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Review Article

Structures, Functions and Therapeutic Potential of Cyclotides

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Abstract

Background and objectives: Plants usually produce a large number of secondary metabolite molecules, including antimicrobial peptides that typically interact with membranes of pathogens and kill these. Cysteine is more abundant in plant antimicrobial peptides than other sources. These phytopeptides can be classified into 5–8 distinct groups, based on the length, secondary structure type, number and model of disulphide bridges, and others. Among the different groups of phytopeptides, cyclotides are end-to-end cyclic with a disulphide bridge in the knot fashion. Cyclotide peptides are primarily known for its anti-nematode, anti-mollusk, and anti-trematode action. This review aims to provide detailed insights on cyclotides, from their origin, and structural and functional characterization, to their therapeutic potential, biotechnological application, and future prospective.

Methods: In order to accomplish the objectives of the present review, available review articles, and research articles correlated to phytopeptides, peptide databases, and Cybase, especially for cyclotides, were consulted.

Results: Several studies have revealed that modifications in the loop regions of the cysteine knot do not change the conformation of cyclotide. This is one of the most important features used in biotechnological applications, such as the delivery of active peptides (intracellular/intercellular) as an inhibitor, an activator of enzymes and different factors, or an agonist or antagonist for different receptors.

Conclusions: Cyclotides are structurally characterized as a peptide of the cysteine-knot motif, with a high tolerance of sequence variability, providing a great scaffold for drug delivery and combinatorial libraries. The molecular characterization revealed that the first identified cyclotide (from uterotonic) selectively targets G-coupled oxytocin and vasopressin receptors, in order to facilitate the movement of the endometrium at the beginning of labor.

Keywords: Antimicrobial Phytopeptides; Cyclotide; Knottin; Mobious; Baracelet; Trypsin Inhibitor.

Abbreviations: AEP, asparginyl endopeptidase; CTR, C-terminal repeat; NMR, nuclear magnetic resonance; NTR, N-terminal repeat; HB5, Hedyotide B5.

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Introduction

Proteins/peptides play varied and complex roles in all other macromolecules. These provide intracellular and extracellular support for biochemical reaction catalysis, generate receptors and channels in membranes, intracellularly and intercellularly transport chemicals, have hormone function, and defends against biotic and abiotic stress, among other functions in organisms.[1] Among the different defense systems, the peptides/proteins produced by almost all organisms provide inherent protection against a wide range of invading organisms.[2] These specific protein-protein/protein-peptide interactions can act as medication candidates with therapeutic po-

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tentials.[3] However, due to instability, low membrane permeability, and inaccessibility to intracellular targets, the therapeutic potential of peptides remains restricted.[4]

Peptides (naturally produced or synthesized) are typically very susceptible to breakdown in the physiological milieu.[5] In order to increase stability, conformation restrictions are introduced on the backbone, and/or side chains of amino acids are modified to provide resistance against enzymatic cleavage. The cyclization and modification of amino acid side chains have been discovered as important tools for the stabilization of peptides in physiological environments. Cyclotides are a family of head-to-tail cyclic phytopeptides with three disulphide bridges of knot topology.[6] Intrinsically, cyclotides have different activities, such as antimicrobial, insecticidal, anti-nematodal, anti-mollusk, anti-trematode, anti-HIV, and protease inhibitory, as well as hormone-like activity.[7–10] The excellent stability and sequential engineering amenability between the knots of cyclotides provide a great scaffold for drug design. As a result, cyclotides can be viewed as a natural scaffold for combinatorial libraries that are structurally constrained by the cysteine knot and head-to-tail cyclization, allowing these to accept all types of mutations, except for conserved cysteine residues involved in knot.[11–13] Furthermore, some cyclotides can interact with intracellular targets by crossing the plasma membrane.[14]

Discovery

Several decades before, the first cyclotide was recognized from the plant *Oldenlandia affinis* of the *Rubiaceae* family, which is being used as traditional tea in an African country. The analysis predicted that the component of uterotonic tea to facilitate birth was a peptide,[15,16] although there is no available modern protein chemistry or other technique to elucidate this in detail. Its structural characterization with cyclic nature and the presence of the cysteine knots were elucidated after 1995, and this was named, kalata B.[6] Subsequently, many more cyclic peptides similar to Kalata B were isolated from other plants of *Rubiaceae* and plants in other families, such as *Violaceae*, *Solanaceae*, *Fabaceae, Cucurbitaceae,* and *Apocynaceae*. Most of the cyclotides have been reported from *Rubiaceae* and *Violaceae.*[17,18] Furthermore, almost all of the plants in the *Violaceae* family contain cyclotides, while merely approximately 5% of plants in the *Rubiaceae* family contain these.[18] Cyclotides have been discovered to be distributed across all types of tissues, including the roots, stems, leaves, flowers, and seeds, in some plants.[10,19] Furthermore, a plant encompasses 10–160 different cyclotides. An *in silico* transcriptomic and proteomic study reported that the plant *Viola tricolor* of *Violaceae* contained 168 cyclotides, and it was extrapolated that *Violaceae* can contain approximately 150,000 different cyclotides.[20,21] In order to facilitate access to various types of identified cyclotides, a database called, CyBase, was recently developed, and this is publicly available on the website, CyBase.org.au. More than 300 cyclotides have been reported, and these are available on the aforementioned website for the further understanding of proteomics and other characteristics.[22]

Structural characteristics

A cyclotide, similar to other defense peptides, is derived from a precursor peptide that contains a signal domain/ER domain, an N-terminal prodomain, cyclotide, and a C-terminal prodomain. Sometimes, the flanking sequences of the N and C terminals exhibit a type of repeat known as, the N-terminal repeat (NTR) and C-terminal repeat (CTR), which presumably play important roles in the process of N to C cyclization.[23] The indispensable hypothesis of Asparginyl endopeptidase (AEP) suggested that NTR, CTR, and more especially, N-terminal Gly/Ala and C-terminal Asn/Asp may principally be involved in the AEP-mediated N to C amidation. Furthermore, the AEP hypothesis has been validated in the cyclotide-producing plant *Momordica cochinchinensis* by changing the Asn/Asp amino acid sequence or knocking out the AEP gene.[24–27] The recent discovery of linearized cyclotides or acyclotides, with the missing Asn/Asp of the C-terminus or improper CTR, further strengthens the AEP hypothesis of the cyclization of cyclotides.[24,28] In the chemical synthesis of cyclotides, disulphide bridges are formed during N to C peptization, while in the biological synthesis, the oxidation of cysteine begins in the precursor peptide, bringing the N and C termini nearby, and facilitating the end-to-end cyclization.[27,29] However, the detailed process for the biosynthesis of cyclotides has not been endeavored.

Nuclear magnetic resonance (NMR) spectroscopy is generally used to characterize the three-dimensional structure of small proteins/peptides, although this cannot be used for the characterization of all kinds of proteins/peptides, because a number of peptides remain unordered in the solution state. NMR is an excellent technique for cyclotides, because the highly constrained and knotted structure reinforces the ability of the cyclotide to remain ordered, even in the solution state. Furthermore, NMR can also be employed as a non-invasive technique to evaluate the position of the cysteine, which forms a disulphide knot. However, its validation would require invasive catalytic methods, such as reduction and alkylation.

Cyclotides are plant-origin defense peptides in the cyclic protein/peptide family, which have a higher number, when compared to all other cyclic groups. Furthermore, these are a unique class of defense molecules that comprise of various potential properties. Cyclotides usually have 27–40 amino acids, and head-to-tail cyclic peptides, and these are further stabilized with three disulphide bridges. In cysteine knot topology, disulphide bridges form as a ladder between CysI-CysIV, CysII-CysV and CysIII-CysVI (Fig. 1b). Since cyclization and the cysteine knot provide resilience against chemicals, thermal, and even enzymatic degradation, cyclotides are the only naturally occurring peptides that have been reported to be orally active among peptide therapeutic leads/candidates.[22,30–34] Except for the cysteine position, cyclotides are highly tolerant to sequence variations, in terms of topology. Furthermore, cyclotides are structurally and highly constrained, are able to cross the cellular membrane, and have specific affinity for ligands or proteins. The very unusual stability, highly constrained topology, and versatile scaffold of cyclotides fuel great interest to develop potential therapeutic and diagnostic reagents.[29,35,36]

The distinctive property of proline, *cis* or *trans*, provides a special feature in the stereometric conformation of proteins/peptides. Cyclotides are largely categorized into the subfamilies of Mobius and Bracelet, based on the *cis* or *trans* proline. Cyclotides that contain the *cis*-proline produce a twisted backbone of 180°, and these are known as Möbiöus, while those that contain the *trans*-proline are known as Bracelet (Fig. 1).[37,38] In known cyclotides, approximately two-thirds belong to Bracelet, and nearly one-third belong to Mobius. Apart from these two subfamilies, there is another minor class of cyclotides known as, trypsin inhibitor.[28,37,38] Trypsin inhibitor cyclotides cross cell membranes by utilizing different endocytic pathways, and interacting with various kinds of proteins and ligands. Furthermore, trypsin inhibitors are mainly isolated from the seeds of *Momordica cochinchinensis*, with a potent inhibition property of K_i = 20–30 pM. Apart from the knot structure, the amino acid contents of trypsin inhibitor cyclotides quite vary. In addition, these cyclotides (trypsin inhibitors) have

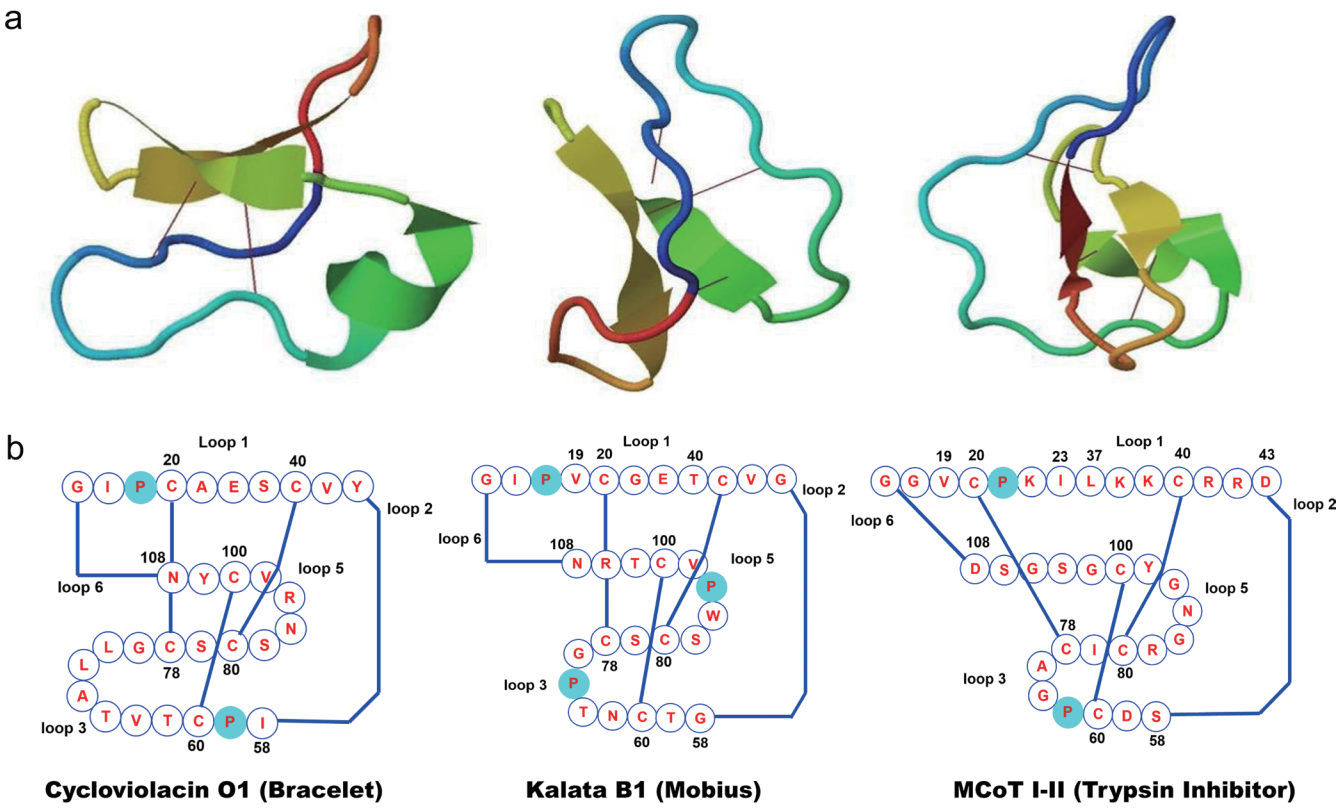

Fig. 1. Tertiary structure and the corresponding schematic representation loop formation in different classes of cyclotides. (a). Tertiary structure: the different size loops provide the differential activity of class Bracelet, Mobius and Trypsin inhibitor, and the connecting line defines the disulphides, which generate the loops. (b). Schematic representation of the loop: C denotes Cys, and the lines that connect these define the pattern of the disulphide bridge, which created loops of variable sizes. The tertiary structure of the different classes of cyclotides and the schematic representation were illustrated using CyBase (CyBase.org.au).

relatively higher sequencing homology, when compared to the linear cysteine knot squash trypsin inhibitor, which has been often referred to earlier as, acyclic knottins.[28] Although all three subfamilies have similar knot topology, except for loop composition, cyclotides in the Bracelet subfamily are larger and more diverse, when compared to those in the Mobius subfamily. Furthermore, compared to both Mobius and Trypsin inhibitors, Bracelet cyclotides offer a more complex situation in *in vitro* conditions for correct folding. As a result, Bracelet is less thoroughly investigated and employed in various biotechnological applications and characterization, when compared to Mobius and trypsin inhibitors.[28]

Functional assessment

Cyclotides in the Mobius and Bracelet subfamilies are known as defense peptides in plant systems. The defense peptides of this group mainly have anti-nematode, anti-trematode, and anti-mollusk activity. Similar to other defense peptides, cyclotides exhibit its activity through physical interaction with the membrane, disturbing the normal integrity. As previously documented, when the larvae of Lepidopteran species ingest plant tissues that contain cyclotides, the membrane integrity of midgut line cells becomes damaged, halting the proliferation. Different kalata cyclotides have varying inhibitory activities against the growth of nematode larvae (*Haemonchus contortus* and *Trichostrongylus colubriformis*), and ranks from the most potent kalata B6 to the least kalata B3. In addition, cyclotide cycloviolacin, such as O2, O3, O8 and O13, inhibit larval growth, similar to kalata B1 and B2, but lesser than B6.[39] Furthermore, regardless of whether these are from the Mobious and Bracelet groups, cyclotides with 3–4 basic residues usually have higher inhibitory activity in larvae. Mechanistically, cyclotide Kalata B can specifically bind to phosphatidylethanolamine, and promote aggregation, leading to pore formation-mediated cell death.

Cyclotides have antimicrobial and antitumor activity. Similar to most antimicrobial peptides, cyclotides are basically amphipathic, and possess patches of hydrophobic and hydrophilic amino acids. Kalata B1 has potent activity against both Gram-positive and -negative bacteria,[7,28] and cycloviolacin O2 has potent activity against *Staphylococcus aureus* in mice.[40] In the *in vitro* analysis of anti-bacterial testing, the antimicrobial activity of cyclotides was impaired when the bacterial growth media contained high levels of salt.

The detailed molecular characterization revealed that uterotonic cyclotide selectively targets G-coupled oxytocin and vasopressin receptors to promote endometrium movement at the start of labour.[21] Cyclotides usually have anticancer activity with adverse hemolytic and cytotoxicity, but the modification of the primary structure or linearization would reduce the adverse toxicity. For example, in two variants of cyclovalcacins (O2 and O13), O2 has a serine and O13 has an alanine at the same position, and O13 has approximately 3–4 fold more hemolytic activity than O2.[41] Furthermore, several cyclotides have selective activity against cancerous cells.[23,42,43] For example, vingo 5 acts against cervical HeLa cells,[44] and a cyclotide isolated from the Chinese plant *Hedyotis*

diffusa (*Rubaceae*) has potent anti-proliferative and anti-metastasis activity against various prostate cell lines and tumor growth.[45] Similarly, the HB7 obtained from *Hippocrepis biflora* and MCoTI-PMI, which was engineered from MCoTI-I, exhibited tumor suppressor activity in a xenograft model of prostate cancer.[46]

Therapeutic potential of cyclotides

Cyclotides have gained increasing attention due to its diverse biological properties and potential applications in agronomic and pharmaceutical industries. Furthermore, cyclotides have a common mode of action, and its activity is evaluated by its capacity to bind to target biological membranes, generate pores, and disrupt these. Cyclotides from *Fabaceae, Poaceae, Rubiaceae* and *Violaceae* exhibit antibacterial, anti-cancer, hemolytic, nematocidal, antifungal, anti-HIV, insecticidal and molluscicidal activities.[47]

Anticancer potential of cyclotides

There is a huge library of cyclotides available for screening the anticancer potential against various types of cancer cell lines. Interestingly, cyclotides are a rapidly emerging class of plant-derived cyclic peptides, which exhibit great toxicity against cancerous cell lines. The present study discussed the anti-cancer properties of several cyclotides against a variety of tumors, and the limited information on its mode of action. In addition, the data was tabulated to provide concise information on anticancer cyclotides (Table 1).[23,48–59]

A previous study revealed that nucleotide T1 and T4 can suppress HeLa cell proliferation, with an IC_{50} value of 0.6 μM.[48] Hedyotide B5 (HB5), HB6, HB7, HB8 and HB9 were isolated from the leaves and root of *Hedyotis biflora*, and were found to have cytotoxicity against four pancreatic cancer cell lines. In particular, HB7 inhibited the migration and invasion of capan2 cells and suppressed the tumor growth by reducing the tumor size and weight in a xenograft model.[49] A recent study reported that several cyclotides from *Viola tricolor*, such as verve peptide A, CyO2, CyO13 and kalata B1, suppress the proliferation of glioblastoma U-87MG and SH-SY5Y cells, with IC_{50} values ranging within 2.15–7.92 μM. Further investigations have revealed that the combination of verve peptide A or CyO2 with temozolomide (TMZ) can enhance the apoptosis of U-87MG cells, suggesting that these cyclotides may increase the efficacy of TMZ chemotherapy against glioblastoma.[60] Hyen D is the most abundant cyclotide of the medicinal plant *Hybanthus enneaspermus*, and has been shown to exert cytotoxic effects on Hela cells, with an IC_{50} value of 0.92 μM.[61] Two cyclotides, Poca A and B, were isolated from the root of *Pombalia calceolaria*, and it was observed that these can reduce MDA-MB-231 cell viability, while CyO4 can inhibit the proliferation and migration of breast cancer cells.[50] Oligopeptides obtained from *Momordica charantia*, such as MCLO-12, inhibit the proliferation and induce apoptosis in non-small cell lung A549 cancer cells in a dose-dependent manner by suppressing the MAPK-p38 and JNK pathways.[62] Recently, a bioactive peptide, IM-7, was reported to suppress the proliferation of leukemia MOLT-4 and NB4 cells in a dose-dependent manner, and induce autophagy and apoptosis by modulating the beclin1, caspase-3 and Bcl-2 expression, and it was also found that IM-7 can enhance the chemotherapeutic effects of daunorubicin.[63] Diffusa cyclotide (DC) 1, DC2 and DC3 were purified from the root and leaves of *Hedyotis diffusa*, and were found to have potent cytotoxicity against prostate cancer PC3, DU145 and LNCaP cells *in vitro*. In particular, DC3 suppressed the proliferation, migration and invasion of LNCaP cells, and inhibited tumor growth in a xenograft model.[64] Previously, the CyO2 of *Viola odorata* was reported to have the ability to selectively kill highly proliferative tumor cells, and cause cell death by membrane permeabilization. Further investigations have indicated that the combination of CyO2 and doxorubicin can inhibit the proliferation of MCF-7 and drug-resistant MCF-7/ADR cells, highlighting the chemosensitization potential of CyO2 against doxorubicin-resistant breast cancer cells.[51] Furthermore, several novel chassatides were isolated from *Chassatide chartacea*, and chassatide C7, C8 and C11 inhibited the proliferation of HeLa cells, with IC_{50} values of 1.2, 1.0 and 1.2 μM, respectively.[52] Cyclotides obtained from *Clitoria ternatea* were evaluated for its anticancer and chemosensitizing potential against the A549 and A549/paclitaxel cell lines, and the results indicated that some of these cyclotides can significantly reduce the IC_{50} of paclitaxel by many folds against lung cancer cells.[53] The linoorbitides (LOB) 1, LOB2 and LOB3 obtained from flaxseed have potent anticancer activities against breast cancer MCF-7 and Sk-Br-3, and melanoma A375 cells, in which LOB3 has the most cytotoxic and selective activity.[65]

Anti-HIV potential of cyclotides

HIV is a potentially fatal virus that targets CD4+ T lymphocytes and macrophages. HIV infection can dramatically reduce CD4+ cells, leading to the onset of AIDS with a set of symptoms. More than 100 cyclotides have been identified for its activities against HIV infection.[66] Concise details on anti-HIV cyclotides have been collated, in order to allow readers to view these all at once (Table 2).[9,66–71]

The EC_{50} value is usually used to evaluate the effectiveness of cyclotides in anti-HIV activity. Various cyclotides have varying anti-HIV activities, with EC_{50} values ranging within 0.04–1.21 μM. Crude extracts of *Chassalia parviflora*, circulin A and circulin B have exhibited strong activity against HIV, with an EC_{50} ranging within 0.04–0.26 μM.[67] Subsequently, the *in vitro* anti-viral activity against HIV of another four cyclotides obtained from *Chassalia parviflora,* circulin C–F exhibited a similar activity, with an EC_{50} ranging within 0.05–0.27 μM.[9] The cycloviolins A–D obtained from *Leonia cymosa* exhibit anti-HIV activity, with an EC_{50} of 0.13 μM.[68] Cycloviolacins O13, O14 and O24, and kalata B1 have also exhibited significant anti-HIV activity, with an EC_{50} value of 0.32, 0.44, 0.30 and 0.66 μM respectively.[66,69] The anti-HIV activity of cycloviolacins Y1/Y4/Y5 and kalata B1 exhibit an EC_{50} of 1.21, 0.20, 0.04 and 0.66 μM, respectively.[69] The leaf-specific cyclotide Vhl-1 obtained from *Viola hederacea* exhibit anti-HIV activity, with an EC_{50} of 0.87 μM.[70] Furthermore, cyclotide palicourein was isolated from *Palicourea condensata*, and exhibited significant activity against viral strain HIV-1RF in CEM-SS cells, with an EC_{50} of 0.10 μM.[71]

Bio-technological applications

Cyclotides can be applied in the industry, such as the utilization of naturally active cyclotides as a bio-insecticide, given that several plants produce insecticidal cyclotides. For instance, cyclotides from the areal part of *butterfly pea* have potent membrane leakage activity for the insect gut mimicking membrane.[32] Subsequently, the butterfly pea areal part contains potential insecticidal cyclotides.[34] Recently, the extract obtained from *butterfly pea* has been demonstrated to contain a bioinsecticide in Australia, and this bioinsecticide (Sero-X) can be commercially used on maca-

Table 1. Anti-cancer cyclotides and details, such as source, family/subfamily and IC_{50}

Cytotoxic activity of cyclotides					
S.N.	**Cyclotides**	**Plant**	**Family/Subfamily**	**IC_{50} (μM)**	**Reference**
1	Chassatide C2	*Chassalia chartacea*	Rubiaceae/Bracelet	2.44	52
2	Chassatide C7			1.20	52
3	Chassatide C			1.00	52
4	Chassatide C10		Rubiaceae/Hybrid	5.00	52
5	Chassatide C11		Rubiaceae/Bracelet	1.20	52
6	Cliotide T1	*Clitoria ternatea*	Fabaceae/Bracelet	0.60	48
7	Cliotide T10			0.70	53
8	Cliotide T12			0.78	53
9	Cliotide T2		Fabaceae/Möbius	8.00	48,53
10	Cliotide T3			2.00	48,53
11	Cliotide T7		Fabaceae/Bracelet	0.73	53
12	Cter B			3.50	54
13	Cter E			2.50	54
14	Cter G			3.00	54
15	Cycloviolacin O19	*Viola odorata*	Violaceae/Bracelet	0.52	54,55
16	Cycloviolacin O3			0.42	54
17	Cycloviolacin O4	*Pombalia calceolaria Viola odorata Hedyotis diffusa*		9.80	50
18	Cycloviolacin O8			0.80–1.15	56
19	Hedyotide B5		Rubiaceae/Bracelet	1.03–1.32	49
20	Hedyotide B6			1.85–2.33	49
21	Hedyotide B7			0.33–0.68	49
22	Hedyotide B8			1.88–3.11	49
23	Hedyotide B9			1.14–2.01	49
24	Mela 1	*Melicytus latifolius*	Violaceae/Möbius	2.09–9.83	57
25	Mela 2			1.30–19.26	57
26	Mela 3			2.04–18.73	57
27	Mela 4			2.04–18.73	57
28	Mela 5			1.58–11.42	57
29	Mela 6			1.58–11.42	51
30	Tricyclon A	*Viola tricolor*	Violaceae/Bracelet	8.70	54
31	Poca A	*Pombalia calceolaria*		1.80	50
32	Poca B			2.70	50
33	Psyle A	*Psychotria leptothyrsa*	Rubiaceae/Bracelet	7.770	51
34	Psyle E			0.64–1.73	51
35	Mram 8	*Viola philippica*		1.75–15.5	57
36	Vaby A	*Viola abyssinica*	Violaceae/Möbius	7.60	58
37	Vaby D			2.60	58
38	Vibi E	*Viola biflora*	Violaceae/Bracelet	3.20	23
39	Vibi G			0.96	23
40	Vibi H			1.60	23
41	Viphi A	*Viola philippica*		1.75–15.5	23
42	Viphi D			1.55–5.24	59
43	Viphi E			1.55–5.24	59
44	Viphi F			1.03–6.35	59
45	Viphi G			1.03–6.35	59

Table 2. Anti-HIV activity exhibiting cyclotides and details, including source, family/subfmaily and EC_{50}

Anti-HIV activity of cyclotides					
S.N.	**Cyclotides**	**Plant**	**Family/Subfamily**	**EC50 (μM)**	**Reference**
1	Circulin A	*Chassalia parvifolia*	Rubiaceae/Bracelet	0.04–0.26	67
2	Circulin B				
3	Circulin C			0.05–0.275	9
4	Circulin D				
5	Circulin E				
6	Circulin F				
7	Cycloviolin A	*Leonia cymosa*	Violacea/Bracelet	0.13	68
8	Cycloviolin B				
9	Cycloviolin C				
10	Cycloviolin D				
11	Cycloviolacin O13	*Viola odorata*		0.32	66
12	Cycloviolacin O14		Violacea/Möbius	0.44	
13	Cycloviolacin O24			0.30	
14	Cycloviolacin Y1	*Viola yedoensis*	Violaceae/Bracelet	1.21	69
15	Cycloviolacin Y4			0.20	
16	Cycloviolacin Y5			0.04	
17	Kalata B1		Violacea/Möbius	0.66	
18	Vhl-1	*Viola hederacea*	Violaceae/Bracelet	0.87	70
19	Palicourein	*Palicourea condensate*	Rubiaceae/Bracelet	0.10	71

damia nut and cotton crops. Similarly, due to the inhibitory activity of Kalata-B1 against activated peripheral lymphocytes, the direct plausibility of this promising pharmaceutical has led to its application as an immunosuppressant. The subsequent study of the lysine scanning of kalata B1 and the analysis of the antiproliferative activity of prominent active mutant T20K revealed the inhibition of T cell proliferation by downregulating the expression of interleukin-2, its receptor, interferon-γ and tumor necrosis factor (TNF-α).[72,73] After several successive *in vivo* experiments with T20K kalata B1, Cyxone was recently produced, and this is presently being tested in a phase 1 clinical trial as an immunosuppressant.[74] Furthermore, cyclotides obtained from *Palicourea sessilis* were identified as pase A–E, and some of these cyclotides, such as pase A–D, inhibit the proliferation of human lymphocytes in a dose-dependent manner, suggesting that pase cyclotides may work as immunosuppressants.[75]

As a consequence of high tolerance of sequence variability, cyclotides and other knottin peptides have been developed for the diagnosis, regulation of expression or inhibition, and specific delivery of candidate drugs through the introduction of multiple specific epitopes.[29,76] Grafting bioactive epitopes onto specific cyclotide frames can help to stabilize the bioactive epitope and enhance the ability to cross membranes.[14] The first pharmacologically active construct of cyclotides was designed by incorporating the vascular endothelial growth factor receptor on multiple loops of kalata B1.[77] The goal of the growth factor receptor grafting was to competitively inhibit the interaction between the growth factor and its receptor, in order to stop the proliferation of cancerous cells in the vascular system. In a similar approach, several antagonists were designed by utilizing cell-permeating trypsin inhibitor cyclotides to target cytokine-associated cancers. For instance, angiotensin-1-7 (Ang 1–7) is a peptide hormone that counters against angiotensin II *via* the MAS receptor, and exhibits vasodilator, anti-proliferative and anti-angiogenic activity. However, its poor stability in serum affects its clinical application. Interestingly, Ang 1–7 grafted in MCoTI-I has good stability, and a comparable affinity for the MAS receptor.[33] In addition, several constructs of cyclotides were designed to circumvent cardiovascular problems, neurodegenerative diseases, autoimmune disorders, such as multiple sclerosis, and other inflammatory response, which were probably executed through the modulation of protein-protein interaction.[33,78,79]

Stabilized scaffolds due to the presence of disulphide knots and cyclic nature offer a window to incorporate biologically active peptides between the backbone of the intra-cysteine sequence, in order to avoid enzymatic degradation. Recently, the oral natural disulphide knot construct, Linzess (guanylate cyclase C agonist), has been approved by the FDA to combat chronic constipation and petulant bowel syndrome,[80] bringing great interest for the development of other knottin-based oral drug candidates. The incorporation of bioactive peptides between the cysteines loop of cyclotides or knottin peptides can lead to several successful constructs, such as the orally active analgesic Bradikynin B1 construct developed by grafting the active peptide into kalata B1.[81] Similarly, the anti-obesity antagonist candidate molecule was developed through the incorporation of a group of peptide hormones, Melanocortin, with kalata B1.[82] Angiogenic peptides, such as laminin and osteopontin, were incorporated into the trypsin inhibitor, MCoTI-II, to develop an antagonist of the vascular endothelial growth factor receptor.[83] All these constructs have significant potency and great stability in physiologic conditions. Therefore, the unique characteristic of

the very high sequence tolerance of cyclotides can provide a great lead scaffold to construct stable candidate drugs or diagnostic molecules, opening a window for the development of various lead molecules.

Future perspectives

In Australia, the butterfly pea extract known as, Sero-X, has recently been approved as a bioinsecticide for use on macadamia nut and cotton crops. The first cyclotide pharmacologically active construct was created by incorporating the vascular endothelial growth factor receptor on the multiple loops of kalata B1. The recent FDA approval of the oral natural disulphide knot construct, Linzess, against constipation and petulant bowel syndrome has triggered great interest on the development of oral knottin-based drug constructs. In this line, several constructs have been reported, such as analgesic Bradikynin B1, an anti-obesity antagonist by peptide hormone Melanocortin with kalata B1, angiogenic peptides, such as laminin and osteopontin incorporated into trypsin inhibitor MCoTI-II, and others. Due to the numerous biological properties and high tolerance for sequence variability, cyclotides are attracting a lot of attention for its potential applications in the pharmaceutical and agricultural sectors.

Conclusions

In contrast to the animal system, the majority of plant antimicrobial peptides comprise of multiple disulphide bridges, which provide compactness and stability in adverse environments, and even against metabolic enzymes. Cyclotides have high stability against high-temperature and enzymatic degradation. Due to its structure nature, cyclotides can act as stabilized scaffolds to open a window for inserting biologically active peptides between inter-cysteine sequences (loop), in order to avoid enzymatic degradation.

To date, the majority of plant diversity remains unexplored, and cyclotides have only been reported from a few of plant families. Therefore, the present status demands more intensive researches, in order to identify more biologically functional, stable and safe cyclotides, which may benefit humans. Identifying cyclotides can provide the clues and footsteps to the synthetic biology of peptides. At present, the majority of studies have used plant leaves and roots for identifying novel cyclotides. However, it remains unclear whether plant seeds contain novel cyclotides, because cyclotides linoorbitides was reported in the seeds of *Linum usitatissimum*.

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Conflict of interest

Dr. Sarfuddin Azmi has been an editorial board member of *Journal of Exploratory Research in Pharmacology* since February 2022. The authors have no other conflicts of interest to declare.

Author contributions

Study concept and design: SA and MKH; acquisition of data: MM and SS; drafting of the manuscript: SA, MM, SS and MKH; critical revision of the manuscript for important intellectual content: SA and MKH; administrative, technical and material support: MM and SA; study supervision: SA, MM, MKH and SS. All authors have made a significant contribution to the study, and approved the final manuscript.

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