



# Advances in the Study of Structural Modification and Biological Activities of Anoplin

Ye Wu, Rui Huang, Jin-Mei Jin, Li-Jun Zhang, Hong Zhang, Hong-Zhuan Chen, Li-Li Chen\* and Xin Luan\*

Institute of Interdisciplinary Integrative Medicine Research, Shanghai University of Traditional Chinese Medicine, Shanghai, China

Anoplin is an amphipathic,  $\alpha$ -helical bioactive peptide from wasp venom. In recent years, pharmaceutical and organic chemists discovered that anoplin and its derivatives showed multiple pharmacological activities in antibacterial, antitumor, antifungal, and antimalarial activities. Owing to the simple and unique structure and diverse biological activities, anoplin has attracted considerable research interests. This review highlights the advances in structural modification, biological activities, and the outlook of anoplin in order to provide a basis for new drug design and delivery.

**Keywords:** anoplin, structural modification, structure–activity relationship, antimicrobial activity, antitumor activity

## OPEN ACCESS

### Edited by:

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### \*Correspondence:

Li-Li Chen  
llchen@shutcm.edu.cn  
Xin Luan  
luanxin@shutcm.edu.cn

### Specialty section:

This article was submitted to  
Medicinal and Pharmaceutical  
Chemistry,  
a section of the journal  
Frontiers in Chemistry

**Received:** 20 March 2020

**Accepted:** 19 May 2020

**Published:** 07 July 2020

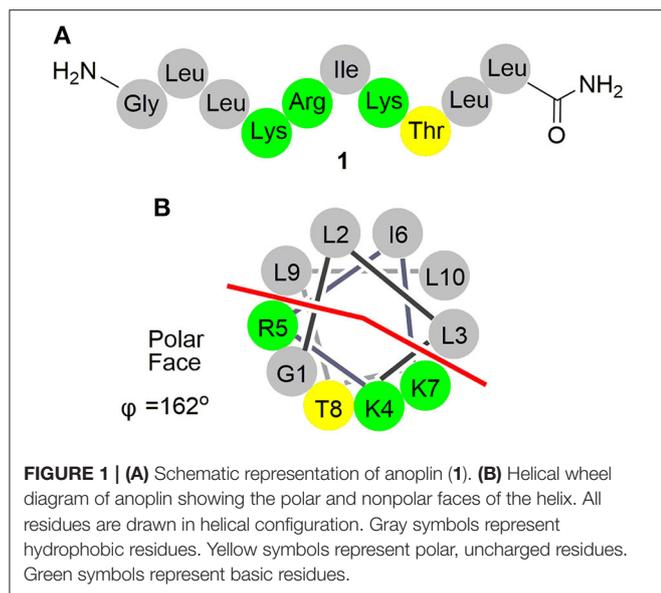
### Citation:

Wu Y, Huang R, Jin J-M, Zhang L-J,  
Zhang H, Chen H-Z, Chen L-L and  
Luan X (2020) Advances in the Study  
of Structural Modification and  
Biological Activities of Anoplin.  
*Front. Chem.* 8:519.  
doi: 10.3389/fchem.2020.00519

## INTRODUCTION

Wasp venom is the light yellow, transparent liquid secreted by the venom glands and accessory glands of wasp; it is stored in a poison sac and discharged by sting (Monteiro et al., 2009). In general, venom is used as a protective measure against the invaders that threaten their survival or colony (Hou et al., 2014). It is worth mentioning that, for wasps, venom is also used during the hunting of prey (Yan et al., 2011). Wasp venom is mainly composed of a few biological active peptides (like mast cell degranulating peptides and mastoparan) (Argiolas and Pisano, 1985; Monteiro et al., 2009), active enzymes (phospholipase A2 and hyaluronidase) (Tsai et al., 2011), histamine, 5-hydroxytryptamine, choline, glycerin, amino acids, and so on (Schmidt, 1982). Among them, peptides in the wasp venom usually exhibit superior pharmacological effects and a promising clinical value (Arifuzzaman et al., 2019; Li et al., 2019).

As a wasp venom peptide, anoplin (**1**, **Figure 1**) was isolated from the venom sac of the Japanese solitary spider wasp *Anoplius samariensis* (Hisada et al., 2000). Anoplin is the shortest, amphipathic, linear  $\alpha$ -helical antimicrobial peptide (AMP) with only 10 residues (Gly-Leu-Leu-Lys-Arg-Ile-Lys-Thr-Leu-Leu-NH<sub>2</sub>) (Konno et al., 2001; Jittikoon, 2015); it also exhibits a wide range of biological activities including antibacterial (Konno et al., 2001; Monincová et al., 2010), mast cell degranulating (Cabrera et al., 2009), antitumor (Zhu et al., 2013; Da Silva et al., 2018; Kai et al., 2018), antimalarial (Carter et al., 2013), antifungal (Jindrichova et al., 2014), and anti-inflammatory activities (Zhong et al., 2020b). Anoplin exerts its functions by direct interaction with anionic bilayers and biological membranes via ion channels (Cabrera et al., 2008; Leung et al., 2011), selectively binding to the bacterial DNA or inhibiting ATP synthase (Syed et al., 2018). Owing to its extremely simple structure and nonhemolytic toxicity, anoplin exhibits superiority in chemical manipulation, structure–activity relationship studies, mechanisms of action, and medical application, which has a great potential as a novel class of drugs for antibiotics and anticancer applications. Given the fact that there is no report on research progress



about structural modification of anoplin so far, we have summarized the advances in structural modification and relevant pharmaceutical activities to push the further application of anoplin.

## STRUCTURAL MODIFICATION OF ANOPLIN

### Point Mutation and Truncation

The Hansen lab (Ibrahim et al., 2005) firstly conducted the structure–function and structure–toxicity relationships research of anoplin by point mutation, C- and N-terminal truncation. Terminus truncations indicated that the full length of decapeptide is necessary for antibacterial activity toward *Escherichia coli* or *Staphylococcus aureus*. Then standard Ala-scan revealed Gly-1, Leu-2, Leu-3, Ile-6, Leu-9, and Leu-10 are critical residues. A series of studies of analogs with different residue substitutions showed that hydrophobic anoplin derivatives generally have a lower minimum inhibitory concentration (MIC). On the other hand, it will also have higher hemolytic activity and reduce or reverse the selectivity between *E. coli* and *S. aureus*. More importantly, the overall charge of +4 is essential for anoplin to differentiate bacteria and normal cells.

After Meinike et al. verified the substitution of Arg-5 with different hydrophobic amino acids that resulted in potent analogs, they further replaced Arg-5 with peptoid monomers. Among the tests, the therapeutic index (TI; larger values indicate an improved activity specificity) of analogs containing *N*-(2,2-diphenylethyl) Gly or *N*-(1-naphthalenemethyl) Gly (**2** or **3**, **Figure 2A**) is commendably comparable with that of anoplin, but the selectivity was reversed toward *E. coli* and *S. aureus* (Meinike and Hansen, 2009).

To further explore suggested activity-improving discoveries, the Hansen group (Munk et al., 2013) presented 19 new anoplin analogs that substituted in amino acid positions 2, 3, 5, 6, 8,

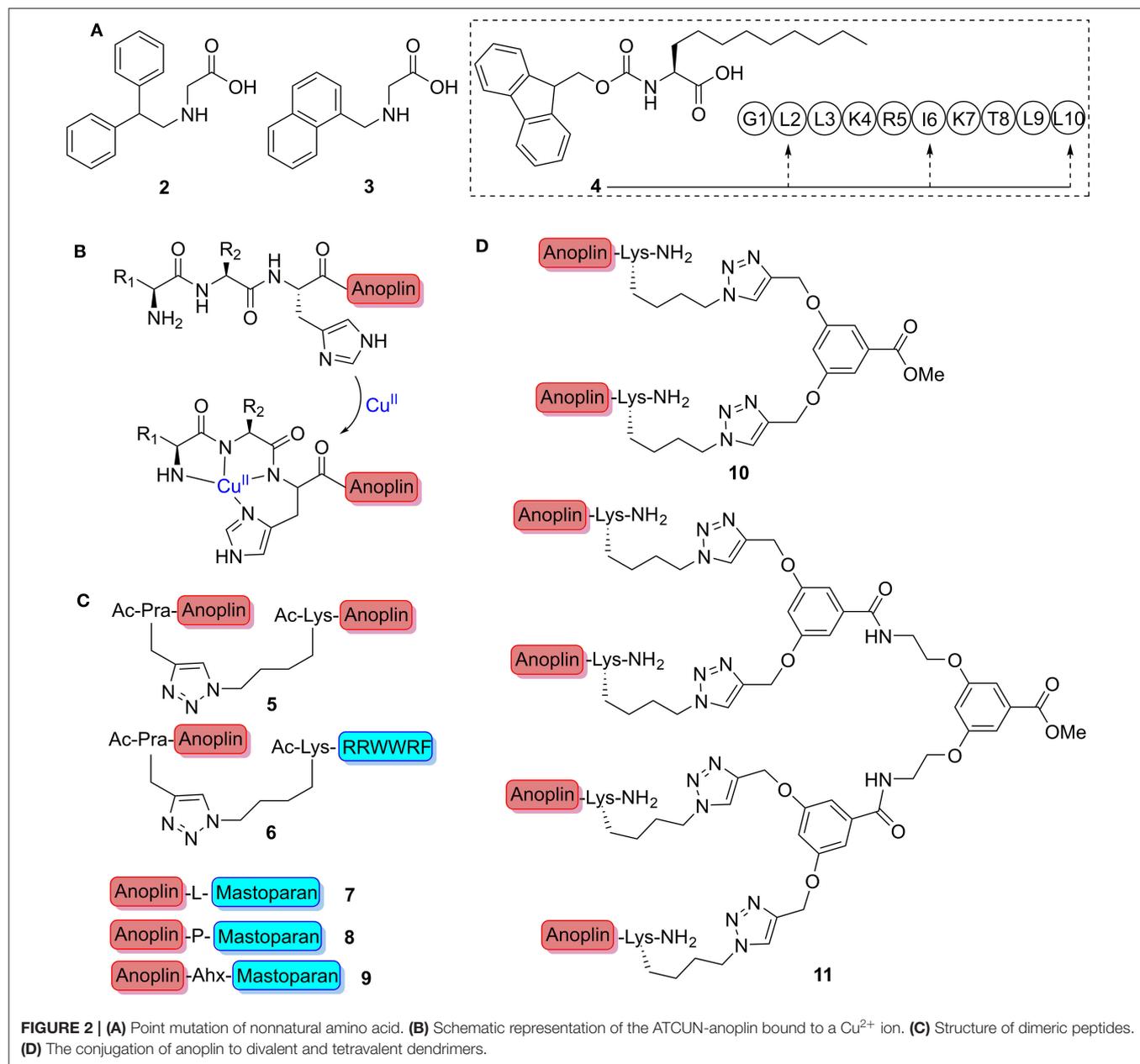
9, and 10 with Lys, Trp, Phe,  $\beta$ -2-naphthylalanine (2Nal), and  $\beta$ -cyclohexylalanine (Cha). Some discrete substitutions using Lys showed some promise, but Trp and Phe did not provide anoplin analogs with improved microbial specificity or lessened hemolytic activity (Braun and Heijne, 1999; Chen et al., 2005; Schmitt et al., 2007; Wiradharma et al., 2011). This was probably because Trp and Phe in those sites hindered the interaction between membrane and peptides owing to the structural distortions. It was gratifying that the use of 2Nal<sup>6</sup> and Cha<sup>3</sup> exhibited promising TI against all strains tested. Afterwards, they successfully developed analysis of variance models that describe the relationship between peptide properties (high-performance liquid chromatography retention time, hemolytic property, and MIC) and the structural characteristics of the anoplin analogs by point mutation, and they verified the properties predicted by the mathematical models, which were in reasonable agreement with the measurements (Munk et al., 2014).

Cabrera et al. (2008) synthesized deamidated analog of anoplin to explore the effect of deamidation at the C-terminus, and they found that although anoplin-OH still retained the same  $\alpha$ -helical content, it lost the characteristic antimicrobial and mast cell degranulating activities of anoplin. A reasonable explanation was that carboxylation correlated with electrostatic repulsion and amphipathic character. Subsequently, Pripotnev et al. confirmed that C-terminal amide group was important for the structural stabilization, extra charge and function of anoplin, and the physicochemical properties of peptides defined its AMP activity (Pripotnev et al., 2010). However, the amidation of anoplin was not essential for its antifungal and plant defense stimulating activities (Jindrichova et al., 2014).

Won et al. (2011b) applied several biophysical techniques (UV resonance Raman spectroscopy in combination with Langmuir–Blodgett monolayer technique vesicle leakage assay) to study the relationship between physicochemical properties (secondary structure and surface activity) with biological activities of anoplin and its two derivatives-anoplin-8K (GLLKRIKKLL-NH<sub>2</sub>) and anoplin-1K5V8K (KLLKVIKLL-NH<sub>2</sub>). These results indicated that higher helical tendency, stronger amphipathicity, and extra positive charges could be beneficial for higher antimicrobial activity with weak membrane lytic activity.

As a membrane anchor, the length of lipophilic alkyl chain of lipopeptides is considered highly vital for the bioactivity of peptides (Taft and Selitrennikoff, 1990). To increase the membrane affinity of anoplin without sacrificing important positively charged side chains, Slootweg et al. incorporated the lipophilic amino acid, (*S*)-2-aminoundecanoic acid (**4**, **Figure 2A**), which they synthesized into the peptide sequence (Leu-2, Ile-6, and Leu-10) of anoplin (Slootweg et al., 2013). As expected, all the derivatives exhibited enhanced activities and selectivity toward microbial membranes, while the effect of hemolysis showed considerably increase.

To better comprehend the structure–activity relationships of short cationic  $\alpha$ -helical AMPs, and to achieve a generally applicable set of guidelines on how to increase AMP activity without concomitant introduction of strong hemolytic activity, Wimmer and the Hansen group (Uggerhoj et al., 2015) presented the NMR structure of anoplin in a micellar environment



and systematically set up a vast library of substitutions for antimicrobial activity, hemolytic activity, and changes in structure and lipid interactions. They successfully proposed a do's and don'ts list with the core concept of subtle hydrophobicity increase in the hydrophobic face and the polarity increase in the hydrophilic face, including the following: (1) do not rely on the helical wheel model to design AMP derivatives, especially for nonconservative point mutations; (2) do identify whether each residue interacts with the membrane interior, with lipid head groups, or with the bulk solvent; (3) do not substitute those hydrophobic residues that interact with the lipid head; (4) do increase the polarity of side chains that interact with bulk solvent; and (5) do increase the hydrophobicity of the hydrophobic face in

small steps and a few positions. Based on these findings, it is not hard to see that the stronger amphipathicity plays an important role for the activity of anoplin.

### N-Terminal Auxiliary

Amino-terminal copper and nickel (ATCUN) binding motifs,  $\text{H}_2\text{N-AA}_1\text{-AA}_2\text{-His}$ , are known to actively form reactive oxygen species (ROS) upon metal ions binding, and ROS is primarily a result of  $\text{Cu(II)}$  binding (Harford and Sarkar, 1997; Donaldson et al., 2001; Du et al., 2013) (Figure 2B). The formed ROS can render the bacteria more susceptibility to antimicrobial agents (Fang, 2004). The Angeles-Boza group (Libardo et al., 2014) prepared three ATCUN binding motifs containing derivatives

of anoplin (Asp-Ala-His, Gly-Gly-His, and Val-Ile-His), which were found to be more active than the parent anoplin against a panel of clinically relevant bacteria with non-hemolysis through ROS-induced membrane damage. Whereafter, they found two additional ATCUN sequences (Arg-Thr-His and Leu-Lys-His), which produce  $\cdot\text{OH}$  and other ROS at faster rates than did other ATCUN complexes. When evaluated by the degree of oxidative stress brought induced by new ATCUN-anoplin conjugates, the Arg-Thr-His-anoplin peptide was up to four times more potent than is anoplin alone against standard test bacteria (Libardo et al., 2015). The metal binding tripeptide motifs provided a simple approach to increase potency of anoplin by conferring a secondary action.

Similar with lipophilic alkyl chain of lipopeptides, acylation of the N-terminus with fatty acid moiety can also increase the membrane affinity and the activity of AMPs (Avrahami and Shai, 2002). So Chionis et al. (2016) incorporated the lipophilic octanoic, decanoic, and dodecanoic acid residues into the N-terminus of anoplin. Those results indicated that fatty acid moiety was good for the membrane affinity, antimicrobial potency, and the higher helical content of the peptides, and these peptides were nontoxic to erythrocytes and stable to proteolysis. In addition, the increase of the length of the lipophilic moiety did not further affect the above properties. Salas et al. (2018) thought that the loss of activity upon truncation of anoplin in the literature (Ifrah et al., 2005) was correlated to lowering of hydrophobicity, so they synthesized and investigated the effects of truncated palmitoylated anoplin analogs, and their results demonstrated that palmitoylation of truncated anoplin analogs can increase antimicrobial activity, and even helicity, in water.

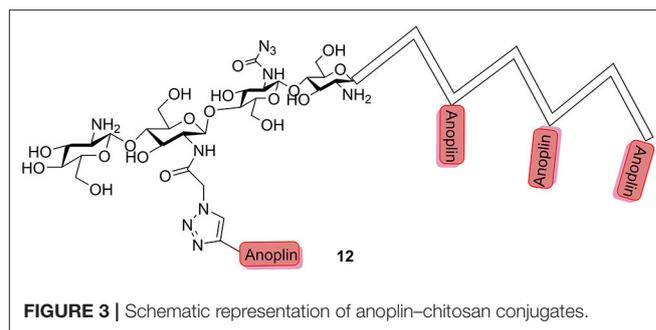
## D-Amino Acid Conversion

D-Peptide and D-protein composed of D-amino acids are potential therapeutic agents with longer half-lives or oral possibility *in vivo* (He et al., 1996; Weinstock et al., 2014). Therefore, D-amino acid substitution is a useful method to improve the enzymatic stability and activity of AMPs. Won et al. (2011a) found that the replacement of all amino acids with their D-stereoisomers did not change bactericidal activity of anoplin, so the antibacterial mechanism is through nonspecific interaction.

Inspired by the experience of Ifrah et al. (2005), Wang et al. (2014) designed and synthesized a group of anoplin analogs by multiple residue substitutions with D-amino acids. Owing to the increased charge, hydrophobicity, and amphiphilicity, anoplin-4 (kllkwkll-NH<sub>2</sub>) composed of D-amino acids displayed the characteristics of the highest antimicrobial activity *in vivo*, higher proteolytic stability, and lower toxicity to normal cells, which make anoplin-4 a great candidate for future optimization and treatment of infection.

## Dimerization and Multivalent Presentation

Hybrid peptide analogs can effectively combine the therapeutic advantages of different peptides and achieve collaborative treatments (Liu et al., 2013; Kamysz et al., 2015; Le et al., 2015). Hexapeptide, RRWRF (named RW), is a cationic peptide derived from the screening of a hexapeptide combinatorial

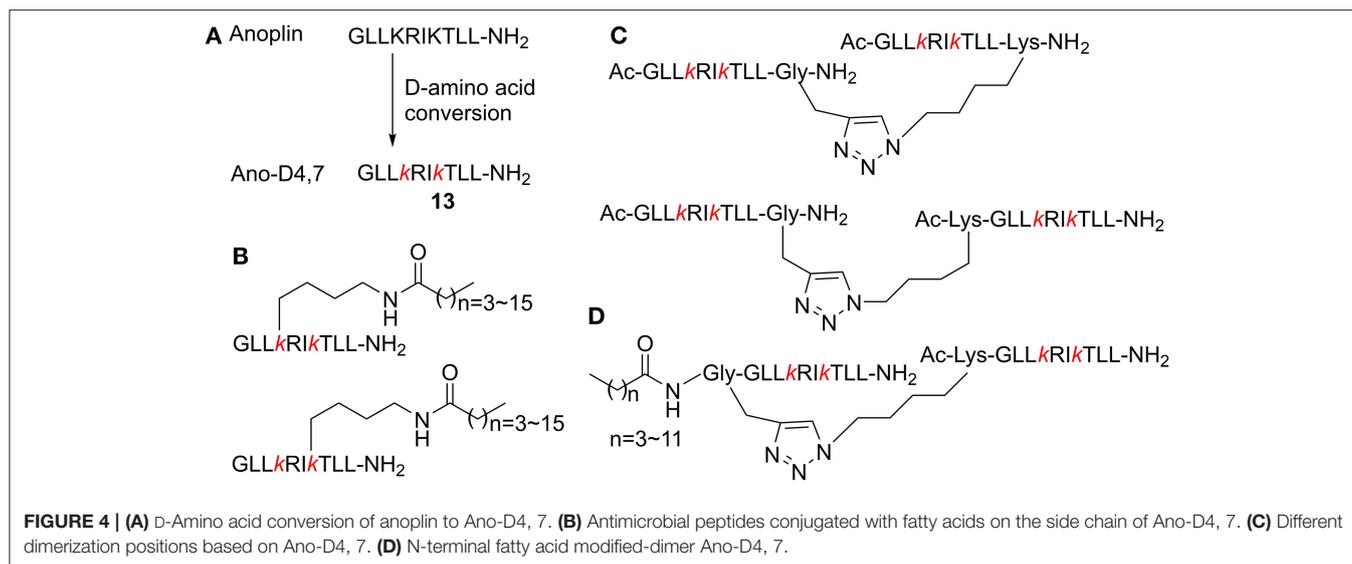


library; it is beneficial to interact with the negatively charged components of the bacterial membrane (Haug et al., 2008). Liu et al. (2017) successfully synthesized the homodimeric peptide **5** and the heterodimeric peptide **6** by copper(I) catalyzed azide-alkyne cycloaddition (CuAAC “click chemistry”) (Figure 2C); the dimer peptides not only had significantly enhanced antimicrobial activity against multidrug-resistant (MDR) bacteria *in vitro* and *in vivo* but also showed synergy and additivity effects when used in combination with conventional antibiotics rifampin or penicillin.

To break  $\alpha$ -helix and obtain greater flexibility of heterodimeric AMPs, Kai et al. (2018) used different amino acids (7–9, Figure 2C), including Leu, Pro, and Ahx (aminocaproic acid), as linker to connect anoplin and mastoparan, another  $\alpha$ -helical AMP from *Vespula lewisii* venom. Findings manifested that Pro and Ahx contributed to the design of ideal dimer AMPs with high selectivity and potency while reducing lytic activity with respect to red blood cells. However, Leu cannot confer increased flexibility to heterodimeric AMPs. It is flexible heterodimeric structure induced by Pro and Ahx that improved the potency of heterodimeric AMPs for negatively charged membranes while reducing interaction with zwitterionic membranes. As can be seen, dimerization offers an effective strategy to screen for the development of novel antimicrobial agents.

Given the toxicity, the short half-life *in vivo*, and stimulation of an immune response of AMPs, multivalent AMPs formed by attaching several peptide monomers to reactive polymer scaffolds via naturally occurring intermolecular disulfide bridges or unnatural scaffold linkers have been used to overcome the problems mentioned above (Liu et al., 2010). Chamorro et al. showed that anoplin was successfully conjugated via click chemistry to divalent and tetravalent dendrimers (**10** and **11**, Figure 2D) (Chamorro et al., 2012). Compounds **10** and **11** clearly resulted in enhanced pore formation of anoplin.

Chitosan is a natural polymer comprised of  $\beta$ -(1-4)-linked glucosamine and some degree of N-acetyl glucosamine with low antimicrobial activity and has been widely applied in the field of biomedicine. Sahariah et al. (2015) reported that grafting of anoplin to chitosan polymers was a rational design for abolishing the hemolytic propensity and increasing the activity of the parent peptide; at the same time, the anoplin–chitosan conjugates had a high degree of control over the resulting peptide grafting density (**12**, Figure 3).



## Combined Modification

Combination of multiple modification methods may represent more effective strategies with their respective advantages. Based on the substitutive anoplins sequence Ano-D4, 7 (13, Figure 4A), GLLkRIkTLL-NH<sub>2</sub> (lowercase letters indicate the D-enantiomer), further modifications were carried out to systematically screen excellent AMPs with optimal therapy potential. Zhong et al. (2020b) conjugated various lengths of fatty acid chains ranging from 4 to 16 carbons onto the side chain of the position 4 or 7 D-amino acid of Ano-D4, 7 (Figure 4B). The antimicrobial activity of those new peptides was highly correlated with the lengths of the fatty acid chain. When the length of the fatty acid chain was  $\leq 12$  carbons, there was a remarkable increase in the MIC values of the new peptides after the hydrophobicity went beyond preceding threshold. Interestingly, the peptides conjugated with the same fatty acid chain ( $\leq 12$  carbons) at position 7 of Ano-D4, 7, exhibited better activity than the corresponding peptide with the modification at position 4. This is probably because side chains of Arg-5 and Thr-8 shielded the fatty acid on the position 4 D-Lys side chain. These peptides also significantly reduced the bacterial load in mice without obvious adverse reactions or any death at the effective dose.

According to previous studies on dimeric modification, Zhong et al. (2020a) further inquired the effects of different dimerization positions on biological activity. They found that the anti-biofilm activity of the C- and N-terminal dimer peptides was better than C-C terminal ones (Figure 4C). Meanwhile, new peptides combining D-amino acid conversion and dimerization displayed higher TI without hemolytic activity. In addition, on the basis of the previous two reports, they designed dimer peptide conjugated fatty acids at the N-terminus of Ano-D4, 7 and dimerization (Figure 4D) (Zhong et al., 2019). The multi-modified analogs were more inclined to present  $\beta$ -strand structure; this pre-assembled state of the dimer peptides led to fast interaction without the demand for extra aggregation process, which caused more efficient permeabilization. Furthermore, the previous

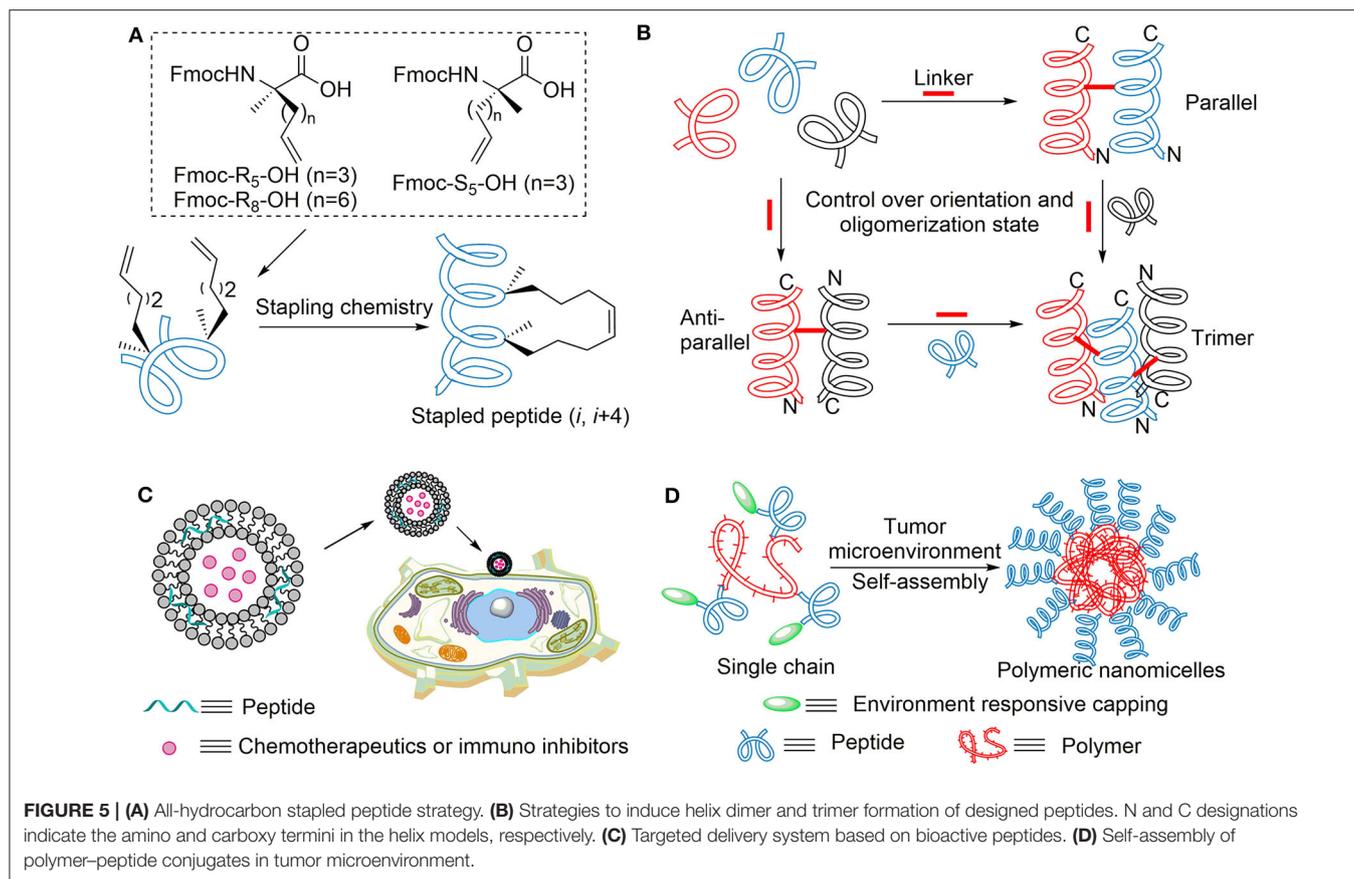
peptides also exerted high stability toward protease, serum, salts, and different pH environments, which proved that combined modification techniques provided more flexible approaches to develop novel AMPs.

## POTENTIAL DEVELOPMENT DIRECTION OF ANOPLIN

### Conformational Constraint

Despite the above notable advances, there are still many disadvantages in the previous modifications. For example, residue substitutions cannot solve the low stability toward protease, and there also may be metabolic problems for D-peptide *in vivo* compared with natural amino acid. The synthesis of dimerization and multivalent presentation are difficult and tedious. A promising alternative approach would constrain  $\alpha$ -helical conformation by stapling chemistry (Schafmeister et al., 2000; Walensky et al., 2004; Moellering et al., 2009; Walensky and Bird, 2014) (Figure 5A). The first stapled peptide, ALRN-6924, is currently in phase II trials to treat advanced solid tumors or lymphomas in connection with MDM2 and MDMX (Carvajal et al., 2018; Ng et al., 2018). We have previously reported the design and synthesis of a series of hydrocarbon stapled melittin peptides of which, some analogs showed remarkable enhancement not merely in antihepatoma activity but also in  $\alpha$ -helicity and protease resistance (Wu et al., 2017a). Subsequently, we developed a new series of stapling amino acids, which contained the native amino acid side chains to expand the scope of the all-hydrocarbon stapled peptide strategy (Wu et al., 2017b).

In addition to  $\alpha$ -helical constraint of single short peptide, nature actually exhibits its own approach to maintain the structural integrity of proteins and protect natural peptides from enzymolysis. A noteworthy example was the self-assembly of  $\beta$ -sheet peptides into amyloid- $\beta$  protein by inter-strand hydrophobic interactions, which exhibited extreme resistance



toward intracellular proteases (Barz et al., 2018). Inspired by this, the Wu group reported that crosslinked helix dimers can be constructed by two disulfide bonds for ultrahigh proteolytic stability (Chen et al., 2015). To overcome the instability of disulfide linkage under the intracellular reducing condition, Wuo et al. developed more judicious covalent bonds like bis-triazole linkers (Wuo et al., 2015) and hexafluoroisopropanol-based bishioether crosslinkers (Wuo et al., 2018; Chen et al., 2019) (Figure 5B). We expect these effective strategies be used to modify anoplin with preferable biological function.

### Targeted Delivery System

Bio-functional nanosystems are also regarded as novel strategies to address some drawbacks of peptides (Aronson et al., 2018). Through the widely accepted enhanced permeability and retention (ERP) effect (He J. Y. et al., 2019), drug delivery platforms based on cytotoxic peptides possessed prolonged circulation time and targeting capability, which improve its therapeutic utility and reduce side effects. More importantly, this technology can achieve co-delivery of multiple drugs for the purpose of combination therapy in precision medicine (Jin et al., 2018) (Figure 5C). To obtain deeper solid-tumor penetration and perfuse more homogeneously within tumor tissue, one ingenious design is tumor microenvironment (TME)-induced *in situ* self-assembly of polymer-peptide conjugates (Cong et al., 2019) (Figure 5D); this transformation-enhanced accumulation and retention (TEAR) effect showed

higher anticancer activity than the ERP effect of traditional nanoparticles (He P. P. et al., 2019).

### CONCLUSION AND OUTLOOK

The emergence of MDR bacteria and the markedly declining supply of safe and efficacious antibiotics have made it urgent to develop new antibiotics. On the other hand, MDR malignant tumor is the major threat to public health. In this context, natural AMPs have been considered as promising strategy for these two disease threats because of the lower potency of resistance for bacteria and tumor toward AMPs (Felício et al., 2017; Mishra et al., 2017). Owing to typical non-receptor-mediated membrane mechanisms (Sveinbjornsson et al., 2017), anoplin possesses more particular superiority than do conventional antibiotics and chemotherapeutics. In addition, anoplin offers great advantages with simple structure and broad-spectrum activity, especially nonhemolytic toxicity. With the anoplin in hand, it is more convenient to obtain lead compounds for potential translational research. We hope that anoplin and its derivatives will inspire the advancement of AMPs as a novel class of drug candidates.

### AUTHOR CONTRIBUTIONS

YW and RH wrote the manuscript. J-MJ and L-JZ contributed to the creation of most figures in this manuscript. HZ

and H-ZC contributed to the editing of this manuscript. L-LC and XL revised and edited the final manuscript. All authors contributed to reference collection, selection, and final proof.

## FUNDING

This work was supported by funds from the National Natural Science Foundation of China (No. 81903654), Program for Professor of Special Appointment (Young Eastern Scholar)

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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