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Peptides As Therapeutics with Enhanced Bioactivity

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Abstract: The development of techniques for efficient peptide production renewed interest in peptides as therapeutics. Numerous modifications for improving stability, transport and affinity profiles now exist. Several new adjuvant and carrier systems have also been developed, enhancing the immunogenicity of peptides thus allowing their development as vaccines. This review describes the established and experimental approaches for manufacturing peptide drugs and highlights the techniques currently used for improving their drug like properties.

Keywords: Peptide, drug delivery, vaccine, manufacture, bioavailability, peptide therapeutic, immunogenicity, peptide drug, peptide synthesis, clinical trials.

INTRODUCTION

Natural and synthetic peptides have shown promise as pharmaceutics with the potential to treat a wide variety of diseases. This potential is often overshadowed by the inability of the peptides to reach their targets in an active form *in vivo*. The delivery of active peptides is challenging due to inadequate absorption through the mucosa and rapid breakdown by proteolytic enzymes. Peptides are usually selective and efficacious, therefore need only be present in low concentrations to act on their targets. The metabolism of peptides is superior to other small molecules due to the limited possibility for accumulation and result in relatively non-toxic amino acid, peptide metabolites. These properties contribute towards the overall low toxicity of peptides, with a limited risk of adverse interactions.

The most important classes of peptides that have been investigated include insulin, gonadotropin-releasing hormone, calcitonin, the enkephalins, the glucagons, other hormone analogs, enzyme inhibitors and vaccines. The majority of peptides were targeted towards treatment of cancers and metabolic disorders, with a large number targeting G-protein coupled receptors. In 2010, there were over 50 peptide drugs approved for marketing, with annual global sales nearing/surpassing US$ 1 billion for the following peptide drugs: ciclosporin (e.g. Neoral®, Novartis), goserelin acetate (Zoladex®, AstraZeneca), glatiramer acetate (Copaxone®, Teva Pharmaceuticals), leuprolide acetate (e.g. Lupron®, Abbott Laboratories), and octreotide acetate (Sandostatin®, Novartis). Additionally, in 2010, more than 100 peptide drug candidates were reported to be in clinical development [1].

This review describes novel approaches in peptide synthesis and the use of peptides as therapeutics.

PEPTIDE THERAPEUTICS

Initially, inefficient and expensive manufacturing processes hampered the development of peptide-based therapeutics. Nevertheless, the production of the peptide drug enfuvirtide (Fuzeon®, Roche, a human immunodeficiency virus (HIV) membrane fusion inhibitor), demonstrated that preparing a 36-amino acid peptide on a ton-scale annually, could be cost effective [2].

The establishment of industrial-scale peptide manufacturers meant that the chemical industry had to supply increased demands, which caused a decrease in prices, increased availability of amino acids and consequently, market growth [3].

The methods used for the preparation of peptide therapeutics have depended on a number of important criteria: sequence length, side-chain reactivity, degree of modification, incorporation of unnatural components, in addition to the required purity, solubility, stability and scale. There are two strategies for peptide production - chemical synthesis and biological manufacturing.

Chemical Peptide Synthesis

Chemical synthesis has been used for the production of peptides in both research and industry and led to the development of the majority of peptide drugs [2]. These chemical-based strategies were usually favored due to their unique ability to incorporate any number of non-natural components. Two chemical methodologies-solution-phase (SPS), and solid-phase peptide synthesis (SPPS) [4] - are the predominant methods of chemical synthesis, but many other approaches, including hybrid synthesis and chemoselective ligation, have also been studied.

Solution-Phase Peptide Synthesis

First described by du Vigneud in 1953, the SPS method was used for the production of most commercial peptide therapeutics [5]. The method was based on the coupling of peptide fragments or a single amino acid in solution Fig. (**1**). The main advantage of SPS was economic because of the use of relatively inexpensive building blocks and reagents. Each intermediate could be analyzed and purified, leading to products of higher purity and easier final purifications. The main limitation of SPS was the need to modify each intermediate reaction step to synthesize a required peptide thus, the development of each synthetic scheme was long, laborious, and relatively expensive. Peptide pharmaceuticals from as few as 3 amino acids (e.g. thyrotropin-releasing hormone) to peptides of 32 residues (e.g. calcitonin) have been synthesized for clinical use using SPS [2].

Solid-Phase Peptide Synthesis

Ten years after the development of SPS, Merrifield introduced SPPS [6]. This method was based on the coupling of protected amino acids to an insoluble support Fig. (**1**). The simplicity of this process was compatible with automated synthesis for the large scale production of peptides. SPPS proved to be faster and less laborious than SPS. Two forms of SPPS methods were developed based on the protection of the α-amino group of an amino acid, the *tert*-butyloxycarbonyl (Boc) group was deprotected by acid (e.g. trifluoroacetic acid) [7], while 9-fluorenylmethyloxycarbonyl (Fmoc) group was removed by base (e.g. piperidine) [8].

The most important limitation to the use of SPPS on an industrial scale was the requirement for excess starting material [2]. However, several peptide therapeutics such as ziconotide (Prialt®, Elan), exanatide (Byetta®, Amylin/Eli Lilly Co.), pramlintide (Symlin®, Amylin) and degarelix (Firmagon®, Ferring) were synthesized by SPPS and approved by the U.S. Food and Drug Administration (FDA) [9, 10]. Since 1992, microwave irradiation has been used extensively to accelerate SPPS by providing a con-

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venient rapid heating method [11]. SPPS was used to make Gramicidin A (peptide antibiotic) and CSF114(Glc) (a glycopeptide immunological probe) with a good yield and high purity [12].

Hybrid Synthesis

Due to the time limitations and adverse side-reactions present in traditional SPS and SPPS protocols, synthesis of some peptide sequences has often proven difficult. Hybrid synthesis, which combines SPS and SPPS, merged the benefits of both protocols Fig. (**1**) [4]. The production of enfurvirtide, established using SPPS, was changed to a hybrid strategy for manufacture in the scale of metric tons annually from three or more fragments [2].

Chemoselective Ligation

Another option for the elongation of peptide chains is through chemoselective ligation techniques, which offer the ability to couple unprotected peptide fragments Fig. (**1**). Native chemical ligation was described as one example of chemoselective ligation, which, through a thiol exchange and spontaneous rearrangement, ultimately formed natural peptide bonds between peptide fragments [13]. The limitation of this strategy was the requirement of an N-terminal cysteine and C-terminal thioester. A modified method used auxillary thiols in place of the cysteine residues, which were removed at the site of ligation and allowed the formation of a non-cysteine linkage [14, 15].

Improving Chemical Synthesis

Due to ecological concerns about the impact of chemical synthesis, there has been pressure to move towards more environmentally friendly methods, or 'green' chemistries. The elimination of traditional organic solvents, such as dimethylformamide and dichloromethane, can save considerable toxic waste. Hojo *et al.* developed a new, water soluble N-protecting group, 2-[phenyl (methyl)sulfonio]ethoxycarbonyl tetrafluoroborate, which was used to successfully synthesize Leu- and Met-enkephalin peptides in aqueous solution on a TentaGel resin [16]. The same group reported the ability of Boc-protected amino acids to be nanodispersed in water and used in SPS. These nanodispersed Boc-amino acids coupled with 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride and N-methylmorpholine produced the Leu-enkephalin pentapeptide in 89% yield [17].

Pseudo-proline (ψPro) modified amino acid residues were designed to minimize aggregation and racemization when synthesizing difficult peptide sequences [18]. Pseudo-prolines (ψPro) are serine- or theonine-derived oxazolidines or cysteine-derived thiozolidines with proline-like ring structures Fig. (**2**). A fragment condensation of the complex $RNase_{1\text{-}39}$ glycopeptide made with ψPro fragments was carried out successfully using SPPS without racemization [19]. The use of polyethylene glycol (PEG)-based resins, particularly ChemMatrix® (CM), showed benefits over traditional polystyrene-based resins [20, 21]. CM was very stabile with a high loading capacity and has been used in both aqueous solutions and during the synthesis of difficult hydrophobic peptides [21]. The use of ψPro in combination with CM was successful in the synthesis of a 68-amino acid chemokine, which was unsuccessful using a polystyrene resin due to aggregation [22]. Kessam *et al.* suggested that a newly developed resin incorporating PEG polymerized onto a poly(vinyl alcohol) core may be even more valuable, economically and environmentally, after demonstrating its promise against other resins in synthesis of a section of acyl carrier protein [23].

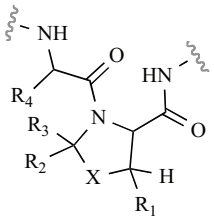

Fig. (2). General structure of a pseudo-proline (ψPro) residue.

Biological Peptide Synthesis

Biological methods of peptide production countered some of the shortfalls of traditional chemical synthesis (e.g. sequence length and timeframe concerns). Enzymatic synthesis and native isolation were widely used for the assembly of shorter active peptide sequences [24]. Fermentation was used for production of short peptides, such as cyclosporine [25]. *In vivo* and *in vitro* recombinant systems utilized generated fragments of DNA or plasmid DNA to ultimately translate the protein or large peptide of interest. These methods are limited by difficulties in purifying the peptide of

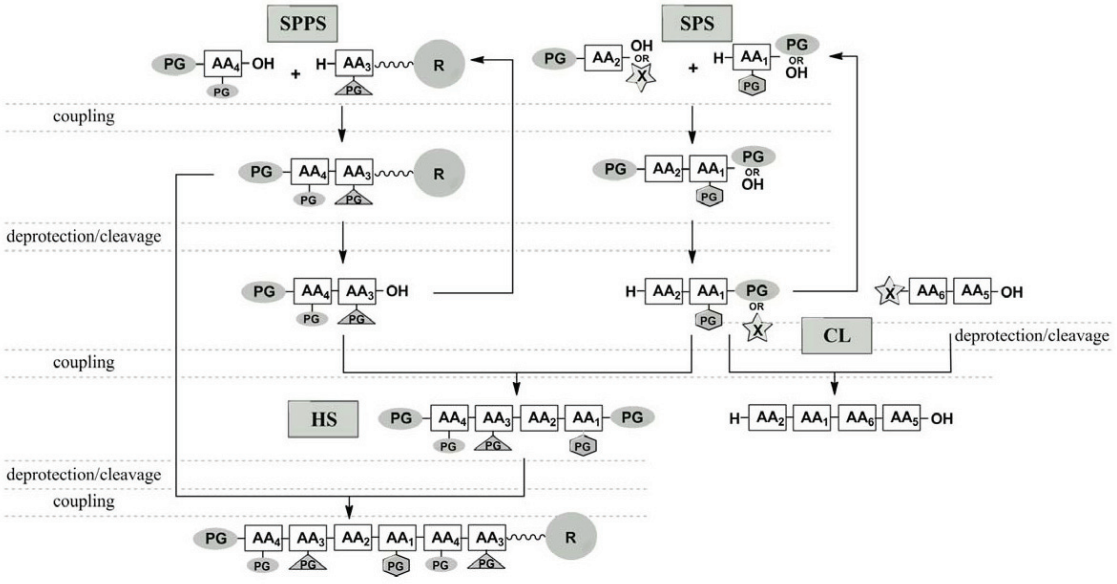

Fig. (1). Principals of chemical syntheses. Each method involved a cycle of deprotection and coupling to form a chain of amino acids. PG: protecting group; AA: amino acid; X: (activated) functional group; R: solid support resin; SPPS: solid-phase peptide synthesis; SPS: solution-phase peptide synthesis; HS: hybrid synthesis; CL: chemoselective ligation.

interest from the other peptides and proteins from the expression system, and the inability to incorporate a large range of unnatural amino acids.

Recombinant Synthesis

Recombinant synthesis involves the expression of the peptide within a specialized system from an artificial gene. *In vivo*, this is commonly prepared in microorganisms like *Eschericia coli* (*E. coli*) [26]. In some cases, *in vivo* production took several weeks but the significant benefit was the ease of upscaling to high-volume fermentation. In these expression systems, the peptides were usually fused to carrier domains (e.g. protein) that facilitate their targeting into the periplasm or inclusion bodies. This method allowed for a simpler, rapid purification (e.g. low-speed centrifugation followed by nickel chelation chromatography) of the expressed peptide from the proteins and peptides already existing within the organism. A number of peptide therapeutics prepared by *in vivo* methods are currently available on the market. For instance, ecallantide (Kalbitor®, Dyax Corp.) and desirudin (Iprivask®, Canyon Pharmaceuticals), which are 65 and 60 amino acid residue peptides expressed in yeast, and teriparatide (Forteo®, Eli Lilly Co.), 34 residues expressed in *E. coli* [27-29]. The assembly of a polymeric, tandem-repeat cDNA sequence coding for peptide units separated by stable or cleavable linkers has been used for expressing smaller peptides [30].

A more recent advance in recombinant expression technology was the use of *in vitro* (cell-free) systems, in which, fragments of DNA were transcribed and translated in a one-pot reaction Fig. (**3**) [31]. The advantage of these systems is that peptides and proteins can be generated in a short time (minutes to hours). Autonomous cells were used traditionally but a number of alternative organisms such as *Leishmania tarentolae* (a protozoan parasite of lizards) were developed [32] and have been shown to be cheaper than previous systems [33].

The major limitation of biological peptide synthesis is the inability to incorporate unnatural amino acids. However, this can be overcome using a range of *in vitro* expression techniques. Extended anticodon tRNAs (pre-aminoacylated with unnatural amino acids) substituted natural tRNAs to selectively include these amino acids in a number of sequences [34]. One example was the introduction of genetically programmed cross-linking by the presentation of two unnatural residues. Neumann *et al.* incorporated alkyne- and azide-containing amino acids to facilitate a rapid cycloaddition, to form a triazole cross-linkage for enhanced proteolytic and structural stability [35].

Other

An alternative to using expression vectors is the production of peptides as hydrolysates from food proteins. This technique has resulted in the production of a range of bioactive peptides. Peptides were derived from bovine milk proteins that had angiotensin converting enzyme inhibitory, immunomodulatory, antithrombotic and hypocholesterolemic activities [36-38]. Enzymes were also applied to add functional modifications to peptide precursors (e.g. glycosylation [39], amidation [40]). Although biological methods have shown considerable promise, these approaches are still generally expensive and are not necessarily viable for production in industrial scales.

Combined Chemical and Biological Synthesis: Peptide Semi-synthesis

Semi-synthesis was developed as the combination of biological and chemical peptide synthesis methods. Peptide sequences produced by recombinant methods were further altered chemically and/or enzymatically. One of these examples is liraglutide, approved by the FDA in 2010 for the glycemic control of type two diabetes mellitus in adults. Liraglutide is an analogue of glucagon-like peptide 1, recombinantly expressed in yeast with a 16-carbon lipid chain chemically attached to Lys^{26} via a glutamic acid spacer [41, 42]. Televancin (Vibativ®, Theravance/Astellas Pharma) is a second example of peptide semi-synthesis. Used to overcome resistance and to improve ADME properties, televancin is made by the lipidation of vancomycin (a glycopeptide antibiotic synthesized using fermentation techniques) [43]. In another example of complicated semi-synthesis, the aforementioned ψPro $RNase_{1-39}$ glycopeptide fragment condensation product was coupled to a recombinantly-expressed $RNase_{40-124}$ peptide fragment using native chemical ligation, producing the full protein [19].

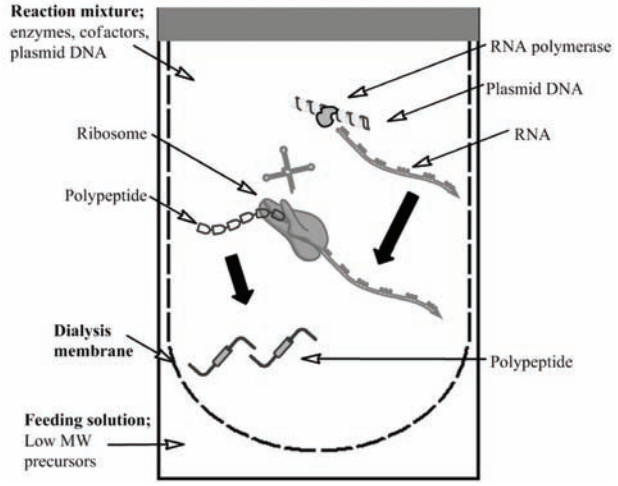

Fig. (3). Cell-free peptide expression system. Low molecular weight (MW) precursors fed through the dialysis membrane to the molecular machinery derived from the cell lysate. The template DNA was added inside the membrane with the lysate and translated and expressed in a one-pot reaction.

PEPTIDE VACCINES

Compared with traditional attenuated vaccines, modern subunit peptide vaccines have increased safety, higher specificity, greater purity and are more cost-effective to produce. Subunit peptide vaccines only include the minimal antigenic components required for vaccine efficacy [44]. These peptide-based vaccines are easily modified, non-infectious and highly stabile against enzymatic degradation when altered and/or incorporated into carrier [45]. Unlike some other formulations, peptide-based subunit vaccines exist as one entity inclusive of all components essential for a successful vaccine, incorporating adjuvant, carrier and antigen [46, 47]. This eliminated the need to co-administer other compounds (e.g. adjuvant) that could be associated with adverse effects in the host.

The inherent instability of peptides in peptide-based vaccines has the potential to induce non-specific immune responses or may fail to stimulate immunity. Natural peptide epitopes have been substituted with peptidomimetics and peptide-peptidomimetic hybrids to enhance specificity and immunogenicity since the early 1990s. The use of peptidomimetics for designing antigens is comprehensively reviewed by Croft and Purcell [48].

Lipopeptide Vaccines

The immunogenicity of peptide vaccines can be improved by the use of lipopeptides. The N-terminal moiety of a lipoprotein found in *E. coli*, tripalmitoyl-S-glyceryl cysteine or Pam3Cys, was elucidated in 1983 [49]. The conjugation of bacterial lipid constructs, such as Pam3Cys, to synthetic peptides induced high antibody titers [50, 51]. The mechanism of action remains unclear,

though it was suggested that they assisted in the anchoring, internalization and presentation of the attached antigens [52]. This was due to the action of cell-surface receptors, such as the Toll-like receptors, which recognized structurally conserved pathogen-associated molecular patterns [45].

Another strategy to improve the immunogenicity of peptides was the incorporation of multiple copies of the peptide in an antigenic peptide system with a polylysine core [47]. The addition of lipids to this system led to the development of the lipid-polylysine core-peptide, LCP Fig. (**4**). The lipidic tail of the LCP system elicited Toll-like receptor activity, which was observed *in vitro* by its action on the receptor-transfected human embryonic kidney cells [53]. It also induced *in vitro* maturation of splenic dendritic cells. Of particular interest, the LCP constructs exhibited significant immunogenic effects without the need for any additional adjuvant [54, 55]. LCPs were shown to have an additional benefit of storage in freeze-dried form with resistance to temperature and peptidases, due to qualities of lipidation and the multiple antigenic peptide system [45]. The versatility of the LCP system has been tested using antigens from *Chlamydia trachomatis* (*C. trachomatis*) [56], human papillomavirus [57], and *Streptococcus pyogenes* [58-62]. In particular, the incorporation of a monomeric peptide derived from *C. trachomatis* incorporated into the LCP system conferred an increase of immunogenicity by up to 3200-fold [56]. When administered subcutaneously in mice, these vaccine candidates elicited high levels of IgG antibodies. Intranasal administration was also shown to be successful with this system [63].

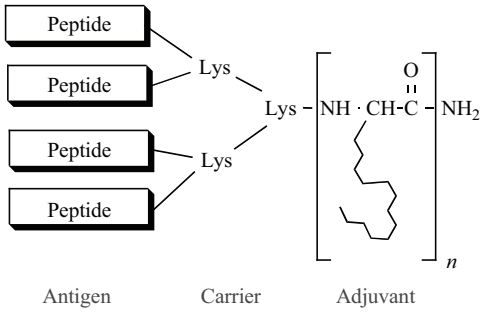

Fig. (4). Lipid-core peptide (LCP) system.

The LCP contains all elements of a successful vaccine system: antigen (peptide), carrier (polylysine) and adjuvant (lipidic tail).

Carbohydrate-Lipopeptide Vaccines

In addition to the use of lipopeptides, carbohydrates can also improve the immunogenicity of peptide adjuvants. The conjugation of carbohydrate moieties to ovalbumin (OVA, derived from egg white protein) enhanced antigen presentation [64], while coating liposome-protamine-DNA nanoparticles with mannan enhanced the immunogenicity of a human papilloma virus-derived peptide epitope [65]. Carbohydrates have also been modified to act as vaccine carriers, providing variable orientations for the attachment of peptide antigens. Changing the structure of the carbohydrate carrier could lead to optimization of the antigen conformation, thus enhancing recognition by the immune system. Carbohydrate-based LCPs showed promising results; eliciting high IgG antibody titers after subcutaneous administration in mice [57, 61].

Nanovaccines

Organic and inorganic polymers, helical peptides, β-sheet forming peptides and carbon nanotubes were applied in the design of successful peptide-based nanoparticle vaccines. The use of these systems was recently reviewed by our group [66]. A Group A Streptococcus peptide epitope was attached to a self-adjuvanting polyacrylate dendrimer nanovaccine, which produced high epitope-specific antibody titers after intranasal administration. These antibodies were able to opsonize Group A Streptococci [67]. High antibody titers were achieved following subcutaneous administration in mice [68]. Promisingly, nanoparticle vaccines were shown to provide some degree of protective immunity after mucosal vaccination [69].

MODIFICATIONS TO ENHANCE BIOLOGICAL ACTIVITY OF PEPTIDE THERAPEUTICS

Although peptides have the potential to act as therapeutic drugs, their physico-chemical properties often limit their progression from research lead to use in the clinic. Fortunately, many physico-chemical properties can be altered to improve drug delivery and enhance biological activity of therapeutic peptides (e.g. stability, transportation and distribution, affinity and targeting, controlled release, and immunogenicity). A particular challenge is the development of peptide therapeutics suitable for oral delivery. The gastrointestinal tract (GI) impedes delivery of peptide drugs. Along with cellular peptidases from mucosal cells, the brush-border membranes of epithelial cells form a major enzymatic blockade [70]. Exopeptidases, aminopeptidases and carboxypeptidases in the GI, break down sequences from the N- and C-termini, whereas endopeptidases recognized cleavage sites within amino acid sequences. The combination of these types of enzymes facilitates rapid and efficient degradation and is a significant barrier to the administration of peptide therapeutics via the GI route. Acid-labile peptide bonds, such as that of an aspartyl-proline bond [71], contribute to a poor oral bioavailability. Chemical modifications Fig. (**5**) at positions known to be susceptible to enzymatic cleavage were reported to increase *in vivo* stability of peptides-based therapeutics dramatically. Substitutions with D-amino acids and cyclization enhanced peptide stability profiles. Other approaches included the use of other unnatural residues, modification of peptide bonds, termini protection/modifications and conjugation to carrier constructs, such as biocompatible polymers [72].

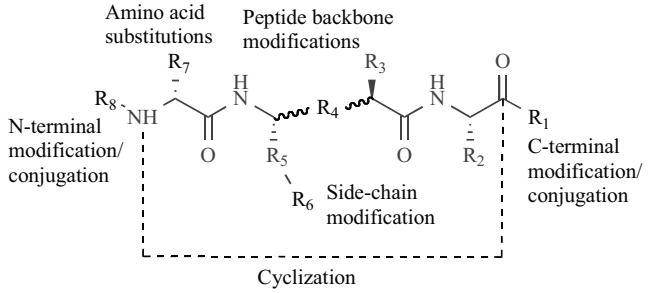

Fig. (5). Examples of chemical peptide modifications. R2, R3, R5, R7 = Amino acid side chains, proteinogenic or otherwise. Possible modifications: R1, R8 = lipids, sugars, acetyls, polymers. R4 = reduced, substituted peptidomimetic bonds. R6 = methylation, acetylation, hydroxylation. R7 = D-conformation, *tetra*-substituted amino acids.

Solubility, lipophilicity, molecular weight and ionization contribute most to biodistribution of therapeutics. However, peptides with strong overall positive charges interact with the phosphate heads of lipids, thus are better able to cross biological membranes [73]. Most tested peptides are cleared from circulation within minutes and oral delivery is rarely possible. Rapid hepatic and renal clearance, aggregation and poor absorption due to hydrophilicity are the major challenges observed in peptide transport.

Peptides were protected from exopeptideases by modification at their N- and C-termini. Examples of modifications were the use of N-pyroglutamate, C-amidation, acetylation [74], pegylation (PEG) [75] and conjugation of carbohydrate moieties [76-78]. Glycosylation at both N- and C-termini conferred improved stability to peptides [76-78].

Route of Administration

The route of administration affects the pharmacokinetics of peptide drug candidates. Parenteral, oral, mucosal and transdermal routes have been explored, with the parental route being most common. However, a number of commercial liposome-based technologies have evidenced the viability of peptide drugs for transdermal and pulmonary delivery [79, 80].

Several non-parenteral routes for peptide delivery bypass the gastrointestinal tract, BBB or liver first-pass metabolism. Formulation strategies are important in these delivery routes for enhanced stability *in vivo* [81]. Excipients, such as salts, cyclodextrins, chelating agents and protease inhibitors altered the natural environment, slowing down the peptide degradation.

The skin is an excellent barrier against large, polar, hydrophilic compounds such as peptides. Nevertheless, transdermal delivery has an advantage over the parental route, enabling control of drug concentration in a non-invasive manner. The modification of lipophilicity, addition of solvents and a number of physical methods, (ultrasound, iontophoresis and electroporation), have all increased peptide permeability [82]. Patches containing microprojections have also shown their effectiveness. A successful example of peptide vaccine transdermal delivery was the use of dry-coated microprojection patches, which required only 1/30 of the dose needed for intramuscular administration [83]. These technologies facilitated the passage of peptides into the epidermis, where it was absorbed into systemic circulation via microcapillaries. A liposome-based transdermal system for the delivery of interferon α-2b recently progressed through to phase II/III clinical trials.

The large, absorptive surface of the alveoli made pulmonary delivery another possible delivery route [84]. Permeation enhancers (e.g. salicylates, fatty acids, bile salts and surfactants) were used to facilitate greater absorption. The potential for long-term negative effects, such as irritation and lung damage, and the cost-benefit implications of the recently withdrawn inhalable insulin peptide drug Exubera® (Pfizer) made this method less favorable [85].

Due to its vascularized subepithelium, the intranasal route provided a large surface area with high permeability and rapid absorption to the systemic circulation. The mucus gel layer and mucociliary apparatus have proven to be considerable barriers to intranasal delivery. Absorption enhancers have been used with some success for overcoming these barriers, however many have been shown to damage the sensitive nasal mucosa [86]. Intranasal delivery of insulin has been thoroughly investigated and a number of nanoparticle systems have recently shown promise in animal models [87, 88]. In one example, vasoactive intestinal peptide was shown to reach the brain when delivered intranasally, whereas intravenous delivery was unsuccessful, indicating a fraction passed through a direct transport pathway [89]. A number of agonists based on natural peptides, such as calcitonin (e.g. Miacalcin®, Novartis), desmopressin (e.g. Desmospray®, Ferring), luteinizing hormone-releasing hormone (LHRH) (e.g. Kryptocur®, Sanofi-Aventis), and oxytocin (e.g. Syntocinon®, Novartis), has been approved for intranasal delivery [90, 91].

Oral delivery is the preferred delivery route due to the ease of administration and greater patient compliance. GI delivery is hampered by concentrated enzymatic activity, the need to pass across the intestinal musical epithelia, and the range of pH (1.2-8.0) encountered along the GI tract. Oral delivery of insulin using polymers and emulsions have reached clinical trials [81] and a number of formulations of ciclosporin (e.g. Sandimmune®/Neoral®, Novartis) and desmopressin (e.g. DDAVP®, Ferring) have been successfully carried through to the clinic too.

Lipidation

The attachment of a lipid moiety protected an unstable peptide from enzymatic degradation [92]. The lipid was attached via either a stable linkage, or a labile one, creating a pro-drug [93, 94]. This use of a pro-drug strategy enhanced the pharmacological characteristics of the peptide without decreasing its activity or selectivity [95]. The use of lipidation to form a pro-drug is a useful strategy to improve the uptake of peptide therapeutics. However, lipidation may impair peptide delivery by causing poor water solubility.

N-terminal conjugation with lipidic amino acids increased the half-life of LHRH by approximately 30-fold in Caco-2 cell homogenates [92]. The lipidic moiety was cleaved first, releasing the active parent peptide, acting in a pro-drug manner. This specific cleavage mechanism was also observed in plasma and kidney preparations [92, 96]. Due to association of the lipid-modified antidiabetic liraglutide with plasma proteins, plasma half-life of the peptide increased from 1.2 to 13 hours after subcutaneous administration [41]. The peptide drug tesamorelin was approved by the FDA in 2010, and used for the treatment of visceral fat accumulation in HIV-infected patients with lipodystrophy. Modification of tesamorelin with a hexenoyl moiety enhanced resistance to dipeptidyl peptidase 4, which deactivated the parent peptide: growth hormone-releasing hormone [97].

Lipidation of hydrophilic compounds has been used extensively to improve absorption by passive diffusion across epithelial barriers, notably in the gastrointestinal tract and the central nervous system [76, 98]. Lipid moieties coupled to peptides conferred the characteristics essential for intestinal delivery [99]. In studies using a more stable enkephalin analogue, [D-Ala^2, D-Leu^5]-enkephalin, it was observed that an increased lipid moiety chain length enhanced its permeability as long as the solubility was not affected [100]. However, Fredholt *et al.* observed a decrease in permeability with increased lipophilicity in ester pro-drugs [101]. Kellam *et al.* studied the influence of the carbon chain length on the activity of [D-Ala^2, D-Leu^5]-enkephalin and found that the increased chain length led to a decreased activity in mouse vas deferens and guinea pig ileum models [102].

Glycosylation

Liposaccharide-based delivery systems were designed to improve bioactive peptides [76, 98] and were shown to enhance their bioavailability [77, 98, 103, 104]. Somatostatin, a peptide with poor pharmacokinetics, was modified with lipids and with or without a monosaccharide moiety. The liposaccharide-modified somatostatin increased the permeability of the parent peptide when tested *in vitro* across a Caco-2 cell monolayer [105] without eliminating the activity of the lead compound. Another example was C-terminal conjugation of a lipid and a monosaccharide attachment on the N-terminus of a dipeptide, which increased permeability 60-fold [76].

Glycosylation was used to target receptors that naturally recognize specific carbohydrates, such as lectins. Glycosylated peptides were conveniently targeted to particular tissues or organs, or even specific cells for distinct actions. Arg-vasopressin was used as a model peptide to explore the possibility of renal targeting. The carbohydrate-modified arg-vasopressin peptide bound selectively to microsomal fractions of the liver and kidney [106]. In another study, OVA was used to demonstrate the efficacy of glycosylation for the targeting of dentritic cells, increasing antigen presentation by 50-fold for CD4+ T cells and 10-fold for CD8+ *in vitro* [64]. Nevertheless, liposaccharide modification does not guarantee ideal ADME properties in peptides.

Unnatural Amino Acids

The physico-chemical properties of peptides can be improved by the substitution of natural amino acids with unnatural ones: D-conformation, N-methylation, *tetra*-substitution, β-amino acids, and side chain methylation(s). Unnatural amino acids are rarely the

substrates of proteolytic enzymes, therefore their inclusion into the sequence increased the stability of the modified peptide. For example, a D-peptide analogue of C-X-C chemokine receptor type 4 demonstrated dramatically improved stability. The natural L-peptide was degraded within 24 h, whereas the D-peptide showed no signs of degradation, even after 72 h [107]. The antimicrobial peptide heptaibin primarily consisted of unnatural amino acids and was intact after 12 h treatment with proteolytic enzyme pronase E in Tris buffer [108]. Icatibant (Firazyr®, Shire), a bradykinin B_2 receptor antagonist decapeptide authorized by the European Commission in 2008 and the FDA in 2011 for treatment of hereditary angioedema, also contained unnatural amino acids [109]. D-amino acids have been used to make large oligomeric mimics of natural peptides. Using D-amino acids and synthesizing the peptide in reverse sequence order resulted in a similar side chain topology to the parent peptide. These "retro-inverso" (RI) peptides, exhibited similar binding activity with enhanced resistance to proteolysis, therefore increased stability. A peptide with the potential for treatment of Alzheimer's (OR2) was synthesized as an RI peptide. Taylor *et al.* demonstrated that the RI analogue remained intact after 24 h, by which time the parent peptide has been completely degraded [110].

The use of unnatural amino acids has enhanced interactions with peptide targets. D-Amino acid substitutions in β-casomorphin were shown to enhance μ-opioid receptor binding and analgesia [111]. A more complicated modification of a tumor-suppressing p53-derived peptide, including the substitution of unnatural amino acids, led to a more stabilized helix, with an increase of affinity to the Hdm2 oncoprotein up to 63-fold [112].

Peptidomimetics

In addition to the substitution of unnatural amino acids, modifications to peptide bonds improved peptide stability. Chemical modifications to peptide bonds ranged from simple reductions, to the replacement of the carbonyl or amide groups with esters, sulfides and alkyls. This group of peptide analogues was classified as 'peptidomimetics' [113].

Zuckermann and colleagues introduced a family of peptidomimetics called 'peptoids' [114]. The difference between peptides and peptoids was that the amino acid side chain shifted to the α-amino group [115]. An antimicrobial 'KLW' peptide (Lys, Leu and Trp residues) was synthesized as a peptoid and was resistant to trypsin, without the loss of antimicrobial activity. The L-peptide, however, lost all activity after the enzymatic treatment [116]. Other important classes of oligomeric peptidomimetics include urea peptidomimetics, peptide-carbamates and peptido-sulfonamides Fig. (**6**). Most peptidomimetics were synthesized using altered SPPS protocols [117-119]. Peptidomimetics and peptidomimetic-peptide hybrids were used to alter peptide conformation by changing the bond rotations. Urea peptidomimetics and their hybrids mimicked the helical structure of host-defense peptides for use as antimicrobials [120]. β-Peptidomimetics also assumed defined helical structures, with as few as six residues able to form a helix [121]. However, the properties of peptoids have conferred increased flexiblility-thus decreasing affinity-and sulfonamides have been shown to break α-helical and β-strand secondary structures [122].

Another example of natural bond modification was the substitution of disulfide bond(s) in cyclic peptides (e.g. α-conotoxin). In this case, selenocystein technology (based on the replacement of sulfur with selenium) was used to produce more stable and potent nicotinic antagonists [123].

Peptide Carrier/Delivery Systems

In addition to increasing the stability of peptides, peptide carriers or delivery systems such as microspheres, nanoparticles, PEG and polyvinylpyrrolidone (PVP) polymers, along with lipid-based (liposomes, micelles) carriers, a affect the release characteristics via diffusion through, and degradation of, the encapsulating polymer Fig. (**7**) [75, 124]. These particles effected bioadhesion (residence time and absorption), biodegradability (release kinetics), and biocompatibility. Lanreotide, which has been used extensively in Europe (approved by the FDA in 2007), was prepared by a microsphere encapsulation of biodegradable polymer to form a prolonged release system [125]. Furthermore, nanoparticles were effective in delivering dalargin, an enkephalin analog, through the blood brain barrier (BBB) [126]. Steric shielding proved successful for the prevention of peptide degradation on the surface of carrier molecules. A HIV cell-penetrating TAT peptide was shielded successfully using PEG chains attached to the surface of micelles [127].

Some peptides were used as vectors for enhanced transport. Delivery of the peptide dalargin to the brain was enhanced by the conjugation to the peptide vectors SynB1 and SynB3. These chimeric peptides provided enhanced antinociception in mice and passed through the BBB by adsorptive-mediated endocytosis [128].

Conjugation to carrier molecules stabilized the structure of peptides and/or acted as a vector to target specific cells/organs. For example, desmorphin coupled to the OX26 carrier or β-endorphin coupled to cationized albumin for delivery across the BBB [129, 130]. Peptides have shown promise for use as a targeted anti-tumor therapy. An anticancer drug, camptothecin, was conjugated to a PEG carrier with an LHRH analogue (tumor targeting moiety). This multifunctional polymer conferred enhanced antitumor activity over the camptothecin-PEG conjugate alone [131]. Another peptide, IF7, targeted tumor vasculature within minutes, as demonstrated by conjugation to a florescent moiety and enhanced action after conjugation to the anticancer drug, SN-38 [132]. In some cases, vectors have also been shown to lower the toxicity of cationic cytotoxic peptides. An arginylglycylaspartic acid peptide recognizing $\alpha_v\beta_3$ integrin, which is overexpressed in tumour cells and vasculature, coupled to a proapoptotic peptide killed tumor cells with lower nonspecific cytoxicity than expected from cationic proapoptotic peptides [133].

Cyclization

Cyclization of peptide sequences improved peptide stability by eliminating a degree of proteolysis. Since the 1940s, the interest in cyclic peptides has grown substantially [134]. One of the cyclic polypeptide drugs reported in 1945 was bacitracin, which was recently approved by FDA for intramuscular treatment of infants with staphylococcal pneumonia. Depending on the functional groups, peptides were cyclized head-to-tail, head/tail-to-side-chain or side-chain-to-side-chain. Most commonly, cyclization was performed by lactamization, lactonization and sulfide-based linkages. Cyclization of herpes simplex virus by glycoprotein D, was shown to greatly increase enzymatic stability [135]. A thioether linkage provided complete resistance to degradation in 50% human sera, even after 96 h, while the linear peptide was completely degraded during this time. Cyclic variants including a disulfide bridge and peptide bond were demonstrated to increase resistance to degradation with 29% and 73% of the peptide remaining, respectively [135].

Cyclization produced peptides with conformational restrictions. Neurotoxin peptides (e.g. the conotoxins), somatostatin, oxytocin [123], LHRH [136] and insulin with sulfide-based bridges were all shown to be potent peptides. Ring closing metathesis (RCM) was used in peptides, providing alkene staples as substitutes to sulfides and provided alternative constraints. Enkephalin [137], oxytocin [138], α-conotoxin IMI [139] and lanreotide [140] were subjected to RCM modifications. Backbone cyclization of natural conotoxin was shown to be effective for the development of an orally active peptide for the treatment of neuropathic pain [141]. Stapled peptides, such as inhibitors of induced myeloid leukemia cell different-

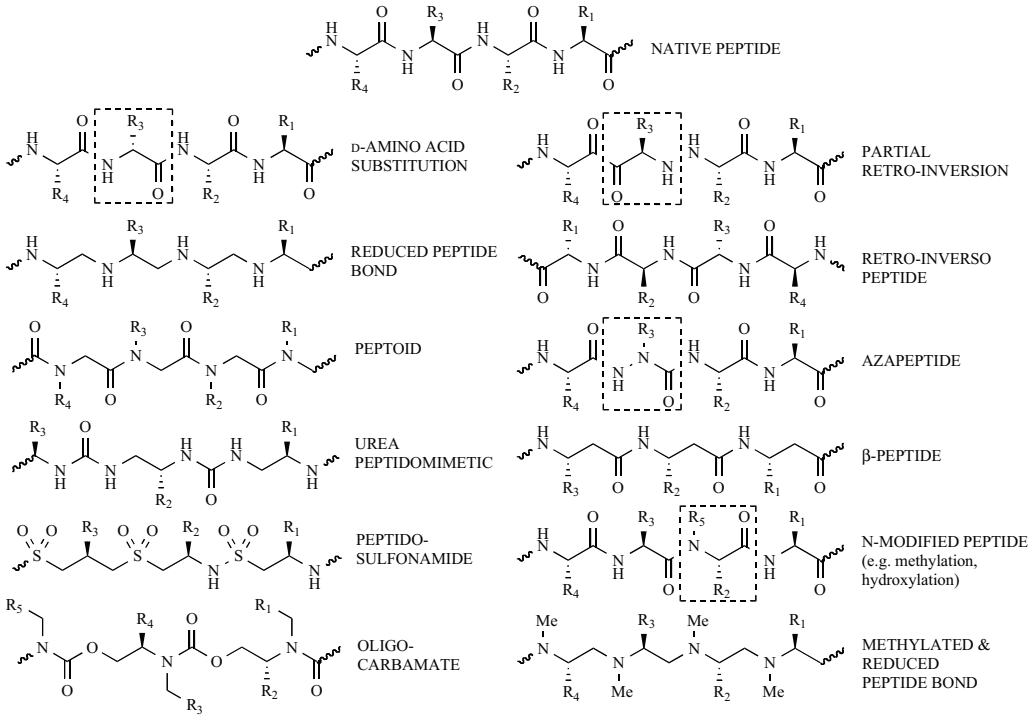

Fig. (6). Examples of peptidomimetics. Modifications range from simple reductions to the replacement of the carbonyl or amide groups with esters, sulfides and alkyls, as well as the use of D-confirmations and β-amino acids.

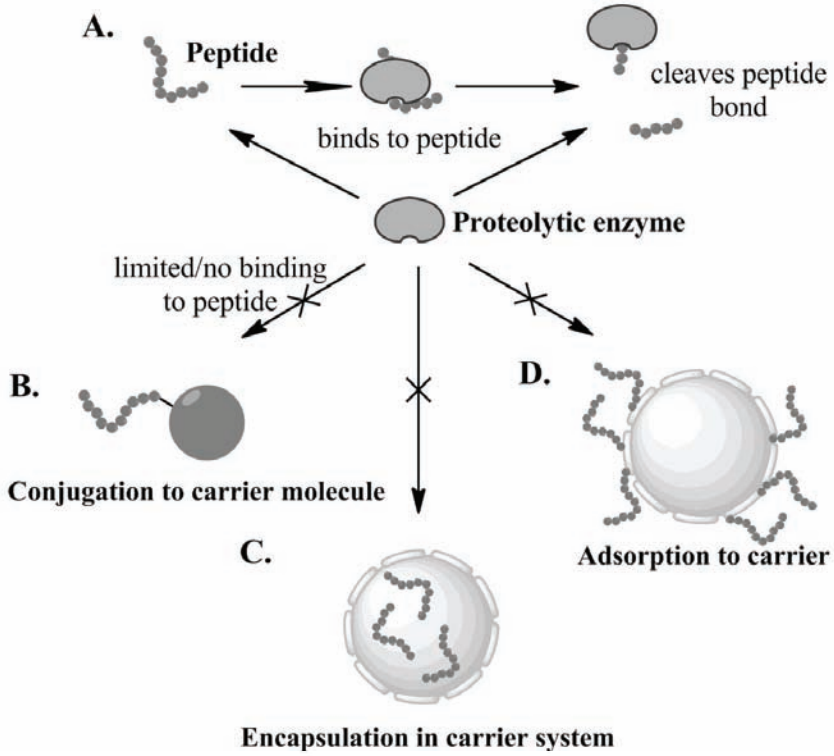

Fig. (7). Conjugation and encapsulation of peptide therapeutics.
A free peptide is degraded rapidly by proteolytic enzymes (**A**). A number of carrier-based transport systems block enzymes from binding to the peptide cargo and cleaving peptide bonds (**B-D**).

tiation protein, showed enhanced selectivity to the target [142]. Formed by simple "click chemistries", 1,2,3-triazoles can mimic trans- and cis-amide bonds, depending on substitutions. Cyclotides, cyclic plant peptides with three conserved disulfide-bonds, were found to be active against HIV. The cyclotide activity was independent of a protein receptor and dependent on peptide oligomerization at the membrane surface [143].

Covalently "stapling" sections of the peptide chains enhanced stability in serum. For example, isomer A, the stapled α-helix of BH3 (previously shown to suppress leukemia cell growth), had

enhanced stability over the unmodified BID BH3 peptide in mouse serum [142]. The stapling of a tumor-suppressing, p53-derived peptide conferred a 4-fold increase over its wild-type [144]. Enfuvirtide was recently hydrocarbon stapled to remedy its instability. It was suggested that these staples create a "proteolytic shield" by reinforcing an α-helix and blocking proteolysis at the staple sites completely [145].

Peptide Receptor and Transporter Targeting

Conjugation to molecules that are recognized by a transporter or specific transport pathway facilitated transport across biological barriers. For example dermorphin (a natural opioid receptor agonist), bound by a cleavable disulfide bridge to OX26 (a monoclonal antibody that binds the transferrin receptor), was shown to produce analgesia, confirming that it passed the BBB [129]. Further investigation identified the Na1-dependent glucose transporter was responsible for the improved transportation of a glycosylated tetrapeptide. The permeation of the β-anomer was 5-times greater than that of the α-conformation [77]. Glycosylation of Leu-enkephalin at the N- or C-termini enhanced transport profiles in a number of cases [102, 146, 147]. Addition of carbohydrates to the side chain of the serine on the N-terminus of the peptide was the most successful modification for retaining its activity. Disaccharide conjugates had considerable antinociceptive properties, with profiles similar to that of morphine [148].

Synthetic peptides were demonstrated by Stoermer *et al.* to bind to protease-activated G-protein coupled receptors. These short peptide sequences were of therapeutic interest because they activated the receptor on immunoinflammatory and cancer cells, amongst others [149].

Scanning, making libraries of peptides with substituted amino acids to determine the necessity and function of each bond and side chain and potential cleavage sites, has led to the simplification and optimization of peptide chains. These methods generated analogues with improved physico-chemical characteristics. Scanning was usually performed using alanine (Ala-scan) [150] and D-amino acids (D-scan) [151]. Ala-scans determined the residues important for signaling and binding purposes and directed mutations were performed to enhance affinity. An Ala-scan resolved the locations for modifying the proinflammatory cytokine, IL-15. A simple amino acid substitution in the $IL\text{-}15_{36\text{-}45}$ peptide led to a change in the half maximal inhibitory concentration from 130μM to 24μM [152].

CONCLUSIONS

Along with biological aspects of developing peptides as therapeutics, manufacturing processes have been improved, making peptides more attractive to the commercial market. A large number of potential peptide therapeutics have not been FDA approved because of their poor physico-chemical characteristics, thus further exploration of techniques aimed at improving the properties of potential peptide therapeutics is critical to the success of this field. The addition of lipidic (e.g. lipoamino acids) and/or hydrophilic units (e.g. carbohydrates, PEG) to potential peptide therapeutics represents an effective strategy for enhanced drug delivery *in vivo*, especially for oral administration. The properties of lipids, carbohydrates and polymers were exploited to render peptide sequences immunogenic, resulting in novel peptide-based vaccine systems. Polymers were also extensively researched for the delivery of peptides, either by their attachment or encapsulation. The use of peptidomimetics, unnatural amino acids and cyclization technologies provided peptides with enhanced characteristics. With the promising *in vitro* and *in vivo* results that all of these delivery systems displayed, many of these analogues and conjugates will progress into clinical trials. It is foreseeable that the synthesis and modification techniques described herein will augment the potential of peptide therapeutics to come.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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