Journal of Enzyme Inhibition and Medicinal Chemistry

ISSN: 1475-6366 (Print) 1475-6374 (Online) Journal homepage: informahealthcare.com/journals/ienz20

Cyclotides: a natural combinatorial peptide library or a bioactive sequence player?

Adriano Mollica, Roberto Costante, Azzurra Stefanucci & Ettore Novellino

To cite this article: Adriano Mollica, Roberto Costante, Azzurra Stefanucci & Ettore Novellino (2015) Cyclotides: a natural combinatorial peptide library or a bioactive sequence player?, Journal of Enzyme Inhibition and Medicinal Chemistry, 30:4, 575-580, DOI: 10.3109/14756366.2014.954108

To link to this article: https://doi.org/10.3109/14756366.2014.954108

Journal of Enzyme Inhibition and Medicinal Chemistry

http://informahealthcare.com/enz
ISSN: 1475-6366 (print), 1475-6374 (electronic)

J Enzyme Inhib Med Chem, 2015; 30(4): 575–580
 DOI: 10.3109/14756366.2014.954108

informa healthcare

REVIEW ARTICLE

Cyclotides: a natural combinatorial peptide library or a bioactive sequence player?

Adriano Mollica[1], Roberto Costante[1], Azzurra Stefanucci[2], and Ettore Novellino[3]

[1]*Dipartimento di Farmacia, Università di Chieti-Pescara "G. d'Annunzio", Chieti, Italy,* [2]*Dipartimento di Chimica, Sapienza, Università di Roma, Rome, Italy, and* [3]*Dipartimento di Farmacia, Università di Napoli "Federico II", Naples, Italy*

Abstract

In this perspective review, we focalized our attention on the application of cyclotides in drug discovery. To date, two principal approaches have been explored since now: (i) the use of cyclotides as scaffold in which bioactive peptides can be grafted to improve stability, oral bioactivity and binding to GPCRs; (ii) their application as natural peptides library. For these reasons, cyclotides probably represent today a step further in the development of new tools in drug design.

Keywords

Cyclic peptides, cyclotides, peptides

History

Received 4 August 2014
Accepted 9 August 2014
Published online 22 September 2014

Introduction

Peptides are a class of organic substances widely diffused in the biosphere. They act as hormons, neurotransmitters and structural biopolymers. Medicinal chemists are constantly learning from nature how peptide signaling works and how to use natural peptide messengers as *lead compounds* for the development of new molecular entities. One of the most important step further in this field was the discovery of a rational hierarchical approach to the peptidomimetics design developed several years ago by eminent scientists such as V. J. Hruby, F. Albericio, D. Seebach, C. Toniolo, etc[1].

This approach consists in the identification of the natural bioactive peptide sequence followed by a series of studies aimed to find the minimum pharmacophore (Ala scan, residues deletion, substitution, truncations) and its optimization to maximize the biological activity and stability. In a physiological environment native peptides are usually synthesized (or released) and degradated directly at the site of action, thus they are very sensitive to cleavage and poorly selective. Then the optimization process usually is based on the introduction of conformational restrictions on the backbone and on the amino acids side chains, in order to stabilize a rigid bioactive structure, with the aim to gain the resistence against the enzymatic degradation. Cyclization was found to be a major tool to stabilize a peptide in a certain bioactive conformation. Many techniques have been developed for peptide cyclization, but the most common are:

(i) The formation of a disulphide bridge between two cysteine residues (–S–S–).
(ii) The amide bond formation (–C(=O) –NH–), namely: ''*head-to-tail*'', ''*side chain-to-tail*'' and ''*side chain-to-side chain*'' cyclizations.

Lately the double bond (–C=C–) formation between two allylglycine residues has also been explored by several researchers since the Grubbs catalyst became easily available[2].

In particular, nature itself is a source of ispiration for the use of disulphide bonds as feature to stabilize complex structures, due to the amazing multitude of native peptides such as insuline, vasopressine, oxytocin, various proteins and polypeptides (Figure 1).

In 1970 Gran et al. discovered cysteine-rich cyclopeptides in *Oldenlandia affinis*[3]. The African women used the decotion of this plant for its oxytocic activity. The plant extract contained many cyclopeptides and some linear fragments then from the analysis of the bioactive extract, a new class of peptides was identified: the cyclotides.

Other cyclotides were found in the violet family (Violaceae), coffee family (Rubiaceae), cucurbit family (Cucurbitaceae) and recently in the Fabaceae and Solanaceae[4].

The great biodiversity and abundance of cyclotides (it has been estimated that there are more than 50 000 different molecules)[4] allows them to be considered, with a good approximation, as a *natural combinatorial peptide library.*

Today, it is not clear the exact role of these complex compounds in plants but their insecticidal, antimicrobial and cytotoxic activities could suggest an involvement in plant's defence against external attacks. Cyclotides were also tested for a number of human pathologies, i.e. for their potential pharmaceutical properties against cancer and HIV, with good results.

Address for correspondence: Dr. Adriano Mollica, Dipartimento di Farmacia, Università di Chieti-Pescara ''G. d'Annunzio'', Via dei Vestini 31, 66100, Chieti, Italy. Tel/Fax: 08713554476. E-mail: a.mollica@unich.it

The good bioactivity and oral bioavailability, together with the high enzymatic stability, make cyclotides good candidates for pharmaceutical applications[3,5,6], and excellent starting point for the *de-novo* peptidomimetics design.

Structure

Cyclotides are *head-to-tail* cyclized peptides, with six cysteine residues oxidized into three disulphide bonds forming a conserved tertiary *supra*-organization called *cystine-knot* motif (CCK)[7], a well-defined 3D shape that confers to the entire molecule great stability against chemical and enzymatic degradation[8]. Although they are interesting scaffolds for drug design in medicinal chemistry[9], unfortunately the screening process for the occurrence of cyclopeptides is often trivial and laborious due to the great differentiation of plant species and the scarse availability of samples[10].

Craik and co-workers defined the *Möebius* and *bracelet* subfamilies for the classification of cyclopeptides[11], which differ for the presence of a *cis* amide bond (Möebius type) centered at the Pro residue in loop five (Figure 2). Approximately 2/3 of the total known cyclotides belong to the bracelet subfamily, however, there are other non-classical structures with characteristics from both the families.

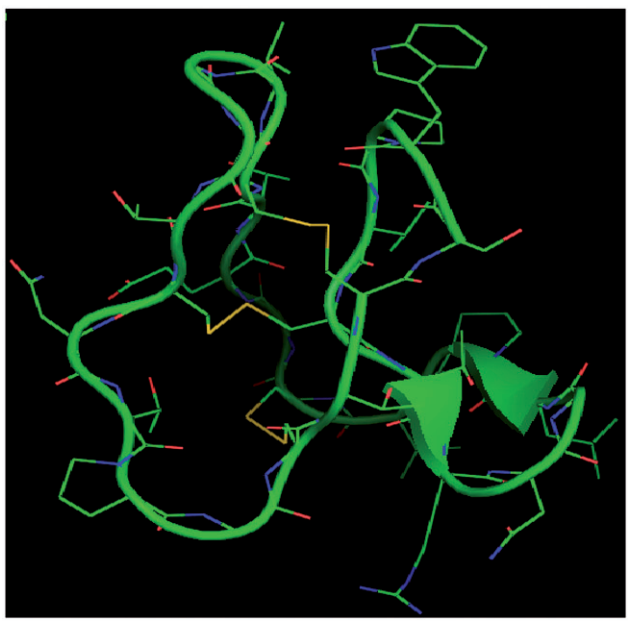


Figure 1. Pymol generated view of the uterotonic polypeptide kalata B1structure characterized by three disulphide bonds.

Until now, cyclotides' structures are not completely clear, expecially for the CCK motif which has been wide elucidated by NMR[3,7,12], with the help of total synthesis[13] and X-ray crystallography[14].

Structure–activity relationships

Natural cyclotides are formed by 28–37 residues and possess a variable net charge (from −2 to +3). The positive charge confers generally a certain affinity for the cell membranes, with a possible lytic or cytotoxic activity[15]. Cationic residues in the bracelet family, are the most responsible for the membrane interaction of cyclotides, as demonstrated by the progressive charge neutralization on cycloviolacin O2 (Figure 2), followed by a decreasing in tumor cell cytotoxicity[16]. The side chain of glutamine residue presents in the bracelet family, is important for membrane adsorption and aggregation acting on the structure of the cyclotide itself; in fact the Gln residue interacts with the backbone of loop 3 through hydrogen bonds, stabilizing a helical structure; in case of the möebius type, these interactions are also present but loop 3 has a polar nature and is not directly involved in membrane interactions. Structural studies revealed that the glutamate residue interacts with the center of the cyclotide and is responsible for the overall structure of the peptide rather than electrostatic interactions[4].

The Ala insertion in the highly conserved sequence of loop 1 (including Glu) abolished the lytic activity of kalata B1, similarly to the substitutions of lipophilic residues[17] in loop 5. The tryptophan residue (at loop 2 in bracelet type and in loop 5 in Möebius family) acts as a membrane interface anchoring residue and is responsible of the membrane adsorption process.

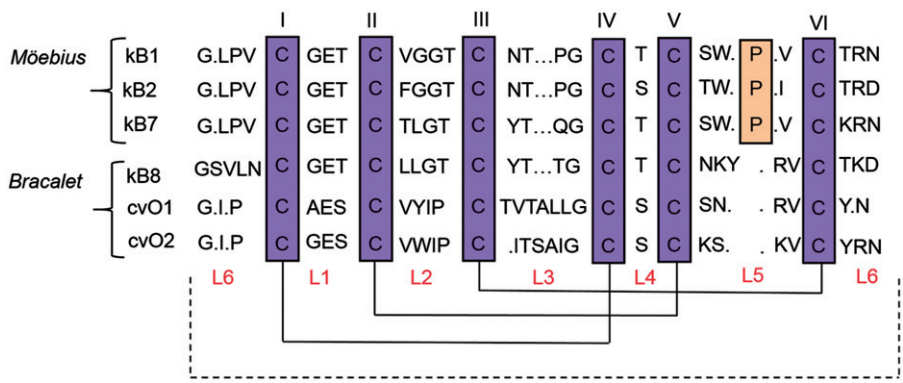
Synthetic approach

Disulphide-rich cyclic peptides synthesis still remains a challenging task due to the complexity level imposed by the 3D structure-stabilizing disulphide cross-links[18].

Recently Cheneval et al[19]. described a straightforward solution cyclization approach to obtain 26–34 size complex disulphide-rich cyclic peptides, using the mild-acid cleavable 2-chlorotrityl linker to generate partially protected peptides, which have been cyclized *head-to-tail* in solution using well established protocols, resulting in good yields (∼10%, based on quantity set up to be cyclized) of isolated peptide (Figure 3). The use of latest generation uronium-type coupling reagents (HATU) and sterically hindered bases (DIPEA) reduces racemization (epimerization) of the *C*-terminal residue during the cyclization step.

The products were folded by random oxidation of cysteines yielding the correct disulphide-bonded isomer in modest yield. In some cases additional isomers were observed and for [L6-RGDS]kalata B1 only the misfolded product was recovered.

Figure 2. Sequences of significative cyclotides belong to the two subfamilies *Möebius* type and *Bracelet* type.



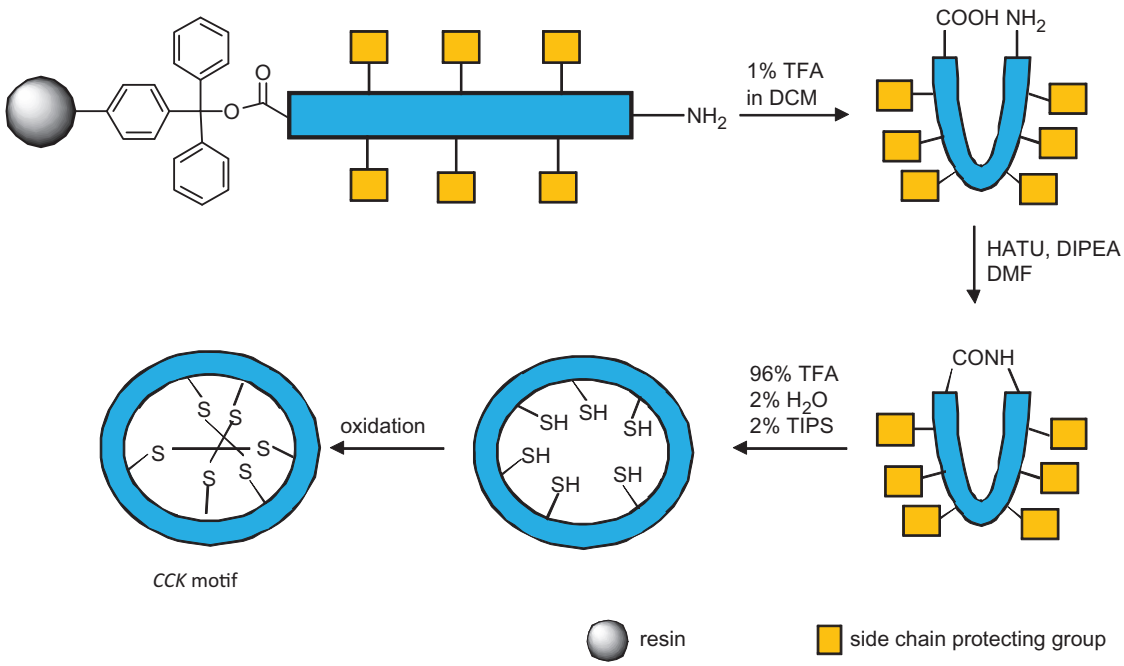


Figure 3. Cyclotides' synthetic strategy based on the random oxidation of deprotected cysteines.

In general, the presence of more than one set of disulphide-bond pairings during oxidation is quite common, as peptides containing six cysteine residues can potentially give rise to 15 different disulphide bonded arrangements[19].

In most cases orthogonally protecting strategies are not essential for the synthesis of Möebius and trypsin inhibitor cyclotides because the major isomer normally contains the desired native connectivity. The orthogonal approach to the synthesis of cyclotides involving different pairs of Cys-protecting groups was recently proposed and reviewed by Burman et al[8].

The role of cyclotides in drug discovery process is dual and must be considered under two different points of view:

(i) As *natural peptides library*. The identification of a bioactive fragment presents in the isolated cyclotide can be used as lead compound for futher modification as new lead compound;

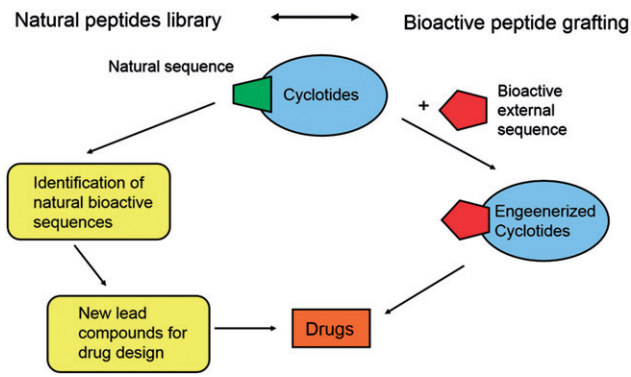
(ii) As *a bioactive sequences player*. A bioactive peptide previously optimized can be inserted into the scaffold of a cyclotide, in order to maintain its activity and to improve stability and oral bioavailability.

These two approaches (Scheme 1) will be separately discussed in sections ''Cyclotide bioactive sequences as lead compounds for the design of novel GPCR ligands'' and ''Peptide-grafting strategy'' of this article, citing few examples.

Cyclotide bioactive sequences as lead compounds for the design of novel GPCR ligands

A great number of drugs binds to receptors coupled with a G protein and cyclotides belong to these substances. Recently Koehbach et al.[20] identified a series of peptides from plants that acts similarly to oxytocin; Kalata B7, a cyclotide of the Möebius family, has been found to posses a strong oxytocic activity.

As expected, active peptides contained in the extracts of *O affinis* were separated and identified to obtain different cyclotides. Kalata B7 exerts its oxytocic activity in a similar way as oxytocin and vasopressin at their clonate receptors, and activates the G protein cascade associated to the oxytocin receptors.



Scheme 1. Schematic representation of the double use of cyclotides; on the right: natural sources of bioactive compounds; on the left: scaffold engineered bearing an external bioactive peptide sequence.

Lately, the sequence of loop 3 alone was found to stimulate the isolated myometrium contractions. After the identification of the peptide portion responsible for this activity, a certain omology of this fragment (–CYTQGC–) and oxytocin (CYIQNCPLG) appeared to be clear (Table 1).

Oxytocin binds to V1a receptors (which are up-regulated during pregnancy) producing contraction in the pregnant females[21]. Kalata B7 acts as a partial agonist at V1a receptors[22], in fact Atosiban, a delaying preterm birth agent is used clinically as both antagonist of oxytocin receptor and V1a receptor[23]. The tyrosine and glutamine residues present in Kalata B7 are organized in a type II β-turn capable to interact with the oxytocin receptor. Those residues are also present in oxytocin. Tyrosine at position 2 is important for receptor–ligand interaction of oxytocin with Tyr^{209} and Phe^{284} of the oxytocin receptor[24].

Thus several cyclopeptides were designed using the kalata B7 loop 3 as template, then kB7-OT1 ([G5, T7, S9])-oxytocin resulted to be a selective agonist at the oxytocin receptor capable to intact human myometrium in the submicromolar range, neglecting other subtype related receptors (i.e. V1a, V1b and

V2 receptors) at concentrations higher than 10 μM. The significative similarity in overall structures between kB7-OT_1 and oxytocin has been demonstrated by NMR studies. Amino acid residues Tyr^2, Ile^3 and Leu^8 are important for the binding at the receptor[25] and substitution of Asp^5 with Gly does not affect the binding ability and receptor activation, despite a significative influence on receptor selectivity[22].

Peptide-grafting strategy

Example A: bradykinin C1 receptor antagonist grafted in Loop 6 of Kalata-B1

The application of cyclotides as scaffold in the study of biologically active compounds has been recently reported. This approach consists of grafting an external peptide sequence into a cyclotide loops, so as to obtain chimeric molecules able to bind G protein-coupled receptors[26,27], e.g. to inactivate VEGF[28], to stimulate angiogenesis[29], to block the entry of HIV via CXCR4[30], and to inhibit serine proteases[31]. The exceptional resistence to enzymatic, chemical and thermal degradation is conferred by a unique well-defined three-dimensional conformation of cyclotides, making these peptides potent drug templates for medicinal and therapeutic applications[32], as peptide-grafting (i.e. grafting of bioactive amino acid sequences onto the cyclotide scaffold)[33], resulting in the synthesis of orally bioavailable and active receptor agonist or antagonist for successful therapeutic use (Figure 4)[34].

Peptide-grafting strategy was successfully performed by Wong et al[26]. incorporating bradykinin (BK) antagonist into Kalata-B1 scaffold. Two original orally bioactive BK-peptide analgesics were obtained by grafting *des*-Arg^{10}-[Leu^9]-kallidin (DALK) and *des*-Arg^9-kinestatin (DAK) onto the extended loop 6 of the kalata B1 (ckb) scaffold. (Table 2, part A)

Two new hybrid peptides were synthetized, ckb-kal and ckb-kin respectively by SPPS (Solid-Phase-Peptide-Synthesis) using the conventional *tert*-butoxycarbonyl strategy. The thioester precursors were purified by RP-HPLC followed by cyclization at the backbone. The final products with the CCK motif were obtained in good yields after oxidation. Enzymatic stability was tested for all the hybrid compounds and was found to be extremely higher than that of the parent BK antagonists. The engineered BK antagonists were also tested for bioactivity; ckb-kal resulted to be a potent antagonist while ckb-kin was the weaker one.

Table 1. Kalata B7 and loop 3 fragment analogues with oxytocic and vasopressin like activity[20].

Name	Sequences
Kalata B7	Cyclo-GLPVCGETCTLGTCYTQGCTCSWPICKRN
[T3,G5,T7, S8,S9]-OT	CYTQGCTSS-NH_2
[T3,P4,G5,S7, S8,T9]-OT	CYTPGCSST-NH_2
[P4,G5,S7,T9]-OT	CYIPGCSLT-NH_2
kB7-OT_1 [Y2A]	CAIQGCTLS-NH_2
kB7-OT_1 [Q4A]	CYIAGCTLS-NH_2
Oxytocin	CYIQNCPLG-NH_2
Vasopressin	CYFQNCPRG-NH_2

Example B: graft of melanocortin agonists

A second exemplificative paper on the insertion of a bioactive peptide sequence in the Kalata-B1 was carried out by

Table 2. Part A[26]: two relevant requences used for grafting in place of loop 6 with bradychinin activity. Part B[27]: four sequences used for grafting in place of loop 6 of Kalata B1 with MCR activity.

Example	Name	Sequence
	Kalata B1	Cyclo-CGETCVGGTCNTPGCTCSWPVCTRNGLPV
A	ckb-kal	KRPPGFSPL
	ckb-kin	QIPGLGPLG
B	Graft 1	GHFRWGV
	Graft 2	GHfRWGV
	Graft 3	THFRWPV
	Graft 4	THfRWPV

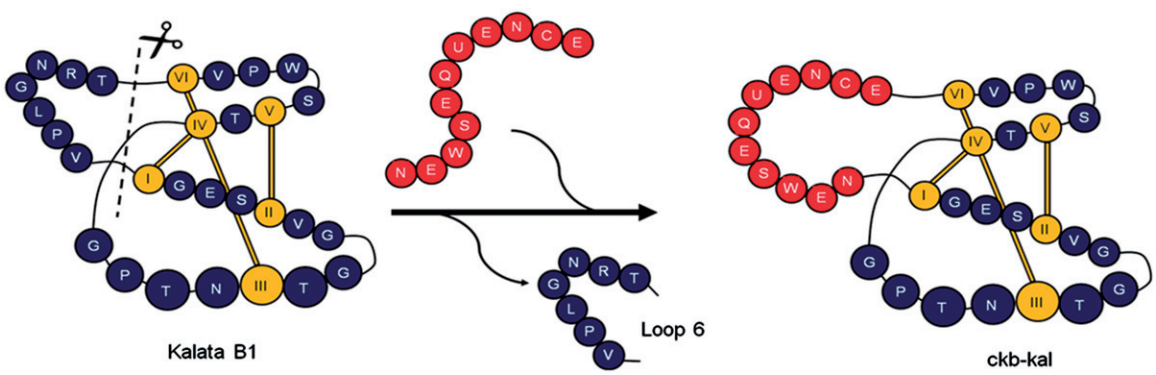


Figure 4. Bioactive peptide (*new sequence*) grafting in place of the native loop 6 of cyclotide scaffold.

Eliasen et al[27]. Melanocortin receptors (MCR)[35], namely, MCR1–5[36] are involved in homeostasis; targeting the MCR4 with an agonist represents an alternative approach for the treatment of obesity. MCR4 has been studied for years in order to develop a drug for weight control, against obesity. Several agonists of MCR4 have been developed and revealed to be effective in rodent models, even if actually no compounds have been approved for the market or clinical trial[37]. Probably, the low selectivity and the intrinsic poor stability of peptide molecules are the main problematic steps for the development of chemical entities for clinical trials[38].

Recently, the discovery of cyclotides stimulates the use of their features to solve some of the pharmacokinetic limitations related to peptides; inspired by the use of kalata B1 as scaffold for bradikinine analogues (example A), Eliasen et al.[27] proposed Kalata-B1 for the insertion of previously optimized melanocortin agonist sequences (Table 2, part B).

Four melanocortin analogues were synthesized, taking into account that a direct replacement of the native sequence with a new sequence different in lenght could force the bioactive (tetrapeptide) in a non-active conformation. Thus other similar sequences were chosen for grafting and two terminal glycine residues were also added. The analogue kB1(GHFRWG) showed the higher selectivity for MCR4, confirming the potential application of this method for targeting specific bioactive molecules implicated in many physiological processes. The observation that kB1(GHFRWG) is very stable to chymotrypsin digestion, demonstrates that the strong stability toward enzymatic cleavage of kalata B1 is systematically transferred to the grafted analogues.

Conclusions

With the recent growth of the research activity in the field of peptides, the concept of proteins as fragile biomolecules is profoundly changed in the last decade. The cyclic backbone and the CCK motif confer to cyclotides a non-common structural rigidity and stability, thus cyclotides have been manipulated by researchers in many ways. In this mini-review, we have described the dual use of the cyclotides: the grafting approach and their use as natural source of bioactive compounds.

Due to the extremely high biodiversity, there are thousands of cyclotides with different bioactivity, just waiting to be tested, similarly to the library-guided drug discovery process, adopted by pharmaceutical corporations, consisting of testing large small-molecules libraries at new biological targets. The interesting point is that in the past, cyclotides can be only derived with enormous difficulties from plant extracts and then tested, whereas today, thanks to the perfectioning of SPPS, HPLC purification and Mass spectroscopy advances, the epitopes responsible for the bioactivity can be isolated, analyzed, synthesized and used as lead compounds for further developments.

However other critical aspects about cyclotides need to be elucidated other than the enzymatic stability, biological profile and the possible immunogenicity of these compounds after oral administration.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

References

1. (a) Hruby VJ. Peptide science: exploring the use of chemical principles and interdisciplinary collaboration for understanding life processes. J Med Chem 2003;46:4215–31.
(b) Subiros-Funosas R, El-Faham A, Albericio F. PyOxP and PyOxB: the Oxyma-based novel family of phosphonium salts. Org Biomol Chem 2010;8:3665–73.
(c) Hoyer D, Bartfai T. Neuropeptides and neuropeptide receptors: drug targets, and peptide and non-peptide ligands: a tribute to Prof. Dieter Seebach. Chem Biodivers 2012;9:2367–87.
(d) Moretto A, Crisma M, Formaggio F, Toniolo C. Building a bridge between peptide chemistry and organic chemistry: intramolecular macrocyclization reactions and supramolecular chemistry with helical peptide substrates. Pept Sci 2010;94:721–32.
(e) Mollica A, Pinnen F, Stefanucci A, Costante R. The evolution of peptide synthesis: from early days to small molecular machines. Curr Bioact Comp 2013;9:184–202.
2. Mollica A, Guardiani G, Davis P, et al. Synthesis of stable and potent δ/μ opioid peptides: analogues of H-Tyr-c[d-Cys-Gly-Phe-d-Cys]-OH by ring-closing metathesis. J Med Chem 2007;50:3138–42.
3. (a) Gran L. An oxytocic principle found in Oldenlandia affinis DC. Medd Medd Nor Farm Selsk 1970;12:173–80.
(b) Gran L. On the effect of a polypeptide isolated from ''Kalata-Kalata'' (*Oldenlandia affinis* DC) on the dominated uterus. Acta Pharmacol Toxicol 1973;33:400–8.
(c) Gran L. Oxytocic principles of *Oldenlandia affinis*. Lloydia 1973;36:174–8.
4. (a) Mulvenna JP, Wang C, Craik DJ. CyBase: a database of cyclic protein sequence and structure. Nucleic Acids Res 2007;34:D192–4.
(b) Rosengren KJ, Daly NL, Plan MR, et al. Twists, knots, and rings in proteins. Structural definition of the cyclotide framework. J Biol Chem 2003;278:8606–16.
(c) Göransson U, Herrmann A, Burman R, et al. The conserved Glu in the cyclotide cycloviolacine O2 has a key structural role. Chem Bio Chem 2009;10:2354–60.
(d) Jennings C, West J, Waine C, et al. The conserved glu in the cyclotide cycloviolacin O2 has a key structural role. Proc Natl Acad Sci USA 2001;98:10614–19.
(e) Ireland DC, Colgrave ML, Nguyencong P, et al. Discovery and characterization of a linear cyclotide from *Viola odorata*: implications for the processing of circular proteins. J Mol Biol 2006;357:1522–35.
5. (a) Plan MRR, Saska I, Cagauan AG, Craik DJ. Backbone cyclised peptides from plants show molluscicidal activity against the rice pest *Pomacea canaliculata* (golden apple snail). J Agric Food Chem 2008;56:5237–41.
(b) Gran L, Sandberg F, Sletten K. *Oldenlandia affinis* (R&S) DC. A plant containing uteroactive peptides used in African traditional medicine. J Ethnopharmacol 2000;70:197–203.
(c) Gran L, Sletten K, Skjeldal L. Cyclic peptides from *Oldenlandia affinis* DC. Molecular and biological properties. Chem Biodivers 2008;5:2014–22.
6. (a) Witherup KM, Bogusky MJ, Anderson PS, et al. Cyclopsychotride A, a biologically active, 31-residue cyclic peptide isolated from Psychotria longipes. J Nat Prod 1994;57:1619–25.
(b) Pranting M, Loov C, Burman R, et al. The cyclotide cycloviolacin O2 from *Viola odorata* has potent bactericidal activity against Gram-negative bacteria. J Antimicrob Chemother 2010;65:1964–71.
7. Ireland DC, Clark RJ, Daly NL, Craik DJ. Isolation, sequencing, and structure-activity relationships of cyclotides. J Nat Prod 2010;73:1610–22.
8. (a) Göransson U, Luijendijk T, Johansson S, et al. Seven novel macrocyclic polypeptides from *Viola arvensis*. J Nat Prod 1999;62:283–6.
(b) Burman R, Gunasekera S, Strömstedt AA, Göransson U. Chemistry and biology of cyclotides: circular plant peptides outside the box. J Nat Prod 2014;77:724–36.
9. Wang CK, Kaas Q, Chiche L, Craik DJ. CyBase: a database of cyclic protein sequences and structures, with applications in protein discovery and engineering. Nucleic Acids Res 2008;36:D206–10.
10. (a) Mulvenna JP, Wang C, Craik DJ. CyBase: a database of cyclic protein sequence and structure. Nucleic Acids Res 2006;34:D192–4.
(b) Chiche L, Heitz A, Gelly JC, et al. Squash inhibitors: from structural motifs to macrocyclic knottins. Curr Protein Pept Sci 2004;5:341–9.
11. Gruber CW, Muttenthaler M, Freissmuth M. Ligand-based peptide design and combinatorial peptide libraries to target G protein-coupled receptors. Curr Pharm Des 2010;16:3071–88.
12. (a) Göransson U, Burman R, Gunasekera S, et al. Circular proteins from plants and fungi. J Biol Chem 2012;287:27001–6.
(b) Colgrave ML, Poth A, Kaas Q, Craik DJ. A new ''era'' for cyclotide sequencing. Biopolymers 2010;94:592–601.

13. Nomura T, Quesada AL, Kutchan TM. The new beta-D-glucosidase in terpenoid-isoquinoline alkaloid biosynthesis in *Psychotria ipecacuanha*. J Biol Chem 2008;283:34650–9.
14. Johnson MT, Carpenter EJ, Tian Z, et al. Evaluating methods for isolating total RNA and predicting the success of sequencing phylogenetically diverse plant transcriptomes. PLoS One 2012;7: 1–12.
15. (a) Ireland DC, Colgrave ML, Craik DJ. A novel suite of cyclotides from *Viola odorata*: sequence variation and the implications for structure, function and stability. Biochem J 2006;400:1–12.
(b) Lindholm P, Göransson U, Johansson S, et al. Cyclotides: a novel type of cytotoxic agents. Mol Cancer Ther 2002;1:365–9.
(c) Strömstedt A, Ringstad L, Schmidtchen A, Malmsten M. Interaction between amphiphilic peptides and phospholipid membranes. Curr Opin Colloid Interface Sci 2010;15:467–78.
(d) Svangård E, Göransson U, Hocaoglu Z, et al. Cytotoxic cyclotides from *Viola tricolor*. J Nat Prod 2004;67:144–7.
16. Herrmann A, Svangård E, Claeson P, et al. Key role of glutamic acid for the cytotoxic activity of the cyclotide cycloviolacin O2. Cell Mol Life Sci 2006;63:235–45.
17. (a) Simonsen SM, Sando L, Rosengren KJ, et al. Alanine scanning mutagenesis of the prototypic cyclotide reveals a cluster of residues essential for bioactivity. J Biol Chem 2008;283:9805–13.
(b) Huang YH, Colgrave ML, Daly NL, et al. The biological activity of the prototypic cyclotide kalata B1 is modulated by the formation of multimeric pores. J Biol Chem 2009;284:20699–707.
18. (a) Wang CK, Hu SH, Martin JL, et al. Combined X-ray and NMR analysis of the stability of the cyclotide cystine knot fold that underpins its insecticidal activity and potential use as a drug scaffold. J Biol Chem 2009;284:10672–83.
(b) Nguyen GK, Zhang S, Nguyen NT, et al. Discovery and characterization of novel cyclotides originated from chimeric precursors consisting of albumin-1 chain a and cyclotide domains in the Fabaceae family. J Biol Chem 2011;286:24275–87.
19. Cheneval O, Schroeder CI, Durek T, et al. Fmoc-based synthesis of disulfide-rich cyclic peptides. J Org Chem 2014;79:5538–44.
20. Koehbach J, O'Brien M, Muttenthaler M, et al. Oxytocic plant cyclotides as templates for peptide G protein-coupled receptor ligand design. Proc Natl Acad Sci USA 2013;110:21183–8.
21. Maggi M. Human myometrium during pregnancy contains and responds to V1 vasopressin receptors as well as oxytocin receptors. J Clin Endocrinol Metab 1990;70:1142–54.
22. Chan WY, Wo NC, Cheng LL, Manning M. Isosteric substitution of Asn5 in antagonists of oxytocin and vasopressin leads to highly selective and potent oxytocin and V1a receptor antagonists: new approaches for the design of potential tocolytics for preterm labor. J Pharmacol Exp Ther 1996;277:999–1003.
23. Manning M, Misicka A, Olma A, et al. Oxytocin and vasopressin agonists and antagonists as research tools and potential therapeutics. J Neuroendocrinol 2012;24:609–28.
24. (a) Chini B, Mouillac B, Balestre MN, et al. Two aromatic residues regulate the response of the human oxytocin receptor to the partial agonist arginine vasopressin. FEBS Lett 1996;397:201–6.
(b) Koehbach J, Stockner T, Bergmayr C, et al. Insights into the molecular evolution of oxytocin receptor ligand binding. Biochem Soc Trans 2013;41:197–204.
(c) Zingg HH, Laporte SA. The oxytocin receptor. Trends Endocrinol Metab 2003;14:222–7.
25. Gruber CW, Koehbach J, Muttenthaler M. Exploring bioactive peptides from natural sources for oxytocin and vasopressin drug discovery. Future Med Chem 2012;4:1791–8.
26. Wong CT, Rowlands DK, Wong CH, et al. Orally active peptidic bradykinin B1 receptor antagonists engineered from a cyclotide scaffold for inflammatory pain treatment. Angew Chem Int Ed 2012; 51:5620–4.
27. Eliasen R, Daly NL, Wulff BS, et al. Design, synthesis, structural and functional characterization of novel melanocortin agonists based on the cyclotide kalata B1. J Biol Chem 2012;287: 40493–501.
28. Gunasekera S, Foley FM, Clark RJ, et al. Engineering stabilized vascular endothelial growth factor-A antagonists: synthesis, structural characterization, and bioactivity of grafted analogues of cyclotides. J Med Chem 2008;51:7697–704.
29. Chan LY, Gunasekera S, Henriques ST, et al. Engineering pro-angiogenic peptides using stable, disulfide-rich cyclic scaffolds. Blood 2011;118:6709–17.
30. Aboye TL, Ha H, Majumder S, et al. Design of a novel cyclotide based CXCR4 antagonist with anti-human immunodeficiency virus (HIV)-1 activity. J Med Chem 2012;55:10729–34.
31. Thongyoo P, Bonomelli C, Leatherbarrow RJ, Tate EW. Potent inhibitors of beta-tryptase and human leukocyte elastase based on the MCoTI-II scaffold. J Med Chem 2009;52:6197–200.
32. (a) Craik DJ. Cyclotides as a basis for drug design. Exp Opin Drug Discov 2012;7:179–94.
(b) Colgrave ML, Craik DJ. Thermal, chemical, and enzymatic stability of the cyclotide kalata B1: the importance of the cyclic cystine knot. Biochemistry 2004;43:5965–75.
33. Ji Y. In vivo activation of the p53 tumor suppressor pathway by an engineered cyclotide. J Am Chem Soc 2013;135: 11623–33.
34. (a) Craik DJ. Chemistry. Seamless proteins tie up their loose ends. Science 2006;311:1563–4.
(b) Gilon C. Backbone cyclization: a new method for conferring conformational constraint on peptides. Biopolymers 1991;31:745–50.
(c) Smith AB. Cyclotides: a patent review. Expert Opin Ther Pat 2011;21:1657–72.
35. Wannamethee SG, Shaper AG, Durrington PN, Perry IJ. Hypertension, serum insulin, obesity, and the metabolic syndrome. J Hum Hypertens 1998;12:735–41.
36. (a) Mountjoy KG, Robbins LS, Mortrud MT, Cone RD. The cloning of a family of genes that encode the melanocortin receptors. Science 1992;257:1248–51.
(b) Chhajlani V, Wikberg JE. Molecular cloning and expression of the human melanocyte-stimulating hormone receptor cDNA. FEBS Lett 1992;309:417–20.
(c) Gantz I, Konda Y, Tashiro T, et al. Molecular cloning of a novel melanocortin receptor. J Biol Chem 1993;268:8246–50.
(d) Gantz I, Miwa H, Konda Y, et al. Molecular cloning, expression, and gene localization of a fourth melanocortin receptor. J Biol Chem 1993;268:15174–9.
(e) Chhajlani V, Muceniece R, Wikberg JE. Molecular cloning of a novel human melanocortin receptor. Biochem Biophys Res Commun 1993;195:866–73.
37. He S, Ye Z, Dobbelaar PH, et al. Discovery of highly potent and efficacious MC4R agonists with spiroindane N-Me-1,2,4-triazole privileged structures for the treatment of obesity. Bioorg Med Chem Lett 2010;20:6524–65.
38. Emmerson PJ, Fisher MJ, Yan LZ, Mayer JP. Melanocortin-4 receptor agonists for the treatment of obesity. Curr Top Med Chem 2007;7:1121–30.