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Cyclotides as Potential Bioactive Cyclic Peptides: An Overview

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Abstract

Peptides are macromolecules composed of few amino acids starting from a dipeptide structure to a polypeptide chain. Selection, isolation, and characterization of peptides with bioactive potential is the need of the day to combat against rapidly growing deteriorating agents affecting major areas of food, health, and agriculture. Peptides are pharmacologically rich candidates as they can serve up as antagonists, agonists, or allosteric modulators against wide range of target classes. Based on size and structural complexity the cyclic peptides have been placed at a reasonable place in drug discovery & development field as they provide useful scaffolds to modulate protein-protein interactions and allosteric binding sites. A unique bioactive family of plant cyclic peptides called 'cyclotides' with its cyclic cysteine knot structure imparts it ultra-stability and different associated bioactivities such as immunosuppressive, cytotoxicity, vasorelaxant, anti-cancerous, antimicrobial etc. In present review discovery, sources, and mechanism of action of cyclotides is discussed along with research on candidate genes (PDI & AEP) involved in the synthesis of cyclotide variants and related features. To overcome the limitations of linear peptides in clinical applications, production strategies of cyclotides like expression in yeast cells, labelling of cyclotides through click chemistry and incorporation of non-natural amino acids is focused for their application in peptide-based drug development. Emphasis is laid on discovering distinct novel peptide structures like cyclotides with drug potential by investigating their structures based on evolutionary studies and their naturally existing activities.

Keywords: Cyclotides, Cytotoxicity, Drug development, Peptide structures, Cyclic peptides, Bioactivities

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1. Introduction

Plants are persistently exposed to a large group of organisms that are damaging, pathogenic in nature and their surviving conditions need quick defense replies from the plants by synthesizing agents like ROS, phenolics, phytoalexins, jasmonic, salicyclics, and sometimes antimicrobial peptides and proteins. Many peptides are produced by plants, many of which have main function as host defense agents these include defensins, thionins, protenase inhibitors and glycosidase inhibitors [1]. These defensive responses comprise novel candidates that could be engineered to develop disease related resistance in plants [2]. Antimicrobial peptides are need of the day to combat against rapidly growing resistant strains affecting adversely the major areas of food, health, and agriculture. This review is mainly focused on the development of different strategies for selection, isolation, and characterization of compounds with bioactive potential, like peptides. Emphasis is laid on discovering distinctive novel chemical structures with drug potential, and to

expose unknown targets, by investigating the structure based on evolutionary studies and optimizing their naturally existing activity [3] [4].

Major yield losses, losses in quality and safety of fresh and processed foods in agricultural production are due to fungal or microbial diseases of plants. Recently the control of fungal or microbial diseases relied mainly on chemical fungicides, however, public concerns about their side effects on human health and the environment, has motivated research to develop new antimicrobial agents that meet current safety and health standards [5] . Analysis in early 2013 has also revealed that almost 70% of the developmental pharmaceutical agents in clinical trials were of smaller size out of which more than 21% were antibodies i.e. may be proteins in nature. Apart from vast development in the field of antibodies and small molecules, peptide-based drug development is still limited and underestimated. Peptides are potentially rich pharmacological candidates as they can serve up as antagonists, agonists, or allosteric modulators across a wide range of target classes. They can also serve us as outstanding targeting agents to deliver grafted items and as transporters to create therapeutics that are bifunctional. Successful use of plant peptides in drug design and therapeutics is credited to several favorable properties i-e. they have large surface area which provide better selectivity and greater affinity for protein targets. The restrained conformational change/flexibility of their structure enhances the favorable binding properties and reduced entropy because of binding. Due to their amino acid arrangement cyclic peptides have very less or no toxicity. Third important property of cyclic peptides to be used as therapeutics includes simple modifications, easy handling, and characterization [6]. Limitations like poor membrane permeability & oral bioavailability, susceptibility to proteolytic degradation and little spans of circulating half-life are often hurdles considered for developing peptide therapeutics.

2.1. Cyclic peptides and cyclotides

A number of cyclic peptides with potential pharmacological applications have been isolated from seeds, latex and roots of various plants. Such peptides possess different bioactivities such as immunosuppressive, cytotoxicity, vasorelaxant, antimalarial, acetylcholine esterase, cyclooxygenase, and tyrosinase inhibitor [7]. Nature of the cyclic peptides (CPs) itself is the basic reason of designing them, there are different approaches for designing and modifying cyclic peptides depending on the purpose for which they are prepared [8]. Cyclization in macrocyclics can also bring better selectivity, potency and permeabilities especially when unique target profiles are considered. Moreover, leading optimizations over a broad range of cyclization chemistries are needed. Synthetically accessible, amenable to analoging and property-based strategies need to be focused [9]. Cyclotides, sunflower trypsin inhibitor like peptides, known as PaWS-derived peptide (PDPs) and orbitides are three main classes of ribosomally synthesized cyclic plant peptides. Orbitides are the cyclic peptides with no disulfide bond and approximately 6 amino acids, PDPs have only one disulfide bond and range in size to 14 amino acids. Third most important plant cyclic peptides are cyclotides with three disulfide bonds [1].

2.2. Types of Cyclic peptides

Because of continuously increasing number of peptides that are cyclic a brief categorization of cyclopeptides is described as discussed here.

2.2.1. Cyclic peptides are divided on the basis of linkages they have as discussed below;

- Homodetic peptides, cyclic structure containing only peptide linkages such as 2, 5- piperazinediones.
- Heterodetic peptides where cyclic structure contains both peptide bond and other linkages.

2.2.2. Cyclopeptides can be grouped into two types on the basis of number of amino acids from terrestrial plants.

- One is cyclopeptides, in which the number of amino acids is less than 14 and there is no presence of any type of disulfide bonds. These types of cyclopeptides are present in the plant families of the *Caryophyllaceae* and *Rhamnaceae*. Some cyclic petides have shown powerful antitumor activities, such as, dianthins E a cyclic hexopeptide in *Dianthus superbus*; cherimolacyclopeptide C, a cycloheptapeptide from *Annona cherimola* seeds; RA-XVII, a bicyclic

hexapeptide in *Rubiaceae*, longicalycinin A, a cyclic heptopeptidein *Dianthus superbus.*

- Another form of cyclopeptide is the recently discovered family of proteins, in which 28-37 amino acid residues are present. There is also the presence of disulfide bonds in these cyclotides. They have well-defined secondary structures and also show similarity to proteins due to the formation of compact three-dimensional folded structure [10].

2.2.3. Cyclic peptides are of two types based on their synthesis;

- Ribosomally and non-ribosomally synthesized peptides. The sirolimus, cyclosporine A (CsA) and tracrolimus are the examples of non-ribosomally synthesized peptides that play a significant role in interference of cytokine signaling. Ribosomally synthesized are gene-encoded, synthesized as precursors and post-translationally modified small peptides (RiPPs) also occur in different taxa.

2.3. Sources of cyclic peptides

Cyclic peptides can be obtained from different sources which include marine, plant, animals, microorganisms, and food sources. *Violaceae, Cucurbitaceae* and *Rubiaceae* families of plants are the richest sources of peptides. Therapeutics derived from marine sources comprises molecules which have antiviral, antiphrastic, antibiotic, anticancer and analgesic activity. Various anticancer compounds have been isolated from marine sources with diverse modes of action, such as anti-microtubule, anti-proliferative and antioxidant. A number of side effects like gastrointestinal pain, fatigue and depression of immune system are associated with traditional chemotherapeutic agents for which natural anticancer drug discovery is necessary. The reason behind the structural stability of these types of peptides indicate the presence of unusual amino acid residues. For the treatment and prevention of cancer the use of peptides that are obtained from marine sources are gaining importance. Marine cyanobacterial compounds show remarkable cytotoxic effects on tumor cell lines of human while the effects of some of these compounds will be at minimum range. For example, by the inhibition of cell cycle Apratoxin A, from *Lyngbya majuscula* possess cytotoxic effects on cultured Human HeLa cells of cervical carcinoma and it is a desipeptide in nature [11].

Cytotoxic or antitumor peptides comprise of about 3.5% of the total population of the marine plants. Due to the presence of some types of modifications such as D-amino acids, some new α-and β-amino acids, hydroxyl acid, as well as oxazole and thiophene in the structures of antitumor peptides the peptides from marine plants are normally stable and have an efficient bioavailability. *Nostoc* sp. GSV224 cyanobacterium only, a class of cyclic depsipeptides comprising 25 analogues have been isolated. Some strongly cytotoxic peptides have also been isolated from *Cyanobacterium Lyngbya majuscule* (legunamides, Homodolastatin, Wewakpeptins and Malevamide D) and *L. semiplena* (algae) [10].

Cyclopeptides in which the number of amino acid residues are less than 14 and disulfide bond is not found, are present in plants of the *Caryophyllaceae* and *Rhamnaceae* families. Some of those cyclopetides are reported to be strongly antitumor. A cycloheptapeptide named cherimolacyclopeptide from *Annona cherimola* seeds; a cyclic hexopeptide named dianthins E present in *Dianthus superbus*; another cyclic heptopeptide known as longicalycinin A isolated from *Dianthus superbus var. longicalycinus*; a bicyclic hexapeptide called RA-XVII (in rheumatoid arthritis) inhabitant of plants of *Rubiaceae* family and a family of cyclopentapeptides named astia isolated from the roots of a medicinal plant *Aster tataricus* are the examples of cyclopeptides.The other type of cyclopeptide involves the discovered cyclotides of viola, poaceae, fabaceae and many more plant families with unique structural features and bioactivities [12].

Peptides derived from microbial sources bind stably with its target sites and have tolerance to hydrolysis when exposed to proteases as compared to its linear precursors due to the absence of carboxyl and amino terminals. They also possess favorable degradability property under field conditions thus preventing accumulation of cyclic compounds to a potentially harmful level**,** which makes these peptides an attractive substitution for

use in agriculture as fungicides. Antimicrobial cyclic peptides are of different types based on bond types within the ring structures; homo or heterodetic and complex cyclic peptides, with diversity in their physicochemical features. Mostly cyclic antimicrobial peptides act by affecting the cell envelope's integrity through direct interaction or disturbance of the biosynthesis of components such as glucan, chitin, and sphingolipid. Cyclic antimicrobial peptides are effective against a range or broad spectrum of plant pathogens e.g., *Cochliobolus*, *Alternaria*, *Geotrichum*, *Botrytis*, *Penicillium*, *Fusarium* species, *Sclerotinia* and *M. grisea*) which might be due to similarities present in fungal envelopes, where these peptides are active. Such mechanisms of action are believed to reduce the chance of resistance that can be developed in microbial populations. These features are ideal merits of using cyclic peptides as antimicrobials or fungicides in the control of diseases affecting plants. Despite merits, there are issues to consider that are associated with the practical applications of mentioned cyclic antimicrobial peptides e.g. the effectiveness under certain physiological conditions, the selective toxicity to pathogen over their hosts, and the synergistic effects with other fungicides needs to be resolved. Hexapeptide PAF19 Ac-RKTWFW-NH_2 obtained through screening of combinatorial library of a synthetic peptide showed anti-fungal activity that cause postharvest damage to fruits, results were similar to that of the hemolytic 26-amino acid melittin that was not actually toxic for bacteria or yeast [13]. Several peptide fragments after digestion by proteolytic enzymes in the gut possess antiviral properties giving food proteins an advantage over synthetic pharmacologic compounds as being non-toxic and well accepted by the consumers, future therapeutics [14]. The antitumor effect of these peptides causes interruption of cell microtubular activity, induction of cell apoptosis, cell cycle blockade, anti-multi drug resistance follows mechanisms like Interruption in cellular microtubules processes can stop the process of division during cell mitosis, due to which the prolongation of the cell cycle occurs. Scytonema pseudohofmanni, is a microtubule dissociation-inducing compound. *In vitro*, it also has various types of inhibitory effects on murine leukemia, human KB cells and lung cancer cells. A new method of cancer therapy is the induction of programmed death (apoptosis) for tumor cell. A group of cyclopeptides called astis can perform its activity on KB cells *in vitro* and *in vivo* in mice on lymphocytic leukemia [10].

Depsipeptide (FR901228, NSC 630176). NSC630167 is an inhibitor of histone deacetylase and it causes the cell cycle to arrest in the G_0/G_1 phase by suppressing the expression of oncogene c-myc mRNA during the study in some human solid-tumor cell lines *in vitro*. The over expression of gene encoded P-glycoprotein is responsible for the synthesis of drug resistance via tumors. It reduces the toxicity of drug by changing the configuration and mechanism of action. Cryptophycin-1 is the effective peptide that play role in this regard [10]. Based on their applications in drug targeting Asn-Gly-Arg (NGR) peptides are of high interest due to recognition of CD13 receptors. Structural analysis of thioether bond-linked novel cyclic NGR peptides has shown that a proto-epitope containing S, R and F amino acids can regulate negatively TNF secretion via macrophages. The interaction between VEGF (vascular endothelial growth factor) and VEGFRs (receptors of VEGF) that is actually inhibition is responsible for anti-cancer treatment. Blockage of the kinase activity on VEGFR indirectly with inhibitors of protein-protein interactions showed great interests in studying oncology. Recently a recombinant humanized monoclonal antibody, approved agent ramucirumab (Cyramza) that specifically binds to VEGFR2, another anti-VEGFR strategy and reported to have greatly increased VEGFR1 binding affinity by using the ELISA-based test [15].

2.4. Cyclic peptides as stable bioactive compounds

Cyclization in peptides eliminates charged termini, which can favor both membrane permeability and metabolic stability. Cyclization of the peptide molecule leads to the development of several medicinal compounds with potent biological activities. It is also thought to reduce conformational entropy losses when it binds target, although some studies have shown the positive impact of cyclization on this entropy [16]. Cyclic peptides are formed into a ring like structure due to the presence of amide ester or disulfide bonds. Because there is no exposure of CN-terminal groups to exopeptides, that is why cyclic peptides are stable enough to degradation by enzymes [17]. Most of the cyclic peptides isolated from natural

sources like plants and marine sources but as only minute quantities are obtained from these sources, attempts have been made towards the synthesis of these cyclic peptides and their derivatives by various methods [18].

2.5. Cyclotides

Besides a lot of success stories related to peptides and specially to cyclic peptides still unique structural configurations and bioactivities are hidden. A group of highly stable and unique class of bioactive cyclic peptides named cyclotides is most versatile of all other peptides and is currently the target of researchers for peptide-based drug targeting and drug development. A lot of prominent properties of cyclotides include their anti-HIV, cytotoxicity, insecticidal, molluscicidal or anthelmintic, uterotonic, neurotensin inhibitory activity, anti-microbial, anti-tumor, antifouling, hemolytic and trypsin inhibition [18]. Small disulfide-rich, cyclotide bioactive compounds are prominent as protease inhibitors, toxins, hormones, and growth factors. Many of these peptides contain a three-disulfide knotted structure formed by two disulfide bonds making a cystine-knot (CK) motif, together with the connecting backbones and forming an embedded ring through which the third bond penetrates. Of particular interest in drug development is the knottin family (CK peptides) contain 25–45 residues, and often possess protease inhibitory activities, from which the name was derived. Knottins also form compact and well-defined structures with extensive internal hydrogen bonding, endowing them with resistance to damage by proteolytic endopeptidases and denaturation by chemicals or heat, as shown by extensive studies, including those using sequencing for determination of their primary structures. Certain CK peptides of the knottin family have further evolved as macrocyclics such as cyclotides, harboring cyclic CKs (CCKs) with no termini, a feature that has made them resistant to exopeptidases [19]. Cyclotides are a family of head-to-tail cyclized small proteins with quite a remarkable structure. They consist of 28-37 amino acids and are characterized by a cyclic cystine knot (CCK) formed by the interactions of 6 cysteine residues. These cysteine residues are oxidized to form a cystine knot core, in which an embedded ring, formed by two disulfide bonds (Cys1-Cys4 and Cys2-Cys5), is threaded by the 3rd disulfide bond (Cys3-Cys6) (Fig 4). That knot, together with the head-to-tail cyclic amide backbone defines the cyclic cysteine knot motif [20]. This CCK motif is shared by all cyclotides and gives an exceptional rigid and stable structure, which makes it extraordinarily resistant towards physical, chemical, and enzymatic degradation. Since the molecular core of this tightly bound knot is occupied by disulfide bonds, the propensity for amino acid side chains that are not associated with the cystine knot protrudes outwards, where they constitute the molecular surface. These amino acids when solvent exposed include a number of residues that form patches of hydrophobic nature in an otherwise hydrophilic surface, making them soluble in both organic and aqueous solvents. This surface-exposed patch of hydrophobic nature contributes to some of the major biophysical properties of the cyclotide, including their late elution in HPLC runs [21]. The amino acids not allied with the cystine knot, are arranged in six intercysteine loops, which are successively numbered 1-6, starting at cysteine 1 (Fig 1, adapted with permission from American Chemical Society). Cyclotides exhibit extensive variations in the size and composition of their loops between the cysteine residues. Loop 1 is the least variable, containing typically three amino acids including a glutamic acid in the middle of the loop. Loop 4 is similarly highly conserved, always comprising just a single residue and almost invariably containing a single hydroxyl bearing residue (serine, threonine or lysine). These two loops constitute segments the backbone that together with their connecting disulfide bonds form the embedded ring that is threaded by the third disulfide bond. The high degree of conservation in these two loops quite possibly reflects the fact that they form a central core of cystine knot CK. By contrast there is much more variation in both size and sequence of the other loops. The most highly conserved element within the loops is an asparagine, or occasionally an aspartic acid residue in loop 6 which seems to play a functional role in the cyclization. Cyclotides feature the regular elements of secondary structure typically seen in larger proteins. Generally, cyclotides are formed by a cystine knot reinforced β-hairpin, comprising loop 4, 5 and part of loop 6 [22]. This β-hairpin is connected with a third more disordered β-strand that is constituted of parts of both loops 1 and 6. Together these structural elements make up a distorted β-sheet. The

secondary structure is stabilized by extensive hydrogen bonding that is like the fold itself, is highly conserved and particularly so within different cyclotide subfamilies.

The structure of tricyclon A describes that the presence of a loop that is disordered in other cyclotides forms a protruding sheet out of the globular core. It was found that the cyclotide folding was amenable to an extent that a range of structural elements can be added to it without affecting the CCK. Tricyclon A, unlike other cyclotides, deprives a hydrophobic patch and has least hemolytic power that makes it attractive from pharmaceutical point of view. A 22 kDa protein provided clues of being processed [23].

2.5.1. The Möbius, bracelet and trypsin inhibitor cyclotides

Cyclotides are divided into three subfamilies. The two large subfamilies Möbius and bracelet, and a minor subfamily of trypsin inhibitors. The bracelet and Mobius subfamilies are distinguished by the presence (Mobius) or absence (bracelet) of a cis-Pro peptide bond in loop 5 [24]. Although the presence of this cis-Pro amino acid residue is the defining feature of the two subfamilies, there also tend to be high sequence similarities for several loops within subfamilies [25]. Furthermore, the two subfamilies also differ in terms of distribution of surface-exposed hydrophobic amino acid residues and their overall net charge, with one of the main differences being the presence of a group of charged residues in loop 5 of bracelets [24]. In addition to the general β-sheet, members of the bracelet family also feature a short α-helical segment in loop 3. Recently the division into these subfamilies has been blurred with the emergence of chimeric cyclotides which displays properties of both Möbius and bracelet families. Trypsin inhibitor cyclotides are the smallest of the three subfamilies. They are classified as cyclotides on the basis that they are cyclic and contain a CCK motif and are otherwise quite distinct from the Möbius and bracelet subfamilies, both in terms of structure and sequence homology. In addition to the CCK motif, the two members of this subfamily are dominated by a series of well-defined turns, a turn of helix and a small beta-hairpin. As their name implies, they are potent trypsin inhibitors [25]. In between subfamilies, loop numbers 2, 3, 5, and 6 show extensive sequence variations in their composition and size. The variation in the residues in these loops, superimposed on an otherwise highly conserved framework, makes cyclotides an excellent template to examine structure-activity relationships [21].

2.6. Sources of cyclotides

Cyclotides have been reported from almost all kingdoms including bacteria and cyanobacteria. Cyclotides have been found from different plant families like Cucurbitaceae, Violaceae, Fabaceae, asteraceae and apocynaceae. The plant cyclotides are found in different plant parts like nodules, seeds, pods, shoots, flowers, leaves, roots and stems, with individual plants expressing a variety of cyclotides [26], [27]. Cyclotides possess insecticidal, molluscicidal or anthelmintic activities, uterotonic, anti-HIV, cytotoxic, neurotensin inhibitory activity, anti-microbial, anti-tumor, antifouling, hemolytic and trypsin inhibition [28], [26], [29] and still much more to be known in future regarding drug designing. Recently cyclotide analogues and variants were also tested for inhibition against dengue being specific and competitive [30]. Studies shows that distributed hydrophobic and hydrophilic patches of amino acids in cyclotides are one of the reasons of its antimicrobial or insecticidal properties causing membrane disruptions [26].

2.7. Cyclotide isolations

A few potential cyclotides each having different source and mechanism of action or synthesis is summarized in sub sections below.

2.7.1. Kalata B1

The cyclotides are investigated to be involved in disruption of biological membranes in herbivorous pests, presenting cyclotides for its potential in various applications as pesticidal molecules [31]. By examining the gut of the Lepidopteran species *Helicoverpa armigera* larvae of cyclotide Kalata B1 ingested through scanning, light, and transmission electron microscopy it was found to induce swelling, blebbing, ultimately rupture cells of the epithelia and disruption of microvilli. This histological reaction was

comparable to that observed in *H. armigera* larvae to delta-endotoxin produced by *Bacillus thuringiensis* that controlled cotton crops pests [32]. Similar findings were reported by Jennings and his co-workers (2001) [33] about the isolation of knotted cyclotides from Rubiaceae and Violaceae families focusing on machainsm of synthesis of kalata B1 from *Oldenlandia affinis* (the African plant).

2.7.2. Labaditin

Cyclotide labaditin and its synthetic open chain analogs have the phenomenon of great membrane insertion. The mechanism of this is based on the initial interactions of hydrophobic nature with the lipid membrane followed by change in confirmation, peptide adsorption of peptide and internalization hence, native labaditin reduced the viability in Gram +ve bacteria [11].

2.7.3. Cycloviolacin

Potentially stable (Chemically and biologically) cyclotides like varv A, varv F, and cycloviolacin O2 from *Viola arvensis* and *Viola odorata* L., besides being anti-microbial were investigated for cytotoxicity against tumor cell lines having defined type of cytotoxic drugs resistance and this activity was dose dependent when compared with normal lymphocytes. This reflected cyclotides as potential pharmacological agent with a unique mode of action [34]. *Viola tricolor* extracts (hydroalcoholic, ethyl acetate (EtOAc). *Viola tricolor* is also a family which is repository of cyclotides that also has the same properties [35]. Eight known & eight new cyclotides (Viphi A–H) were isolated from *Viola philippica*, a member of traditional Chinese medicines and family Violaceae. In addition, Mram 8 and Viba 17 were reported as mature peptides for the first time. Sequences were elucidated by reduction, enzymatic digestion and tandem mass spectroscopy sequencing. Cytotoxic activities were shown against the cancer cell lines MM96L, BGC-823 and HeLa [36]. Sixteen peptides from extracts of *O. affinis, Viola hederaceae,* and *V. odorata* were studied for the 3-D structure of a novel peptide, cycloviolacin O1, using NMR based spectroscopy [37]. The structure elucidated by these studies showed a distorted triple-stranded cystine-knot arrangement of disulphide (S-S) bonds and ß-sheet. This structure is like kalata B1 and circulin A and available structure suggested that sequence variation exist all the way through the peptide family although folding pattern seems to be conserved. Sequence examination revealed that two subfamilies of cyclotides exist, one with a circularization of the backbone like bracelet containing a number of residues positively charged and second Mobius that may be taken as a Mobius strip shaped with a backbone twisting seen due to the presence of *cis*-Pro peptide bond which was absent in case of bracelet.

2.7.4. Cyclotides from Oldenlandia affinis

Although cyclotides are believed to be one of the largest family of naturally occurring cyclic peptides yet cyclotide-encoding cDNAs when isolated from *Viola odorata* and compared with evolutionarily distinct plant *Oldenlandia affinis* showed that they possess one to three cyclotide domains preceded by highly conserved NTRs (N-terminal repeat regions) within species and differ between different species. Besides having no homology at peptide sequence level still they shared structurally conserved helical motif reflecting their possible role in processing, folding and detoxifying cyclotide domains from their precursor proteins [38]. The discovery of Kalata B8 from *O. affinis* reported that isomerization at the site of Asp-Gly sequence which was involved in cyclization and it showed flexibility in the core CCK. This finding was previously found in an unrelated knottins (cyclic) from *Momordica cochinchinensi*. Kalata B8 a hybrid between Mobius and bracelet classes showed structure activity relationship also as it showed anti-HIV activity with no hemolytic activity due to unusual hydrophilic nature [39]. Moreover, kalata B8 also shows disordered loop 6 and isomerization in its cyclic peptide backbone which is similar to what was observed in MCoTI-II that is a completely different family of cyclotides named trypsin inhibitor cyclotides.

2.7.5. Chassatides and panitides

Nguyen et al [40] characterized nine novel linear cyclotides from monocot plant *Panicum laxum* giving an evidence of their existence at the protein level in the Poaceae family, also an evidence of existence of ancient linear analogs that

could have been existed before the divergence of dicots and monocots. Similarly, also discovered four more uncyclotides and 14 novel cyclotides from *Chassalia chartacea*. Precursors of these cyclotides were shortest of all known cyclotides at that time. Originally members of this family like *Chassalia kolly* (Schumach.) and Hepper (family Rubiaceae) are medicinally important plants used in nigeria and west African countries for the treatment of diseases like typhoid and as insect repellent. Antifungal & antibacterial activities were observed against *Candida albicans, Escherica coli, Salmonella typhii, Pseudomonas* aeruginosa, *Staphylococcus aureus, Bacillus subtilis*.

2.7.6. Cliotides

Many bioactive peptides including stable and versatile cyclotides have been isolated from *Clitoria ternatea*. The cyclotides concentration from *C. ternatea* is usually somewhat higher than that regarding nearly all cyclotide containing plants from the Violacecae and Rubiaceae families [41]. Abundance of cyclotides (named Cliotides in *Clitoria*) have been produced from this medicinal plant. The enzyme named butelase 1 was isolated recently from *Clitoria ternatea* which is liable for the biosynthesis and cyclization of backbone of cliotides. Bunga Telang Ligase is the local name commonly used in Singapore. This enzyme has extraordinary efficiency of cyclization of peptides of plant and animal origin and the yield is greater than 95%. K_{cat} value for Butelase 1 is up to 17 s^{-1} and its catalytic efficiency is 542,000 M^{-1} s^{-1}, therefore, it is known as the fastest peptide ligase. Butelase 1 is the first asparagine/aspartate (Asx) peptide ligase. The sequence similarity between Butelase 1 and legumain proteases is 71%. The only difference is that Butelase 1 does not hydrolyze the legumain protease substrate. As butelase 1 mostly targets the NH_2 terminal amino acids of the peptide substrate, so Carboxy terminus specific intermolecular peptide ligations can be checked with the help of it [19]. A lectin found in the seeds of *Clitoria ternatea* was reported its lectin is useful in cancer studies and efforts have been made to get high yield of *Clitoria ternatea* lectin (CTL) [41].

2.8. Genetics of cyclotides

More than 200 cyclotide genes have so far been discovered and expected by some scientists to have more than 50,000 members as genes in only plants. Perhaps believed to be one of the largest genes encoding plant peptides. The cyclotide genes encode linear 11–14 kDa precursor proteins, that contains 1-3 domains of mature cyclotides, having a pro-domain, endoplasmic reticulum (ER) signal sequence and a region of hydrophobic amino acid residues at C-terminal [26]. Some genes encode only a few linear variants that occur naturally are known as uncyclotide. Cyclotide variants with shortest transcripts from petunia of Solanaceae [29] and linear variants panitides are reported from *panicum* of family poaceae [42]. Cyclotide genes are known to be differentially expressed either in inducible or constitutive forms in different parts [29]. A dozen gene sequences exists as hybrid sequences between two subfamilies mobius and bracelet [40]. studies by [42] some genes of the cyclotides like *Clitoria* of Fabaceae family encodes for precursors are chimeras, cyclotide domain and A1b domain (albumin-1). These genes might be emerged from either horizontal gene transfer or convergent evolution in plant genomes.

2.9. Candidates for enzymatic synthesis of cyclotides in plants

Cyclotides cyclization occurs as a post-translational modification although poorly understood. Extensive studies have recognized two major enzymes named AEP & PDI (asparaginyl endopeptidases & protein disulfide isomerases) that are believed to be involved in the processing of cyclotide, from cyclization towards folding events [43,44]. Vacuolar processing enzyme VPEs or AEP are present in large plant vacuole which is present in vegetative parts whose major role is to degrade or immobilize protein by using different proteases and to recycle nutrients. This enzyme is also involved in the conversions of proproteins to their respective mature functional/structural forms [45]. AEP, a ubiquitous enzyme in plants, involves in normal role in catabolism of protein substrates after Asn residues hijacked by cyclotide precursors utilize them for the formation of peptide bond also. These VPE / AEPs can also serve us as a tool to regulate or alter different plant growth stages along with maturation of different plant peptides and proteins a genetically encoded enzyme is reported from potato, sweet potato asparaginyl

endopeptidases (SPAE), transformed in model plant *Arabidopsis* through *Agrobacterium* mediated transformation. AEP is responsible for catalyzing a single processing event both peptide bond cleavage and ligation of cyclotides. Evidence of such catalysis by AEP was seen in *Niotiana benthamiana* (not producer of cyclic peptides) when transformed by KB1 cDNA produced mature cyclotide and no linear forms were present [43]. Such methods can be useful in future by making transgenics to manipulate plants to produce peptides like cyclotides where these are not naturally present [45].

Protein disulphide isomerase (PDI) is an oxidoreductase enzyme produced in higher amounts in the endoplasmic reticulum. Proteins like prolamins that are >90% of the wheat grain protein content are dependent on maturation for this type of enzyme [46]. Interestingly PDI enzymes enhanced the proper folding yield of cyclotide-like molecules, including a linear cyclic molecule and a reduced cyclotide [47] and mopping up some misfolded cyclotide proteins and refold them appropriately [26]. It is speculated that the NTRs are involved in the proper folding of precursor protein before cleavage and cyclization. Experiments explained the mechanism of enzyme dependent folding of plant cyclotides by comparing the absence and presence of folding of cyclotide kalata B1 derivatives in *O. affinis* PDI (OaPDI) which improved the correct oxidative folding of cyclotide at physiological pH. S-S isomerization is one of the key roles of plant PDI to produce insecticidal cyclotides. The *in vivo* relevance of this mechanism still remains to be established. Presumably the PDI interacts usually with the precursor protein, rather than the processed cyclotide domains [26].

2.10. Cyclotides bioactive action and Mechanisms

Cyclotides proved to be versatile in their bioactivities besides being stable drug delivery tool. These peptides behave as excellent antimicrobial agents, anti helminthetic, hormone like behavior, regulate autoimmune responses, anti-HIV, ant cancerous, nematocidal and many more.

2.10.1. Regulation of autoimmune responses

The immunological system is very crucial for detecting threats to the body and malfunctioning of which down-regulate the homeostasis, which may lead to autoimmunity, frequently linked with malfunctioning T lymphocyte cells signaling. Therefore, several medications target to suppress T lymphocytes that become over-reactive but many of them have life-threatening and severe side-effects. Peptides that are ribosomally synthesized are gaining attention due to decreased toxicity effects and enhanced selectivities as compared to small molecules in pharmaceutical industry where particularly circular peptides are unique in these properties with better oral administration. Plant cyclotides experimentally proved effective option to inhibit T lymphocyte cells proliferation with a cystine-knot motif, which confers them highly stable small molecules, thus becoming an attractive pharmaceutical tool [48].

2.10.2. Role in signal transduction like plant hormones

Plant peptides or polypeptides also exist as members of plant signal transduction mechanism in contrary to the concept of classical phytohormones like cytokinins, gibberellins etc. Peptides involved in cell proliferation, wound signal transduction and in the regulation of water / salt homeostasis, i.e. enod40, systemins, phytosulfokines and natriuretic peptides, but still much more are likely to be discovered. In a research study CVX15 based peptides were grafted on loop 6 of MCOTI, a novel peptide that was stable in human serum and inhibited the viral replication of HIV-1 by targeting cytokine receptor CXCR4. An active CXCR4 antagonistic and HIV-1-cell-entry blocker were produced in this study. CXCR4 a chemokine (GPCRs protein) as it behaves as a co-receptor involved in the HIV viral entry into cell and its overexpression is associated with multiple types of cancers. Thus, novel therapeutic peptide-based drug design approach can be of great pharmaceutical importance [25].

2.10.3. Anthelminthic activities

Cyclotides have anthelminthic activities against *Trichostrongylus colubriformis* and *Haemonchus contortus*, important sheep's gastrointestinal nematodes. For this purpose,

investigation was done to check the interaction of kalata B1 a prototypic cyclotide with the external surface of *H. contortus* adult worms and larvae. Cyclotides showed toxicity without being ingested by the worms rather they interact with the external surfaces alone to show toxicity. Evidence for this work came when this cyclotide was labeled. For which a mutant of kB1 with synthetic lysine at position 29 was replaced with asparagine. The inclusion containing mutant kB1 labeled by probing with fluorescein-conjugated avidin with biotin using NHS chemistry of this cyclotide, which is a deficient primary amine. The nematocidal activity of labeled i.e. biotinylated [N29K]-kB1 using a larval development assay was determined which helped in the understanding one action of the cyclotides of being anthelminthic by being toxic without ingestion and by interacting with the surface of the worm's lipid-rich epicuticle layer [49].

Kalata B1 is toxic to 2 important nematodes *Haemonchus contortus* and *Trichostrongylus colubriformis* of sheep. A lysine scan was conducted that incorporated positive charges in kalata framework. From 29 total residues each non-cysteine residue was changed to lysine that decreased that decreased hemolytic and nematocidal activities whereas those residues which resisted lysine incorporation when given positive charge increase the same bioactivities that was 13 folds more than the non-mutant form [50].

2.10.4. Targeting GPCRs

Cyclotides possessing CCK framework that provides it remarkable stability and highly bioactive protein still lacks a lot in explaining about mechanisms involved in its activities. More specifically no receptor for cyclotide native to plants has been reported yet. Recent studies on kalata B7 was reported to induce contractions in human uterine muscle cells. Further techniques like second messenger-based reporter assays and Radioligand displacement confirmed the receptors of vasopressin V1a and oxytocin as part of the family of G protein coupled receptors which were found to be molecular targets cyclotide Kalata B7. Moreover, Koehbach and his coworkers generated an oxytocic nonapeptide with high affinity for the receptor of oxytocin that were cyclotide based that enhanced uterine contractions thus strengthening the concept for developing peptide ligands that are cyclotide-based. Since targeting GPCRs that are 30% target of pharmaceutical companies and is >10% already marketed target of drugs opens the hidden potential of cyclotides and cyclotide based peptide grafted libraries to be screened for drugs development purposes [51].

A cyclotide extract derived from the root powder of the South American medicinal plant ipecac (Carapichea ipecacuanha) is studied for its GPCR modulating activity of the corticotropin-releasing factor type 1 receptor (CRF1R). seven novel cyclotides are identified and characterized One cyclotide, caripe 8 cyclotide is isolated from the most active fraction, was further analyzed and found to antagonize the CRF1R. In contrast, caripe 8 did not inhibit forskolin-, or vasopressin-stimulated cAMP responses at the vasopressin V2 receptor, suggesting a CRF1R-specific mode-of-action. These results in conjunction with other findings establish cyclotides as modulators of class A and B GPCRs. other cyclotide-GPCR interactions are potentially important sources of drug-like molecules [52].

2.10.5. Antimicrobial activity

Staphylococcus aureus is an important causative agent of most skin infections, occurring via surgical wounds. A lot of emphasis is on the importance of identifying new compounds with antimicrobial potential where cyclotides gained a lot of interest owing to its high multifunctional properties and stability. Both kalataB2 (KB2) and cycloviolacin 2 (CyO2) proved to be anti-staphylococcal at 25 mM for CyO2 and 50 mM for KB2 with no cytotoxicity against monocytes suggesting that an increase in the phagocytotic index *in vivo* may possibly be associated with anti-pathogenic behavior [53]. Due to continuous threat of resistant pathogens around us stress on the development of antimicrobial agents from pharmaceutical point of view is emphasized. Cyclotides are versatile in this regard being antimicrobial in nature also but still targeted antimicrobial effects need to be explored. Therefore, semi purified (fractionation & SPE-C18 column chromatography) cyclotides from the Iranian plant *Viola odorata* were tested against plant and human pathogenic bacteria *S. aureus*, *P. aeruginosa*, *Xanthomonas oryzae*, *alstonia*

solanacearum, E. coli, R. cicil and *Bacillus Sp.* Methods like minimal inhibitory concentration (MIC), radial diffusion assays (RDAs) and minimal bactericidal concentration (MBC) was used for assessment of antimicrobial activities. MIC value of semipurified cyclotides was 16 mg/ mL *S. Aureus* (gram-negative). Studies showed that plant pathogens are more susceptible than human ones [54].

Another cyclotide VarvA is identified previously from *Viola arvensis*. MALDI-TOF mass spectrometry determined the correct peptide amino acid sequence and the cyclization sites-critical in this multicyclic compound. The cyclotide showed antimicrobial activity against various Gram-negative bacteria. The highest antimicrobial activity was found to be against *Flavobacterium psychrophilum* [55].

2.10.6. Anti-cancerous activity

Studies on tumor cells usually make use of overexpression of two proteins Hdm2 and HdmX inorder to promote cell survival by inactivating the pathway of p53 tumor suppressor and targeting the interaction of these 2 proteins is a part of therapeutic strategy for treating cancers. Previously reports showed that small linear peptides linked at p53 protein's N-terminal fragment proved to be potent antagonists of Hdm2/HdmX. The poor stability and bioavailability of linear variants has been resolved by engineering of stable cyclotide MCoTI-I (MCo-PMI) for antagonizing intracellular p53 degradation pathway by binding with very low nanomolar affinity with both HdmX and Hdm2 with cytotoxic to wild-type p53 cancer cell lines and high stability in human serum. Thus, cyclotide MCoTI-I becomes an optimized scaffold for studying protein-protein interactions within cells [56].

2.11. A bioinformatics-based approach for cyclotides variants and features

Studies are now getting attractions at genes level due to highly increasing number of cyclotide gene variants and types as this gene encoded peptide family has shown to be among the largest number of genetically encoded family of plants. Thus, there is continuously increasing data and information regarding conserved sequences and variations also that are directly related to structure-function relationships. Bioinformatics based studies on cyclotides are focus of bioinformaticians now, as there is a need to develop databases and tools to handle them for informative analysis. An approach of bioinformatics based, and combined expression analysis showed discovery of cyclotide-like sequences in Poaceae family such as *Zea mays* (maize), *Oryza sativa* (rice) and *Triticum aestivum* (wheat), whereas *Hordeum vulgare* (barley) showed tissue-specific expression. Elevan species of Australian Hybanthus (Violaceae) were sampled, and twenty-six novel sequences were characterized alongwith 246 new cyclotides that added the number of cyclotides in the Violaceae to be >9000. Recent investigations made on >200 Rubiaceae species screened cyclotides in only 22 species, moreover a few also reported cyclotides in the Apocynaceae. Precursor gene sequence analysis and phylogenetic studies predicted that cyclotides evolved independently in different plant families after divergence from Asterids and Rosids (~125 million years ago). This evidence was also supported by the ubiquitous proteolytic enzyme's machinery involved in post-translational cyclization of cyclotides in all plants where found [57].

Furthermore, Nguyen and his co-workers (2011b) also found different evolutionary patterns (horizontal/ convergent) supported by the discovery of A1b (Albumin 1 chain b) domain along with cyclotide domains (cliotide T1–T12) that separate during displacement from precursor structures that were novel in fabaceae plant *Clitoria ternatea* that originally possess antimicrobial and cytotoxic properties. Another recent study on fabaceae plant species which are one of the largest families and significantly playing role in our lives, a study on *Clitoria ternatea*, the Cter M precursor gene transcript to be found in all plant parts, it also proved to be insecticidal to cotton budworm *Helicoverpa armigera* [58]. Latter 12 novel cyclotides from the seed extracts of *C. ternatea* were analyzed and characterized by utilizing Nano spray and MALDI-TOF. Cyclotides with novel sequence motifs were discovered and their discovery helped to know more about cyclotide biosynthesis. MS analysis of these new cyclotides showed that at cyclization site, Asn to Asp variants are more common in contrast to those reported earlier [59]. *In vitro* cell-based assays

proved that cyclotides possess sufficient cytotoxic activities through which cell membrane integrity can be disrupted. Whereas, using a xenograft model *in vivo*, no significant anticancer effect has been seen even by the most potent cyclotide, cycloviolacin O2. Chemosensitization for treating cancer makes use of cyclotides from *C. ternatea*. The work of Gerlach et al proved that when cyclotides are combined with other anticancer reagents, their chemosensitizing abilities become more pronounced [41]. Cliotides belonging to the cyclotides family are also known to have strong antimicrobial activity against *K. pneumonia*, *E. coli*, *P. aeruginosa* and cytotoxicity against HeLa cells. The cliotide (a cyclotide from *Clitoria*), cT19 has shown significant antibacterial and immunomodulatory activities [60].

2.11.1. CyBase

Increasing bioinformatics-based information about one of the largest genetically encoded peptides mainly cyclic ones urged to develop a database CyBase cyclic proteins that serves as repository of sequences/structures/functions. CyBase was redesigned with increased management to growing protein data and improved user-interactivities. This manages synthetic circular proteins data also providing database search and display capabilities for synthetic sequences, structures and their respective function that doubles the amount of data when compared to the initial version, hosts a novel suite of tools that are useful for the characterization, visualization, and engineering proteins. The tool 'Diversity Wheel' is helpful for analyzing circular protein sequence variations and another tool 'Predict Linker' helps in the engineering of cyclic proteins from their linear targets [61]. The tools include 2D representations of sequence/structure, a summary of mutational studies of synthetic analogues and about grafting, information of N- to C-terminal distances of known protein structures and a tool for structural modeling to predict that which linker length to cyclize a protein is the best. Cybase can help to accelerate the discovery of natural cyclic proteins and their engineering assist in drug development projects.

2.12. Production strategies of cyclotide

All the three classes of plant peptides have a common sequence of synthesis, first is the production of precursor protein which is produced ribosomally, next step is the enzymatic synthesis of mature cyclic peptide. In all the reaction steps asperginyl endopeptidase enzyme (AEP) plays major role in biosynthesis because it causes cyclisation of peptides [1]. Cyclotides are mostly ribosomally synthesized such as Kalata of B series in several violaceae plants like *Viola uliginosa*, are becoming scarce and endangered. So, to preserve such a rich potential source *in vitro* culturing and direct or indirect organogenesis techniques are employed using different MS based culturing methods. When observed by AFLP, polymorphism of such tetraploid plant was significantly low, but flow cytometry revealed that a lot is still shared between the 2 ploidies. Surprisingly the eleven different cyclotides that were originally reported in the diploid aerial maternal parts were found to be significantly in very high amounts in the tetraploid parts. Thus, this opens another option of enhanced cyclotide production methods [62].

2.12.1. Production of recombinant Cyclotide

Even though the synthesis of several cyclotides chemically by using solid-phase peptide synthesis has been already reported yet biosynthesis of cyclotides can be done by recombinant DNA technology. An attempt to produce a cyclotide KB1 by recombinant methods was unsuccessful due to issues related to isolation of the same folded cyclotide from the soluble cellular fractions that were too complex. Despite KB1 was the first cyclotide to be produced by recombinant DNA technology and structurally well characterized, its binding partners for affinity chromatography for purification were not discovered. To do so cyclotide trypsin inhibitor II named MCoTI-II (from *Momordica cochinchinensis* plant) was used instead. Biological synthesis of a completely functional MCoTI-II inside living *E. coli* strain BL21 (DE3) cells was the first successful story of producing cyclotide recombinantly. This was done by utilizing an intein-mediated cyclization approach, engineering *E. coli* cell with lot of mutations for glutathione reductases and thioredoxins to promote disulfide bridge formation which increased MCoTI-II yield. This permits modifications with introduction of specific biophysical or chemical probes.

Moreover, cyclotides can now be biosynthesized in bacterial cells like *E. coli* using modified protein splicing units. These characteristics of cyclotides makes them ideal for the production of libraries based on the CCK cyclotide framework that are genetically encoded [63]. Cyclotides can now be screened for its ability to facilitate processes occurring inside living cells for drug development purposes [64,62]. Different approaches can now be used for the production of cyclotides like for MCOTI-I using protein trans splicing PTS and expressed proteins ligations methods in *E. coli* as well as in eukaryotic systems like unicellular yeasts [63,65]. Another approach for recombinant synthesis of cyclotides was developed using "nonsense suppressing orthogonal tRNA/synthetase technology". This technology has also allowed the genetic encoding and incorporation of a large multiplicity of unnatural amino acids (Uaas) in proteins [66]. Previously scientists have tried a lot the same thing to incorporate unnatural amino acids *in vivo* for site specific incorporation into proteins or peptides. This was done by evolving the *E. coli* orthogonal t-RNA synthetases for its specificities to add unnatural amino acids of our choice by generating that are not endogenous to the host. An orthogonal suppressor t-RNA/amino acyl t-RNA synthatase pair was used in *E. coli* that is specific for the t-RNA^{asp} originally present in *Saccharomyces cerevisae* and its partner aspartyl-tRNA synthetases. Both these were used invitro and invivo and were well characterized [67]. The recombinant cyclotide KB1was first time biosynthesized in *E. coli* by Kimura and his co-workers (2005). Biosynthetic approach using PTS (protein trans-splicing) very efficiently generates natural KB1 as well as its several mutants that generated cyclotide-based libraries that could be screened for their bioactivities *in vitro*. Native chemical ligation method made use of adding N-termini Cys teine rsidue and methionine at the C-terimini of KB1 in the engineered plasmid with a modified VMA intein (vacuolar membrane ATPase). Thus, a mechanism (Figure 2) has been elaborated for the *in vivo* biosynthesis of cyclotides in which cellular environments were not more reductive than *E. coli*'s cytoplasm [68]. Method of Intein-mediated backbone cyclization as shown above in Figure 2 has also been used for making Bowman-Birk inhibitor, sunflower trypsin inhibitor 1 (SFTI-1) which was a cyclic peptide. This method is also used for the biosynthesis of other circular peptides like backbone-cyclized naturally occurring θ-defensins and α-defensins. *In-vivo* biosynthesis of wild-type SFTI-1 inside *E. coli*, a small library of the cyclic peptide containing multiple Ala mutants was synthesized and trypsin-binding was estimated. Trypsin binding now has been a property of cyclotide to inhibit trypsin by binding to it such as in case of MCOTI. Results demonstrates evolutionary technologies using the cyclic peptide like SFTI-1 as a molecular scaffold [69]. Thus, synthesis of cyclotides both in bacterial species and by plants is very important for sufficient production of completely folded and cyclized cyclotide for the understanding the hidden roles of these peptides within plants and explore the benefits that could help mankind.

2.12.2. Cyclotide expression in yeast cells

Expressing recombinant bioactive and fully functional cyclotides in eukaryotic cells like *Saccharomyces cerevisiae* was a challenge. Yeast cells are good models to study different human based functions like many proteins important to understand human biology, signaling proteins, cell cycle proteins, and enzymes for protein-processing were all discovered initially by studying their homologs in *Saccharomyces*. For the first time an attempt of cyclotide production was successfully done inside yeast cells by using intracellular PTS (protein trans-splicing) technique in combination with a highly efficient split-intein. α-syn (α-synuclein), a small human lipid-binding protein related to Parkinson's disease. A cyclic peptide cyclo-CLATWAVG (CP4) previously reported to reduce cytotoxicity by α-synuclein-induction in a yeast synucleopathy model, was used with modifications using linear derivative where Cys residue was replaced with Ser, grafted on loop 6 of MCoTI-I. Natural cyclotide MCoTI-I and its engineered bioactive form MCoCP4 were both expressed to a yield of 50 µg/L to 60 µg/L and confirmed by Mass spec. We used MCoTI-I (our unpublished research), which is a trypsin inhibitor because these are interesting candidates for designing drugs since they can be used as natural scaffolds to create novel biological activities and possess low toxicities to mammalian cells. MCoCP4 showed reduction in the toxicity of α – synuclein (human origin) in live yeast cells. This attempt is successful beginning of using yeast for the screening of genetically encoded libraries of

active cyclotides to understand cyclotide interactions and mechanisms in a eukaryotic system [65]. Moreover, it was found that the expressed constructs of peptides also prevented dopaminergic loss of neuron in a nematode animal model *Caenorhabditis elegans* established as Parkinson's model. This work was a step ahead to a previous phage display studies that revealed the possibility of utilizing cyclic peptides as protein ligands in general but had a problem that they can't access proteins into the eukaryotic cells [70]. This work on yeast cells will improve the efficiency of using libraries of cyclic peptides like cyclotides for forward chemical genetics in model organisms of human disease representing eukaryotic system.

2.12.3. Engineered scaffold of cyclotides with grafted epitopes for enhanced bioactive role

Cyclotide kalata B1 (KB1) can also provide engineered superior natural scaffold for active peptides that are active orally and are useful therapeutics. The Bradykinin (BK)-antagonist peptides DAK or DALK were grafted into the KB1 scaffold whose stability was enhanced. Intracellular levels of Ca^{2+} showed that antagonists designed are specific blockers of bradykinin B1 receptor, but not for B2. Abdominal constriction assay done *in vivo* revealed significantly pain inhibition response in the model animal. This advantage was observed only for cyclic analogues as compared to linear ones when administered orally as well as for the antagonistic peptide alone with no response. The combined effect of cystine-knot and cyclization proved to be stronger [71]. The cyclotide scaffold that cross cell membranes through macropinocytosis and can be evolved or engineered using methods of molecular evolution to hinder protein-protein interactions involved in different diseases like cancer or in the designing of new antimicrobial. For example, development of inhibitors of the β-tryptase (serine protease) and human leukocyte elastase (HLE) using the CCK backbone of cyclotide MCoTI-II. β-Tryptase is associated with different inflammatory and allergic problems, and the HLE implicated in pulmonary and respiratory disorders. Moreover MCoTI-based peptides can cross cell membranes in breast cancer cell lines and macrophage through macropinocytosis. It was also seen that grafted helix region from the MCV (*molluscum contagiosum* virus) FLICE-inhibitory protein (FLIP) into MCoTI-I (loop 6) that triggered apoptosis of virally infected cells. Similarly squash trypsin inhibitor a homolog of cyclotide the RGD sequence was grafted into the 1st loop of EETI-II possessed platelet inhibitory activity. The engineered cyclotides showed more potential of avoiding platelet aggregation than linear peptides variants grafted. In another example, Cyclopsychotride (Cpt) A is natural cyclotide of *Psychotropia longipes* that was found to be neurotensin inhibitor. Cpt A inhibited neurotensin binding with its receptor to HT-29 cell membranes and increased intracellular levels of Ca^{2+} which no other neurotensin antagonists can block [13].

2.12.4. Incorporation of non-natural amino acids by orthogonal t-RNA in peptides

Scientists have tried a lot to incorporate non-natural amino acids *in vivo* for site specific incorporation into proteins or peptides. This is done by evolving the *E. coli* orthogonal t-RNA synthetases for their specificities to add unnatural amino acids of once's choice by generating that are not endogenous to the host. An orthogonal suppressor t-RNA/amino acyl t-RNA synthatase pair was used in *E. coli* that is specific for the t-RNA^{asp} originally present in *Saccharomyces cerevisae* and its partner aspartyl-tRNA synthatases. Both these were used *in vitro* and *in vivo* and well characterized. Antimicrobial resistance was also investigated for using this pair [67]. Non-natural amino acids if site-specifically incorporated become a powerful tool to manipulate or alter proteins for functional and structural studies, or to produce proteins with different properties. This can be done by either labeling pre-synthesized proteins with probes at reactive side chains such as lysine or cysteine residues or by probes incorporation into newly developing proteins in the presence of an altered aminoacyl-tRNA (aa-tRNA). The first approach generally requires mutagenesis extensively. In contrary, using an mRNA with a unique cognate codon and modified aa-tRNAs allows incorporation by co-translational techniques, diverse probes will then be positioned at almost any site with minimal perturbation and high specificity. The second method usually uses an amber suppressor aa-tRNA that can recognize unique nonsense codons (like UAG). However, now a variety of engineered and synthetic aa-tRNAs have also been produced for

this purpose, including those that can recognize unique four-base codons. For co-translational incorporation to happen, the non-native amino acid must be bonded to tRNA with high efficiency and specificity, by using an orthogonal aa-tRNA synthetase (aaRS) that is engineered to recognize both tRNA and modified amino acid [72]. Thus, amber suppressor tRNAs are widely used to incorporate multiple probes using non-natural amino acids simultaneously incorporated at different locations within the same protein without being modified. Certain tRNACys derivatives by synthesis, aminoacylation, and modification are developed like amber, ochre and opal suppressor tRNAs originally derived from yeast and *E. coli* [73]. tRNACys can incorporate modified cysteine (chemical) residue selectively at the cognate UGA, UAG and UAA stop codons using an *in-vitro* translation system. These synthetic tRNAs are aminoacylated *in vitro* and further bond was stabilized by attaching covalently a fluorescent dye to sulfhydryl group of cysteine. Read through efficiency in sequence is amber > opal >ochre which was further improved by inhibiting eRF1/eRF3 with an RNA aptamer, thus restricting in higher eukaryotic translation systems the intrinsic hierarchy in stop codon selection limited to UGA and UAA termination suppression. This approach significantly expands the chances for incorporating non-natural amino acids for studying protein structure/function [72].

2.12.5. Labeling of cyclotides

Besides studying cyclotides as a stable peptide-based drug that is bioavailable orally as well and well-studied from different perspectives there was also a need to study the cyclotide drug in a bifunctional manner. That is being itself a harbor of activities and unique features. It may also serve as a stable drug delivery tool with different grafted epitopes of medicinal or agricultural importance [63]. For this purpose, cyclotide have to be labeled *in vitro* as well as *in vivo* using different strategies. Labeling peptides was one of the major needs of science in earlier times for immune assays like an ultrasensitive chemiluminoenzyme immunoassay (CLEIA) where digoxigenin-labeled bradykinin was used as tracer for quantifying kinins. This assay was so sensitive that it can detect bradykinin levels upto 0.1 fmol/mL in its lower limit with ED50 of 0.78 pmol/mL in extracts of carrageenan inflamed and normal tissues [74]. Peptides have also been labeled for ELISA based studies for chemiluminescence detection. Practices of peptides labeling with the stable ^{15}N isotope and NMR-active helps in NMR based studies that illustrates structural and dynamics studies and use it as tracers. However, labeled cyclotides being head-to-tail cyclized peptides are not amenable by conventional strategies using recombinant labeling. This limitation was overcomed by growing *Oldenlandia affinis*, a cyclotide-bearing plant on a medium containing nitrogen-free agar that was supplemented with ^{15}N salts and got complete labeling with no detrimental effect to plant biomass. Kalata B1 and kalata B2 were labeled by this method for NMR studies [26]. The uptake of cyclotide MCoTI-I in live HeLa cells was also an application of labeling peptide with fluorescent probes which was monitored using real time confocal fluorescence microscopy imaging. Results showed that MCoTI-I was internalized in HeLa cells readily depending on temperature with easy access to general lysosomal/endosomal pathways. In their studies they also used labeled markers for clathrin-mediated and cholesterol-dependent endocytosis of both EGFP and cholera toxin B respectively for such endocytotic studies.

Backbone thermodynamics studies were done on MCoTI-I when it binds to Trypsin as trypsin protease inhibitor. A competition experiment of labeled trypsin-[15N]-MCoTI-I with unlabeled MCoTI-I was used to indicate that the backbone structure of MCoTI-I remained unchanged on trypsin binding and chemical changes that resulted in loop1 & 6 helped to accommodate the increased flexibility of the binding loops and are part of entropic penalties/adjustments. Such interesting results were already observed in other protein-protein interactions of high-affinity that involved protease inhibitors. Colgrave et al used mutant of kB1 based on synthetic lysine in which the residue asparagine at 29^{th} position was replaced with lysine that enabled labeling with biotin utilizing the NHS chemistry of such cyclotide, which is normally not present in a primary amine. The biotinylated kB1 was also tested for its nematocidal activity. Camarero et al reported the biological synthesis of a natively folded cyclotide in *E. coli*, MCoTI-II, intracellular backbone cyclization of a linear uncyclotide was through intein-based fusion of

precursor and then correctly folding of cyclotides permits the likelihood of generating cell-based combinatorial libraries that can be simply screened within living cells, for their ability to inhibit or modulate cellular processes. Jagadish and his colleagues in 2013 used Expressed protein ligation EPL for the in-cell generation of MCoTI-I-based cyclotides containing different Uaas using stop codon orthogonal t-RNA synthatase technology to incorporate two different non-natural amino acids in recombinant MCOTI-I for allowing site-specific incorporation of fluorescent probes into this scaffold inside the living cell using *E. coli* as host. In-cell production, however, is less efficient using EPL i.e. only 4 µg/L & 14 µg/L MCoTI-OmeF & MCoTI-AziF respectively. However, PTS-mediated using the efficient Npu DnaE split-inteins of *Nostoc puntiforme* PCC73102 origin gave a production of around 7 times more efficient, thereby giving an attractive alternative to produce these types of peptides and polypeptides. This could help using optical properties to screen or probe the cyclotides with Uaas with a fluorescent material like DBCO-AMCA a fluorescent probe with a dibenzo-cyclooctyne (DBCO)-derivative of the fluorescent dye amino-methyl-coumarin acetate (AMCA) to provide in-cell fluorescently labeled cyclotides. FRET can be used measuring distances between molecules and their dynamics especially among molecules such as proteins in a few nanometer ranges. The labeled cyclotide was used in fluorescence resonance energy transfer (FRET) to visualize the interaction between modified trypsin that was fused at N-terminus with green fluorescent protein (EGFP). AMCA-labeled cyclotide MCoTI-AziF efficiently binds trypsin-S195AEGFP (KD of 1.8 ± 0.7 nM*in vitro*, and cyclotide-protein interaction was monitored by intermolecular FRET shown by the simultaneous increase and decrease of the fluorescence signal at 445 and 515 nm from donor to acceptor, respectively. This finding opens the possibility for in-vitro and potentially also in-cell screening of genetically encoded libraries of cyclotides for the rapid selection of novel cyclotide sequences able to bind a specific bait protein using high throughput cell-based optical screening approaches [63].

2.12.5.1. Labeling cyclotide using unnatural amino acid Uaa

Biosynthesis of cyclotides with Uaas can be possible by using different intein-based methods like EPL (expressed protein ligation) and PTS (protein trans-splicing) but with different yields in both the cases [63]. Jagadish and his coworkers reported the MCOTI-I, a trypsin inhibitory cyclotide, expression with *p*-methoxyphenylalanine (OmeF) and *p*-azidophenylalanaine non-natural amino acid incorporated for site specific incorporation of coumarin fluorescent dye as probe and used it in cell production for natively folded cyclotides (MCoTI-AziF & MCoTI-OmeF) for the first time. The efficient PTS-mediated cyclization was combined with nonsense suppressing orthogonal tRNA/synthetase technology used the in-cell cyclotides production containing Uaas possible. Of particular interest was the introduction of azido-containing Uaas *p*-azidophenylalanine (AziF) which reacted with DBCO-AMCA a fluorescent probe (dibenzo-cyclooctyne DBCO and amino-methyl-coumarin acetate AMCA) and produced in-cell fluorescently labeled cyclotides. This approach for in-cell production of fluorescent-labeled protein was not easily applicable to cyclotides previously due to their restricted backbone-cyclized topology and small size. Thus, now cyclotides containing the Uaa AziF expressed in living bacterial cells and easily labeled with fluorescent DBCO-AMCA can be used to observe cyclotide-protein interactions. The interactions were estimated by fluorescence resonance energy transfer (FRET) with modified trypsin fused at N-terminus with green fluorescent protein (EGFP). This finding opens the possibility for in-vitro and potentially also in-cell screening of genetically-encoded libraries of cyclotides for the rapid selection of novel cyclotide sequences able to bind a specific bait protein using high throughput cell-based optical screening approaches [63]. Protein libraries can now be screened *in vivo* as well as invitro for selection of bioactive members that can be useful for treating different drug targets like proteins of toxins or pathogens or even cancers. Florescent labeling is one the best strategy to screen and understand cyclotide interactions with other agents like other labeled proteins by FRET. For this a fluorescent dye is needed with no overlapping with the spectrums of other interacting labeled member like EGFP, therefore it is labelled with Texas Red that has a negligible background which can interfere in studying protein interaction

both *in vitro* and *in vivo* and by selection and screening through optical approaches. Moreover, it will add new approaches towards peptide-based drug development and targeting studies. Besides studies available on the recombinant peptides production from yeast cell there are no such studies available of cyclotide production and studies for eukaryotic cell drug targeting. A diagrammatic representation of an approach shown in Figure 3 (our unpublished research), as an overview of cyclotide labeling with Texas red succinamyl ester with MCoTI-AziF.

2.12.5.2. Click chemistry for proteins labeling

Those reactions that meet the necessary criteria of being high yielding, selective, and possessing good reaction kinetics are well known as Click reactions. Whereas a subclass of click chemistry reactions whose components are inert to the surrounding biological milieu is called *biorthogonal*. Because of the added complications of biocompatibility, it goes one step higher than the typical terminology of click reaction. Within the group of biorthogonal reactions there are cycloadditions lacking exogenous metals as catalysts, so-called *Cu-free click reactions*. Exogenous metals can have mild to severe cytotoxic effects when used in biological systems and can thus interfere with the delicate metabolic balance of the systems being studied [75].

Mimicking nature in organic synthesis may help the invention of new pharmaceuticals given the large number of possible structures. Click chemistry in combination with combinatorial chemistry, building chemical libraries and high-throughput screening speeds up new drug discoveries by making every reaction during multistep synthesis faster, predictable, and efficient. One of the most popular reactions is the azide alkyne known as Huisgen cycloaddition reaction using a Copper (Cu^{+2}) catalyst at room temperature (Fig 4). Click chemistry has also been used for selective labeling of biomolecules within living systems. A Click reaction that is to be performed in a biological system must meet an even more precise set of criteria than in an *in vitro* reaction. It must be biorthogonal, meaning the reagents used may not interact with the biological system in any way, nor may they be toxic to cell. The reaction must also occur at neutral pH or around physiological pH and at or around body temperature. Most click reactions have high energy content and are irreversible and involve carbon-hetero atom bonding processes. An example of it is the Staudinger ligation of azides. Click chemistry has diverse applications like in two-dimensional gel electrophoresis separation, modification of peptide function with triazoles, preparative organic synthesis of 1,4-substituted triazoles, drug discovery, modification of natural products and synthetic pharmaceuticals, natural product discovery, macrocyclizations using Cu(I) catalyzed triazole couplings, modification of nucleotides and DNA by triazole ligation, supramolecular chemistry: rotaxanes, calixarenes, and catenanes, dendrimer design, Polymers and Biopolymers, carbohydrate clusters and carbohydrate conjugation by Cu(I) catalyzed triazole ligation reactions, surfaces, material science, nanotechnology and bioconjugation e.g. azido coumarin [76]. Biorthogonal chemical reactions are leading the way for new inventions in biology. These reactions possess extreme selectivity and biocompatibility in a way that reacting reagents can form covalent bonds within a rich functionalized biological system in some cases, living organisms. Now Cu-free click chemistry has been tailored to be biorthogonal by eliminating a cytotoxic copper catalyst, allowing reaction to proceed fastly and without live cell toxicity. Cu-free click reaction has been applied within cultured cells, live zebrafish, and mice. For example, the binding of specific DNA or molecular activity may be retained for zinc finger proteins labeled through copper free click chemistry reaction [77]. Residue specific labeling of zinc finger proteins which can be useful conducting *in vitro* screens for assembled zinc finger proteins through FRET assays based on solution and microarray-based hybridization. Copper catalyzed azide alkyne cycloaddition help to revealed reaction and chemistry between protein-DNA interaction and protein-protein binding with the use of surfaced based assay. Fluorescent labeling strategies provide a useful tool for the quantitative measure of dynamics in protein and transcriptional factor to visualized protein interaction and their specific orthogonal chemistries with the use of different fluorescent dyes [22]. Another example of such biorthogonal reaction includes the incorporation of phenyl azide chemistry into proteins through the use of p-azidophenylalanine (azF). The phenyl azide moiety opens different routes to non-natural Post-

Translational Modifications (nnPTM): photochemical transformations and Click chemistry adduct addition.

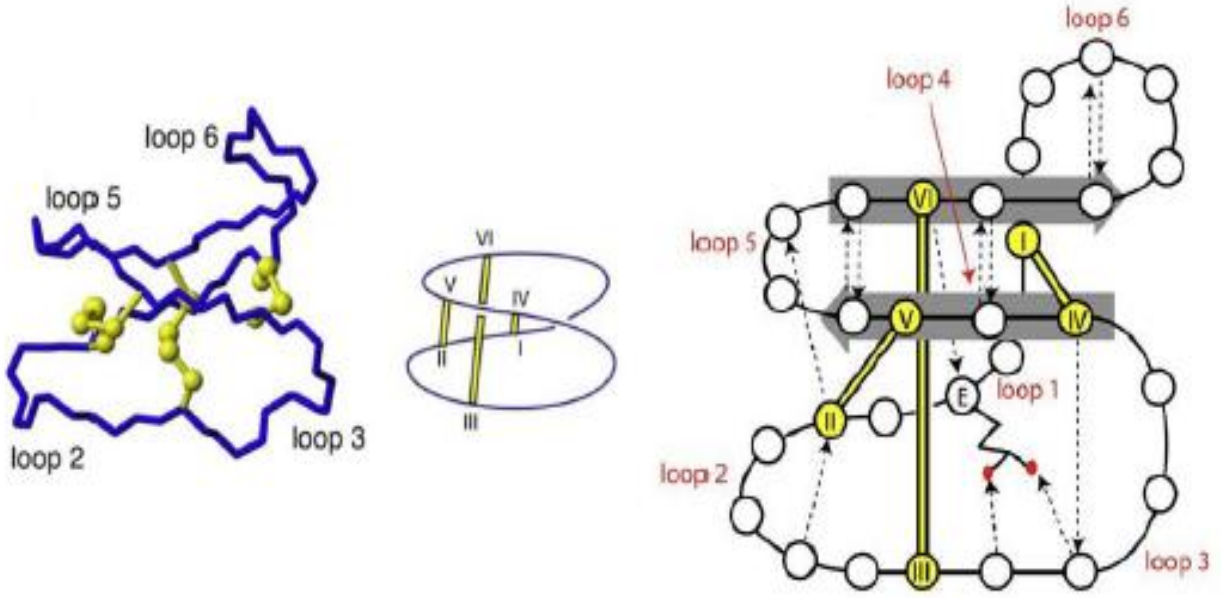

Figure 1: Structural scaffold and features of Cyclotide with loops presented in different ways.

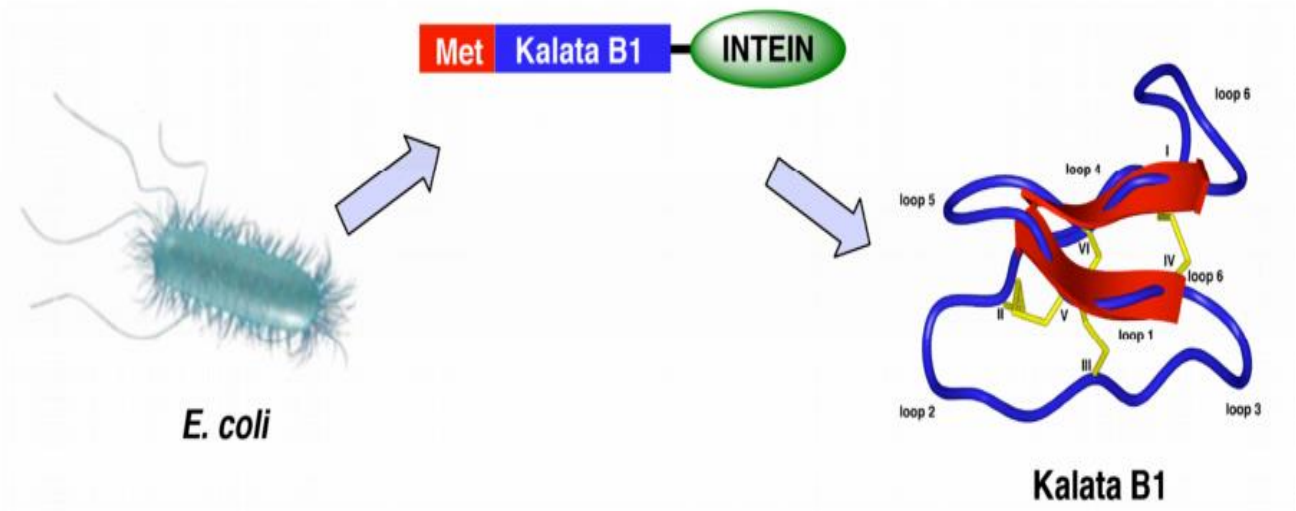

Figure 2: The *in vivo* biosynthesis of cyclotides in bacterial cell. Synthesis of cyclotide Kalata B1 into completely folded and cyclized functional form with reactive inteins that on cleavage causes cyclization and reduced environment in the cell causes folding by disulphide bonds formation, figure reproduced from [69].

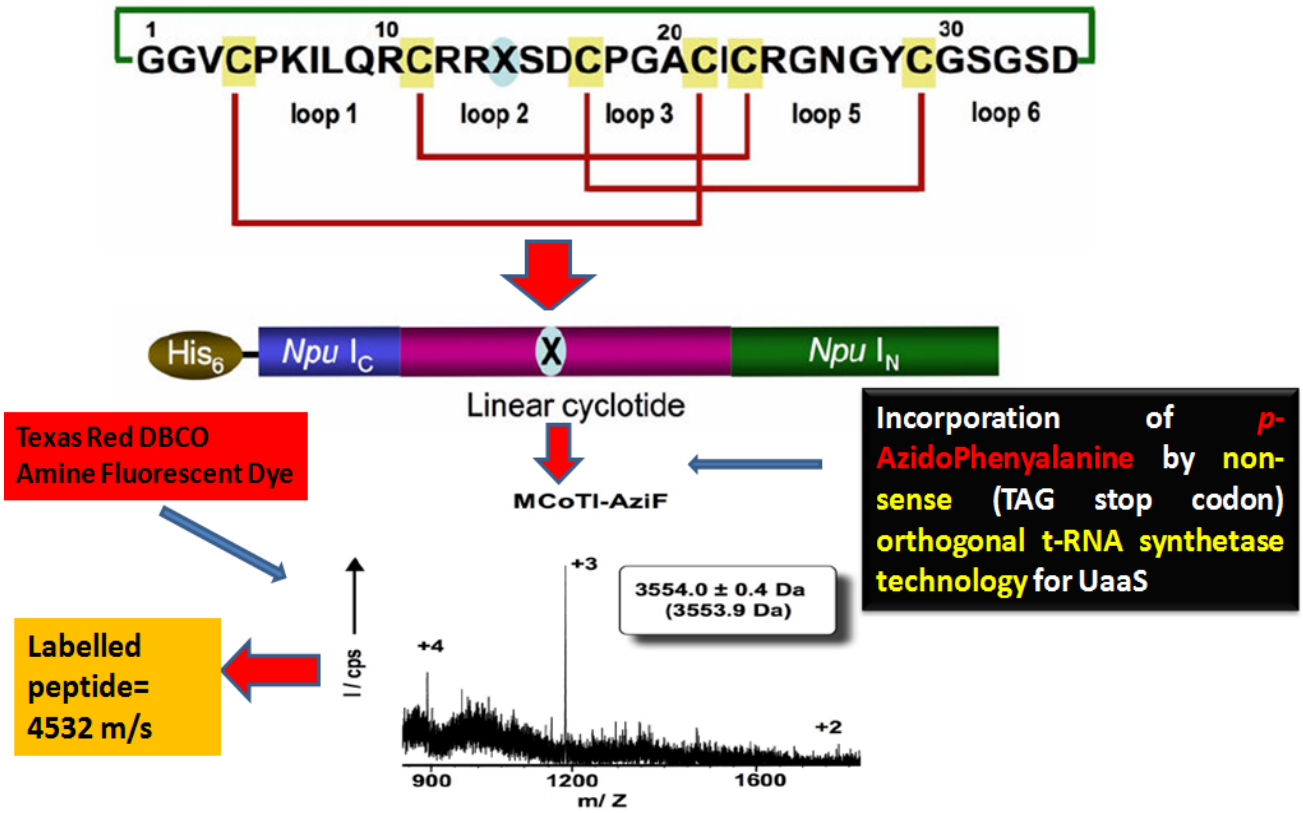

Figure 3: Overview of Cyclotide labeling with Texas red dye. Cyclotide MCoTI-AziF labeled with Texas red-DBCO amine dye and it's in cell folding using intein-mediated protein trans-splicing (PTS) technique combined with non-sense suppressing orthogonal t-RNA synthetase technology.

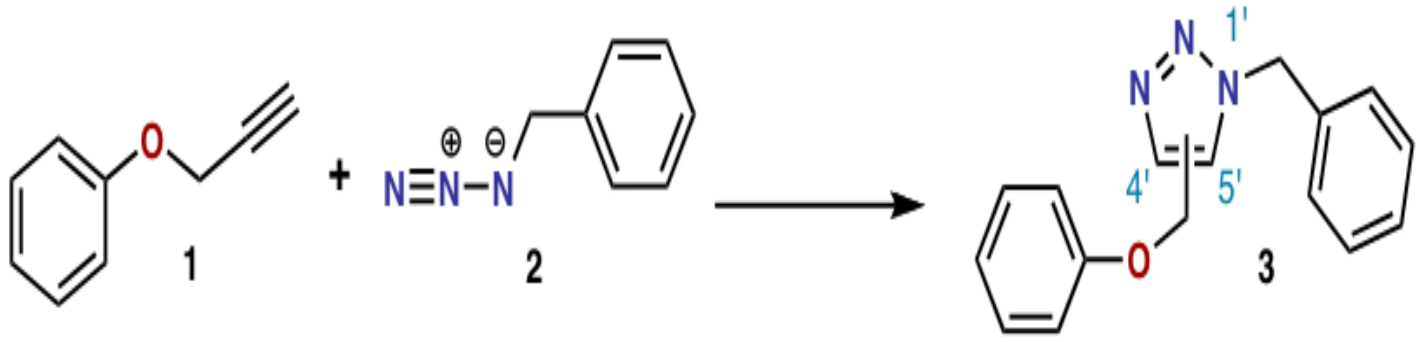

Figure 4: A Cu free click reaction between an azide and Alkyne. In the reaction above azide **2** reacts neatly with alkyne **1** to afford the triazole **3** as a mixture of 1,4-adduct and 1,5-adduct at 98 °C in 18 hours (Kolb *et al*., 2003).

Table 1: Advantages and disadvantages of peptides as drugs [78].

Advantages	Disadvantages
✓ High potency ✓ High selectivity ✓ Broad range of targets ✓ Potentially lower toxicity than small molecules ✓ Low accumulation in tissues High chemical and biological diversity	• Poor metabolic stability • Poor membrane permeability • Poor oral bioavailability • High production costs • Rapid clearance • Sometimes poor solubility

Azide–alkyne cycloaddition is fast, becoming an important method for orthogonal biomolecule conjugation but has been mostly used in a passive manner, for example, to label proteins. Baskin and his coworkers reported this type of reaction for labeling proteins with a core dibenzyl cyclooctyne (DBCO) reactive handle that was chosen to have very distinct properties like it is an amine derivative of DBCO that has hydrogen donor and acceptor groups opening up the potential to form H-bonds between residues not normally close enough to each other in the protein structure secondly they made use of a rhodamine dye (Texas Red) which is a planar, large and hydrophobic dye that has proved valuable in labeling proteins for fluorescent imaging [78].

2.13. The Future of peptide-based drugs

Historically plants have been a rich source for drug discovery. For example, salicylic acid, one of the most well-known antipyretic, anti-inflammatory and analgesic drugs, was originally derived from willow bark (Salix alba). Similarly, the discovery of artemisinin, a highly effective compound against the Malaria parasite was first isolated from Artemisia annua led to the award of the Nobel Prize in Physiology and Medicine in 2015, such discoveries have commonly involved small molecules. However, plant peptides are gaining consideration for new opportunities in drug discovery and development [52].

For the development of peptide-based drug there is a need to find out new sources which contain lead materials in the future. Disadvantages and advantages of peptides as drugs are summarized in Table 1.

There are some limitations in the development of peptide-based drugs, firstly there is a need of some type of reagents like resins and amino acids which are protected that are very expensive for the development of small peptides. For purification and production of these drugs there should be development of methods that are cheaper. By using chemical synthesis or molecular biology techniques this type of limitation can be overcome. Second type of modifications will be necessary to enhance the permeability of membranes. Due to increase in number of peptides in clinical applications there is a need for the development of methods to improve the delivery and transport. Recently, peptides and small molecule drugs are conjugated to antibodies (to improve targeting), to carbohydrates (to improve solubility, protection from degradation or conformational rearrangements) to PEGs and lipids (to improve uptake and permeability). For the delivery and development of peptide-based drugs there are more common procedures that are used as alternative methods. For example, recently the discovery of some type of cyclic peptides that are structurally stable. These types of peptides are produced in larger amount and reported to play important role in the peptide-based drug design as valuable scaffolds.

3. Conclusions

The peptide-based drugs may be produced to a greater extent by using plants as main source for their production. Different types of technologies which include protein splicing and genetic code reprogramming also play a significant

role for the development of cyclic peptide-based drugs. Besides extensive research on cyclotides we are still lacking the knowledge of the bioactive potential of indigenous plants possessing cyclotides and can still find new genetic variants of genes of cyclotides and correlate them. Present review finds gaps in study of cyclotides for optical studies to trace the path of cyclotides within cell or towards the target when epitopes are attached for specific purpose to it and thus better understand its mechanism of action within cells Moreover, there is need for opening the possibility for *in vitro* and potentially in-cell screening of genetically-encoded libraries of cyclotides. For rapid selection of novel cyclotide sequences able to bind a specific bait protein that are fluorescently labeled using high throughput cell-based optical screening approaches are required to work on for peptide-based drugs development and targeting studies.

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