Review Article

The bountiful biological activities of cyclotides

Abstract

Cyclotides are exceptionally stable circular peptides (28–37 amino acid residues) with a unique cyclic cystine knot (CCK) motif that were originally discovered through ethnobotanical investigations and bioassay-directed natural products screenings. They have been isolated from four angiosperm families (Violaceae, Rubiaceae, Curcurbitaceae, and Fabaceae), and they exhibit a wide range of bioactivities including antibacterial/antimicrobial, nematocidal, molluscicidal, antifouling, insecticidal, antineurotensin, trypsin inhibiting, hemolytic, cytotoxic, antitumor, and anti-HIV properties. Reports indicate that the mechanism of cyclotide bioactivity is the ability to target and interact with lipid membranes via the development of pores. Additionally, the nature of their surface-exposed hydrophobic patch and CCK play integral roles in the potency of cyclotides. Their extraordinary stability and flexibility have recently allowed for the successful grafting of analogs with therapeutic properties onto their CCK framework. This achievement, coupled with the myriad of useful naturally occurring bioactivities displayed by cyclotides, makes them appealing candidates in drug design and crop management.

Key words:
Bioactivity, cancer, cyclotides, HIV, host defense

Discovering Cyclotides

The discovery of cyclotides is attributed to ethnobotanical investigations and bioassay-directed screenings of potentially therapeutic plants. In 1965, a professor of Pharmacognosy at Uppsala University, Dr. Finn Sandberg, reported his observations of indigenous plant use in the Central African Republic. A remedy from the plant "Wetegere" (Gbaya language), later identified as *Oldenlandia affinis* (Roem. & Schult.) DC (Rubiaceae), was administered to hasten uterine contractions.[1] In the 1970s, the Norwegian physician Lorents Gran participated in a Red Cross Relief Mission which included harvesting medicinal plants in the northern Congo of Africa. Dr. Gran observed women of the Lulua tribe (Tsjiluba language) harvesting the above-ground tissues of a plant called "kalata-kalata" which subsequently was taxonomically verified as *O. affinis*. Elder healers prepared an aqueous decoction (~1 part powdered aerial tissue to 1 part boiling water) and then ingested the "tea" to induce labor. Use of the plant as an uterotonic was surrounded by a degree of secrecy among the women, and although the decoction produced rapid deliveries, in some cases severe spasms ensued and emergency caesarian sections were required.[2-5]

Upon returning to his native country, Dr. Gran isolated several polypeptides in samples of *O. affinis* extracts that exhibited remarkably strong uterotonic activity. With the aid of protein chemist, Dr. Knut Sletten, the principal bioactive peptide, now named kalata B1, was identified and almost fully sequenced.[6] This peptide was speculated to be a cyclic structure; however, it was exceptionally resistant to degradation and N-terminal amino acid sequencing, and at the time the available enzymatic tests were insufficient to provide conclusive proof of the cyclic nature of kalata B1. Therefore, the complete sequence of the prototypic cyclotide, kalata B1, was not reported until the three-dimensional solution structure was confirmed using two-dimensional magnetic resonance (NMR) spectroscopy and distance-restrained simulated annealing.[7]

At around this time (mid-1990), three independent research facilities reported the discovery of macrocyclic peptides with

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six cystine residues isolated from violaceous and rubiaceous plants. During a screening for new saponins, the hemolytic violapeptide I (from *Viola* sp.; Violaceae) was isolated, and the finding was published in a German specialist trade journal.[8] In 1994, the National Cancer Institute in the USA was evaluating a collection of plants for anti-HIV activity; the cyclotides, circulin A and circulin B, were characterized from extracts of the tropical tree *Chassalia parvifolia* K. Schum (Rubiaceae).[9] Finally, Merck Laboratory Researchers (USA) identified cyclopsychotride A from extracts of *Psychotria vellosiana* Benth. (Rubiaceae) while testing natural products for neurotensin antagonistic activity.[10] During the next decade, additional reports on the isolation of polypeptides with a circular nature and unique cyclic cystine knot (CCK) motif from violaceous, rubiaceous, and cucurbitaceous plants were reported which prompted the formal designation of the cyclotides as a plant protein family in 1999.[11-13]

Cyclotide-Producing Plant Families

Violaceae

Roughly 198 cyclotides have been discovered from 36 species in the Violaceae, Rubiaceae, Cucurbitaceae, and Fabaceae plant families [Table 1]. Seventy-two percent of sequenced cyclotides have been characterized from 24 species of Violaceae, and cyclotides are present in every violaceous species analyzed. The family comprises ~23 genera and 800 species of cosmopolitan shrubs, herbs, and rarely trees[16] and takes its name from the genus *Viola*, the violets/pansies, which are tiny herbaceous perennials. In terms of economic revenue, violaceous flowers are frequently used in the fragrance and cuisine industries. Traditional Chinese medicine routinely incorporates the violets into healing practices, as several species have antioxidant anthocyanins, vitamin A and C, glycosides, saponins, flavonoids, carotenoids, and cyclotides. Furthermore, extracts from *Viola odorata* L., a species rich in cyclotides, display antineoplastic, antiviral, anti-HIV, and antitumor effects.[17]

Rubiaceae

The distribution of cyclotides in Rubiaceae is more limited (i.e., many species screened for cyclotides do not express them) compared with Violaceae, yet the extent to which cyclotides are present in Rubiaceae remains unclear, in part because fewer than 10% of the existing rubiaceous species have been evaluated for cyclotide expression. Rubiaceae is the fourth largest angiosperm family with ~650 genera and 13,000 species of shrubs and small trees that occur mostly in tropical and subtropical regions of the world.[18] Alkaloids are prevalent throughout the family, and familial members are an important source of coffee, timber, dyes, ornamentals, and prescription medicines.[19-21]

Cucurbitaceae

Only two cucurbitaceous cyclotides have been isolated from *Momordica cochinchinensis* (Lour.) Spreng. These cyclotides, MCoT-I and MCoT-II, inhibit trypsin, an enzyme essential for nutrition in mammalian systems, and are circular with the CCK motif yet they share no further sequence similarity to other cyclotides; therefore, these peptides have been described as cyclic knottins, trypsin inhibitors, or cyclotides.[22,23] The family of the melons and squashes, Cucurbitaceae, comprises ~125 genera and 960 species of predominantly annual vines.[24] The genus *Momordica* consists of ~60 species of climbing herbs and lianas that have a history of use in Chinese folk medicine.[25] A systematic search for cyclotides in Cucurbitaceae is warranted to explain their distribution.

Fabaceae

The most recent addition to the cyclotide-expressing plant families is Fabaceae, the family of the legumes;[26] 24 novel cyclotides have been isolated from *Clitoria ternatea* L. Fabaceae is the third largest family of angiosperms with ~730 genera and over 19,400 species of mainly herbs and large trees. Throughout history, humans have heavily relied upon fabaceous plants for agricultural and medicinal purposes. Fabaceous species provide one-third the global crop production. The cyclotide-expressing genus *Clitoria* consists of ~60 species of woody plants with papilionaceous flowers and leguminous fruits. Remedies of *C. ternatea* have been used to enhance fertility, control menstruation, treat gonorrhea, induce vomiting, and provide an antidote to animal bites in traditional healing systems throughout Asia, Africa, and South America.[27]

Cyclotide Structure

Cyclotides are circular proteins characterized by 27–38 amino acids and a unique cystine knot topology of six highly conserved cystine residues linked via three disulfide bonds as illustrated in Figure 1. The disulfide bonds (in yellow) connect cystine residues (Roman numerals I–VI) to create a ring and knotted configuration that generates six backbone segments (loops 1–6) between the successive residues. All cyclotides have an associated secondary structure involving a β-hairpin centered in loop 5.[13,28]

Cyclotides can be divided into three subfamilies. Möbius cyclotides have a *cis*-peptide bond prior to the Proline (Pro, P) in loop 5 which creates a twist in the conceptual ribbon of the peptide backbone; bracelet cyclotides lack this bond. Members of the Möbius subfamily generally show less variation in loop size and amino acid sequence, have fewer positively charged residues, and are less hydrophobic compared with bracelet cyclotides. The third subfamily, trypsin inhibitors, has been suggested, but as mentioned only two trypsin inhibitor cyclotide sequences have been discovered. Although structural variations provide the basis for subfamily delineation, a few natural chimeras (i.e., cyclotides containing some loops with characteristics of the Möbius subfamily and others with characteristics of the

Table 1: Known taxonomic distribution and abundance of cyclotides in angiosperms

Family	Taxa	Cyclotide
Violaceae	*Gloeospermum blakeanum* (Standl.) Hekking	Globa A-G
	G. pauciflorum Hekking	Glopa A-G
	Hybanthus denticulatus Ballard, Wetter, Zamora	Hyde A
	H. floribundus (Lindl.) F. Muell.	hyfl A–F; hyfl I–M
	H. parviflorus Baill.	Hypa A
	Hymanthera obovata Kirk	Hobo A
	Leonia cymosa Mart.	Cycloviolin A–D
	Melicytus ramiflorus J.R. & G. Forster	mram 5, 10, 11
	M. macrophyllus A. Cunn	Mema A; Mema B
	Orthion oblanceolatum Lundell	Orto A
	Rinorea gracilipes Engl.	Rigra A
	R. lindeniana Kuntze	Rili A; Rili B
	Viola arvensis Murr.	Varv peptide A–H; violapeptide 1; tricyclon B
	V. abyssinica Steud. ex Oliv.	Vaby A–E
	V. biflora L.	Vibi A–K
	V. cotyledon Ging.	Vico A–B
	V. decumbens L.f.	Vide A
	V. hederacea Labill.	Cycloviolacin H1–H4; vhr1; vhl1-2
	V. labridorica Schrank	Vila A–D
	V. nivalis Roem. & Schult.	Vini A
	V. odorata L.	Cycloviolacin O1-O25; Vodo M,
	V. philippica Cav.	N, O; violacin A Viphi A-H
	V. tricolor L.	Vitri A-F; tricyclon A; Varv Hm; Varv He
	V. yedoensis Makino	Cycloviolacin Y1–Y5
Rubiaceae	*Chassalia parvifolia* Schum.	Circulin A - F
	Chassalia discolor K. Schum.	CD-1
	Oldenlandia affinis (Roem. & Schult) DC	Kalata B1–B17; S
	Hedyotis biflora	Hedyotide B1, B2
	Palicourea condensata Standl.	Palicourein
	Palicourea rigida Kunth	Parigidin-br1
	Psychotria leptothyrsa Miq.	Psyle A-F
	Psychotria suterella Müll. Arg	PS-1
	Psychotria vellosiana Benth.	Cyclopsychotride A
Cucurbitaceae	*Momordica cochinchinensis* Spreng.	MCoTI-I, MCoTI-II
Fabaceae	*Clitoria ternatea* L.	Cter A-L; cliotide T1–T12

Family	Violaceae	Rubiaceae	Cucurbitaceae	Fabaceae	Total
Genera	8	5	1	1	15
Species	24	10	1	1	36
Cyclotides	134	38	2	24	198

The table provides a list of known cyclotides and the taxa from which they were originally isolated accumulated from Cybase and SciFinder Scholar searches[14,15]

bracelet subfamily) have been described.[29] As additional cyclotides are discovered, subfamily classifications may require evaluation.

The primary structural elements of cyclotides include a cystine knot associated with a distorted triple-stranded β-sheet stabilized by a number of hydrogen bonds, an almost strictly conserved glutamic acid (Glu, E) in loop 1 that is involved in hydrogen bonding interactions with loop 3, and a surface-exposed hydrophobic patch that influences retention time on RP-HPLC and bioactivity.[30,31] The highly conserved asparagine (Asn, N) or occasionally aspartic acid (Asp, D) in loop 6 is thought to be necessary for cyclization.[32] Almost all cyclotides have a glycine (Gly, G) residue preceding Cys IV[33] which readily adopts a positive ϕ angle required for the type II β-turn needed to connect loop 3 to the cystine knot.[30]

Biologically Active Properties of Cyclotides

In general, the use of peptides as pharmaceuticals has been limited due to inadequate stability and bioavailability under physiological conditions. However, the exceptional stability, sequence plasticity, and framework flexibility of cyclotides, coupled with their numerous potent bioactivities resulting from their ability to target lipid membranes, emphasize the assertion that these cyclic polypeptides are ideal candidates for studies in the development of

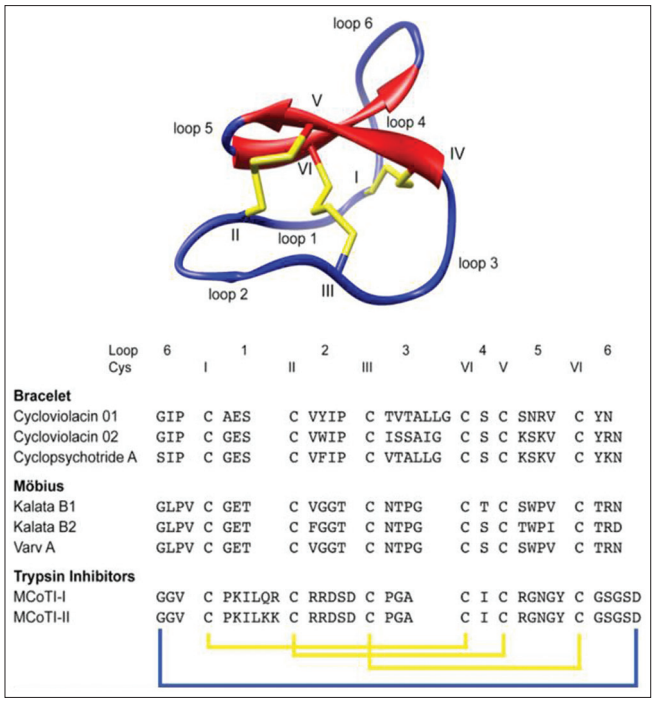

Figure 1: A representation of cyclotide structure and sequence. Kalata B1 (PDB ID 1nb1) has a seamless peptide backbone with three disulfides (yellow) connecting cysteine residues (Roman numerals). Backbone segments are labeled loops 1–6. Amino acid sequences are provided.

novel drugs and biopesticides.[34,35] The speculated natural function of cyclotides is in plant defense as illustrated by several reports of their antibacterial/antimicrobial,[36] insecticidal,[37,38] antihelmintic,[39-41] nematocidal,[42] antifouling,[43] and molluscicidal properties.[44] The use of cyclotides in human health applications was first explored during the discovery of kalata B1, and although its uterotonic activity was established in rat, rabbit, and human uteri, it is not recommended as an oxytocic agent because of the severe side effects.[4] During the past decade, a profusion of bioactivity-directed research demonstrates that cyclotides display an assortment of activities, including antineurotensin,[10] trypsin inhibiting,[22,23] hemolytic,[30,31] cytotoxic/antitumor,[45-56] and anti-HIV activities;[9,29] several of these properties have prospective therapeutic relevance.

Table construction to assemble cyclotide bioactivities

In an effort to amass the literature available pertaining to cyclotide biological activity and concisely illustrate it using informative reference tables, a series of SciFinder Scholar searches was first performed, and Table 2 shows the number of articles retrieved when using the search key word function and then searching either cyclotide by itself or cyclotide plus a biological activity. After reviewing each abstract and eliminating irrelevant publications, the decision was made to summarize those bioactivities that may have the greatest potential in agricultural and pharmaceutical applications

Table 2: Results of SciFinder Scholar searches for cyclotide bioactivity literature

Key word(s) searched	Number of articles
Cyclotide	449
Cyclotide and uterotonic or uteroactive	38
Cyclotide and hemolysis or hemolytic	52
Cyclotide and neurotensin	11
Cyclotide and antimicrobial	71
Cyclotide and antifungal	3
Cyclotide and insecticidal	77
Cyclotide and cancer	34
Cyclotide and antitumor	43
Cyclotide and cytotoxic	9
Cyclotide and anticancer	1
Cyclotide and HIV	80
Cyclotide and AIDS	12
Cyclotide and infectious disease	24

(i.e., host defense, anticancer, and anti-HIV properties); any manuscripts that had not been read were obtained for review. Tables 3-6 provide a wealth of information summarizing the bioassays and potency of evaluated cyclotides in/against the specified bioactivity. The remainder of this review highlights the factors affecting cyclotide potency and describes their potential use as natural defense agents and in the treatment of cancer and HIV.

Structural and Molecular Features of Cyclotides Impact Bioactivity

The mechanism of cyclotide bioactivity is membrane interaction

Changes in cyclotide bioactivity have been reported when different targets or cyclotides are compared which may indicate that a variety of membrane and cyclotide characteristics influence bioactivity. An increasing body of evidence suggests that membrane interactions and the formation of pores are responsible for cyclotide bioactivity.[47,50,52,69-73] For instance, reports indicate that kalata peptides selectively bind to bacterial membranes,[69,74] and some cyclotides may form discrete pores on the external surface of nematodes and thereby interact with the lipid-rich epicuticle layer at the surface of the worm.[42]

Furthermore, the antitumor properties of cyclotide, cycloviolacin O_2 (CyO_2) are caused first by the disruption of lipid membranes followed by leakage of contents from whole cells as well as liposomes.[70] CyO_2 causes rapid (within 5 minutes) disruption of lipid bilayers and is selectively cytotoxic in a dose-dependent manner. As a result of their pore-forming properties, CyO_2 and psyle cyclotides chemosensitize drug-resistant breast cancer cells to doxorubicin.[50,70] As illustrated in Table 5, cyclotides display potent cytotoxic properties against a range of cancer cell types, and a recent study evaluating neutral (zwitterionic)

Table 3: Cyclotides exhibiting antibacterial, antimicrobial, insecticidal, or molluscicidal activity

Activity	Cyclotide	Taxa or Cell line	Assay	Bioactivity	Ref.
Antibacterial and antimicrobial	Circulin A	*Staphylococcus (St) aureus; Candida kefyr; Candida tropicalis*	ARD	0.19, 18.6, & 19.4*	[36]
	Circulin B	*Escherichia coli; Proteus (Pr) vulgaris; Klebsiella oxytoca; St. aureus*	ARD	0.41, 6.80, 8.20, & 13.5*	[36]
	Cyclopsychotride A	*E. coli; K. oxytoca; Pr. vulgaris; Pseudomonas (Ps) aeruginosa*	ARD	1.55, 5.8, 13.2, 13.5*	[36]
	Cycloviolacin O2	*E. coli ; K. pneumonia; Ps. Aeruginosa; Salmonella (S) enterica* servoar Typhimuium LT2	ARD, MIC, TK	2.2 to >50**	[56,57]
	Kalata B1	*E. coli; S. enterica* servoar Typhimuium LT2; *St. aureus; C. kefyr*	ARD	0.26 to21.4*, >100**	[36,57]
	Kalata B2	*E. coli; St. aureus; St. enterica*	MIC	>35**	[57]
	Hedyotide B1	*E. coli; St. salivarius*	ARD	3.4 & 5.9*	[58]
	Vaby A	*E. coli; St. aureus; St. enterica*	MIC	32.5 to >90	[56,57]
	Vaby D	*E. coli; St. aureus; St. enterica*	MIC	50 to >90**	[56,57]
Insecticidal	Kalata B1	*Helicoverpa punctigera* (cotton budworm)	FT, MIC	†,**	[37]
	Kalata B2	*Helicoverpa armigera* (cotton bollworm)	FT, MIC	†;> 35**	[38]
	Kalta B1	*H. armigera*	FT	‡	[59]
	Hypa A	*Ceratitis capitata* (Medfly)	MT	58–100%	[60]
	Parigidin-br-1	*Diatraea saccharali* (sugarcane borer) *Spodoptera frugiperda* (SF-9)	FT, CVT	60%†, 1.7¶	[61]
Reverse antifouling	Cycloviolacin O2	*Balanus improvises* (barnacle)	LAB	0.25§	[43]
Molluscicidal	Kalata B2	*Pomacea (Po) canaliculata* (golden apple snail)	MAA, MA	53¶, 78%	[44]
	Kalata B1	*Po. canaliculata*	MA	68%	[44]
	Cycloviolacin O1	*Po. canaliculata*	MA	100%	[44]

ARD = antimicrobial radial diffusion assays; CVT = cell viability tests using an insect cell line; FT = feeding trials; MT = mortality trials; LAB = larval attachment bioassays; MAA = molluscicidal activity assays; MA = mortality assays; MIC = minimum inhibitory concentration; Ref. = reference and; TK = time-kill kinetics; Percentages indicate percent mortality; *The values reflect the inhibitory concentration 50 (IC_{50}) (μM) from respective references; †The cyclotide significantly inhibited growth and increased mortality; ‡The cyclotide inhibited growth and caused swelling and lysis of midgut cells; §The concentration (μM) required for complete inhibition of settlement; ¶The concentration (μM) required to cause 50% mortality or cytotoxicity; **The values reflect the MIC (μM)

Table 4: Cyclotides displaying antihelminthic activity

Cyclotide	Taxa or Cell line	Level of bioactivity (IC_{50} μM)*	Ref.
Cycloviolacin O1	*Haemonchus contortus, Trichostronglyus columbriformis* (sheep nematodes)	2.82, 3.89	[39]
Cycloviolacin O2	*H. contortus*†, *T. columbriformis*	0.12, 0.24	[39]
Cycloviolacin O3	*H. contortus, T. columbriformis*	0.21, 0.23	[39]
Cycloviolacin O8	*H. contortus, T. columbriformis*	0.24, 0.22	[39]
Cycloviolacin O13	*H. contortus, T. columbriformis*	0.21, 0.19	[39]
Cycloviolacin O14	*Necator americanus* (human hookworm), *Ancylostoma caninum* (dog hookworm), *H. contortus*†, *T. columbriformis*	1.40, 0.37, 0.41, 0.64	[39,41]
Cycloviolacin O15	*H. contortus, T. columbriformis*	0.38, 0.41	[39]
Cycloviolacin O16	*H. contortus, T. columbriformis*	0.27, 0.45	[39]
Cycloviolacin O24	*H. contortus, T. columbriformis*	1.74, 2.99	[39]
Cycloviolacin H3	*H. contortus, T. columbriformis*	0.85, 5.90	[39]
Cycloviolacin Y4	*H. contortus, T. columbriformis*	2.01, 2.27	[39]
Cycloviolacin Y5	*H. contortus, T. columbriformis*	2.28, 2.40	[39]
Kalata B1	*H. contortus*†, *T. columbriformis, N. americanus, A. caninum*‡	2.26, 5.22, 3.63, 1.57	[39,41]
Kalata B2	*H. contortus, T. columbriformis*	1.59, 5.69	[39]
Kalata B6	*H. contortus, T. columbriformis, A. caninum*	0.87, 2.62, 7.13	[39,41]
Kalata B7	*H. contortus, T. columbriformis*	6.29, 5.64	[39]

*The level of bioactivity (inhibitory concentration 50 = IC_{50}) was evaluated in larval development assays. †Significant decreases in motility were observed in adult worm motility assays. ‡Significant decreases in motility and survival were observed in adult worm motility assays. Ref. = reference

membranes with and without cholesterol and/or anionic lipids demonstrates that the membrane binding and disrupting properties of cyclotides are dependent on lipid composition since CyO_2, a member of the bracelet subfamily, was a potent membrane disrupter with selectivity toward anionic membranes, while Möbius cyclotides, kalata B1 and kalata B2, display significantly less lytic activity toward those membranes.[72,75]

Table 5: Cyclotides exhibiting cytotoxic or antitumor activity

Taxa	Cyclotide	Cell line or type	IC_{50} (μM)	Ref.
Psychotria leptothyrsa	Psyle A - F	U-937GTB, MCF-7, MCF-7/ADR	0.72 to > 10	[51,52]
Viola odorata	CyO2	A549, ACHN, BEL-7402, BGC-823, BEL-7402, DU145, CCRF-CEM, CCRF-CEM/VM-1, CLL,NCI-H66, NCI-H69AR, MDA-MB-231, OVCA, PBMC, RPMI-8226/s, RPMI-8226/ Dox40, RPMI-8226/LR-5, U251, U-937GTB, U-937VcR	0.1 to 17.05	[45,49,52,55]
V. abyssinica	Vaby A	U-937 GTB	7.6	[56]
	Vaby D	A549, BEL-7402, BGC-823, DU145, MDA-MB-231, U251, U-937 GTB	2.8 to 46.62	[53,56]
V. arvensis	Varv A	ACHN, CCRF-CEM, CCRF-CEM/VM-1, CLL,NCI-H66, NCI-H69AR, OVCA, PBMC, RPMI-8226/s, RPMI-8226/ Dox40, RPMI-8226/LR-5, U-937GTB, U-937VcR	2.7 to 12.1	[45]
	Varv F	ACHN, CCRF-CEM, CCRF-CEM/VM-1, CLL,NCI-H66, NCI-H69AR, OVCA, PBMC, RPMI-8226/s, RPMI-8226/ Dox40, RPMI-8226/LR-5, U-937GTB, U-937VcR	2.6 to 7.4	[45]
V. labridorica	Vila A	A549, BEL-7402, BGC-823, DU145, MDA-MS-123, U251	5.08 to > 10	[49]
	Vila B	A549, BEL-7402, BGC-823, DU145, MDA-MS-123, U251	6.25 to 34.65	[49]
	Vila D	A549, BEL-7402, BGC-823, DU145, MDA-MS-123, U251	> 10 to 49.59	[49]
V. philippica	Viphi A–G	BGC-823, HeLa, HFF-1, MM96L	1.03 to 6.35	[55]
	Viba 15, 17, Varv A, Kalata B1	BGC-823, HeLa, HFF-1, MM96L	1.32 to 10.21	[55]
V. tricolor	Varv A,D, E, F, H, He, Hm	RPMI-8226/s, U-937GTB	4 to 74.39	[46,54]
	Vitri A–F	A549, BEL-7402, DU145, MDA-MB-231, RPMI-8226/s, U251, U-937GTB	0.6 to 54.39	[46,54]
V. biflora	Vibi D	U-937GTB	> 30	[49]
	Vibi E	U-937GTB	3.2	[49]
	Vibi G	U-937GTB	1	[49]
	Vibi H	U-937GTB	1.6	[49]

A549 (human lung carcinoma); ACHN (renal adenocarcinoma); BEL-7402 (human hepatocellular carcinoma); BGC-823 (gastric carcinoma); CCRF-CEM and CCRF-CEM/VM-1 (T-cell leukemia and drug resistant sub-line); CLL – Chronic lymphocytic leukemia; Hela – human epithelial carcinoma; HFF-1 – Human foreskin fibroblasts; MDA-MB-231 (human breast carcinoma); MM96L (human melanoma); NCI-H66 and NCI-H69/AR (small cell lung cancer and resistant sub-line); OVCA – Ovarian carcinoma; PBMC – Peripheral blood mononuclear cells; RPMI-8226/s and RPMI-8226/Dox40 and RPMI-8226/LR-5 (myeloma and two drug resistant sub-lines); U-937GTB and U-937VcR (histiocytic lymphoma and drug resistant sub-line); MCF-7 and MCF-7/ADR (human adenocarcinoma breast cancer and drug resistant sub-line) and U251 (human glioblastoma). CyO_2 = cycloviolacin O_2 and Ref. = reference

The potency of anti-HIV activity for cyclotides (as exemplified in Table 6) is also influenced via membrane interactions. For example, kalata B1 is sequestered on the membrane most likely through self-association and then forms conductive pores with channel-like activity via the insertion of oligomers into the lipid bilayers of membranes.[76] Furthermore, specific interactions with phospholipids containing phosphatidylethanolamine (PE) headgroups and nonspecific lipid hydrophobic interactions can alter anti-HIV potency. Kalata B1 can target and disrupt HIV particles that have raft-like membranes and are rich in PE phospholipids.[71] The activity is not dependent on the recognition of chiral receptors since the all D-enantiomer of kalata B1 that was synthesized was still active in cytotoxic and hemolytic assays.[73] Taken together, this body of evidence indicates that the mechanism of cyclotide bioactivity is membrane interactions via pore formation.

A defined hydrophobic patch influences bioactive potency

One prominent characteristic of cyclotides is a hydrophobic patch which is formed by solvent-exposed amino acids that protrude outward to the molecular surface due to the occupation of the core by the disulfide bonds of the cystine knot; this feature impacts antibacterial,[36,57] insecticidal,[38] cytotoxic,[75] anti-HIV,[64,65] and hemolytic activities[77,78] such that increases in the hydrophobic surface area correlate with enhanced bioactivities.[64] As mentioned, the cyclotide subfamilies, bracelet and Möbius, differ in the absence and presence of a *cis*-Pro peptide bond in loop 5, respectively. These subfamilies also differ in their orientation in the membrane due to variations in the distribution of surface-exposed hydrophobic amino acid residues and their net charge. For instance, the bracelet cyclotide, CyO_2, interacts with the lipid bilayers via the hydrophobic segments of loops 2 and 3, while the hydrophobic loops 5 and 6 are buried in the membrane of the Möbius peptide varv A. This feature impacts bioactivity in that bracelet cyclotides tend to be more potent. Interestingly, chimeric cyclotides such as kalata B8 and psyle A demonstrate the importance of the amphiphatic structure of cyclotides in that they resemble Möbius cyclotides except for their loop 5 composition. In this loop, the loss of hydrophobic residues generally seen in Möbius cyclotides disrupts their amphipathicity and decreases cytotoxicity by greater than 30-fold.[72,75]

Table 6: Cyclotides exhibiting anti-HIV activity

Taxa	Cyclotide	IC_{50} (μM)	EC_{50} (μM)	Ref.
Chassalia parvifolia	Circulin A	0.04–0.26	0.05	[9]
	Circulin B	0.04–0.26	0.05	[9]
	Circulin C	0.05–0.275	*	[62]
	Circulin D	0.05–0.275	*	[62]
	Circulin E	0.05–0.275	*	[62]
	Circulin F	0.05–0.275	*	[62]
Leonia cymosa	Cycloviolin A	0.13	0.56	[63]
	Cycloviolin B	0.13	0.56	[63]
	Cycloviolin C	0.13	0.56	[63]
	Cycloviolin D	0.13	0.56	[63]
Viola odorata	Cycloviolacin O2[†]	*	*	[50]
	Cycloviolin O13	0.32	6.4	[64]
	Cycloviolacin O14	0.44	4.8	[64]
	Cycloviolacin O24	0.308	6.17	[64]
Viola yedoensis	Cycloviolin Y1	1.21	4.47	[65]
	Cycloviolin Y4	0.12	1.72	[65]
	Cycloviolin Y5	0.04	1.76	[65]
Oldenlandia affinis	Kalata B1	0.14	3.5	[66]
	Kalata B8	2.5	11	[67]
Palicourea condensata	Palicourein	0.1	1.5	[29]
Viola tricolor	Varv E	0.35	3.98	[65]
Viola hederacea	Vhl-1	0.87	*	[68]

IC_{50} refers to the cytotoxicity to target cells, and EC_{50} refers to the cytopathic inhibitory activity in XTT (tetrazolium salt) cell proliferation assays. Ref. = Reference. *Not tested. [†]Cycloviolacin O_2 (1.5 μM) caused ~60% membrane disruption on HIV type 1 HTLVIIIB cells which was four-fold greater than uninfected human T-cell lymphoma HuT78 cells[50]

Two amino acid residues that can impact potency of several bioactivities are the conserved Glu in loop 1 and tryptophan (Trp, W). For instance, the esterification of Glu in the bracelet cyclotide, CyO_2, results in a 48-fold decrease in cytotoxic potency[48] and a near loss of antibacterial activity against *Salmonella* sp.[57] Apparently Glu does not similarly affect the Möbius cyclotide, varv A, which displays only a three-fold decrease in potency when the residue is esterified. The hydrophobic Trp residue common in many cyclotides plays an important role in bioactivity because when peptides containing Trp bind to the membrane, Trp is buried into the lipid bilayers and enhances cytotoxicity. Hydroxlyation of Trp in models of varv A and CyO_2 results in dramatic decreases in cytotoxicity.[72,75]

An intact circular backbone is essential for several cyclotide bioactivities

The unique features of cyclotides (i.e., circular structure and CCK motif) are considered crucial traits impacting their bioactivity. Indeed, the reduction/alkylation of disulfide bonds and/or linearization of the circular backbone can result in a complete loss of antibacterial, cytotoxic, or hemolytic activity.[30,48,58,79] Reduced peptides in general are significantly more susceptible to denaturation via enzymes or chemicals compared with oxidized species.[80,81] However, psyle C is a linear peptide (or "uncyclotide" as suggested by Nguyen and colleagues)[58] that retains the other unique features of cyclotides, and it is active against lymphoma, breast cancer, drug-resistant breast cancer, and chemosensitizes cells to the anticancer drug doxorubicin.[50-52] Violacin A is also an uncyclotide although it has dramatically reduced hemolytic activity compared with other cyclotides which is thought to be attributed to its atypically low hydrophobicity and linear nature.[78] Hedyotide B2, a third naturally occurring uncyclotide, has no bacterial activity.[58] Thus, it appears that forced reduction results in a loss of activity, and further studies on the retained cytotoxicity of psyle C and bioactivity potency of the other uncyclotides may shed new insight on the importance of the CCK.

Cyclotides may be therapeutic and useful scaffolds in drug design

Recently, analogs with vascular endothelial growth factor (VEGF) antagonism were successfully grafted onto the CCK framework of kalata B1. The normal function of VEGF is the creation of new blood vessels; however, when VEGF is overexpressed, it can contribute to disease. Solid cancers will not grow beyond a limited size without a supply of blood. Cancers that express VEGF have an ample source of blood and are able to grow and metastasize. Therefore, the development of stable peptide analogs with VEGF antagonism which are grafted onto cyclotides is a novel approach that may be useful in the treatment of diseases where angiogenesis is an important component.[35]

Additionally, cyclotides display potent, salt-dependent antibacterial properties against both Gram-negative and Gram-positive bacteria as illustrated in Table 3.[36] Since

the cyclic structure and cystine knot motif of cyclotides closely resembles current antimicrobial drug leads, such as microcin J25, cyclotides may be useful templates for designing novel antibiotics. Tables 3 and 4 also emphasize the fact that cyclotides can inhibit the movement, growth, and development of insect larva and parasites and increase mortality.[37,38,59-61] Taken together, these reports support the supposition that cyclotides alone or as scaffolds can deter or be engineered to inhibit interactions associated with cancer, infectious disease, and pest management.

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