The role of the cyclic peptide backbone in the anti-HIV activity of the cyclotide kalata B1

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Abstract The plant cyclotides, the largest known family of circular proteins, have tightly folded structures and a range of biological activities that lend themselves to potential pharmaceutical and agricultural applications. Based on sequence homology, they are classified into the bracelet and Möbius subfamilies. The bracelet subfamily has previously been shown to display anti-HIV activity. We show here that a member of the Möbius subfamily, kalata B1, also exhibits anti-HIV activity despite extensive sequence differences between the subfamilies. In addition, acyclic permutants of kalata B1 displayed no anti-HIV activity, suggesting that this activity is critically dependent on an intact circular backbone.

Keywords: Cyclotide; Cystine knot; Circular protein; Acyclic permutant

1. Introduction

It has become evident in recent years that circular proteins are present in a wide range of organisms and that backbone circularization is not restricted to small non-ribosomally synthesized peptides [1,2]. Recently discovered circular proteins have been isolated from plants, bacteria and even mammals, with one group forming a particularly large family of related proteins named the cyclotides [3]. The cyclotides are plant derived circular proteins containing approximately 30 residues and three disulfide bonds. Approximately 50 sequences have been published and many more are expected to be discovered in the near future [3–11].

A range of biological activities have been reported for the cyclotides, including uterotonic [12], anti-HIV [8] and inhibition of neurotensin binding [9], but it appears likely that the peptides are present in plants for defense related purposes based on their antimicrobial [13] and insecticidal activities [14]. The mechanism of action and the structure–activity relationships associated with these various activities have not been elucidated.

Analysis of the primary sequences of the cyclotides has revealed the presence of two major subfamilies, namely the bracelet and Möbius subfamilies [3]. The terminology for the subfamilies is derived from analysis of the three-dimensional structures that reveal the presence of a *cis* proline in the Möbius subfamily that introduces a conceptual twist in the circular backbone. Although the three-dimensional structures of only six cyclotides have been reported [3,10,15–18], a highly conserved framework has emerged, comprising an inhibitor cystine knot [19] arrangement of the three disulfide bonds and β-sheet secondary structure as shown in Fig. 1. This structural motif has been termed the "cyclic cystine knot" (CCK) and appears to confer extreme structural stability to the molecules containing it [3]. A small region of helix is present in some members of the bracelet subfamily.

The sequences of selected members of both subfamilies [8,10,20] are shown in Fig. 1 together with their disulfide connectivity [17,21–23]. The six backbone loops between the six cysteine residues have varying degrees of conservation both within and between the subfamilies. The most highly conserved are loops 1 and 4, which comprise backbone segments that are directly involved in the cystine knot motif. We have previously shown that breaking the backbone in these loops prevents folding into a native-like conformation. By contrast, a break in the backbone in any of the other four loops leads to acyclic permutants that display a native fold, albeit without a cyclic backbone [24].

The cyclotides have potential applications in the pharmaceutical and agricultural fields based on their intrinsic activities. In particular, the worldwide AIDS epidemic has intensified interest in identifying naturally occurring antiviral molecules [25]. Several cyclotides have been reported to have anti-HIV activity [6–8,26] and indeed these cyclotides were first identified in screening programs directed towards such activity. Furthermore, their potent insecticidal activity suggests that the cyclotides may be useful as "natural" insecticides if applied to crop plants. Given that the cyclotides are gene encoded, it may be possible to produce transgenic plants engineered to have built in resistance to *Helicoverpa* and related pests, much in the same way as the *Bacillus thuringiensis* toxin has been used to protect corn, cotton and soy beans [27]. In addition to making use of their intrinsic activity, the stable framework of the cyclotides might also prove useful as a molecular scaffold onto which novel bioactivities may be grafted [28,29].

To realize the potential of cyclotides, a thorough analysis of their structure–activity relationships is required. The natural sequence variation observed in the cyclotides provides a means

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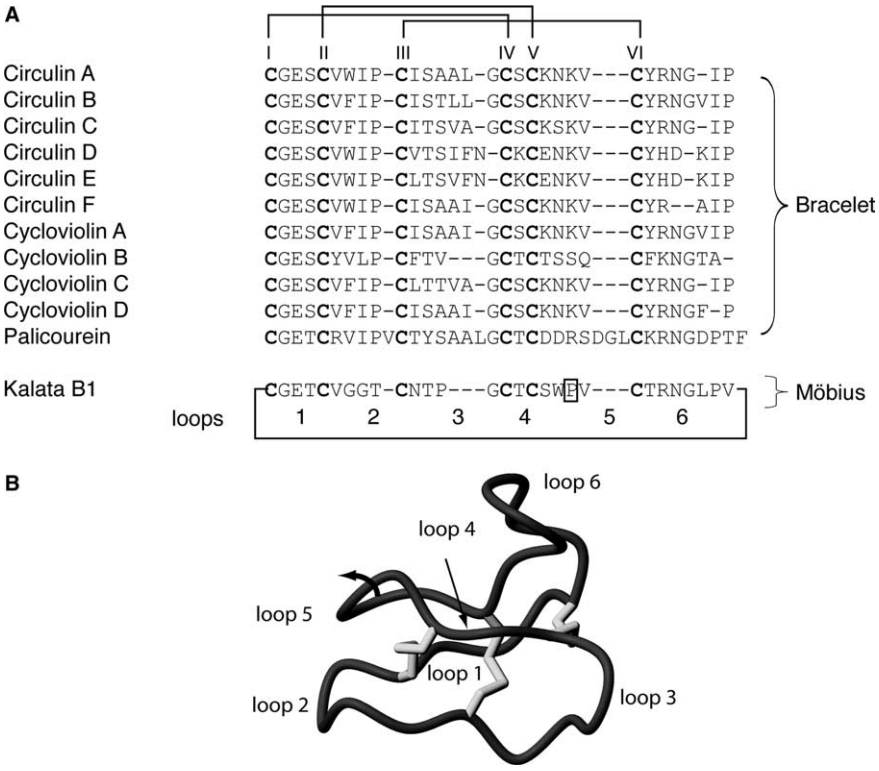

Fig. 1. Amino acid sequences of cyclotides tested for anti-HIV activity. (A) The six-cysteine residues are highlighted in bold and the disulfide connectivity is shown by lines connecting the cysteine residues. The six backbone loops between the cysteine residues are labeled loops 1–6. The circular backbone is represented by a line connecting loops 1 and 6. The proline in loop 5 of kalata B1 is boxed to highlight the fact that the nomenclature for the Möbius subfamily is based on this proline being in a *cis* conformation. (B) The three-dimensional structure of the prototypic cyclotide kalata B1 [pdb code-1NB1]. The disulfide bonds are shown with gray lines and the loops are labeled 1–6. The location of the backbone twist due to a *cis*-Pro peptide bond in the Möbius subfamily is highlighted by a curved arrow in loop 5.

of exploring such relationships but so far relatively few of the known cyclotides have been tested in a wide range of assays. Selected members of both families have been tested in antimicrobial assays and the greater number of positively charged residues in the bracelet cyclotides has been suggested to result in the broader spectrum of antimicrobial activity compared to kalata B1, a member of the Möbius subfamily [13]. So far, anti-HIV activity has only been demonstrated for bracelet cyclotides [6–8].

To address the question of whether anti-HIV activity is specific to the bracelet cyclotides, we have examined the anti-HIV activity of kalata B1, the prototypic member of the Möbius subfamily. We show that despite its reduced cationic character, kalata B1 also exhibits anti-HIV activity. Furthermore, acyclic permutants of kalata B1 (i.e., molecules with the backbone opened in each of the six loops between the cysteine residues as shown in Fig. 2) were inactive, indicating that anti-HIV activity is critically dependent on the presence of an intact cyclic backbone.

2. Materials and methods

Synthesis of kalata B1 and acyclic permutants – Kalata B1 and its acyclic permutants were synthesized as previously described [24,30]. Briefly, peptides were assembled using manual solid phase peptide synthesis with Boc chemistry on a 0.5 mmole scale. MBHA or PAM resin was used (Applied Biosystems, Foster City, CA) and amino acids added to the resin using HBTU with in situ neutralization [31]. N-terminal acetylation was performed on resin for one of the permutants with a vast excess of acetic anhydride and DIEA in DMF. Cleavage of the peptide from the resin was achieved using hydrogen fluoride (HF) with cresol and thiocresol as scavengers (HF:cresol:thiocresol; 9:1:1 v/v). The reaction was allowed to proceed at −5 to 0 °C for 1 h. Following cleavage, the peptides were dissolved in 50% acetonitrile, 0.1% TFA and lyophilized. The crude, reduced peptides were purified using preparative reverse-phase HPLC (RP-HPLC) on a Vydac C18 column. Gradients of 0.1% aqueous TFA and 90% acetonitrile/0.09% TFA were employed with a flow rate of 8 ml/min and the eluant monitored at 230 nm. These conditions were used in the subsequent purification

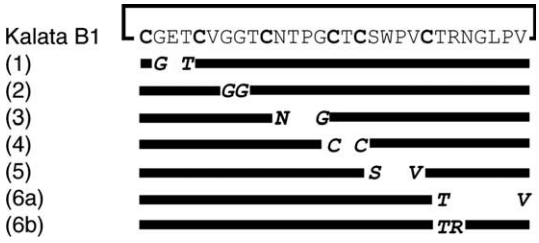

Fig. 2. Acyclic permutants of kalata B1 in which the backbone is opened in each of the six loops. The peptides are numbered according to the backbone loop that is broken in the acyclic derivative. The introduced N- and C-termini of the acyclic permutants are shown underneath the native sequence, with the broad black lines indicating where the native sequence is retained. In most cases, a few residues of the original circular peptide sequence were truncated at the break sites. Permutants 2, 3 5, 6a and 6b folded in native-like conformations and were analyzed for anti-HIV activity.

steps. Mass analysis was performed on a Sciex (Thornhill, Ontario) triple quadrupole mass spectrometer using electrospray sample ionization.

Disulfide formation – Oxidation reactions on the permutants were performed using the optimum conditions established for the cyclic peptide [30]. The purified reduced peptides were dissolved in 50% 2-propanol, in the presence of 1–10 mM reduced glutathione in 0.1 M ammonium bicarbonate (pH 8.5). The reactions were left at room temperature for 24 h. The pH was lowered with TFA prior to purification with RP-HPLC.

Anti-HIV activity assays – An in vitro XTT-based anti-HIV assay was used as described previously [32] to examine the effect of kalata B1 and its acyclic permutants on virus-induced cell killing in HIV-infected cultures.

3. Results and discussion

In the current study, we show for the first time that a member of the Möbius subfamily of cyclotides exhibits anti-HIV activity. Kalata B1 was synthesized and the disulfide bonds formed as previously described [30]. The synthetic peptide has been shown by NMR spectroscopy to be identical to the native form [30]. Anti-HIV assays were performed on synthetic kalata B1 with the highest concentration of sample tested being 3500 nM. Kalata B1 effectively inhibited the cytopathic effects of HIV-1 infection in cultured human T-lymphoblast (CEM-SS) cells. The antiviral cytoprotective concentration (EC_{50}) of synthetic kalata B1 is approximately 140 nM, while the cytotoxic concentration (IC_{50}) was greater than 3500 nM. Table 1 summarizes the results and compares them with data for other cyclotides.

The level of activity displayed by kalata B1 is comparable to the other cyclotides that have been tested, including circulins A–F [7,8] cycloviolins A–D [6] and palicourein [26]. These peptides displayed EC_{50} values of ~40–275 nM, depending on the type of cell line used (Table 1). Interestingly, the IC_{50} of kalata B1 was >3500 nM, whereas IC_{50}'s of ≈500–1500 nM were observed for the circulins, cycloviolins and palicourein [6–8]. Thus, the "therapeutic index" of kalata B1 is substantially higher than other cyclotides. There is a relatively high degree of sequence similarity between the circulins and cycloviolins. However, cycloviolin B diverges from the other sequences with significant variations in loops 2 and 5, as shown in Fig. 1. Kalata B1 also differs significantly from the circulins and cycloviolins, particularly in loops 2, 3 and 5. Given the similarity in the assay results for the various cyclotides tested, including those coming from different subfamilies, it appears that sequence variation does not significantly influence the anti-HIV activity.

Table 1
Anti-HIV activity of cyclotides

Peptide	EC_{50}[a]	IC_{50}[b]	Reference
Kalata B1	~140 nM	>3500 nM	This study
Acyclic permutants of kalata B1	No activity	No activity	This study
Circulins A–F	40–275 nM	~500 nM[c]	[7,8]
Cycloviolins A–D	~130 nM	~560 nM	[6]
Palicourein	100 nM	1500 nM	[26]

[a] Values for HIV-infected cell lines.
[b] Values for uninfected cell lines.
[c] IC_{50} values are for circulins A and B.

One of the most significant sequence variations observed between the bracelet and Möbius subfamilies is the presence of a larger number of cationic residues in the bracelet family. This feature has been highlighted in a previous study on the antimicrobial activity of cyclotides [13] in which the arginine residue of both kalata B1 and circulin A was modified. The study suggested that at least one cationic residue is required for electrostatic interactions with bacterial surfaces for activity [13]. This observation is consistent with the current analysis because kalata B1 and cycloviolin B, both of which contain a single positive charge, display anti-HIV activity. It will be of interest to determine the activity of mutants not containing this positive charge or of those with additional charges included in the sequence. Alanine mutagenesis studies will provide a better understanding of the critical residues for activity.

Acyclic permutants of kalata B1 were also tested in the anti-HIV assay to examine the effect of opening the backbone on this activity. Acyclic permutants involving backbone breaks in loops 2, 3, 5, and 6 were used in the study as they retained the native fold [24]. By contrast, opening the backbone in loops 1 and 4 prevented folding into the native conformation and so these derivatives could not be studied. None of the acyclic permutants tested, i.e., compounds (2), (3), (5), (6a), and (6b) in Fig. 2, were active at concentrations of ~3.5 to 20 μM. All of the permutants, with the exception of (2), were synthesized with C-terminal amides and thus negative charges were not introduced. Permutant (3) was also synthesized with an acetylated N-terminus, but this variant still did not display anti-HIV activity, indicating that the lack of anti-HIV activity is not due to the introduction of a positive charge in the permutant. The lack of activity of the acyclic permutants suggests that the critical residues for activity are not localized to one region of the molecule. Although the overall native fold is retained in the acyclic permutants as evidenced by a chemical shift analysis and determination of the three-dimensional structure of one of the loop 6 analogs [33], differences have been observed in the amide exchange rates [24]. Specifically, an increase in the amide exchange rates was observed for the acyclic permutants relative to the native, cyclic peptide, indicating increased accessibility of solvent to the hydrogen bond network of the cyclotides. In turn, this is likely associated with increased flexibility and a decrease in stability upon opening the circular backbone. It is possible that such differences in backbone "breathing" motions are related to the lack of anti-HIV activity of the acyclic permutants. However, further mutagenesis studies are required to confirm a role for the circular backbone and discern why the activity does not appear to be localized.

The trends in anti-HIV activity seen in the current study correlate well with previous results where the native peptide displayed hemolytic activity but not the acyclic permutants [24]. It is possible that a common mechanism is involved in both these activities, although further analysis is required to elucidate the mechanism. Given the range of activities displayed by the cyclotides, it appears likely that their mode of action may involve some type of membrane disruption.

In summary, we have shown that anti-HIV activity is not restricted to the bracelet subfamily of the cyclotides as a member of the Möbius subfamily also displays it. The sequence variation between the subfamilies does not appear to influence the level of activity, suggesting that the overall fold is

important for activity rather than individual residues. However, the dynamics of the backbone may also play a role, as acyclic permutants that are structurally similar to the native peptide, but are potentially more flexible, do not have anti-HIV activity. The fact that kalata B1 has similar anti-HIV activity, but decreased cytotoxicity suggests that it may be a more promising lead in anti-HIV therapy than previously tested cyclotides. Further testing of a range of naturally occurring cyclotides and synthetic mutants will provide valuable information on the structure–activity relationships of this fascinating family of macrocyclic peptides.

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