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Disulfide-Rich Peptides in Drug Development

İlaç Geliştirmede Disülfit-Zengin Peptidler

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ABSTRACT

Peptides are important biomolecules in drug development with their high specificities to their targets. Many peptide-based drug candidates have been increasingly involved in clinical and preclinical studies. Unfortunately, peptides have some disadvantages such as poor metabolic stability, poor oral bioavailability and high production costs. These problems can be overcome by modifications that have been inspired from highly stable disulfide-rich peptides already found in nature. This review describes the structure and bioactivity of disulfide-rich peptides and their development with various modifications to become candidate molecules in drug design and development studies.

Key Words

Therapeutic peptides, drug development, cyclotides, peptide stability.

ÖZ

Peptidler, ilaç geliştirmede hedeflerine yüksek özgüllükleri olan önemli biyomoleküllerdir. Birçok peptid-bazlı ilaç adayı, klinik ve preklinik çalışmalarda giderek daha fazla yer almaktadır. Ne yazık ki, peptidlerin zayıf metabolik stabilite, zayıf oral biyoyararlanım ve yüksek üretim maliyetleri gibi bazı dezavantajları vardır. Bu problemler, hali hazırda doğada bulunan oldukça stabil disülfit bakımından zengin peptidlerden ilham alınarak yapılan modifikasyonlar ile çözülebilir. Bu derlemede disülfit-zengin peptidlerin yapı ve biyoaktiviteleri ve ilaç tasarım ve geliştirme çalışmalarında aday moleküller haline gelmek için çeşitli modifikasyonlarla geliştirilmeleri anlatılmaktadır.

Anahtar Kelimeler

Terapötik peptidler, ilaç geliştirme, siklotitler, peptid stabilitesi.

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THERAPEUTIC PEPTIDES

Therapeutic molecules are divided into two main classes as molecules with less than 500 Da molecular weight and protein-structured biologicals with molecular weight greater than 5000 Da. Although small molecule therapeutics are known to be stable molecules as they do not degrade when taken into the body, they have low specificity to their targets and hence side effects, as they can create undesirable interactions with other regions outside the active site of the target molecule. On the other hand, because of their large molecular structures, biologicals bind to their targets with high specificity and thus produce less side effects. Therefore, the number of biologicals approved by drug authorities has increased in recent years [1-3]. However, biologicals are easily degraded by proteases because of their protein-based structures, resulting in reduced bioavailability and hence a more limited effect. In today's pharmaceutical research and development, many studies have been carried out to eliminate these disadvantages of small molecule therapeutics and biologicals. In recent years, peptides have become molecules of interest in such studies. Peptides ranging in molecular weight from 500 Da to 5000 Da have advantages such as high target specificity, less toxicity and less deposition in tissues as biologicals (Figure 1). They can also easily pass through the cell walls as small molecules and interact with their target molecules.

Peptides are biomolecules formed by binding of 40 and/or fewer amino acids to each other by peptide bonds. The therapeutic efficacy of the peptides has been extensively studied and is of great interest in the diagnosis and treatment of many diseases [4].

To date, peptides have been isolated from many organisms, particularly from microorganisms, animals and plants, and their therapeutic efficacy has been studied by many research groups [5-8]. These peptides are used in today's drug screening, design and development studies, and some have already been approved as drugs. For example, cyclosporin A, isolated from *Tolypocladium inflatumizole* fungus, is a peptide having 11 residues and has oral bioavailability [9-11]. Although its antifungal activity is known at first, it is currently used as an immunosuppressant in organ transplants [12]. Linaclotide, another peptide drug that can be taken orally, interacts

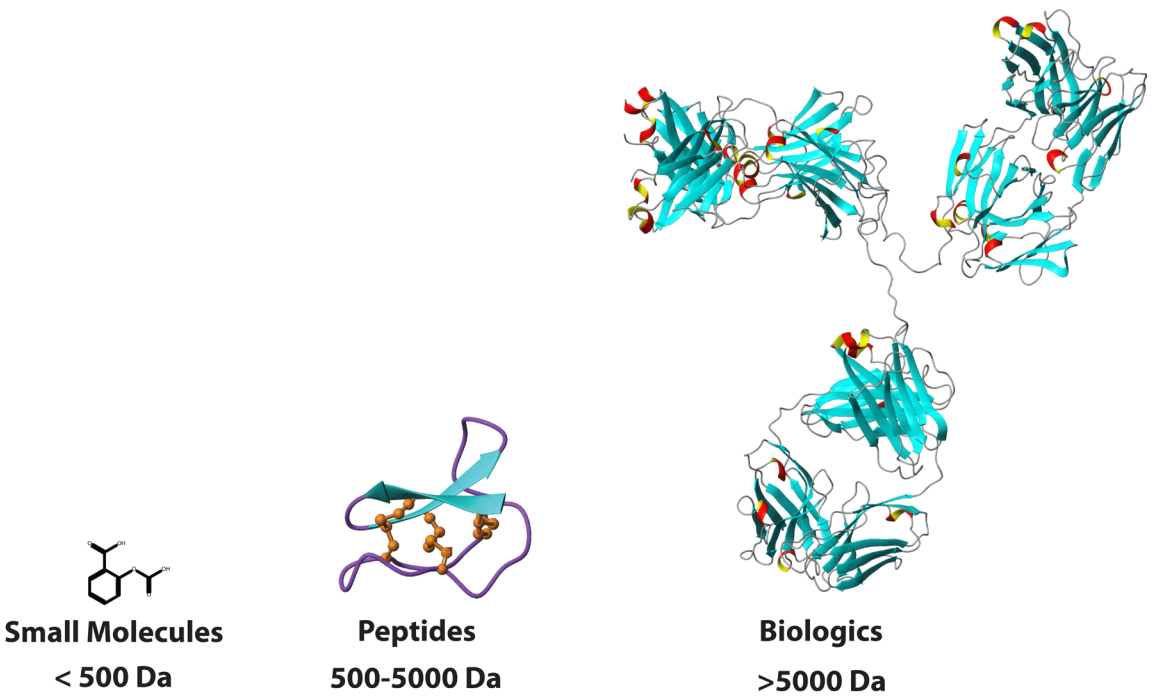

Figure 1. Illustration of therapeutic molecule structures by molecular weight. Peptides are therapeutic biomolecules between small molecules and biologicals based on their molecular weight and are used in today's pharmaceutical research and development. Aspirin, kalata B1 (PDB code: 1NB1) and IgG2a (PDB code: 1IGT) monoclonal antibody are given as examples for small molecules, peptides and biologicals, respectively. Kalata B1 and IgG2a molecular structures were prepared with MOLMOL [Figure was adapted from reference 6].

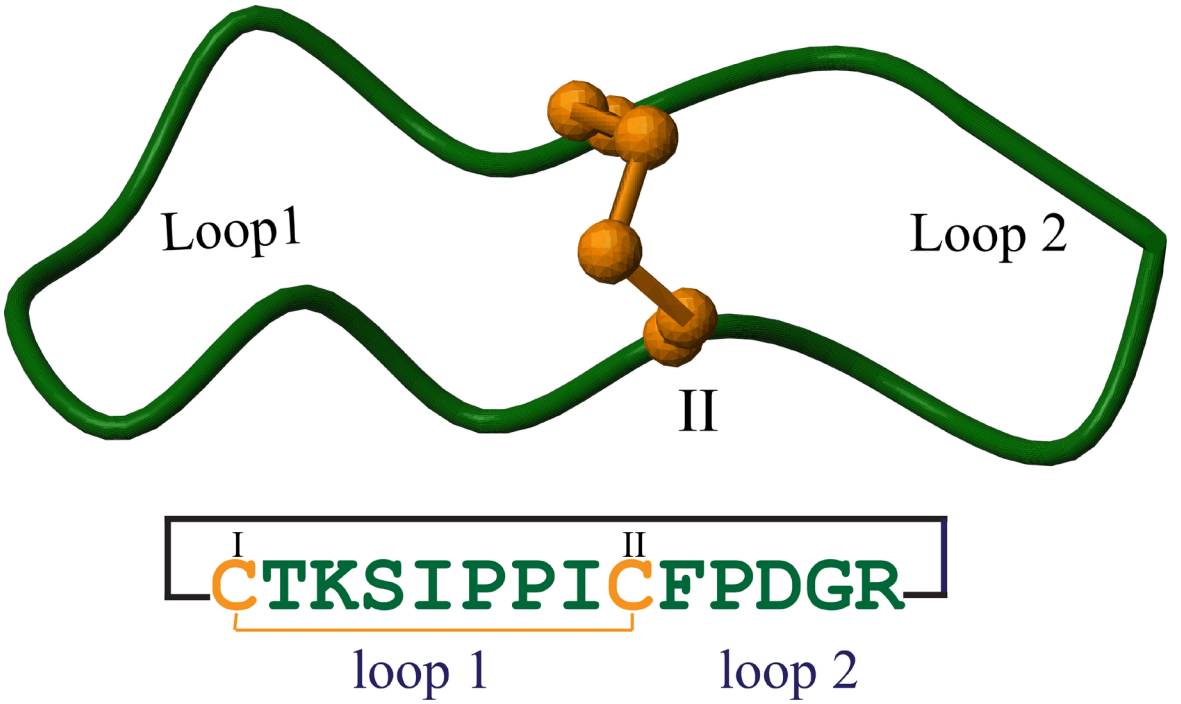

Figure 2. The three-dimensional structure and amino acid sequence of SFTI-1 peptide (PDB code: 1JBL). The peptide has 14 residues and a disulfide bridged cyclic backbone structure. The peptide backbone is shown in green, and the Cys amino acids forming the disulfide bridge are shown in orange. Molecular structure was prepared with MOLMOL [Figure was adapted from reference 6].

with guanylate cyclase C and is used in the treatment of irritable bowel syndrome. This peptide drug has 14 residues and three disulfide bridges. Colistin, a peptide drug isolated from *Paenibacillus polymyxa*, is used in infectious diseases caused by gram (-) bacteria. Another drug with antimicrobial activity is daptomycin which was approved in 2013 by the American Food and Drug Administration (FDA), is a lipopeptide isolated from *Streptomyces roseosporus*.

Peptides, particularly from venoms, saliva and skin secretions of animals, have an important place in the development of peptide-based drugs because of their high therapeutic potentials. Eptifibatide, isolated from snake venom has been used clinically since 1998 for its anticoagulant activity [13]. Captopril, another peptide drug isolated from snake venom, is used in the treatment of hypertension with angiotensin converting enzyme (ACE) inhibitor activity [14]. Tirofiban, Batroxobin, Hemocoagulase, Ximelagatran are other peptide drugs in the market derived from snake venoms [15-18]. Another therapeutic peptide of animal origin is Exenatide, which is isolated and developed from the saliva of the Gila monster lizard. This C-terminal amidated and 39 amino acid long peptide was approved by FDA in 2005 for the treatment of Type 2 diabetes [19].

Peptides derived from conus species of marine snails living in tropical oceans are called conotoxins [20,21]. Conotoxins are disulfide-rich linear biomolecules with 12-40 amino acids. Because of their high potency and selectivity on potassium, sodium, calcium and chloride ion channels and receptors, conotoxins are considered as important therapeutics [22,23]. The synthetic version of 25 amino acid-long MVIIA peptide derived from the venom of the *C. magus* cone snail is the first conotoxin-type peptide drug used for the treatment of chronic pain under the generic name of Ziconotide (Prialt®) [24,25]. Another promising conotoxin is Vc1.1 peptide from the venom of *C. victoria* and has 16 amino acids with two disulfide bonds. It can inhibit nicotinic acetylcholine receptors (nAChR) and its synthetically cyclized version has higher stability compared to lineer version [26].

Chlorotoxin is a 36 amino acids long scorpion venom peptide isolated from *Leiurus quinquestriatus* venom. Three-dimensional structure of chlorotoxin has been identified by Nuclear Magnetic Resonance (NMR) spectroscopy [27]. It has an α-helix and three β-sheets structure with four disulfide bonds. A synthetic iodine derivative of chlorotoxin, ^{131}I-TM-601, was reported as

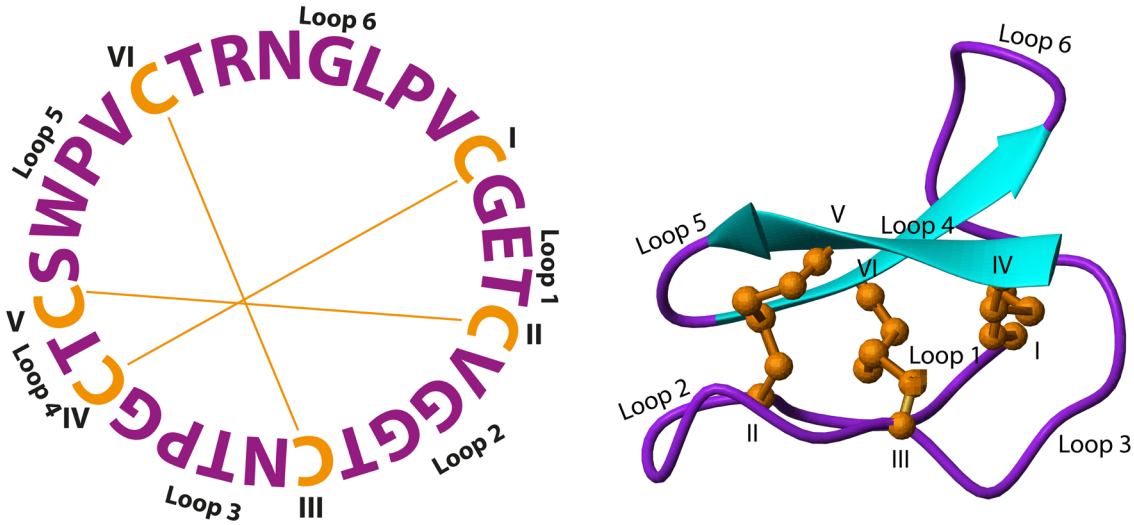

Figure 3. Amino acid sequence and three-dimensional structure of Kalata B1 peptide (PDB code: 1NB1). The peptide has a cyclic backbone structure and six Cys residues forming the three disulfide bridges. The peptide backbone is shown in purple, and the Cys amino acids and the disulfide bonds are shown in orange. The three-dimensional structure on the right was prepared with MOLMOL [Figure was adapted from reference 6].

it can bind to glioma, but not to normal tissues [28]. It has been reported that chlorotoxin can also bind Annexin 2 which is an extracellular matrix protein of cancer cells. This cancer cell selectivity of chlorotoxin has been utilized to develop a conjugated molecule of the peptide with a fluorescent dye. The conjugate is called "tumor paint" which can highlight brain tumor cells under fluorescent light during surgical resection [29,30] and help surgeons to distinguish cancer cells from normal cells more precisely. Tozuleristide (BLZ-100) is a version of tumor paint developed by conjugating chlorotoxin with the near-infrared fluorophore indocyanine green for imaging pediatric brain tumors and it is now in phase I clinical trials [31].

In addition to conotoxins and chlorotoxin and other venom peptides, macaque and baboon leukocytes, frog skin secretions, spider hemocytes and bee venoms are also other rich sources of bioactive peptides that have been under investigation for the development of peptide therapeutics for many decades [32-35].

Plants are other important sources of bioactive peptides that are frequently used in drug research and development studies due to their high stability and cell penetration abilities. They have also antimicrobial, anti-HIV and anticancer bioactivities [36]. For example, trypsin inhibitor SFTI-1 peptide isolated from sunflower plant is one of the most studied plant-derived peptides with high stability. It is a 14 amino acid long single disulfide-bridged cyclic peptide with a lysine residue which provide the trypsin inhibitor activity. In addition to the cyclic backbone and the single disulfide bridge, hydrogen bonds between the two antiparallel β-sheets also have a significant effect on the stability of SFTI-1 [37]. While having a homologous sequence to Bowman-Birk inhibitors that inhibit trypsin/chymotrypsin enzymes, it has also been reported to inhibit the matriptase enzyme that induces cancer cell metastasis [38,39]. In Figure 2, the three-dimensional solution structure and amino acid sequence of SFTI-1 are shown.

Because of their diverse and invaluable bioactivities, peptides have an important role in pharmaceutical research and development studies. However, the major challenges in the development of peptide-based drugs are their poor stability and consequently poor oral bioavailability. In order to overcome this problem, the drug development studies firstly discover the peptide or peptide epitope and aims to increase the stability and bioavailability of this epitope by various strategies. Strategies for increasing peptide stability has been initially inspired by the structure and bioactivity of plant derived disulfide-rich peptides (e.g. cyclotides) found in nature.

Disulfide Rich Peptides; Cyclotides

In the 1960s, Norwegian Doctor Lorents Gran observed that women living in the Democratic Republic of Congo in Africa boiled *Oldenlandia affinis* plant and drank its

tea to facilitate childbirth. It has been reported that the main active ingredient in this uterotonic plant extract is the kalata-kalata peptide, which does not denature despite boiling in water and is resistant to proteolytic enzymes when administered orally [40]. Approximately 25 years later, the peptide was renamed as kalata B1 and was reported to have a cyclic peptide backbone and a cystine knot motif arranged by its three disulfide bonds (Figure 3) [41,42].

Cyclotides are found in Rubiaceae, Violaceae, Cucurbitacea, Solanaceae and Fabaceae plant families [43]. In the following years, new cyclotides were isolated from *Panicum laxum*, a member of the single-core Poaceae plant family [44,45]. They are cyclic peptides with 28-37 residues, of which six are Cys residues. The peptide sequence between the two Cys amino acids is called "loop". In loop 1, the cyclotides generally contain a total of 3-4 amino acids with a Glu residue, 4-8 amino acids in loop 2, 3-7 amino acids in loop 3, only one amino acid (Ser, Thr or Ile) in loop 4, 4-5 amino acids in loop 5, and 5-8 amino acids in loop six [46-48]. The lists and activities of cyclotides are deposited in a database called Cybase (http://www.cybase.org.au/) [49].

The three disulfide bonds of cyclotides are arranged in themselves to form the cyclic cystine knot (CCK) structural motif. This CCK motif, which is the most characteristic feature of cyclotides, is the ring structure formed by Cys1-Cys4 and Cys2-Cys5 disulfide bridges together and the third disulfide bridge formed by Cys3-Cys6 embed this ring to form CCK motif (Figure 4). This motif is extremely important as it gives chemical, thermal and enzymatic resistance to the peptide structure [50]. Cyclotides have an important role in drug research and development due to the outstanding stability of CCK motif and the cyclic backbone, their tolerance to sequence modifications, and the ability of some cyclotides to cross the cell membrane.

Cyclotides are divided into three sub-classes as Möbius, bracelet and trypsin inhibitors. The Möbius and bracelet cyclotides are similar but are separated by the presence or absence of a cis-Trp-Pro bond in loop 5, respectively. Examples of the most commonly used cyclotides in drug design studies as Möbius and Trypsin inhibitors are kalata B1 and MCoTI-II peptides, respectively. Bracelet subfamily peptides are not preferred very much in the studies due to the lack of conformation in the natural form of the peptide after they are synthetically produced [51,52].

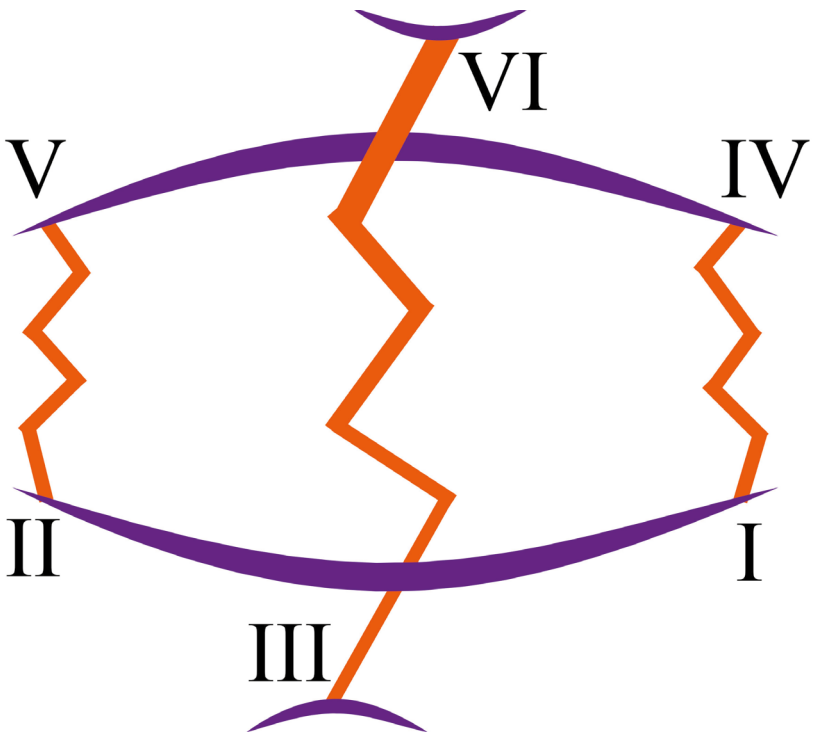

Figure 4. Cyclic cystine knot (CCK) motif. Three disulfide bonds are arranged in themselves in which two of them form a ring and the third one embeds this ring to form a knot motif. The CCK motif provides outstanding resistance to peptides against chemical, thermal and enzymatic effects. The peptide backbone is shown in purple and disulfide bridges in orange [Figure was adapted from reference Figure was adapted from reference].

In addition to the high stability of cyclotides, it has been shown that they also have many important bioactivities. The most important task of these peptides is to protect the plant against insects that was reported in a study in which cyclotides can inhibit the growth of *Helicoverpa larvae* (insecticide) [53]. Cyclotides have also been reported in many studies to have nematicide, molluscicide, anti-HIV and antimicrobial activities [54-57]. Because of these remarkable pharmacological properties, many research groups have been investigating cyclotides for a couple of decades.

The positive charge that gives the antimicrobial effect to cyclotides is extremely important for their membrane activities. The positively charged cyclotides can electrostatically interact with the negative charge on the membranes of tumor cells, bacteria and pathogens, break down the membranes of the cells and enter them. The bioactivities of Möbius and bracelet cyclotides change in relation to the presence or absence of phosphotylethanolamine (PE), one of the membrane phospholipids found in the membrane, and high affinities of these cyclotides against PE have been reported [58,59]. However, MCoTI-II, which does not have an affinity for PE, can enter the cell as a result of its interaction with phosphatidylinositol (PI) and phosphate acids (PA) in the membrane [60,61]. Furthermore, the first loop of cyclotides, particularly the Glu residue, is extremely important for membrane degrading activity [62]. The cytotoxic effects of cyclotides varv A and varv F belonging to the Möbius subfamily and cycloviolacin O2 from the bracelet subfamily were also examined and these cyclotides showed selective cytotoxic effects by interacting with cancer cells containing more negatively charged phosphatidylserine (PS) in their membranes compared to normal cells [63-65].

Strategies for Enhancing Peptide Stability

Although peptides have significant bioactivities, they degrade as proteins when taken orally due to the low pH of stomach content and their susceptibility to proteases such as trypsin, chymotrypsin, pepsin and carboxy peptidases. If the peptide medications that should be taken frequently for treatment would be taken by injection which is not preferred by the patients. Peptide drugs are given by parenteral injection due to their poor oral bioavailability. For example, the peptide hormone insulin is administered by injection to treat diabetes because of its susceptibility to various proteases in the body. Therefore, the stability of the peptide-based drug leads needs to be enhanced with various strategies to be resistant to proteases without affecting peptide bioactivity. Cyclization of peptide backbone, grafting and addition of unnatural amino acids to the peptide sequence or modifications of amino acids are important strategies for this purpose. In Figure 5 below, strategies that can be applied to increase peptide stability are given.

Cyclization of Peptide Backbone

Perhaps the most important strategy to improve the peptide stability is the cyclization of linear peptide backbone that has been inspired by the emergence of cyclic peptides with high stability (e.g. cyclotides) [66-70]. It has been possible to obtain peptide structures that are more resistant to proteolytic enzymes by using bridges formed between amino acids side chains or joining the peptide backbone N- and C-terminals using linker residues that has an appropriate length of the distance between the terminals of the peptide without disrupting the overall three-dimensional structures but to increase the peptide stability.

Conotoxins have limited stability to proteolytic enzymes due to their linear frameworks. The cyclization method has been one of the most preferred method for increasing the stability of these peptides. The most famous example is the cyclization of α-conotoxin Vc1.1 peptide backbone reported in 2010 [71]. In this study, considering the three-dimensional structure, the peptide was cyclized with six linker residues (GGAAGG) in accordance with the distance between the N- and C-terminals of Vc1.1. After cyclization, the three-dimensional structure of the peptide was preserved as native fold and the peptide stability was increased by 46% compared to linear Vc1.1. cVc1.1 has been also more stable in simulated gastric fluid and simulated intestinal fluid compared to linear Vc1.1 and orally active in rat model used in the study. Another example is the cyclization of an 18 amino acid long Gomesin peptide isolated from hemocytes of spider *Acanthoscurria gomesiana*. After cyclization, it's found that stability and cytotoxicity of this antimicrobial peptide to HeLa cells increased compared to its linear version [72].

Peptide backbone cyclization has been successfully applied on many other linear bioactive peptides, particularly on conotoxins, scorpion venom peptide chlorotoxin (CTX) and wasp venom mastoparan (MP-C) peptide [30,73-76].

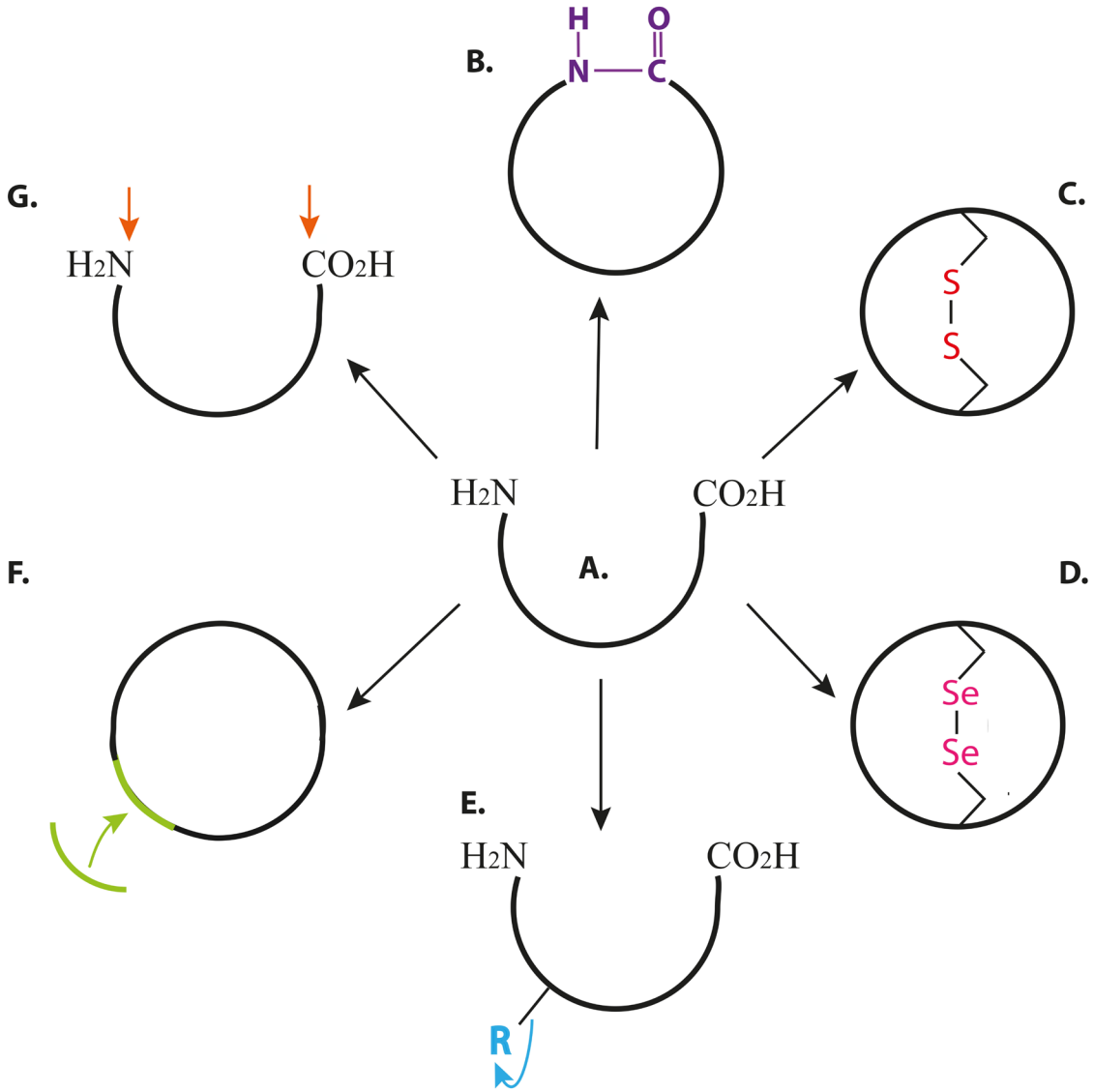

Figure 5. Strategies to increase peptide stability to make peptides resistant to proteases without affecting their bioactivities. **A.** linear peptide structure. **B.** head-to-tail peptide backbone cyclization. **C.** addition of disulfide bridges. **D.** addition of diselenide bridges. **E.** stereochemically inversion of amino acids (use of D-amino acids). **F.** grafting of an active peptide epitope and **G.** capping the N- and C-terminals [Figure was adapted from reference 6].

Grafting of Peptide Epitopes

Placing bioactive short linear peptide epitopes on the peptide scaffolds with proven stability is known as grafting. It has been reported in many studies that the stability of the bioactive linear epitope can be increased by grafting [77-80]. Since the plant-derived peptides kalata B1, MCoTI-II and SFTI-1 peptides are highly resistant to chemicals, heat and enzymes, they have been used more in many peptide-based drug development studies than other peptides to date. Since these prototypic peptides can also be synthesized by solid phase peptide synthesis (SPPS) methods and can easily be folded correctly as their native forms, peptide epitope grafting studies in the literature have generally been applied by utilizing these three peptides.

In the literature, grafting studies are mainly carried out on the sixth loop of cyclotides, which appears to be tolerant to sequence replacements. Because the first and fourth loops of cyclotides form the main center and contain a limited number of amino acids (e.g. loop 1 has 3-4 residues and loop 4 has only one residue). The second loop is important for peptide folding as it can form salt bridges. The structure and properties of the

peptide epitope are as important as the loop where the grafting will be performed. Abdul Ghani et al. studied the effect of structure and bioactivity by altering some mutual loops of kalata B1 and MCoTI-II and reported that the CCK motif is resistant to intact if covalent bond interactions do not occur, but bioactivity can vary greatly even with minor changes, including point mutations. Therefore, the previous literature data should be carefully examined before any grafting studies [67,68].

Thrombospondin-1 (TSP-1) is a fragment of a seven-amino acid peptide with an anti-antigenogenic effect. In order to increase the stability of this peptide, grafting of the seven-residue bioactive epitope (GVITRIR) of TSP-1 onto MCoTI-II and SFTI-1 was reported in 2015. For both synthesized grafted peptides, the stability of the peptide epitope was increased while maintaining its bioactivity inherent as in native TSP-1 [81].

In another study, the bradykinin B1 receptor antagonists DALK peptide with nine amino acid residues was grafted onto loop 6 of kalata B1 for inflammatory pain treatment and reported notable stable in human serum for more than six hours with almost 90% remained intact while DALK was degraded completely in this time period. In this study, it was also demonstrated that the grafted peptide was orally active [82]. In a following study, the peptide was also grafted separately on both loops of SFTI-1. The peptides were synthesized in both linear and cyclic forms [83]. The stability of each synthesized peptide was significantly increased, and the linear peptides exhibited less trypsin activity than bradykinin peptide epitope and cyclic grafted peptides.

Peptide Analogs

Peptide analogs harboring unnatural amino acids in the sequence are one of the strategies that can be used to enhance peptide stability against proteases. Polybia-CP, isolated from *Polybia paulista* wasp venom, is a 12 residue C-terminal amidated antimicrobial peptide. In 2017, Jia et al. synthesized its analog with D-amino acids in order to optimize the stability of this peptide and reported that the stability of the modified peptide was enhanced against trypsin and chymotrypsin enzymes. In the same study, the hemolytic effect of the peptide was significantly reduced compared to native Polybia-CP, although the antimicrobial effect was slightly reduced [84].

The plant-derived kalata B1 peptide achieves many important bioactivities by its electrostatic interaction with the cell membrane. In 2011, Sando and colleagues synthesized kalata B1 with D-amino acids and investigated its effects on cell membrane compared to kalata B1 in its native form. D-kalata B1 was reported to have lower hemolytic activity. In addition, although the membrane affinity of D-kalata B1 was less, its anti-HIV activity, cytotoxicity, and hemolytic effect continued even they have slightly decreased [85].

In addition to cyclization, grafting and the use of unnatural amino acids, replacement of disulfide bonds by diselenide bonds, covalent coupling of the peptide with polyethyleneglycol (PEG) or lipids and truncation or capping of the N- and C-terminals of the peptide have also been reported to increase the stability of the peptides [86-89].

In conclusion, disulfide-rich peptides found in plants and animals are promising drug scaffolds due to their high stabilities and diverse bioactivities including antimicrobial, anti-HIV, anticancer, analgesia, hypertension and more. Furthermore, although peptides have some drawbacks, they can be chemically modified to overcome the disadvantages such as stability and hemolytic activity and to contribute novel bioactivities.

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