# Cyclotides as templates in drug design

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**Cyclotides are remarkably stable proteins from plants that have a range of pharmaceutical and agricultural applications based on both their various bioactivities and their potential for use as stable protein-engineering templates. This article discusses literature on pharmaceutically relevant activities of cyclotides, including anti-HIV, antimicrobial and cytotoxic activities, and evaluates their potential therapeutic applications. Their applications as templates for the design of antiangiogenic agents for the treatment of cancer and as anti-infective agents are also described. Toxic effects of cyclotides, whose native function is as insecticidal agents, can be removed by simple mutagenesis, thus rationalizing the apparent conundrum of proposing insecticidal agents as leads for human therapeutics.**

Cyclotides are disulfide-rich proteins from plants that are characterized by a unique combination of a head-to-tail cyclized backbone and a knotted arrangement of three conserved disulfide bonds [1]. Their natural function in plants seems to be as host-defense agents, as deduced from their potent insecticidal activity and their high expression levels in many plant tissues [2,3]. However, interest in cyclotides extends beyond their natural defense function because their unique structures engender them with a range of valuable biophysical and chemical properties, including exceptional resistance to thermal, chemical and enzymatic treatments [4–6].

The exceptional properties of cyclotides first came to notice because of the discovery of what is now known to be their prototypic member, kalata B1, as a bioactive agent in indigenous medicine in Africa [7]. Gran reported the use of the plant *Oldenlandia affinis* to make a tea that was used by women in the Congo, during labor, to accelerate childbirth [8]. Kalata B1 is a peptide of 29 amino acids (Fig. 1a) that seems to survive boiling and ingestion before the expression of uterotonic activity associated with its medicinal applications. The three-dimensional structure of kalata B1 was reported in 1995 [9] and comprises a cystine knot motif [10,11] in which two disulfide bonds and their connecting backbone segments form a ring that is penetrated by a third disulfide bond (Fig. 1b). In addition to this knotted disulfide topology, kalata B1 was, at the time of its discovery, unique because it had neither the N- nor the C-termini of conventional proteins. The termini are absent because of the head-to-tail cyclization of the peptide backbone.

At around the same time that the structure of kalata B1 was reported, independent studies from three groups described stable bioactive peptides in plants with a range of activities, including hemolytic [12], neurotensin antagonist [13] and anti-HIV activity [14]. In the following few years, several dozen related macrocyclic peptides with a range of activities were discovered [1,15,16], and in 1999, the name 'cyclotides' was introduced to refer to this family [1]. Since then, a large number of cyclotides have been reported, and the complete list of currently known sequences is collected in Cybase, a database devoted to circular proteins (http://www.cybase.org.au/). Other examples of circular proteins occur in bacteria, plants and animals. Cyclotides are the largest of the currently known families of circular proteins and are generally divided into two major subfamilies: the bracelet and Möbius cyclotides (see Fig. 1a for amino acid sequences of examples belonging to these two subfamilies). A third subfamily, the trypsin inhibitor cyclotides [17] (also known as 'cyclic knottins' [18]), contains only two members, MCoTI-I and MCoTI-II [19], which are of particular interest from a pharmaceutical perspective because of their ability to penetrate cells and, thus, potentially interact with intracellular targets.

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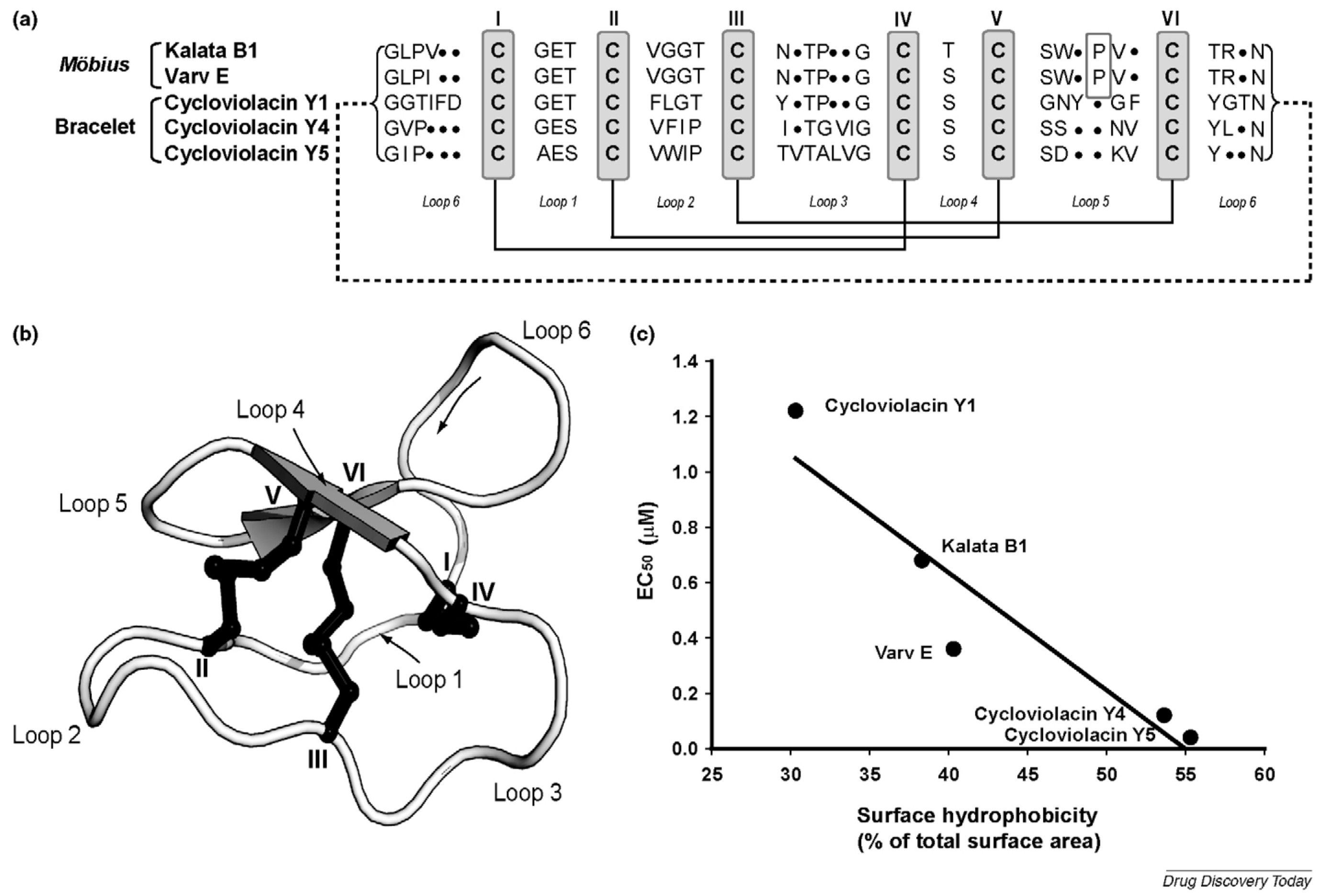

**FIGURE 1**

Anti-HIV activity of cyclotides isolated from *Viola yedoensis*. **(a)** Amino acid sequences of the cyclotides isolated from *V. yedoensis* [34]. Cyclotides have a well-defined three-dimensional structure with a cyclic backbone and six conserved cysteine residues. The cyclic structure is represented by the dashed line, the cysteine residues are labeled I–VI, the three disulfide bonds are shown with black lines and the six loops are labeled 1–6. The subfamily classification is based on either the presence or the absence of a *cis*-Pro residue in loop 5; cyclotides belonging to the Möbius subfamily possess a conceptual twist owing to the presence of this *cis*-Pro residue. **(b)** Structure of the prototypic cyclotide kalata B1 (PDB ID: 1nb1 [9]). **(c)** Correlation between cyclotide surface hydrophobicity and their anti-HIV activity [34].

All cyclotides contain six conserved cysteine residues that comprise the cystine knot core. The backbone segments between these residues are termed 'loops' and, in the naturally occurring derivatives discovered so far, contain one to eight amino acids. Many of these loops are variable in amino acid sequence, leading to the description of cyclotides as naturally occurring combinatorial templates in which a conserved cystine knot core is surrounded by loops of varying amino acid size and content [20]. These loops display the residues responsible for the bioactivity of cyclotides and can be synthetically modified to incorporate new sequences [21]. Examination of the sequences of all currently known cyclotides has shown that in addition to the conserved cysteine residues, there are two other sites of conservation: a Glu in loop 3, which has an important role in defining structure [22–24] and bioactivity [25,26], and an Asn (or Asp) in loop 6, which is crucially involved in the biosynthetic pathway for cyclotides in plants [27,28].

Aside from their insecticidal activity, the other reported activities of cyclotides are of potential pharmaceutical interest and include antimicrobial, anti-HIV and antitumor activities. This article reports on these various pharmaceutical activities and evaluates the present status of cyclotides as potential lead molecules for drug development.

## Pharmaceutically relevant activities of cyclotides

### *Anti-HIV activity*

The most extensively investigated biological activity of cyclotides is their anti-HIV activity, which was first reported by Gustafson *et al.* [14] as part of a screening program at the U.S. National Cancer Institute for novel anti-HIV natural products. In this study, two macrocyclic peptides (circulin A and B) from the bark of an African tree were shown to inhibit the HIV infection of different host cell lines [14]. Subsequent reports showed that a range of other cyclotides, belonging to bracelet or Möbius subfamilies and of native or synthetic origin, also had anti-HIV activity [29–34]. *Prima facie*, these reports suggest that cyclotides have potential application as anti-HIV drugs, but there are several factors that have limited their progression to the clinic.

The exact mechanism of anti-HIV action of these compounds is still unclear. A concentration-dependent increase in cytoprotection, together with a decreased level of infectious virions and an inability to affect HIV-reverse transcriptase activity [14], suggest that the protective effect occurs before the entry of the virus into the cell. The HIV infection process is initiated by virus particles binding to receptors at the surface of target cells, followed by fusion and mixing of the viral and host cell membranes. Therefore, it seems probable that the anti-HIV action of cyclotides occurs through a mechanism that affects the binding and/or the fusion of the virus with the target membrane. Other peptides have been reported to inhibit HIV entry into the cell, including enfuvirtide, or T20 [35], commercialized by Roche (Fuzeon®). The mechanism of action of T20 and other HIV fusion inhibitor peptides seems to be related to their ability to bind to either the target cell membrane [36] or the virus membrane [37], inhibiting the binding and fusion of the virus with the target cell.

Recent studies have suggested a correlation between the anti-HIV activity of cyclotides and their surface hydrophobicity [33,34]. This correlation is shown in Fig. 1c, in which the cytoprotective anti-HIV activity of five cyclotides recently isolated from *Viola yedoensis* (a plant widely used in Chinese traditional medicine) is plotted against their degree of surface hydrophobicity. Although the precise mechanism of action is not known for anti-HIV and for other individual bioactivities, in general, cyclotide bioactivities seem to be broadly correlated with membrane binding. The membrane-binding hypothesis is supported by surface plasmon resonance studies showing cyclotides binding to phospholipid membranes [38] and nuclear magnetic resonance (NMR) reports demonstrating that cyclotide binding occurs primarily through their surface-exposed hydrophobic patches [23,39,40]. In addition, a strong correlation between insecticidal activity, hemolytic properties and model membrane leakage efficiency was found in an Ala-scanning study on kalata B1 [41]. Together, these reports support the hypothesis that the anti-HIV protective effect is modulated by membrane-binding affinity, which is enhanced by increases in the size of the hydrophobic patch on the cyclotide surface; nevertheless, it would be premature to conclude whether the HIV inhibition mediated by cyclotides occurs via their binding at the virus membrane, at the target cell membrane or at both.

At this stage, cyclotides are not being considered as candidates for anti-HIV therapy because their therapeutic index (i.e. the ratio of their therapeutic effects to toxic effects) is typically too small (<10) to be clinically applicable [33]. Nevertheless, recent results obtained with kalata B1 and cycloviolacin Y5 show higher therapeutic indices of 25 and 44, respectively [34]. That only a handful of cyclotides have been tested for anti-HIV activity, together with the estimated existence of more than 50,000 cyclotides [42], means it is possible that other untested analogs might be more selective in their toxicity. A better understanding of their anti-HIV mechanism of action could also accelerate the development of a cyclotide-based anti-HIV drug.

### *Antimicrobial activity*

Cyclotides have hydrophilic and hydrophobic patches located in different regions of their surfaces and, thus, to some extent, resemble the amphipathic nature of many classical antimicrobial peptides. This structural characteristic was possibly a motivating factor for the initial study, reported by Tam *et al.* [43], to evaluate whether cyclotides had antimicrobial activity. In this study, the synthetically derived cyclotides (kalata B1, circulin A, circulin B and cyclopsychotride A) were tested against a range of human pathogenic bacteria and fungi, and promising activity was reported (Table 1). Low micromolar activity was observed against both Gram-positive and Gram-negative bacteria in the absence of salt, but much of their activity was abolished by salt concentrations having more physiological relevance.

Since the publication of the 1999 study, there have been no follow-up reports on antimicrobial activities, except one paper published by Gran *et al.* on three cyclotides isolated from *O. affinis* [44]. Table 1 summarizes the results from the two studies; kalata B1 is the only peptide common to both studies. Tam *et al.* considered kalata B1 inactive against *E. coli* and active against *Staphylococcus aureus*, whereas Gran *et al.* reported the opposite trend. In the study by Tam *et al.*, the lack of antimicrobial activity of kalata B1 against *E. coli* was concluded based on the minimum inhibitory concentration (MIC) against *E. coli* being >500 μM in a one-day incubation study [43]. In the study carried out by Gran *et al.*, antibacterial activity was observed after incubation of ~1700 μM of kalata B1 over three days [44]. Differing peptide concentrations and incubation times might explain the different conclusions. More surprisingly, the results obtained in an antibacterial assay against *S. aureus* were discordant; the MIC reported by Tam *et al.* was 0.26 μM for kalata B1 in the absence of salt [43], whereas Gran *et al.* reported no antibacterial activity at concentrations up to ~5.5 μM kalata B1 either in the presence or in the absence of salt [44] (see Table 1 for more details on experimental conditions). Whereas Tam *et al.* used synthetically produced kalata B1, Gran *et al.* conducted their studies with extracted native peptide, raising the possibility that the varying results might reflect a difference between synthetic and natural material. However, this seems improbable because another study has shown that the disulfide connectivities and *cis*-proline configuration of a synthetic kalata B1 analog are identical to the native form [45]. The study by Tam *et al.* did not report a direct comparison of native and synthetic kalata B1 but did compare the native and the synthetic forms of three other cyclotides (circulin A, circulin B and cyclopsychotride) and found them to be identical. Overall, the reasons for the differences in the two studies are not fully resolved.

Antimicrobial peptides that act by membrane targeting typically have an amphipathic structure, possess a large number of basic amino acids and have a preference for negatively charged membranes, which explains their targeting negatively charged bacterial membranes over neutral mammalian cell membranes. Cyclotides have an amphipathic structure with separate hydrophobic and hydrophilic faces, but their global charge is normally zero (e.g. kalata B1) or close to zero (e.g. kalata B2). In contrast to classical antimicrobial peptides, at physiological ionic strength, kalata B1 does not have a preference for negatively charged membranes [41]. Further work is required to define the antimicrobial activity of cyclotides and their clinical relevance, but given the widely recognized need for new classes of antibiotics to overcome the growing problem of resistance of micro-organisms to existing drugs and the unique structures and stabilities of cyclotides, their potential antimicrobial activities are certainly worth further investigation.

**TABLE 1**

**Antimicrobial activity measured for cycloti des under high- and low-salt conditions**[a]

| *Organism* | MIC[b] (μM) | | | | | | | | Activity[c] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Circulin A | | Circulin B | | Cyclopsychotride | | Kalata B1 | | Kalata B1 | | Kalata B2 | | Kalata B7 | |
| | Salt | No salt | Salt | No salt | Salt | No salt | Salt | No salt | Salt | No salt | Salt | No salt | Salt | No salt |
| **Gram-negative** | | | | | | | | | | | | | | |
| ***Escherichia coli*** | >500 | >500 | >500 | 0.41 | >500 | 1.55 | >500[d] | >500 | ✓ | ✓ | X | X | ✓ | ✓ |
| ***Pseudomonas aeruginosa*** | >500 | >500 | 48.0 | 25.5 | 50.2 | 13.5 | >500 | >500 | | | | | | |
| ***Proteus vulgaris*** | >500 | 54.6 | >500 | 6.80 | >500 | 13.2 | >500 | >500 | | | | | | |
| ***Klebsiella oxytoca*** | >500 | >500 | 15.6 | 8.20 | 13.2 | 5.80 | >500 | 54.8 | | | | | | |
| ***Haemophilus influenzae*** | | | | | | | | | X | X | X | X | X | X |
| **Gram-positive** | | | | | | | | | | | | | | |
| ***Staphylococcus aureus*** | >500 | 0.19 | >500 | 13.5 | >500 | 39.0 | >500 | 0.26 | X | X | X | X | X | X |
| ***Micrococcus luteus*** | >500 | >500 | >500 | >500 | >500 | 48.0 | >500 | 40.4 | | | | | | |
| **Fungi** | | | | | | | | | | | | | | |
| ***Candida kefyr*** | >500 | 18.6 | >500 | 29.0 | 48.0 | 14.0 | >500 | 21.4 | | | | | | |
| ***Candida tropicalis*** | >500 | 19.4 | >500 | >500 | >500 | 56.5 | >500 | >500 | | | | | | |
| ***Candida albicans*** | >500 | >500 | >500 | >500 | >500 | >500 | >500 | >500 | | | | | | |

[a] This table is a comparison of results obtained in two independent studies [43,44].

[b] Minimum inhibitory concentration (MIC) was determined after testing seven concentrations of peptide in radial diffusion assay with underlay gel containing 1% agarose and 10 mM phosphate buffer without salt (no salt) or with 100 mM NaCl (salt); 5 μl of each concentration were incubated at 37°C for 3 hours + 16–24 hours. The bacteria clear zones were measured and MICs were determined from the dose–response curves [43].

[c] Antibiotic effect on *E. coli* was tested after the addition of 10 μl of 5 mg/mL peptide stock solution to agar plates. Peptides were incubated at 37°C for three days. Antibacterial effects on *S. aureus* and on *H. influenzae* were evaluated by adding peptide directly to the growth medium; 2, 4, 8 or 16 mg/L peptide concentrations were incorporated and plates were inoculated with either *S. aureus* or *H. influenzae* and incubated at 37°C for one day [44].

[d] Gray shading highlights contradictory results between the two independent studies.

### *Antitumor activity*

Selective cytotoxic activity of several cyclotides against cancer cells compared with normal mammalian cells, with similar potency to clinically used anticancer drugs, has been reported over the past few years [46–48]. The cytotoxicity of the tested cyclotides was reported not only against different human cancer cell lines [46–48] but also against solid tumors from small-cell lung cancer, for which many established chemotherapeutic drugs are not very effective [46].

Although the mechanism has not been elucidated, the disturbance of membrane integrity seems to cause cytotoxicity, as supported by the ability of cycloviolacin O2 to disrupt tumor cell membranes [49]. The cell membrane properties of cancer cells differ from those of normal cells in several ways, including in fluidity, in surface area and in the global net charge owing to changes in the lipid and glycoprotein composition. Typically, cancer cells have a larger expression of anionic phosphatidylserine phospholipids and *O*-glycosylated mucins [50]. Such differences are known to contribute to the preferential targeting of peptides with anticancer properties [50]. As noted earlier in this article, several studies have shown that cyclotides bind to biological membranes, where the affinity for the membrane seems to be dependent on both the peptide and the membrane properties [38,41]. Differences in affinity might explain the observed differences in cytotoxicity toward cancer cell lines over normal human cells; nevertheless, this hypothesis awaits confirmation because a correlation between cyclotide affinity and the varying properties of cancer cells versus healthy cells has not yet been reported.

Cytotoxicity dose–response curves obtained in the presence of cyclotides have a very steep profile [25,47–49], as shown for cycloviolacin O2 against a human lymphoma cell line in Fig. 2. This steep profile is consistent with a mechanism that involves a concentration threshold and/or an aggregation phenomenon being required for inducing membrane disruption and cytotoxic effects, as has been observed for some other membrane-active peptides [51,52]. It is noteworthy that cytotoxicity is dependent not only on the native three-dimensional structure of cyclotides [25] but also on particular residues in their sequence [25,47,48]. Small differences in some residues seem to have a great impact on cytotoxicity, as exemplified by recent chemical modification studies for charged side chain residues in cycloviolacin O2 [25] (Fig. 2). Modification of the three positively charged amino acids only decreased the cytotoxic activity sevenfold, whereas Glu modification resulted in a 48-fold decrease [25] (Fig. 2). The affected Glu residue is highly conserved throughout the cyclotide family and was recently reported, on the basis of an Ala-scanning study, to be crucial for the insecticidal activity of kalata B1 [26]. This residue is believed to participate in a hydrogen bond network that stabilizes the native cyclotide fold [23,24], which has been demonstrated to be crucial for the bioactivity of the cyclotides.

As noted previously, NMR studies show that cyclotides bind to membranes through their hydrophobic patch [23,39,40]. Membrane-binding studies, together with cyclotide cytotoxicity studies, suggest that differences in membrane composition and cyclotide primary structure modulate the membrane affinity of cyclotides and their toxic effects. A comprehensive study on cyclotide-membrane-binding properties is currently being carried out in our laboratory and, we hope, will help to elucidate their

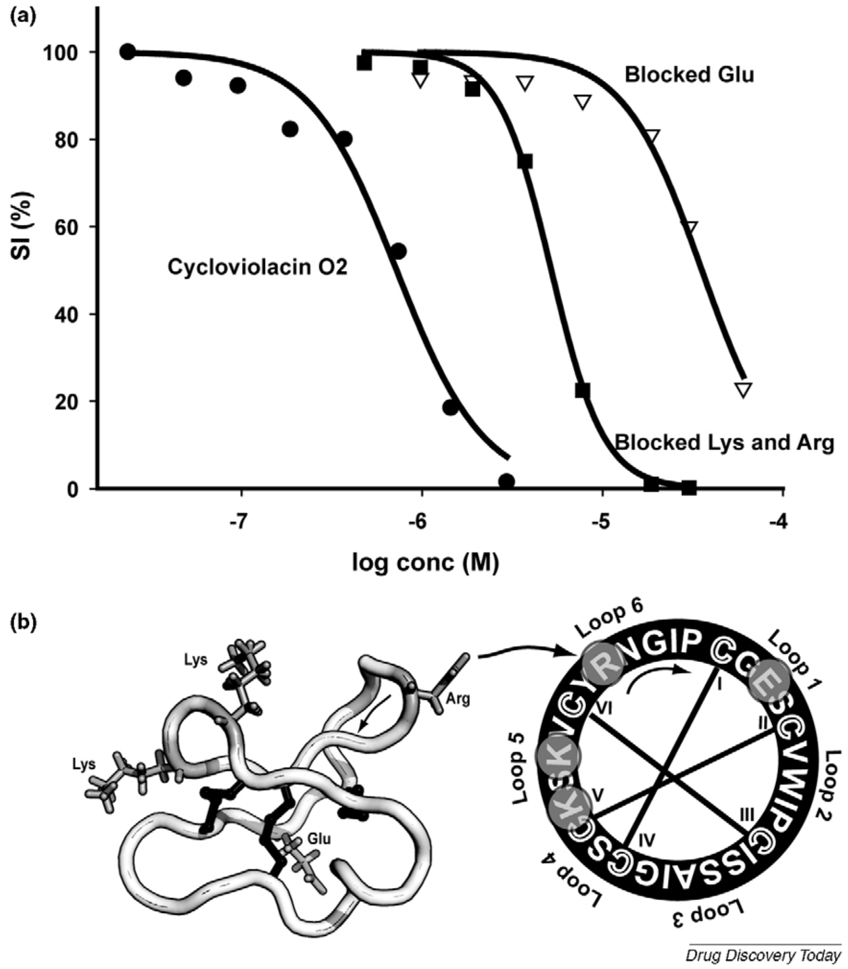

**FIGURE 2**

Cytotoxicity of cycloviolacin O2 and its analogs to the human lymphoma cell line U-937 GTB. **(a)** Concentration–response curves of native cycloviolacin O2 (black circles) and two chemically modified analogs. In the analog [Glu(Me)]cycloviolacin O2 (clear triangles), the Glu side chain was blocked by methylation; in the compound [Lys(Ac)$_2$Arg(CHD)]cycloviolacin O2 (black squares), the three positive charges were blocked, the Lys side chains were acetylated and the Arg residue was modified with cyclohexanodione (CHD). The activity is expressed as percentage of survival index (SI). **(b)** Structure and amino acid sequence of cycloviolacin O2 (PDB ID: 2kcg [68]). The disulfide connectivity is shown in black, the cysteine residues are labeled I–VI, and the six loops are identified. The side chains of Glu, Lys and Arg are shown in the structure to identify the location of the negative and positive amino acid residues. Figure prepared using data reported in Ref. [25].

mechanism of action and understand their different biological activities.

### *Neurotensin antagonism*

In 1994, the macrocyclic peptide cyclopsychotride A was isolated from the South American tree *Psychotria longipes* and reported to possess a neurotensin antagonist effect [13]. Neurotensin exerts its effects by interacting with specific membrane receptors, stimulating an increase in intracellular Ca$^{2+}$ levels. Witherup *et al.* [13] observed a reduced binding of neurotensin to the cell membrane in the presence of cyclopsychotride A. However, a direct neurotensin antagonist effect of cyclopsychotride A was contradicted by increased intracellular levels of Ca$^{2+}$ and failure of a known neurotensin antagonist to interfere with the effects of cyclopsychotride A. In addition, similar results were obtained from cell lines that do not express neurotensin receptors [13]. Thus, this report does not provide strong evidence for a direct neurotensin antagonism effect, but it does show that cyclopsychotride A can modulate membrane permeability to Ca$^{2+}$. Cyclotides have been reported to bind metals (e.g. Mn$^{2+}$) [40,53], and a direct binding of cyclopsychotride A to Ca$^{2+}$ could be a possible explanation for the reported findings. However, binding to metals other than Mn$^{2+}$ has not been reported so far. Furthermore, a recent Ala-scan study showed that metal binding was present even in inactive cyclotides, suggesting that a link between cyclotide activity and metal binding is tenuous [26]. It was shown recently in a study with kalata B1 that cyclotides modulate membrane permeability by the formation of a membrane pore with channel-like activity and without selectivity for specific cations [41]. The formation of such channels could explain the reported effect of cyclopsychotride A on intracellular Ca$^{2+}$ levels.

The reason for the interest in this area is that because neurotensin is known to have a role in neurotransmission and neuromodulation through its interaction with specific receptors, modulation of its effects by cyclotides could prove to be of valuable pharmaceutical potential; unfortunately, there has been no follow-up to the report on a neurotensin antagonist effect, so it seems that there is little current interest in pursuing the cyclotides as antineurotensin agents.

## Toxicity

Some cyclotides have been reported to have several toxic effects, not least of which is their insecticidal activity [2,3,54], as well as activity against a range of other organisms, including mollusks [55], barnacles [56] and nematodes [57–59]. Because none of these toxic activities are directed at mammals, they are not necessarily of concern for the development of cyclotides as human therapeutics, although the existence of any degree of toxicity needs to be taken into consideration for their further development. Where mammalian toxicity is concerned, the lethal dose ($LD_{50}$) of kalata B1 injected intravenously in rats and rabbits is reported to be 1.0 mg/kg and 1.2 mg/kg, respectively [60]. As far as we are aware, no quantitative toxicity data have been reported for other cyclotides. Cyclotides have also been reported to have hemolytic activity against rat and human erythrocytes, with $HD_{50}$ values in the range 5–300 μM [34,43,61]. The wide variation reflects differing incubation times and temperatures. Interestingly, the hemolytic activity of kalata B1 can be abolished via any one of approximately eight point mutations to alanine for residues located in a discrete surface patch of the molecule [26]. Similarly, some naturally occurring cyclotides have markedly reduced hemolytic activity compared with kalata B1; MCoTI-II is nonhemolytic, for example. Thus, it is important to note that toxicity data for one cyclotide cannot be extrapolated to others.

Cardiotoxic effects in rats, associated with ventricular tachycardia and increased arterial blood pressure, have been reported for kalata B1 [60]. Anecdotal reports from native medicine applications in Africa noted that there was a high incidence of emergency caesarean interventions because of cervical spasms during births associated with the uterotonic use of plant extracts containing kalata B1 [8]. Indeed, one of the early reports on the pharmacological characterization of kalata B1 recommended that *O. affinis* extracts not be used as a uterotonic agent because of these complications [60]. However, that hemolytic effects and insecticidal activity of kalata B1 can be removed by simple single amino acid substitutions [26] suggests that the other toxic effects, such as cardiotoxicity or spasms, might also be ameliorated, although this has not yet been tested. Overall, we believe that toxic effects of native kalata B1 are not necessarily an impediment for the further pharmaceutical development of synthetically modified (bioengineered) cyclotides.

## Bioengineering of cyclotides

In our view, the most promising application of cyclotides as therapeutics will probably derive not from the exploitation of the bioactivities of naturally occurring examples but from the use of cyclotide structures as peptide templates for the stabilization of bioactive peptide epitopes. The potential use of cyclotides as a scaffold for therapeutic purposes has emerged, not only because of their high resistance to thermal, enzymatic and chemical degradation [5] but also because of the rapidly increasing number of reported cyclotide sequences that have effectively demonstrated the plasticity of the cyclotide framework and its tolerance to substitution [62]. The tolerance to substitution of the cyclotide framework was first exemplified in a study in which proof of concept for the grafting of new sequences onto kalata B1 was established by replacing some hydrophobic residues in loop 5 of the sequence with charged and polar residues [21]. In this study, the stable native fold was maintained and the undesired hemolytic activity was reduced [21], but the most important finding was that cyclotides can be engineered to introduce foreign sequences into

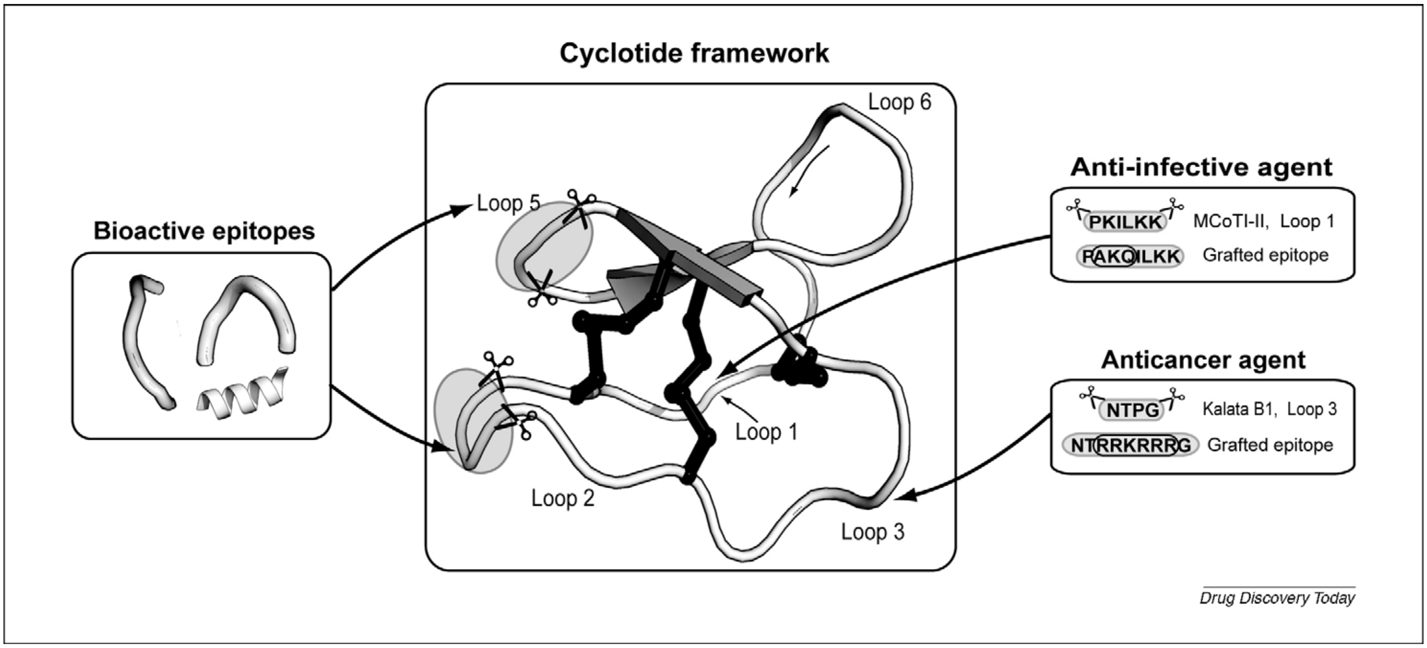

**FIGURE 3**

Schematic illustration of the principle of grafting bioactive epitopes onto the cyclotide framework. Here, we use the term 'epitope' loosely to refer to any bioactive peptide sequence, rather than just those that elicit an immunological response. The epitopes might be derived from larger proteins or might be isolated peptides. In recent studies, two bioactive epitopes were successfully grafted onto loop 3 of kalata B1 [63] and loop 1 of MCoTI-II [64], leading to the development of novel antiangiogenic and anti-infective agents. The location of these epitopes in the cyclotide scaffold is illustrated using the kalata B1 structure.

them and yet still fold into their characteristic cyclic cystine knot motif. Figure 3 highlights the principle of grafting foreign peptide epitopes onto a cyclotide framework.

Potential pharmaceutical applications of grafted cyclotides were demonstrated in two recent reports, one relating to the design of novel anticancer agents [63] and the second relating to the development of novel anti-infective agents [64]. The sequences and locations of the grafts are shown in Fig. 3. The anticancer application involved the grafting of a peptide epitope reported to have antiangiogenic properties onto the kalata B1 cyclotide framework [63]. Unregulated angiogenesis is associated with tumor growth and so antiangiogenesis agents have potential applications in cancer treatment. The selected epitope, an arginine-rich hexapeptide referred to as poly-Arg, was known to inhibit the interaction of the angiogenesis regulator, vascular endothelial growth factor A (VEGF-A), with its receptor. Like most small linear peptides, however, such a hexapeptide sequence is unlikely to be useful as a drug in its own right and would probably suffer from poor proteolytic stability and poor bioavailability. To overcome these limitations, the poly-Arg peptide sequence was individually grafted into loops 2, 3, 5 and 6 of the kalata B1 framework [63]. Loops 1 and 4 form part of the cystine knot and, therefore, were not modified because it was rationalized that alterations there might disrupt the stability of the cystine knot. The analog with the poly-Arg sequence grafted into loop 3 showed the highest activity in inhibiting VEGF-A receptor binding ($IC_{50}$ of 12 $\mu$M) when compared with the linear poly-Arg epitope and the other grafted analogs. Moreover, the hemolytic activity of native kalata B1 was absent in the grafted derivative, and the susceptibility of the poly-Arg sequence to proteolysis was reduced in the grafted analog compared with the isolated peptide sequence. Overall, this study demonstrated that a pharmaceutically relevant activity could be grafted onto a cyclotide and detected in a cell-based assay, where the activity of the grafted molecule is similar to that of the target epitope but is placed in a molecular context in which it is more stable.

It is important to point out that one of the potential problems with such grafting approaches is that the epitope might potentially be constrained to a nonactive conformation when placed in the cyclotide framework. In practice, because there are multiple possible grafting sites available in the various loops, it is usually possible to choose one that will mimic the native conformation of the epitope. Overall, we believe that this potential disadvantage is counterbalanced by other advantages of the grafting approach, including the synthetic flexibility of the cyclotide scaffold, the possibility of preparing libraries and the possibility of including non-natural amino acids or other elements in the design of bioactive molecules.

Members of the trypsin inhibitor subfamily of cyclotides, by definition, contain a trypsin inhibitor site. Interestingly, this site is in loop 1 of the structure and, thus, forms part of the embedded ring of the cystine knot [19]. Leatherbarrow and colleagues [64] recently showed that this active-site loop could be tailored in its specificity for proteases other than trypsin and, in particular, designed a modified MCoTI-II cyclotide to inhibit the 3C protease from the foot-and-mouth disease virus (FMDV). This protease is implicated in replication of the FMDV, and its inhibition thus represents one approach to inhibiting the virus. The synthetically modified cyclotide showed low micromolar inhibition of the protease [64]. This loop 1 engineering study effectively demonstrated the tolerance of the cyclotide framework even to modifications of a loop that is part of the cystine knot core. Moreover, MCoTI-II has been reported to be internalized into cells efficiently [65], enabling the potential use of this scaffold for intracellular targets.

Differential protease expression has been associated with many diseases, including inflammatory and pulmonary diseases, cancer and cardiovascular conditions. Therefore, proteases are recognized as primary drug targets [66]. Although only one example has been reported so far, re-engineering the activity of cyclotides belonging to the trypsin inhibitor subfamily to modulate their specificity for proteases other than trypsin has potential applications in drug development for protease targeting.

For the sake of balance, we note that the framework grafting approach is not necessarily the only solution to the problem of stabilizing peptide epitopes but can be used as an alternative or in parallel with other approaches, such as the introduction of non-natural amino acids, D-amino acids, PEGylation or encapsulation approaches [67].

## Concluding remarks

Cyclotides are still at an early stage of development as therapeutics, but there are promising indications that they might prove useful in the not-too-distant future. So far, several therapeutically relevant bioactivities have been reported for a range of naturally occurring cyclotides but, in all cases, the potencies are not sufficient to justify progression into human trials, and combined with residual toxicity concerns, native cyclotides have not yet reached the clinic. A much more promising approach seems to be the use of cyclotides as templates for protein-engineering applications to stabilize biologically active epitopes. It has been clearly established that the native hemolytic effects of cyclotides can be eliminated using simple point mutations, thus ameliorating concerns about toxicity for synthetic cyclotide frameworks. The success of grafting epitopes into the stable cyclotide framework has been demonstrated so far for potential anti-infective and anticancer agents.

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