Pharmaceutical applications of cyclotides

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Cyclotides are cyclic peptides, present in several plant families, that show diverse biological properties. Structurally, cyclotides share a distinctive head-to-tail circular knotted topology of three disulfide bonds. This framework provides cyclotides with extraordinary resistance to thermal and chemical denaturation. There is increasing interest in the therapeutic potential of cyclotides, which combine several promising pharmaceutical properties, including binding affinity, target selectivity, and low toxicity towards healthy mammalian cells. Recently, cyclotides have been reported to be orally bioavailable and have proved to be amenable to modifications. Here, we provide an overview of the structure, properties, and pharmaceutical applications of cyclotides.

Introduction

Circular peptides can be found in all life kingdoms, including bacteria, plants, and mammals [1]. Peptides have evolved and become a starting point for the development of novel peptide-based drugs. Some already have a role as modulators, including the human defensins [2], whereas others have proved their value as drugs (e.g., the fungal cyclosporine A, which is a cyclic fungal peptide with immunosuppressive activities) [3]. Among natural molecules with therapeutic purposes, the discovery of cyclotides (cyclic peptides from plants) opened a new source of natural leads for the development of new drugs [4]. Cyclotides have been highlighted as outstanding molecules because of their high stability under different biological conditions, thus facilitating their translation to the clinic [5]. This family displays exceptional stability and sequence plasticity, with a range of pharmaceutical and agrochemical activities, making them potentially bioactive compounds that can represent innovative lead molecules [6]. Over the past decade, several reviews have described the discovery, optimization, and potential of cyclotides as a scaffold for drug development and biotechnological applications [5,7–9]. Here, we provide an overview of cyclotide discovery, structure and recent publications regarding their biosynthesis, as well as their latest pharmaceutical applications.

Discovery and structure

Cyclotides were initially reported by Seather *et al.* in 1995, although they had been used in traditional medicine long before that [10,11]. The first reported cyclotide, kalata B1 (kB1), is an active compound in the plant *Oldenlandia affinis*, used in traditional indigenous medicine in Africa to accelerate childbirth [10]. Since then, cyclotides have been reported in plants from the Violaceae, Rubiaceae, Cucurbitaceae, Solanaceae, Poaceae, and Fabaceae, with >280 different cyclotides reported so far [12,13]. Moreover, it has been estimated that a single cyclotide family, for example from the Violaceae, might have upto 150 000 members [12,14,15], and a single plant might contain over 160 different cyclotides [15,16].

The first efforts to isolate cyclotides from plants included purification and structural characterization studies at the proteome level. Chemical approaches to extract, purify, and characterize cyclotides combine techniques such as high-performance liquid chromatography (HPLC), liquid chromatography associated with

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mass spectroscopy (LC-MS/MS), matrix-assisted laser desorption/ionization time of flight tandem (MALDI-TOF), electrospray ionization mass spectroscopy (ESI-MS), nuclear magnetic resonance spectroscopy (NMR), and enzymatic digestion [17]. The sequences of cyclotides are determined using a combination of mass spectrometry methods [18]. Finally, the tridimensional structure is determined using NMR [19]. However, given that a single plant can contain up to 160 different cyclotides, the proteome approach to discovering cyclotides can be laborious. Thus, researchers have started to study peptides at the genome level [20]. A combination of next-generation sequencing (NGS) and bioinformatics has emerged as an alternative for cyclotide discovery [15,21]. Usually, cDNA libraries are created from mRNAs extracted from the plant using NGS, and then homology studies using computational tools are performed to identify cyclotides [22]. Finally, the new sequences are deposited in GenBank [15]. In addition, cyclotides along with flavonoids and coumarins were used as chemotaxonomic biomarkers to identify the phylogeny of closely related violets, highlighting misclassification issues associated with this family [23].

Cyclotides are ~30 amino acids long and, structurally, have a unique motif. This motif is known as the cyclic cysteine knot (CCK), which corresponds to a head-to-tail circular knotted topology with three disulfide bonds, with one disulfide penetrating a ring formed by the two other disulfide bonds (Fig. 1). The disulfides Cys^{I}–Cys^{IV} and Cys^{II}–Cys^{V} form a ring, which the disulfide Cys^{III}–Cys^{VI} goes through [24]. Cyclotides also have six loops. These loops correspond to the six backbone segments between the cysteine residues. Interestingly, these loops can be modified in length and amino acid content without affecting the stability of the cyclotides [25]. By contrast, removing one type of disulfide connectivity can affect their stability and structure [26]. Most 3D structures of cyclotides have been elucidated using solution NMR spectroscopy [27]. The structural arrangement adopted by this class of mini proteins makes cyclotides stable and rigid, allowing them to resist thermal and chemical denaturation, as well as biological degradation [26]. An exception is the acyclotide sequences described recently, which have the cysteine knot motif, although the cyclic backbone is missing. For example, the peptide paragidin-br3 from *Palicourea rigida* has an acyclotide structure

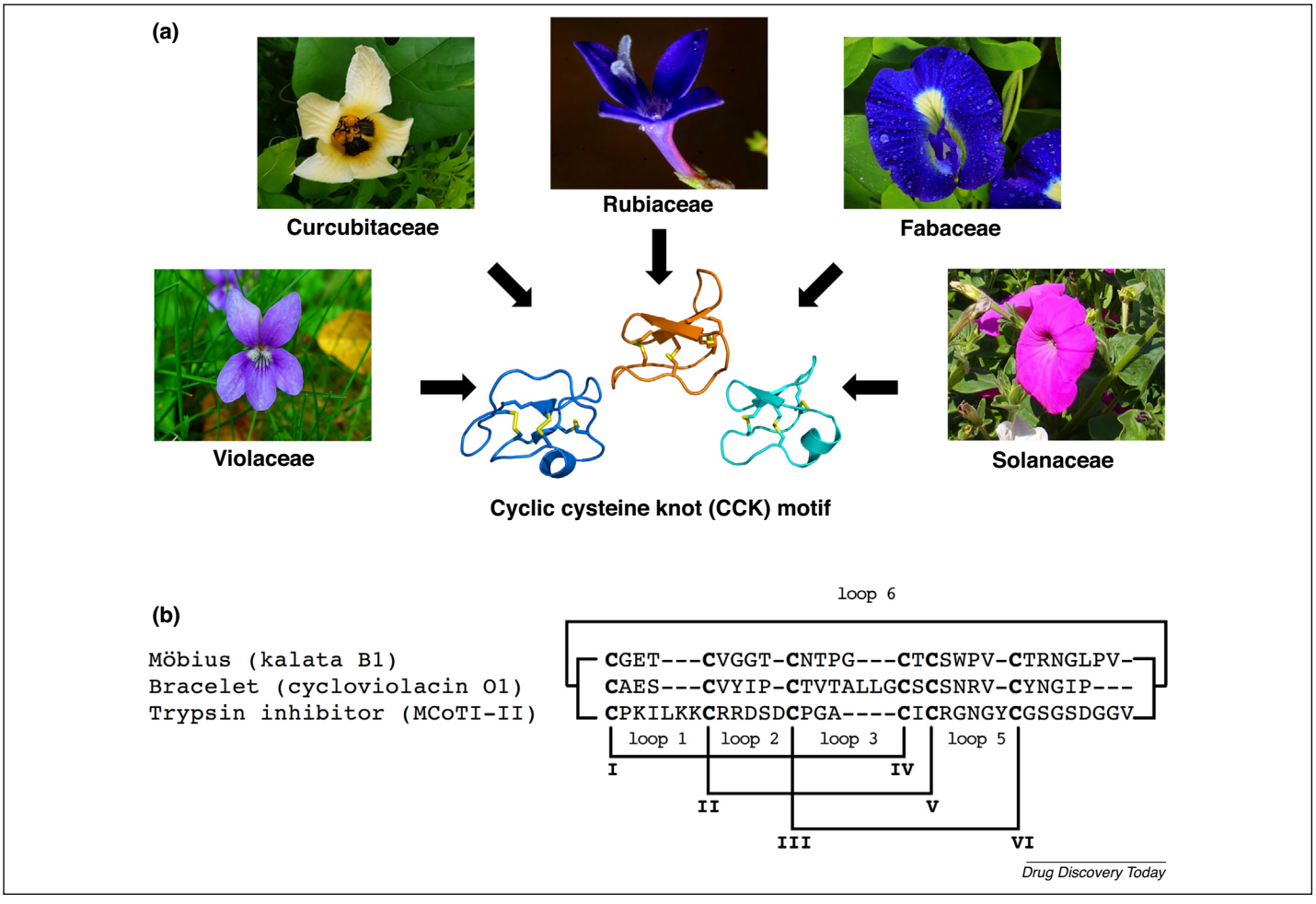

FIGURE 1

Primary and tertiary structures of cyclotides from the Möbius, bracelet, and trypsin inhibitor subfamilies of cyclotides. **(a)** Plant families from which these cyclotides have been isolated. 3D structures for kalata B1 [Protein Data Bank (PDB) identifier: 1nb1], cycloviolacin O1 (PDB identifier: 1nbj) and MCoTI-II (PDB identifier: 1ib9). The disulfide bonds are shown as yellow sticks. **(b)** Amino acid sequences of the selected cyclotides. Conserved cysteine residues are in bold type and connected by brackets.

(free N and C termini) but retains cytotoxic activity against cancer cell lines (MCF-7 and CACO2) [22].

There are three subfamilies of cyclotides described so far, the Möbius, bracelet, and trypsin inhibitor subfamilies (Fig. 1). Although the CCK topology is shared among all subfamilies, the amino acid composition of the six loops differs notably, although changes in the loop sequences minimally affect their 3D structure [25]. Structurally, the Möbius and bracelet subfamilies differ in the presence or absence of a 180º twist in the peptide structure produced by a *cis* tryptophan-proline peptide bond in loop 5 in the Möbius subclass, which is lacking in the bracelet subfamily [28]. By contrast, the trypsin inhibitor subfamily has a longer sequence in loop 1 compared with the other two subfamilies. In terms of their biological activities, the trypsin inhibitor cyclotides have more limited activities than the Möbius and bracelet subfamilies.

In silico screening of cyclotides in databases

As mentioned earlier, the discovery of novel cyclotides can be laborious and, consequently, has encouraged the application and development of bioinformatics tools aiming at cyclotide screening. Since the postgenomic era, the number of sequences deposited in public databases has increased greatly, providing a rich source of biological information. Thus, studies have focused on the screening of cyclotides in public databases [20], also using this information to shed light on the evolution and distribution of these peptides in the plant kingdom [29].

Mulvenna *et al*. [29] were among the first to use bioinformatics tools to screen for cyclotide sequences in graminaceous crops. Homology searches with BLAST were performed, also considering the cysteine patterns already described for cyclotides. As a result, it was observed that cyclotide-like sequences are widespread in the Poaceae, with a total of 22 nonredundant (NR) hits obtained from economically important plants, including maize (*Zea mays*) and wheat (*Triticum aestivum*) [29]. These findings also suggested that cyclotide-like sequences might be found in a wider variety of plants than previously reported. Moreover, in terms of evolution and distribution, this work [29] also reinforced the presence of cyclotides in monocots [30], raising the hypothesis that cyclotides are derived from a common ancestral gene (150 million years ago) before monocots and dicots diverged [29].

An increasing number of studies has focused on developing more accurate strategies for the identification of cyclotide sequences. Thus, in addition to the BLAST-dependent approach described earlier [29], two novel BLAST-independent tools have recently been reported, CyPerl and CyExcel [31]. These have been used to harvest novel cyclotides and analogs from plant genomes and protein databases. By using these programs, >200 cyclotide analogs have been identified from 13 plant families, out of which seven families were described for the first time as cyclotide producers [31]. More recently, another data-mining computational approach (regular expression – REGEX) was applied for screening cyclotides from the National Center for Biotechnology Information (NCBI) NR protein database, resulting in the identification of six novel cyclotide-like precursors from Poaceae [32]. Taken together, these strategies highlight the importance of NGS studies for the continuous growth of public databases aiming at the identification of novel cyclotide sequences, along with a better understanding of how this class of cyclic peptides has evolved.

Cyclotide biosynthesis

Cyclotides are naturally biosynthesized ribosomally from genetically encoded precursor proteins [33]. Cyclotide precursors have endoplasmic reticulum (ER)-targeting sequences that permit entrance into the secretory pathway, where disulfide bonding occurs through the action of disulfide isomerases [34], resulting in cyclotide folding [35] (for a more extensive review on this topic, see [33,36]). Diverse mechanisms of gene regulation have been reported. The most significant are alternative mRNA splicing, selective translation by ribosome instability, and differential precursor processing [37]. Furthermore, cyclotide genes present a characteristic and highly conserved genetic organization, which encodes linear precursors constituted of up to 200 amino acid residues [38]. The gene encodes a protein containing an ER signal-coding sequence, which is followed by a single N-terminal pro-domain (NTPD) coding sequence, a highly conserved N-terminal repeat (NTR) region, a mature cyclotide domain (MCD), and a hydrophobic C-terminal repeat (CTR) [39]. A single gene can encode more than one cyclotide or, more interestingly, different genes can encode the same cyclotide. For instance, kB1 is encoded by both *Oak1* and *Oak2* [37,40].

The post-translational modifications involved in peptide cyclization are not fully understood. However, the participation of an asparaginyl endopeptidase (AEP)-like ligase is important in C-terminal cleavage and cyclization [41]. Sequence analysis and mutagenesis studies have revealed the importance of a conserved Asn-Gly (or Asp-Gly) motif at the end of the cyclotide domain in loop 6 [35,42]. Furthermore, the lack of Asn/Asp residues produces a linear cyclotide-like peptide, thus supporting the relevance of these residues in cyclization [43]. So far, the mechanism implicated in the proteolytic processing of the N-terminal domain has remained elusive and, given that N-terminal processing is an important step for AEP-mediated backbone peptide cyclization, identifying the enzymes involved in this step has relevance for cyclotide production. Recently, members of the papain-like cysteine protease (PLCP) family were identified as responsible for the N-terminal proteolytic release of the peptide. The enzyme (NbCysP6) was able to produce an acyclic peptide from a kB1 synthetic precursor (LQLK-kB1) [44]. These authors identified PLCPs from *O. affinis* responsible for the N-terminal processing of kB1. They proposed the name kalatase A for OaRD21A, highlighting the role in kalata biosynthesis in the plant. Finally, to see whether OaEAP1b and OaRD21A could produce a fully folded cyclotide, the synthetic precursor LQLK-kB1-GI was used as substrate. The incubation of the synthetic peptide with both enzymes generated a cyclic kB1 [44].

In vitro cyclotide synthesis

Cyclotides are relatively small and, therefore, linear precursors can be synthesized by using chemical methods (SPPS). After the peptide is cleaved from the resin, it can be cyclized, oxidized, and folded. The backbone cyclization and folding of the linear precursors can be completed in aqueous solution under physiological conditions using native chemical ligation (NCL), where the linear peptide presents an N-terminal cysteine and an α-thioester group at the C terminus. Both tert-butylcarbonyl (Boc)-based and 9-fluorenylmethoxycarbonyl (Fmoc)-based chemistry have been used to incorporate C-terminal thioester groups during the peptide

synthesis required for cyclization [45–47]. For a more extensive review of this topic see [48] or to read about the advantages and limitation of both Boc-and Fmoc-based chemistry, see [49]. Most cyclotides from the Möbius and trypsin inhibitor families are produced using Fmoc-based chemistry, because this method involves less hazardous chemicals than other techniques. Despite the progress regarding chemical cyclotide synthesis, their production on a large scale remains a challenge. Recently, enzymatic strategies have been investigated to cyclize disulfide-rich peptides to optimize the process of synthesis. Proteases, peptide ligases, and transpeptidases have been used to cyclize polypeptides using linear peptides synthesized by SPPS as substrates. For example, the ligases butelase 1, omniligase-1, and sortase A were recently described as useful tools to cyclize peptides *in vitro* [50]. The peptide MCoTI-II has also been cyclized using an immobilized trypsin with a yield of 92% solid-phase peptide synthesis [51]. kB1 has been cyclized using butelase 1, with yields of ~95% [52], and sortase-mediated backbone cyclization converted linear kB1 to a cyclic form in ~50% of cases [53]. Thus, enzymatic peptide cyclization strategies represent a promising methodology for the large-scale production of fully cyclic peptides.

Techniques of molecular biology and recombinant DNA technology have allowed the heterologous expression of peptides. The biological synthesis of cyclotides offers advantages over the aforementioned chemical synthesis of peptides. For example, there is no size limitation, whereas chemical synthesis is limited to a maximum peptide size of 150 residues [54]. Microorganisms, including *Escherichia coli* or *Saccharomyces cerevisiae*, have been used to express fully cyclic and folded peptides. Different approaches have been reported to produce cyclic peptides, including expressed protein ligation (EPL) [55], intein-mediated protein *trans*-splicing (PTS) [56], protease-catalyzed transpeptidation [53], and genetic code reprogramming [57]. For a detailed method of recombinant expression of cyclotides using split inteins see [58] and, for a more extensive review on chemical and biological production of cyclotides, see [59]. In addition, PTS-mediated backbone cyclization has been used to produce cyclotides containing unnatural amino acids in *E. coli*. These experiments were performed to produce cyclotides to be easily labeled and used as probes for imaging purposes [56]. Moreover, not only *E. coli*, but also eukaryotic cells have been used to express cyclotides, including native and engineered cyclotides. For example, the native cyclotide MCoTI-I and the grafted cyclotide MCoCP4 were successfully expressed in Baker's yeast (*S. cerevisiae*). The engineered peptide chosen was α-synuclein, a small lipid-binding protein that has been linked to Parkinson's disease [60].

Moreover, plant-based production could be an attractive alternative to produce desired cyclotides. As discussed earlier, understanding of the mechanism of cyclotide synthesis in plants has improved over the past decade, opening routes to produce cyclotides faster, more cheaply, and with safer techniques compared with traditional methods of synthesis, including chemical or heterologous expression in microbial or mammalian cells [61]. Plants can produce levels of cyclotides up to 1 g per kg of wet plant [62]. Therefore, using plants as factories to produce folded cyclotides for agricultural or therapeutic applications has been highlighted as a promising strategy. Some efforts in this regard have been published recently [63].

Native activities of cyclotides

As mentioned earlier, a single plant can express >150 different cyclotides [15], and a plant family could contain up to 150 000 different sequences [14,15]. Thus, plants must have a biological purpose for the synthesis of cyclic peptides in nature. The first studies to discover biological activities of cyclotides reported a range of activities, including antimicrobial, insecticidal, anthelmintic [64], and pesticidal activities (Table 1) [65,66]. However, the natural purpose of cyclotides is likely to be to act as self-defense molecules in plants against pests and herbivores [37,67–69]. However, why a plant family expresses hundreds of different cyclotides and with high levels of sequence diversity remains to be fully understood, but recent publications suggest that this diversity is a defensive strategy against different kinds of herbivores [70]. The first study regarding insecticidal activities of cyclotides reported the ability of kB1 to inhibit the growth and development of lepidopteran *Helicoverpa punctigera* larvae [37,71]. kB1 was found to be a potent insecticidal molecule at concentrations of 0.8 $\mu mol.g^{-1}$, which is the same concentration found naturally in plants. The insecticidal activity of cyclotides has been also reported for kB2, Cter M, and parigidin-br1 (Table 1) [72,73]. However, the mechanism of insecticidal activity is not well understood, but is known to involve the disruption of the mid-gut membranes when ingested by the larvae [74]. Recently, it was reported that the possible mechanism of membrane disruption for kB1 is through the direct interaction with phosphotidylethanolamine phospholipids (PE) as the first step of binding to cellular membranes, causing the formation of pores and leading to cell death [75]. Additionally, phospholipid/cyclotide interactions studies have also proposed that cyclotides can interact with insect receptors. kB7 has been shown to weakly interact with inotocin receptors from *Tribolium castaneum* and *Lasius niger*, acting as an agonist. Although more studies are needed to confirm the relevance of this molecular mechanism, these findings are a promising starting point to clarify the role of cyclotides in the neuropeptide signaling systems of insects [76].

Another native activity described for cyclotides is their ability to inhibit microbial cell growth. The antimicrobial activity of cyclotides was first reported by Tam and coworkers in 1999 [77]. In this study, synthetic kB1 was tested against Gram-positive and Gram-negative bacteria, resulting in a minimum inhibition concentration (MIC) of 0.2 μM for *Staphylococcus aureus*. By contrast, kB1 was ineffective against *E. coli* and *Pseudomonas aeruginosa*. Other studies also confirmed the antimicrobial activity of cyclotides [78]. However, these experiments were performed under low-salt conditions and increasing the amount of salt resulted in the abolition of the antimicrobial activity. A later study showed that semipure cyclotides present higher antimicrobial activity against plant-pathogenic Gram-negative bacteria compared with human-related pathogenic bacteria [79], emphasizing the role of cyclotides as host-defense compounds in plants. In a recent study, a set of 18 cyclotides were investigated under low-salt conditions and their antimicrobial properties were compared with melittin and LL-37, both known for their antibacterial properties [80]. All cyclotides belonging to the bracelet or Möbius subfamilies reported antimicrobial activity at 40 μM or below, whereas the cyclotides from the trypsin inhibitor subfamily (MCoTI) did not show antimicrobial activity [71]. Although the antimicrobial activity of cyclotides is

TABLE 1

Summary of the cyclotides and their grafted analogs described in this article in terms of source, application, and structural arrangement[a].

Cyclotide	Source	Bioactivities		3D Structures (PDB ID)	Refs
		Native	Engineered		
Möbius subfamily					
kB1	*Oldenlandia affinis*	Insecticidal; hemolytic; nematocidal; antimicrobial; uterotonic; anthelmintic	Anti-HIV; protease inhibition; antiangiogenic; chronic and inflammatory pain; MS	1NB1; 4TTM; 1JJZ;1ZNU	[11,64,65,77,89,98,119,120]
kB2	*Oldenlandia affinis*	Antimicrobial; nematocidal; molluscicidal; insecticidal; anthelmintic	Analgesic; protease inhibition; antiviral	1PT4; 2KCH	[37,65,72,81]
Bracelet subfamily					
Cycloviolacin	*Viola Spp*			1NBJ; 2GJ0; 2KCG;	[71,78,81,82]
parigidin-br1-br3	*Palicourea rigida*	Antimicrobial; insecticidal; molluscicidal; nematocidal	Anticancer; hemolytic; anti-HIV	2KNM; 1DF6; 2KNN	[22,73]
Trypsin inhibitor subfamily					
MCo-TI	*Momordica cochinchinensis*	Protease inhibition	Anticancer; antiangiogenic	1IB9; 2MT8; 2PO8; 5WOV; 5WOW; 1HA9;	[87,98]
MCo-TII	*Momordica cochinchinensis*	Protease inhibition	Antiangiogenic; immunogenic; antithrombotic; anti-HIV; bioimaging	2IT8; 4GUX	[88,93,96,106–108]

[a] Data were retrieved from CyBase [117], Protein Data Bank (PDB), and Antimicrobial Peptides Database (APD).

not a common activity for all three subfamilies, cycloviolacin peptides (bracelet subfamily) appear to have the most well-known antimicrobial activity both *in vitro* and *in vivo* (Table 1) [71,78,81]. Nevertheless, classifying cyclotides as antimicrobial peptides should consider studies reporting similar levels of toxicity towards mammalian cells [82].

An innovative method to enhance the biological activity of peptides is by using nanobiotechnology [83,84]. Nanoparticles increase protection from proteolysis and favor specificity. Moreover, they allow a controlled release of peptides near the target and also improve native antimicrobial properties. Nanoformulations can also enhance limited pharmaceutical characteristics, including short half-life in blood stream, toxicity, and bioavailability. Thus, this strategy represents a new tool that could be explored to design new peptide-based antibiotics that exhibit a broad spectrum of activity. In this regard, the cyclotides kB2 and parigidin-br1 have been nanoencapsulated to improved their anticancer properties and sustained release. As a result, it was observed that nanoencapsulated cyclotides were capable of partially compromising colorectal adenocarcinoma (CACO2) and breast cancer (MCF-7) cell viability [85]. This was an innovative strategy for cyclotide administration and represents a starting point for further studies, especially given the lack of nanoencapsulated cyclotides reported to date.

Engineered cyclotides with novel activities

Many efforts have focused on the exploitation of cyclotide stability properties for drug development. The potential of cyclotides as scaffolds for the development of therapeutics has been reported previously. Grafting studies, which comprise peptide epitopes stabilized in the cyclotide structure, are a powerful tool to design new drugs (Fig. 2a). The six backbone loops formed between cysteine residues vary in size and sequence diversity. Usually, cyclotide loops contain from three to eight amino acid residues, except for loop 4, with only one residue (Fig. 1). In addition, loops 1 and 4 are the most conserved, probably because they comprise the ICK structural motif and, therefore, are less appropriate for cyclotide engineering. Loops 2, 3, 5, or 6 have been modified and used to insert small peptides aiming at novel biological properties. These loops are amenable to modifications with some structural limitations [6]. From a single-point residue mutation to a 21-residue substitution, cyclotides have been able to tolerate the incorporation of bioactive epitopes for the treatment of cancer, pain, and multiple sclerosis (MS), as described here.

Since Clark and coworkers [86] reported for the first time the manipulation of a CCK peptide (kB1, loop 5), the concept of cyclotide engineering and design drastically changed. In their study, mutations in loop 5 of kB1 were performed, resulting in two nonhemolytic analogs with improved pharmacological potential and conserved folding compared with the native peptide [86]. These findings, along with several other studies in the field of cyclic peptides, reinforce the advantages of using the highly stable CCK scaffold of cyclotides as a framework in which peptide fragments (epitopes) can be grafted (Fig. 2a), leading to increased potency, selectivity, and stability towards specific targets. This strategy has been widely reported, and proof-of-concept studies have shown the application of grafted cyclotides in cancer [87], angiogenesis [88], inflammatory pain treatment [89], and cardiovascular diseases (Fig. 2b,c; Table 1) [90].

An increasing number of studies has focused on the development of new molecules and strategies to target cancer-related genes, transcripts, and proteins. Oncogenic proteins, including Hdm2 and HdmX, for example, have a crucial role in the negative regulation of the transcription factor p53 [91]. This transcription factor is well known for its regulatory role in preventing tumorous cells from replicating by inducing apoptosis. Hdm2 and HdmX

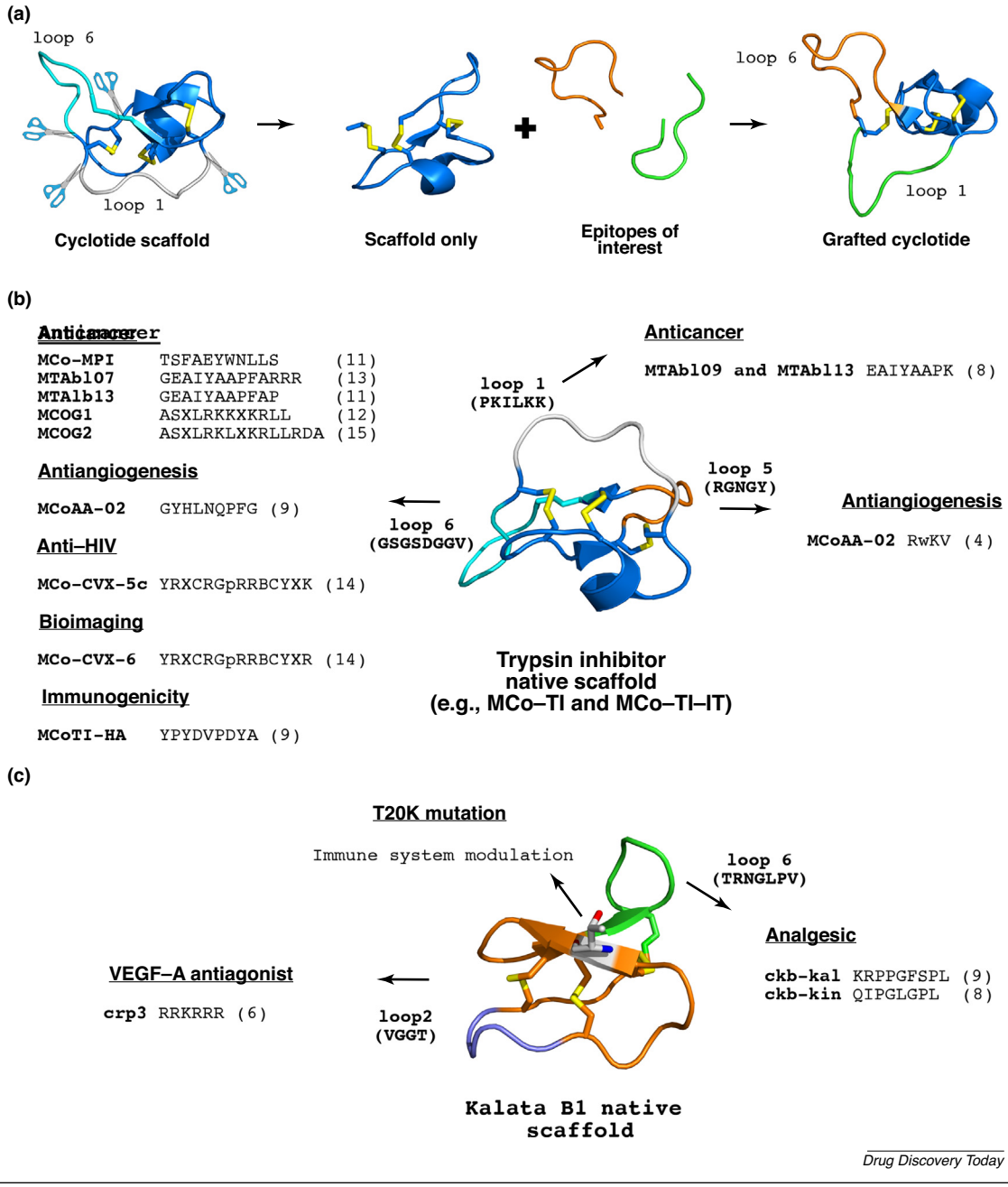

FIGURE 2

Schematic representation of the highly stable cyclic cystine knot (CCK) scaffold of cytokines as a framework in which peptide fragments (epitopes) can be grafted, aiming at novel biological activities. (a) The 3D structure of MCoTI-II [Protein Data Bank (PDB) identifier: 1IB9] was used to illustrate the grafting process. The disulfide bonds are shown as yellow sticks. (b) Graphical representation of grafted MCoTI-II with different engineered bioactivities. (c) Graphical representation of kalata B1 (PDB identifier: 1nb1) with engineered bioactivities.

have been described as key factors in the degradation of p53 and the inhibition of p53 transactivation, respectively [91]. As a consequence, both Hdm2 and HdmX inactivate p53 tumor suppressor pathways, favoring the survival of tumor cells [87]. In this context, the development of novel Hdm2/HdmX antagonists appears as a promising alternative in cancer therapies [91,92]. With that in mind, Ji and coworkers [87] engineered a cyclotide variant (MCo-PMI) by grafting a p53-derived helical peptide (PMI) into loop 6 of MCoTI-I (Fig. 2b). It was observed that MCo-PMI works as an Hdm2/HdmX antagonist at nanomolar concentrations both *in vitro* and *in vivo*, leading to the activation of the p53 tumor suppressor pathway (Table 1). In addition, MCo-PMI was cytotoxic towards p53 cancer cell lines, also presenting high structural stability [87].

Similarly to MCoTI-I, the cell-penetrating MCoTI-II has been used as a framework for the development of peptide-based inhibitors targeting cancer-related proteins, including the BCR-ABL tyrosine kinase [93]. BCR-ABL tyrosine kinase is one of the main causes of chronic myeloid leukemia (CML), and tyrosine kinase inhibitors are usually used as treatment [94]. These inhibitors

target the ATP-binding site of BCR-ABL; however, mutations in this site appear as a recurrent obstacle in CML therapies [94]. In this context, the sequence of an optimal substrate of Abl kinase (abltide) has been grafted into loops 1 and 6 of the cyclotide MCoTI-II [93] (Fig. 2b; Table 1). *In vitro* assays have shown that abltide-grafted MCoTI-II peptides inhibit Abl kinases (both the native and the multidrug resistant [T315I]Abl) at micromolar concentrations, also revealing remarkable stability in serum [93]. Despite these promising findings, abltide-grafted MCoTI-II peptides were not cytotoxic towards the K562 cell line (a model cell line obtained from a 53-year-old female with CML), which could be explained by the trapping of these peptides in cell compartments, leading to the inhibition of small amounts of BCR-ABL [93].

The MCo-TI-II scaffold has also been used for the development of a stable cyclic peptide agonist of SET (Fig. 2b; Table 1), a human nuclear oncoprotein usually overexpressed in diverse types of cancerous cell, including acute myeloid leukemia and CML [95,96]. This oncoprotein inhibits the tumor suppressor activity of a serine/threonine phosphatase, PP2A, thus interfering with the regulation of cell growth and signaling [97]. Taking into account the relevance of SET as a molecular target for cancer treatments, D'Souza and coworkers [96] designed novel cyclotide variants containing a peptide derived from apolipoprotein E (apoE) (COG peptide), which is a potent antagonist of SET. COG was grafted into loop 6 of MCo-TI-II, and the two variants, MCOG1 and MCOG2, were evaluated regarding their structure and function. It was observed that both the MCo-TI-II scaffold and COG helical structure were conserved in the grafted variants. Surface plasmon resonance studies were carried out, revealing that the variants bound to SET with higher affinity than the native MCo-TI-II. Moreover, both MCOG1 and MCOG2 were toxic (IC50) to K562 cancerous cells (described earlier) at 2.9 and 5 μM, respectively (Table 1). As for the other grafted cyclotides here described, MCOG1 and MCOG2 were highly stable in serum [96].

Apart from the oncoprotein cited earlier, angiogenesis inhibitors have been considered important in cancer studies. Angiogenesis is a complex process in which new blood vessels are formed, thus increasing the proliferation and migration of endothelial cells [98]. However, disturbances in angiogenesis regulation provide nutrients and oxygen to tumorous cells and, thus, angiogenesis inhibitors have been explored as an alternative to regulate tumor formation [99]. Vascular endothelial growth factor (VEGF) is one of the main regulators of angiogenesis and is usually upregulated in human tumors, thus representing a possible molecular target for engineered VEGF antagonists [100]. Gunasekera and coworkers [98] grafted an arginine-rich epitope (RRKRRR) responsible for VEGF-A antagonism into the loops 2, 3, 5, and 6 of kB1 (Fig. 2c; Table 1). Among the generated analogs, cpr3 displayed higher inhibitory activities against VEGF-A (IC_{50} = 12 μM) [98]. Structural analyses revealed that the cpr3 grafted region is disordered and extends beyond the core of the peptide compared with the nonactive analog cpr6. This difference in cpr3 structural arrangement was then described as a protagonist in antiangiogenesis (Table 1). In addition, all active and nonactive grafted peptides abolished the cytotoxic properties of kB1 [98].

Interestingly, studies have also described 2-in-1 antiangiogenesis grafted cyclotides with specificity to bind more than one molecular target in cancerous cells [88]. Compared with grafted cyclotides containing only one epitope (e.g.,cpr3, described earlier), the insertion of two epitopes is supposed to display synergistic properties, leading to improved antiangiogenesis activities (Fig. 2b; Table 1). As an example, Chan and coworkers [88] reported the design of two generations of MCo-TI-II grafted peptides (Table 1) in which epitopes derived from somastatin (SST-01 and -02), a pigment epithelium-derived factor (PEDF) and a poly-Arg-derived peptide antagonist to VEGF were inserted in different combinations. To develop dual-targeting grafted MCo-TI-II, two main variants were designed, MCoAA-01 and -02, containing the SST-01 epitope in loop 5, and the poly-Arg and PEDF epitopes in loops 1 and 6, respectively [88]. *In vitro* and *in vivo* studies were performed, revealing that MCoAA-02 was the most potent variant in terms of cell migration and proliferation, as well as in regulation of VEGF. These findings led the authors to suggest that MCoAA-02 inhibits blood vessel growth around cancerous cells resulting, ultimately, in the blockage of nutrients around tumors [88].

Indeed, most studies regarding grafted cyclotides have mainly focused on cancer therapies. Nevertheless, studies have shown the advantages of using these peptides for other purposes. For example, Wong and coworkers [89] designed two novel kB1 variants, named ckb-KAL and ckb-KIN, in which bradykinin B1 receptor antagonists were grafted into loop 6 (Fig. 2c; Table 1). Bradykinin has a crucial role in numerous pathophysiological injuries, also acting as a potent inducer of endogenous pain [101]. The authors observed that both ckb-KAL and ckb-KIN acted as antagonists of the bradykinin B1 receptor in HeLa cells [89]. Moreover, the analgesic effect of ckb-KAL and ckb-KIN (and also the linear control) was evaluated in *in vivo* models through the measurement of abdominal constriction in mice. As result, the authors observed that all variants decreased the number of writhing movements significantly, and ckb-KAL was the most active cyclic peptide with the highest oral availability [89]. Recently, the pharmacokinetic characterization of kB1 and grafted analogs, ckb-KAL and ckb-KIN, was reported in rats when administered orally and intravenously [102]. The comparative study for natural and grafted cyclotides using different routes of administration supported the activity of ckb-KAL, which displays the best volume of distribution of all peptides studied [102]. This study demonstrated that cyclotides display appropriate drug efficiency and are comparable to peptide drugs already on the market [102].

Vascular occlusion events caused by thrombosis are a common occurrence and are usually associated with cardiovascular diseases [103]. Factor XIIa (FXIIa) has been studied because of its importance in thrombosis and, consequently, FXIIa inhibitors capable of blocking the intrinsic coagulation pathways have been investigated [e.g., the FXIIa inhibitory antibody (3F7)] [104,105]. Recently, Swedberg and coworkers [106] showed that epitopes resulting from the cleavage of tetrapeptide *para*-nitroanilide (*p*NA) substrates by FXIIa and factor Xa (FXa) can be grafted into the canonical loop of MCo-TI-II to generate stable FXIIa inhibitors as an alternative to costly antibody-based FXIIa inhibitors (Fig. 2b; Table 1) [106]. Cardiovascular diseases caused by thrombosis include myocardial infarction [103], which is also related to the renin angiotensin system. Thus, grafted cyclotides have also been developed as angiotensin receptor (AT1-7) activators for the treatment of both lung cancer and myocardial infarction [90].

Anti- HIV-1 activity has also been investigated in grafted cyclotides. Aboye and coworkers [107] reported, for the first time, the engineering of a MCoTI-I variant, named MCo-CVX-5c, capable of inhibiting HIV-1 viral replication by targeting the G-protein-coupled chemokine receptor 4 (CXCR4) (Fig. 2b; Table 1). Previous reports highlighted the importance of CXCR4 in the regulation of several biological functions, including angiogenesis, metastasis, embryonic development, HIV replication, among others, thus suggesting the relevance of CXCR4 antagonists [108,109]. MCo-CVX-5c was generated through the grafting of a polyphemusin-derived peptide CVX15 into loop 6 of MCoTI-I [108]. *In vitro* assays revealed that MCo-CVX-5c acts as a potent CXCR4 antagonist, being capable of blocking the entry of HIV-1 in human lymphocytes [107].

Besides the therapeutic application MCo-CVX-5c, this grafted cyclotide was recently studied for bioimaging purposes, providing guidance for the treatment of diseases [108]. Lesniak and coworkers [108] performed a single mutation in loop 6 of MCo-CVX-5c, replacing a lysine with an arginine, generating MCo-CVX-6. This mutated peptide was further modified, resulting in the radiolabeled cyclotide [^{64}Cu]MCo-CVX-6D. [^{64}Cu]MCo-CVX-6D, similar to its parent cyclotide, presented high binding specificity for CXCR4, which was subsequently reported as a crucial mechanism for the efficient detection of CXCR4-expressing cells in tumors [108]. These findings provide a new perspective regarding the therapeutic application of cyclotides as imaging agents (Fig. 2b; Table 1).

Although grafted cyclotides have been demonstrated as potential drug candidates with diverse applications, the immunogenicity and immunological effects of these peptides remain unknown. Thus, Kwon *et al*. [110] recently described the immunogenicity of MCoTI-I in its native form and with a HA epitope (YPYDVPDYA) grafted into loop 6 (MCoTI-HA) (Fig. 2B = b; Table 1). To evaluate the immunogenicity of these peptides, cyclotide-protein carrier conjugates were developed, aiming at the delivery of these peptides to antigen-presenting cells (APCs). For this, unnatural amino acids were included within the sequences of the cyclotides to allow a 'click' reaction with the carrier protein. In this case, a dibenzocyclooctyne (DBCO)-modified anti-class II MHC VHH antibody, named VHH7 [110] was used. As a result, the authors observed that cyclotide-specific antibodies against both MCoTI-I cyclic and linear versions were elicited by MCoTI-I + VHH7. Similar findings were reported with MCoTI-HA + VHH7. Nevertheless, no reactivity was observed for the linear HA epitope alone, indicating the importance of using a cyclotide scaffold for the production of antibodies that recognize engrafted epitopes [110]. Therefore, it was concluded that cyclotide-VHH7 conjugates successfully led to the target delivery of this peptide to APCs, also opening a new field of research on cellular targeting of cyclotides.

Apart from the immunogenicity activity of MCoTI-I cited earlier, kB1 has been reported for its immune system modulation activity, specifically as an immunosuppressant agent. Gruber and colleagues (2012) showed that kB1 was capable of inhibiting primary activated human lymphocytes [111]. A year later, this same research group identified the silencing of T cell proliferation *in vitro* via an interleukin-2 dependent mechanism as the cause of immunomodulation [112]. Based on those reports, Gruber's lab developed a point-mutated kB1, called (T20 K)kB1 (Fig. 2C), which is orally stable, and tested it in a mouse model of MS [113]. MS is the most common autoimmune disease and treatments options remain limited [114]. (T20 K)kB1 showed a significant reduction in the symptoms in an experimental mouse model for autoimmune encephalomyelitis [113]. Moreover, both parenteral and oral administration routes were tested and substantially impaired the disease progression with moderate adverse effects [113]. Currently, positive results have been reported in preclinical studies and Phase I clinical trials with (T20 K)kB1, encouraging the use of point-mutated kB1 in more advanced clinical trials [115]. Similarly, another study of kB1 reported that, by grafting central nervous system peptide epitopes [e.g., myelin oligodendrocyte glycoprotein (MOG 35-55)] into kB1, an effective variant named MOG3 was generated, which prevented MS development in a mouse model [116]. Taken together, these findings demonstrate the potential use of these cyclotides for peptide-based therapies aiming at treating MS.

Concluding remarks

Currently, >700 cyclotides are deposited in CyBase [117], a database of cyclic protein sequences and structures, with applications in protein discovery and engineering. This family of cyclic plant peptides has been widely investigated as drug candidates and/or drug scaffolds, mainly because of their remarkable stability. Moreover, considering their high-cost and time-consuming chemical synthesis, an increasing number of bioprocessing strategies have been developed aiming at the feasible and economical production of cyclotides as pharmaceuticals [118]. Indeed, cyclotides have been characterized as self-defense molecules in plants, leading to their application as pesticides and antimicrobial agents. In addition, the study of cyclotides as scaffolds for the generation of improved variants (grafted cyclotides) with specific activities has attracted attention in alternative areas, including cancer, angiogenesis, inflammatory pain treatment, cardiovascular diseases, and cellular targeting. Therefore, the development of grafted cyclotides has enabled the insertion of bioactive epitopes into highly stable structures, thus supporting health programs aiming at peptide-based therapies.

The prospect of developing a cyclotide-based drug or a cyclotide-based insecticide in the near future is high. Considering the promising example of (T20 K)kB1 moving to Phase I clinical trials to treat MS or the Sero-X product commercialized as a bioinsecticide (https://innovate-ag.com.au/our-product/), we can expect that cyclotides will shortly become active ingredients for many pharmaceutical products. Indeed, some questions regarding the role of cyclotides in plants remain, as well as cyclotide engineering for novel biological activities on humans. However, over the past decade, research on cyclotides has emphasized these molecules as orally effective, and with the potential to be produced by advanced technologies aiming at reduced costs. Moreover, cyclotides can also be expressed in plants. Thus, considering all the advantages and disadvantages of cyclotides, cyclotides represent fascinating molecules that offer a huge potential in many fields, including biomedicine, agriculture, and biotechnology.

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