Discovery and mechanistic studies of cytotoxic cyclotides from the medicinal herb *Hybanthus enneaspermus*

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ABSTRACT

Cyclotides are plant-derived peptides characterized by a ~30-amino-acid-long cyclic backbone and a cystine knot motif. Cyclotides have diverse bioactivities, and their cytotoxicity has attracted significant attention for its potential anticancer applications. *Hybanthus enneaspermus* (Linn) F. Muell is a medicinal herb widely used in India as a libido enhancer, and a previous study has reported that it may contain cyclotides. In the current study, we isolated 11 novel cyclotides and one known cyclotide (cycloviolacin O2) from *H. enneaspermus* and used tandem MS to determine their amino acid sequences. We found that among these cyclotides, hyen C comprises a unique sequence in loops 1, 2, 3, 4, and 6 compared with known cyclotides. The most abundant cyclotide in this plant, hyen D, had anticancer activity comparable to that of cycloviolacin O2, one of the most cytotoxic known cyclotides. We also provide mechanistic insights into how these novel cyclotides interact with and permeabilize cell membranes. Results from surface plasmon resonance experiments revealed that hyen D, E, L, M, and cycloviolacin O2 preferentially interact with model lipid membranes that contain phospholipids with phosphatidyl-ethanolamine headgroups. The results of a lactate dehydrogenase assay indicated that exposure to these cyclotides compromises cell membrane integrity. Using live-cell imaging, we show that hyen D induces rapid membrane blebbing and cell necrosis. Cyclotide–membrane interactions correlated with the observed cytotoxicity, suggesting that membrane permeabilization and disintegration underpin cyclotide cytotoxicity. These findings broaden our knowledge on the indigenous Indian herb *H. enneaspermus* and have uncovered cyclotides with potential anticancer activity.

INTRODUCTION

Cyclotides are a large family of cysteine-rich peptides, ~30 amino acids in size, derived from plants (1). They have attracted great attention from both academia and industry since they were first reported in the 1990s. In 2017, Sero-X, which is a cyclotide-based eco-friendly pesticide extracted from *Clitioria ternatea* (2), was approved to market in Australia for the protection of cotton and macadamia nut crops (https://innovate-ag.com.au). In 2019, a single-residue mutated variant of cyclotide kalata B1, [T20K]kalata B1, entered Phase I clinical trials for multiple sclerosis (3). These examples foreshadow the great potential of cyclotides in both agriculture and medicine.
Cyclotides have a role in plant defense, acting as insecticides or anti-pathogenic agents (4,5). They also exhibit a diverse range of other biological properties (6), including anti-fouling (7), molluscicidal (8), uterotonic (9), anti-HIV (10), antimicrobial (11), cytotoxic-anticancer (12), immunosuppressive (13), and protease inhibitory activities (14,15). Cyclotides are characterized by a head-to-tail cyclic backbone and a cystine knot motif, which renders them ultra-stable against degradation by harsh conditions and are also amenable to residue substitutions (16-18). These properties make cyclotides ideal scaffolds for grafting bioactive epitopes (19), with potential applications in the agricultural and pharmaceutical industries.

To date, cyclotides have been identified in five major plant families, namely the Violaceae, Rubiaceae, Cucurbitaceae, Solanaceae, and Fabaceae (20-22). They are categorized into three subfamilies (bracelet, Möbius, and trypsin inhibitor), with archetypical cyclotides (23-25) shown in Fig. 1. A distinctive structural feature of the Möbius subfamily is a 180° twist in the main chain structure, which originates from a Pro in loop 5 adopting a cis conformation (16). The majority of cyclotides discovered to date are from the bracelet and Möbius subfamilies; trypsin inhibitor cyclotides have been found only in the *Momordica* genus from the Cucurbitaceae family (14,26).

In contrast to other plant families, every species in the Violaceae family seems to produce cyclotides (27,28). Of the 433 cyclotides described in CyBase (http://www.cybase.org.au/), 259 occur in violaceous plants (29,30). The violaceous herb *Hybanthus enneaspermus* Linn F. Muell (*H. enneaspermus*) is an Indian traditional medicinal herb with a wide range of medicinal applications, including anticonvulsant (31), free radical scavenging (31), nephroprotective (32), antiarthritic (33), larvicidal (34), anti-anemic (35), antidiabetic (36), hepatoprotective (37,38), and aphrodisiac activities (39). A previous study suggested the existence of cyclotides in this plant based on mRNA analysis, but only two partial sequences were reported (40). Therefore, we aimed to further explore the cyclotide content of *H. enneaspermus* and to evaluate their bioactivity, specifically, their cytotoxic activity against transformed cells.

The cytotoxic activity of cyclotides has attracted interest for their potential anti-cancer applications (41,42). Two Möbius cyclotides (varv A and varv F), and a bracelet cyclotide (cycloviolacin O2, cyO2), have been identified as having strong cytotoxic activity against 10 different human tumor cell lines (43). Other cyclotides with anti-tumor effects include vitri A (44), vibi (E, G and H) (12), psyle (A, C and E) (42), viphi (A, D–G) (45), mram 8, viba (15 and 17), varv (A and E) (44,45), vitri F (46), mela (1–7), mech (2 and 3) (47), vila (A and B) (48), Poca (A and B), cyO4 (49), kalata B1 and B2 (50). Previous studies indicate cyclotides exert their cytotoxic activity by disrupting the lipid bilayer of cell membranes (51,52). In particular, cyclotides interact with cells by specifically binding to phospholipids containing phosphatidylethanolamine (PE)-headgroups via interaction with a conserved patch of amino acid residues, called a bioactive patch, and insert into the lipid bilayer via a hydrophobic patch on their surface (50,53-55).

In this study, we sequenced 12 cyclotides in plant material from *H. enneaspermus* for structural and activity characterization, of which 11 were novel. Five of the novel cyclotides demonstrated potent cytotoxicity, which correlated with their affinity for lipid membrane bilayers. This work demonstrates that the medicinal plant *H. enneaspermus* is a rich source of cyclotides with cytotoxic activity.

**RESULTS**

**Isolation and sequencing of cyclotides from *H. enneaspermus*** — A total of 12 cyclotides were isolated from the leaves, stems, and seeds of *H. enneaspermus*. Each cyclotide showed a mass increment of 348 Da after reduction and S-alkylation with iodoacetamide, suggesting the existence of three disulfide bonds, and had a mass increment of 18 Da after digestion with endoproteinase Glu-C, indicative of a cyclic backbone. They were fully sequenced using tandem MS (Table 1). Eleven of these cyclotides have novel sequences and were named ‘hyen’ (*Hybanthus enneaspermus*) according to a previously reported nomenclature system (40,56), and designated as hyen C to hyen M in order of their appearance on RP-HPLC (Fig. 2). One of these peptides has an identical sequence to the previously characterized cyclotide cyO2. Hyen A and hyen B are two partial mRNA transcripts reported earlier from the same plant (40); however, due to their low expression levels, only the sequence of hyen B could be partially identified in this study. Hyen C was the only Möbius cyclotide isolated from *H. enneaspermus*. To illustrate the sequencing of hyen peptides, hyen D, the most abundant cyclotide in this plant, is shown as a representative example (Fig. 3).
We assembled the transcriptome using reported RNA-Seq data of *H. enneaspermus* roots and shoots (57), and identified 93 putative cyclotide sequences (Table S1). Most (71/93) of these putative cyclotides do not contain a Pro residue in loop 5 and were classified as bracelet cyclotides; only six putative Möbius cyclotides were identified in the transcriptome, with another 16 acyclic cyclotides that cannot be classified into either subfamily. We further explored the peptidome of *H. enneaspermus* by matching the masses of proteinase fragments of crude extracts with the transcriptome using ProteinPilot™ software. The sequences of 13 cyclotides were fully matched and 34 were partially identified (labeled in dark green and blue, respectively, in Table S1), representing approximately 50% of the 93 putative cyclotides identified from our transcriptome analysis. Of the 12 cyclotides isolated and sequenced de novo using MS from *H. enneaspermus* (Table 1 and the first 12 in Table S1), hyen C, D, E, F, M, and cyO2 were fully found in the transcriptome and the other six were partially identified in the transcriptome (labeled in light green in Table S1). We postulate that hyen G could be a post-translational modification of hyen F through the deamidation of Asn to Asp in the last position of its sequence. Overall, our analysis suggests *H. enneaspermus* might express up to 53 cyclotides (the first 53 in Table S1).

**Structural analysis of cyclotides from *H. enneaspermus* by NMR** — Hyen C is the only Möbius cyclotide identified at the peptide level in *H. enneaspermus*. It has a unique sequence in loops 1, 2, 3, 4, and 6 compared to other known cyclotides, making it particularly interesting as it expands the diversity of cyclotide sequences. We consequently decided to examine its structure using NMR spectroscopy. As hyen C could not be isolated from the plant in sufficient quantity to allow this analysis, we produced it using solid-phase peptide synthesis. Synthetic hyen C showed a similar profile on RP-HPLC to its native counterpart, suggesting that the synthetic variant achieved its native fold (data not shown). Of the 12 cyclotides that were identified using de novo sequencing, only five of them, namely hyen D, E, L, M, and cyO2, could be isolated in relatively high amounts from *H. enneaspermus*. These five peptides, as well as synthetic hyen C, were further studied as representatives of hyen cyclotides.

Total correlation spectroscopy (TOCSY) and NOESY NMR spectra were recorded for synthetic hyen C and isolated hyen D, E, L, and M, as well as cyO2. The peaks in the amide region were dispersed, suggesting the corresponding peptides adopt a well-defined fold (examples of two-dimensional spectra of hyen D are shown in Fig. S1). The secondary αH chemical shifts were calculated by subtracting the 1H NMR chemical shifts from those measured in random coil peptides (58), providing information on the secondary structure content of the hyen cyclotides. Hyen D and E have similar secondary structure elements to cyO2 (24), with which they share 67–80% sequence identity, except for loop 3 that is more variable in sequence. The secondary αH chemical shifts of hyen L and M were similar to those reported for cyO1 (16), consistent with the high sequence identity shared between these peptides (93% and 89%, respectively; Fig. 4). CyO2 and cyO1 are both 30 amino acids in length; however, cyO2 has six residues in loop 3 and six residues in loop 6, whereas cyO1 has seven residues in loop 3 and five residues in loop 6. Hyen C shares 65% and 60.9% sequence identity with kalata B7 (kB7) and kB1, respectively. As shown in Fig. S2, the overall secondary chemical shifts of hyen C were similar to those reported for kB7 (59) and kB1 (60), except for loop 2, which has a different sequence and length. Finally, the secondary αH chemical shifts of the Cys residues for hyen C, D, E, L, and M were similar to kB1, cyO2, and cyO1, indicating they all have stable structure and the cystine knot motif (CysⅠ–CysⅣ, CysⅡ–CysⅤ, and CysⅢ–CysⅥ) is highly conserved.

Since hyen D is the most abundant cyclotide in *H. enneaspermus*, it was studied as a representative to further understand the involvement of hydrogen bonds in stabilizing the overall structure. Amide proton temperature coefficients (ΔαNH/ΔT) were determined at pH 3.66 and 5.75 (Fig. S3). A total of 15 residues were observed to be involved in the formation of hydrogen bonds and acted as hydrogen donors. A homology model of hyen D was made based on the cyO2 structure (24) in Modeller 9v20 and visualised in PyMOL; the hydrogen bond acceptors of these residues are presented in Table S2. Overall, the predicted hydrogen bonds in the hyen D homology model were consistent with the amide temperature coefficients. These hydrogen bonds are thought to be critical for stabilizing the overall structure of hyen D. We also built homology models of the other hyen peptides based on cyO1 (16), cyO2 (24) and kB1 (23) structures and calculated their electrostatic potential, as shown in Fig. 6 and 7.

**Cytotoxic and hemolytic activity of cyclotides from *H. enneaspermus* —** The cytotoxicity of hyen C, D, E, L, M, cyO2, and kB1 (the prototypical cyclotide with known toxicity) against cancer (MCF-7, HeLa, and K562) and normal (HUVEC) cells was evaluated (Table 2 and Fig.
S4). Hyen D exhibited slightly stronger cytotoxicity than cyO2, which is one of the most cytotoxic cyclotides reported so far (61). Hyen D displayed more than three-fold higher cytotoxic activity compared to hyen E in all cell lines tested, and more than two or three-fold higher cytotoxic activity compared to hyen L and M against K562 or MCF-7 cell lines, respectively. With regard to selectivity, no obvious cancerous versus non-cancerous cell selectivity was observed in the cyclotides tested. Hyen C was not cytotoxic to any of the cell lines in the tested concentration range. In summary, the six tested cyclotides can be ranked according to their cytotoxic activity as follows: hyen D > cyO2 > hyen M > hyen L > hyen E > kB1 > hyen C.

Some cyclotides are known to lyse human red blood cells (RBCs) (62), which reduces their potential therapeutic value as anti-cancer agents. Therefore, we determined the hemolytic activity of hyen D, E, L, M, and cyO2 (Table 2 and Fig. S5). Among the cyclotides tested, hyen D exhibited the highest hemolytic activity, followed by cyO2; other cyclotides exhibited less than 50% hemolysis at 50 μM. These hemolytic results were congruent to the cytotoxicity assay, as the two most cytotoxic cyclotides, hyen D and cyO2, were also the two most hemolytic. Despite this, hyen D and cyO2 exhibited approximately 20-fold higher potency against cancer cells than that for RBCs, suggesting that they have potential for therapeutic applications.

Membrane binding properties of cyclotides from H. enneaspermus — The main structural lipids in eukaryotic membranes are glycerophospholipids, including phospholipids containing phosphatidylcholine (PC)-, phosphatidylethanolamine (PE)-, phosphatidylserine (PS)-, phosphatidylinositol (PI)-, and phosphatidic acid (PA)-headgroups. Phospholipids containing PC-headgroups account for over 50% of the phospholipids in the eukaryotic membranes (63), hence the zwitterionic phospholipid 1-palmitoyloleoylphosphatidylcholine (POPC), which is commonly found in mammalian cell membranes and forms bilayers in fluid phase at 25°C, was chosen to model the overall neutral charge and fluidity of the outer leaflet of healthy mammalian cell membranes. In addition, several other model membranes were used to investigate the influence of other lipids on cyclotide-membrane interactions. In particular, POPE was included in our experiments, as PE-containing phospholipids have been suggested to be crucial for membrane binding of other cyclotides (62). The anionic phospholipid POPS was also included in our tests to evaluate whether electrostatic interactions facilitate binding of cyclotides onto lipid bilayers. Peptide-membrane binding affinity was compared by the peptide-to-lipid maximum binding (P/Lmax, mol/mol), the peptide concentration required to achieve half-maximum binding at equilibrium, i.e., the KD value (μM), and the dissociation rate (kd, s⁻¹) (Fig. 5 and Table 3).

All tested cyclotides, except hyen C, exhibited a strong preference for membranes containing POPE (Fig. 5C), as previously reported for other cyclotides (62,64), with a linear increase in P/Lmax as the POPE composition increases (Fig. 5D). Meanwhile, the cyclotides did not bind well to membranes composed either of pure POPC or POPC/POPS (80:20 molar ratio) (Fig. S6). To further investigate whether anionic lipids facilitate cyclotide binding, cyO2, hyen D and hyen L were evaluated with model membranes containing both POPE and POPS (POPC/POPE/POPS, 60:20:20) (Fig. 5F). There was a slight increase in P/Lmax and a decrease in KD of three tested cyclotides in binding onto POPC/POPE/POPE, compared to POPC/POPE membranes. In conclusion, all the tested cyclotides showed a preference for lipid bilayers containing POPE phospholipids, and the incorporation of POPS facilitate the binding of cyclotides, potentially through electrostatic attractions. Hyen C is an exception and did not bind to any of the lipid membrane compositions tested. Overall, the cyclotides from H. enneaspermus can be ranked according to their ability to bind POPC/POPE (80:20) membranes as follows: hyen D > cyO2 > hyen M ≈ hyen L > hyen E > hyen C.

LDH assay and SYTOX Green microscopy imaging — To investigate whether these cyclotides compromise cell membrane integrity, HeLa and HUVEC cells were treated with cyclotides for 2 h and the enzymatic activity of cytosolic lactate dehydrogenase (LDH) leaked into medium was measured. The results are shown in Table 2 and Fig. S4B. The LDH results suggest that hyen D, E, L, M, cyO2 and kB1 induce cell membrane leakage within 2 h. To gain insights into the mechanism of membrane disruption, the morphology of HeLa cells upon treatment with hyen D, cyO2 or kB1 at 1 or 2 μM was monitored using live-cell microscopy for 6 h with 1 μM of SYTOX® Green, a membrane impermeable dye that stains the nuclei of cells with disrupted membranes. The time-lapse movies are included in the supplementary videos, in which rapid plasma membrane blebbing was observed in the presence of hyen D or cyO2, and then the nuclei of cells with compromised membranes were stained with
SYTOX® green (Video S1, 2). Cells treated with hyen D or cyO2 become fluorescent soon after addition of the peptides, whereas no significant increase in fluorescence intensity was observed from cells treated with kB1 at the same concentration or with water as controls (Video S3, 4). A custom FIJI macro was created to select individual nuclei and monitor the cell death through recording the mean nuclear green fluorescence intensity at each time point. The average intensity of 200 cells from three positions of each well was plotted against time of observation (Fig. 8). Hyen D displayed the same trend in cell death profile as cyO2 at 1 and 2 μM while no visible signs of toxicity were observed for kB1 at concentrations that were well below its CC50. This finding further demonstrates how the cyclotides induce cell death via cell membrane disruption.

Cell internalization evaluated using flow cytometry and confocal imaging – The internalization efficiency of hyen D was evaluated and compared to the gold standard cell-penetrating peptide TAT at 0.25, 0.5 and 1 μM. The selected concentrations covered the range from low-cytotoxic to cytotoxic concentrations. Alexa Fluor® 488 (A488) conjugated peptides were incubated with HeLa cells for 1 h, and then the mean fluorescence emission and fluorescent cell percentage were measured using flow cytometry before and after the addition of Trypan Blue (TB). The fluorescence from peptide binding on the cell surface or from cells with compromised membranes were quenched after treatment with TB. The results are shown in Figs. 9A, B, and C. TAT, which has similar profiles before and after the addition of TB, was shown to penetrate cells without causing membrane leakage. At 1 μM, the cellular uptake of hyen D was approximately eight-fold and three-fold higher than TAT before and after addition of TB, respectively. The significant drop in the fluorescence signal upon the treatment with TB was also observed at 0.5 μM on hyen D, suggesting that at cytotoxic concentrations, hyen D binds to and/or compromises cell membranes, with some of the hyen D internalized into cells. Moreover, there was an 8–10% drop of the percentage of fluorescent cells upon addition of TB (data not shown), further indicating that cells have their membrane compromised. At 0.25 μM, hyen D did not exhibit a significant drop neither in mean fluorescence signal nor percentage of fluorescence cell upon TB addition, suggesting that at this concentration, most hyen D is internalized into the cells. The hyen D membrane binding activity was further confirmed with HeLa cells treated with hyen D using confocal microscopy imaging at 0.5 μM, as shown in Fig. 9D. A488-labeled hyen D mainly bound to and lit up the cell membranes (red arrow, Fig. 9D), but some hyen D entered cells through endosomes (white arrow, Fig. 9D), as suggested by the punctate distribution within the cells.

DISCUSSION

In this study we showed that *H. enneaspermus* is a rich source of cyclotides by isolating and characterizing 12 cyclotides from extracted plant material. Cyclotides exhibit a wide range of pharmaceutical properties, including cytotoxic activity. CyO2 was isolated from *H. enneaspermus* and is one of the most cytotoxic cyclotides reported, affecting almost every studied cancer cell line (42,43). We discovered that hyen D, the most abundant cyclotide isolated from *H. enneaspermus*, is slightly more potent than cyO2, and is the most cytotoxic among the seven cyclotides tested. The potent cytotoxicity of hyen D is probably due to the presence of a hydrophobic patch and three positively-charged Lys residues, which contribute to a total net charge of +2 (53,64).

We used the homology structures we built to further explore the molecular basis of the cyclotide cytotoxicity (Fig. 6 and 7). Hyen E was the least cytotoxic among the five tested bracelet cyclotides. It is slightly more hydrophobic than hyen D, but its molecular surface is less positively charged than those of hyen D, which provides a potential explanation for its lower activity in the cellular assays. Similarly, a previous study showed that vibi E, a neutral net charge bracelet cyclotide derived from *Viola biflora* (12), was three-fold less cytotoxic than vibi G, which has a net charge of +2. The other two novel cyclotides hyen L and M have similar sequences to that of cyO1. They have lower cytotoxicity compared to cyO2 and hyen D. In addition to a lack of positive net charge, they also have a different distribution of their hydrophobic patch compared to cyO2 (Fig. 6), which also could affect the cytotoxic potency. Hyen M was slightly more cytotoxic than hyen L, perhaps due to its higher hydrophobicity (via substitution of Trp to Tyr at position 10 in loop 2).

Previous studies have shown that bracelet cyclotides such as cyO2 are typically more cytotoxic than Möbius cyclotides (12,43), probably because bracelet cyclotides contain more positively charged residues than Möbius cyclotides, which facilitates peptide-membrane interactions (61). In addition, the distribution of hydrophobic residues forming a large patch at the surface of the molecule in bracelet and
Möbius cyclotides is different (46, 48, 55). In this study we also found all the tested bracelet cyclotides are more potent in cytotoxicity than kB1. Hyen C, the only Möbius cyclotide and the most hydrophilic cyclotide among all the 12 sequenced peptides, lacked cytotoxic activity, which may be due to the amino acid sequence of loop 6. A previous study demonstrated the importance of hydrophobic residues in loop 6 which form part of the hydrophobic patch of kB1, with a single Lys mutation of Leu-2, Pro-3, or Val-4 in loop 6 resulting in a three- to five-fold decrease in cytotoxicity (55). The lack of hydrophobic residues in loop 6 of hyen C breaks the hydrophobic patch therefore explaining its non-toxic activity (Fig. 7). Our study reinforces that the hydrophobic patch of cyclotides is essential for their cytotoxicity, and that positively charged residues can facilitate their cytotoxicity (61).

We then studied whether the cytotoxic activities of cyclotides correlates with their membrane-binding properties through the SPR technique. Hyen D exhibited the strongest affinity among all peptides in POPC/POPE (80:20) and POPC/POPE/POPS (60:20:20) membrane models, with a P/L max value of 0.32 (Table 3). Hyen D had a slow membrane-dissociation rate, further supporting that it binds tightly to the lipid bilayers. By contrast, hyen E displayed the lowest P/L max, which correlates with its low cytotoxicity. However, hyen E had the lowest dissociation rate in the series, shown by the $k_d$ values in Fig. 5A. The slow dissociation rate of hyen D and E may be attributed to two conserved residues in loop 3 (Phe14 and Thr15), which form part of the hydrophobic patch and the bioactive face previously shown to be important for peptide-membrane insertion and interaction, respectively (24, 55). CyO2 showed a similar P/L max to hyen L and M, but exhibited a higher P/L ratio at lower concentrations and the smallest $K_0$, which supports its higher cytotoxicity than hyen L and M. Finally, hyen C displayed no binding against POPC/POPE (80:20) membranes in the tested concentration range, explaining its lack of cytotoxic activity. Overall, our data indicate that the cytotoxic potency of cyclotides from *H. enneaaspermus* correlates with their membrane binding affinity, suggesting that the cytotoxicity mechanism is mediated by their ability to bind to lipid membranes.

The membrane binding properties of the Möbius cyclotide kB1 have been extensively investigated via lysine scanning (55), alanine scanning (65), and other single residue (50, 54) mutagenesis studies, with three crucial regions identified: the amendable face, the bioactive patch, and the hydrophobic patch. The bioactive patch of kB1, formed by Glu-7 and adjacent amino acid residues, was later shown to be involved in recognition of PE-headgroups; while the hydrophobic patch is responsible for membrane insertion (54). The amendable face is where the residues could be modified without impairing biological activity (55). According to the bioactive patch of kB1, which is formed by Gly-6, Glu-7, Thr-8, Asn-15, Thr-16, and Arg-28, the corresponding bioactive patch of hyen C should be formed by Gln-6, Glu-7, Thr-8, Ser-16, Thr-17, and Lys-29. The previous study shows [G6A]kB1 and [G6K]kB1 lost their inhibition to insect and nematode larvae, respectively (55, 65). Another study (66) suggests Gly6 in kB1 is very crucial for the PE-headgroup interaction, because [G6A]kB1 could not bind with PE-containing membrane. This could probably be because Gly-6 is at the edge of hydrophobic patch and plays a critical role on the effective surface topography. Here we predict that having Gln-6 instead of Gly-6 makes hyen C unable to interact with PE-headgroups. Furthermore, the hydrophobic patch of hyen C has two residues less than kB1, providing further explanation for its lack of affinity for the POPC/POPE (80:20) model membrane (Fig. 7).

The crucial regions of bracelet cyclotides with important membrane binding properties have been less studied because of the difficulty to make them and their variants synthetically. Although bracelet and Möbius cyclotides were previously shown to bind lipids in different orientations (i.e., the bracelet cyclotide cyO2 uses loops 2 and 3, while the Möbius cyclotide kB1 uses loops 5 and 6 (24)), bracelet cyclotides also contain a hydrophobic patch and a bioactive patch similar to those of Möbius cyclotides. The hydrophobic patch of bracelet hyen cyclotides comprises loop 2 and some residues in loop 3, and the bioactive patch is presumably formed by Glu-6 and adjacent residues, as well as the positively charged residues in loops 5 and 6. This theoretical identification of important residues is supported by previous studies of cyO2, where the methylation of Glu and the acetylation of Lys in loop 5 and Arg in loop 6 caused significant loss in cytotoxic (67), anthelmintic (68), and antibacterial activity (69). Indeed, the most cytotoxic cyclotides discovered thus far, including psyle E (70), vitri A, vitri F (46), vibi G (12), cyO2, cyO19 (61), and hyen D, all share the same feature in the bioactive patch, with two positively charged residues in loop 5 and one positively charged residue in loop 6. Our findings are consistent with the fact that the amphiphilic nature of these peptides is
thought to modulate their membrane binding properties, which underpin their cytotoxic potency (61,71).

As cancer cell membranes have more exposed PE phospholipids than healthy cells, and are usually more negatively charged, it was proposed that cyclotides may preferentially target tumor cells (50). However, like the results in this study, no obvious selectivity was observed in the previous study when both bracelet and Mōbius cyclotides were tested on a non-cancerous cell line (foreskin fibroblast cells) along with cancer cell lines (45,47,50). One explanation is that healthy cells are more dynamic compared to cancer cells, and this property facilitates cyclotide insertion (50). Therefore, more studies are required to explore their therapeutic potential and fine tune their selectivity for cancer cells.

Further mechanistic studies on several cyclotides were performed to understand the potential mechanisms of their cytotoxicity. Detecting the enzymatic activity of the cytosolic enzyme LDH released from the tested cell lines upon adding cyclotides is a way to investigate whether cytotoxicity correlated with cell membrane disruption. The LC50 indicated by LDH assay are consistent with the results from their cytotoxicity assay, indicating that cell membrane permeabilization may be responsible for the observed cytotoxicity. From the live-cell imaging assay using time-lapse microscopy, hyen D and cyO2 induced morphological changes on HeLa at both tested concentrations. Specifically, plasma membrane blebbing was observed at multiple positions upon addition of the peptides, followed by membrane rupture which led to the influx of SYTOX Green dye (Video S1 and S2). The cell necrosis induced by cyclotides is characterized by a general swelling of the cell and rapid loss of plasma membrane integrity (72). A previous report claimed that cyO2 induces cell necrosis at 4 μM on human lymphoma cells, but did not disrupt the membrane of Hela cells loaded with calcein at 1 μM measured by a calcein cell assay (52). Our results suggest that hyen D and cyO2 induce cell necrosis of HeLa at 1 and 2 μM. The observed inconsistency across the results is probably due to different evaluation methods. Moreover, cell internalization assays and confocal microscopy were conducted to further explore how hyen D entered and localized in HeLa cells. At 0.5 and 1 μM, a large portion of A488-labeled hyen D bound on the surface of membrane, with some entering cells as indicated by the flow cytometry results (Fig. 9B, C), whereas a higher percentage of hyen D entered cells at a sublethal concentration (0.25 μM, Fig. 9A). However, it is not clear whether hyen D that internalized into cells would induce cell apoptosis as reported in an earlier study (73).

In conclusion, we have identified five novel cyclotides from H. enneaspermus with potent cytotoxicity and the underlying mechanism of the observed activity was confirmed through a series of mechanistic studies. In particular, cyclotides interact with PE-containing phospholipids, accumulate on the cell membrane, inducing necrotic blebbing which leads to cell death. The newly discovered cyclotide hyen D can be internalized by HeLa cells better than TAT and localize in endosomes at a non-toxic concentration. Overall, our findings suggest membrane binding and disruption contribute to the cytotoxic activity of cyclotides.

**EXPERIMENTAL PROCEDURES**

**Plant material – H. enneaspermus** was collected from a roadside near the Calliope River (Gladstone, Queensland, Australia) and grown under LED lamps of the AP67 spectra at 170 μmol m−2 s−1 (Valoya Oy, Helsinky) with 16 h daylength at an ambient temperature of 28°C in a plant growth room. Plants were raised from self-fertilized seeds in Jiffy pots.

**Extraction, purification, and isolation of cyclotide candidates** – The plant was separated into three parts: leaves, stems, and seeds. Each part was frozen in liquid nitrogen and ground into a fine powder with a mortar and pestle, then extracted with 50% acetonitrile (MeCN) and 1% formic acid (FA) at 4°C overnight. The plant extract solution was centrifuged, and the supernatant collected, concentrated, and lyophilized. The dry sample was dissolved and then fractionated using a Strata C18-E solid phase extract (SPE) cartridge (20%–80% MeCN; 1% FA). Masses from all eluted SPE fractions were detected with MALDI-MS and later freeze-dried. Peptide-containing fractions were purified with a Phenomenex C18 RP-HPLC column and Shimadzu RP-HPLC unit (buffer A: 0.05% TFA in H2O; buffer B: 90% MeCN in H2O with 0.05% TFA).

**Reduction, alkylation, and preliminary analysis of hyen-like cyclotides** – Isolated peptides were dissolved in 0.1 M NH4HCO3 buffer (pH 8.0), reduced with DTT (10 mM) at 60°C for 30 min, and then alkylated with iodoacetamide (25 mM) under nitrogen at room temperature for 30 min. Reduced and alkylated peptides were then digested overnight with trypsin, chymotrypsin, endoproteinase Glu-C, and a mixed trypsin and endoproteinase Glu-C cocktail at 37°C. The reaction was quenched with 10% FA. The masses of
alkylated and digested peptides were detected by a 5800 MALDI-TOF/TOF MS (AB SCIEX, Canada) and a 5600 TripleTOF LC-MS/MS (AB SCIEX, Canada). The sequences were analyzed and annotated manually on MALDI-TOF/TOF MS (e.g. Fig. 3) along with assistance from the ProteinPilot™ search engine (searching LC-MS/MS data against a database adapted from Cybase containing 760 sequences) (version 4.0.8085). Detected protein threshold [Unused ProtScore (Conf)] is > 0.05 (10%). The reduction, alkylation, and digestion scheme of hyen D, and the enzyme digestion fragments are shown in Figure S7 and Table S4, respectively.

Transcriptomic analysis and peptidomic analysis—RNA-Seq data for H. enneaspermus was previously reported (57), with the raw data for the roots and shoots deposited in NCBI-SRA under the accession numbers SRR6394743 and SRR6394745, respectively. For this work, a refined transcriptome assembly pipeline was used that was optimized to facilitate assembly of multigene family sequences in Trinity v2.8.4 to create the Henn4.2 transcriptome assembly (74). Trinity parameters on filtered data were: --kmer_size 27 --min_kmer_cov 2 --path_reinforcement_distance 30. Putative cyclotide sequences were obtained from assembled data by tblastn search against the Henn4.2 transcriptome using a list of query peptides given in Table S3 (75). The resulting list of non-redundant transcript hits were manually annotated to identify the coding sequence of the precursor and cyclotide domain. From the annotated sequences, unique complete and partial cyclotide and linear cyclotide sequences were identified. We then explored the peptidome of H. enneaspermus by searching reduced, alkylated and digested crude extracts (same method as for isolated peptides) with the transcriptome as a database using ProteinPilot™ software (version 4.0.8085) (Detected protein threshold [Unused ProtScore (Conf)] is > 0.05 (10%).)

Solid-phase synthesis for cyclic and linear peptides—Fmoc solid-phase synthesis of hyen C was carried out as described previously (76). Briefly, the linear precursor of hyen C with sidechain protecting groups and the 2-chlorotrityl chloride (CTC) resin was synthesized on an automated peptide synthesizer. Linear hyen C was cleaved from the 2-CTC resin using 1% TFA in dichloromethane (DCM). After lyophilizing, the sidechain protected crude cyclotides were dissolved in DMF at 2mM concentration; then N, N, N', N'-tetramethyl-O-(7-azabenzo-triazol-1-yl) uranium hexafluorophosphate (HATU) and N, N-diisopropylethylamine (DIPEA) was added to give a final concentration of 5 mM and 10 mM, respectively. The solution was stirred for 6 h and lyophilized overnight after diluting twice with 50% MeCN (0.05% TFA). Sidechain deprotection was carried out by adding 50 mL of trisopropylsilane (TIPS): H2O: TFA (2:2:96, v/v/v) and stirring for 2.5 h. The crude hyen C was purified via RP-HPLC, and then folded using 0.1 M ammonium bicarbonate (pH 8.5, 2 mM reduced glutathione, and 0.5 mM oxidized glutathione. TAT (sequence: YGRKKRRQRRPPQG) and TAT-G (sequence: YGGGKGGQGGGPPQG) were also synthesized using same method as linear hyen C and chosen as positive and negative control in hyen D cell internalization assay. TAT, TAT-G and the correctly folded hyen C was purified via several RP-HPLC runs.

Structural analysis of hyen cyclotides by NMR—Hyen D, E, L, M, and cyO2 were dissolved in 500 μL H2O and 50 μL D2O; 1 μL (10 mg/mL) of 4, 4-dimethyl-4-silapentane-1-sulfonic acid (DSS) was added as a reference. The sample pH was kept within the range of 3 to 5. One-dimensional 1H spectra were acquired using a Bruker ARX 600 NMR spectrometer at 298 K, followed by two-dimensional spectra (TOCSY and NOESY). All NMR spectra were assigned using the CCPNMR program (version 2.4.2). Secondary αH chemical shifts were calculated by subtracting the observed shifts from corresponding random coil 1H NMR chemical shifts (58); they were also compared to cyO1 (BMRB code: 4461) (16) and cyO2 (BMRB code: 16073) (24). The temperature coefficient experiment was carried out only on the most abundant peptide, hyen D. TOCSY and NOESY spectra were recorded at 288, 293, 298, 303, 308, and 313 K (pH 3.66 and 5.75). Amide proton chemical shifts in the temperature coefficient were plotted in the above temperature range, as previously described (77,78). A value more than -4.60 ppb/K indicates the hydrogen is involved in a hydrogen bond (78). The hydrogen bonds were also predicted using PyMOL (version 2.2.2). Electrostatic potential was computed in PyMOL using APBS and generated for cyO2, hyen D, hyen E, hyen L, and hyen M using homology modelling based on previously reported cyO1 (16) and cyO2 (24) structures. Chemical shifts of hyen C, D, E, L and M are reported in the BMRB (codes: 50165, 50161, 50163, 50162, and 50164 respectively).

Cytotoxic assay—The cytotoxicity of the cyclotides was evaluated using the resazurin assay. Assays were performed as described previously (50) by adding isolated peptides to cells grown in vitro. All cells were
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seeded at 5,000 cells/well (100 μL/well) into a 96-well flat-bottomed plate one day before adding the peptides, except for K562, which was seeded at 10,000 cells per well (100 μL/well) on the day of the experiment. All test peptides were dissolved in water and tested at different concentrations ranging from 12 to 0.0625 μM (2-fold dilution; final concentrations) in triplicate. Cells were treated with hyen C, D, E, L, M, cyO2, kalata B1, water, and 1% Triton X (TX). After 24 h of incubation, 10 μL of 0.05% (w/v) resazurin was added and incubated with cells for another 18 h. Absorbance was measured at 540 nm and 620 nm (Tecan M1000 Pro plate reader, Labsystems). The percentage of dead cells was quantified by calculating the absorbance ratio, R (R = absorbance at 620/540), and applying the following equation: % Cell death = (Rsample − RH2O) / (RTX − RH2O) × 100. Data was analyzed with GraphPad Prism software (version 8.3.1) using “Specific binding with Hill slope” algorithm.

**Hemolytic assay** – Cyclotides and melittin were prepared and tested at concentrations ranging from 50 to 0.39 μM and 6.67 to 0.05 μM, respectively (two-fold serial dilutions in water) using the previously published method (55). Briefly, RBCs were obtained through repeated wash and centrifugation, and were diluted to 0.25% with PBS solution prior plating (100 μL/well). 20 μL of cyclotide solution, melittin, and 1% (v/v) Triton X-100 was added in plate and incubated at 37°C for 1 h. After centrifuging, 100 μL of supernatant was transferred to a flat-bottomed plate and absorbance at 415 nm (A) was measured on Multiskan Ascent plate reader (Labsystems). Hemolysis was calculated using the following equation: % Hemolysis = (A_{sample} − A_{H2O}) / (A_{TX} − A_{H2O}) × 100. Data was analyzed with GraphPad Prism software.

**Peptide-lipid binding assay** – Synthetic lipids were obtained from Avanti Polar-Lipids. Six lipid combinations were prepared: POPC, POPC/POPE 80:20, POPC/POPE 90:10, POPC/POPE 95:5, POPC/POPS 80:20, and POPE/POPC/POPS 60:20:20. The assay was performed as previously described (62). Briefly, homogenous small unilamellar vesicles (SUVs) dispersed in buffer (10 mM HEPES, 150 mM NaCl, pH 7.4, filtered) were obtained by extruding through a polycarbonate membrane with a 50 nm pore size diameter. Membrane binding of native cyclotides was evaluated using an L1 sensor chip on a Biacore 3000 instrument. A suspension of SUVs (0.5 mM lipid) was injected over an L1 chip surface at 2 μL/min for 40 min, followed by a short pulse of 10 mM NaOH (50 μL/min, 36 s). Then, cyclotide solutions were injected over the lipid surfaces (5 μL/min, 180 s) and the dissociation process was monitored for 10 min. The chip surface was regenerated by several washing cycle described previously (79). All cyclotides were evaluated at 0, 4, 6, 8, 16, 24, and 32 μM. Peptide-to-lipid molar ratio was calculated to evaluate the binding ability of the tested cyclotides (62). Ko and kD value was calculated through GraphPad Prism software. Ko was determined by fitting in specific binding with Hill slope, whereas kD was fitted in a dissociation equation using a single sensorgram at 32 μM.

**LDH enzymatic activity assay** – Cell membrane permeabilization was evaluated using a LDH assay. HeLa and HUVEC cells were grown and treated with peptides as in the cytotoxicity assay. LDH assays were performed as previously reported using a kit (CytoTox96®, Promega) (80). After 2 h of treatment with peptides, 10 μL of supernatant was collected and incubated with LDH substrate for 30 min at room temperature in the dark. The reaction was stopped using 10 μL of stop solution before the absorbance was read at 490 nm in a microplate reader (Tecan M1000 Pro plate reader, Labsystems). The percentage of cells with their membrane compromised was calculated using the equation: (A_{sample} − A_{H2O}) / (A_{TX} − A_{H2O}) × 100.

**Cell membrane disruption visualized using live cell imaging** – HeLa cells were seeded in a 96-well black clear glass bottom plate (ibidi cat#89621) at 10,000 cells/well and grown overnight. Before the assay, the medium was replaced by phenol red DMEM supplemented with 1% (v/v) Penicillin-Streptomycin and spiked with 1 μM SYTOX Green. Live cell imaging started immediately after HeLa cells were treated with cyclotides hyen D, cyO2 and kB1 at 1 or 2 μM individually. Images were acquired using a 20× 0.45NA S Plan Fluor ELWD objective, and SYTOX Green signal was detected using the 485 nm line of a Lumencor LED array and filtered with a 525/30 nm emission filter, with capture using a Hamamatsu Orca Flash 4.0 sCMOS camera running NIS Elements AR. Images were captured every 77 s for 6 h using a custom built inverted widefield microscope with a Nikon TiE inverted stand with the Perfect Focus System, and OKO laboratories incubation. The mean fluorescence of 200 cells in three positions of each sample was measured by running a custom-written script on FIJI software and plotted against time.

**Cell internalization assay followed by confocal microscopy** – A cell internalization assay was conducted as reported previously (81). Briefly, the
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single A488-conjugated TAT, TAT-G and hyen D were purified to >95% purity. HeLa cells were seeded in a 24-well plate at 1×10⁵ cells/well one day prior to the assay. Labeled TAT, TAT-G and hyen D at 0.25, 0.5 and 1 μM were incubated with HeLa cells for 1 h at 37°C in medium without serum. Cell mean fluorescence emission was measured using flow cytometry (BD FACSCanto™ II) by analyzing 10,000 cells per sample with excitation at 488 nm and emission at 530/30 nm, before and after the addition of trypan blue (160 μg/mL). Experiments were repeated on three independent days. To visualize cellular uptake of hyen D, HeLa cells were seeded in an 8-well Borosilicate plates (Thermo Fisher Scientific) at 15,000 cells/well one day prior to the assay. Right before the experiment, the medium was replaced with phenol red free DME medium containing wheat germ agglutinin (WGA)-Alexa-647 conjugate (1:500, v/v), and cells were treated with A488-labeled hyen D or TAT at 0.5 μM. Images were captured after 1 h treatment on Zeiss 880 LSM confocal equipped with a 63x 1.4 NA Plan Apochromat objective running Zeiss Zen Black software. All the microscopy images were analyzed using FIJI software (82). Cells were incubated at 37°C with 5% CO₂ during all the imaging process.

**DATA AVAILABILITY**

1. The 12 novel protein sequence data reported in this study will appear in the UniProt Knowledgebase under accession number C0HLN7~N9 and C0HLP0~8. (confidential until paper published).
2. Hα chemical shifts of hyen C, D, E, L and M are reported in the BMRB (codes: 50165, 50161, 50163, 50162, and 50164).
3. Raw mass spectrometry data for digested crude plant extract and search against transcriptome data is deposited in PRIDE (Project accession: PXD017924).

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**CONFLICT OF INTEREST**

The authors declare that they have no conflicts of interest with the contents of this article.

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### TABLES and FIGURES

#### Table 1. Cyclotides isolated and sequenced from *H. enneaspermus*

| Cyclotide | Experimental Mass (Monoisotopic) (Da) | From | Sequence | UniprotKB accession numbers<sup>h</sup> | Overall charge |
|-----------|--------------------------------------|------|----------|------------------------------------------|---------------|
| hyen A<sup>a,b</sup> | n.d. | RNA(40) | XXXCGESCVYIP-CTVTALLGCSCKDV-CYKN | n.d. |
| hyen B<sup>a,b</sup> | n.d. | RNA(40) | XXXCGETKVTKRCSGQG---CSCLKGRSCYD | n.d. |
| hyen C | 3316.4 | st, l<sup>c</sup> | GTHCPQETVTSTRCTSTQG---CSNWPI-CFKN<sup>e</sup> | C0HLN7 | +1 |
| hyen D | 3154.5 | st, l | GF-PGESCVYIP-CFTAAIG-CSCKSV-CYKNS<sup>g</sup> | C0HLN8 | +2 |
| hyen E | 3232.2 | st | GV-PGESCVYIP-CFTGIIIN-CSRDKV-CYNN<sup>g</sup> | C0HLN9 | 0 |
| hyen F | 3143.2 | s<sup>d</sup> | GL-PGESCVYIP-CISTVLGCSDKNV-CYRN<sup>e</sup> | C0HLP0 | +1 |
| hyen G | 3144.2 | st, l | GL-PGESCVYIP-CISTVLGCSCKNV-CYRD<sup>g</sup> | C0HLP1 | 0 |
| hyen H | 3173.3 | st, l | KI-PGESCVYIP-CISSVLGCSCKNV-CYKD<sup>g</sup> | C0HLP2 | +1 |
| hyen I | 3173.2 | s | GSTPGSECVWIP-CISGIVG-CSCKNV-CYMD<sup>g</sup> | C0HLP3 | -1 |
| hyen J | 3187.4 | s | GSVPGESCVWIP-CITSIAG-CSCKNV-CYMD<sup>g</sup> | C0HLP4 | -1 |
| hyen K | 3100.4 | s | GI-PGESCFIFIP-CITTVVG-CSCKNV-CYDN<sup>g</sup> | C0HLP5 | -1 |
| hyen L | 3087.4 | st | GI-PICESCVYIP-CTVTALLGCSCKDV-CY<sup>f</sup> | C0HLP6 | -1 |
| hyen M | 3141.3 | st, l, s | SI-PICESCVWIP-CTVTALLGCSCKDV-CYN<sup>f</sup> | C0HLP7 | -1 |
| cyO2 | 3138.3 | st, s | GI-PGESCVWIP-CISSAIG-CSCKSV-CYRN<sup>f</sup> | C0HLP8 | +2 |

<sup>a,b</sup>Two partial sequences reported in the literature as Hyen A and Hyen B.  
<sup>c</sup>st: stem; l: leaf.  
<sup>d</sup>s: seed.  
<sup>e</sup>Isobaric residues determined by transcriptome.  
<sup>f</sup>Isobaric residues determined by NMR spectroscopy analysis.  
<sup>g</sup>Isobaric residues deduced by sequence homology.  
<sup>h</sup>The protein sequence data reported here will appear in the UniProt Knowledgebase under these accession numbers.
Table 2. Cytotoxic (CC\textsubscript{50} ± SEM\textsuperscript{a}) and hemolytic activity (HC\textsubscript{50} ± SEM\textsuperscript{b}) of cyclotides from \textit{H. enneaspermus}

| Cell lines | cyO2   | hyen C | hyen D | hyen E | hyen L | hyen M | kalata B1 |
|------------|--------|--------|--------|--------|--------|--------|-----------|
| K562\textsuperscript{c} | 0.64 ± 0.02 | n.d.   | 0.52 ± 0.03 | 2.85 ± 0.09 | 1.54 ± 0.08 | 1.16 ± 0.03 | n.d. |
| HeLa\textsuperscript{d} | 0.82 ± 0.2 | >8     | 0.88 ± 0.05 | 2.64 ± 0.09 | 2.00 ± 0.04 | 1.34 ± 0.04 | 5.88 ± 0.21 |
| MCF-7\textsuperscript{e} | 1.77 ± 0.10 | n.d.   | 0.75 ± 0.05 | 3.73 ± 0.25 | 3.34 ± 0.28 | 2.36 ± 0.16 | 8.31 ± 0.62 |
| HUVEC\textsuperscript{f} | 0.80 ± 0.03 | n.d.   | 0.62 ± 0.01 | 2.06 ± 0.08 | 1.71 ± 0.04 | 1.38 ± 0.03 | 4.30 ± 0.07 |
| RBC\textsuperscript{g} | 25.60 ± 1.93 | n.d.   | 24.56 ± 2.16 | >50     | >50     | >50     | 65 ± 8\textsuperscript{h} |

\textsuperscript{a}Data represent the mean ± standard error of the mean (SEM) from triplicates. \textsuperscript{b}Data represent the mean ± SEM from duplicates. \textsuperscript{c}K562: leukemia cell line. \textsuperscript{d}HeLa: cervical cancer cell line. \textsuperscript{e}MCF-7: breast cancer cell line. \textsuperscript{f}HUVEC: human umbilical vein endothelial cells. \textsuperscript{g}RBC: red blood cell. \textsuperscript{h}The EC\textsubscript{50} of kalata B1 on RBCs is from the report by Henriques et.al (62) based on the same method. n.d. not determined.
Table 3. Binding affinity of cyclotides against chosen model membranes measured by SPR<sup>a</sup>

| Peptide | POPC/POPE (80:20) | POPC/POPE (90:10) | POPC/POPE (95:5) | POPC/POPE/POPS (60:20:20) |
|---------|--------------------|--------------------|-------------------|---------------------------|
|         | P/L max<sup>b</sup> (mol/mol) | K<sub>D</sub><sup>c</sup> (μM) | k<sub>d</sub><sup>d</sup> (μM<sup>-1</sup>s<sup>-1</sup>) | P/L max | K<sub>D</sub> | P/L max | K<sub>D</sub> | P/L max | K<sub>D</sub> |
| cyO2    | 0.25 ± 0.01        | 6.1 ± 0.4          | 4.2 ± 0.04        | 0.16 ± 0.00 | 7.1 ± 0.2 | 0.051 ± 0.002 | 10.0 ± 0.7 | 0.27 ± 0.01 | 5.2 ± 0.3 |
| hyen C  | 0.02 ± 0.01        | n.a.               | n.a.              | n.d.         | n.d.     | n.d.         | n.d.       | n.d.       | n.d.       |
| hyen D  | 0.32 ± 0.00        | 6.8 ± 0.1          | 2.6 ± 0.03        | 0.16 ± 0.00 | 5.3 ± 0.3 | 0.080 ± 0.003 | 5.3 ± 0.3 | 0.32 ± 0.00 | 5.1 ± 0.2 |
| hyen E  | 0.19 ± 0.00        | 8.4 ± 0.3          | 2.3 ± 0.02        | n.d.         | n.d.     | n.d.         | n.d.       | n.d.       | n.d.       |
| hyen L  | 0.25 ± 0.01        | 7.6 ± 0.2          | 3.0 ± 0.06        | n.d.         | n.d.     | n.d.         | n.d.       | 0.26 ± 0.01 | 6.8 ± 0.4 |
| hyen M  | 0.26 ± 0.00        | 10.0 ± 0.3         | 2.2 ± 0.05        | n.d.         | n.d.     | n.d.         | n.d.       | n.d.       | n.d.       |

<sup>a</sup>The binding of cyclotides to membranes lacking PE-phospholipids (i.e. POPC and POPC/POPS) is weak; and the response curves did not reach a plateau in the concentration range tested, and therefore the P/L max and K<sub>D</sub> value could not be defined (data is not shown). <sup>b</sup>maximum peptide-to-lipid molar binding ratio. <sup>c</sup>peptide concentration required to achieve half-maximum binding at equilibrium. <sup>d</sup>dissociation rate constant at 32 μM. The standard error was determined by fitting a non-linear equation (specific binding with Hill slope). n.d. not determined. n.a. not available.
Figure 1. Three-dimensional structures and sequences of the archetypical cyclotides of the three cyclotide subfamilies. (A) kalata B1 (PDB ID: 1NB1) (23) from the Möbius subfamily; (B) cycloviolacin O2 (PDB ID: 2KCG) (24) from the bracelet family; (C) MCoTI-II (PDB ID: 1IB9) (25) from the trypsin inhibitor family. The peptide structures (top) are in cartoon representation; arrows represent β-sheets. Disulfide bonds are shown as yellow sticks. In the sequence alignment (bottom), the yellow and black lines represent the disulfide bonds and peptide bond forming the circular backbone, respectively. Cysteine residues are on a yellow background. The Pro residue in the loop 5 of kalata B1 that adopts a cis conformation is in red.
Figure 2. LC-MS trace of extracted *H. enneaspermus* stem material. (hyen F, I, J, K are from seed extract, which is not shown).
Figure 3. Enzyme digestion and MALDI-MS/MS sequencing of hyen D. (A) MS/MS spectrum of the precursor mass 3521.67$^+$ [M+H]$^+$. (B) MS profile of hyen D after S-alkylation with iodoacetamide and digestion with endoproteinase Glu-C. (C) MS profile of hyen D after S-alkylation with iodoacetamide and digestion with a mixture of endoproteinase Glu-C and trypsin. (D) MS/MS spectrum of peptide fragment A with a mass of 1994.07$^+$. 
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Figure 4. Secondary $\alpha$H chemical shifts of tested cyclotides and literature reported cycloviolacin O2 (cyO2) and cycloviolacin O1 (cyO1). (A) Secondary $\alpha$H chemical shifts of hyen D, E, and cyO2 isolated from $H$. enneaspermus were compared to cyO2 (BMRB code: 16073) and the 3D structure of cyO2 (PDB ID: 2KCG) (24); (B) secondary $\alpha$H shifts of hyen L and M were compared to cyO1 (BMRB code: 4461) and the 3D structure of cyO1 (PDB ID: 1DF6) (16). The purple helical wheels represent $\alpha$-helices, the yellow sticks represent disulfide bonds, and the pink arrows represent $\beta$-sheets.
Figure 5. Membrane binding properties of hyen cyclotides evaluated using surface plasmon resonance. Peptides were injected over lipid bilayers deposited onto and L1 chip for 180 s and dissociation was followed for 600 s. A) Sensorgrams obtained with 32 μM of tested cyclotides on POPC/POPE (80:20) bilayers, showing distinct dissociation rates. The response units reach equilibrium within the association phase: a reporting point at the end of association (at 180 s after sample injection) was used to plot dose-response curves (B, C, E, F). Response units obtained at 180 s were converted into mol of peptide (1 RU = 1 pg/mm² of peptide or lipid) and normalized to the amount of lipid deposited on the chip (62). The sigmoidal curves were fitted using a one-site specific binding model with Hill slope in GraphPad prism. The binding affinity of selected cyclotides against different lipid compositions is shown in panels B (POPC), C (POPC/POPE, 80:20), E (POPC/POPS, 80:20), and F (POPC/POPE/POPS, 60:20:20). Panel D illustrates the linear relationship of cyO2 and hyen D at 32 μM between maximum peptide-lipid molar binding ratio (P/L max) and the percentage of POPE present in model membranes with POPC as matrix.
Figure 6. Electrostatic potential generated by cyO2, hyen D, hyen E, hyen L and hyen M, and mapped on their accessible solvent surface. The electrostatic potential was computed using APBS. The surface are colored from red to blue standing for electrostatic potential from -5 (and below) to +5 kT/e (and above). The hydrophobic patch formed by hydrophobic residues in loops 2 and 3 are highlighted in green.
Figure 7. Electrostatic potential generated by hyen C and kalata B1, mapped on their accessible solvent surface. The hydrophobic patch is highlighted in green; the bioactive face is highlighted in pink.
Figure 8. The mean SYTOX Green fluorescence intensity of HeLa cells monitored using time lapse microscopy in real time. (A) Mean fluorescence intensity on HeLa cells treated with hyen D or kB1 at 1 μM. (B) Mean fluorescence intensity on HeLa cells treated with cyO2 at 1 μM or water. Scale bar, 100 μm.
Figure 9. Internalization of hyen D into HeLa cells. Cells were incubated for 1 h with Alexa Fluor® 488-labeled hyen D and monitored with flow cytometry. (A, B, C) Mean fluorescence emission intensity of cells treated with peptides at 0.25, 0.5 and 1 μM, before and after treatment of TB. The intensity values were normalized to the fluorescence intensity obtained with cells treated with the same concentration of TAT after addition of TB. All experiments were repeated in triplicate, and the results are represented as mean ± SD. (D) A488-labeled hyen D (0.5 μM) were incubated with HeLa cells for 1 h and monitored by confocal microscopy. The cell membrane was labeled with wheat germ agglutinin conjugated with Alexa Fluor® 647 (A647-WGA). Live cell images from left to right are bright-field image (gray, upper panel), A647-WGA (blue, lower panel), A488-labeled hyen D (green, middle panel), and the merged image of A488-hyen D and A647-WGA (right panel). Scale bar, 20 μm.
Discovery and mechanistic studies of cytotoxic cyclotides from the medicinal herb *Hybanthus enneaspermus*
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