjugates as transport substrates, essentially as described before (13, 27). Briefly, a suspension of Colo-205 cells was used, and biotinylated BSA-SV40 T-antigen NLS, biotinylated BSA-Tat NLS, and biotinylated BSA-ARM mimetic BC peptides conjugates were used as transport substrates. The cells were permeabilized with digitonin, and then the reticulocyte extract was added at 4 °C (when needed); the transport was initiated by the addition of the substrate (biotinylated-BSA-NLS), and then the reaction mixture was transferred to 30 °C for 30 min. All other experimental conditions were as described before (13, 27). The results given are an average of triplicate ELISA determinations whose standard deviation never exceeded ±20%.

### Fluorescence Anisotropy Measurements

A Rev-RRE complex was formed by mixing 10 nM Rev-FI with 8.5 nM of the prefolded 67-nucleotide RRE (heated to 90 °C then cooled slowly to room temperature). All displacement experiments were done at 20 °C in a buffer containing 30 mM HEPES (pH 7.5), KCl (100 mM), sodium phosphate (10 mM), NH$_4$OAc (10 mM), guanidinium hydrochloride (10 mM), MgCl$_2$ (2 mM), NaCl (20 mM), EDTA (0.5 mM), and Nonidet P-40 (0.001%). Upon binding the RRE, only minor changes in the emission spectrum of fluorescein were seen (about 10% quenching of Rev-FI). We have taken the change in anisotropy as being directly proportional to the fraction of Rev-FI bound (25). At the starting point of each titration there is 5 nM of unbound Rev-FI, 5 nM Rev-FI-RRE complex, and 3.5 nM free Rev-FI. Following complex formation, an inhibitor [I] is titrated into the thermocontrolled cuvette, and a decrease in anisotropy is observed. At high concentrations of inhibitor, anisotropy saturates at 0.081 (the same value observed for the unbound Rev-FI peptide). Sufficient mixing time was always provided to allow for equilibrium to be reached. At the concentration of inhibitor, which disrupts half of the formed Rev-RRE complex, there must be 2.5 nM of the Rev-FI-RRE complex, 7.5 nM of free Rev-FI, 5.23 nM of the RRE-inhibitor complex, and 0.77 nM of free RRE (calculated from the $K_D$ of Rev-FI-RRE). From a simple, three component, competitive binding equilibrium, the following equation (Equation 1) can be derived.

$$
\frac{[\text{RRE}]}{[\text{RevFI} - \text{RRE}]} + [\text{RevFI}] + K_D = K_I [\text{RevFI} - \text{RRE}] [1]
$$

$$
K_I = K_D \frac{[\text{RevFI} - \text{RRE}] [1]}{[\text{RevFI}] [1 - \text{RRE}]}
$$

**EQUATION 1**
The $K_d$ of the Rev-RRE interaction is calculated by titration of the prefolded 67-nucleotide RRE fragment into a solution of 10 nM Rev-Fl. The following equation was used to calculate the $K_d$ for RRE-Rev-Fl binding:

$$A = A_0 + \Delta A ([RNA]_{total} + [Rev-Fl]_{total} + K_d) - ([RNA]_{total} + [Rev-Fl]_{total} + K_d)^2 - 4[RNA]_{total}[Rev-Fl]_{total}^{1/2}/2[Rev-Fl]_{total},$$

where $A$, observed anisotropy; $A_0$, anisotropy in the absence of RNA; and $\Delta A$, total change in anisotropy upon saturation of Rev-Fl. The $K_d$ obtained for Rev-Fl binding to the RRE was used to calculate the $K_i$ values for all inhibitors with known binding stoichiometry (28, 29). In the case of the cyclic ARM peptides, a 1:1 binding stoichiometry to RRE has been observed using gel electrophoresis (not shown).

**RESULTS**

Structure-based Design of the Tat ARM Mimetic Backbone Cyclic Peptide Library—The Tat ARM sequence, RKKRRQRRR, was used as a template for the design of the ARM mimetic library according to the BC-P approach (1). Based on the NMR structure of HIV-1 Tat (23), we have determined the best side chain to be derivatized into the backbone cyclization ring. The HIV-1 Tat lacks any defined secondary structure, and its ARM, which is located between residues 48–57, is exposed on the protein surface and bears a flexible disordered conformation (see Fig. 1). The six arginine residues (Arg49, Arg52, Arg53, Arg55, Arg56, and Arg57) face the solvent and are likely to interact with importin $\beta$, the nuclear import receptor, as well as with the RNA. Gln54, on the other hand, faces the interior of the protein and is unlikely to interact with other molecules. This residue forms several potential hydrogen bonds with other...
residues in the Tat protein, which can stabilize the ARM in its bioactive conformation(s). Whereas the arginine residues are all essential for TAR RNA binding, Gln\textsuperscript{54} is unnecessary and can be mutated to other residues without loss of the TAR binding ability (30). This allowed us to replace Gln\textsuperscript{54} by the backbone cyclization ring (see Fig. 1).

To maintain the original peptide sequence, we have covalently connected the backbone amide nitrogen in the Gln position to the N terminus of the ARM peptide. We have included a cysteine residue at the C terminus of all peptides, as an attachment point to BSA for the nuclear transport studies. To maximize the number of possible conformations that can potentially support NLS activity and/or RNA binding activity, two variable methylene linker chains have been incorporated into the backbone cyclization ring (see Fig. 1). The final ARM mimetic library contained 16 peptides (see Fig. 2) that were synthesized using the simultaneous multiple peptide synthesis methodology (25), as described under “Experimental Procedures.”

Screening of the ARM Mimetic Library for Nuclear Import Ability—The ability of the peptides to mediate nuclear import was tested in permeabilized cells using the ELISA-based assay system (13, 27). The peptides were conjugated to biotinylated BSA either as mixtures or separately. The amount of the conjugates in the nuclei of permeabilized colo cells was determined quantitatively (13, 27) (see “Experimental Procedures”). Out of the library, only peptide Tat11 (n = 4, m = 4 in Fig. 2) mediated nuclear import of its BSA conjugate.

The nuclear import of the Tat11-BSA conjugate was characterized according to several parameters that demonstrate a specific receptor-mediated nuclear uptake (Fig. 3). Nuclear import of Tat11-BSA did not require the addition of cytosolic factors, indicating an importin \alpha-independent pathway. Furthermore, the addition of cytosolic extract caused inhibition of its nuclear import. Nuclear uptake of Tat11-BSA was found to be ATP dependent and was inhibited by free Tat11 peptide (1 mg/ml). Such competitive inhibition indicates a receptor-mediated transport.

We have repeated the experiments in the absence of external cytosolic extract. We assumed that in these experiments the added importin \alpha does not compete with the NLS-BSA conjugate for the importin \beta binding site, relieving importin \beta for NLS binding. Nuclear import without cytosolic extract was not observed, again, under ATP depletion. It was also inhibited by the free Tat11 peptide and free Tat-NLS peptide, both of which interact with importin \beta (see Fig. 4). As can be seen, the inhibition of nuclear import by the addition of cytosolic extract was even more impressive.

Another characteristic of nuclear import is its partial inhibition by wheat germ agglutinin (WGA). WGA is known to interact with the NPC and block importin \alpha-mediated nuclear import, which is carried out via the pores. In our experiments, WGA did not inhibit importin \beta-mediated nuclear import (Fig. 3) and in some cases even increased it (Fig. 4). This may suggest that importin \alpha-independent nuclear import is carried out via a different site in the NPC than the WGA binding site.
FIG. 4. Characterization of nuclear transport of Tat11-BSA without the addition of cytosolic factors. Nuclear transport of Tat11-BSA (green) was determined under various experimental conditions without the addition of cytosolic factors, compared with nuclear uptake of Tat-NLS-BSA (red) under the same conditions. Nuclear uptake of the conjugates in permeabilized cells was determined using the ELISA-based assay system. For experimental details see Refs. 13 and 27 and “Experimental Procedures.”

We have compared the nuclear import characteristics of Tat11-BSA to the Tat-NLS-BSA, whose nuclear import is mediated by importin β (16) and to the SV40-NLS-BSA, whose nuclear import is mediated by importin α (15). As expected, nuclear import of SV40-NLS-BSA was dependent on the addition of cytosolic extract and was inhibited by the addition of WGA but was almost not inhibited by the free Tat11 and Tat-NLS peptides (Fig. 3). In contrast, nuclear import of Tat11-BSA was facilitated by the absence of cytosolic extract or by the addition of WGA and was inhibited by free Tat11 peptide, showing similar characteristics to those of Tat-NLS-BSA. This suggests that they both utilize the same nuclear import pathway, probably importin β. The nuclear import characteristics of the Tat-NLS-BSA and Tat11-BSA conjugates were similar also when the experiment was carried out without the addition of cytosolic extract (Fig. 4).

Screening of the ARM Mimetic Library for Inhibition of Nuclear Import in Vitro—The ability of the BC ARM-derived peptides to inhibit nuclear uptake of various transport substrates was determined using the ELISA-based assay system. The transport substrates used were Tat-NLS-BSA (which uses the importin β pathway) and SV40-NLS-BSA (which uses the classic importin α pathway). The inhibition of Tat-NLS-BSA import was determined with and without the addition of cytosolic extract, because its nuclear uptake does not involve importin α (see above). Most of the peptides inhibited nuclear import of Tat-NLS-BSA but did not inhibit nuclear import of SV40-NLS-BSA (Fig. 5). In almost all cases, inhibition of Tat-NLS-BSA nuclear uptake was more significant in the absence of cytosolic factors (compare the green and blue bars in Fig. 5).

Inhibition of Rev-RRE Interaction by the ARM Mimetic Peptides—As mentioned above, the ARM, in addition to its NLS properties, functions as the RNA binding domain of the Tat and Rev proteins. We have determined whether the BC ARM mimetic peptides also exhibit RNA binding. Similar to the Tat-ARM, the Rev-ARM is responsible for nuclear uptake and for the specific binding of the protein to its corresponding RNA element, the RRE. The HIV-1 Rev protein facilitates the nuclear export of unspliced and singly spliced viral RNA by binding to the RRE. (31). We have used the Rev-RRE experimental system to determine the RNA binding activity of the ARM mimetic peptides (32). The ability of each peptide to displace a fluorescein-labeled Rev-ARM peptide (Rev-Fl) from the RRE is measured by monitoring changes in fluorescence anisotropy. All peptides tested are potent inhibitors of Rev-RRE binding (Table I) and are about two orders of magnitude more active than neomycin B (a well studied in vivo and in vitro inhibitor of Rev-RRE complex formation) (33).

DISCUSSION

We describe the application of the BC-P approach for the development of a BC peptide that functionally mimics the Tat-NLS. This peptide, “Tat11,” is able to mediate nuclear import of its BSA conjugate, probably by activating the importin β receptor. In the past, the BC-P approach yielded peptides that were inhibitory, displaying only antagonistic properties (1, 13). Also, in the present work most of the BC Tat peptides displayed antagonistic properties, as demonstrated by their ability to inhibit receptor-mediated nuclear transport. However, only peptide Tat11 displayed agonistic properties as shown by its ability to mediate nuclear import. This is the first time that the BC-P approach has produced an agonistic peptide, which can activate a receptor. Because all BC Tat peptides have the same primary sequence, we assume that Tat11 is the only peptide that adopts a conformation capable of activating the importin β receptor, whereas the other antagonistic peptides might bind the receptor in conformations sufficient to inhibit but not to activate it.

Peptide Tat11 is the first example of an NLS-proteinomimetic. Its BSA conjugate shows all the features of specific nuclear import; it is ATP-dependent and is inhibited by the corresponding free peptide. Tat11-mediated nuclear import is different from that of SV40-NLS-BSA import (which takes place in the common importin α pathway). It shows, however, similar characteristics to nuclear import mediated by the Tat-NLS, which proceeds via the importin β pathway (16). Both are energy-dependent processes and are competitively inhibited by the free Tat-NLS and Tat11 peptides, and both do not require the addition of cytosolic factors. The effect of adding cytosolic extract can yield information regarding the nuclear uptake machinery utilized by the NLS tested. During cell permeabilization, the cytosol, which contains soluble proteins (including importin α), leaks out of the cell. To reconstitute the transport

An HIV-1 Tat-NLS Mimetic

Fig. 5. Screening of the ARM mimic library for inhibition of nuclear uptake in vitro. The peptides (1 mg/ml) were screened for their ability to inhibit nuclear import of various transport substrates: SV40-NLS-BSA (red), Tat-NLS-BSA with cytosol (green), and without cytosol (blue). Percentage inhibition was determined by the ELISA-based assay system as described under “Experimental Procedures.”

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Ki (nM)</th>
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<tbody>
<tr>
<td>Tat-1</td>
<td>20 ± 10</td>
</tr>
<tr>
<td>Tat-4</td>
<td>5 ± 4</td>
</tr>
<tr>
<td>Tat-7</td>
<td>5 ± 4</td>
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<tr>
<td>TAT11</td>
<td>5 ± 4</td>
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<tr>
<td>Tat-15</td>
<td>5 ± 4</td>
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<tr>
<td>Tat-16</td>
<td>5 ± 4</td>
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<tr>
<td>Rev ARM</td>
<td>1.4 ± 1</td>
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<td>Tat ARM</td>
<td>20 ± 10</td>
</tr>
<tr>
<td>Neomycin</td>
<td>1100 ± 250</td>
</tr>
<tr>
<td>Kanamycin B</td>
<td>15000 ± 200</td>
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</tbody>
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TABLE 1

BC ARM-mimetic peptides inhibit Rev ARM peptide-RRE interaction

Kᵢ values were determined using fluorescence anisotropy techniques as described under “Experimental Procedures.” Because the binding stoichiometry for neomycin and kanamycin is unknown, only their IC₅₀ values can be reported.

The rapid emergence of HIV variants that are resistant to protease and reverse-transcriptase inhibitors requires new strategies for antiretroviral therapy (35, 36). The Tat protein is a key regulatory protein of HIV-1, and thus inhibition of its function is of therapeutic potential. Inhibition of Tat activity has been extensively researched and several families of small molecules that inhibit Tat activity via inhibition of the Tat-TAR binding have been reported (37–41). Our results indeed show (Fig. 5) that most of the BC peptides are inhibitors of nuclear uptake of Tat-NLS-BSA (the importin β pathway) but not of SV40-NLS-BSA (the importin α pathway). Thus, we have achieved selectivity and found inhibitors that are optimized only for the distinct pathway used by the Tat protein. Such inhibitors may serve as lead compounds for the development of molecules, which will selectively block nuclear import of the Tat protein, and thus might serve as anti-HIV lead compounds.

In addition to NLS activity, Tat11 also has a high affinity for the RRE RNA (Kᵢ = 5 nM). This presents the intriguing possibility that small molecules that bind RNA can be actively concentrated within the nucleus. Many small proteins (including Tat and Rev) are well under the molecular weight cut-off for passive diffusion but still enter the nucleus using active transport. The pharmacological use of small molecules that take advantage of active transport machinery could become an effective method to “target” nuclear RNA. Thus, Tat11 is a promising anti-HIV lead compound that can inhibit two critical protein-protein and protein-RNA interactions crucial to the viral life cycle 1) inhibition of Tat nuclear uptake and 2) inhibition of Rev-RRE binding.

It is interesting to note that all cyclic peptides tested inhibited the Rev ARM-RRE interaction with Kᵢ values in the low nanomolar range (Table 1) and are more potent than the linear Tat-ARM and the known inhibitors neomycin and kanamycin. Although the peptides are derived from the Tat-ARM, they inhibited the interaction between the Rev-ARM and its corresponding RNA element. This is not surprising, because the Tat and Rev ARMs are homologous sequences. The observation that most of the peptides displayed a similar affinity to the RRE RNA might be explained by nonspecific electrostatic effects. However, the diminished activity of Tat1 (relative to the other Tat peptides) indicates that some conformational factors are important for RRE binding of the BC peptides. The selectivity of these peptides (relative to other RNA sequences) is currently being tested.

The ARM is a protein sequence with a dual functionality. It functions both as an NLS and as an RNA binding domain. We assume that the different functions within the same segment...
protein, similar to small linear peptides, arise from conformational flexibility. The BC-P approach, which is based on the discovery of conformationally constrained bioactive peptides, is an appropriate solution to achieve selectivity between different functions. This was demonstrated here for the HIV-1 Tat ARM. Only peptide Tat-11 is an active NLS, whereas all of the peptides bound to RNA. Moreover, the bioactive conformation of the Tat NLS is not known, and solving the NMR structure of the mimetic Tat11 can shed light on it.

Summary—In summary we have expanded the scope of the BC-P approach in the following ways: (a) We have developed a functional mimic of a protein sequence using this method. (b) We have succeeded to develop a selective mimic of a protein sequence with a dual function. 2) Tat11 is a lead compound, which could potentially inhibit HIV-1 life cycle by a dual mechanism: inhibition of nuclear import and of RNA binding. It has been demonstrated that the Tat-ARM is able to penetrate through the cell plasma membrane (42), and thus it is expected that the bioavailability of the ARM mimetic compounds will be high. The optimization of the ARM mimetic Tat11 as a lead compound for the development of anti-HIV compounds is underway.

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REFERENCES