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Cyclotides, a versatile ultrastable micro-protein scaffold for biotechnological applications

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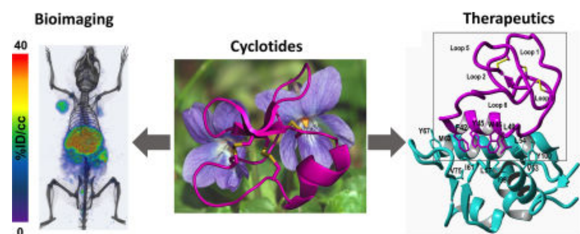
Abstract

Cyclotides are fascinating microproteins (≈30–40 residues long) with a unique head-to-tail cyclized backbone, stabilized by three disulfide bonds forming a cystine knot. This unique topology makes them exceptionally stable to chemical, thermal and biological degradation compared to other peptides of similar size. Cyclotides have been also found to be highly tolerant to sequence variability, aside from the conserved residues forming the cystine knot, able to cross cellular membranes and modulate intracellular protein-protein interactions both *in vitro* and *in vivo*. These properties make them ideal scaffolds for many biotechnological applications. This article provides and overview of the properties of cyclotides and their applications as molecular imaging agents and peptide-based therapeutics.

TOC



Keywords

cyclotide; cystine knot; circular protein; drug design; bioimaging agents; MCoTI-I; kalata B1

The success of protein-based therapeutics is revolutionizing drug development. Peptide and protein-based therapeutics can target with high selectivity and specificity defective protein-protein interactions involved in human disease. Despite their success, however, there are still numerous stability and delivery issues associated with their use as therapeutic agents. For example, monoclonal antibodies -one of the most successful protein-based therapeutics with several blockbuster drugs on the market and many more in clinical development- can only

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target extracellular molecular targets due to their inability to cross biological membranes. They are also extremely expensive to produce and are not bioavailable due to their susceptibility to proteolytic degradation. These issues have led to the exploration of alternative protein scaffolds as a source for novel types of protein-based therapeutics.[1,2]

Special attention has been recently given to the use of highly constrained poly-peptides as extremely stable and versatile scaffolds for production of high affinity ligands for specific protein capture and/or development of therapeutics.[3,4] Cyclotides represent a new emerging family of large plant-derived backbone-cyclized polypeptides (≈30–40 amino acids long) that share a 3 disulfide-stabilized core characterized by an unusual knotted structure (Fig. 1).[5] Cyclotides have several characteristics that make them ideal as drug development and bioimaging tools (see recent reviews[6,7]). For example, they are remarkably stable to chemical, thermal, and proteolytic degradation due to the circular topology and cystine knot.[8] Their relative small size makes them also readily accessible by chemical synthesis and can also be encoded within standard cloning vectors, and expressed in bacteria or animal cells (see recent reviews on the production of cyclotides[9]). Cyclotides from the trypsin inhibitor subfamily, can cross the cellular membranes of mammalian cells through different endocytic pathways[10,11] being able to target intracellular protein interactions both *in vitro* and *in vivo*.[12] The molecular/structural determinants of membrane crossing for the MCoTI-based cyclotides has not been completely elucidated yet, although it is likely related to their high positive charge at physiological pH. Finally, cyclotides have been shown to be orally active.[13] For example, the first cyclotide to be discovered, kalata B1, is an orally effective uterotonic,[8] and several other kalata B1-based cyclotides have also shown to be orally active.[13,14] Altogether, these characteristics make the cyclotide scaffold an ideal substrate for molecular evolution strategies to enable generation and selection of novel peptide-based diagnostics, therapeutics and research tools. This article provides a brief overview of their properties and their use as molecular frameworks for the design of peptide-based diagnostic and therapeutic tools.

Discovery and distribution

The first cyclotide cyclotide was discovered in the late 1960s by Gran when studying an indigenous medicine in central Africa that was used to facilitate childbirth.[15] This traditional remedy was based on a tea obtained from the plant *Oldelandia affinis* from the *Rubiaceae* family.[16] Analysis of the tea revealed that the main component with uterotonic activity was a peptide around 30 residues long that was named kalata B1. Due to the limitations of the protein chemistry techniques available in the early 1970s, it was not until 1995 when the Cys-knot backbone-cyclized nature of kalata B1 was first elucidated (Fig. 1).[8] Around the same time, other macrocyclic peptides of similar size, sequence and structure that kalata B1, were also isolated from plants of the *Rubiaceae* and *Violaceae* families (Fig. 1).[17–19] These findings lead to the definition of the cyclotide family of microproteins in 1999 based on the sequence and structure homology of these new discovered type of backbone-cyclized Cys-knotted polypeptides.[20] Cyclotides have been also found in plants from the *Cucurbitaceae, Fabaceae, Solanaceae* and *Apocynaceae* families in addition to *Rubiaceae* and *Violaceae*, with the latter two families providing the majority of the cyclotides known thus far (Fig. 2).[21]

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Even though most of the cyclotides have been isolated from the plants of the coffee and violet families, the distribution of cyclotides within these two families is quite different. For example, only around 5% of plants from the coffee family that have been analyzed so far have shown to contain cyclotides; in contrast all of the plants studied from the violet family have been found to contain cyclotides.[21] Cyclotides are usually distributed across all tissues, including flowers, leaves, stems, roots, and in some cases even seeds, with a single plant typically producing multiple cyclotides (≈10–160).[22–24]

Initially, the discovery of novel cyclotides was almost exclusively based on their isolation from the plant followed by chemical characterization of the corresponding peptides.[25] The use of more modern chemical approaches has reduced the amount of plant material required for a full characterization of the cyclotides contained in the sample when compared to the early methods. For example, with the use of MALDI-TOF/TOF and LC-MS/MS techniques is not uncommon to discover novel cyclotides from plant samples as small as 1 cm^2 of leaf tissue.[26–28] Since naturally-occurring cyclotides are ribosomally produced from genetically encoded protein precursors the use of *in silico* screening approaches can be used for the identification of cyclotides in plants.[29]In a recent study, the use of *in silico* transcriptomic and proteomic screening identified 164 cyclotides in *Viola tricolor*, which led to the authors of this study to estimate the total number of cyclotides in the *Violaceae* family alone to be around 150,000.[30] The CyBase database was recently created to allow easy access to the large number of cyclotide sequences that have been found thus far.[31] This database can be accessed through a publicly available website (http://CyBase.org.au) and contains above 300 known cyclotides as well as useful proteomic tools for their analysis.

Structure

All naturally-occurring cyclotides are 27–37 amino acid long, contain six Cys residues and are backbone-cyclized (Fig. 1). The Cys residues are forming three disulfide bonds that adopt Cys-knot topology, where the disulfides Cys^{I}-Cys^{IV} and Cys^{II}-Cys^{V} form a ladder arrangement with the disulfide Cys^{III}-Cys^{VI} running through them (Fig. 3A). The highly interlocked structure of this cyclic cysteine knot (CCK) motif makes the cyclotide backbone very compact and rigid.[32] Accordingly, cyclotides are extremely stable compounds that are highly resistant to thermal and chemical denaturation as well as biological degradation.[33,34] The fact that the first cyclotide, kalata B1, which was able to remain structurally intact and biologically active after being extracted by boiling water to make a medicinal tea, is proof of the extraordinary stability of these type of cyclic polypeptides.

Cyclotides have been classified into three subfamilies known as the Möbius, bracelet, and trypsin inhibitor cyclotide subfamilies.[35] Although all the subfamilies share the same cyclic Cys-knot topology, the composition of the loops is different in each subfamily. For example, Möbius cyclotides, such as kalata B1, contain a *cis*-Pro bond at loop 5 resulting in a slight twist of the backbone, while bracelet cyclotides do not have it (Fig. 3B). Bracelet cyclotides are by far the more abundant making up approximately two-thirds of the sequenced cyclotides known thus far.[36] These cyclotides are slightly larger and more structurally diverse than Möbius cyclotides. Despite its abundance in nature, bracelet cyclotides are more difficult to fold *in vitro* than either Möbius or trypsin inhibitor cyclotides, which makes them

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more challenging to obtain by synthetic methods using standard peptide synthesis protocols. Accordingly, bracelet cyclotides are less used in the development of the biotechnological applications that will be described later in this review.

The trypsin inhibitor subfamily of cyclotides consists of only a small number cyclotides isolated from the seeds of the plant *Momocordica cochinchinesis* (*Cucurbitaceae* family).[37,38] They are potent trypsin inhibitors ($K_i \approx 20 - 30$ pM) and do not share significant sequence homology with the other cyclotides beyond the presence of the three-cystine bridges that adopt a similar backbone-cyclic cystine-knot topology (Fig. 1). These cyclotides are more sequence-related to linear cystine-knot squash trypsin inhibitors and for this reason are sometimes referred as cyclic knottins.[39]

More recently, a novel type of cyclotides possessing novel sequence features, including a lysine-rich nature, have been isolated from two species of Australasian plants from the *Violaceae* family.[40] However, there is still limited information on how easy these novel cyclotides can be prepared by chemical means, which should dictate its potential to be used as useful molecular scaffolds for biotechnological applications.

Biological activities of naturally-occurring cyclotides

The biological function of the naturally-occurring cyclotides of the Möbius and bracelet sub-families in plants seems to be primarily as host-defense agents as deduced from their activity against insects.[5,41–43] For example there are numerous reports in the literature showing that cyclotides can efficiently inhibit the growth and development of nematodes and trematodes,[44–46] and of mollusks.[47]

The biological activity of these type of cyclotides involves the interaction with cellular membranes disrupting their normal function. For example, ingestion of cyclotides by the larvae of Lepidopteran species disrupts their midgut membranes.[48] The mechanism of how these type of cyclotides work has been widely studied and for example, for kalata B1, is well established that the first step on the interaction with the membranes involves specific binding of the cyclotide to the phosphatidylethanolamine phospholipids present in the cellular membrane. This step compromises the integrity of the membrane causing the formation of pores and/or leakage of cell contents (see a recent review in this topic[49]).

In addition to their insecticidal and nematocidal activities, cyclotides have been also shown antimicrobial and anti-tumor activities. The cyclotides from the Möbius and bracelet sub-families have shown to possess amphipathic properties due to the presence hydrophobic and hydrophilic patches located in different regions of their surface, resembling to some extent the nature of classical antimicrobial peptides. The cyclotide balata B1 shows antimicrobial activity against Gram-negative and Gram-positive bacteria.[50] Similar antimicrobial activities have been also found in cyclotide from *Hedyota biflora* (*Rubiaceae* family)[51,52] and *Clitoria ternatea* (*Fabaceae* family).[53] The most active cyclotide tested so far is the bracelet cyclotide cycloviolacin O2, which was shown to have antimicrobial activity against *Staphylococcus aureus* in a mouse infection model.[54] Despite these encouraging results, it should be noted that the antimicrobial activity of most cyclotides when tested *in vitro* seems to depend on the

buffer composition occurring only under non-physiological conditions when low ionic strength buffers are used. This seriously limits its potential on the design of effective antimicrobial therapeutics. Several cyclotides have been described to possess selective cytotoxicity against several cancer cell lines, including primary cancer cell lines, when compared to normal cells.[55–57] For example, a recent publication described the cytotoxic activity of cyclotide vingo 5 from *Viola ignobilis* in HeLa cells.[58] In addition, three new cyclotides isolated from *Hedyotis* diffusa, a Chinese medicinal plant from the *Rubiaceae* family, have been shown to induce apoptosis, and inhibit proliferation and migration of several prostate cancer cell lines.[59] The most active cyclotide of the three, cyclotide DC3, inhibited tumor growth in a mouse xenograft model.

Although, these results are promising, the therapeutic index (i.e. the ratio between the dose required for therapeutic effects versus toxic effects on normal cells) of cytotoxic cyclotides is not optimal yet indicating that will require optimization before they can be developed into effective anti-cancer agents.

It is worth noting, that recent studies on identification of the molecular targets of labor-accelerating kalata B7 and analogs were shown to be the G protein-coupled oxytocin and vasopressin V_{1a} receptors.[60]

Biosynthesis of cyclotides

Naturally-occurring cyclotides are enzymatically processed from ribosomally produced precursor proteins. In many cases, cyclotides possess dedicated genes that encode multiple copies of the same cyclotide, and in others, mixtures of different cyclotide sequences.[61]

The first genes encoding cyclotide precursor proteins were found in the plant *O. affinis* (*Rubiaceae* family) for the kalata cyclotides (Fig. 2).[41] The gene encodes a protein containing an endoplasmic reticulum (ER)-targeting sequence, a pro-region, a highly conserved N-terminal repeat (NTR) region, a mature cyclotide domain, and a hydrophobic C-terminal tail (Fig. 4).[62]Similar genes have also discovered in other plants from the *Violacea* and *Rubiaceae*,[64,6] and more recently also in plants from the *Solanaceae, Fabaceae* and *Cucurbitaceae* families (Fig. 2).[5,38,53,63] These new genes provide novel protein precursor architectures, indicating a high diversity in the way cyclotides are produced in nature.

The post-translational modifications involved in the biosynthesis of cyclotides is not fully understood yet (Fig. 4). Recent studies, however, have indicated that asparaginyl endopeptidase (AEP)-like ligases are a key element in the C-terminal cleavage and cyclization of cyclotides. The transpeptidation reaction involves an acyl-transfer step from the acyl-AEP intermediate to the N-terminal residue of the cyclotide domain.[64,65] AEPs are Cys proteases that are very abundant in plants, where they specifically cleave the peptide bond at the C-terminus of Asn and, less efficiently, Asp residues. All the cyclotide precursor proteins identified so far contain a well-conserved Asn/Asp residue at the C-terminus of the cyclotide domain in loop 6. Two AEP-like ligases have been identified so far, rO*a*AEP1_b and butelase-1, which were cloned from the cyclotide-producing plants *O. affinis* and *C.*

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ternatea, respectively.[66,67] These ligases have shown efficient capabilities in cyclizing various peptides, including linear cyclotide precursors containing the C-terminal recognitions sequence and even D-peptides.[66,68–71] Despite the tremendous progress done on understanding at the mechanistic level of how cyclotides are produced in plants, there is still is not too much known about the N-terminal cleavage process and the protease involved at that step.

Chemical synthesis of cyclotides

Due to the relatively small size of cyclotides (≈30–40 amino acids long), the corresponding linear precursors can be readily synthesized by chemical methods using solid-phase peptide synthesis (SPPS) (for a recent review in this topic see[9]). The backbone cyclization step of the linear precursor can be easily accomplished in aqueous buffers under physiological conditions by using an intramolecular version of native chemical ligation (NCL).[72] The only requirement is for the linear precursor to contain an N-terminal cysteine and an α-thioester group at the C-terminus (Fig. 5).[9] Peptide α-thioesters can be easily generated using standard solid-phase peptide synthesis approaches using either Boc- or Fmoc-based chemistry.[9] The linear precursor once cleaved from the resin can be cyclized and folded sequentially. The cyclization and folding steps can be also carried out in a 'single pot' reaction by using glutathione (GSH) as a thiol additive.[73] This approach has successfully been used to chemically generate many native and engineered cyclotides.[6]

Chemoenzymatic cyclization of the corresponding synthetic linear precursors using AEP-like ligases has been also described for the synthesis of cyclotides.[66,67] Intriguingly, these enzymes do not require the cyclotide linear precursor to be natively folded for the cyclization to proceed efficiently.[66]

The serine protease trypsin has also been used to produce several cyclotides based on the naturally occurring trypsin inhibitor cyclotide MCoTI-II.[74] In this case, folded linear cyclotide precursors bearing the P1 and P1′ residues at the C- and N-termini respectively were used as a viable substrate for trypsin-mediated cyclization. The use of trypsin-mediated cyclization provides a very efficient route for obtaining cyclotides with trypsin inhibitory properties with yields close to 92% for cyclotide MCoTI-II.[74] It should be noted, however, that the introduction of mutations that affect the binding to the proteolytic enzyme may affect the cyclization yield.[9] The transpeptidase like sortase A (SrtA) enzyme has been also used for the production of cyclotides from synthetic linear precursors.[75] Due to the SrtA sequence requirements to work efficiently, the heptapeptide motif (LPVTGGG) is left at the ligation site, which should be taken into consideration when producing biologically active cyclotides.

Recombinant expression of cyclotides

The discovery of intein-mediated protein splicing both in *cis* and *trans* has made possible the generation of backbone-cyclized polypeptides using standard expression systems (for a recent review in this topic see[9]) (Fig. 6). Our group pioneered the use of intein-mediated backbone cyclization for the biosynthesis of fully folded cyclotides inside bacterial cells by

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making use of an intramolecular version of intein-mediated ligation (also called Expressed Protein Ligation (EPL)).[9]

More recently, our group made use of intein-mediated protein *trans*-splicing (PTS) for the efficient production of naturally-occurring and engineered cyclotides in prokaryotic and eukaryotic expression systems (Fig. 6).[76,77] This approach is quite efficient, providing in-cell production of folded cyclotides that can reach intracellular concentrations in the range of 20–40 μM. In *E. coli*, this level of expression equals to ≈10 mg of folded cyclotide per 100 g of wet cells for cyclotide MCoTI-I.[78]

Efficient in-cell production of cyclotide allows the generation of large genetically-encoded libraries of cyclotides inside live cells that can be rapidly screened for the selection of novel sequences able to modulate or inhibit the biological activities of selected biomolecular targets. In addition, it also facilitates the production of cyclotides labeled with NMR active isotopes such as ^{15}N and/or ^{13}C in a very inexpensive fashion. The production of ^{15}N and/or ^{13}C-labeled cyclotides makes possible the use of heteronuclear NMR spectroscopy to study structure-activity relationships of any biologically active cyclotides and their molecular targets. For example, this was recently demonstrated in the structural studies carried out on a cyclotide engineered to bind the p53 binding domain of the E3-ligases Hdm2 and HdmX (Fig. 7A).[12]

Engineered cyclotides with novel biological activities

The unique properties associated with the cyclotide scaffold make this molecular framework extremely valuable in the development of novel peptide-based therapeutics (see Table 1).[34,79] As mentioned earlier, the CCK topology found in cyclotides provides them with a highly rigid and compact structure that is extremely resistant to physical, chemical and biological degradation. In addition, the cyclotide scaffold is highly tolerant to mutations making it an ideal molecular framework for molecular evolution and grafting for production of novel cyclotides with new biological activities. Cyclotides from the trypsin inhibitor subfamily are also not toxic to mammalian cells *in vitro* up to concentrations of 100 μM[12] or *in vivo* with daily doses of cyclotides of up to 40 mg/kg,[12] and have been shown to be able to cross cellular membranes[10,11] for targeting intracellular cytosolic protein-protein interactions.[12] The trypsin inhibitory properties of these cyclotides can be easily removed by mutating the residue Lys4 located in the loop 1 to a non-positively charged residue. The pharmacologic potential of engineered cyclotides grafted with biological active peptide sequences was first demonstrated in two early studies aimed to develop novel anti-cancer[80,81] and anti-viral peptide-based therapeutics.[74] Molecules with anti-angiogenic activity have potential applications in cancer treatment due to the fact the tumor growth is usually associated with unregulated angiogenesis. In this early case, an Arg-rich peptide antagonist for the interaction of vascular endothelial growth factor A (VEGF-A) and its receptor was grafted into several loops of cyclotide kalata B1 producing cyclotides with anti-VEGF activity.[80] The most active cyclotide was able to block VEGF-A receptor binding with an IC_{50} of ≈ 12 μM, which although is the first example of a successful functional redesign of a cyclotide, its biological activity would still need to be improved by several orders of magnitude for a potential pharmacological application *in vivo*. A similar approach

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was also used recently to target the bradykinin and melanocortin 4 receptors for pain and obesity management, respectively.[13,82] The kalata B1-based bradykinin antagonist was shown to be orally active[13] highlighting the potential of the cyclotide scaffold for the development of orally-bioavailable peptide-based therapeutics. The cyclotide MCoTI-I from the trypsin inhibitor subfamily has been also employed for the design of a potent CXCR4 antagonist.[83] This cytokine receptor is associated with multiple types of cancers where its overexpression/activation promotes metastasis, angiogenesis, and tumor growth and/or survival.[84]

The trypsin inhibitor subfamily of cyclotides has been also used for the design for protease inhibitors with potential pharmacological relevance. Proteases are well-recognized drug targets as they are involved many human diseases including inflammatory diseases, cancer, cardiovascular and neurodegenerative conditions.[85,86] For example, mutated versions of the cyclotide MCoTI-II were transformed into potent and selective foot-and-mouth-disease (FMDV) 3C protease, β-tryptase and human leukocyte elastase inhibitors.[74,81,87] β-Tryptase and human leukocyte elastase are validated targets for inflammatory disorders.

In a recent work, a point mutated version of cyclotide kalata B1 was reported to have oral activity in a mouse model of multiple sclerosis.[88] The pharmacological potential of grafted cyclotides in the context of multiple sclerosis has been also explored by grafting peptide sequences from the MOG35-55 epitope onto the cyclotide kalata B1.[89]

Without any question, one of the most exciting features of the cyclotide scaffold is that some cyclotides, those from the trypsin inhibitor subfamily, can penetrate cells and access the cytosolic cellular fraction. This exceptional property found in some cyclotides makes possible the cellular delivery of biologically active cyclotides to target intracellular protein-protein interactions. In a recent report, the cyclotide MCoTI-I was engineered to produce a potent inhibitor for the interaction between p53 and the proteins Hdm2/HdmX (Fig. 7). [12] The engineered cyclotide, MCo-PMI, was able to bind with low nanomolar affinity to both Hdm2 and HdmX, showed high stability in human serum, and was cytotoxic to wild-type p53 cancer cell lines by activating the p53 tumor suppressor pathway both *in vitro* and *in vivo* (Fig. 7).[12] This report constitutes the first example of an engineered cyclotide being able to target an intracellular protein-protein interaction in an animal model of human colon carcinoma therefore highlighting the therapeutic potential of MCoTI-cyclotides for targeting intracellular protein-protein interactions. An identical approach but using cyclotide MCoTI-II instead was also reported to antagonize *in vitro* the SET protein, which is overexpressed in some human cancers.[90]

MCoTI-grafted cyclotides have been also used to inhibit α-synuclein-induced cytotoxicity when expressed in yeast *S. cerevisiae*.[77] α-Synuclein is a small lipid-binding protein that is prone to misfolding and aggregation, that has been linked to Parkinson's disease by genetic evidence and its abundance in the Parkinson's disease-associated intracellular aggregates known as Lewy bodies, and therefore it is a validated therapeutic target for Parkinson's disease.

Given the good *in vivo* biological activity of some MCoTI-cyclotides, the biodistribution and potential to cross the blood brain barrier of cyclotides MCoTI-I/II have been recently studied.[91,92] These two reports indicated that MCoTI-cyclotides are distributed predominantly to the serum and kidneys, confirming that they are stable in serum and suggesting that they are eliminated from the blood through renal clearance.[91,92] In addition, cyclotide MCoTI-II showed no significant uptake into the brain.[91]

Screening of cyclotide-based libraries

As discussed earlier, in-cell production of natively folded cyclotides makes possible the production of large libraries of genetically-encoded cyclotides, potentially containing billions of members. The generation of this tremendous molecular diversity should allow the selection of strategies that mimic the evolutionary processes found in nature to select novel cyclotide sequences able to target specific molecular targets. As a proof of principle, a genetically-encoded library based on cyclotide MCoTI-I was generated to mutate every single amino acid in loops 1, 2, 3, 4 and 5 in order to explore the effects on folding and trypsin binding activity of the resulting mutants.[93] The results obtained in this early work showed that most of the mutants were able to fold efficiently therefore emphasizing the high plasticity and sequence tolerance of MCoTI-based cyclotides.[93]

Cyclotide-based libraries have been also used for phenotypic screening in eukaryotic cells.[77] In this work, an engineered cyclotide (MCoCP4) that was designed to reduce toxicity of human α-synuclein in live yeast cells was selected by phenotypic screening from cells transformed with a mixture of plasmids encoding MCoCP4 and inactive cyclotide MCoTI-I in a ratio of 1 to 50,000. These results show the potential for performing phenotypic screening using genetically encoded cyclotide-based libraries in eukaryotic cells for the rapid selection of novel bioactive cyclotides. Moreover, heterologous production of cyclotides in eukaryotic expression systems should also allow the introduction different post-translational modifications not available in bacterial expression systems, like phosphorylation and/or glycosylation.

In addition to the use of biological expression systems for screening purposes, the development of efficient methods for the chemical synthesis, cyclization and folding of cyclotide-based libraries also allows to perform high throughput screening on chemically-generated libraries of cyclotides.[73] A recent report showed that bioactive folded MCoTI-based cyclotides can be efficiently produced in parallel using a 'tea-bag' approach in combination with high efficient cyclization-folding protocols.[73] The approach described in this work also included an efficient purification procedure to rapidly remove non-folded or partially folded cyclotides from the cyclization-folding crude. This method can be used for the purification of cyclotide mixtures, therefore making it compatible with the synthesis of amino acid and positional scanning libraries to perform efficient screening of large chemical-generated libraries.

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Cyclotides as molecular imaging probes

Developing suitable diagnostic molecular tools is key for early detection and monitoring in the successful treatment of many diseases, including cancer.[1,6] The technological advances in imaging instrumentation during the past two decades has dramatically increased our bioimaging capabilities and has also increased the need for improved molecular imaging agents. Optimal imaging agents should provide high affinity to provide high selectivity over healthy tissue, rapid clearance from healthy tissue to reduce background signal, and high chemical and biological stability.

Properly functionalized engineered linear squash trypsin inhibitors, which share sequence homology and structure with the trypsin inhibitor subfamily of cyclotides but are not backbone-cyclized, have been shown to be excellent bioimaging and detection tools in cancer (recently reviewed in [1]). Integrin-binding variants based on the *Ecballium elaterium* trypsin inhibitor II (EETI) have been extensively used as bio-imaging agents. They have been shown to provide significant tumor accumulation and low imaging signals in kidney, liver, and other organs. More recently, the cyclotide MCoTI-I from the trypsin inhibitor subfamily has been also shown to be an excellent bioimaging tool to visualize CXCR4-overexpressing cancer cells in a mouse model (Table 1 and Fig. 8).[92] In this work, a [^{64}Cu]-DOTA-labeled version of cyclotide MCo-CVX-6D, which is a potent CXCR4 antagonist with a remarkable *in vivo* resistance to biological degradation in serum, was used for the efficient detection of tumors containing CXCR4-expressing cells in mice using positron emission tomography–computed tomography (PET-CT) (Fig. 8A).[92]. The biodistribution of [^{64}Cu]MCo-CVX-6D confirmed the detection of tumors with high CXCR4 expression and demonstrated favorable kinetics by PET imaging (Fig. 8B). In contrast, the [^{64}Cu]-DOTA-labeled version of cyclotide MCoTI-I showed major accumulation in the kidneys which is consistent with renal clearance of this cyclotide (Fig. 8B). Rapid and non-invasive detection of CXCR4 overexpression in all malignant lesions in entirety should provide unprecedented opportunities to stratify patients for CXCR4-based therapies as well as testing efficacy of the corresponding treatment.

Concluding remarks

We can say with confidence that cyclotides are now becoming to be a well-studied family of micro-proteins that given their unique properties are also starting to gain acceptance as molecular scaffolds for the potential design of novel peptide-based therapeutics and diagnostic tools. Their unique knotted arrangement of three disulfide bonds and circular backbone topology provides a unique molecular framework that confers exceptional resistance to thermal/chemical denaturation, and enzymatic degradation. Cyclotides can also cross mammalian cellular membranes to target protein-protein interactions *in vitro* but also and more importantly in animal models. The fact that cyclotides can target extracellular as well as intracellular targets highlights the high stability of the Cys-knot to be degraded/oxidized under complex biological conditions. The relative small size of cyclotides facilitates their chemical synthesis allowing the introduction of chemical modifications such as non-natural amino acids and PEGylation to improve their pharmacological properties. Moreover, cyclotides are amenable to substantial sequence variation and can be produced in

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several heterologous expression systems making them ideal substrates for molecular evolution strategies to enable generation and selection of compounds with optimal binding and inhibitory characteristics. All these unique properties make them promising leads or frameworks for the design of novel peptide-based therapeutics as well as bioimaging reagents.

No cyclotides have reached human clinical trials yet, however, the results obtained with several bioactive cyclotides in animal models may hint that this could occur in a not too distant future. One the main challenges that affect cyclotides, if they want to compete with small-molecule therapeutics, is their oral bioavailability. Although some cyclotides have proven to be orally active, there is still little information about their oral bioavailability. It is anticipated, however, that more studies on the biopharmaceutical properties of these exciting new micro-proteins may be available very soon.

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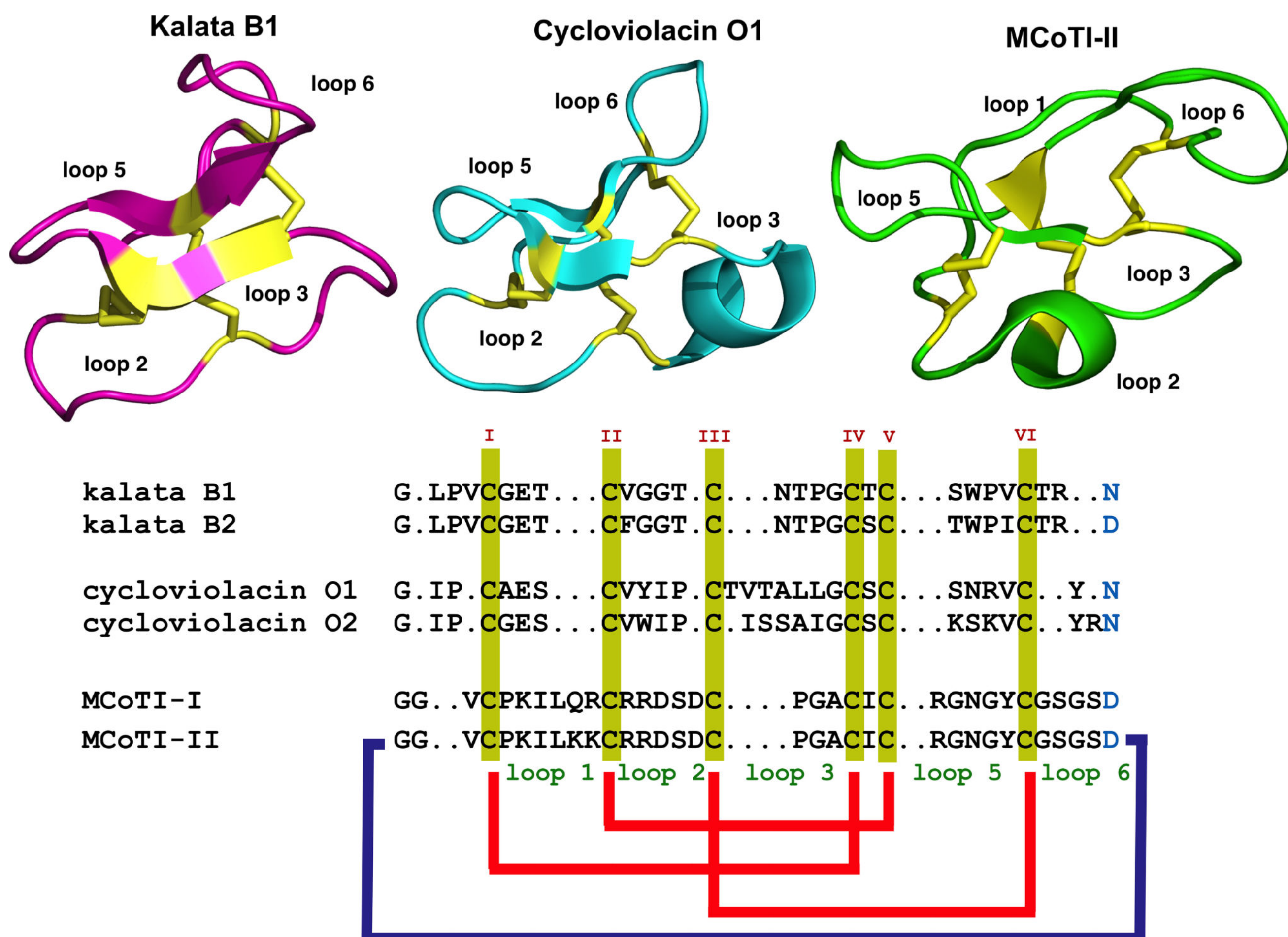

Figure 1.

Sequence alignment and structures of different cyclotides belonging to the Möbius (kalata B1, pdb: 1NB1), bracelet (cycloviolacin O1, pdb: 1NBJ) and trypsin inhibitor (MCoTI-II, pdb: 1IB9) subfamilies. Conserved Cys and Asp/Asn (required for cyclization) residues are marked in yellow and light blue, respectively. Disulfide connectivities and backbone-cyclization are shown in red and a dark blue line, respectively. The six Cys residues are labeled with roman numerals whereas loops connecting the different Cys residues are designated with arabic numerals. Molecular graphics were created using PyMol.

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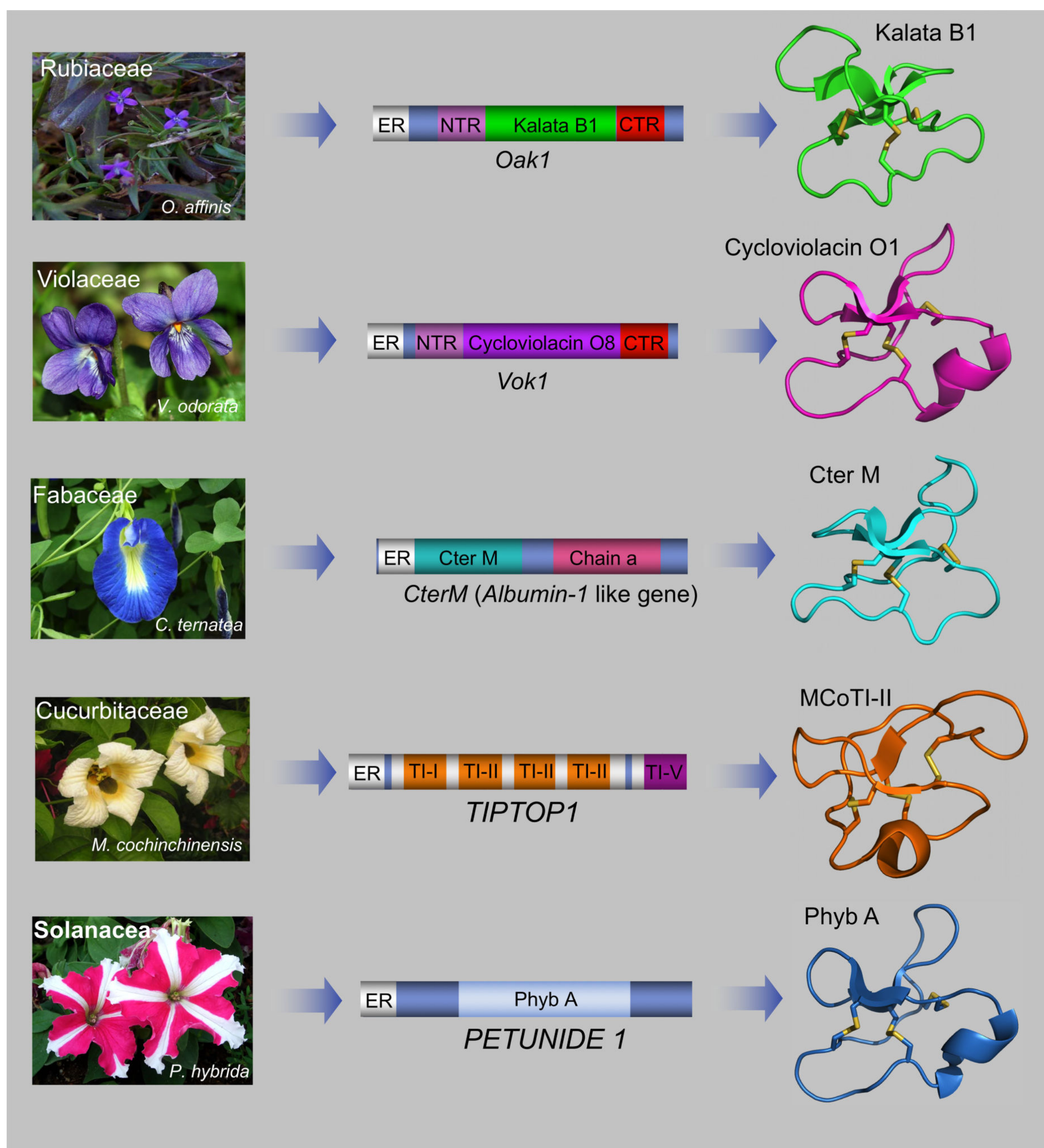

Figure 2.

Genetic origin of cyclotides in plants. Some plants from the *Rubiaceae* and *Violaceae* families have dedicated genes to the production of cyclotides. These genes encode protein precursors containing an ER signal peptide, an N-terminal pro-region, the N-terminal repeat (NTR), the mature cyclotide domain and a C-terminal flanking region (CTR).[61] Cyclotides recently isolated from *C. ternatea* (*Fabaceae* family), are produced from precursor proteins containing an ER signal peptide immediately followed by the cyclotide domain, which is flanked at the C-terminus by a peptide linker and the albumin a-chain. The cyclotide domain, in this case, replaces the albumin-1 b-chain.[5] The trypsin inhibitor subfamily of

cyclotides are produced from TIPTOP proteins, which contain a tandem series of cyclic trypsin inhibitors terminating with an acyclic trypsin inhibitor.[38] The protein precursors for cyclotides from the *Solanaceae* family are encoded in genes similar to those found in the *Rubiaceae* and *Violaceae* plants with dedicated precursor proteins that have an ER signal, a pro-region, the linear peptide precursor, and end with a hydrophobic tail.[63] Molecular graphics were created using PyMol.

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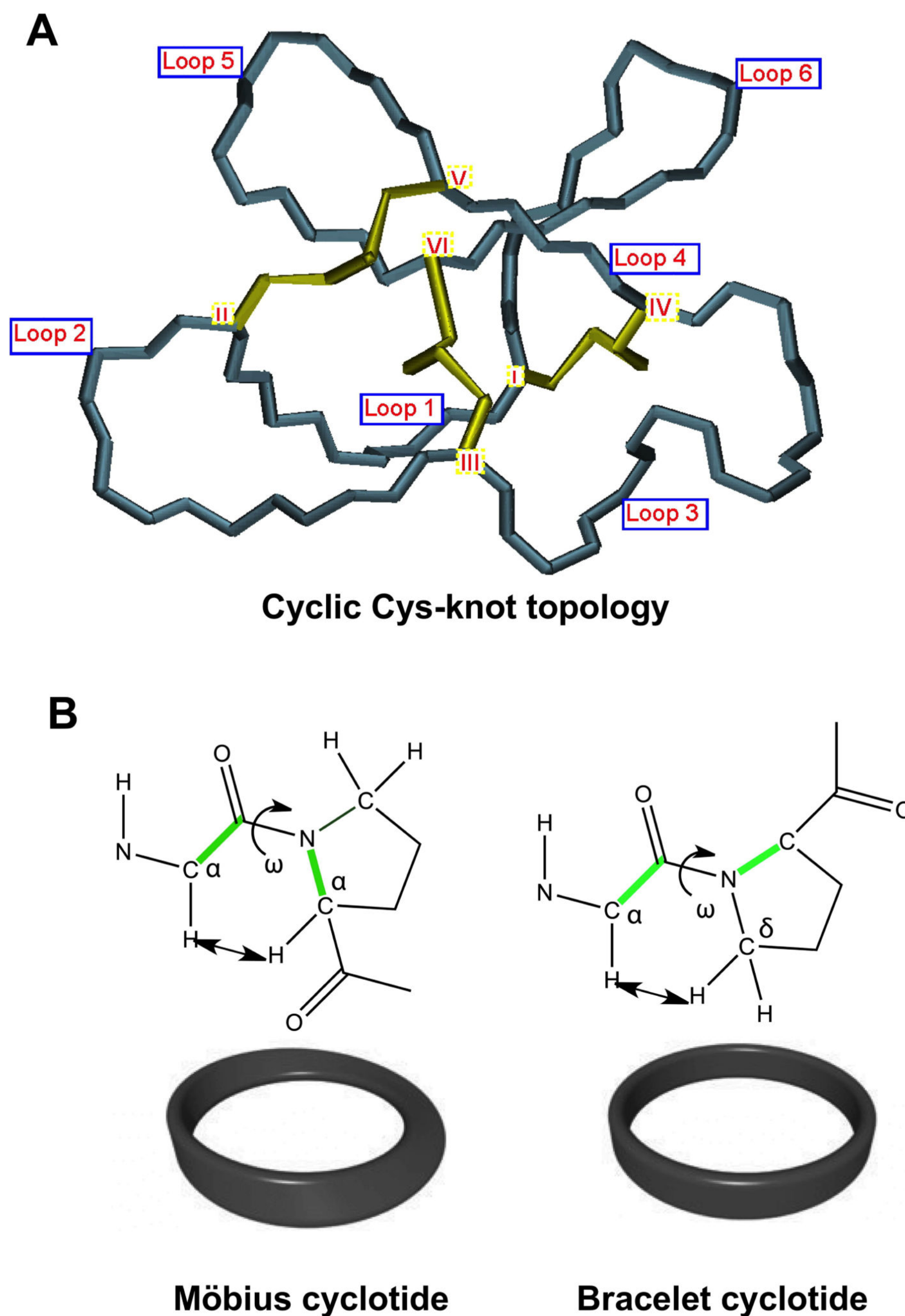

Figure 3.

Structural characteristics of the cyclic cysteine know (CCK) topology found in all cyclotides. **A.** Detailed three-dimensional structure of the cyclic cystine knot (CCK) topology and the connecting loops found in cyclotides. The six Cys residues are labeled with roman numerals whereas loops connecting the different Cys residues are designated with arabic numerals. **B.** Möbius cyclotides contain a *cis*-Pro residue in loop 5 that induces a local 180° backbone twist, whereas bracelet cyclotides do not possess this structural feature.

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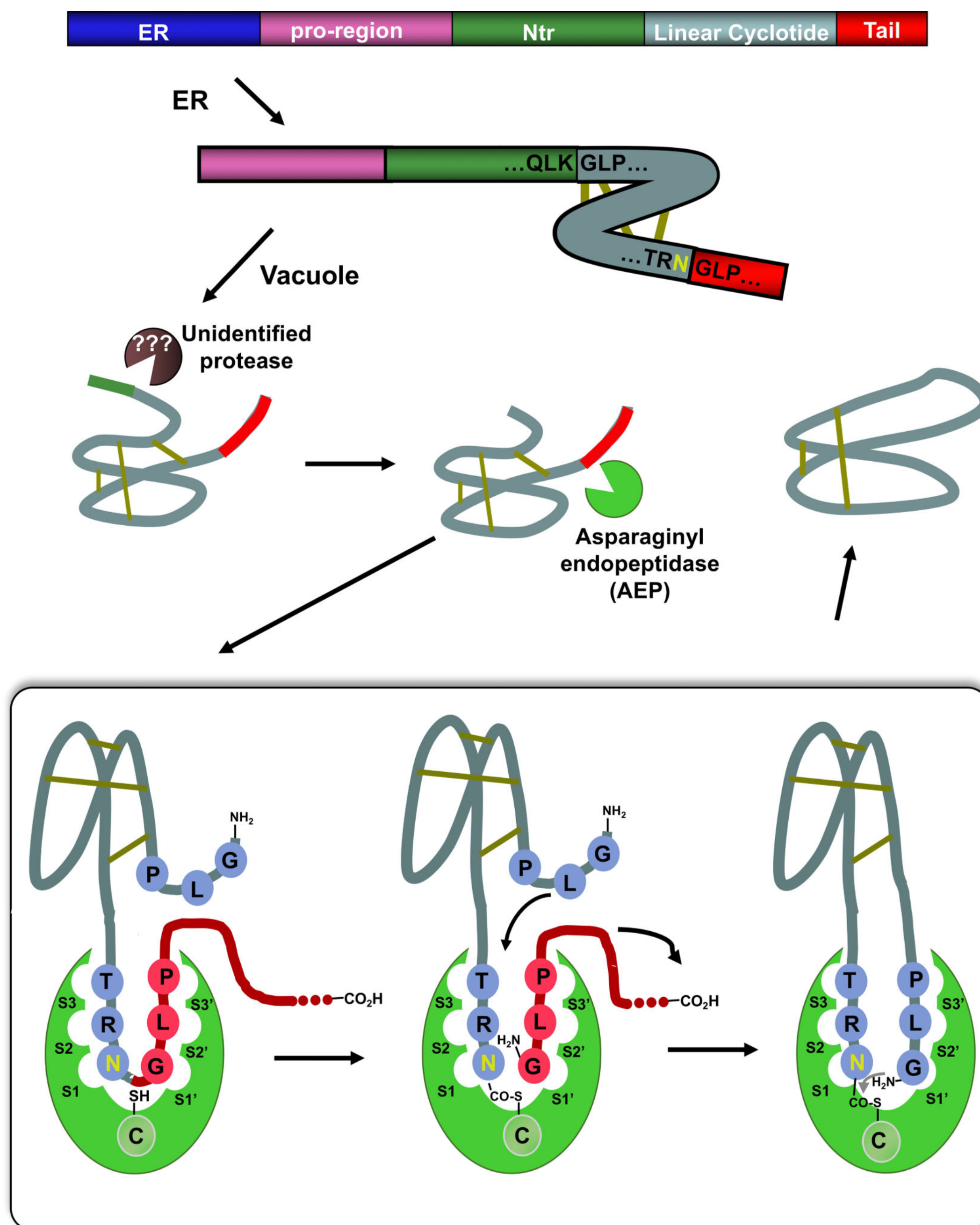

Figure 4.

Scheme summarizing the proposed mechanism for the biosynthesis of cyclotide kalata B1. It is now well accepted that the cyclization step is mediated by an asparaginyl endopeptidase (AEP), a common Cys protease found in plants. The cyclization takes place at the same time as the cleavage of the C-terminal pro-peptide from the cyclotide precursor protein through a transpeptidation reaction. The transpeptidation reaction involves an acyl-transfer step from the acyl-AEP intermediate to the N-terminal residue of the cyclotide domain.[61] The kalata B1 protein precursor contains an ER signal peptide, an N-terminal pro-region, the N-

terminal repeat (NTR), the mature cyclotide domain and a C-terminal flanking region (tail, also known as CTR).

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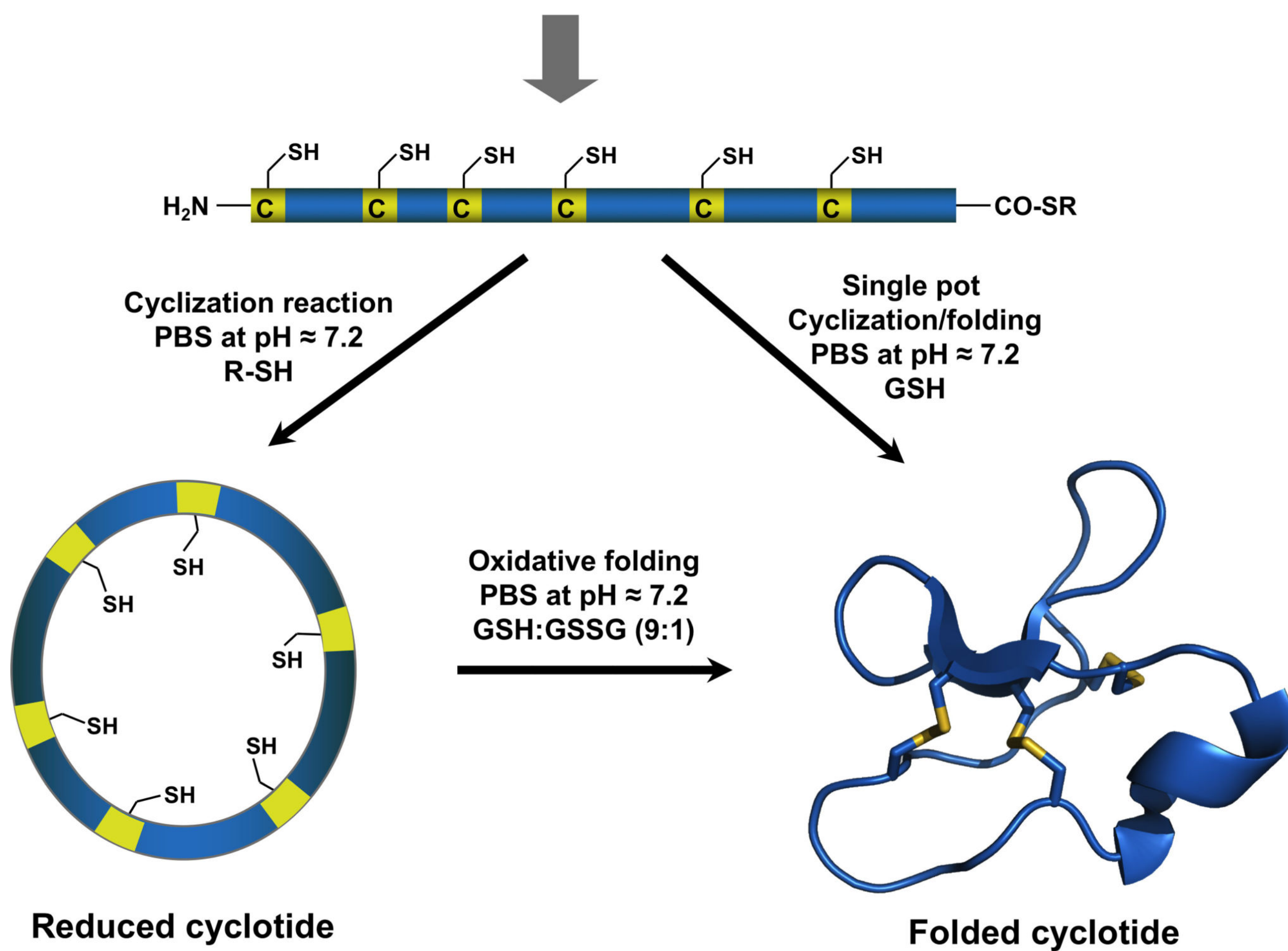

Figure 5.

Chemical synthesis of cyclotides by making use of an intramolecular native chemical ligation (NCL). This method requires the chemical synthesis of a linear precursor bearing an N-terminal Cys residue and an α-thioester moiety at the C-terminus. The linear precursor can be cyclized first under reductive conditions and then folded using a redox buffer containing reduced and oxidized glutathione (GSH).[9] Alternatively, the cyclization and folding can be efficiently accomplished also in a 'single pot' reaction when the cyclization is carried out in the presence of reduced GSH as the thiol cofactor.[9]

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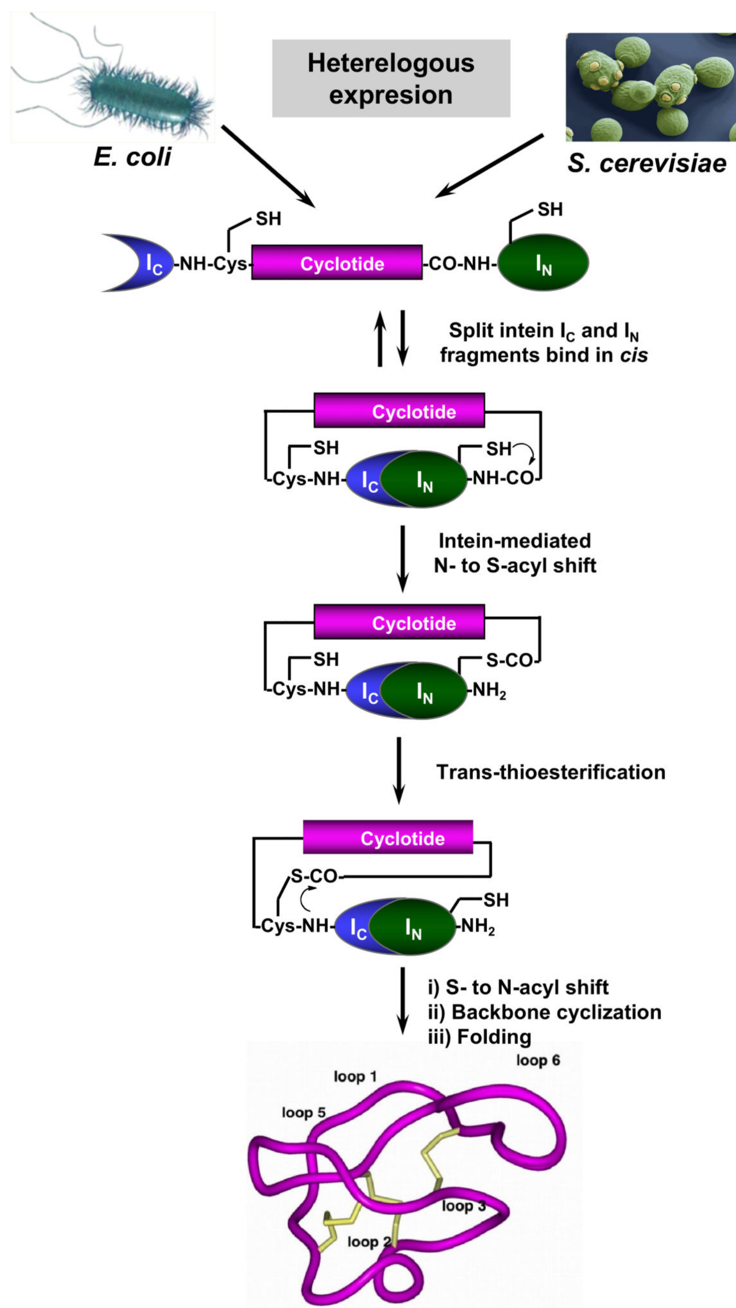

Figure 6.

Heterologous expression of cyclotides by protein trans-splicing (PTS).[76–78] To facilitate the backbone cyclization the linear cyclotide precursor is fused in-frame at the C- and N-termini directly to the I_N and I_C polypeptides of the *Npu* DnaE split intein. This approach has been used for the generation of MCoTI-cyclotides, where the native Cys residue located at the beginning of loop 6 was used to facilitate backbone cyclization. During the intramolecular processing of this construct the N- and C-termini of the linear cyclotide precursor are linked together through a native peptide bond through a transpeptidation reaction mediated by the

self-processing domains of the split intein. This approach has been successfully used to produce bioactive cyclotides in eukaryotic and prokaryotic expression systems.[76–78]

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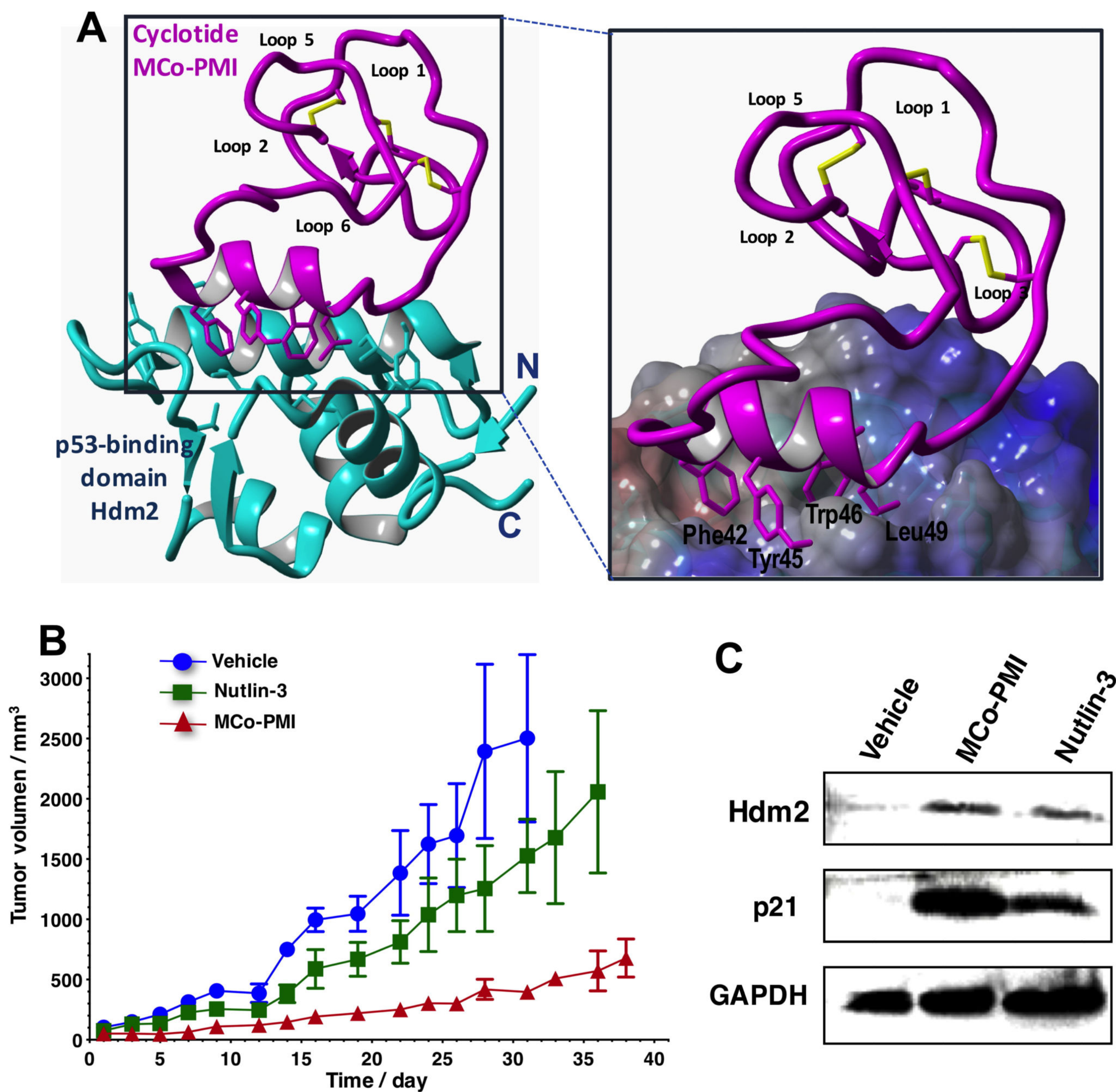

Figure 7.

Structure and *in vivo* activity of the first cyclotide designed to antagonize an intracellular protein-protein interaction *in vivo*. **A.** Solution structure of the engineered cyclotide MCo-PMI (magenta) and its intracellular molecular target, the p53 binding domain of oncogene Hdm2 (blue). The cyclotide binds with low nM affinity to both the p53-binding domains of Hdm2 and HdmX. **B.** Cyclotide MCo-PMI activates the p53 tumor suppressor pathway and blocks tumor growth in a human colorectal carcinoma xenograft mouse model. HCT116 $p53^{+/+}$ xenografts mice were treated with vehicle (5% dextrose in water), nutlin 3 (10 mg/kg) or cyclotide (40 mg/kg, 7.6 mmol/kg) by intravenous injection daily for up to 38 days. Tumor volume was monitored by caliper measurement. **C.** Tumors samples were also

subjected to SDS-PAGE and analyzed by western blotting for p53, Hdm2 and p21, indicating activation of p53 on tumor tissue.

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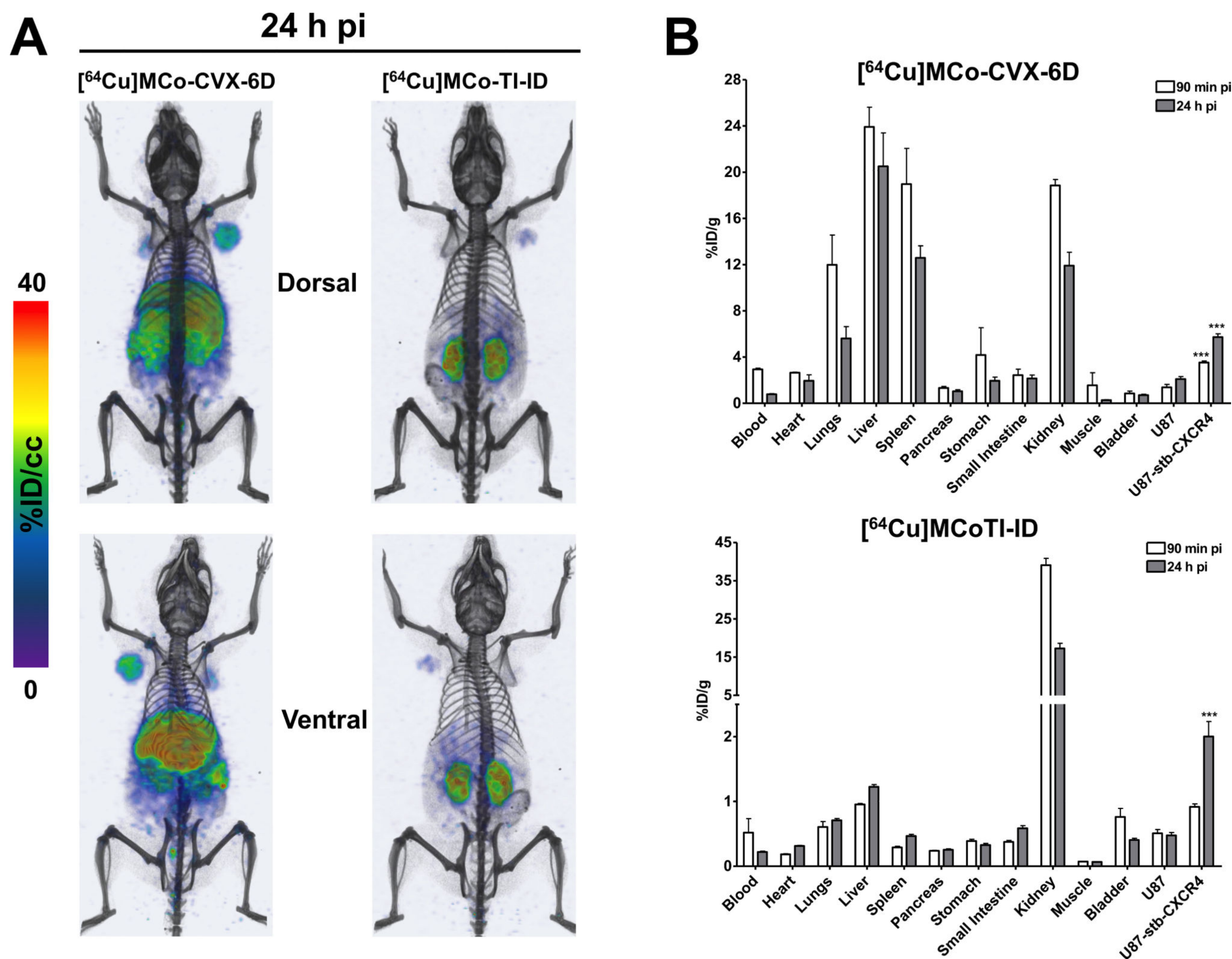

Figure 8.

Use of a CXCR4-targeting cyclotide as a bioimaging tool for detecting CXCR4-overexpressing tumor cells in animal models.[92] **A.** Distribution of [^{64}Cu]MCo-CVX-6D (CXCR4-targetign cyclotide) and [^{64}Cu]MCoTI-ID (a DOTA-labeled variant of native trypsin inhibitor cyclotide MCoTI-I) in NOD/SCID mice bearing U87 and U87-stb-CXCR4 tumors with PET-CT. Representative PET-CT volume rendered images of mice (n=3) injected with 250 μCi of [^{64}Cu]MCo-CVX-6D or [^{64}Cu]MCoTI-ID recorded 24 h post tracers injection demonstrating high accumulation of [^{64}Cu]MCo-CVX-6D in the U87-stb-CXCR4 tumor and [^{64}Cu]MCoTI-ID showed mostly kidney uptake, suggesting renal clearance for the non-CXCR4-targetign cyclotide. **B.** *Ex vivo* evaluation of [^{64}Cu]MCo-CVX-6D and [^{64}Cu]MCoTI-ID distribution and specificity in NOD/SCID mice bearing U87 and U87-stb-CXCR4 tumors. E*x vivo* biodistribution analysis was performed at 90 min and 24 h after post tracers injection.

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Table 1

Summary of work published in engineered cyclotides with novel biological activities leading to therapeutic and bioimaging applications.

Cyclotide	Biological activity	Loop Modified	Application	Ref.
Möbius subfamily				
Kalata B1	VEGF-A antagonist	2, 3, 5 & 6	Anti-angiogenic, potential anti-cancer activity	80
Kalata B1	Dengue NS2B-NS3 Protease inhibitor	2 & 5	Anti-viral for Dengue virus infections	94
Kalata B1	Bradikynin B1 receptor antagonist	6	Chronic and inflammatory pain	13
Kalata B1	Melanocortin 4 receptor Agonist	6	Obesity	82
Kalata B1	Neuropilin-1/2 antagonist	5 & 6	Inhibition of endothelial cell migration and angiogenesis	95
Kalata B1	Immunomodulator	5 & 6	Protecting against multiple sclerosis	89
Kalata B1	Immunomodulator	4	Protecting against multiple Sclerosis	14
Trypsin inhibitor subfamily				
MCoTI-I	CXCR4 antagonist	6	Anti-metastatic and anti-HIV PET-CT imaging	73,92,96
MCoTI-I	p53-Hdm2/HdmX Antagonist	6	Anti-tumor by activation of p53 pathway	12
MCoTI-II	FMDV 3C protease Inhibitor	1	Antiviral for foot-and-mouth disease	74
MCoTI-II	β-Tryptase inhibitor	3, 5 & 6	Inflammation disorders	87
MCoTI-II	β-Tryptase inhibitor Human elastase inhibitor	1	Inflammation disorders	81
MCoTI-II	CTLA-4 antagonist	1,3 & 6	Immunotherapy for cancer	97
MCoTI-II	Tryptase inhibitor	1	Anticancer	98
MCoTI-II	VEGF receptor agonist	6	Wound healing and cardiovascular damage	99
MCoTI-I	α-Synuclein-induced cytotoxicity inhibitor	6	Parkinson's disease Validate phenotypic screening of genetically-encoded cyclotide libraries	77
MCoTI-II	BCR-Abl kinase Inhibitor	1 & 6	Chronic myeloid leukemia Attempt to graft both a cell penetrating peptide and kinase inhibitor	100
MCoTI-I	MAS1 receptor agonist	6	Lung cancer and myocardial infarction	101
MCoTI-II	SET antagonist	6	Potential anticancer	90
MCoTI-II	FXIIa and FXa inhibitors	1 & 6	Antithrombotic and cardiovascular disease	102
MCoTI-II	Thrombospondin-1 (TSP-1) agonist	6	Microvascular endothelial cell migration inhibition Anti-angiogenesis	103
MCoTI-II	Antiangiogenic	5 & 6	Anti-cancer	104