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# Importance of the Cell Membrane on the Mechanism of Action of Cyclotides

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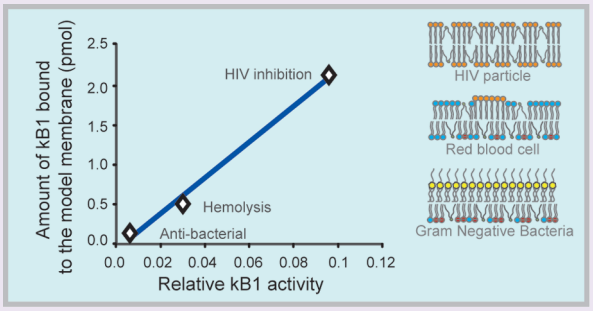
**ABSTRACT:** Their distinctive structures, diverse range of bioactivities, and potential for pharmaceutical or agricultural applications make cyclotides an intriguing family of cyclic peptides. Together with the physiological role in plant host defense, cyclotides possess antimicrobial, anticancer, and anti-HIV activities. In all of the reported activities, cell membranes seem to be the primary target for cyclotide binding. This article examines recent literature on cyclotide-membrane studies and highlights the hypothesis that the activity of cyclotides is dependent on their affinity for lipid bilayers and enhanced by the presence of specific lipids, *i.e.*, phospholipids containing phosphatidylethanolamine headgroups. There is growing evidence that the lipid composition of target cell membranes dictates the amount of cyclotides bound to the cell and the extent of their activity. After membrane targeting and insertion in the bilayer core, cyclotides induce disruption of membranes by a pore formation mechanism. This proposed mechanism of action is supported by biophysical studies with model membranes and by studies on natural biological membranes of known lipid compositions.




## CYCLOTIDES: DISCOVERY, STRUCTURE, AND DIVERSITY

Cyclotides are a fascinating family of plant peptides that were first discovered in an indigenous traditional medicine, used in the Congo, to accelerate childbirth.[1,2] The active uterotonic compound was found to be a 29-amino-acid peptide[3] that is able to resist boiling and ingestion before exerting its medicinal activity as a uterotonic agent. The peptide was named kalata B1 (kB1) and is now regarded as the prototypic member of the cyclotide family.

The remarkable resistance of kB1 to degradation is due to its unusual structure, as revealed by solution-state NMR studies.[4] The topology of kB1 is characterized by a head-to-tail cyclic structure and three disulfide bonds forming a cyclic cystine knot motif (CCK). The cystine knot is formed when the $Cys^{III}$-$Cys^{VI}$ disulfide bond penetrates a ring formed by the disulfide bonds $Cys^{I}$–$Cys^{IV}$ and $Cys^{II}$–$Cys^{V}$ and their inter-Cys backbone segments (Figure 1). The cystine knot occupies the molecular core, whereas the majority of the other amino acids side chains are exposed on the molecular surface. The backbone segments between the Cys residues are referred to as loops, and their exposure on the molecular surface suggests that they are important for the bioactivity of kB1.

Around the time of the report of the kB1 structure, several other macrocyclic peptides from plants were also identified[5−9] and are now known to share the CCK structure. The term "cyclotide" (**cycl**ic pep**tide**) was proposed in 1999 to characterize this new family of plant proteins, in which the unique CCK structure was defined as the signature motif of the family.[10] Cyclotides are therefore distinguished from other proteins and peptides, as they possess a unique structure that confers on them resistance to high temperatures, protease degradation, and chemical chaotropes.[11,12]

Extensive work has been done to identify and characterize a wide range of cyclotides, and it was found in early studies that an individual plant can express multiple cyclotides.[13,14] To date more than 200 cyclotide sequences have been reported in plants from the Rubiaceae, Violaceae, Fabaceae, and Cucurbitaceae families, but many more are expected to be found, with 50,000 members predicted to exist.[15] Some examples of sequences of known cyclotides are included in Table 1, and a complete list of all cyclotides characterized to date is available in the online database on circular proteins, CyBase http://www.cybase.org.au).[16] CyBase also documents the gene sequences that encode cyclotide precursor proteins.[17,18]

The cyclotide family was originally divided into the subfamilies Möbius and bracelet,[10] defined on the basis of the presence or absence of a conceptual twist in the circular backbone, respectively. Cyclotides belonging to the Möbius subfamily have a *cis*-Pro residue in loop 5 (see Table 1), which is responsible for a local 180° twist in the peptide bond angle; by contrast, all backbone peptide bonds in bracelet cyclotides have a *trans* configuration. There is high sequence homology

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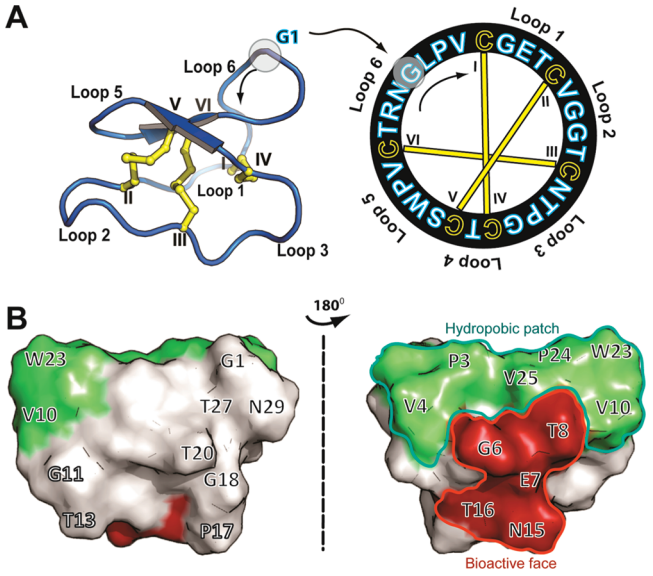


**Figure 1.** Structure of kB1. (A) Three-dimensional structure (PDB ID: 1nb1) and sequence of kB1. As for other cyclotides, kB1 contains six conserved Cys, which together with the cyclic backbone form a cyclic cystine knot. The backbone segments between Cys residues are termed loops. Disulfide bonds are shown in yellow, the Cys are labeled I–VI, and the six loops are identified. The black arrow indicates the direction of the peptide chain N–C. The position of $Gly^{1}$ is indicated in the structure; this residue is ligated to $Asn^{29}$ during the biosynthesis of the circular backbone. (B) Surface representation of kB1 in two views showing the bioactive face, identified from an Ala scan[62] (in red), and the hydrophobic patch (in green). In the left view, the molecule has the same orientation as the three-dimensional structure represented in panel A). The bioactive face and hydrophobic patch are localized on the same side of the molecule, shown in the right view. The hemolytic and insecticidal activities of kB1 are ablated when any of the residues in the hydrophobic patch or in the bioactive face are substituted with a Lys residue.[63]

within each of these two subfamilies, but lower homology between the subfamilies.

A third subfamily of cyclotides, the trypsin inhibitor subfamily, was recently proposed,[19,20] with two members identified to date (*Momordica cochinchinensis* trypsin inhibitors I and II, MCoTI-I and MCoTI-II).[21] These peptides were isolated from the dormant seeds of *M. cochinchinensis* and have powerful trypsin inhibitory activities.[21] The sequences of this third subfamily show very little similarity with the other two subfamilies, but the members do have the CCK structure, which defines them as cyclotides (see Table 1). These trypsin inhibitors are also referred to as cyclic knottins.[22] Consistent with this categorization, MCoTI-I and MCoTI-II show high sequence homology with acyclic cystine-knotted squash trypsin inhibitors.[21]

Although there is extensive variation in the sequences of members of cyclotide family, there are homologies between members belonging to the bracelet and Möbius subfamilies that are important for their physicochemical properties and biological activities: the six Cys and the disulfide connectivity, forming the CCK, the presence of a conserved Glu residue in loop 1, and the presence of a conserved Asn (or Asp) in the last position in loop 6 (see Table 1). In addition to the cystine knot, the conserved Glu residue in loop 1 also has an important role in structure stabilization of cyclotides belonging to both Möbius and bracelet subfamilies;[23−25] whereas the Asn (or Asp) residue is important for the backbone cyclization by an asparagine endopeptidase.[26,27] Cyclotides also typically have a cluster of surface-exposed hydrophobic amino acids (see kB1 example, Figure 1B) that appears to be involved in their biological activities.[28−30]

In summary, cyclotides are a large family of natural peptides from plants that can be regarded as a natural combinatorial peptide template,[31] onto which a range of sequences are displayed. This array of sequences on the CCK signature motif confers cyclotides with a wide range of activities. Like other naturally occurring cyclic peptides from bacteria, plants, and animals, cyclotides are renowned for their exceptional stability.[32]

## BIOLOGICAL ACTIVITIES AND APPLICATIONS OF CYCLOTIDES

Cyclotides probably evolved to protect plants against pests, a function that is consistent with the presence of a large number of different cyclotides in an individual species,[13,14] their high abundance in aerial plant tissues,[33] and most importantly their inhibition of the growth and development of insects, *e.g.*,

**Table 1. Sequences of Selected Natural Cyclotides Belonging to the Möbius, Bracelet, and Trypsin Inhibitor Subfamilies**

| | loop 6 | | loop 1 | | loop 2 | | loop 3 | | 4 | | loop 5 | | loop 6 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Möbius** | | I | | II | | III | | IV | V | | VI | | |
| kalata B1 | G - L P V | C | G - - - E T | C | V G - G T | C | N T - - - P G | C | T | C | S W - P V | C | T R - - N |
| kalata B2 | G - L P V | C | G - - - E T | C | F G - G T | C | N T - - - P G | C | S | C | T W - P I | C | T R - - D |
| kalata B7 | G - L P V | C | G - - - E T | C | T L - G T | C | Y T - - - Q G | C | T | C | S W - P I | C | K R - - N |
| varv F | G - V P I | C | G - - - E T | C | T L - G T | C | Y T - - - A G | C | S | C | S W - P V | C | T R - - N |
| **Bracelet** | | | | | | | | | | | | | |
| cycloviolacin O1 | G - I P - | C | A - - - E S | C | V Y - I P | C | T V T A L L G | C | S | C | S N - R V | C | Y - - - N |
| cycloviolacin O2 | G - I P - | C | G - - - E S | C | V W - I P | C | I S S - A I G | C | S | C | K S - K V | C | Y R - - N |
| kalata B5 | G - T P - | C | G - - - E S | C | V Y - I P | C | I S G - V I G | C | S | C | T D - K V | C | Y L - - N |
| kalata B8 | G S V L N | C | G - - - E T | C | L L - G T | C | Y T - - - T G | C | T | C | N K Y R V | C | T K - - D |
| **Trypsin Inhibitor** | | | | | | | | | | | | | |
| MCoTI-I | G - - G V | C | P K I L Q R | C | R R D S D | C | P G - - - - A | C | I | C | R G N G Y | C | G S G S D |
| MCoTI-II | G - - G V | C | P K I L K K | C | R R D S D | C | P G - - - - A | C | I | C | R G N G Y | C | G S G S D |


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*Helicoverpa* species, upon ingestion.[18,34] In addition to their insecticidal activity, cyclotides have nematocidal activity against two important gastrointestinal parasites of livestock, *Hemochus contortus* and *Trichostrongylus colubriformis*,[35] and have molluscidal activity against *Pomacea canaliculata*,[36] a pest of rice crops. These activities suggest that cyclotides potentially have a wide range of applications in agriculture for pest control in species that intrinsically do not produce cyclotides.

In addition to the pesticidal activities, a range of other activities have been reported for cyclotides, as recently reviewed.[37] Their anti-HIV activity was first discovered as part of a screening program at the U.S. National Cancer Institute for novel anti-HIV natural products[5] and is one of the most extensively investigated activities of cyclotides. Several cyclotides belonging to the bracelet or Möbius subfamilies possess anti-HIV activity.[28,38−42] The hemolytic activity of cyclotides has also been extensively studied after first being reported for viola peptide-1.[7] Cyclotides typically have only mild hemolytic potency ($EC_{50}$ >10 $\mu$M), but hemolysis is often used as a convenient screening bioassay to evaluate the possible cytotoxic activity of newly discovered cyclotides.[43,44]

A study in 1999 reporting that cyclotides have antimicrobial properties[45] attracted much attention, and cyclotides are now sometimes referred to in the literature as plant antimicrobial peptides (AMPs).[46−48] Tam and colleagues tested four synthetically made cyclotides against Gram-positive and Gram-negative bacteria as well as fungal strains, and promising antimicrobial activity was reported against some species.[45] Nevertheless, the antimicrobial activity was observed only in low salt conditions, and the use of more physiologically relevant salt concentrations in the assay buffers seemed to ablate the antimicrobial activity.[45] This observation, together with the fact that cyclotides typically have a total charge close to zero and lack a preference for negatively charged membranes,[49] suggests that in general cyclotides have a poor antibacterial profile.[50] Consistent with this suggestion, a recent study demonstrated that antimicrobial activity is very much dependent on the specific cyclotide,[51] with cycloviolacin O2 showing high potency and most others being ineffective. In addition, another recent study extensively evaluated the antimicrobial activity of native kB1 and found it to be weak.[52] Therefore, the suggestion that cyclotides are plant AMPs should be carefully qualified and evaluated on a case-by-case basis. Nevertheless, the worldwide problem of bacterial resistance is encouraging alternatives to conventional antibiotics. Peptide-based antibiotics are strong candidates as they are potentially less susceptible to the development of bacterial resistance, but they have several limitations, including low *in vivo* stability and bioavailability. The exceptional stability of cyclotides could potentially overcome the drawbacks associated with traditional AMPs, and if a suitably active cyclotide analogue can be found, it might be an attractive alternative to conventional antibiotics.

Along with the various biological activities reported for cyclotides, their stable structure and tolerance for amino acid substitutions[53] has attracted interest in them as scaffolds for protein engineering.[54] In particular, their tolerance for the insertion of biologically active peptide sequences into the CCK framework is an exciting approach to stabilize peptides drug leads. As a proof-of-concept, several bioactive peptide sequences have been successfully grafted into the cyclotide scaffold and maintained biological activity.[55,56] The engineered molecules have the advantage of being stable to acidic conditions and digestive enzymes such as pepsin[56] and are potentially suitable for oral administration.

Some cyclotides have cell-penetrating properties.[57−59] MCoTI-II was the first cyclotide discovered to enter human cells,[57] but recently MCoTI-I[58] and kB1[59] have also been shown to be able to traverse cell membranes. In general, cell-penetrating peptides are regarded as an attractive strategy to deliver molecular cargoes inside cells. The tolerance of cyclotides to substitutions, together with their outstanding stability and cell-penetrating properties, make them particularly attractive as scaffolds to deliver active epitopes that target intracellular sites.

In summary, cyclotides have a range of pharmaceutical applications, not only due to the intrinsic pharmaceutically relevant activities of many native cyclotide sequences, but also as a template for the insertion of biologically active epitopes into their stable molecular framework. Furthermore, some cyclotides are able to penetrate cell membranes, providing a means to reach intracellular targets. Cyclotide bioengineering by rational design will benefit from an elucidation of the mode(s) of action behind the various activities reported for cyclotides. Specifically, it will be important to decrease toxic effects (*e.g.*, hemolysis), optimize biologically desired effects, and improve the delivery and targeting inside cells. In this article we review the mechanism of action of cyclotides, with particular emphasis on membrane interactions.

## ■ ARE THERE COMMON LINKS BETWEEN THE VARIOUS ACTIVITIES?

Besides sharing structural similarities, cyclotides of the bracelet and Möbius subfamilies share some biological activities, including anti-HIV and hemolytic activities.[28] This observation suggests that they might possess common mechanism(s) of action. Conversely, a single cyclotide can have a number of different activities; for instance, kB1 has insecticidal,[18] hemolytic,[60] and anti-HIV properties.[40] Interestingly, when the cyclic backbone of kB1 is disrupted, the molecule loses anti-HIV[40] and hemolytic activities,[61] supporting the suggestion that kB1 might have a common mode of action that leads to these two biological activities. This hypothesis is further supported by an alanine scan of kB1,[62] in which a localized set of single Ala mutations (see Figure 1B) resulted in loss of both hemolytic and insecticidal activities. Overall, an array of evidence supports the conclusion that the anti-HIV, hemolytic, and insecticidal properties of kB1 are correlated and that they share a common mode of action. Both the integrity of the backbone structure and the peptide sequence are important for the biological activities of cyclotides.

The majority of cyclotides have an exposed hydrophobic patch on their surface, and the integrity of this hydrophobic patch has been shown to be necessary for activity, as demonstrated for kB1[63] and varv A.[64] When several natural cyclotides are compared for their cytoprotective effects against HIV infection, a broad correlation between hydrophobic patch geometry and anti-HIV efficiency is evident,[28] suggesting that the hydrophobic properties of cyclotides contribute, in some fashion, to their biological activities.

Studies with dodecylphosphocholine micelles using NMR spectroscopy show that kB1 binds to the micelle surface mainly *via* its surface-exposed patch of hydrophobic residues.[29] A similar binding mode has also been observed for kalata B2,[65] kalata B7,[66] cycloviolacin O2,[65] and varv F.[25] These findings suggest that the importance of the surface hydrophobic patch to


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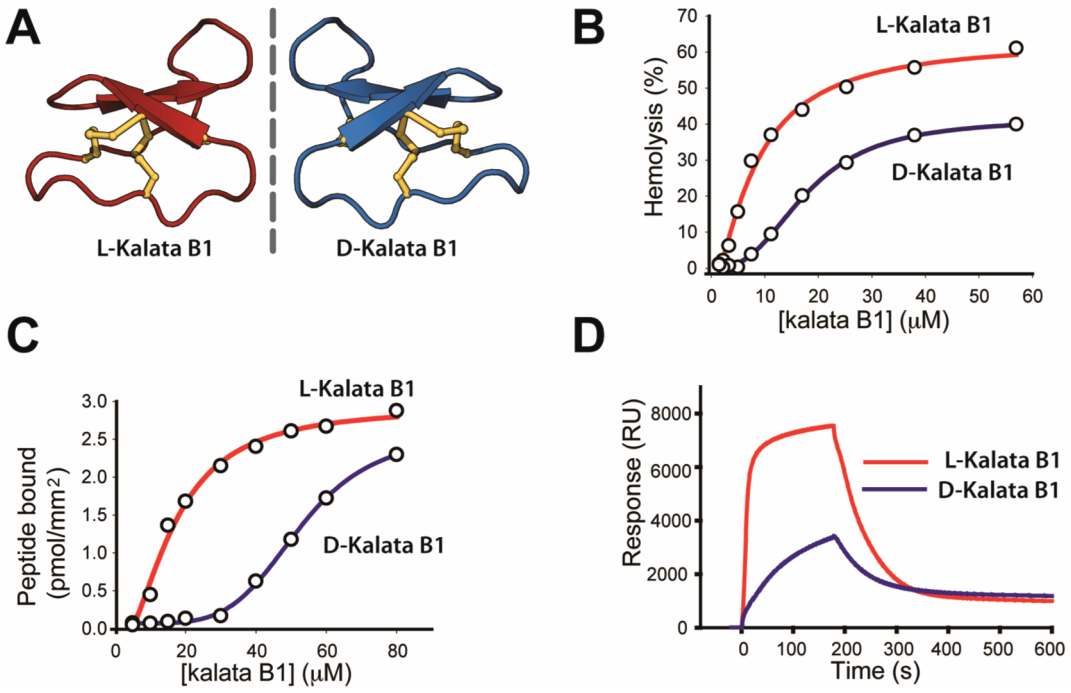


**Figure 2.** Comparison of L-kB1 and D-kB1 peptides with regard to their structure, activity, and membrane-binding affinity. (A) L-kB1 (PDB ID: 1nb1) and D-kB1 (PDB ID: 2jue) are mirror images as determined by $^{1}$H NMR. (B) Hemolytic dose–response curves of the two enantiomers. (C) Amount of L- and D-kB1 bound to POPC/POPE (80:20 molar ratio) lipid membrane. Membrane binding of native kB1 and D-kB1 was studied by SPR. The amount of peptide bound to the membrane was calculated at the end of association phase, as a function of peptide concentration. (D) Comparison of 50 $\mu$M L- or D-kB1 injected for 180 s (association phase) over POPC/POPE (80:20) lipid surfaces deposited on an L1 chip. Dissociation was monitored after the cessation of the injection (dissociation phase). Figure was adapted from ref 70.

biological function might be correlated with the ability of cyclotides to target and disrupt cell membranes.

A mechanism dependent on cell membrane integrity is consistent with the hemolytic properties of cyclotides. Peptides with hemolytic properties are known to insert into the hydrophobic core of erythrocyte membranes.[67] Insertion of cyclotides in insect membranes is supported by transmission electron micrographs of the midgut cells in lepidopteron larvae, in which severe membrane disruption is observed after ingestion of cyclotides.[68] In addition, biophysical studies with model membranes clearly show that cyclotides bind to and disrupt phospholipid bilayers. Surface plasmon resonance (SPR) studies have shown that kB1 binds to pure phospholipid bilayers,[69] and consistent with this binding, fluorescence and electrophysiological measurements reveal disruption of bilayers by a pore-forming mechanism.[49] Moreover, a strong correlation between the membrane-disrupting ability and the bioactivity of cyclotides was demonstrated in a comparison of hemolytic and membrane-lytic properties of Lys mutants of kB1.[49] Similarly, cycloviolacin O2, a member of the bracelet subfamily, has been shown to disrupt membranes in both cultured cancer cells and model membranes.[30]

From the studies conducted to date it is clear that the activities of cyclotides broadly correlate with the presence of a surface-exposed hydrophobic patch and an ability to disrupt cell membranes. Cyclotides belonging to the trypsin inhibitor subfamily are distinguished from Möbius and bracelet cyclotides not only by their lack of sequence homology but also by unrelated and distinct bioactivities. In particular, MCoTI-II is not cytotoxic and does not bind to model membranes,[59] in agreement with a lack of a hydrophobic patch on its surface.

## IS A CHIRAL RECEPTOR INVOLVED IN THE BIOLOGICAL ACTIVITIES OF CYCLOTIDES?

The observation that the hemolytic or insecticidal activities of kB1 can be eliminated or greatly reduced by a single residue mutation anywhere within a localized set of residues[62] could be interpreted to suggest that a recognition process through a receptor might be involved in the activities of kB1. These "bioactive" residues form a patch located on one face of the molecule, known as the bioactive face (see Figure 1B).

To assess the possible involvement of a receptor, a study with an all-D-enantiomer of kB1, a mirror image of the native kB1 (Figure 2A), was recently undertaken.[70] When a peptide–membrane interaction occurs *via* a receptor-dependent mechanism, a specific chirality is required for molecular recognition; therefore, D-enantiomers are not recognized and are inactive. On the contrary, if a peptide–membrane interaction is governed by hydrophobic interactions and is independent of a chiral receptor, both the L- and D-enantiomers are active.[71] Native kB1 was compared with all-D-kB1, and the nematocidal,[35] anti-HIV, cytotoxic and hemolytic properties[70] were evaluated. The all-D-kB1 was active in all of the bioassays, indicating that these biological activities of kB1 are independent of chiral recognition.[35,70] Interestingly, the all-D-kB1 was slightly less effective than all-L-kB1 in all cases tested, illustrated, for example, by the hemolytic response curves in Figure 2B. Nevertheless, the dose–response curves show similar profiles, suggesting that L- and D-isomers promote membrane disruption by an identical mechanism.[70]

The small differences in the biological effectiveness of the L- and D-isomers of kB1 were explained when their ability to bind to membranes was evaluated. Studies using model membranes suggested that kB1 has a preference for phospholipids


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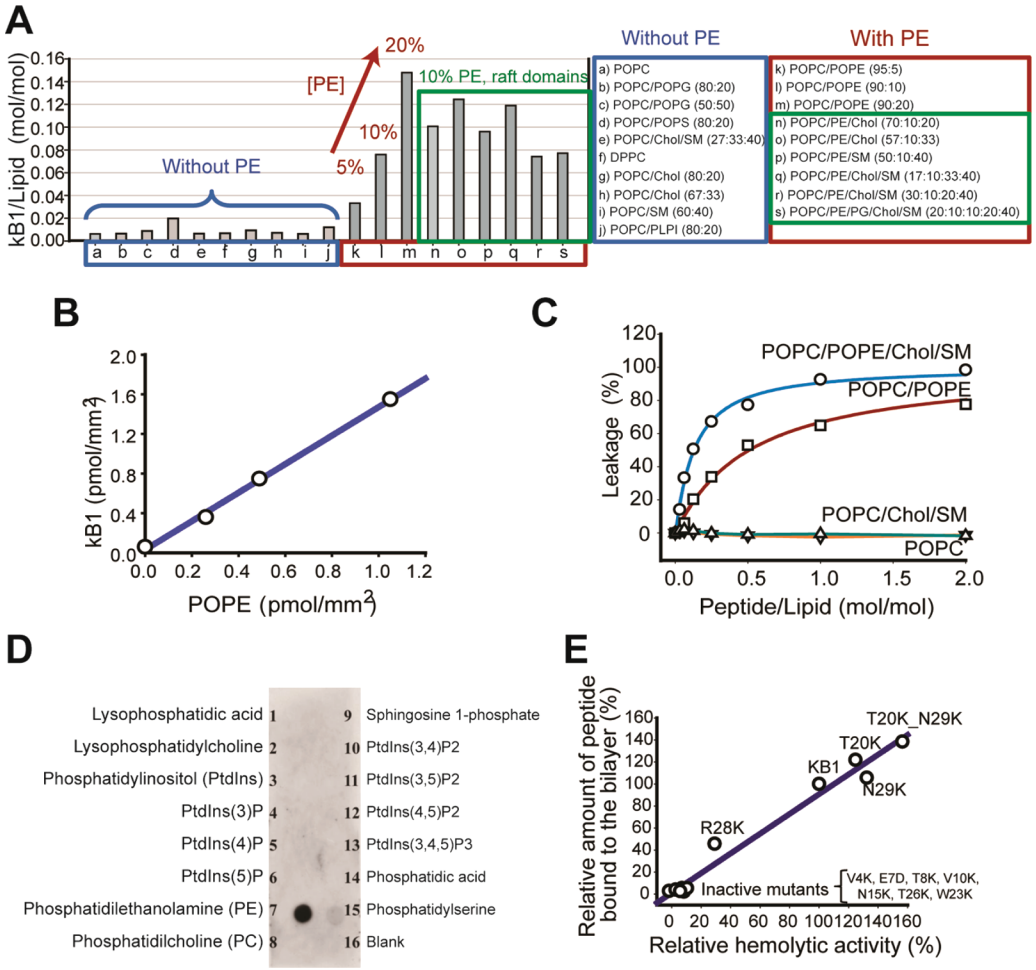


**Figure 3.** Lipid selectivity of kalata B1. (A) The affinity of kB1 for various lipids as followed by SPR is shown. The molar ratio of each lipid in a mixture is indicated. The response was normalized to the total amount of lipid deposited on the chip by calculating peptide-to-lipid ratio on the membrane; see ref 52 for more details on experimental data. The amount of peptide bound to the membrane was low in the absence of POPE (see lipid systems in the blue box) but increases when POPE is present (see lipid systems in the red box). Lipid systems with a fixed amount of POPE (*i.e.*, 10% POPE) that incorporate Chol and/or SM to modulate raft-like domain properties are shown in the green box; see ref 52 for more details. A larger amount of kB1 was found to bind to membranes with properties of raft-like domains when compared with fluid membranes (*e.g.*, POPC/POPE (90:10) in fluid phase *versus* POPC/POPE/Chol/SM (17:10:33:40), a model of raft-like membrane). (B) The amount of kB1 bound to POPC or POPC/POPE (95:5, 90:10 or 80:20) bilayers is plotted *versus* the amount of POPE on the chip surface. In these conditions, all of the lipid systems are in the fluid phase and a strong linear correlation is seen. (C) Vesicle leakage efficiency induced by kB1 for various lipid compositions (POPC, POPC/Chol/CM (27:33:40), POPC/POPE (90:10), and POPC/POPE/Chol/SM (17:10:33:40)). (D) Binding of kB1 with a nitrocellulose membrane containing various lipids (PIP strip, see ref 59 for more details). (E) The affinity of kB1 mutants for the lipid bilayer dictates its hemolytic efficiency. The amount of peptide bound to model membranes composed of POPC/POPE/Chol/SM (17:10:33:40) is plotted against the relative hemolytic activity. Native kB1 was compared with either Lys mutants that were more active (T20K, N29K, and T20K–N29K) than kB1 or less active (R28K) than kB1 or were inactive (V4K, E7D, T8K, V10K, N15K, V16K, W23K, and V25K). Figure adapted from ref 52.

containing phosphatidylethanolamine (PE) headgroups.[49,69] Therefore, membranes in the fluid phase composed of pure palmitoyloleoylphosphatidylcholine (POPC) or a mixture of POPC/palmitoyloleoylethanolamine POPE (80:20 molar ratio) were compared.[70] Weak binding to POPC was observed; however, a significant increase in the binding to POPC/POPE bilayers was evident for both isomers, confirming the importance of PE headgroups for kB1 binding. Although the two mirror image kB1 isomers have identical sequences, hydrophobicity, and three-dimensional structures, there are differences in binding properties to the POPC/POPE bilayers (Figure 2C and D). Specifically, the L-kB1 binds faster and has an increased affinity for the bilayer compared with the D-isomer.[70] The differences in the binding affinity for the native and all-D-kB1 isomer suggest that the weak chiral environment in the phospholipid bilayer is sufficient to induce some chiral discrimination. In particular, phospholipids containing PE headgroups, which possess one chiral center, might be responsible for this discrimination.[70] These studies collectively exclude a chiral protein as a requirement for kB1 activity and instead suggest that efficiency is dependent on affinity for the lipid membrane.[70]

## HOW IMPORTANT IS THE LIPID COMPOSITION FOR THE BIOLOGICAL ACTIVITIES OF CYCLOTIDES?

**Affinity for Lipid Model Membranes and Lipid Selectivity.** Studies with model membranes suggest that cyclotides bind to membranes but display lipid selectivity.[49,69] Recently, kB1–membrane binding affinity was systematically


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scrutinized,[52] and the effects of the phospholipid headgroup, charge, fluidity, and membrane organization were evaluated, as well as lipid selectivity being examined. The presence of PE phospholipids was shown to be a requirement for kB1 membrane binding (Figure 3A and B), and an increase in the binding with the amount of PE was evident, for membranes in a fluid phase environment. When membranes with a similar PE content but different fluidities were compared, kB1 had a higher affinity for membranes in the liquid disordered phase (*i.e.*, more rigid membranes rich in cholesterol (Chol) and sphingomyelin (SM) that are raft-like domains) than fluid bilayers (Figure 3A, see POPC/POPE (90:10) in fluid phase *vs* POPC/POPE/Chol/SM (17:10:33:40) a model of a raft-like domain), revealing that the environment surrounding PE phospholipids can further modulate the membrane-binding affinity of kB1. This lipid selectivity was confirmed by membrane leakage studies (Figure 3C). Specific binding with PE-containing phospholipids, but not with other tested phospholipids, was further supported by studies of binding to nitrocellulose membranes containing phospholipids with a range of headgroups (Figure 3D).[59] In summary, whereas a specific interaction between kB1 and phospholipids containing PE headgroups is essential for efficient membrane targeting, the surrounding lipid composition further modulates the insertion of more peptide.[52]

A parallel between membrane-affinity and activity was clearly established when kB1 analogues with different hemolytic efficiencies were compared.[52] Nonhemolytic kB1 analogues were unable to bind to lipid membranes even when PE phospholipids are present, whereas kB1 analogues with stronger hemolytic properties had an increased affinity for the membrane.[52] Figure 3E exemplifies the correlation between hemolytic efficiency of a series of kB1 mutants and affinity for a phospholipid membrane of a given lipid composition. Interestingly, both the surface-exposed hydrophobic patch and the bioactive patch on kB1 seem to be important for membrane-targeting, as mutations in the hydrophobic properties of kB1 (*e.g.*, W23K or V10K) and in the bioactive patch (*e.g.*, E7D or T16K) not only affect the hemolytic properties of kB1 but also make the peptide unable to bind to the lipid membrane.[52] NMR studies suggest that the bioactive patch is important to target PE headgroups,[52] whereas the hydrophobic patch is required for insertion in the hydrophobic core of the membrane.[29] Kalata B1 is a Möbius cyclotide. Another recent study on the bracelet cycloviolacin O2[72] showed that it has the ability to extract PE phospholipids from model membranes. This result suggests that PE headgroups are also involved in the mechanism of action of cyclotides belonging to the bracelet subfamily and further supports the hypothesis that there are similarities between the mechanisms of action of a wide range of cyclotides.

Overall, biophysical studies with model membranes show that (i) kB1 has membrane selectivity; (ii) the mechanism of action of kB1 correlates with its ability to target and disrupt the lipid bilayer; and (iii) the membrane-binding affinity of kB1 and its analogues is strongly correlated with their biological potency.

**Model Membranes *versus* Biological Membranes.** Different organisms have different membrane characteristics, and we proposed the hypothesis that differences in the biological efficiency of cyclotides might be explained by differences in affinity for the cell membrane of target organisms. To test this hypothesis, the ability of kB1 to target organisms with distinct biological membranes, including mammalian (red blood cells, RBCs), bacterial, and viral (HIV) species, was evaluated and correlated with the studies using model membranes.[52]

The hemolytic properties of kB1 have been reported in several studies, which confirm its ability to target, insert, and permeabilize RBCs. As noted above, kB1 membrane-active analogues have hemolytic activity, whereas membrane-inactive analogies are not hemolytic (*e.g.*, N29K *vs* V25K mutants in Figure 4A). The RBC membrane is asymmetric whereby the

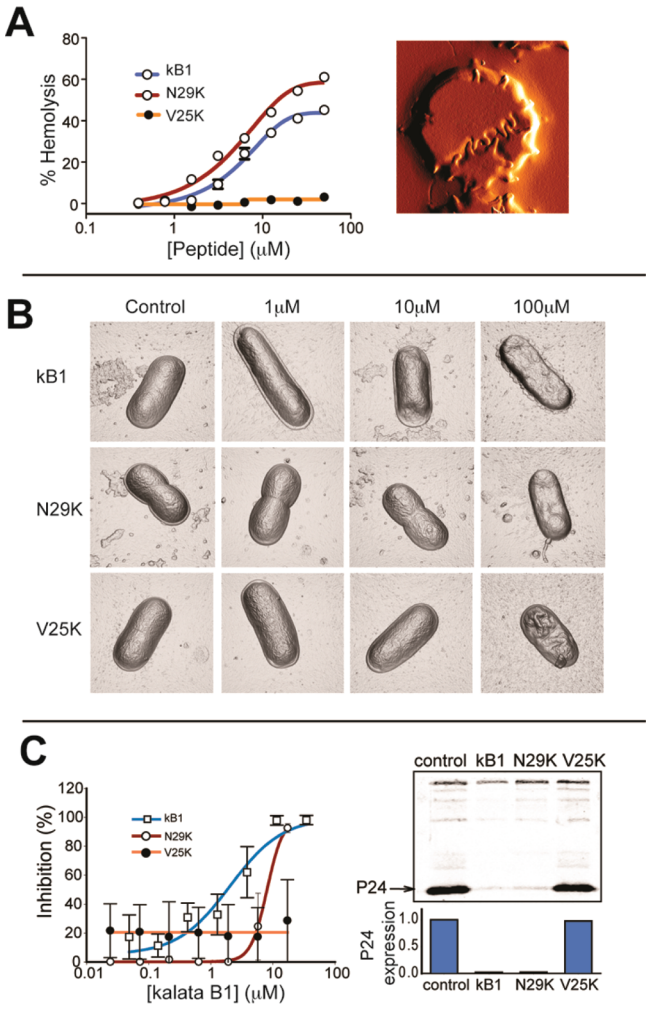


**Figure 4.** Binding of kB1 to biological membranes. (A) Hemolysis dose−response curves for kB1 and its analogues N29K and V25K. Insert shows an AFM image of a RBC after incubation with kB1. (B) The effect of kB1 and its analogues on the shape of *Escherichia coli* followed by AFM. (C) Inhibition activity of kB1 and its analogues against HIV-1 NL4.3. The Western blot shows the detection of the protein P24 after treatment of NL4.3 virus particles with 50 $\mu$g/mL kB1 and its analogues. A low level of viral capsid protein p24 indicates removal of membrane envelope and disruption of the particle. Figure adapted from ref 52.

largest amount of PE-containing phospholipids is located in the inner layer, with only a small proportion of PE phospholipids exposed in the outer layer.[73] However, RBCs have a large amount of Chol and SM in the outer leaflet, which segregate and form more rigid domains (raft-like domains) in the main fluid phase of the cell membrane.[74,75] Based on the membrane properties of RBCs, our hypothesis is that kB1 targets the


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membrane of RBCs through the small percentage of PE exposed in the outer layer and that the raft domains facilitate the insertion of more peptide molecules in the lipid membrane. This hypothesis is supported by atomic force microscopy (AFM) imaging and model membrane studies.[52]

Upon addition of kB1 to RBCs, a change from the typical round biconcave shape to a crenated shape is evident, as shown in Figure 4A.[52] The crenated shape reveals that kB1 inserts into the outer layer, inducing expansion and redistribution of phospholipids. This loss in the physiological lipid membrane asymmetry implies that a larger amount of PE is exposed in the outer membrane and that PC phospholipids and SM are translocated to the inner layer.[76] Thus, after insertion into the outer membrane, kB1 induces the transbilayer movement of phospholipids, attracting more PE-phospholipids to the outer membrane and promoting the insertion of more kB1 molecules. This suggestion is supported by model membrane studies, as outward movement of PE phospholipids is observed when kB1 is added to a raft-like model membrane composed of a small percentage (1%) of PE headgroups but with Chol and SM. These results show that kB1 is capable of self-promoting its binding by increasing the amount of PE exposed in the outer layer.[52]

With regard to bacterial membranes, we noted above that cyclotides have been described in the literature as AMPs, but few cyclotides have actually been tested for their antimicrobial properties[45,46,51] and potent activity at physiological salt conditions has been obtained only for cycloviolacin O2.[51] Generally, AMPs are cationic peptides with a preference toward negatively charged bacterial membranes over neutral eukaryotic membranes. Membrane-active AMPs exert their action through adsorption at the polar-interface region of the bacterial membrane, mediated by electrostatic attractions. Insertion of AMPs into the hydrophobic core can occur but is not a requirement.[77] Bacterial cells are characterized by an anionic outer cell wall and inner membrane. The inner membrane is negatively charged and can be very rich in PE headgroup phospholipids; nevertheless, this inner membrane is only accessible to AMPs after they have overcome the anionic outer cell wall, enriched with lipopolysaccharide in Gram-negative bacteria or with the anionic peptidoglycan in the Gram-positive bacteria.

A study of kB1 against *Escherichia coli* (Gram-negative) and *Staphylococcus aureus* (Gram-positive) revealed its weak antimicrobial activity, as no effect in inhibition growth of these bacteria was detected in the presence of up to 100 $\mu$M peptide.[52] The inability to target the bacterial membrane was further confirmed by AFM imaging (Figure 4B); effects on bacterial shape were only visible at the highest concentration tested, 100 $\mu$M, but were distinct from the strong effects observed with other AMPs.[78] In addition to a weak effect, the activity is independent of the membrane-activity of kB1 as membrane-active and membrane-inactive analogues have similar effect. This weak antimicrobial activity of kB1 was expected, as this peptide has a neutral global charge and no preference for negatively charged membranes; hypothetically, kB1 can target the bacterial inner membrane rich in PE but cannot bind to the outer membrane, as shown using model membranes that mimic the inner and outer membranes of *E. coli*.[52]

The results obtained with RBCs and bacterial cells confirm the correlation between affinity for a target cell membrane and the biological potency of cyclotides. Briefly, the activity of kB1 is independent of electrostatic attractions between the peptide and the negatively charged components of the cell membrane; however, it is dependent on the presence of exposed PE phospholipids in the outer layer and hydrophobic interactions. After membrane targeting through binding to PE phospholipids, kB1 inserts into the membrane's hydrophobic core.[52]

**Anti-HIV Activity of Cyclotides.** For HIV infection to progress the virus must deliver its genome into the host cell where it will be replicated; the infection process is initiated by receptor recognition at the surface of the host cell and is followed by fusion of the viral and host membranes. Once inside the cell, the HIV reverse transcriptase is responsible for genome replication. From the literature, it is known that cyclotides have no detectable effect on HIV reverse transcriptase activity;[41] the anti-HIV activity of cyclotides is correlated with the presence of the surface-exposed hydrophobic patch;[28] and the all-D-kB1 isomer also has anti-HIV activity.[70] These findings suggest that the cytoprotective effect occurs before the entry of the virus into the cell by a mechanism dependent on cyclotide-membrane binding but independent of receptor inhibition.[50] Nevertheless, until recently it was not clear whether the anti-HIV mechanism was due to cyclotide binding to membranes in the host cell, the virus, or both.

The importance of membranes for the anti-HIV activity of kB1 was delineated when it was shown to have virucidal activity against two different strains of HIV. Similar virucidal activity against two strains was obtained with a membrane-active and hemolytic analogue of kB1, N29K, whereas the membrane-inactive analogue, V25K, had no effect (Figure 4C). Furthermore, kB1 and the active analogue were shown to disrupt the viral particles, whereas the membrane-inactive analogue was unable to do so (Figure 4C). These results are consistent with kB1 targeting the HIV membrane by a mechanism independent of a receptor but dependent on peptide–membrane binding properties.

The membrane of HIV particles is formed from raft domains present in the host cell, and therefore HIV particles have a rigid bulk phase and are often described as a big raft.[79] In addition, the HIV membrane is rich in PE phospholipids.[79,80] As noted already, kB1 is able to target and disrupt model membranes containing PE phospholipids, and this capability is even more pronounced when the membrane is rich in Chol and SM, having properties of a raft-like membrane. These results support the virucidal activity of kB1 and suggest that kB1 will select HIV particles over eukaryotic cells, which have a lower percentage of PE exposed than HIV particles. A preference for HIV particles over eukaryotic cells is supported by model membrane studies and is in agreement with the reported therapeutic index of 25 (*i.e.*, the ratio of therapeutic to toxic effects) for cyclotides.[40]

To the best of our knowledge, kB1 is the first reported peptide with an ability to disrupt HIV particles. Other membrane-active peptides with anti-HIV activity have been described, *e.g.*, T20 or Fuzeon, T1249 or sifuvirtide, but in these cases the membrane-binding aspects[81,82] of the anti-HIV mechanism are *via* inhibition of receptor recognition. That is, they inhibit the interaction of HIV with the gp41 complex and the fusion of the viral membrane to the host cell.[83] That kB1 inactivates HIV by membrane-targeting opens up a range of promising potential applications for this peptide, as well as other cyclotides, as mutation or replacement of lipids by HIV

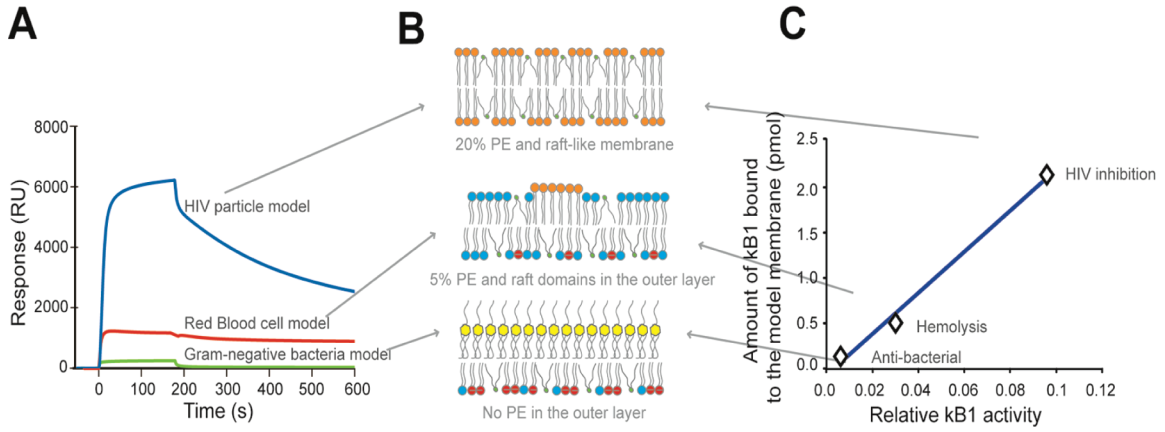


**Figure 5.** The ability of kB1 to target cell membranes determines its biological activity against different organisms. (A) The membrane binding of kB1 for membranes that mimic Gram-negative outer membrane (LPS), red blood cell outer layer (POPC/POPE/Chol/SM (22:5:33:40)), and viral particle membranes (POPC/POPE/Chol/SM (17:20:33:40)) followed by SPR. (B) Models of the lipid composition of HIV particle envelope, RBC, and Gram-negative bacteria outer membrane. Orange headgroups represent a raft-like membrane, green headgroups represent phospholipids with PE headgroups, blue headgroups represent phospholipids with PC headgroups, red headgroups represent phospholipids with negatively charged headgroups, and yellow represents the LPS layer). (C) The amount of peptide bound to the lipid systems was calculated from the sensorgrams in panel A and plotted against their relative bioactivity. The relative activity of kB1 was estimated relative to the activity of peptides with high activity (*i.e.*, mellitin as a hemolytic peptide, BP100 as an antimicrobial and T20 as an inhibitor of HIV).

particles is virtually impossible. Therefore, cyclotide-resistant strains of HIV are unlikely to develop.

Overall, the results obtained with model membranes are corroborated with the activity of kB1 against RBCs, bacteria, or HIV (Figure 5). The membrane properties that were identified to be preferred by kB1, in particular the amount of PE exposed at the membrane surface, the importance of rigid domains in facilitating the hydrophobic interactions, and the weak contribution of electrostatic interactions, are correlated with the efficiency against the three target membranes. In summary, kB1 does not have a preference for the negatively charged bacterial membranes that lack exposed PE phospholipids. By contrast, it is able to target PE phospholipids in RBC membranes, insert into the hydrophobic core, and induce membrane permeabilization. It targets HIV particles because they have a large percentage of PE exposed in their membrane and have a characteristic liquid-ordered phase.

## CONCLUSIONS

There are now many studies suggesting that the mechanism of action of cyclotides broadly correlates with their ability to bind to and disrupt membranes in target cells. Here we have examined the importance of the cell membrane in the biological actions of cyclotides, with particular emphasis on the prototypic cyclotide kB1, whose mechanism of action and membrane activity have been explored in the literature.

The evidence supports the conclusion that the various activities of kB1 are independent of chiral protein recognition[70] but are dependent on its affinity for lipid bilayers (see Figures 2−5) and ability to disrupt cell membranes.[49] Briefly, the mechanism of action of kB1 can be described in four steps: kB1 (i) targets membranes through specific interactions with phospholipids with PE headgroups, (ii) inserts in membranes through nonspecific hydrophobic peptide-lipid interactions, and (iii) promotes outward movement of PE phospholipids, exposing more PE in the outer leaflet and self-promoting the binding of more cyclotide; and (iv) when a threshold concentration is achieved in the membrane, the cyclotides self-aggregate, inducing pore formation and eventual membrane disruption.

Although cyclotides belonging to both bracelet and Möbius subfamilies have been shown to be active against similar cell targets (*e.g.*, hemolytic and anti-HIV activities have been reported for both subfamilies), the efficiency is dependent on both the target organism and on the cyclotide. Therefore, the existence of a single mechanism that explains all of the activities of all of the cyclotides is somewhat simplistic. Different cyclotides have different hydrophobic and electrostatic properties and might, therefore, have different lipid selectivity and diverse membrane-disruption mechanisms. For example, kB1, which is globally neutral, has weak antimicrobial activity, whereas cycloviolacin O2, with a global charge of +2, has antimicrobial activity against Gram-negative bacteria, revealing that electrostatic interactions might be important for cycloviolacin O2 activity. These results suggest that differences in hydrophobicity and hydrophilic properties of cyclotides might preferentially target one organism over another.

The hypothesis that different cyclotides have distinct membrane preferences would explain why an individual plant produces a suite of cyclotides, *i.e.*, to provide the plant with defense molecules that can attack a broad spectrum of offensive organisms, each with distinct membrane properties. The differences in activity of these cyclotides would be modulated by membrane-binding affinity.

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### Notes

The authors declare no competing financial interest.

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## KEYWORDS

Anti-HIV peptide: peptide active against the human immunodeficiency virus; Antimicrobial peptide: peptide active against bacteria or fungi; Circular protein: protein with a cyclic backbone; Cyclic peptide: peptide with a cyclic backbone; Drug design: the process of designing new drugs; Kalata B1: the first cyclotide molecule discovered; Lipid selectivity: the phenomenon of a molecule selectively binding to a particular lipid type; Protein mirror image: a protein which is composed of all D-amino acids; Peptide membrane interactions: interactions between peptides and membranes; Pore formation: the formation of pores in lipid membranes

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