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Rational Design of Hybrid Peptides: A Novel Drug Design Approach*

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Summary: Peptides play crucial roles in various physiological and pathological processes. Consequently, the investigation of peptide-based drugs is a highlight in the research and development of new drugs. However, natural peptides are not always ideal choices for clinical application due to their limited number and sometimes cytotoxicity to normal cells. Aiming to gain stronger or specific or novel biological effects and overcome the disadvantages of natural peptides, artificial hybrid peptides have been designed by combining the sequence of two or more different peptides with varied biological functions. Compared to natural peptides, hybrid peptides have shown better therapeutic potentials against bacteria, tumors, and metabolic diseases. In this review, design strategies, structure features and recent development of hybrid peptides are summarized; future directions for the research and development of hybrid peptide drugs are also discussed.
Key words: hybrid peptides; design strategies; antibacterial, anti-tumor and anti-metabolic diseases; chemical modification

Peptides, which play crucial roles in nearly every physiological process[1–5], are chains of amino acids linked by peptide bonds. Nowadays, more than 7000 natural peptides have been identified, many of which have shown application values[6–12]. Vincent Du Vigneaud synthesized the first peptide drug oxytocin in 1953[13], which won him the Nobel Prize in 1955. Since then, peptide drugs have developed rapidly, and more than 80 peptide drugs have been approved for market worldwide[14]. Advantages of peptide drugs include their good bioactivity and higher stability compared with protein drugs. The development of chemical synthesis and biological expression technology for peptides has considerably reduced the cost of peptide drugs, which are usually much cheaper than the protein/antibody drugs with similar functions. These unique advantages have made peptide drugs an important field of new drug research and development.

The development of peptide drugs has gone through three stages: extraction of natural peptides, chemical or biological synthesis of natural peptides, and chemical modification/artificial design of peptides. As one of the first clinically used peptide drugs, insulin is a good example, which has gone through the extraction from animal sources, recombinant human insulin, and rationally designed fast- or long-acting insulin (insulin Aspart, insulin detemir, insulin glargine, etc.). The main disadvantages of natural peptides include their relatively limited number and sometimes cytotoxicity to normal cells, and some natural antimicrobial peptides (AMPs) may even cause hemolysis[15]. Artificial peptides, however, can provide solutions to these problems. Since clinical treatment sometimes requires the combination of two or more kinds of peptides, artificially designed hybrid peptides are considered to have promising application potentials. Recently, rational designing of hybrid peptides has drawn great attention. The concept of hybrid peptide is simple, which combines the sequence of two or more different peptides (fig. 1) with varied biological functions, with aims to gain stronger or novel biological effects. Hybrid peptides have shown great therapeutic potentials for anti-bacterial, anti-tumor, and anti-metabolic diseases. In this review, design strategies, structure features and recent development of hybrid peptides are reviewed, and future directions for the research and development of hybrid peptide drugs are discussed.

Fig. 1 Diagram that illustrates the concept of a hybrid peptide

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1 Design Strategies of Hybrid Peptides

Rational design of hybrid peptides directly affects the final biological activity. Presently there are two common design strategies for hybrid peptides.

1.1 Fusion of Cell Targeting/Penetrating Peptide with a Functional Peptide

A widely-applied hybrid peptide design strategy is to fuse cell penetrating peptide (CPP) or cell targeting peptide (CTP) with a biological functional peptide to avoid "off-target" effect (fig. 2). This strategy helps hybrid peptides to cross cell membrane or specifically bind to the target cells for higher therapeutic effect with decreased side effects, which is similar to the design strategy of peptide drug conjugates (PDCs)[16]. For example, several lytic peptides, which are rich in D-leucine and D-lysine, show strong capacity to destroy the cell membranes of cancer cells. However, they also cause damages to the cell membranes of normal cells, which limits the anti-cancer applications of these lytic peptides[17]. To overcome this obstacle, a 'TfR' peptide, which specifically binds to transferrin receptor that is overexpressed in cancer cells, is conjugated with lytic peptide by a glycine-rich linker, and the resulting hybrid TfR-lytic peptide demonstrates specific cytotoxicity to cancer cells[18].

1.2 Dual Function or Multi-function Hybrid Peptides

This strategy aims to construct dual/multi-function hybrid peptides by conjugating different functional peptides. All the domains of the hybrid peptides, although coming from different functional peptides, possess biological/therapeutic activities, so as to produce synergistic effects (fig. 3). The theoretical basis for the design of dual function and multi-function hybrid peptides lies on the maintenance of homeostasis *in vivo*, which depends on the participation of multiple peptides. For example, glucose homeostasis is finely tuned by insulin, glucagon, ghrelin, glucagon like peptide-1 (GLP-1) and other peptides. Therefore, better therapeutic effects may be achieved by the combination of two or more peptide drugs. To construct such hybrid peptides, an essential requirement is the formation of synergistic therapeutic effects by the different functional fragments of hybrid peptides. A GLP-1/xenin hybrid peptide was reported recently,

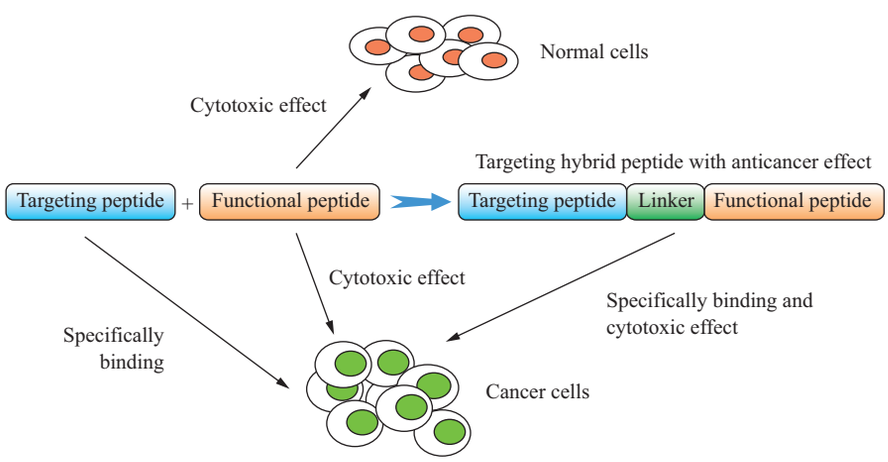

Fig. 2 Fusion of cell targeting/penetrating peptide with functional peptide

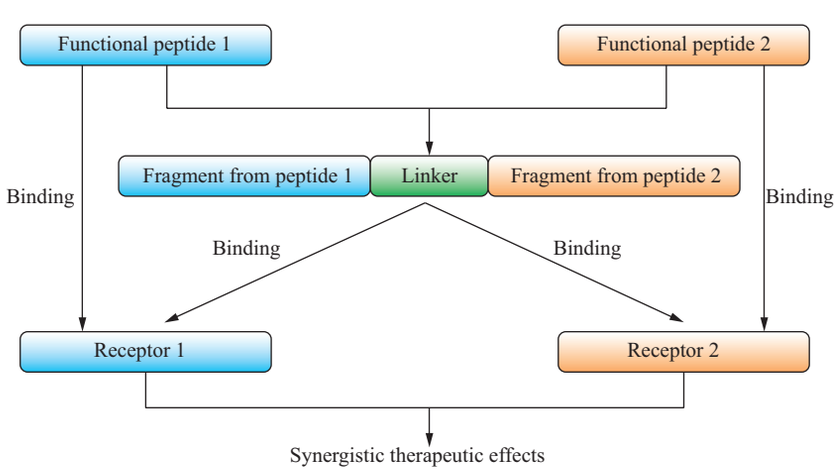

Fig. 3 The design strategy of dual function or multi-function hybrid peptides

which is formed by GLP-1, a peptide that suppresses appetite and promotes the proliferation of pancreatic β-cells in the islets, and Xenin, a peptide which suppresses appetite and improves glucose-induced insulin secretion. Compared to GLP-1, the GLP-1/Xenin hybrid showed better performance in improving glucose homeostasis, circulating lipids and restoring gastric inhibitory peptide (GIP) sensitivity in high fat diet mouse model[19].

1.3 Issues to be Noted

There are some issues that should be noted in the design of hybrid peptides. First of all, behind a successfully-designed hybrid peptide, there usually lies a clear complete analysis of the structure-activity relationships of peptides. Take a GcgR/GLP-1R co-agonist for example, the structure-activity relationships of glucagon and GLP-1 were analyzed through computer-aided drug design, which revealed that the highly-conserved N-terminal sequence of these hormones is the determinant of their different biological activities, and the 2nd, 3rd, 10th and 12th amino acids are the key in maintaining glucagon activity[20, 21]. Based on these analyses, a GcgR/GLP-1R co-agonist, which performed balanced agonism at each receptor, was successfully designed[22].

Secondly, in most hybrid peptides, fragments from different peptides are not conjugated directly, but through a proper linker. Such design helps to keep a certain distance in the space, thus prevents the functional fragments from interfering with each other. With the presence of the linker, fragments of hybrid peptides can recognize and bind with the corresponding molecules, and consequently function in a relatively independent way. Generally, the linker is required to have certain stability in plasma to guarantee the molecular structural integrity of hybrid peptides before the peptides are enriched in targeted cells or tissues. Choices for the components of the linker could be amino acid chain or hydrocarbon chain[23, 24].

Thirdly, the structures of hybrid peptides are becoming more complex. For instance, a hybrid peptide-estrogen conjugation, which is constructed by linking functional fragments of GLP-1, exenatide and estradiol[25], showed powerful activities in treating obesity and type 2 diabetes (T2D) in animal models. Similar results were gained in the case of another hybrid peptide-thyroid hormone conjugation which is designed based upon glucagon, exenatide and thyroid hormone[26]. Moreover, chemical modifications, such as palmitic acid modification, polyethylene glycol modification and D-amino acid substitutions, have helped to effectively improve the stability and pharmacokinetic properties of hybrid peptides[22, 27].

2 Therapeutic Hybrid Peptides

Hybrid peptides have been shown to be effective against bacteria, cancers, and metabolic diseases (tables 1 and 2). Recent progresses of hybrid peptides for therapeutic purposes are summarized below.

2.1 Anti-bacterial Hybrid Peptides

Antimicrobial peptides (AMPs), which were first isolated from insects, are part of the innate immune response found among all classes of life. Natural AMPs are short peptides with typical lengths between 15 and 40 residues. Due to their destructive effect on cell membranes, these peptides are potent, broad spectrum antibiotics on Gram-negative and Gram-positive bacteria, viruses, fungi and even transformed or cancerous cells[28]. Almost all of AMPs have positively charged surface and enriched number of basic amino acids such as Arg, Lys and His[29].

Despite of their great potential in drug development, concerns exist in the clinical application of AMPs considering their potential toxicity to normal cell membranes. Though several AMPs have been applied in clinical practice, most of them are for external use[30]. To address the off-target problem, targeting peptides have been fused with AMPs to construct targeting AMPs. For example, G10KHc is a

Table 1 Representative hybrid peptides with design strategy of CTPs/CPPs plus functional peptides

Name	CTPs/CCPs	Functional peptides	Linker	Target	Application	References
G10KHc	KH peptide	Novinspirin G10	Amino acid chain	*Pseudomonas*	Anti-infection of *Pseudomonas*	[23]
M8G2	M8 peptide	G2 peptide	Amino acid chain	*Streptococcus mutans*	Anti-infection of *Streptococcus mutans*	[31]
M8(KH)-20	KH peptide	Artificial peptide (20aa)	Carbon chain	*Pseudomonas and Streptococcus mutans*	Anti-infection of *Pseudomonas* and *Streptococcus mutans*	[24]
TfR-lytic peptide	TfR peptide	Lytic peptide	Amino acid chain	Transferrin receptor	Anticancer	[18]
RGD-Tachyplesin 1	RGD peptide	Tachyplesin 1	Amino acid chain	Integrin receptor	Treatment of melanoma	[67]
Pro-apoptotic peptides	RGD peptide	AMPs (rich in D-leu and D-lys)	Amino acid chain	Integrin receptor	Anticancer	[68]
Hybrid Antp-TPR peptide	TPR domain	Antp domain	None	Hsp90	Anticancer	[37]
IL-4α-lytic	Peptide fragment targeting IL-4α	Lytic peptide	Amino acid chain	IL-4α	Anticancer	[38]
EGFR-lytic peptide	Peptide fragment targeting EGFR	Lytic peptide	Amino acid chain	EGFR	Anticancer	[39]

Table 2 Representative hybrid peptide designed with dual/multi-function hybrid peptides

Name	Source of functional peptides	Linker	Application	References
CecropinA(1-8)-LL37(17-30)	Cecropin A, LL37 peptide	Amino acid chain	Broad-spectrum antibacteria and low side effects	[32]
P18	Cecropin A, magainin	None	Treatment of melanoma	[40]
IAPP-insulin hybrid peptide	ProhIAPP, insulin C	None	Early detection of type 1 diabetes	[69]
GLP-1/xenin hybrid peptide	GLP-1, xenin	Peptoid chain	Treatment of obesity and type 2 diabetes	[19]
GcgR/GLP-1R co-agonists	Glucagon, GLP-1	None	Treatment of obesity and type 2 diabetes	[49, 50]
GLP-1R/GIPR co-agonists	GLP-1, GIP	None	Treatment of obesity and type 2 diabetes	[52]
GcgR/GLP-1R /GIPR triagonist	Glucagon, GLP-1, GIP	None	Treatment of obesity and type 2 diabetes	[53]
Exendin-4/gastrin/xenin-8-Gln hybrid peptide	Exendin-4, gastrin, xenin	Amino acid chain	Treatment of obesity and type 2 diabetes	[55]
PapMA	Papiliocin, magainin2	None	Antibacteria and anti-inflammation	[56]
LB-PG and CA-PG	Progetrin-1, bovine lactoferricin and cecropin A	None	Anti-inflammation	[57]
PK20	Endomorphin-2 analog, neurotensin	None	Anti-allergy	[58]
BSBHp	α-helix peptide and β-sheet peptide	None	Therapy of Alzheimer's disease	[59, 60]
CecropinA-magainin2 hybrid peptide	CecropinA, magainin2	None	Antivirus	[70]

targeting AMP which is composed of a *Pseudomonas*-specific targeting moiety (KH) and a broad-spectrum AMP (novispirin G10), and the hybrid shows enhanced bactericidal activity and accelerated killing kinetics against *Pseudomonas* compared to G10 alone[23]. Another targeting AMP, M8G2 hybrid peptide, includes a targeting domain which specifically recognizes *Streptococcus mutans* (*S. mutans*), which enables it to selectively eliminate the plaque bacterium *S. mutans* from the normal microflora in humans[31].

Hybridizing different AMPs has been an effective method to obtain novel hybrid AMPs with elevated antibacterial activity but minimized cytotoxicity by controlling the changes in hydrophobicity and charge. A hybrid peptide cecropin A(1-8)-LL37(17-30), which is fused by AMPs LL37 and cecropin A, shows significantly increased antibacterial activity and minimized hemolytic activity compared to LL37 or cecropin A alone, without hemolytic side effect seen in LL37 used alone[32].

2.2 Hybrid Peptides in Cancer Therapy

Peptide drugs for cancer treatment have been extensively investigated for decades. Many peptides have been applied in cancer treatment, such as iRGD, Melittin, luteinizing hormone releasing hormone (LHRH) and magainin[33–36]. To improve the anti-tumor activity of peptide drugs and reduce their side effects, some targeting anti-tumor hybrid peptides have been designed.

In 2001, a "hybrid Antp-TPR peptide" was reported, which is composed of an Hsp90-targeting tetratricopeptide repeat (TPR) domain and a cell-penetrating Antennapedia homeodomain (Antp)[37]. The resulting hybrid Antp-TPR peptide inhibited the interaction of Hsp90 with the TPR2A domain, and induced cell death in the breast, pancreatic, renal, lung, prostate, and gastric cancer cell lines without affecting the viability of normal cells. Since interleukin-4 receptor α (IL-4Rα) is highly expressed on the surface of various human solid tumors, IL-4Rα-lytic peptide, which contains a target moiety to bind IL-4Rα and a cytotoxic lytic peptide, is designed to selectively eliminate cancer cells[38]. EGFR-lytic peptide is a hybrid peptide that targets the epidermal growth factor receptor (EGFR), in which an EGFR-binding peptide is conjugated with a designed lytic-type peptide containing cationic-rich residues[39]. *In vitro* and *in vivo*, this hybrid peptide demonstrated anticancer activity on EGFR-overexpressing cancer cells.

In addition, P18 (KWKLFKKIPKFLHLAKKF) is a helical hybrid antibiotic peptide with prominent cytotoxic activity against melanoma cells and low toxicity to normal NIH-3T3 cells. It is designed by fusing the positively charged N-terminal region of cecropin A with the C-terminal amphipathic region of magainin[40].

2.3 Hybrid Peptides in the Treatment of Metabolic Diseases

Metabolic diseases such as obesity and diabetes are a global health threat and a rapidly increasing economic burden. Treatments for metabolic diseases include medication, diet, exercise and other complementary treatments. The maintenance of homeostasis *in vivo* depends on the participation of multiple peptides, and combination therapy of two or more peptide drugs has therefore been taken into consideration seriously. At present, hybrid peptides used in the treatment of metabolic diseases are mostly dual/multi-function peptides, of which the most successful examples are glucagon receptor subfamily co-agonists.

Glucagon receptor subfamily includes GcgR, GLP-1R, glucagon like peptide-2 receptor (GLP-2R) and gastric inhibitory peptide receptor (GIPR). GLP-1 plays an important role in the mediation of glycemic effects, which is related to bariatric surgery benefits[41–43]. Currently, GLP-1 mono-agonists treatment provides an insufficient yet meaningful body weight loss in most obese patients, primarily through anorectic or satiation properties[44]. Another candidate for constructing such hybrid peptides is glucagon, which is essential in

promoting lipolysis and thermogenesis[45–47]. However, glucagon has the effect of raising blood glucose and is considered as one of the factors promoting T2D[48]. The rationale behind the design of GcgR/GLP-1R co-agonists is to combine the appetite-suppressing effect of GLP-1 with the lipolysis and thermogenesis-promoting effect of glucagon, while inhibiting the glucagon-induced hyperglycemia. Thus GcgR/GLP-1R co-agonists not only antagonize the inherent diabetogenic risk by regulating blood glucose, blood fat and body weight and synergistically reducing insulin-resistance, but also provide therapeutic supplemental efficacy through an independent weight-lowering mechanism.

GcgR/GLP-1R co-agonists and GLP-1R/GIPR co-agonists have already shown better efficacy than GLP-1 alone in treating metabolic diseases[49–52]. In 2015, a GcgR/GLP-1R/GIPR triagonist was reported and demonstrated even better efficacy in treating obesity and T2D[53]. The mechanism behind the function of the triagonist is shown in fig. 4.

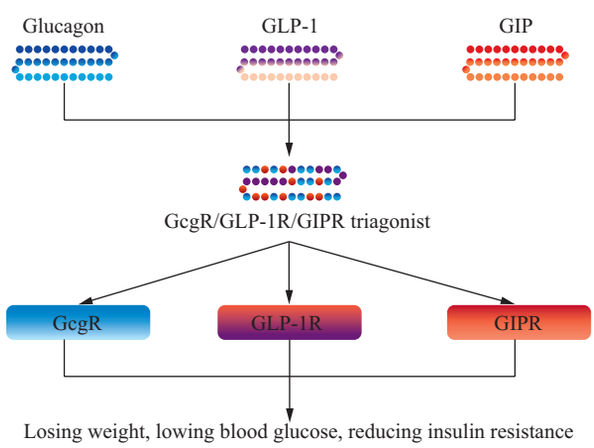

Fig. 4 The mechanism of GcgR/GLP-1R/GIPR triagonist

Although no GcgR-containing co-agonists have been approved to the market, the developments of GcgR/GLP-1R co-agonists, GLP-1R/GIPR co-agonists and GcgR/GLP-1R/GIPR triagonists are highly competitive. At present, several clinical trials with GcgR/GLP-1R co-agonists are on-going[54].

In addition to GcgR/GLP-1R co-agonists, GLP-1R/GIPR co-agonists and GcgR/GLP-1R/GIPR triagonists, together with other hybrid peptides have also been designed and reported for the treatment of metabolic diseases. Examples include GLP-1/xenin hybrid and Exendin-4/gastrin/xenin-8-Gln, both show beneficial effects in controlling blood glucose[19, 55].

2.4 Hybrid Peptides for Other Diseases

Hybrid peptides have also been applied in anti-inflammatory and Alzheimer's disease (AD) therapy. Hybrid peptides including PapMA[56], LB-PG, CA-PG[57] and PK20[58], have been proved to have anti-inflammatory effects. For AD therapy, a designed hybrid peptide BSBHp, which acted as an inhibitor of amyloid-β peptide aggregation, demonstrated positive therapeutic effects[59, 60]. Moreover, it is considered to have potential clinical value to prevent and treat AD and T2D by inhibiting the aggregation of amyloid peptides like amyloid-β[61–66], thus hybrid peptides with anti-aggregation activities are attractive drug candidates for such diseases.

3 Future Directions for Hybrid Peptides

Hybrid peptides have shown great therapeutic potentials for diseases. We summarize and propose three future directions for hybrid peptides as follows. Firstly and probably the most importantly, big data analytics and artificial intelligence (AI) are increasingly involved in the design of hybrid peptides. These fast-developing techniques help to uncover hidden functional correlations among different natural peptides, providing previous unseen or ignored insights in constructing new hybrid peptides. They can also rapidly reveal unknown therapeutic targets by examining large-scale various data sets and stimulating experiments, thus expand the application scope of hybrid peptides.

Secondly, more hybrid peptides are synthesized through novel chemical modification methods, or constructed with unnatural amino acids. Better bioactivity and higher stability can be achieved by introducing these new methods in designing hybrid peptides, the cost can also be reduced by optimizing current synthetic methods.

Finally, the linker can be considered in the modification of hybrid peptides. Currently reported linkers for hybrid peptides are usually stable amino acid chains or hydrocarbon chains. However, unstable linkers may act as a trigger. The design strategy of such hybrid peptides is to construct a linker which degrades under specific conditions, for example, the tumor microenvironment. Along with the degradation of the linker, certain peptide fragments are released to function independently, which may promote therapeutic effects under some clinical cases.

Conflict of Interest Statement

The authors declare that there is no conflict of interest.

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