

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

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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  $\alpha$  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 mimetic of a protein sequence, which activates a nuclear import receptor and (b) a mimetic 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.

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)<sup>1</sup> 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  $\alpha$ . The complex formed is then attached to the receptor importin  $\beta$ , 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

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). Being relatively small, proteinomimetics often solve acute problems associated with the use of proteins as drugs, such as

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<sup>1</sup> The abbreviations used are: BC, backbone cyclic; NLS, nuclear localization signal; NPC, nuclear pore complex; ARM, arginine-rich motif; BSA, bovine serum albumin; BC-P, backbone cyclic proteinomimetics; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; HIV-1, human immunodeficiency virus type 1; HPLC, high pressure liquid chromatography; ELISA, enzyme-linked immunosorbent assay; RRE, Rev response element; WGA, wheat germ agglutinin.



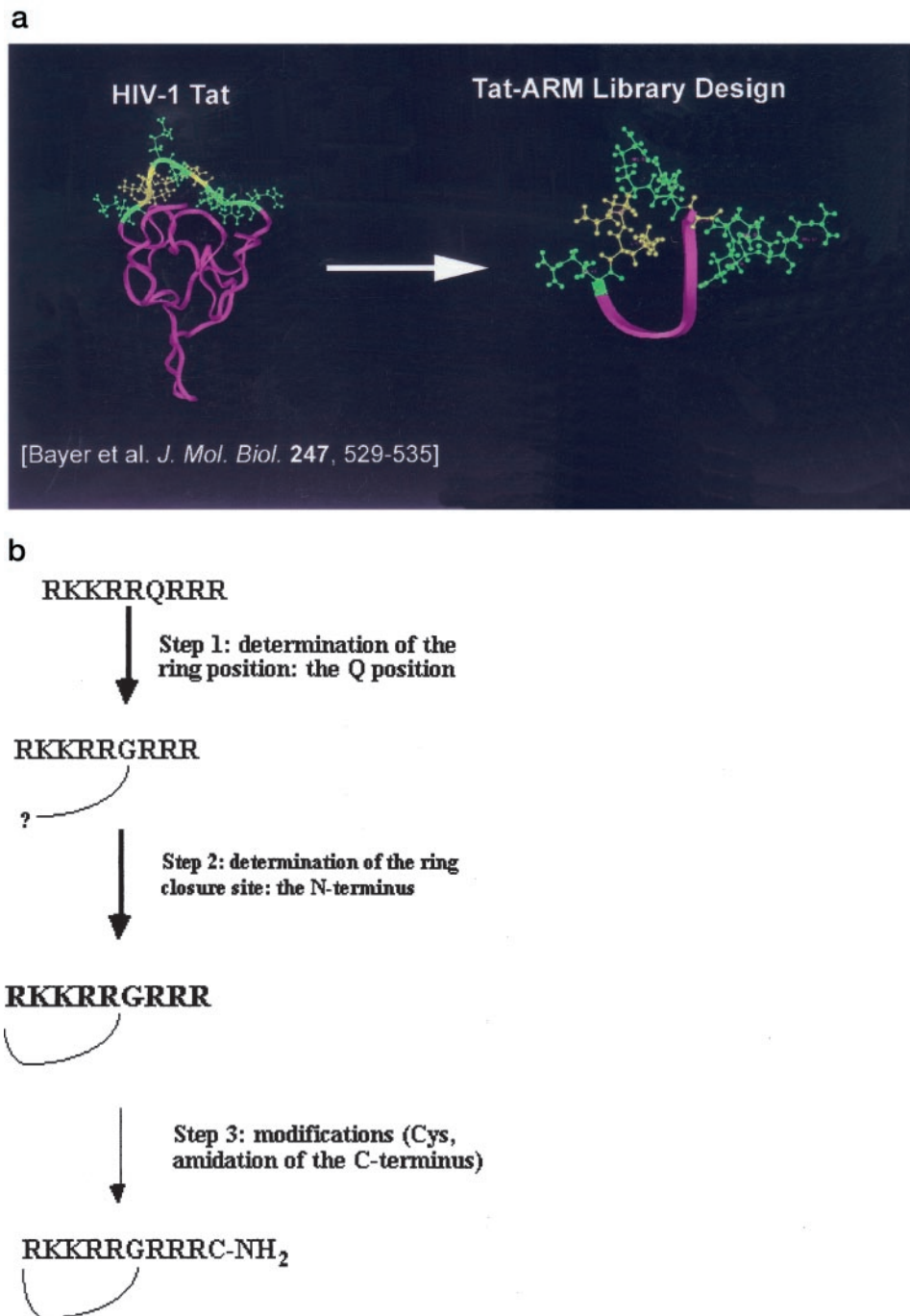


FIG. 1. **The design of the Tat-ARM mimetic library.** *a*, a general scheme of the structure-based BC ARM mimetic library design. The design was based on the NMR structure of HIV-1 Tat protein (23). *b*, the steps of the structure-based BC ARM mimetic library design are: 1) determination of the ring position, 2) determination of the ring closure site, and 3) various modifications.

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-F1. The following equation was used to calculate the  $K_d$  for RRE-Rev-F1 binding.  $A = A_0 + \Delta A \left( \frac{[RNA]_{total} + [Rev-F1]_{total} + K_d}{[RNA]_{total} + [Rev-F1]_{total} + K_d} - \frac{([RNA]_{total} + [Rev-F1]_{total} + K_d)^2 - 4[RNA]_{total}[Rev-F1]_{total}}{2[Rev-F1]_{total}} \right)^{1/2}$ , where  $A$ , observed anisotropy;  $A_0$ , anisotropy in the absence of RNA; and  $\Delta A$ , total change in anisotropy upon saturation of Rev-F1. The  $K_d$  obtained for Rev-F1 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, RKKRRQ-

RRR, 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 (Arg<sup>49</sup>, Arg<sup>52</sup>, Arg<sup>53</sup>, Arg<sup>55</sup>, Arg<sup>56</sup>, and Arg<sup>57</sup>) face the solvent and are likely to interact with importin  $\beta$ , the nuclear import receptor, as well as with the RNA. Gln<sup>54</sup>, 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<sup>54</sup> is unnecessary and can be mutated to other residues without loss of the TAR binding ability (30). This allowed us to replace Gln<sup>54</sup> 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.

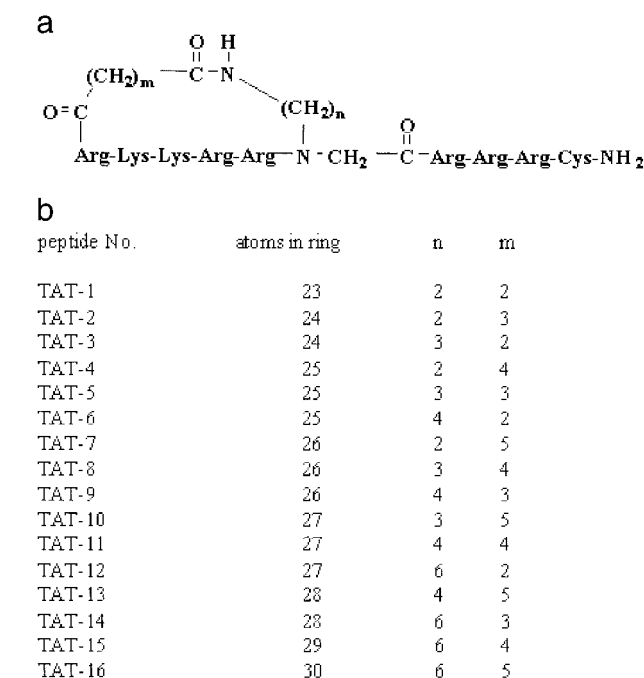


FIG. 2. Structure of the BC ARM mimetic library. *a*, a general formula of the library.  $n = 2, 3, 4, 6$ ;  $m = 2, 3, 4, 5$ . *b*, a detailed description of all the peptides in the library.  $n$  and  $m$  refer to *a*.

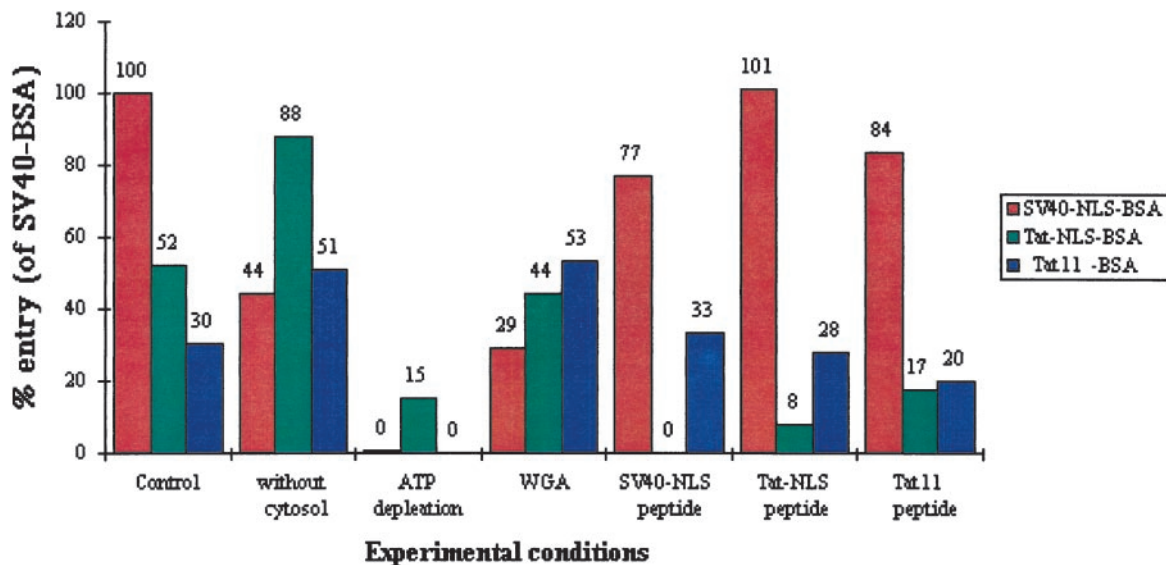
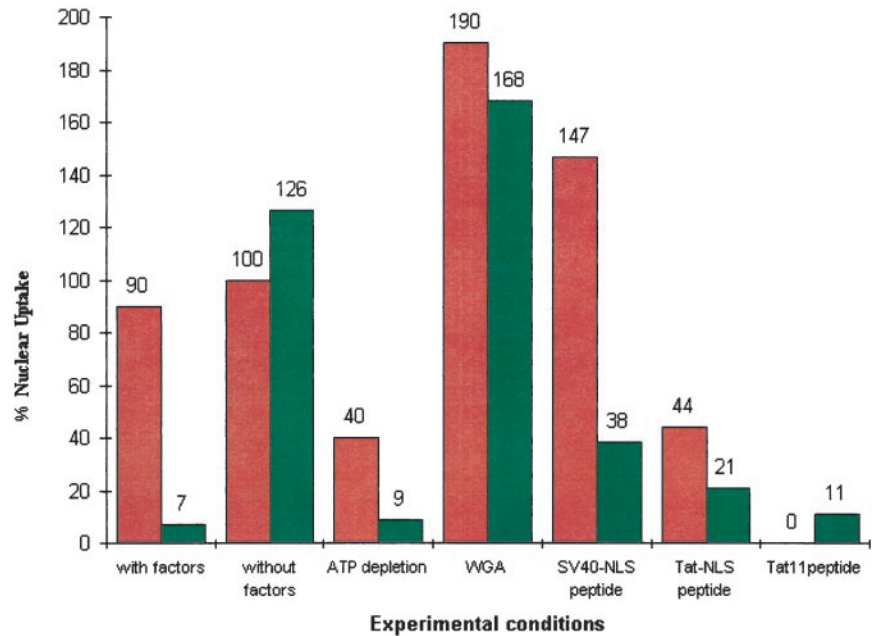


FIG. 3. Characterization of nuclear transport of Tat11-BSA. Nuclear transport of Tat11-BSA (blue) was determined under various experimental conditions in the presence of cytosolic factors, compared with nuclear uptake of Tat-NLS-BSA (green) and SV40-NLS-BSA (red) under the same conditions. Nuclear uptake of the conjugates in permeabilized cells was determined using the ELISA-based assay system. For experimental details see Refs. 13 and 38 and "Experimental Procedures."

**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  $\beta$  (16) and to the SV40-NLS-BSA, whose nuclear import is mediated by importin  $\alpha$  (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  $\beta$ . 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  $\beta$  pathway) and SV40-NLS-BSA (which uses the classic importin  $\alpha$  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  $\alpha$  (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-RRE 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-F1) 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  $\beta$  receptor. In the past, the BC-P approach yielded peptides that were inhibitory, displaying only antagonistic properties (1, 13).<sup>2</sup> 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  $\beta$  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  $\alpha$  pathway). It shows, however, similar characteristics to nuclear import mediated by the Tat-NLS, which proceeds via the importin  $\beta$  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  $\alpha$ ), leaks out of the cell. To reconstitute the transport

<sup>2</sup> E. Hariton-Gazal, D. Friedler, A. Friedler, N. Zakai, A. Loyter, and C. Gilon, unpublished results.

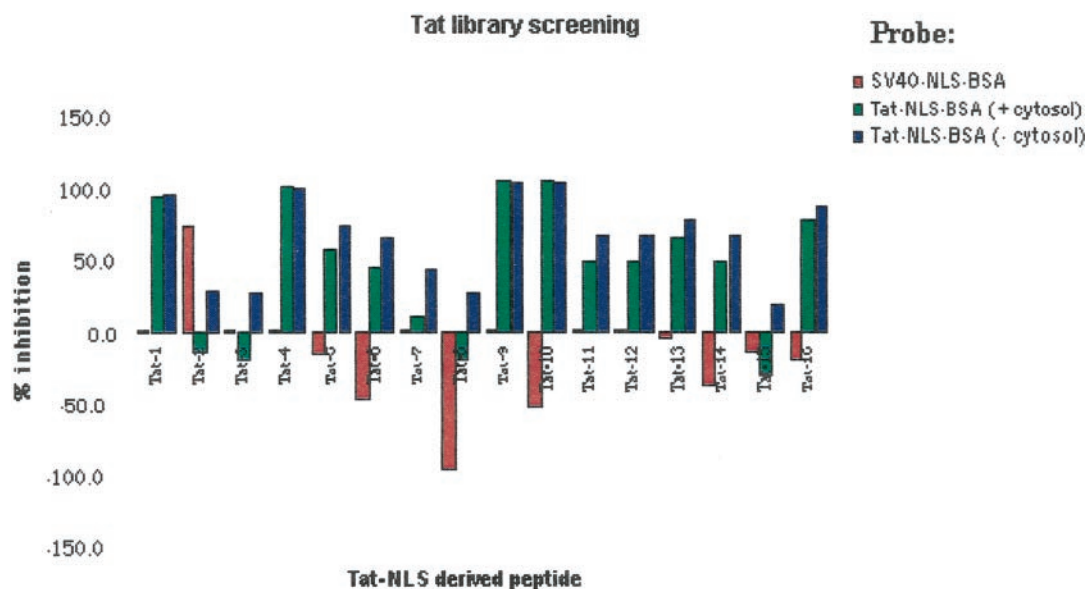


FIG. 5. **Screening of the ARM mimetic library for inhibition of nuclear uptake *in vitro*.** The peptides (1 mg/ml) were screened for their ability to inhibit nuclear import of various transport substrates: SV40-NLS-BSA (red), Tat-NLS-BSA with cytosol (green), and without cytosol (blue). Percentage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

TABLE I

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

$K_i$  values were determined using fluorescence anisotropy techniques as described under “Experimental Procedures.” Because the binding stoichiometry for neomycin and kanamycin is unknown, only their  $IC_{50}$  values can be reported.

Peptide	$K_i$ (nM)
Tat-1	20 ± 10
Tat-4	5 ± 4
Tat-7	5 ± 4
TAT11	5 ± 4
Tat-15	5 ± 4
Tat-16	5 ± 4
Rev ARM	1.4 ± 1
Tat ARM	20 ± 10
Neomycin	1100 ± 250
Kanamycin B	15000 ± 200

system for the determination of importin  $\alpha$ -mediated nuclear import (the “common” pathway), cytosolic extract should be added, otherwise nuclear uptake cannot take place because of lack of importin  $\alpha$ . In contrast, when nuclear import is mediated by a direct binding to importin  $\beta$  (as is the case with the ARM), the addition of cytosolic extract inhibits nuclear import, because the added importin  $\alpha$  competes for binding to importin  $\beta$ . Our results are consistent with those of Jans and co-workers (34) and also with the observations that the nuclear import of Tat proceeds by a direct interaction with importin  $\beta$  (16), assuming that a significant portion of importin  $\beta$  remained attached to the NPC following cell permeabilization. The observation that the addition of cytosolic factors (containing importin  $\alpha$ ) strongly inhibited nuclear import of Tat11-NLS-BSA is explained by assuming that most of the importin  $\beta$  was saturated by importin  $\alpha$  and was not available for binding the Tat11-BSA conjugate and mediate its nuclear uptake. The inhibition by cytosolic factors was much greater and could be observed more easily in the case of Tat11-BSA compared with the linear Tat-NLS-BSA.

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  $\beta$  pathway) but not of SV40-NLS-BSA (the importin  $\alpha$  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 ( $K_i = 5$  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  $K_i$  values in the low nanomolar range (Table I) 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 mimetic of a protein sequence using this method. (b) We have succeeded to develop a selective mimetic 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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