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REVIEW ARTICLE

Therapeutic peptides: chemical strategies fortify peptides for enhanced disease treatment efficacy

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Abstract
Therapeutic peptides, as a unique form of medication composed of orderly arranged sequences of amino acids, are valued for their high affinity, specificity, low immunogenicity, and economical production costs. Currently, more than 100 peptides have already secured market approval. Over 150 are actively undergoing clinical trials, while an additional 400–600 are in the preclinical research stage. Despite this, their clinical application is limited by factors such as salt sensitivity, brief residence in the bloodstream, inadequate cellular uptake, and high structural flexibility. By employing suitable chemical methods to modify peptides, it is possible to regulate important physicochemical factors such as charge, hydrophobicity, conformation, amphiphilicity, and sequence that affect the physicochemical properties and biological activity of peptides. This can overcome the inherent deficiencies of peptides, enhance their pharmacokinetic properties and biological activity, and promote continuous progress in the field of research. A diverse array of modified peptides is currently being developed and investigated across numerous therapeutic fields. Drawing on the latest research, this review encapsulates the essential physicochemical factors and significant chemical modification strategies that influence the properties and biological activity of peptides as pharmaceuticals. It also assesses how physicochemical factors affect the application of peptide drugs in disease treatment and the effectiveness of chemical strategies in disease therapy. Concurrently, this review discusses the prospective advancements in therapeutic peptide development, with the goal of offering guidance for designing and optimizing therapeutic peptides and to delve deeper into the therapeutic potential of peptides for disease intervention.

Keywords Therapeutic peptides · Chemical strategies · Physicochemical factors · Disease treatment strategies · Druggability

Introduction

Peptides are molecules created by the covalent linkage of amino acids via amide bonds and typically contain fewer than 50 amino acids (Hayashi et al. 2012; Henninot et al. 2018). They are essential in fields such as medicine, pharmacy, and biochemistry. Following the landmark synthesis of insulin in 1921 as the inaugural therapeutic peptide, over 100 peptides have gained market approval, over 150 are undergoing clinical trials, and between 400 to 600 are in the preclinical stage of research (Lau and Dunn 2018; Muttenthaler et al. 2021; Otvos 2024). The rapid development of recombinant biotechnology, structural biology, peptide synthesis and purification techniques, and new analytical tools since the twenty-first century has greatly accelerated the research and development process of peptide drugs. It has also promoted the maturation of the peptide drug development pipeline, painted a bright future for the commercial development and large-scale production of peptide drugs, and ushered peptide drugs into a new era of development (Wang et al. 2022). As advancements in peptide pharmaceuticals continue, notably with the breakthrough application of GLP-1 drugs in weight loss, peptide drug sales have seen sustained growth, market expansion, and have become significant in managing a range of conditions such as infectious

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diseases, cancers, diabetes, orthopedic diseases, cardiovascular illnesses, and inflammation (Mozaffarian 2024; Muttenthaler et al. 2021).

Compared to small molecule drugs, peptide drugs exhibit higher target selectivity and specificity, allowing for more precise intervention in the onset and progression of diseases, thereby enhancing therapeutic efficacy and reducing side effects (Pechenov et al. 2021; Thakur et al. 2023). Compared to large molecule protein drugs, peptide drugs have more advanced synthesis technology, are easier to separate from impurities and by-products, have lower immunogenicity and production costs, and are more amenable to modification. Additionally, peptide drugs are favored for their high safety, strong penetration ability, shorter development cycles, and structural diversity. Despite significant advancements in peptide drug development, challenges such as salt sensitivity, brief circulation times, inadequate cellular uptake, and overly flexible conformation have limited their clinical application. These factors result in swift degradation and clearance within the body, preventing the attainment of effective therapeutic levels at the treatment site (Rezende et al. 2021; Zhang et al. 2024).

As a rapidly developing therapeutic method, the physicochemical properties, in vivo stability and biological activity of peptide drugs are closely related to their chemical structures (Aslan and Yuka 2024; Wang et al. 2022). Therefore, to further enhance the drug potential of peptide drugs and improve their shortcomings, chemical modification strategies, as an effective tool for peptide modification, have been widely adopted by researchers. For example, Maybauer et al. (Masri et al. 2020) developed selepressin by chemically modifying the enzymatic cleavage sites of vasopressin, which maintains the same target selectivity as vasopressin but exhibits better serum stability. Additionally, by chemically replacing natural amino acids with their analogs, the affinity and specificity of peptide drugs can be enhanced (Henninot et al. 2018; Masri et al. 2020). For example, homoarginine, β-phenylalanine, homoleucine, and benzyloxytyrosine, which are natural amino acids' analogs and commercially available non-natural amino acids, can be conveniently used for chemical modifications during peptide synthesis (Hone et al. 2019). Liraglutide and semaglutide, two GLP-1 analog drugs, have achieved enhanced affinity and target selectivity through the aforementioned chemical modifications (Del Olmo-Garcia and Merino-Torres 2018; Kelly et al. 2022; Scheen 2024). Moreover, the subtle forces within peptides, including hydrogen bonds, hydrophobic interactions, and van der Waals forces, are often insufficient to maintain their stable secondary structure. Altering the backbone or side chains chemically helps maintain the peptides' natural structure, leading to the development of more promising drug candidates (Li et al. 2020b, 2024). For instance, ALRN-6924 is a lymphoma treatment drug, currently undergoing Phase II clinical trials (Guerlavais et al. 2023; Meric-Bernstam et al. 2017; Saleh et al. 2021; Shustov et al. 2018). The side-chain cyclization in this drug stabilizes its secondary structure, enhances its targeting affinity to the proteins MDM2/MDMX, and boosts its antitumor activity. It can be seen that chemical modification strategies have effectively boosted the biological potency and enzymatic stability of peptide drugs. Among the many chemical modification strategies, amino acid substitution (Pan et al. 2022), lipidation (Gou et al. 2021; Jiang et al. 2018), PEGylation (Kim et al. 2021; Mood et al. 2021), cyclization (Mourao et al. 2020; Zhang et al. 2019), and glycosylation (Bednarska et al. 2017; Dwivedi et al. 2019) have shown significant advantages in improving the conformation, increasing bioavailability, enhancing bioactivity, and compatibility of peptide drugs.

To date, numerous outstanding review articles have delved into the discovery, modification, disease-related applications of therapeutic peptides, as well as the mechanisms of their interactions with host cells and targets (Fetse et al. 2023; Ghadiri et al. 2024; Li et al. 2021a; Wang et al. 2022). In this review, we aim to compile the latest research findings to elucidate the key physicochemical factors and chemical modification strategies that influence the properties and activity of peptide drugs (Fig. 1). We will also summarize how these physicochemical factors affect the peptide drugs' application in disease treatment and evaluate the efficacy of chemical modification strategies in therapeutic applications, with the aim of providing references and guidance for the design and optimization of therapeutic peptides.

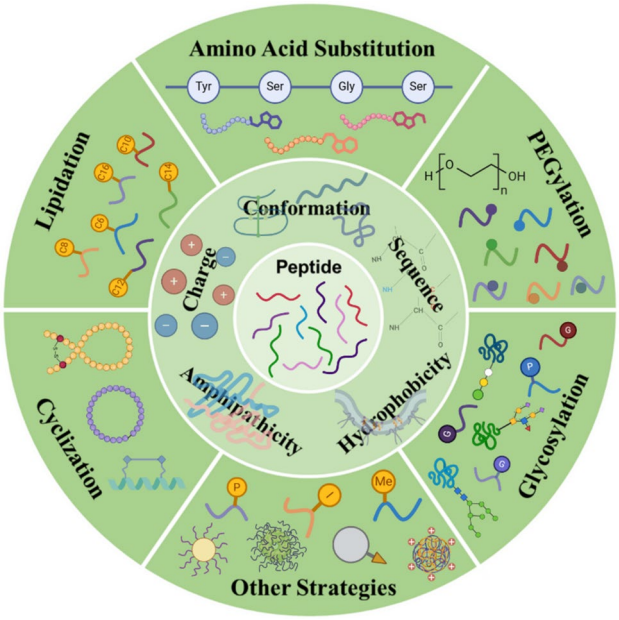

Fig. 1 Strategies for chemically modulating peptide physicochemical factors for disease treatment

Physicochemical factors controlling therapeutic peptides

Based on their antimicrobial mechanisms, therapeutic peptides are divided into two categories: membrane-disruptive peptides and non-membrane-disruptive peptides (Goki et al. 2024; Li et al. 2024). Membrane-disruptive peptides eliminate pathogens or tumor cells by disrupting cellular membrane structures. These peptides generally possess broad-spectrum antimicrobial or antitumor effects and are widely distributed in nature, encompassing both exogenous toxin peptides and endogenous host defense peptides. Owing to the wide range of antimicrobial characteristics and lower resistance risk, membrane-disruptive peptides are considered promising contenders for the next generation of antibiotics to tackle the threat of antibiotic-resistant superbugs (Li et al. 2021a; Thakur et al. 2022). However, they face challenges in development, including salt sensitivity, vulnerability to enzymatic breakdown, high toxicity towards mammalian cells, and lack of selectivity. Non-membrane-disruptive peptides, on the other hand, enter cells without disrupting cellular membrane structures and interact with intracellular proteins, blocking protein–protein interactions (PPIs), making them leading candidates in the field of PPI inhibitors and showing broad application prospects in treating various diseases (Li et al. 2020b, 2024). The main challenges for this class of peptides include susceptibility to enzymatic degradation, poor membrane permeability, and high conformational flexibility. Regardless of the type of peptide, their properties and biological activities are closely related to five physicochemical factors: charge, hydrophobicity, conformation, amphiphilicity, and sequence (Goki et al. 2024; Klepach et al. 2022; Thakur et al. 2022).

Charge

The charge of peptide drugs markedly influences their solubility, cell membrane interactions, transmembrane capabilities, and biocompatibility (Klepach et al. 2022). The isoelectric point (pI) refers to the pH value at which a peptide exhibits a net neutral charge, and this parameter helps predict the net charge state of peptide molecules under different pH conditions. Structural software can facilitate the acquisition of this information through calculations. However, as research into peptide drugs deepens and more complex structures continue to emerge, researchers are being incentivized to develop more advanced computational tools for a deeper analysis of peptide drugs. For example, Gauci et al. (2008) designed a calculator capable of determining the pI values of peptides with modifications including N-terminal acetylation or phosphorylation. Despite this, in the face of numerous modification strategies, there is still a need to develop more complex and accurate computational tools to meet these demands.

Cationic peptide drugs are typically rich in positively charged amino acids, including His, Arg, and Lys (Goki et al. 2024; Stone et al. 2019). These drugs are pivotal in disrupting cell membranes and enhancing membrane permeability and have been extensively studied. Research indicates that peptide drugs with a net charge between +2 and +9 under physiological pH conditions exhibit good membrane-disrupting effects and permeability (Li et al. 2017; Luo and Song 2021). They can interact electrostatically with negatively charged phospholipids, which is also a way to achieve targeted action of peptide drugs. However, peptide drugs with a higher net charge do not always have stronger activity and selectivity, as their strong electrostatic interactions with phospholipids may hinder their deep penetration into biological membranes and promote hemolysis. For instance, studies on magainin analogs have found that peptide drugs with a net charge of +4 exhibit the strongest membrane-disrupting activity, while higher net charges may trigger different mechanisms (Wang et al. 2019; Xiang et al. 2017).

In anionic peptide drugs, common negatively charged residues like Glu and Asp have a net charge between − 1 and − 7, including human dermcidin, mammalian SAPPs, amphibian maximin and cathelicidin, etc. (Luo et al. 2021; Zhang et al. 2021). These peptide drugs can achieve antimicrobial action by forming salt bridges with metal ions (such as Zn^{2+} and Ag^{+}) or by blocking signaling pathways to exhibit anti-inflammatory activity. Furthermore, research suggests that the bactericidal potency of anionic peptides is augmented with NaCl, yet diminished when phosphates or EDTA are present (Harris et al. 2009; Thakur et al. 2022).

Hydrophobicity

In the realm of small molecule and peptide drug design, assessing hydrophobicity has been proven to be of significant importance (Klepach et al. 2022). The hydrophobicity of peptide drugs is a key determinant of their membrane permeability, which is primarily influenced by hydrophobic amino acids, constituting approximately half of the total amino acids. The engagement of peptide drugs with cell membranes is predominantly governed by hydrophobic forces. Peptide drugs with low hydrophobicity struggle to form strong chemical bonds or interactions with cell membranes, affecting their permeability and selectivity. However, in some cases, excessive hydrophobicity may reduce the activity of peptide drugs, as increased aggregation could impede their engagement with cell membranes and cellular uptake. Therefore, it is crucial to differentiate between

the intrinsic hydrophobicity of a peptide calculated from its amino acid composition and the hydrophobicity obtained through molecular dynamics when exposed to a solvent environment, which is essential for drug design and development. In any technique for assessing hydrophobicity, this distinction must be fully considered (Klepach et al. 2022).

Recent research has uncovered an interesting phenomenon: the hydrophobicity of peptide drugs can differentially affect their penetration of mammalian and bacterial cell membranes, a finding that offers new possibilities for the development of antimicrobial drugs specifically targeting bacterial membranes (Andreev et al. 2018). The apparent binding constant (K_{app}) is an important metric for assessing how hydrophobicity affects membrane interactions and permeability, reflecting the binding affinity of different peptides to model membranes. A higher K_{app} value for a peptide with the membrane indicates a stronger affinity, which helps to more accurately predict the efficacy and specificity of peptide drugs (Domingues et al. 2020). Chemical modification strategies that enhance peptide drugs' hydrophobicity through introducing hydrophobic amino acids have, according to numerous studies, been shown to increase their biological activity (Li et al. 2020b, 2018, 2024; Wu et al. 2019). For instance, Li group (Li et al. 2018) and the Wu group (Wu et al. 2019) significantly increased the hydrophobicity of toxin peptides by cyclization and the introduction of hydrophobic amino acid residues, effectively enhancing their antitumor activity.

Conformation

When interacting with cell membranes, peptide drugs can form various structures, including β-sheets, β-turns, α-helices, and loops, with β-structures and α-helices being the predominant form (Li et al. 2020c). Numerous studies have uncovered the conformational changes of peptide drugs under different environments. Peptide drugs containing α-helices typically exhibit random coils in watery solutions, but they transition into α-helices upon encountering membrane-mimicking solvent environments or interact with anionic membranes. It is particularly noteworthy that the negative charges in the phospholipid bilayer of the membrane structure facilitate this transition (Klepach et al. 2022). Research on cecropin analogs has shown that the number of negatively charged phospholipids in the model membrane influences the degree of α-helical structure formation, and the inherent packing of phospholipids also contributes to the folding of α-helices. Peptide drugs containing α-helices must interact with the cell membrane when targeting cell membrane or intracellular targets. Therefore, the formation of α-helical structures is crucial for their activity and is significant for selectively recognizing target surfaces (Haney et al. 2019). However, owing to the constraints imparted by β-sheet backbone or disulfide bond cyclization in peptide drugs, peptide drugs containing β-structures are relatively stable and less prone to conformational changes when transitioning from aqueous solutions to membrane environments.

Beyond the direct effect on biological activity, the secondary structure of peptide drugs also has a profound influence on their stability, bioavailability, pharmacokinetic properties, targeting, and physicochemical properties (Goki et al. 2024; Henninot et al. 2018). Through chemical modification techniques, peptide drugs' secondary structure can be optimized to reduce their polar surface area, thereby enhancing drug stability and decreasing the risk of in vivo degradation by proteases and peptidases (Buckton et al. 2021). The secondary structure stability directly affects the pharmacokinetic properties of peptide drugs, including distribution, metabolism, and excretion. Chemically modified peptide drugs, due to the enhanced structural stability, can reduce degradation in the body, extending their half-life and bioavailability (Lee et al. 2019a; Vinogradov et al. 2019). Moreover, a well-designed secondary structure can boost the binding affinity and specificity of peptide drugs to their specific targets, improve receptor selectivity, reduce binding to off-target receptors, and thus mitigate drug side effects (Ullrich and Nitsche 2024). Additionally, optimization of the secondary structure can also enhance peptide drugs' physicochemical properties, including solubility and stability, which are crucial for drug formulation development and the selection of administration routes (Fosgerau and Hoffmann 2015). Therefore, peptide drugs' secondary structure has a significant impact on their physicochemical characteristics and pharmacological effects, and optimizing secondary structure is a key strategy for improving their druggability.

Amphipathicity

The ratio and distribution of hydrophilic and hydrophobic regions in peptide drugs are known as amphiphilicity (Thakur et al. 2022). Charge, hydrophobicity, and secondary structure are three key factors affecting peptide drugs, which together determine the amphiphilicity of peptides. Any changes in these parameters may lead to changes in the amphiphilicity of peptide drugs. When interacting with bacterial membranes, peptide drugs typically fold into amphiphilic structures. There are various methods to achieve amphiphilicity, among which the amphiphilic helix wheel is one of the most fundamental and widely used methods (Yount and Yeaman 2013). The periodic structure of amphiphilic α-helical peptides with a 3.6 residue period makes them particularly suitable for interacting with bacterial membranes. Moreover, the properties of amphiphilic helices, when interacting with neutral membranes, can significantly affect the hemolytic potential and biological activity. High amphiphilicity can result in the creation of a single

hydrophobic domain, which could increase toxicity towards cells containing neutral lipids (Goki et al. 2024).

The hydrophobic moment (HM), introduced by Eisenberg et al. (Eisenberg et al. 1982) in 1982, serves to quantify amphiphilicity. It characterizes the alignment of hydrophobic residues along the peptide's axis in periodic structures like β-sheets or α-helices. The HM value can reflect the degree of amphiphilicity of peptide molecules, reflecting the spatial segregation between hydrophilic and hydrophobic residues (Mishra et al. 2017). With an escalating HM value in model peptides, there is a corresponding rise in their membrane permeability and hemolytic activity. There is evidence to show that if peptide drugs act on membranes through electrostatic and hydrophobic interactions, an increase of HM value minimally impacts interactions with negatively charged membranes. Conversely, in the case of neutral host cell membranes, toxicity may be enhanced with an increase in HM value, primarily because the reduced electrostatic pull between neutral phospholipids and peptides (Bechinger and Gorr 2017; Maturana et al. 2017). Therefore, an appropriate HM value helps to enrich peptide drugs in specific tissues or cells, reduces toxicity to normal cells, and enhances the efficacy of peptide drugs.

Sequence

The length of peptide drugs greatly influences their biological activity, and typically requires more than 7 amino acids to form peptides with amphiphilic structures (Thakur et al. 2022). For example, membrane-disruptive peptides that follow the barrel-stave model to kill bacteria need to align parallel to the bacterial membrane and eventually penetrate it vertically. This process requires a minimum of eight amino acids for the formation of β-sheet peptides or up to 22 for α-helical peptides to effectively exert their antibacterial action. In contrast, shorter peptides may have weaker membrane binding capabilities, form unstable pores on the membrane, and are less likely to adopt secondary structures like α-helices (Thakur et al. 2022). The peptide length not only affects their conformation and mechanism but may also impact their biocompatibility (Narayana et al. 2021). For instance, the peptide KR12, obtained by truncating LL-37, maintained the same antibacterial activity as LL-37 but had lower cytotoxicity (Narayana et al. 2021).

Peptide drugs comprise a variety of amino acid residues, including charged residues like Lys and Arg, hydrophobic ones such as Val, Phe, and Trp, and hydrophilic ones like Cys, Thr, Asn, and others. Statistical data reveal that Gly, Ala, Lys, and Leu in amphibian peptides, Gly and Ala in bacterial peptides, Gly, Lys, and Ala in insect peptides, and Gly and Cys in plant peptides, are particularly prevalent, each constituting more than 10% of the composition (He and Deber 2024; Travkova et al. 2017). The peptide drugs' physicochemical properties are directly influenced by their amino acid sequences, so their physicochemical properties can be optimized by adjusting the sequences (Cardoso et al. 2018). Moreover, cationic residues like Lys and Arg are prevalent in peptide drugs and significantly contribute to electrostatic binding with the negatively charged elements of biological membranes (Shagaghi et al. 2018). Generally, Arg and Trp are two essential amino acid residues for potent antibacterial effects (Straus 2024; Zhu et al. 2022). The distinctive hydrogen bonding and positive charge of Arg, along with the intricate nature of Trp, enhance the interaction of peptide drugs with membranes. Arg's positive charge enhances peptide drug adsorption to membranes, while hydrogen bonding further support their interaction with cell membranes. Significantly, Arg can engage in cation-π interactions with aromatic residues, forming hydrogen bonds, a capability that Lys lacks. This difference leads to peptide drugs containing Arg being more active than those containing Lys. In addition, many studies have amply confirmed that Trp residues are drawn to the lipid bilayer's interfacial region and are ideal amino acid residues for better binding of peptide drugs to membranes (Straus 2024; Zhu et al. 2022).

Integrating unnatural amino acids into the sequence design of peptide drugs offers considerable benefits. Peptide sequences enriched with unnatural amino acids can effectively resist degradation by proteases, enhancing the metabolic stability of peptide inhibitors and improving their pharmacokinetic properties (Armiento et al. 2020; Ji et al. 2022). Moreover, incorporating unnatural amino acids can reduce peptide drugs' immunogenicity, as the human immune system primarily recognizes peptide segments composed of L-amino acids. Incorporating unnatural amino acids can mitigate immune responses caused by the drug, thereby enhancing the drug's safety and efficacy (Abdulbagi et al. 2021; Miyamoto and Homma 2018). The lower toxicity of metabolites from unnatural amino acid-based peptide sequences typically results in minimal side effects and a higher safety profile for human consumption (Luo et al. 2018). For instance, Zhao's group (Wu et al. 2020) significantly enhanced the enzymatic stability of peptide drugs and improved the druggability of bee venom peptide HYL by employing D-amino acids. Hence, the incorporation of unnatural amino acids into peptide sequences has emerged as a key strategy in peptide drug design.

Chemical modification strategy regulating therapeutic peptides

Peptide post-modification is a key approach in developing new peptides and expanding their functional range. These modifications typically target nucleophilic groups like amino, thiol, and hydroxyl groups to help organisms

dynamically regulate peptide structure and function in different cellular environments (Li et al. 2021a). Chemical modification strategies, as an important means of peptide post-modification, not only provide significant support for studying the mechanisms of peptide post-modification and constructing new peptides in vitro to enrich peptide functions, but also offer indispensable technical means for adjusting the physicochemical factors of peptide drugs, improving their physicochemical properties, enhancing their proteolytic stability, and increasing their biological activity in disease treatment (Gan et al. 2021; Lenci and Trabocchi 2020; Song et al. 2024). A variety of chemical modification tactics have been widely utilized to enhance peptide drugs, including amino acid substitution, lipidation, PEGylation, cyclization, and glycosylation. These strategies have shown significant advantages in regulating the physicochemical factors and properties of peptide drugs to better achieve disease treatment.

Amino acid substitution

Enhancing peptide drugs' physicochemical properties and biological activity can be straightforwardly achieved by substituting one or more amino acids with alternative L-amino acids. For instance, by modifying the natural antimicrobial peptide magainin II (H-GIGKFLHSAKKFGKAFVGEIMNS-OH) with cationic and hydrophobic amino acids, the analog peptide pexiganan (H-GIGKFLKKAKKFGKAFVKILKK-NH_2) was successfully obtained (Gottler and Ramamoorthy 2009). The modified analog pexiganan has shown enhanced antimicrobial activity and significant broad-spectrum antibacterial effects. Similarly, several other synthetic peptide drugs obtained through L-amino acid substitutions have successfully advanced to later stages of clinical trials, including iseganan (Trotti et al. 2004), omiganan (Sader et al. 2004), and P11 (Yip et al. 2009).

Human peptides and proteins predominantly consist of L-amino acids, which are readily targeted and broken down by enzymes. Because of these enzyme specificity, D-amino acids exhibit greater resistance to enzymatic degradation. Thus, replacing L-amino acids with D-amino acids can significantly improve therapeutic peptides' stability against proteolysis (Domhan et al. 2019). Additionally, swapping L-amino acids with D-variants can also decrease the incidence of anti-PEG antibody production and reduce the toxicity associated with peptide-PEG conjugates in animal models (Sylvestre et al. 2021). A substantial amount of research has shown that replacing L-amino acids with D-variants in peptide drugs can effectively prevent proteolysis (Kumar et al. 2018; Shao et al. 2019; Ting et al. 2020). Generally, peptides can undergo complete or partial D-amino acid substitution. In peptide sequences, the complete replacement of L-amino acids with D-variants yields enantiomers, while partial replacement produces different diastereomers (Gomes et al. 2018). However, it's crucial to recognize that while D-amino acid substitution can increase peptide stability, it has the potential to affect peptide's biological activity. This occurs as the D-variant incorporation may change the peptide structure and the ability to engage with targets, contingent on where and how many D-amino acids are substituted. Therefore, it is vital to ensure that D-amino acid incorporation doesn't substantially modify the peptide's secondary structure. For example, Wang et al. (Zhao et al. 2016) extracted a lysine-rich natural antimicrobial peptide from wasp venom, named MPI. They synthesized the D-enantiomer of this peptide, discovering that it notably enhanced protease resistance and preserved its antimicrobial potency against a range of bacteria, encompassing Gram-positive strains, Gram-negative strains, and fungi. Nonetheless, altering the stereochemistry of lysine residues in MPI led to a decrease in antimicrobial activity, primarily attributed to the destabilization of the peptide's secondary structure. Li et al. (2019) discovered a semi-D-amino acid substituted variant, D-Arg-W3R6, of the antimicrobial peptide W3R6, which maintained the parent peptide's antimicrobial activity. In contrast, the fully D-amino acid substituted D-enantiomer D-W3R6 exhibited significantly reduced antimicrobial effects. Both analogs exhibited good proteolytic stability. Over recent years, various strategies have been devised for synthesizing D-peptides with complete D-amino acid substitution, among which the method of directly obtaining D-peptides using mirror phage display technology has received increasing attention (Adaligil et al. 2019; Zhou et al. 2020). For example, Zhou et al. (Zhou et al. 2020) discovered a D-peptide capable of blocking the immune checkpoint TIGIT, potentially positioning it as a candidate for cancer immunotherapy. Additionally, computer-aided design has emerged as a novel approach for the de novo design of D-peptides. For instance, Yang et al. (2019) synthesized a D-peptide that could bind to tumor necrosis factor α (TNF-α) and prevent its binding to the TNFR1 receptor. Although D-variant replacement is frequently employed to lower peptides' susceptibility to proteolysis, it's worth mentioning that D-variants may not have specific receptors within mammalian cells, potentially affecting the immunomodulatory effects of peptide drugs (Gan et al. 2022; Shao et al. 2019).

Beyond the L/D-amino acid replacement strategy, another method to boost the resistance to proteolysis and the efficacy of peptide drugs is incorporating unnatural amino acids. In a study, researchers replaced the Trp of the peptide with the unnatural amino acid Azulenyl-Alanine (AzAla). The modified peptide demonstrated increased resistance to proteases, preserved its antimicrobial properties, and displayed decreased hemolytic toxicity alongside improved cell compatibility than the original peptide (Ridgway et al. 2015). In another study, Wang et al. (2012) substituted an unnatural

amino acid for phenylalanine at the C-terminus of endomorphin and found that the endomorphin analogs, incorporating unnatural amino acids, not only had stronger metabolic stability but also to some extent increased the biological activity of endomorphin on the μ receptor. Furthermore, cationic antimicrobial peptide Pepo5 derivatives designed by combining non-natural amino acids like norleucine, 4-aminobutyric acid (Aib), L-thiazolidine-4-carboxylic acid, L-2,4-diamino-butyric acid (Dab), and L-2,3-diaminopropionic acid (Dap), as well as D-amino acids, showed better proteolytic stability while maintaining their original antimicrobial activity and lower cytotoxicity, indicating better potential for drug development (Lu et al. 2020). TX-109 (Fig. 2A) is a tripeptide composed of a hydrophobic non-natural tryptophan residue and two arginine residues, developed by Lytix Biopharma et al. (Gan et al. 2022; Nilsson et al. 2015), and completed a Phase II clinical trial in 2014 to evaluate its activity and safety. Despite no new trials being registered, the successful case of TX-109 highlights the potential of using non-natural amino acids in the development of therapeutic peptides.

Lipidation

Most naturally occurring therapeutic lipopeptides with significant antimicrobial effects are primarily extracted from various microorganisms, including the *Bacillus genus*, *Paenibacillus genus*, *Pseudomonas genus*, and *Microbispora genus* (Cochrane and Vederas 2016; Sunna et al. 2017). Some naturally sourced lipopeptides, such as polymyxin B, daptomycin, and colistin, have received FDA approval for clinical infection treatment. Although advancements in employing fatty acids for structural modifications have been relatively slow, fatty acids, as an endogenous substance in the body, have always attracted the attention of researchers and have been widely applied in the lipophilic modification of peptide drugs.

Lipidation modification is a process in which fatty acids are introduced at specific sites of peptide drugs through chemical methods. These fatty acids can be combined with peptide drugs in the form of amide bonds, ester bonds (S- or O-), or S-bonds (ether or disulfide) to improve their hydrophobicity, self-assembly characteristics and secondary structure (Zhang and Bulaj 2012). This modification can enhance the stability of peptide drugs, extend their half-life, and is of great importance for improving pharmacokinetic and pharmacodynamic performance. At the same time, due to the similarity of fatty acids to the phospholipid structures on the surface of cell membranes, lipidated peptide drugs can usually increase the lipophilicity of peptides, thereby improving drug absorption in the intestine and mucosal permeability. In addition, fatty acids can bind to serum albumin, and the resulting complexes, due to their increased molecular size, are less likely to be transported, thereby extending the residence time of peptides within the body (Rizzuti et al. 2015).

Multiple studies have indeed confirmed that modifying antimicrobial peptides with lipidation can effectively enhance their antibacterial activity. For instance, research by Metzler-Nolte and colleagues (Albada et al. 2012) found that the lipidation modification at the C-terminus or N-terminus of the peptide $(Arg\text{-}Trp)_3$ significantly enhanced its antibacterial effects against *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Mukhopadhyay and colleagues (Joshi et al. 2018) reported a lipopeptide S-8, which contains hydrophobic aryl and alkyl non-natural amino acids, bookended by Arg and 1-naphthyl-D-alanine (Fig. 2B). S-8, with an "internal" fatty acid chain, showed very low minimum inhibitory concentration (MIC) values against

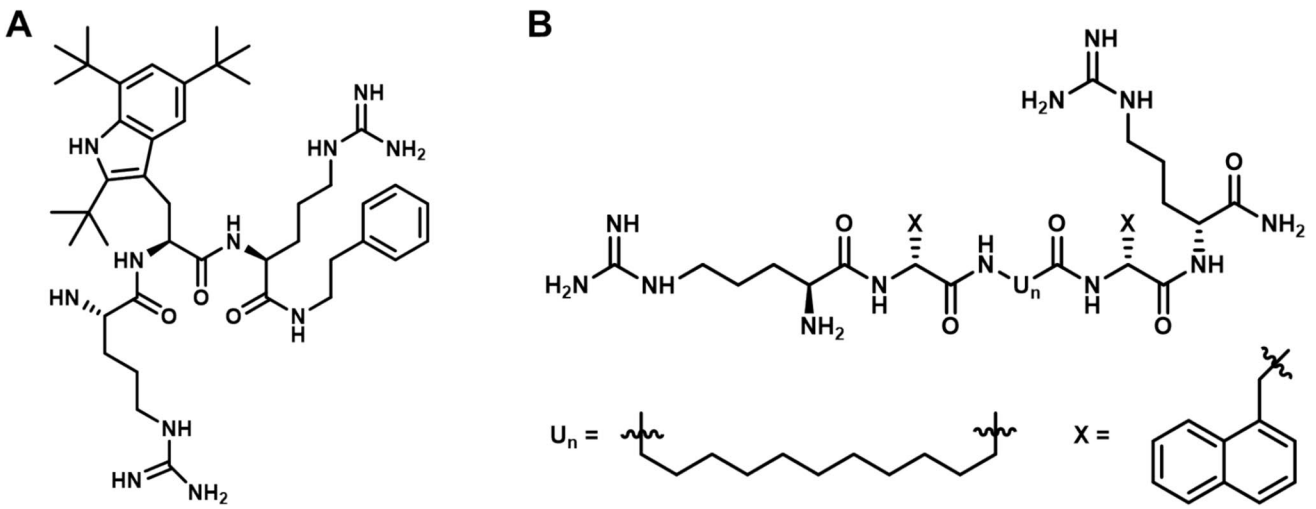

Fig. 2 The structure of LTX-109 (Gan et al. 2022; Nilsson et al. 2015) (**A**) and S-8 (Joshi et al. 2018) (**B**)

clinically relevant *Staphylococcus* strains through membrane depolarization, while exhibiting low hemolysis and activity against vancomycin-resistant bacterial biofilms. Additionally, Toth and colleagues (Azmi et al. 2015, 2016) reported several branched lipidated antimicrobial peptides containing varying compositions and arrangements of Lys and 2-aminododecanoic acid. These analogs, characterized by multiple fatty acid chains and a high positive charge, exhibited strong antibacterial effects against Gram-positive bacteria, low toxicity to human cell lines, and increased sensitivity to trypsin.

Additionally, bivalirudin, a hirudin-derived peptide simplified from the structure of leech venom, is an anticoagulant drug developed by The Medicines Company (Table 1), which gained FDA approval for marketing in December 2000, mainly used for unstable angina in percutaneous transluminal coronary angioplasty and percutaneous coronary intervention (Liu et al. 2015). However, as a peptide drug, bivalirudin has a low exposure (AUC_{0-t} = 23.7 nmol min mL^{-1}) and a short half-life ($t_{1/2}$ = 15.1 min), with less than desirable pharmacokinetic properties. To overcome these shortcomings, researchers chemically modified bivalirudin analog 73 through a lipidation strategy. Comparing the two bivalirudin analogs, 73 and 74, it was found that their pharmacological activities remained essentially unchanged, but peptide 74, which was modified with a fatty acid, showed a significant increase in exposure (AUC_{0-t} = 1371.7 nmol min mL^{-1}) and half-life ($t_{1/2}$ = 212.2 min) compared to the unmodified peptide 73 (AUC_{0-t} = 25.7 nmol min mL^{-1}, $t_{1/2}$ = 13.5 min), with the exposure and half-life increasing by 58 times and 14 times, respectively (Table 1).

Liraglutide (Sjoholm 2010), Semaglutide (Lau et al. 2015) and Tirzepatide (France and Syed 2024), three successfully marketed hypoglycemic peptide drugs, all introduce lipidation modifications derived from the natural glucagon-like peptide-1 (GLP-1). Incorporating fatty acids enhances the drugs' hydrophobicity, masks the dipeptidyl peptidase-4 binding sites, reduces renal excretion, and thereby increases the half-life (Fig. 3). Liraglutide, a long-acting GLP-1 receptor agonist from Novo Nordisk, can form a stable heptamer at the injection site, effectively masking the dipeptidyl peptidase-4 binding sites, and forming a reversible complex with serum albumin. This significantly extends the absorption period of liraglutide within the body and augments the peptide drug's half-life (Sjoholm 2010). Furthermore, Semaglutide introduces an octadecanoic acid through glutamic acid as a linker on the side chain of the 26th lysine, enhancing its hydrophobicity and significantly extending its half-life to a week (Lau et al. 2015). Moreover, in Tirzepatide, the C20 fatty diacid group is linked to the peptide main chain via a γ-Glu linker. This structural modification significantly enhances the drug's stability and bioavailability, while simultaneously reducing its rapid metabolism within the body (France and Syed 2024).

Although lipidation has demonstrated efficacy in boosting the biological potency and physicochemical traits of peptide pharmaceuticals, this modification may also increase the affinity of peptides for various biological membranes (Lee et al. 2019b; Rounds and Straus 2020; Zhong et al. 2020). Therefore, the lipidation of peptide drugs may lead to a decrease in membrane or target protein specificity and an increase in hemolysis rates. However, this disadvantage can be effectively overcome by combining lipidation with other chemical modification techniques.

Table 1 Incorporating fatty acid to enhance the pharmacokinetic properties of bivalirudin analogs (Liu et al. 2015)

Peptide	Sequence	PK parameter	
		AUC_{0-t} (nmol min mL^{-1})	$t_{1/2}$ (min)
Bivalirudin(**72**)	DFPRPGGGGQGDFEEIPEEYL	23.7 ± 2.8	15.1 ± 1.3
73	DFPRPGGGGQGDFEPIPEDAYDE	25.7 ± 2.6	13.5 ± 2.6
74	DFPRPGK(stearic acid)GGQGDFEPI-PEDAYDE	1371.7 ± 207.8	212.2 ± 58.4

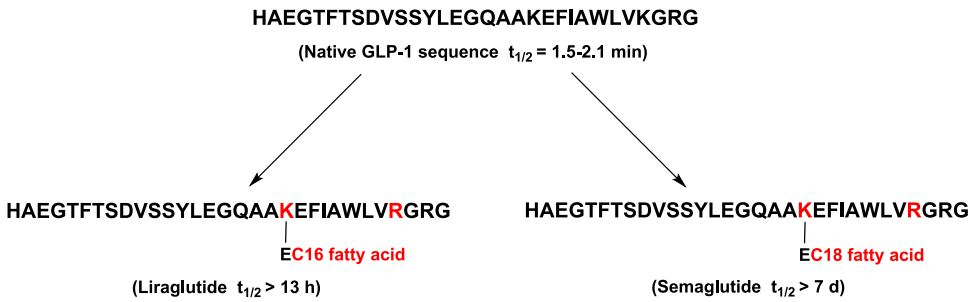

Fig. 3 Incorporating fatty acid to enhance the half-life of GLP-1 analogues (Lau et al. 2015)

PEGylation

Polyethylene glycol (PEG), recognized as an active pharmaceutical polymer, is characterized by its inert nature and high solubility in water, as well as in both polar and non-polar solvents (Sousa et al. 2018). The structure of PEG can be linear, branched, or multi-armed, and the process of conjugating PEG to therapeutic agents is referred to as "PEGylation," which relies on the appropriate selection of molecular weight and the chemical bond used to attach to the target molecule. Additionally, PEG exhibits degradability and low toxicity in the body, making it a common choice for modifying peptide drugs to enhance their properties and biological activity. PEG is typically attached to peptide-based drugs, offering substantial benefits, such as extending serum half-life, improving aqueous solubility, and increasing stability, biocompatibility (Gomes et al. 2018; Roberts et al. 2012). PEGylation can also increase the molecular weight and hydrodynamic radius of peptide drugs, thereby reducing glomerular filtration by the kidneys (Hamley 2014).

Currently, there are various PEG-modified peptide drugs on the market, among which PEG-modified interferon α stands out as a successful example of this structural modification strategy (Fig. 4). Interferon α can effectively inhibit or clear hepatitis B or C viruses, but as a peptide drug, it has the disadvantages of being easily degraded by enzymes, having a short half-life, and requiring frequent injections. To address these issues, Schering-Plough Research Institute (SPRI) introduced PEG into interferon α, enlarging the molecule's size and decreasing the likelihood of glomerular filtration by the kidneys, thereby extending the half-life (Gao et al. 2024). However, PEGylation may reduce the binding capacity of the peptide to target proteins, thereby affecting its therapeutic effect. Consequently, PEG-modified interferon α may reduce receptor binding due to the introduction of PEG, diminishing the antiviral activity of interferon α. To counteract this, SPRI conducted in-depth research on the size of PEG and ultimately determined that using a 12 kDa PEG could extend the half-life while preserving the antiviral activity of interferon α to the greatest extent possible (Gao et al. 2024).

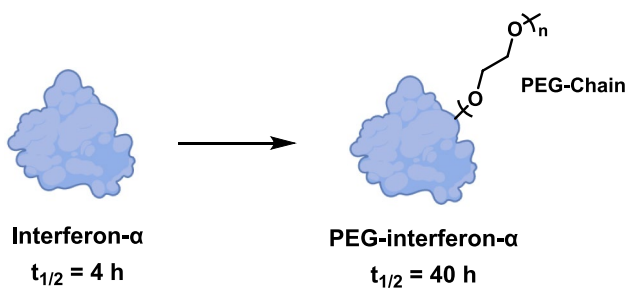

Fig. 4 Enhancing the half-life of interferon-α through PEGylation

PEGylation is also an effective strategy for reducing cytotoxicity and hemolytic effects, as well as improving activity. It has been reported that by PEGylating the N-terminus of an innovative composite peptide (CaLL, composed of cecropin A fragments and LL-37), PEG-CaLL (Fig. 5A) was obtained, which protects the peptide from degradation by serum, reduces hemolytic toxicity, and enhances the therapeutic effect on pulmonary infections (Morris et al. 2012). Furthermore, in experiments with a rat lung model, PEG-CaLL did not damage the integrity of the pulmonary epithelium, indicating its potential safety in managing bacterial pneumonia. In addition, Kim et al. (Kim et al. 2021) designed peptide sequences with a PEG backbone, known as PEGtides, using a modular approach. These sequences exhibit strong bactericidal effects and cell specificity, thereby increasing the therapeutic index of peptide drugs and demonstrating an innovative peptidomimetic PEGylation modification strategy (Fig. 5B).

Additionally, while PEGylation can reduce cytotoxicity and hemolytic effects, it may also diminish the peptide's affinity for biological membranes and intracellular targets, thereby reducing its biological activity (Singh et al. 2014). This phenomenon is likely due to the steric hindrance from PEG molecules, which alters the peptide's structure, disrupts its binding to biological membranes and intracellular targets, and consequently impairs its biological effects (Christian et al. 2009). For instance, compared to the native peptides, PEGylation of tachyplesin and magainin resulted in a significant reduction in cytotoxicity, and it also diminished their efficacy against *Escherichia coli* and *Staphylococcus epidermidis* (Imura et al. 2007). Furthermore, Malmsten and colleagues (Singh et al. 2014) also confirmed that PEGylation of the antimicrobial peptide KYE28 led to a reduction in its antimicrobial potency, an effect that was related to the length of the PEG chain. Nevertheless, the PEGylated peptide drugs demonstrated a marked decrease in hemolytic toxicity and enhanced bacterial selectivity when mixed with bacteria and blood cells. Therefore, when employing PEGylation modification strategies, it is necessary to carefully balance the relationships between the half-life, selectivity, cytotoxicity, and activity of peptide drugs.

Cyclization

Cyclization, a frequently employed and straightforward modification technique for peptide drugs, can bring a series of favorable properties to peptidomimetics and linear peptides, including improving target selectivity and specificity, increasing cell permeability, and enhancing stability (Zorzi et al. 2017). Cyclic peptides, due to their fixed geometric shape, reduce the entropy cost of binding, enabling them to bind to targets more effectively and selectively. In contrast, linear peptides, because of their

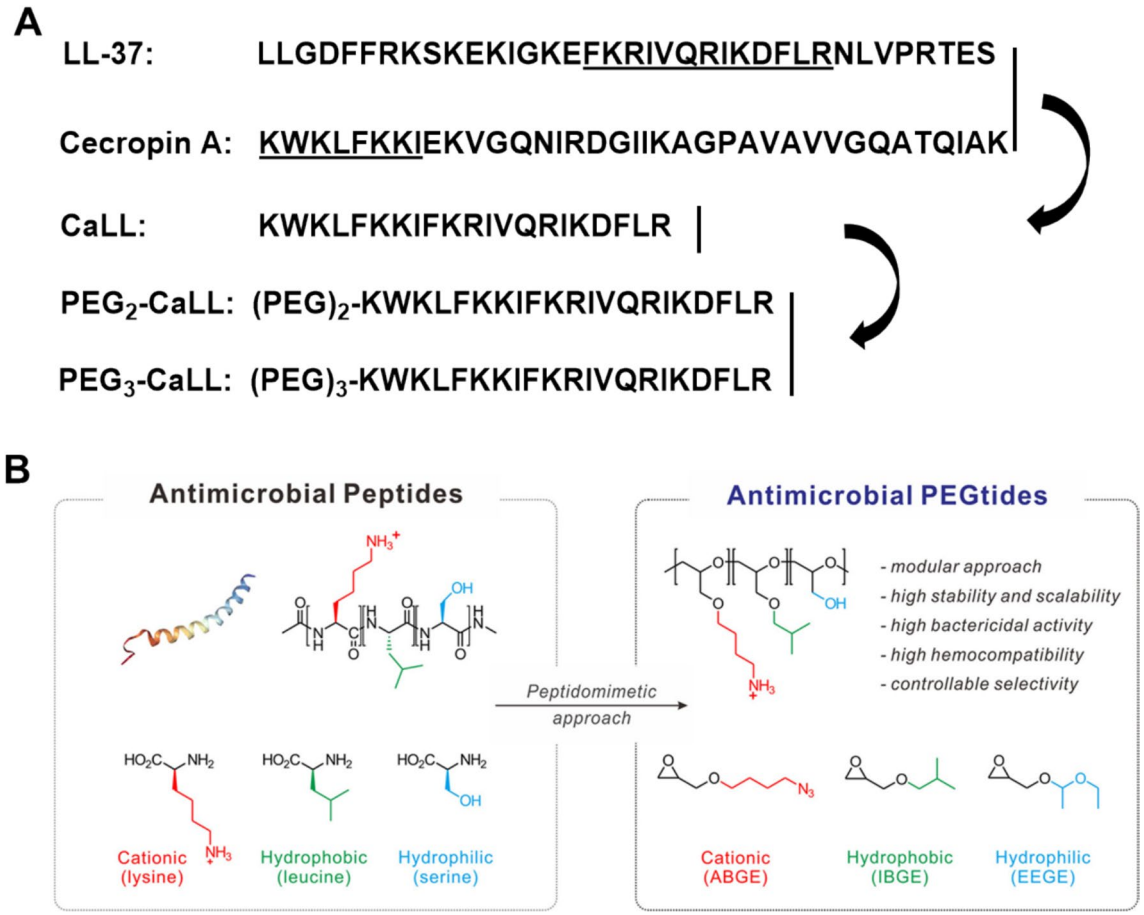

Fig. 5 **A** Sequence data of peptides PEG-CaLL. The underlined amino acids indicate the sequences that are integrated to form CaLL (Morris et al. 2012). **B** The antimicrobial PEGtides with PEG backbone was simulated by modular method through antimicrobial peptides (Kim et al. 2021). Adopted from Kim et al. (2021)

high conformational flexibility, may lead to non-specific and off-target interactions, thereby elevating the risk of adverse side effects. Cyclization can encompass a range of strategies, including backbone—backbone cyclization, side chain—backbone cyclization, and side chain—side chain cyclization (Fig. 6) (Mourao et al. 2020). The cyclization of peptide drugs can increase proteolytic stability (Ricardo et al. 2020; Yang et al. 2018) and cell permeability (Dougherty et al. 2019; Perry et al. 2018; Tian et al. 2017), and effectively rigidify the peptides' secondary configurations. Typically, a single peptide sequence is incapable of forming turn or loop structure on its own, but cyclization strategies promote secondary structure formation by pre-organizing interactions within the peptide molecules (Li et al. 2020a; Miao et al. 2018). Therefore, peptide cyclization is also a common strategy

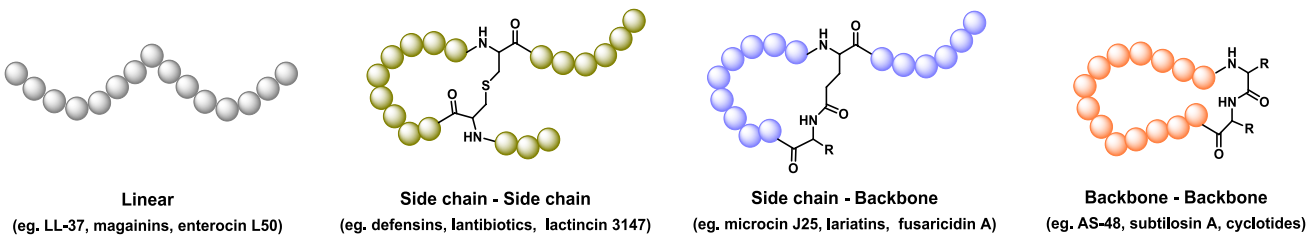

Fig. 6 The methods of peptide cyclization, including side chain—side chain cyclization, side chain—backbone cyclization, and backbone—backbone cyclization (Mourao et al. 2020; White and Yudin 2011)

to stabilize secondary structures, including β-sheets and α-helices (Neukirchen et al. 2016; Ricardo et al. 2019).

Cyclic peptides are increasingly being used in therapeutics to modulate protein–protein interactions, diagnostics, enzyme inhibition, imaging, and RNA binding, leading to an exponential growth trend in the application of cyclization strategies in peptide drug development (Choi and Joo 2020). A multitude of research has shown that cyclic peptides often exhibit excellent physicochemical properties and activity, thanks to their improved resistance to proteolysis and increased conformational rigidity. Moreover, cyclization can also enhance cell selectivity, thereby reducing toxicity to host cells (Ting et al. 2020; Wang 2012). Recently, various innovative methods have recently emerged for designing, synthesizing, and evaluating cyclic peptides. Drawing inspiration from the natural cyclic defensins' intramolecular cysteine disulfide bridges, Lai and colleagues (Mwangi et al. 2019) successfully developed a cyclized antimicrobial peptide formed through disulfide bonds. They introduced two cysteine residues into the peptide cathelicidin-BF15-a3 extracted from snake venom, and the resulting cyclic peptide demonstrated good activity against *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, with minimal hemolysis, enhanced stability, and a reduced propensity for resistance development. Hancock and colleagues (de la Fuente-Núñez et al. 2014; Etayash et al. 2020) studied the effects of various cyclization methods on the antimicrobial, anti-biofilm, and immunomodulatory activities of the linear peptide IDR-1018. The cyclization methods used included head—tail cyclization, cyclization of the Glu carboxyl with the N-terminal amino group, and cyclization through the introduction of cysteine at both ends of the peptide by disulfide bonds. Among the three cyclization methods, the macrocyclization produced by side chain—tail cyclization showed strong anti-inflammatory ability in a mouse skin infection model and significantly reduced bacterial numbers. Additionally, Fetse et al. (Cheng et al. 2022) discovered cyclic peptides capable of antitumor action by inhibiting PD-1/PD-L1 interactions using a macrocyclization screening method. Compared to the parent peptide, the cyclic derivative's PD-L1 blocking activity was increased by 34 times, and serum stability was improved by 4 times. The study indicates that macrocyclization screening is an invaluable technique for peptide drug development, particularly in scenarios where structural details of peptide-receptor interactions are scarce.

α-helices are a common secondary structural feature of many peptide drugs, but synthetic peptides often fail to maintain stable α-helical structures in aqueous solutions. This instability increases the likelihood of peptide hydrolysis, weakens binding to targets, and thus results in reduced peptide stability and biological potency (Li et al. 2020b, 2024). To address this issue, researchers have developed various backbone-restricting methods to preserve the α-helical configuration of peptides. Peptides obtained through these methods are known as stapled peptides, which are essentially a form of cyclization modification strategy (Fig. 7A). ALRN-6924 (Fig. 7B) is a stapled peptide obtained through an all-hydrocarbon stapling approach, serving as a typical representative of MDM2/MDMX antagonists (Guerlavais et al. 2023; Meric-Bernstam et al. 2017; Saleh et al. 2021; Shustov et al. 2018). Currently, the antagonist ALRN-6924 is in Phase II clinical trials, showing promise to treat advanced lymphomas and solid tumors. To boost the pharmacodynamics and pharmacokinetics of peptide PDI, Pentelute et al. (Lautrette et al. 2016) used a lysine N-aromatization stapling strategy, adding two lysine residues at the i and i + 7 positions, retaining PDI's key residues, and synthesized a perfluoroaromatized stapled peptide. Stapled peptide SPDI-14 (Fig. 7C) exhibits higher enzymatic stability compared to linear peptides. Furthermore, compared to unstapled peptides, SPDI-14 binds more effectively to target proteins.

Overall, peptide cyclization strategies have shown significant advantages in enhancing the stability, cell permeability, target affinity, bioavailability, and efficacy of peptide pharmaceuticals, marking a significant trend in peptide drug development. With the continuous advancement of synthetic technology and the emergence of innovative strategies, the future is expected to yield more potent cyclic peptide pharmaceuticals to address a broad spectrum of therapeutic demands for various illnesses.

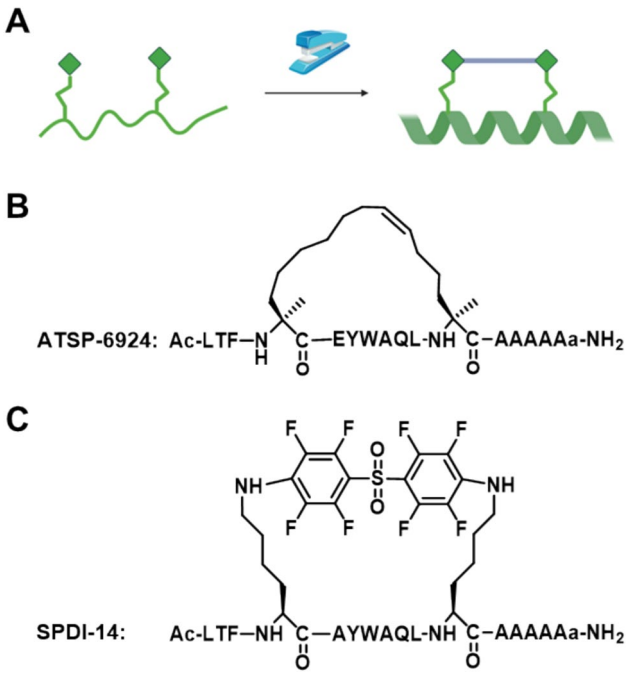

Fig. 7 **A** Schematic illustration of the stapled strategy. **B**, **C** Structure of ALRN-6924 (**B**) and SPDI-14 (**C**)

Glycosylation

Glycosylation of proteins, a key post-translational modification, significantly enriches the diversity of protein structure and function. Glycan groups are covalently attached to different amino acid residues of proteins or peptides, and based on the mode of attachment, they can be categorized into four types: O-glycosylation (attaching to Thr, Ser, or Tyr), N-glycosylation (attaching to Asn), C-glycosylation (attaching to Trp), and S-glycosylation (attaching to Cys) (Fig. 8) (Li et al. 2021a). The presence of glycan groups exhibits a range of favorable physiological and biological properties, including aiding in protein folding, promoting protein recognition, enhancing immune responses, and improving target specificity, thereby amplifying the biological activity of peptide drugs (Moradi et al. 2016). Moreover, glycosylation occurring at specific amino acid positions can affect the stereochemistry, rigidity, and activity of peptide drugs (Lele et al. 2015). At the same time, the nature of glycosylation also hinges on the characteristics of the sugar-peptide bond interactions and can be further varied considering the glycosidic bond, oligosaccharide components, chain length, and overall structure (Bednarska et al. 2017). Therefore, glycosylation strategies are widely regarded as a highly promising method that can increase the hydrophilicity of peptides, improve bioavailability, regulate and stabilize peptide conformation, and enhance their resistance to proteolysis.

O-glycosylation stands out as a prevalent form of post-translational modification found in organisms across the board. Attaching glycosyl moieties to Ser or Thr residues in peptides through covalent bonds can form a spectrum of O-glycosylated peptides, enhancing their biological activities. The types of O-glycosylated peptides include mucin-type O-glycosylation (attaching N-acetylgalactosamine GalNAc), O-glucosylation, O-fucosylation, and O-mannosylation (Maky et al. 2015). Some research groups have found that removing the glycosylation part from the intestinal bacteriocin F4-9 results in the total elimination of its antimicrobial efficacy (Hoffmann et al. 1999; Uttenweiler-Joseph et al. 1998). Considering the significant impact of tryptophan on hemolytic activity, Kaur et al. (Dwivedi et al. 2019) designed an indole derivative, and replaced the aromatic amino acid with O-glycosylated threonine, thereby decreasing cytotoxic effects on mammalian cells while enhancing therapeutic selectivity. The presence of sugar limited the conformational flexibility of O-glycosylated indoles and promoted their interactions in micellar environments. Moreover, the glycosylation of indoles did not alter the peptides' mechanism, cytokine inhibition, and LPS binding, but reduced their toxicity to red blood cells and macrophages. O-glycosylation in peptide drugs generally increases peptide stability as well. Wu et al. (2020) used O-glycosylation strategies with a variety of 12 monosaccharides to investigate their impact on peptide physicochemical properties and antitumor activity. They discovered that several O-glycosylated peptides could improve enzymatic stability and cell selectivity, and enhance the antitumor activity of peptides. Currently reported O-glycosylated peptides indicate that sugar moieties not only boost stability and biological potency but also help receptor recognition without disrupting the membrane. In addition, the amino groups and moderate lipophilicity of sugar moieties also are pivotal to the activity of peptide drugs (Li et al. 2021a).

Natural N-glycosides are predominantly attached to the side chain of Asn and typically occur at the Asn-Xxx-Ser/Thr tripeptide sequences within the body. Chemically synthesized N-glycosylated peptides often include one or more glycosylated amino acids as synthetic units in the peptide assembly process. Guo et al. (Hu et al. 2009) utilized readily available phenyl-protected N-glycosylated amino acids, directly incorporating them into solid-phase peptide synthesis, to produce tyrocidine A analogs with monosaccharides, disaccharides, or lactose attached to the side chain of Asn. They found that the directly attaching the sugar moiety could influence the conformation and biological potency of tyrocidine A, while a short linker between the sugar moiety and asparagine could preserve its effective biological efficacy, resulting in the generation of more effective novel N-glycosylated peptides. Hu and colleagues (Zou et al. 2015) further expanded the N-glycosylated tyrocidine A analogues through attaching various monosaccharides and disaccharides to short linkers. By incorporating N-glycosylated amino acids, they synthesized N-glycosylated tyrocidine A with strong antimicrobial effects against vancomycin-resistant *Enterococci* and methicillin-resistant *Staphylococcus aureus*. Their research indicated that a short linker between tyrocidine A and α-glycosides positively affects their efficacy, whereas

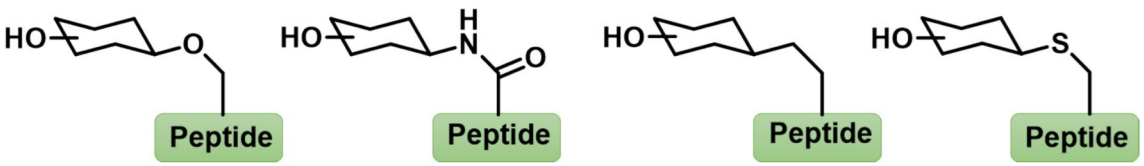

Fig. 8 Forms of peptide glycosylation, including N-glycosylation, O-glycosylation, C-glycosylation, S-glycosylation

a longer linker could be advantageous for β-glycosides. Chan-Park et al. (Li et al. 2012) utilized polymer chemistry techniques to combine poly-L-lysine with chitosan, generating a range of cationic chitosan-poly-L-lysine (CS-g-Kn) through N-carboxy anhydride ring-opening polymerization, which demonstrated considerable antimicrobial effects against Gram-negative bacteria. Glycosylated peptides have structural affinity for cell wall components of fungi and bacteria, leading to strong membrane permeability and membrane disruption capabilities.

S-glycosylation peptides, as thiol-linked analogs of O-glycosides, have been found in human urine. S-glycosides exhibit stability in both chemical and enzymatic aspects, making them a reasonable structural alternative to O-glycosides, and their higher flexibility helps maintain the biological activity of peptide drugs (De Leon et al. 2017). The journal Nature has reported several glycosylated peptides containing S-glycosylation, including glycocin F, ASM1, and thurandacins A/B (Bédard and Biron 2018; Norris and Patchett 2016). In addition, Donk et al. (Biswas et al. 2021) discovered that S-glycosylated sublancin analogs, including galactosylation, mannosylation, and N-acetylglucosaminylation, exhibit antimicrobial activity similar to that of natural S-glycosylated sublancin and have superior activity compared to non-glycosylated analogs. Cationic polysaccharide peptides, similar to peptidoglycans, show high selectivity for microorganisms, but their synthesis involves various chemical syntheses or heavy metal catalysts (Li et al. 2012). To forgo copper catalysts, Li and colleagues (Su et al. 2017) combined various antimicrobial peptides with three distinct biocompatible chitosans through copper-free thiol-ene "click" chemistry. This approach yielded several polysaccharide glycosylated peptides within an environmentally friendly and low-toxicity manner, expanding antimicrobial activity to antifungal activity. Early studies on the structure–activity relationship of enzymatic S-glycosylated peptide drugs indicate that glycosylation is a necessary condition for biological activity, but the stereochemistry of the hexose part is not crucial (Biswas et al. 2021; Wang et al. 2014). Moreover, replacing O-GlcNAc with S-linked GlcNAc enhances bactericidal activity, providing insights for the design of more effective antibiotic peptides (Amso et al. 2018). Detailed studies on the mechanism of glycocin F show that the S-glycosylation part helps peptide drugs target membrane targets (Bisset et al. 2018). Compared with O-/N-glycosylated peptides, S-glycosylated peptides have improved stability against extracellular and bacterial glycosidases and transglycosidases (Triola et al. 2010), and with the latest advancements for synthesizing S-glycosylated amino acids (De Leon et al. 2018) and enzymatic S-glycosylation (Brunsveld et al. 2006; Zhang et al. 2008), the modification and optimization scope of S-glycosylated peptide drugs will be greatly expanded.

In addition to integrating glycosylated amino acids into peptide sequences, carbohydrate groups can also be selectively coupled with peptide sequences. Verly et al. (Junior et al. 2017) utilized the well-established copper(I)-catalyzed azide-alkyne cycloaddition (CuAAc) reaction to develop more efficient antifungal peptides. They coupled monosaccharide fragments to the known antimicrobial peptide hylaseptin-P1 through a triazole linker, and the resulting glycopeptides exhibited higher biological activity, including stronger membrane interaction and membrane disruption capabilities. Furthermore, Gomes and colleagues (Barbosa et al. 2017) covalently linked the arginine-containing antimicrobial peptide Dhvar-5 to chitosan through a copper-promoted click chemical reaction, and this peptide, fixed on the surface of the polysaccharide compound, inhibited bacterial attachment and caused bacterial demise. Like other glycosylation modifications, C-glycosylation modifications help peptide drugs interact more effectively with targets and minimize toxicity to mammalian cells. The modification of glycosyl groups using bioconjugate chemistry techniques can further promote the utility and evolution of effective antimicrobial peptides targeting pathogens.

Glycosylation is an extremely promising tool, which not only enhances the biological activity of drugs but also preserves their specific conformation, crucial for stereospecific receptor recognition (Bisset et al. 2018). Chemical glycosylation can also alter the peptide drugs' physicochemical traits, surmount challenges related to glycosidase resistance, and enhance therapeutic applications through non-natural modifications (Ghaderi et al. 2012). As proteolytically stable drugs, chemical glycopeptides have been developed in various fields such as oncology, vaccine development, and the creation of antifreeze proteins (Szekely et al. 2014). Importantly, chemical glycosylation not only produces more potent peptides against pathogens but also aids in stabilizing peptide conformation for receptor recognition, improves peptide stability against various enzymatic hydrolyses, and uniquely impacts the peptide drug sequence in each instance.

Other strategies

In addition to the aforementioned chemical modification strategies, other methods like N-methylation (McBrayer et al. 2019; Savitha et al. 2020), side-chain halogenation (Kemker et al. 2021; Molchanova et al. 2020), and guanidination (Berthold et al. 2013) have also seen substantial advancements in peptide drug development. These methods are pivotal for improving the stability, bioavailability, and biological effects of peptide drugs and show broad application prospects.

The nitrogen atom alkylation within peptide amide bonds is a key chemical modification that can significantly enhance the druggability and pharmacokinetic properties of

therapeutic peptides, including absorption, half-life, and bioavailability (Li et al. 2021b). Among the many N-alkylation techniques, N-methylation has been the most frequently utilized due to its universality and synthetic convenience. N-methylation modification improves the stability of peptide drugs by increasing steric hindrance, which encourages the adoption of a cis-conformation in peptides. For example, McBrayer et al. (2019) confirmed that N-methylation substantially enhances peptide resistance to proteolysis, extending the half-life of the *Enterococcus faecalis* quorum-sensing regulatory peptide over sixfold. Another study assessed the activity of N-methylated peptide inhibitors against neutral endopeptidase, aminopeptidase N, and angiotensin-converting enzyme amidst their natural substrates. The findings showed that N-methylation made the peptide derivatives exhibit stronger antihypertensive, antihypertrophic, and antifibrotic activities (Savitha et al. 2020).

Halogenation is a rising technique for modulating the pharmacological activity of organic compounds, including peptide drugs (Sana et al. 2022). Of the halogens, fluorine, iodine, chlorine, and bromine are commonly integrated into drug molecules to boost their biological potency. Particularly, chlorine and fluorine have seen extensive use in drug discovery. Halogenation is often linked to enhancing target specificity, improving cell membrane permeability, and reducing side effects. For instance, incorporating halogenated tryptophans into RGD peptides enhances their specificity and binding affinity for integrin αvβ3 (Kemker et al. 2021). Similarly, Molchanova et al. (2020) explored the effects of different halogens on peptide antimicrobial potency and found that chlorine- or bromine-substituted peptides can enhance antimicrobial effects against Gram-positive bacteria, while fluorination had little impact. Although halogenation can bestow beneficial traits upon peptide pharmaceuticals, swapping polar groups for halogens might heighten peptide hydrophobicity, potentially leading to aggregation and loss of activity in peptide drugs.

The quaternary guanidinium group, in guanidination modification, does not directly participate in target inhibition but plays a role due to its significant physical properties and a high pK_a value of 13.8 (Li et al. 2021a). Moreover, the positive charge carried by the ionized guanidinium group can form strong interactions with phosphate groups through hydrogen bonding, a mechanism that further enhances the antimicrobial effects of guanidinated peptides (Peng et al. 2017). Hoffmann and colleagues (Berthold et al. 2013) designed an antimicrobial peptide featuring N, N, N′, N′-tetramethylguanidine, which has the most positive charge among its counterparts and significantly enhances antimicrobial activity, showing effective action against Gram-negative bacteria.

Relative to other techniques, these aforementioned modification methods, though not extensively used in peptide drug development, hold promise to become part of the toolkit in future peptide engineering research to combat various diseases. These strategies open up more possibilities for new therapeutic drug development by enhancing the stability, bioavailability, and biological activity of peptide drugs. With continuous research and technological advancements, it is expected that these strategies will gain wider application in the field of peptide drugs.

Conclusions and perspectives

Interest in using peptides as drugs for treating various diseases has surged in recent years thanks to their distinctive biochemical attributes and broad healing capabilities, making them ideal candidates for treatment. Since 2019, at least 15 therapeutic peptides have been approved for clinical use (Table 2). Although therapeutic peptides have achieved notable success, their broader application is still constrained by challenges such as limited membrane permeability and vulnerability to enzymatic breakdown. By introducing chemical modification methods such as amino acid substitution, lipidation, PEGylation, cyclization, glycosylation, etc., the stability and biological activity of peptide pharmaceuticals can be markedly enhanced. To date, over 80 therapeutic peptide drugs have been commercialized globally, while numerous others are in preclinical research and clinical trials. These peptide pharmaceuticals have seen broad application in diabetes, cardiovascular conditions, gastrointestinal disorders, oncology, infectious diseases, and vaccine creation. The advancements in semaglutide, a GLP-1 receptor agonist by Novo Nordisk, and tirzepatide, a GLP-1/GIP dual agonist by Eli Lilly, for diabetes and weight loss have occupied an important position in the global market and shown strong growth momentum. The global GLP-1 drug market has exceeded 30 billion US dollars and is projected to grow a scale of 165 billion US dollars by 2031 (Long et al. 2024; Mozaffarian 2024; Yue et al. 2024). Considering the huge therapeutic potential, market opportunities, and economic value of peptide drugs, therapeutic peptide will continue garnering investment and research, achieving continuous progress and further success.

The immunogenicity of therapeutic peptide drugs refers to their ability to induce an immune response in the body, which is primarily manifested as the production of anti-drug antibodies (Jankowski et al. 2023; Shankar et al. 2014). Immunogenicity may lead to reduced drug efficacy, altered pharmacokinetic and pharmacodynamic properties, and even serious immune-related adverse reactions. The assessment of immunogenicity risk for therapeutic peptides requires a comprehensive consideration of multiple factors, including: (1) Molecular characteristics: such as molecular size and structure. Typically, peptide drugs with fewer than 8 amino

Table 2 FDA-approved therapeutic peptides in recent years

Peptide	Mechanism	Indication	Modification strategy
Tirzepatide	GLP-1 agonist	Type 2 diabetes	Amino acid substitution, lipidation
Semaglutide	GLP-1 agonist	Type 2 diabetes	Amino acid substitution, lipidation
Melphalan	DNA alkylating agent	Multiple myeloma and amyloid light-chain amyloidosis	Amino acid substitution, drug conjugate
Voclosporin	Calcineurin inhibitor	Lupus nephritis	Amino acid substitution, N-alkylation
Vosoritide	C-type natriuretic peptide analog	Achondroplasia	Cyclization
Pegcetacoplan	Binds to complement protein C3 and its activation fragment C3b	Paroxysmal nocturnal hemoglobinuria	Amino acid substitution, Cyclization, PEGylation, N-alkylation
Dasiglucagon	Glucagon analog	Hypoglycemia in diabetics	Amino acid substitution
Piflufolastat-F18	PSMA targeting	PET of PSMA-positive lesions in men with prostate cancer	Urea-based modification
Difelikefalin	Kappa opioid receptor agonist	Pruritus associated with chronic kidney disease	Amino acid substitution
Odevixibat	Inhibitor of ileal bile acid transporter	Pruritus in patients aged over 3 months with progressive familial intrahepatic cholestasis	Amino acid substitution
Setmelanotide	MC4R activator	Chronic weight management	Amino acid substitution, cyclization
^{64}Cu-DOTATA TE	Somatostatin receptor agonist	Scintigraphic imaging	Amino acid substitution
^{68}Ga-PSMA-11	PSMA	Diagnosis of recurrent prostate carcinoma	Urea-based modification
Afamelanotide	MC1R receptor agonist	Erythropoietic protoporphyria	Amino acid substitution, N-alkylation
Bremelanotide	Melanocortin receptor agonist	HSDD	Amino acid substitution, N-alkylation, cyclization

acids are not expected to be immunogenic unless impurities or polymers are present; (2) Impurities and polymers: Impurities or polymers that may be introduced during the manufacturing process can lead to unexpected immunogenicity; (3) Patient factors: such as disease status, route of administration, and dosing frequency (De Groot et al. 2023; Roberts et al. 2024). Chemical modification strategies not only enhance enzymatic stability and membrane permeability, but some, like the substitution of unnatural amino acids, also reduce immunogenicity. Recent research has shown that peptides with unnatural amino acids exhibit decreased affinity for HLA class II molecules, thereby reducing T-cell activation (Azam et al. 2021).

It's noteworthy that peptide drugs are gaining increasing attention for treating drug-resistant bacterial infections (Gan et al. 2022; Li et al. 2021a; Thakur et al. 2022). Although antibiotic resistance poses a growing threat, there is a measured approach from pharmaceutical companies towards the development of new antibiotics. Currently, a limited number of antibiotics can address infections from drug-resistant superbugs, with colistin—dubbed the "antibiotic of last resort", yet it is recognized for its potential nephrotoxicity in humans (Cai et al. 2020; Whitehead et al. 2023). However, the discovery of the MCR-1 gene, the first plasmid-mediated colistin resistance gene reported in 2016, has compelled the global community to directly confront the antibiotic resistance crisis (Liu et al. 2016). Confronted with the possibility of running out of effective antibiotics to treat drug-resistant bacteria, the quest for new treatment methods as alternatives to traditional drugs becomes particularly urgent. The discovery of antimicrobial peptides provides us with an excellent opportunity for developing potential therapeutic alternatives to replace the current, less effective antibiotics. Antimicrobial peptides, obtained from diverse natural sources, possess unique mechanisms that are less likely to induce resistance, as well as broad-spectrum antimicrobial activity and potent immunomodulatory and anticancer properties, positioning them as feasible substitutes for traditional antimicrobials and immunomodulatory therapies. However, issues including instability, toxicity, and side effects have impeded their clinical advancement. Thus far, research have shown that, in contrast to traditional antibiotics, several natural antimicrobial peptides have not yet successfully evolved into therapeutic entities in clinical trials. Although the challenges in introducing antimicrobial peptides into clinical applications, various chemical methods to enhance their metabolic stability, as well as advanced schemes to reduce the synthesis cost, are anticipated to expedite their development as therapeutic agents in the coming years.

It's important to recognize that every chemical modification approach has its constraints, and no single method can provide the perfect combination of all ideal therapeutic peptide characteristics (Fetse et al. 2023; Maciuszek et al. 2021). For instance, while the D-amino acid substitution

strategy can markedly enhance peptide resistance to proteolysis, it could also affect the conformation and efficacy of the modified peptides. Likewise, lipidation strategies might boost peptide hydrophobicity, thereby raising the likelihood of aggregation within biological contexts. Nonetheless, by combining different chemical modification strategies, peptides with significantly improved properties and biological activity can be produced, thus promoting the deeper entry of peptide drugs into clinical research. Additionally, intrinsic factors such as peptide sequence, charge, hydrophobicity, conformation, and amphiphilicity should be considered in rational design, as these parameters significantly affect antimicrobial potency, hemolytic potential, mammalian cell toxicity, and immunomodulatory effects. Crafting peptide pharmaceuticals is typically a protracted, costly, and resource-intensive endeavor. Employing computational techniques, including machine learning and deep learning, can help predict and optimize peptide drugs' biological effects and stability. With the rapid development of artificial intelligence technology, AI-driven approaches to peptide drug design and optimization are poised to streamline the process, making it faster, more affordable, and highly precise. To encapsulate, peptide therapeutics is a rapidly growing domain within the pharmaceutical sector, holding much promise with numerous breakthroughs on the horizon. This has ignited significant interest among drug companies and researchers, which will likely spur further advancements in peptide drug development.

Author contributions Qingmei Li gathered and organized the literature and drafted the manuscript and Wen Chao assisted with the illustrations and documentation. Lijuan Qiu initiated the writing, and edited the manuscript. All contributors engaged in discussions and made revisions to the manuscript.

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Declarations

Conflict of interest The authors have declared that no competing interest exists.

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