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A comparative study of backbone versus side chain peptide cyclization: Application for HIV-1 integrase inhibitors

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ABSTRACT

Peptide cyclization is an important tool for overcoming the limitations of linear peptides as drugs. Backbone cyclization (BC) has advantages over side chain (SC) cyclization because it combines N-alkylation for extra peptide stability. However, the appropriate building blocks for BC are not yet commercially available. This problem can be overcome by preparing SC cyclic peptide analogs of the most active BC peptide using commercially available building blocks. We have recently developed BC peptides that inhibit the HIV-1 integrase enzyme (IN) activity and HIV-1 replication in infected cells. Here we used this system as a model for systematically comparing the BC and SC cyclization modes using biophysical, biochemical and structural methods. The most potent SC cyclic peptide was active almost as the BC peptide and inhibited IN activity in vitro and blocked IN activity in cells even after 6 days. We conclude that both cyclization types have their respective advantages: The BC peptide is more active and stable, probably due to the N-alkylation, while SC cyclic peptides are easier to synthesize. Due to the high costs and efforts involved in preparing BC peptides, SC may be a more approachable method in many cases. We suggest that both methods are interchangeable.

1. Introduction

Linear peptides have several disadvantages as drug leads, the major of them being lack of stability in cells due to proteolytic digestion. Peptide cyclization is an excellent method for improving peptide stability.[1–4] Side chain to side chain (SC) cyclization utilizes functional groups such as amines, carboxylic acids or thiols, or the amino or carboxy termini of the peptide to form the cyclization through amide or disulfide bonds. Backbone cyclization (BC) combines cyclization with N-alkylation to enhance the stability of peptides.[4] Here we performed an extensive comparative study of BC versus SC cyclization on an anti HIV-1 lead linear peptide developed in our labs (see below).

The Human Immunodeficiency Virus type 1 (HIV-1) integrase (IN) enzyme is an important target for anti-HIV drugs. It catalyzes the integration of viral DNA into the host genome, which is a crucial step in the HIV-1 replication cycle.[5–10] The first FDA-approved IN inhibitor, Raltegravir (MK-0518),[11–17] is used for treating HIV-1 as part of combination antiretroviral therapy. We have previously described an IN-inhibitory peptide derived from residues 361–370 of the IN cellular partner protein LEDGF/p75. LEDGF 361–370 inhibited IN catalytic activity in vitro, shifted the oligomerization equilibrium of IN towards the tetramer and inhibited HIV-1 replication in cells[18] as well as in mice model.[19] LEDGF 361–370 also blocked LEDGF/p75-IN interaction in infected cells.[20] Another study showed that LEDGF 361–370 competes with the full length LEDGF/p75 on IN binding.[21] The stability of LEDGF 361–370 was improved using backbone cyclization. One of the backbone cyclic peptides, *c*(MZ 4-1), was a stable and potent IN inhibitor.[22] *c*(MZ 4-1) bound IN in the low micromolar range, penetrated cells and inhibited IN catalytic activity in vitro and HIV-1 replication in cells with the same potency as the linear parent LEDGF 361–370.

Abbreviations: AIDS, acquired immunodeficiency syndrome; HIV, human immunodeficiency virus; IN, integrase; MAGI, multinuclear activation of a galactosidase indicator; MOI, multiplicity of infection; LEDGF, lens epithelium-derived growth factor; TDW, triple distilled water; BC, backbone cyclization; SC, side chain to side chain; HBTU, (2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; MALDI-TOF, matrix assisted laser desorption ionization time of flight; MBHA, methylbenzhydrylamine; NMR, nuclear magnetic resonance; RMSD, root-mean-square deviation; PyClock, 6-chloro-benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; TFA, trifluoroacetic acid; TIPS, triisopropylsilane.

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c(MZ 4-1) showed significantly improved stability compared to LEDGF 361–370. The solution NMR structure of *c*(MZ 4-1) in association with IN revealed that its bioactive conformation resembles that of the corresponding residues in LEDGF/p75.[22]

BC is a useful method to enhance the stability of peptides since it combines both N-alkylation and cyclization.[4,23–31] However, its major drawbacks are that the building blocks are not commercially available and that the synthesis of BC peptides often results in low yields since coupling to the secondary amine of the non-natural amino acids is synthetically challenging. This problem can be overcome by designing side chain to side chain cyclic peptide analogs of the most active BC peptide. Here, we performed a comparative study of BC versus SC cyclization of LEDGF 361–370 and prepared SC cyclic analogs of *c*(MZ 4-1). The side chain cyclization was performed using commercially available building blocks and an automated peptide synthesizer. We compared the structure and activity of the SC cyclic analogs with the BC peptide and with the parent linear LEDGF 361–370. The SC cyclic peptide *c*(MZ 4K-1) was stable and active in cells almost as the BC *c*(MZ 4-1). We conclude that the BC peptide is more active and stable than the SC cyclic peptide since it is stabilized by the combination of cyclization and N-alkylation while SC cyclic peptides are stabilized only by cyclization. However, when taking into account the cost and duration involved in preparing BC peptides, SC may be an effective solution in many cases. The SC cyclic peptide *c*(MZ 4K-1) may be used as anti-HIV lead compound for further studies.

2. Materials and methods

2.1. Cyclic peptides library synthesis, labeling and purification

The cyclic peptides were synthesized on a Liberty Microwave-Assisted Peptide Synthesizer (CEM) using standard Fmoc chemistry. N-α-Fmoc-L-aspartic acid α-allyl ester was added to the parent LEDGF 361–370 sequence at its N-terminus. Fmoc-Nδ-alloc-L-ornithine or Nα-Fmoc-Nω-alloc-L-lysine were added to the C termini of the peptides *c*(MZ 3O-1) or *c*(MZ 4K-1), respectively. Alloc and allyl were selectively removed manually as described.[22] Peptides were cyclized manually by stirring the resin with the coupling reagent 6-chloro-benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (Pyclock) (7 equiv) (Luxembourg bio technologies, Israel) and DIPEA (14 equiv) in *N*-methyl-2-pyrrolidone overnight. Cyclic peptides were fluorescein-labeled before cleavage as described.[18] The peptides were purified on a Merck Hitachi HPLC using a reverse-phase C8 semi-preparative column (Vydac) with a gradient from 5% to 60% acetonitrile in water (both containing 0.001% (v/v) trifluoroacetic acid) and analyzed using MALDI-TOF MS. Peptide concentrations were determined using a UV spectrophotometer (Shimadzu) as described.[18]

2.2. Protein expression and purification

The full-length histidine tagged IN with five mutations (C56S, W131D, F139D, F185K, and C280S) was expressed and purified essentially as previously described.[32]

2.3. Fluorescence anisotropy

Measurements were performed at 10 °C using a PerkinElmer LS-55 luminescence spectrometer equipped with a Hamilton Microlab 500 dispenser.[33,34] The fluorescein-labeled LEDGF 361–370-derived cyclic peptide (1 ml, 100 nM in 20 mM Tris buffer pH 7.4, 185 mM NaCl) was placed in a cuvette, and the non-labeled IN protein (200 μl, ~50 μM) was titrated into it in 20 aliquots of 10 μl at 1 min intervals. The total fluorescence and anisotropy were measured after each addition using an excitation wavelength of 480 nm and an emission wavelength of 530 nm. Data were fit to the Hill equation:

$$R = R_0 + \frac{\Delta R * (K_a^n * [\mathrm{IN}]^n)}{1 + K_a^n * [\mathrm{IN}]^n}$$

Where R is measured anisotropy, ΔR is the amplitude of the anisotropy change from R_0 (free peptide) to peptide in complex, [IN] is the added concentration of IN, K_a is the association constant and ^{n} is the Hill coeficient.[18]

2.4. Quantitative estimation of IN catalytic activity in vitro

Determination of the IN enzymatic activity by a quantitative assay was performed as described.[6,35] In brief, 20 μM of the linear LEDGF 361–370 parent peptide (IN-peptide molar ratio 1:50) inhibited the IN catalytic activity by ~85%. This molar ratio was used for all experiments with the cyclic peptides.

2.5. NMR measurements

A 1 mM solution of cyclic peptide and 100 μM of IN in 20 mM phosphate buffer, 100 mM sodium chloride, 20.1 mM glycerol with 10% v/v D_2O was prepared from the lyophilized powder. The solution had an apparent pH of 6.87 as described.[22] NMR experiments were performed on a Bruker Avance 600 MHz DMX spectrometer operating at the proton frequency of 600.13 MHz, at 15.0 °C. The transmitter frequency was set to the water signal, which was calibrated at 4.89 ppm. Total correlation spectroscopy (TOCSY) using the MLEV-17 pulse scheme for the spin lock[36,37] and nuclear overhauser effect spectroscopy (NOESY) experiments were acquired under identical conditions for all samples, using gradients for water saturation and a mixing time of 160 ms.[38] Spectra were processed and analyzed with the TopSpin (Bruker Analytische Messtechnik GmbH) and SPARKY3 software (Goddard T. D. and Kneller D. G., SPARKY 3, University of California, San Francisco). Resonance assignment followed the sequential assignment methodology developed by Wüthrich.[39] The three-dimensional structures of the peptides were calculated using XPLOR (version 3.856)[40] by hybrid distance geometry-dynamical simulated annealing. Molmol[41] was used to create the final ensemble of structures. Low energy structures chosen for further analysis had no NOE violations, deviations from ideal bond lengths of less than 0.05 Å, and bond angle deviations from ideality of less than 5 Å. Figures were made using Chimera.[42]

2.6. Cell penetration experiments

The fluorescein-labeled peptides (10 μM in PBS) were incubated with HeLa cells for 2 h at 37 °C. After three washes in PBS, the cells were visualized by confocal microscopy.[18]

2.7. Quantitative estimation of HIV-1 infection by determination of extracellular p24 Lymphoid cells

Cells were incubated with the indicated peptides (12.5 μM) for 2 h and following infection with wild-type HIV-1 at a MOI of 0.01, the cells were incubated for 8 days. The amount of p24 protein was estimated in the cell medium every 2 days exactly as described.[35] For the multiple dose treatment a fresh dose of peptide was added to the medium every 2 days.

3. Results

3.1. Side-chain to side-chain (SC) cyclic analogs of *c*(MZ 4-1): Design and synthesis

The main disadvantage of BC is that the appropriate building blocks are not yet commercially available and the synthesis is not yet fully automated. To solve this problem we designed SC cyclic analogs of the lead BC peptide, *c*(MZ 4-1).[22] This type of cyclization can be performed using commercial building blocks on an automated synthesizer, resulting in higher yields and greater ease of synthesis. Two SC cyclic derivatives of *c*(MZ 4-1) were synthesized, in which the alloc glycine building unit (AGBU) in *c*(MZ 4-1) was replaced by either Nα-Fmoc-Nδ-alloc-L-ornithine or Nα-Fmoc-Nω-alloc-L-lysine to form the cyclic peptides *c*(MZ 3O-1) and *c*(MZ 4K-1), respectively (Scheme 1). *c*(MZ 3O-1) had the same ring size as *c*(MZ 4-1) but had one atom less in the bridge, while *c*(MZ 4K-1) preserved the same bridge size but the ring size was increased by one atom (For illustration see Fig. 1). The ring size is defined as the number of the backbone atoms that are included at the cyclization scaffold (including the bridge). The bridge size

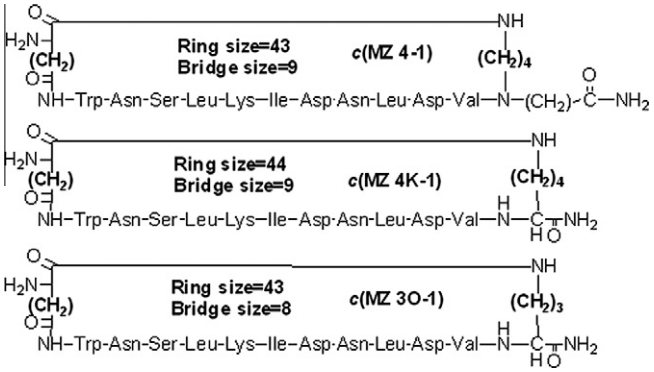
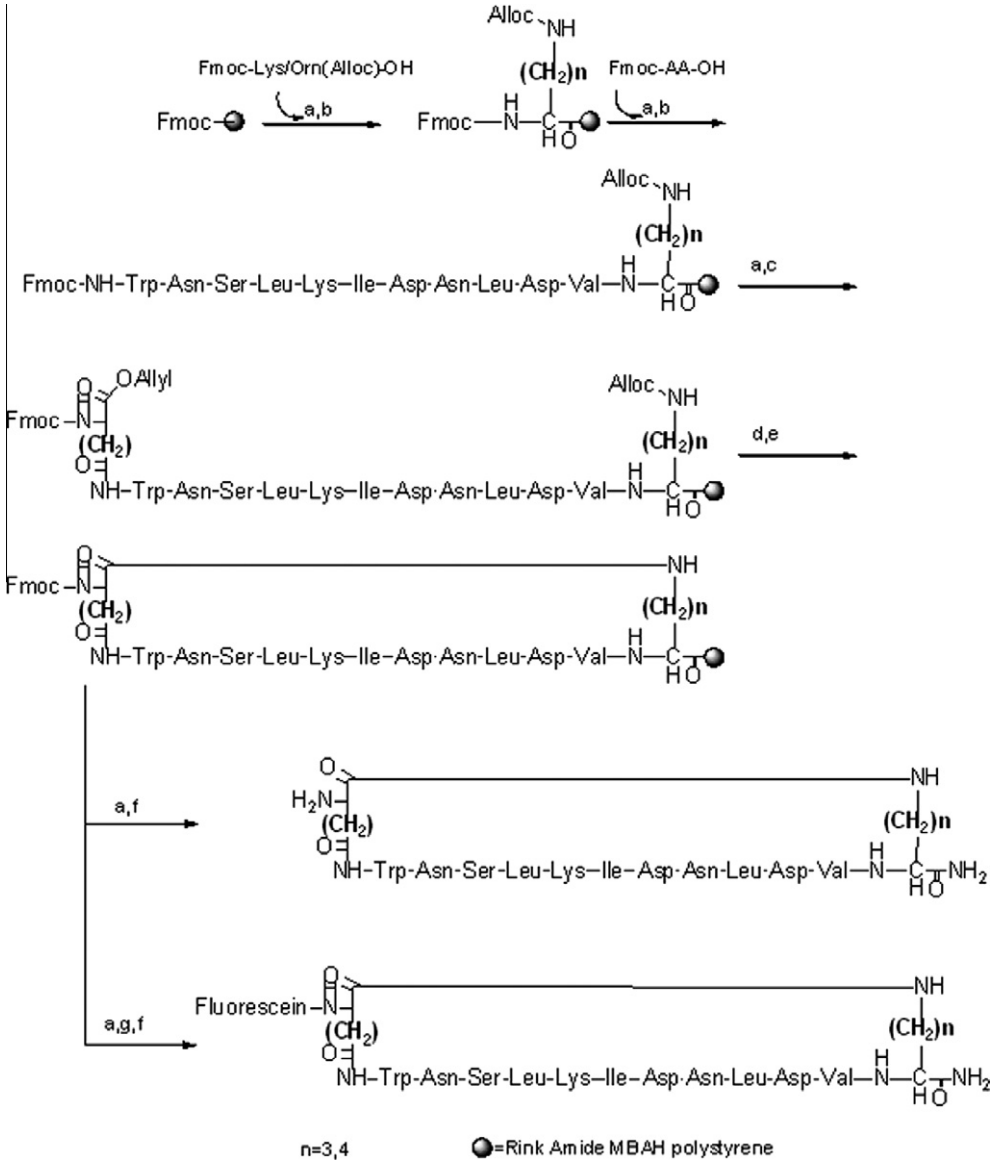


Figure 1. The SC cyclic *c*(MZ 4-1) analogs. The bridge branched from the alpha carbon while in the BC library was branched from the alpha nitrogen.



Scheme 1. Synthesis of the SC LEDGF 361–370 derived cyclic peptides library. The peptides were synthesized using a microwave-assisted peptide synthesizer except for steps e–h that were performed manually. Reagents and conditions: (a) Twenty percentage piperidine/NMP; (b) Fmoc-AA-OH, HBTU, DIPEA; (c) N-α-Fmoc-L-aspartic acid α-allyl ester, HBTU, DIPEA; (d) $(PPh_3)_4Pd(0)$, $PhSiH_3$; (e) PyClock, DIPEA; (f) TFA,TIPS,TDW; (g) fluorescein, HBTU, DIPEA.

represents the number of atoms from the alpha carbon of Nα-Fmoc-Nδ-alloc-L-ornithine or Nα-Fmoc-Nω-alloc-L-lysine to the α-nitrogen of the tryptophan (in this case the N-terminus, see Fig. 1).

3.2. The SC cyclic peptide *c*(MZ 4-1) binds IN and inhibits its activity in vitro and in cells with similar potency to the BC peptide

Fluorescence anisotropy was used to study binding of IN to the fluorescein labeled *c*(MZ 4-1) SC analogs. The two fluorescein labeled analogs bound IN with affinities in the low micromolar range (Fig. 2A). Fluorescein labeled *c*(MZ 4K-1) bound the IN tetramer with a Hill coefficient of around 4, like the original LEDGF 361–370, while fluorescein labeled *c*(MZ 3O-1) bound the IN dimer as indicated from its Hill coefficient (Fig. 2A and Table 1). Both *c*(MZ 3O-1) and *c*(MZ 4K-1) inhibited IN catalytic activity in vitro by ~50%. For comparison, the linear parent peptide LEDGF 361–370 and the BC *c*(MZ 4-1) inhibited IN activity by ~85% at the same concentrations and conditions (Fig. 2B), suggesting that both the bridge and the ring size, along with the cyclization mode, are important for inhibiting enzymatic activity of IN in vitro. *c*(MZ 3O-1) and *c*(MZ

Table 1
LEDGF-derived cyclic peptides binding to IN and their effect on IN catalytic activity

Peptide	*n*[a]	Binding affinity to IN[b] (μM)	Hill coefficient	IN inhibition[c] (%)
LEDGF 361–370		5.1 ± 0.1	3.6 ± 0.3	83
c(MZ 4-1)	4	5.0 ± 0.4	3.4 ± 0.9	90
c(MZ 4K-1)	4	2.5 ± 0.6	4.8 ± 0.9	55
c(MZ 3O-1)	3	2.0 ± 0.2	2.1 ± 0.1	50

[a] *n* Refer to the length of the alkyl chains, as described in Scheme 1.
[b] The binding affinities were determined by fluorescence anisotropy using the linear SC and BC cyclic fluorescein labeled peptides. IN (60 μM) was titrated into the fluorescein-labeled peptides solution (100 nM). Data were fit to the Hill equation.
[c] Values are taken from the data in Figure 2.

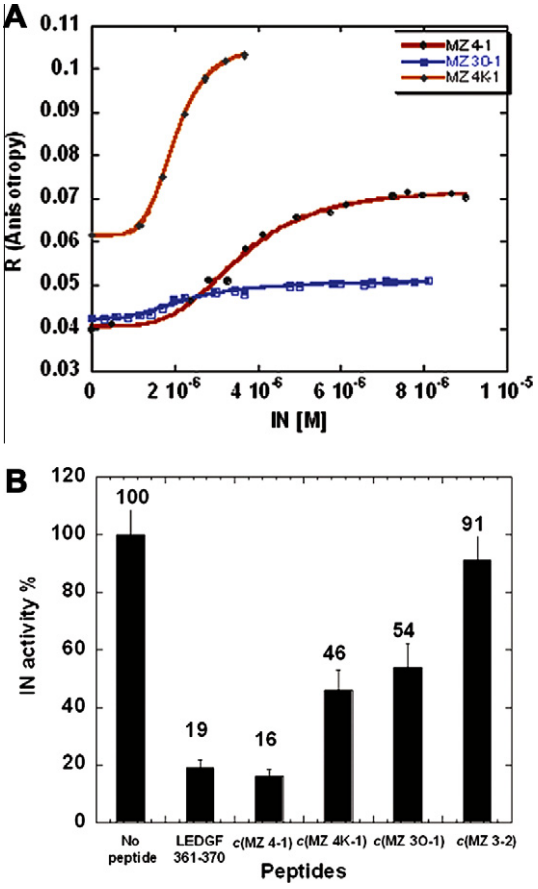


Figure 2. The SC cyclic peptides bind and inhibit IN catalytic activity in vitro. (A) The binding of the SC cyclic peptides to IN was studied using fluorescence anisotropy. IN (60 μM) was titrated into the indicated fluorescein labeled cyclic peptide (100 nM). The binding curves were fit to the Hill equation. Binding affinities and Hill coefficients are shown in Table 1. (B) Inhibition of IN catalytic activity: *c*(MZ 4-1) analogs bind IN at the micromolar range and inhibit IN catalytic activity in vitro. IN (390 nM) was incubated in the presence of LEDGF 361–370 and its cyclic peptides analogs at molar ratio of peptide/IN 50:1 and the overall integration process was monitored as described in materials and methods. *c*(MZ 3-2) was used as a negative control since we previously showed that this cyclic peptide was not active in vitro[22] *c*(MZ 6-1) was used as a negative control since we previously showed that this cyclic peptide was not active in cells.[22]

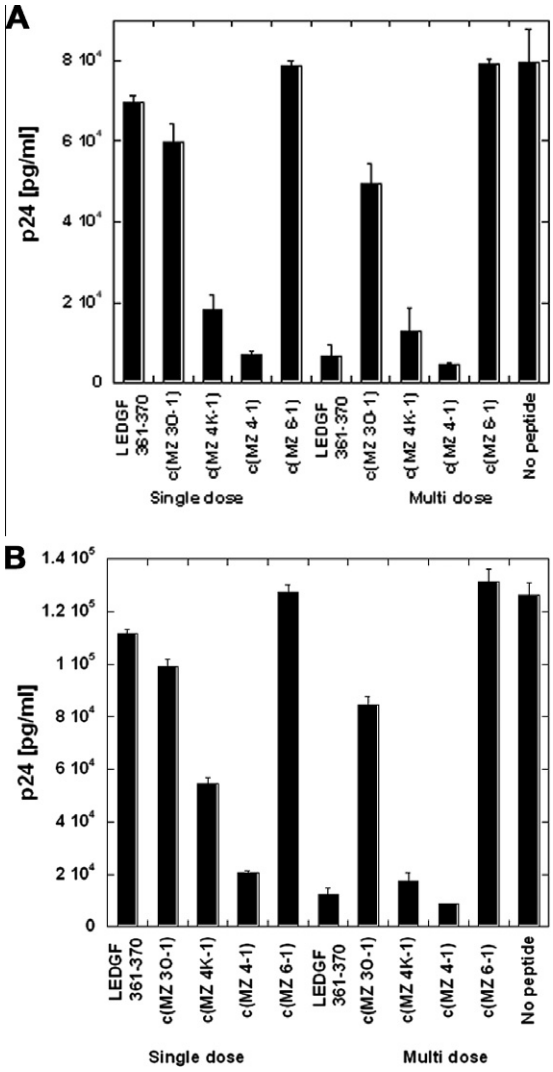


Figure 3. *c*(MZ 4K-1) preserves the *c*(MZ 4-1) long term inhibitory effect on HIV-1 replication. H9 T-lymphoid cells were incubated with *c*(MZ 4K-1), *c*(MZ 3O-1), *c*(MZ 4-1) and LEDGF 361–370 and the total amount of the released virus was estimated based on the p24 protein content at days 6 and 8. (A) *c*(MZ 4-1) and *c*(MZ 4K-1) inhibit HIV-1 replication in cell culture after 6 or 8 days. Shown is the quantification of the inhibition of p24 formation in T-lymphoid cells after 6 days post infection and (B) 8 days post infection. To determine the time dependent inhibitory effect of the peptides were introduced either in single dose at day 0 (single dose) or in multiple dose in which an additional dose was added every 2 days (multiple dose).

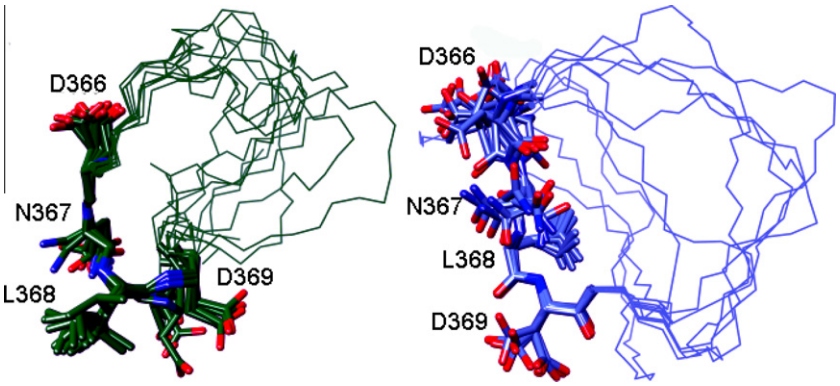


Figure 4. The NMR structures of *c*(MZ 4K-1) and *c*(MZ 3O-1) bound to IN. The conformation of *c*(MZ 4K-1) and *c*(MZ 3O-1) in the presence of IN were determined in solution by NMR. (Green, left panel) The backbone RMSD of *c*(MZ 4K-1) bound to IN was 1.34 Å and the local RMSD of 366–369 (in sticks) was 0.21 Å (Right panel) *c*(MZ 3O-1) bound IN had a backbone RMSD of 1.47 Å and a local RMSD (366–369) of 0.45 Å. All structures had reduced local RMSD values in the region of 366–369 (in sticks) upon binding. Molecular graphics prepared using Chimera.[42]

4K-1) both penetrated cells and their effect on HIV-1 replication in T-lymphoid cells was tested after 6 and 8 days post infection. (Fig. 3 A and B). Following multiple doses treatment, *c*(MZ 3O-1) inhibited HIV-1 replication by ~40% at 6 and 8 days post infection and *c*(MZ 4K-1) showed ~85% inhibition at the same time point as was shown by *c*(MZ 4-1) and LEDGF 361–370. In the single dose treatment, *c*(MZ 4K-1) inhibited HIV-1 replication in cells by 80% 6 days post infection and by 65% 8 days post infection while under these conditions *c*(MZ 4-1) inhibited HIV-1 replication by 90% and the linear LEDGF 361–370 did not have any activity. *c*(MZ 3O-1) inhibited HIV-1 replication by 25% at both 6 and 8 days post infection. These results indicate that *c*(MZ 4K-1) has a long term inhibitory effect that is close to that of *c*(MZ 4-1).

To determine the effect of the cyclization type on the stability of the cyclic peptides, LEDGF 361–370, c(MZ 4-1), *c*(MZ 4K-1) and *c*(MZ 3O-1) were incubated at 37 °C in a mixture of trypsin and chymotrypsin and their degradation rate was monitored by mass spectrometry. LEDGF 361–370 was degraded after 30 min while the three cyclic peptides started to degrade only after 150 min (data not shown). We conclude that both types of cyclization increase the stability of the peptides compared to the linear LEDGF 361–370.

3.3. Solution NMR structures of *c*(MZ 3O-1) and *c*(MZ 4K-1)

The effect of cyclization type and specifically the bridge and ring sizes on the solution structures of *c*(MZ 4-1) SC cyclic analogs was studied by NMR, by comparing solution structures of the free peptide to that in interaction with IN. The backbone RMSD of the IN-associated *c*(MZ 4K-1) was 1.34 Å and the local backbone RMSD of residues 366–369 was 0.21 Å. IN-associated *c*(MZ 3O-1) had a backbone RMSD of 1.47 Å and a local RMSD in residues 366–369 of 0.45 Å. For comparison, the local RMSD of residues 366–369 in *c*(MZ 4-1) was 0.04 Å.[22] Upon introducing IN, all structures showed a reduced local RMSD in the region of 366–369, suggesting that this region is stabilized upon IN interaction in all cases (Fig. 4). The number of restraints in the bound and interacting states were within ±5 of each other (Supplementary Table C.) such that the change in conformational space is not deemed to be a spectral artifact. Residues 366–369 in *c*(MZ 4-1) showed the lowest local RMSD value of all the bound cyclic peptides tested, indicating a more confined conformational space. The higher RMSD values of residues 366–369 in the side chain to side chain cyclic peptides, *c*(MZ 4K-1) and *c*(MZ 3O-1), imply that their IN-associated states are less conformationally restricted. This may explain their lower inhibitory activity. The bridge size had more effect on the structure of the cyclic peptides than the ring size. Both *c*(MZ 4-1) and *c*(MZ 4K-1) have the same bridge size and both have less conformational space compared to *c*(MZ 3O-1).

4. Discussion

In this study the BC and SC cyclization types were extensively compared using biophysical, biochemical and structural methods. The SC cyclic peptide *c*(MZ 4K-1), which was designed based on the active BC peptide *c*(MZ 4-1), emerged as a stable and potent anti HIV lead compound.

The main disadvantages of BC are that the appropriate building blocks are not yet commercially available, the coupling to the secondary amine is difficult and the synthesis is not automated. SC cyclization, however, is well established and the building blocks are readily available. Our results show that the bridge size has a large effect on the structure and the in vivo activity of the cyclic peptides. *c*(MZ 4K-1) and *c*(MZ 4-1), which are both active in cells, have the same bridge size of nine atoms but a different ring size of 44 and 43 atoms, respectively. *c*(MZ 3O-1), which was hardly active in cells, had a different bridge size of only 8 atoms. *c*(MZ 3O-1) also showed the highest RMSD in the 366–369 region in the NMR structures (0.45 Å) while *c*(MZ 4K-1) had a lower local RMSD value of 0.21 Å and *c*(MZ 4-1) had the lowest value of 0.04 Å.[22] This suggests that a bridge size of 9 atoms is required for stabilizing the bioactive structure of the LEDGF—derived cyclic peptides.

We have previously shown that the linear LEDGF 361–370[18] and the BC peptide *c*(MZ 4-1)[22] stabilized the IN tetramer and shifted the IN oligomerization equilibrium towards it. In this study we showed that the SC cyclic peptide *c*(MZ 4K-1) shifted the oligomeric state IN towards the tetramer but *c*(MZ 3O-1) specifically bound the IN dimer. This is consistent with our previous observations, showing that peptides with larger ring sizes stabilized the IN tetramer, while the cyclic peptides with smaller rings bound the IN dimer.[22]

The most active SC analog, *c*(MZ 4K-1), was a slightly less potent IN inhibitor than the BC peptide *c*(MZ 4-1). The NMR solution structure of the active *c*(MZ 4-1) indicated that the conformation of residues 366–369 was stabilized upon IN binding. This suggests that these residues, which are known to bind IN in the parent protein[43] adopted a bioactive conformation in the BC peptide as well. Residues 366–369 in *c*(MZ 4-1) showed the lowest local RMSD value of all the bound cyclic peptides, indicating a more confined conformational space.[22]The higher RMSD values of residues 366–369 in the SC cyclic peptides, *c*(MZ 4K-1) and *c*(MZ 3O-1), imply that their IN-bound states are less able to achieve the biologically

active conformation. The observation that *c*(MZ 4-1) showed the lowest local RMSD while interacting with IN, suggests that it achieves and maintains the bioactive conformation better than the other peptides. The increased rigidity of the ring may be due to the N-alkylation in the alloc glycine building unit, which exists only in the BC peptides.

Previous studies have demonstrated that BC improves the peptides stability towards enzymatic degradation.[44–46] The long term inhibitory effect of *c*(MZ 4-1) and *c*(MZ 4K-1) on HIV-1 replication in the single dose experiment implies that both cyclic peptides are more stable than the linear parent peptide. Both cyclic peptides preserved the inhibitory activity even after long incubation, while the linear peptide was unable to retain any long term effect due to rapid degradation.[22] These findings indicate that both modes of cyclization stabilize the peptide and increase its duration of activity in cells. The BC peptide *c*(MZ 4-1) retained full potency 8 days post infection while the SC analog *c*(MZ 4K-1) showed only partial activity in cells. This may be explained by the nature of their cyclization. In the BC peptide, both N-alkylation and cyclization stabilize the peptide while in the SC cyclic peptide, there is no N-alkylation. Comparing the properties of SC versus BC peptides is essential for drug design since cyclization is one of the most frequently used methods for peptido- and proteinomimetics. The synthetic disadvantages of BC make this method sometimes difficult to approach. Future improvement of coupling methods and building blocks accessibility will contribute tremendously to the popularity of this method. This work proves that, to a certain extent, the BC scaffold can be replaced by the SC one.

5. Conclusions

We describe two types of cyclic peptides that are stable and potent inhibitors of IN enzymatic activity and HIV-1 replication in cells. We showed that an active SC cyclic analog can be designed based on an active BC lead peptide. Both cyclization types have their respective advantages: The BC peptide is more active and stable, probably due to the benefit of N-alkylation, while SC cyclic peptides are easier to synthesize. We suggest that both methods are interchangeable. We conclude that both *c*(MZ 4-1) and *c*(MZ 4K-1) may become anti-HIV lead compounds.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.03.039.

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