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A BROAD REVIEW ON PLANT DERIVED BIOACTIVE PEPTIDES TO COMBAT PATHOGENIC OUTBREAKS A FUTURE PEPTIDE BASED THERAPEUTIC APPROACH[#]

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Abstract

Cyclotides are plant derived protein belongs to globular microproteins (≈ 25 to 30 amino residues long) class with a cyclized head-to-tail backbone and its structure was stabilized by the presence of cystine knot by three disulfide bonds. Cyclotides withstand to biochemical degradation and heat resistant compared to other types of peptides. In addition, conserved amino acids in the region of cystine knot showed strong tolerance towards genetic errors. Interesting, their topology makes them suitable to oral administration and cellular membrane transportation via crossing cell membranes. All these characters shown cyclotides has been strongly potent candidate for designing anti-viral plant derived peptides to combat newly emerged and reemerged outbreaks and also making it has a natural scaffold for drug development template. From the recent reports, cyclotides have been genetically engineered to study protein-peptide interactions for targeted drug delivery methods. In this paper we are undertaking review on discovery, history, role of cyclotides in Plants defense mechanism, tools to characterization of cyclotides, Membrane binding efficiency of cyclotides, antiviral property, cyclotide scaffold designing.

Keywords: Cyclotides, Plants defense mechanism, tools to characterization of cyclotides, Membrane binding efficiency, antiviral property, scaffold designing

[#]Review Article

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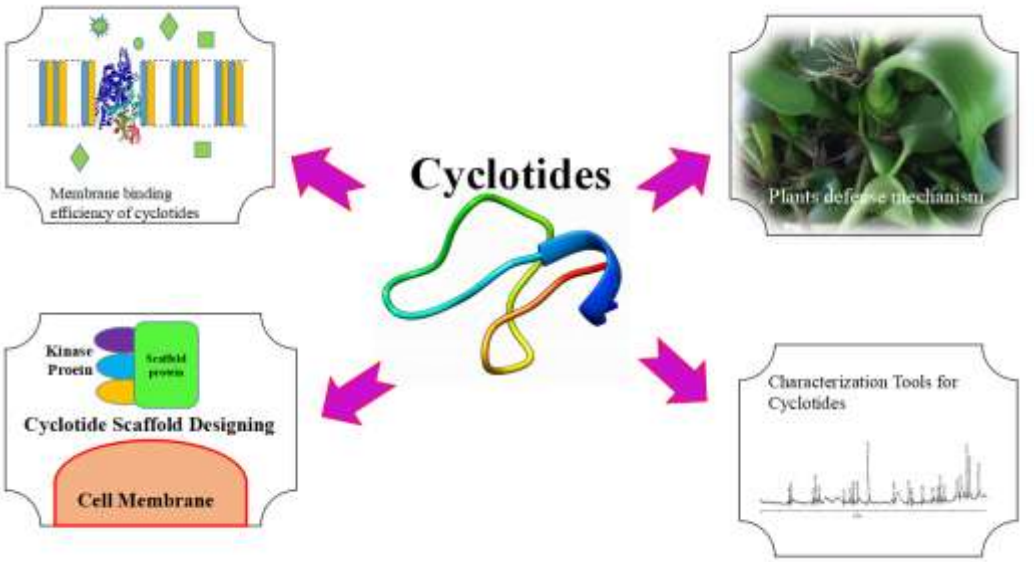
Introduction

Peptides have diverse biological activities such as enzymatic reactions, receptor interaction, membrane channel transportation, intracellular and extracellular transportation from one cell to neighbour cells. Cyclotides are protein-encoded cyclic micro peptide which range of about 25 to 30 amino residues in length that formed by following transcription, translation, isomerization of disuphide due to the presence of cystine amino residues, protein transportation, cleavage and cyclization with the help of Vacuolar processing enzyme (VPE). The VPEs are belongs to Cysteine protease family which involved in cyclization step by the mechanism called proteolysis. The cyclization step by VPEs of cyclotides depend on short read recognition sequence of cyclotide domain within or flanking region [1-4].



Reports from the past 20 years on Cyclotides discovered majorly in *Violaceae, Rubiaceae, Cucurbitaceae, Fabaceae, Solanaceae, and Apocynaceae*plant families. The nucleotide sequence found in cystine knot and cyclization domain site are highly conserved, but variations in nucleotide sequences vary in loop regions by single amino acids variations. The Cyclotide nucleotide sequence are not targeting the genomic architecture of host plant and due to this reason, standard proteomics and genomics methods are not suited to Cyclotides sequencing [5].

Cyclotides have six conserved cysteine residues which is a backbone of knot structure with three disulphide bonds formation [6]. These topologicalies making cyclotides towards attracted scaffold in molecular drug designing and protein engineering framework. It has been reported that monocots have no cyclotides and it lacks aspartate, asparagine residues which is a key amino residues for cyclotide cyclization mechanism [7,8]. The word

cysteine knot was derived to understand the structure of three-dimensional folding of cylotides. In native state, the disulphides bonds were formed between 6 cysteine residues with hydrophobic core and thereby forming a cystine knot [9]. Notably, cyclotides not undergo unfolding step from their native folding with the presence of disulphide bridges without undergoing cleaving or breaking of any one of disulphide covalent bonds.

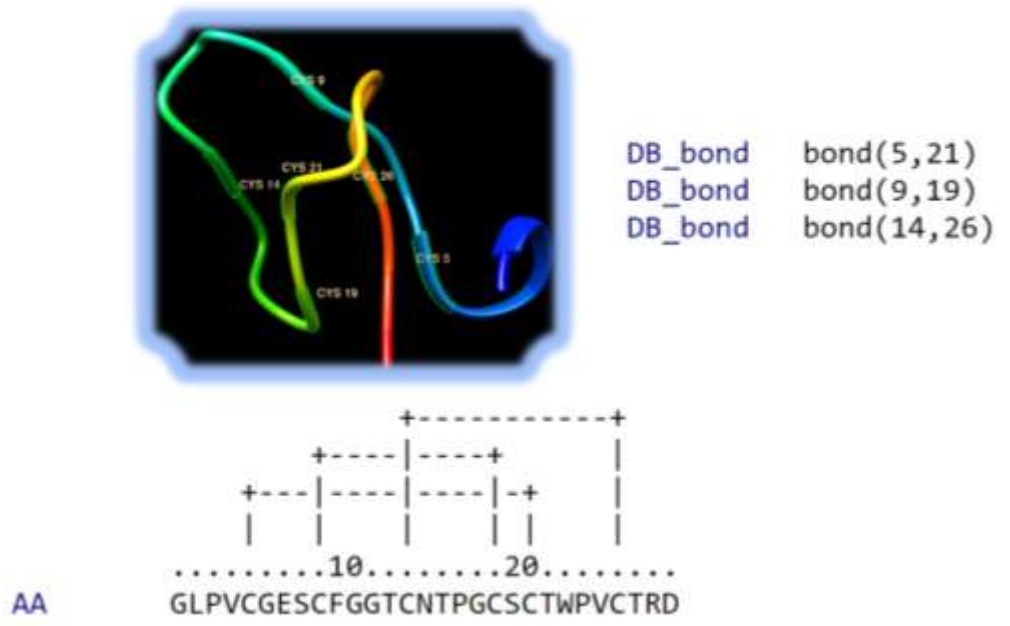


Fig. 1. Disulphide bridge analysis of Cyclotide psyleio D (*Psychotria brachyceras*) [38]

Cyclotides are shown similarities with three subfamilies such as bracelet, mobius and trypsin inhibitor. All subfamilies shared similar cysteine structure with slight changes in loop conformations and amino acids sequences. The bracelet and mobius have similar cyclotide folds with difference in loop 5 [10]. Surprisingly 2/3 rd of naturally occurring cyclotides in plants are belongs to bracelet category and 1/3 rd belongs to mobius and only very small cyclotides are with trypsin inhibitor. Cyclotides display various biological activities against pathogens in order to show defense mechansism of plants such as protease activity, anti-microbial, insecticidal, cytotoxic activity and anti-HIV activity [11]. Cyclotides are classified into 3 types based on their applications which includes natural cyclotides, engineered or grafted cyclotides and single amino mutated cyclotides. Despite of sequence all cyclotides share the similar cystine knot motif. To date many cyclotides were reported in *Rubiaceae, Vioalceae, Cucurbitaceae, Fabaceae and Solanaceae* plant families [12-16].

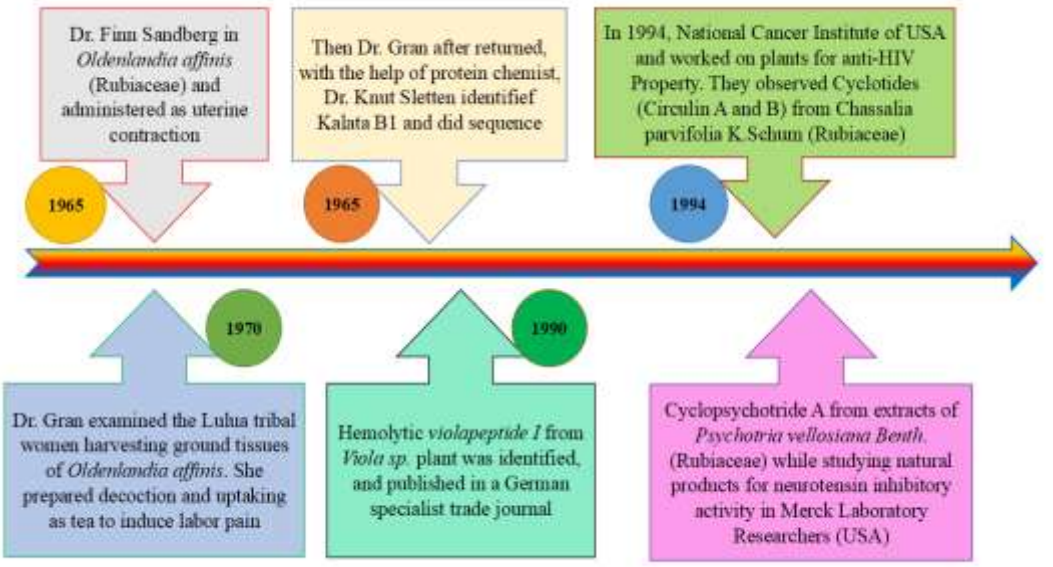


Fig. 2. The flow chart represents the overall initial history and discovery of Cyclotides

Role of Cyclotides In Plants Defense Mechanism

The exceptional characters of cyclotides primary function in host plants to function in plant defense. The defense activity such as anti-helmintic, antagonistic molluscicidal, anti-nematocidal and anti-microbial activity. Plants express wide range variety of cyclotides and currently more than 50,000 cyclotides have been reported in Violaceae and Rubiaceae plant families [17]. The 180° twist in backbone of cyclotides is used to distinguish cyclotides categories (Mobius and bracelet). The trypsin inhibitor cyclotides has dissimilar sequence than Mobius and bracelet [18]. Studies reported that primary function of cyclotides in plants to inhibit the microbial growth and insects [19]. It is still unclear why an single plant species expressing wide range of cyclotides. This unique pattern of expressing cyclotides might have produced for counteracting individual foreign molecules for plant host protection. Not only it involved in antagonistic activity but have reported as signaling cascades and modulators of immune response. The reported cyclotides have role in plant defense such as kalata (B1, B2, B5), Cycloviolacin O14, varv A & F (Mobius subfamily), Circulin (A&B), Cycloviolacin (O2, Y1), vhl-1 (Bracelet subfamily) and MCoT-II (Trypsin subfamily) [19-37]. The mechanism of action by which cylotides under disruption of cellular membrane. To date, cyclotide showed limited genetic diversity and it contrast with other cyclotides includes stability, expression level and hydrophobic nature allowed them as ideal scaffold candidates. The insecticidal activity of cyclotides were reported in 2001 where kalata B1 demonstrated Helicoverpa larvae growth inhibition and concluded as potent anti-insect growth inhibitor. The insecticidal mechanism involved in larvae mid gut membrane disruption when cyclotides ingested (found in plant leaves) and studied using electron microscope [38,39].

Pomacea canaliculata(Golden Apple snail) reported as one of the major rice pest in South East Asia where cyclotides tested for antagonistic activity. Cycloviolacin O1, kalata (B1 &B2) were reported potent toxic against golden apple snail than commercial molluscicide metaldehyde. The median lethal concentration of cyclotides (kalata B2) was 53 μM and metaldehyde133 μM [33]. The cyclotide kalata B2 showed low toxicity against *Oreochromis niloticus*(a control non-target fish in rice fields) when compared with rotenone (a commercial piscicidal compound). These studies suggested that cyclotides could be useful natural molluscicidal molecule.

Cyclotides kalata (B1, B2, B3, B5, B6 and B7) in-vitro studied against pest nematodes *Hemonchus contortus* and *Trichostrongylus colubriformis* egg, larvae and adult life viability. The results proved it has potent inhibitory action against larvae and adult worm motility activities and hence considered as to used for anti-helmintic molecule. Next study was carried out to study sequence effective in larvicial activity and mutation in alanine residue of kalata B1 reduced larvicial activity. The mutation analysis determined importance of alanine residue in cyclotide kalata B1 sequence [23,31,32].

Tools to Characterization of Cyclotides

The collected plant materials were extracted 50% acetonitrile (MeCN) and 1% formic acid (FA) for overnight at 4ºC. Then the extracted solution was proceeded to centrifugation and obtained supernatant was concentrated and lyophilized. The lyophilized sample was fractionated by solid phase extraction or liquid phase extraction. All eluted fractions were detected with MALDI-MS.Peptide-containing fractions were purified using RP-HPLC column.The peptides were isolated from plant materials and dissolved in 0.1 M NH_4HCO_3 buffer with pH of 8.0. The cyclotides cysteine amino acid rich short peptides and represented complex folding structure by forming disulphide bridges. For this complexity, cyclotides 3-D structure was reduced with DTT at optimum temperature for 20-30 minutes. Followed this alkylation was done with iodoacetamide for 30 min under nitrogen at room temperature. Then the prepared peptides were subjected to enzymatic denaturation overnight using trypsin, chymotrypsin, endoproteinase Glu-C, and a mixed trypsin and endoproteinase Glu-C at 37ºC and quenched with Formic acid , stored at 4 °C to 20 °C until further analysis [43].

MALDI−TOF and Peptide Sequencing

The reduced/alkylated sample was analysed in MALDI-TOF analyser. MS experiments were performed using α-cyano-hydroxyl-cinnamic acid matrix at a concentration of 5 mg mL^{-1} in 50 % (v/v) acetonitrile.The samples were mixed with matrix solution and spotted on target plate. Tandem mass spectra was obtained using
Peptidomic analysis laser energy of 1 kV with and without the use of collision-induced dissociation. Then using sequence fragment assembly the cyclotides were analyzed. The MS spectra was examined and sequences based on N-terminal b-ion and C-terminal y-ion series assignment [44].

Reverse Phase-High Performance Liquid Chromatography

The purification of cyclotides were done by RP-HPLC using semi-preparative and analytical Kromasil C_{18}columns (5 μm; 100 Å) with linear gradients of 0.1–1 % min−1 or

isocratic flow of 25–35 % buffer B (90 % acetonitrile in ddH2O, 0.08 % trifluoroacetic acid) at flow rates of 3 and 1 ml min^{-1}. Reduction was carried out and stopped after incubation of 30 min with the addition of concentrated trifluoroacetic acid. Immediately the samples were analysed in HPLC purification. Samples were aliquots in folding buffers (2.5–10 μM peptide, at final concentration of 2 mM reduced (GSH) and 0.1 mM oxidized (GSSG) glutathione) and freeze dried. The freeze dried aliquots were resolved with another folding buffers 25 and 75 % isopropanol and 35 % DMSO/5 % dodecyl-β-maltoside (DBM) in 0.1 M NH_4HCO_3 buffer at pH 8.2. For experimental control purpose with cycloviolacin O2final concentrations of 2 mM GSH and 2 mM cystamine in 35 % DMSO/5 % DBM buffer and GSH/cystamine (2/2 mM) in 0.1 M Tris–HCl buffer (pH 8.5) at 4 °C and 20 °C as a final folding condition. The aliquots were performed at different time intervals such as 15 min, 1hr and 24 hr with after 20 °C. Samples were quenched with concentrated trifluoroacetic acid and analyzed for RP-HPLC. The different folding aliquots were analyzed for peak detection and folding kinetic graphs, rate constant calculations, half time by one-phase association fit were prepared with the help of GraphPad Prism 5 software [44].

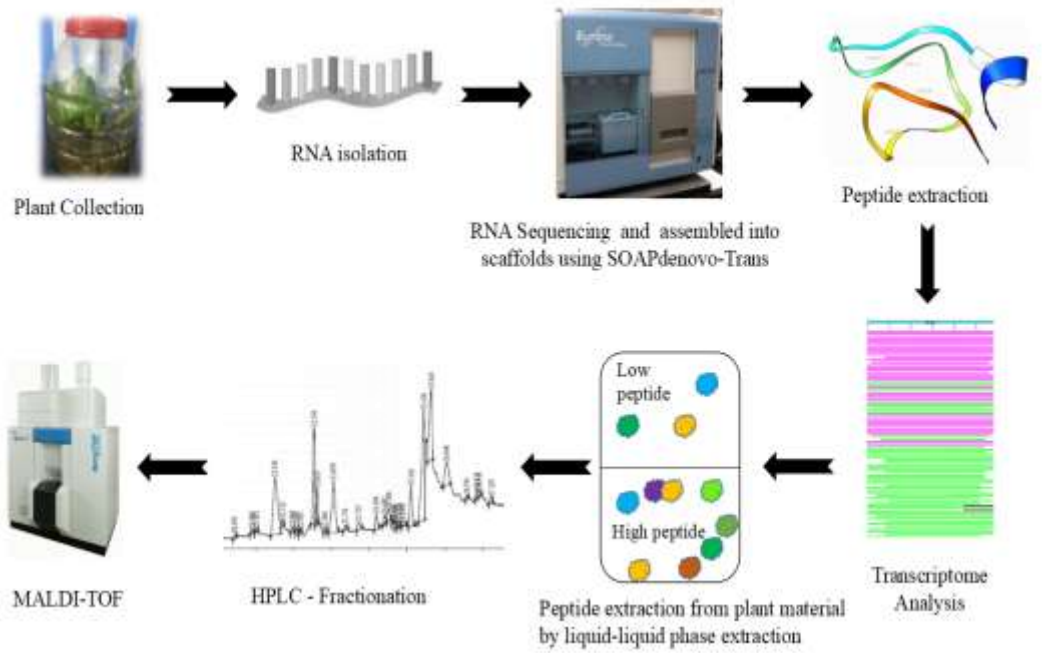


Fig. 4. Cyclotides isolation, extraction and characterization graphical chart [42]

Structural Analysis by Nuclear Magnetic Resonance (NMR)

Nuclear magnetic resonance spectroscopy is a compatible analytical technique for determining structures, molecules interactions and dynamics information for drug designing applications. Previously the NMR was used to characterize drug candidates in

pharmaceutical applications but recently they were also used for analyzing cyclotides. The purified cyclotides were dissolved in 90 % H_2O, D_2O at pH 6 to reach final concentrations of 0.4 mM. A Bruker ARX 500,600 and DMX 750 modelwas used for analysis at 288-328 K. Phase-sensitive recording mode was used for spectra recording with time-proportional phase incrementation [45]. 2-D experiments were obtained by TOCSY [46] along with 80 ms mixing time, NOESY [47] with 200 ms mixing time, DQF-COSY [48], E-COSY [49] in 100 % D_2O. TOCSY and NOESY experiments were undertaken by water suppression mode using modified WATERGATE sequence [50]. Lower power irradiation when relaxation delay used in DQF-COSY method [51].

Membrane Binding Efficiency of Cyclotides

Membrane binding affinity was fully depend on hydrophobic nature of peptides to enter into the cellular membrane. Remarkably cyclotides are used as scaffold in pharmaceutical drug design and have intrinsic bio-activities. Cyclotides belongs to Mobius and bracelt subfamilies are reported to have ability of cross membrane barriers due to the presence of lipid-binding domains on their surface. The lipid-binding domain allowed cyclotides and it is very crucial for cell penetration. In contrast trypsin inhibitor cyclotides lack these lipid-binding domains and their cellular entering mechanism is different [52]. The biological mechanism of action of cyclotides mostly involves membrane interaction followed by cell penetration [53-56]. Trypsin inhibitor cyclotides follow cell penetration and Mobius/bracelet follows cell penetration at low concentration and cellular disruption there by cellular leakage at high concentration.

One study reported that membrane specificity of cyclotides by surface plasmon resonance (SPR) method. The solution of cyclotides was injected over the surface of lipid layer and the dissociation rate was monitored. Control lipid mixtures were used to validate the dilineate calculation and lipid composition of cyclotides. Peptide-to-Lipid molar ratio was evaluated to understand the binding efficiency of cyclotides. The vesicle leakage created by cyclotides was checked and quantified by carboxyfluorescein (CF) dequenching [57].

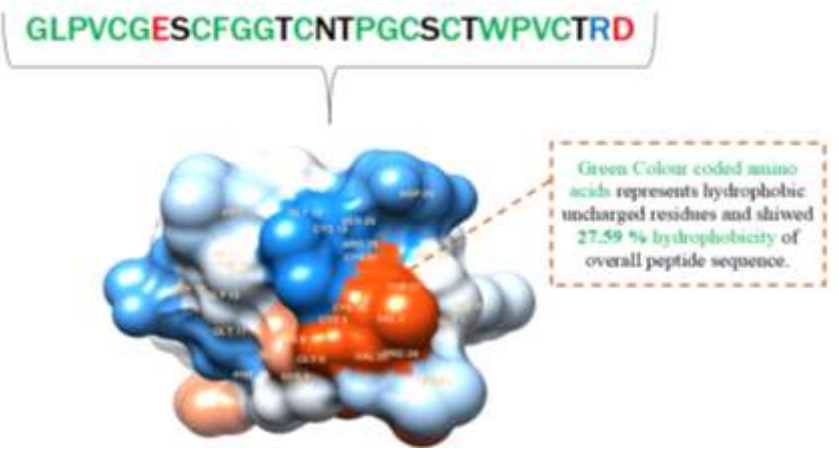


Fig. 3. Cyclotide psyleio D [*Psychotria brachyceras*] hydrophicity of peptide anaysis. Other analysis such as acidic (6.9 %), basic (3.45 %), Neutral (62.07%)

Anti-Viral (Cyclotides) Peptides

The native role of cyclotides in host plant is defense against pathogens and pests [58,59]. However, the discovery of cyclotide kalata B1 was observed by its uterine contractions during delivery of women when it is ingested of decoction made from *Oldenlandia affinis*[60]. This fact rise the attention that plant peptide could resist to denaturation even at boiling temperature that led to the investigation of CCK motif importance in cyclotides [61]. Extensively, cyclotides exhibited anti-HIV activity [62] and further analysis reported that kalata B1 and varv Emembers of the Möbius subfamilies tested against HIV inhibitory activities [63,64]. The cycloviolacins O13, O14 and O24 inhibited HIV-1 infection in human T-lymphoblast (CEM-SS) cells culture. *Circulin A, Circulin B, Circulin C, Circulin D, Circulin E, Circulin F, Cycloviolin A, Cycloviolin B, Cycloviolin C, Cycloviolin D, Cycloviolacin O13, Tricyclon A, Vhl-1, Cycloviolacin Y1, Cycloviolacin Y4, Cycloviolacin Y5* belongs to bracelet subfamily. *Kalata B1, Cycloviolacin O14, Cycloviolacin O24, Varv E* belongs to Mobius subfamily were anti-HIV reported cyclotides [65].In two studies, cyclotides were biologically grafted to develop anti-cancer and anti-viral properties [66, 67].

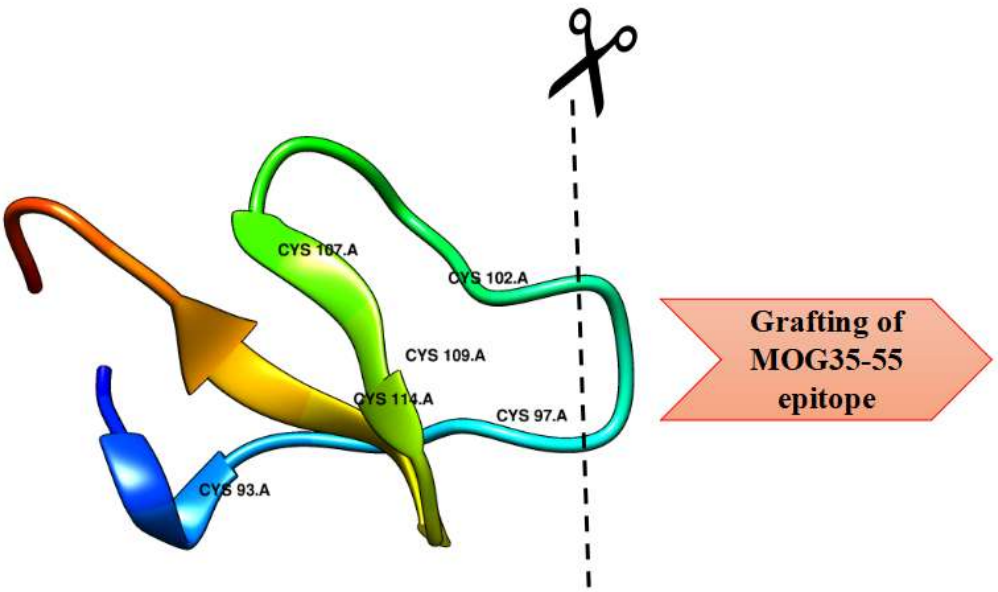


Fig. 4. The pharmacological capability of cyclotides with regards to multiple sclerosis has been additionally investigated by joining peptide groupings from the MOG35-55 epitope onto the cyclotide kalata B1 [77].

Cyclotide Scaffold Designing

The mechanism of action of Mobius kalata B1, involved in binding with cellular membrane by the presence of phosphatidylethanolamine phospholipids [68-70]. This action disrupting the membrane physical integrity and triggering pore formation on cell membrane along with significant cellular organelles leakage [71-74]. To date, Cyclotide MCoTI-I has

been designed for potent antagonistic activity against cytokine receptor [75]. Different mutations created on loop 1 and 6 of MCoTI-II for specific and susceptible against foot-and-mouth-disease (FMDV) 3C protease inhibitor [67]. All the more as of late, cyclotide MCoTI-II was likewise changed into a profoundly strong kallikrein-related peptidase 4 (KLK4) inhibitor (Ki ≈ 0.1 nM) that showed 100,000-overlay selectivity over related KLKs. This was practiced by joining a favored KLK4 cleavage grouping into circles 1 and 6 of cyclotide MCoTI-I [76].

Conclusion Remarks

This paper reviewed bioactivities of cyclotides, extraction strategies and characterization tools. It was found that hydrophobic nature of cyclotides plays a crucial role in membrane binding followed by bioactivity. Cyclotides belongs to Mobius and bracelet sub-families showed both hydrophobic and hydrophilic patches on their surface and resembling like amphipathic nature which can have anti-microbial properties. From the recent study the cyclotides mechanism of action is reported that cyclotides showed inability in inhibition of HIV reverse transcriptase enzymatic activity and suggested that virucidal activity happen before viral entry into the host cell. But however it is unclear whether cyclotides binding with cellular membrane of host cell or envelope of viruses or to the both. The unique characters of cyclotides making them as potent scaffold for novelpeptide based therapeutic purposes. As reported earlier, the CCK motif of cyclotides are exceptionallu high resistant and stable to biochemical degradations. In addition cyclotides also more tolerance to mutations and used them as a molecular framework peptide for grafting with bioactive short stretch amino acids to graft new bioactive peptides to combat outbreaks when no significant medicines available.

Although cyclotides have unique properties bu not yet reached any human clinical trial process and still reports have mentioned bioactivities in animal models. It is anticipated that future grafting of viral antigen determinant epitopes on cyclotides may compete with other small therapeutics peptides in order to develop a immune response in humans.

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