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Research review paper

Designing improved active peptides for therapeutic approaches against infectious diseases

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ABSTRACT

Infectious diseases are one of the main causes of human morbidity and mortality. In the last few decades, pathogenic microorganisms' resistance to conventional drugs has been increasing, and it is now pinpointed as a major worldwide health concern. The need to search for new therapeutic options, as well as improved treatment outcomes, has therefore increased significantly, with biologically active peptides representing a new alternative. A substantial research effort is being dedicated towards their development, especially due to improved biocompatibility and target selectivity. However, the inherent limitations of peptide drugs are restricting their application. In this review, we summarize the current status of peptide drug development, focusing on antiviral and antimicrobial peptide activities, highlighting the design improvements needed, and those already being used, to overcome the drawbacks of the therapeutic application of biologically active peptides.

1. Introduction

In the present century, healthcare is facing diverse challenges, in particular the increasing number of antibiotic resistance cases (Arias and Murray, 2009) and diagnosed cancers (with some of them directly related to chronic infections) (Arnold et al., 2015; Attiê, 2014), as well as the spread of novel viral strains (Bhatt et al., 2013; Skalickova et al., 2015). The emergence of multi-resistant bacterial strains belonging to the *ESKAPE* pathogen group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa* and *Enterobacter* species) (Edwards et al., 2016), and high rates of viral spread, associated mostly with tropical and subtropical regions (Maharajan et al., 2016), have massively contributed to this public health problem (Dickey et al., 2017; McKenna, 2013).

Pharmaceutical companies are facing major challenges in terms of drug production, costs, research and development. This has caused a substantial delay in launching new antibiotics on the market, not only due to limitations on their development, but also because of the ongoing evolution of those infectious agents that led to the rise in resistance to the drugs most widely used in the last decades. This phenomenon is not only related to human healthcare, but can also be observed in agriculture and veterinary applications (Chen et al., 2005; Gordon et al., 2005). As a strategy to overcome these problems, peptide drugs have been gaining interest, mostly due to their advantages in biocompatibility and target selectivity over conventional drugs, as it can be noticed based on a significant approval rate since the beginning of this century (Ahrens et al., 2012; Fotouhi, 2015). Even presenting high safety and ease of handling or storage, the limitations associated with peptide drugs are restricting their potential application (Cruz et al., 2004). Besides their high proteolytic degradation, limited pharmacodynamic and pharmacokinetic properties, the costs of production and the issues related to intellectual property tend to limit the development of such drugs (Uhlig et al., 2014). In this review, singularities and advances in peptide drug development will be assessed, especially focusing on antiviral and antimicrobial activities, in order to better understand the drawbacks that need to be overcome in the next few years.

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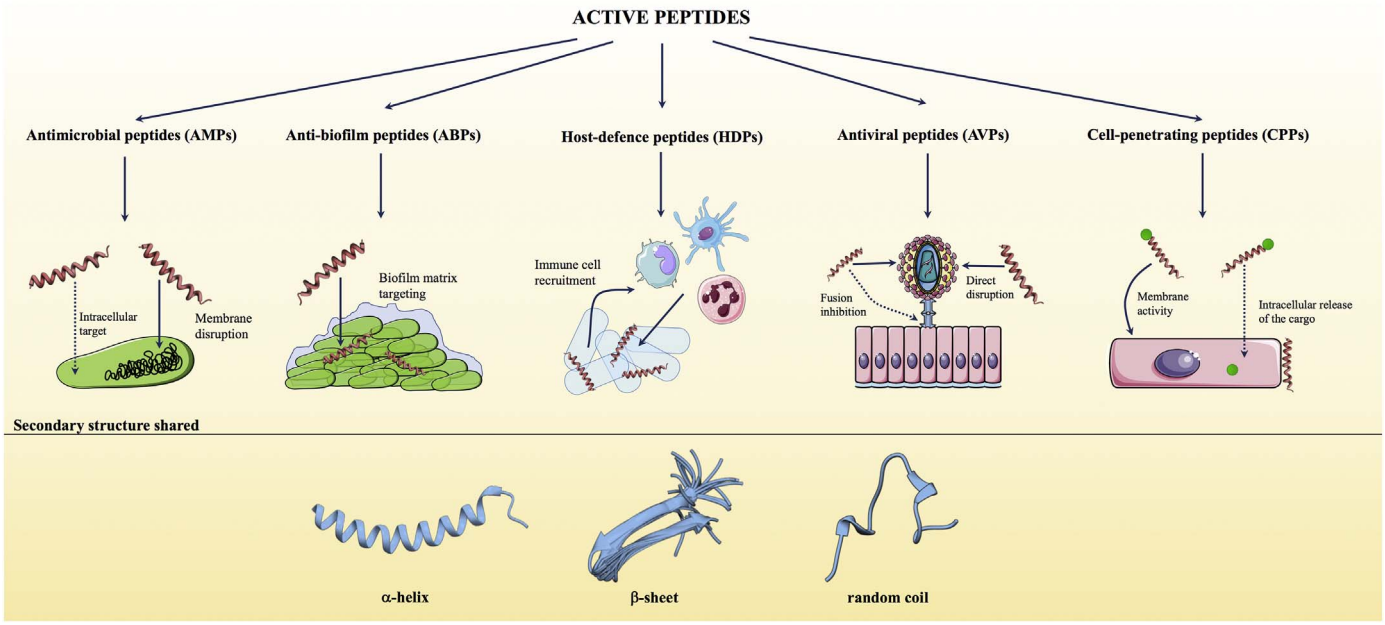

Fig. 1. Distinct categories of active peptides, separated by classification and mechanism of action. Secondary structure is shared by most of the peptides in the different categories.

2. Peptide properties

Peptides are among the ideal candidates to be used as an alternative therapeutic option, alongside conventional drugs, in different therapeutic fields (Lohner and Hilpert, 2016). As already mentioned, peptides have strong potential in terms of drug design and application, but even so, limitations regarding pharmacodynamics and kinetics properties have restricted their applicability in the pharmaceutical, agriculture and quality control markets (Fotouhi, 2015; Li et al., 2008; Lohner and Hilpert, 2016). Even with these obstacles, research regarding peptide activity has been extensive, with the identification of different small amino acid sequences that are active towards viruses, bacteria, fungi or cancer cells (Falcao et al., 2014; Felício et al., 2017; Franquelim et al., 2010, 2008; Matos et al., 2010; Rautenbach et al., 2016; Wang et al., 2008). Continuous research has been focused on simplifying the amino acid sequence, to increase stability and activity, as well as improving peptide pharmacological properties, facilitating potential application and commercialization by the pharmaceutical companies (Ramesh et al., 2016).

Antimicrobial peptides (AMPs) belong to a class of peptides that was first described as natural peptides with activity towards different pathogens, and that have their origin in the innate immune system of different organisms (Brogden, 2005; Smith and Dyrynda, 2015). Most of these peptides have direct activity against bacteria, especially planktonic bacteria, regardless of their origin (Drusano, 2004). When bacteria are not planktonic, they tend to form organized complexes at the infection sites, where they all communicate, improving their survival rates (Batoni et al., 2016). These complexes, known as biofilms, may well have high resistance to antibiotics, but they have shown to be susceptible to peptides (antibiofilm peptides - ABPs) (Ribeiro et al., 2016). Peptides can also present activity against viruses or can actively prevent viral infection of healthy cells (antiviral peptides - AVPs) (Hsieh and Hartshorn, 2016). These different types of peptides may also show activity towards the pathogen by recruiting immune cells (indirect activity), regardless of the peptide's main action. In this situation, they are named host-defense peptides (HDPs) (Hancock et al., 2016). Another class of peptides that has been gaining interest is that of cell-penetrating peptides (CPPs), which share some characteristics with AMPs (Henriques et al., 2006). CPPs have membrane translocation activity, with the ability to transport a small molecule cargo, which could be another peptide, protein, RNA or DNA (Bahnsen et al., 2015; Freire et al., 2017; Rodrigues et al., 2011). Peptides frequently exhibit several of these properties, resulting in poorly defined frontiers in terms of properties and mechanisms (Hale and Hancock, 2007).

2.1. Antimicrobial peptides

The class of AMPs has been refined in recent decades, and most peptides are considered less prone to promote multi-resistance in different pathogens (Brogden, 2005; Chen et al., 2005). Initially described as peptides produced by immune cells, such as neutrophils, eosinophils and macrophages, to fight infections (Friedrich et al., 2001; Mattar et al., 2016; Padhi et al., 2014; Perron et al., 2006), these peptides with a natural origin were later synthetically produced, as an approach to improve their bioactivity, by changing their amino acid sequences (Schr, 2010). Considering those with natural sources, peptides can be isolated from animals, plants and bacteria, but the large amount of biological sample required and the high difficulty of expression led researchers to synthesize those peptides chemically, with the advantage of having a higher purity and yield (Meneguetti et al., 2017; Schr, 2010; Zasloff, 2002). This strategy allowed the emergence of synthetic AMP design by two different approaches. The first was to use natural AMPs as templates and, with small changes considering the optimal properties for a highly active AMP, redesign their sequence, either changing amino acid residues or shortening them (Cardoso et al., 2016; Migliolo et al., 2016, 2012). The other approach was to use bioinformatics tools in the design of purely synthetic *de novo* AMPs (Hilpert et al., 2009). The number of AMP databases has increased, which can help researchers in the design process or in the screening of designed sequences (Seshadri Sundararajan et al., 2012; Waghu et al., 2016). More recently, mathematical algorithms that consider desirable antimicrobial peptide properties for improving activity have also been implemented, using for that the sequences already described in the databases (Melo et al., 2011; Porto et al., 2017).

Despite their origin, antimicrobial peptides are described as small molecules (sequence between 5 and 50 amino acid residues), amphipathic, highly hydrophobic and with a positive net charge (Kang et al., 2017). A few AMPs present negative net charge, and are also highly active, but these are less frequent and have been poorly described in terms of mechanisms of action (Falcao et al., 2014). Regarding their

structure, AMPs can be divided in α-helix, β-sheet or random coil (Broekman et al., 2011). Most AMPs have a random coil structure when in solution, adopting a well-defined conformation (usually α-helical) after interaction with the cell membrane of their targets (Fig. 1) (Brogden, 2005). An example of a peptide with an α-helical structure after membrane interaction is LL-37. This human-cathelicidin peptide, isolated from human neutrophils, is one of the most studied AMPs (Turner et al., 1998). Protegrin-1, isolated from porcine leukocytes, is an example of an AMP with β-sheet structure (Dong et al., 2014). Both AMPs showed high activity towards different pathogens, despite their different structure, which indicates that the structure alone does not determine their efficiency. In fact, properties such as size, charge, sequence, structure, hydrophobicity and amphipathicity are all important in defining AMP activity, and a proper balance between them is essential (Brogden, 2005; Hollmann et al., 2016). Additionally, this balance seems to be important for the lipid selectivity of the peptides, which may result in their antibacterial activity or cytotoxic effects (Alves et al., 2010; Domingues et al., 2014; Gonçalves et al., 2012a, 2012b; Maturana et al., 2017). Even so, the relations between AMP properties, mode of action and target membrane composition are not well understood, which is noted in several studies focused only on the properties of similar AMP sequences (Juba et al., 2015).

It is important to differentiate two general mechanisms of action of AMPs: direct membrane disruption, the most studied and the first to be identified, and an activity not involving membrane disruption (Brogden, 2005; da Cunha et al., 2016; Fjell et al., 2012). Among the membrane disruptive mechanisms, the toroidal pore, carpet, aggregate and barrel stave models were the representations developed to explain the different AMP modes of action at the membrane level (Hancock and Sahl, 2006; Melo et al., 2009; Zasloff, 2002). For instance, magainin 2 is an AMP that is known to promote pathogen destruction by the toroidal model (Campagna et al., 2007; Yang et al., 1998). Other AMPs, such as the extensively studied melittin or PGLa, are known to have a detergent-like effect on pathogen membranes (carpet model) (da Silva and Teschke, 2003; Naito et al., 2000). The similarity between these models is because the AMP leads to the membrane destabilization without any specific pathway or intracellular target to promote these effects, which could be translated in direct activity against the pathogens. In contrast, non-membrane disruptive mechanisms are characterized by pathogen targeting, leading to their destruction, without membrane disruption (Bechinger, 1999; da Cunha et al., 2016; Hale and Hancock, 2007). Even though the initial electrostatic interaction of the peptide with the membrane is essential to drive them towards the pathogen, these AMPs have specific intracellular targets, usually involved in protein, DNA and/or RNA synthesis and regulation, promoting the failure of specific metabolic pathways or fundamental cell biology processes (Brogden, 2005). Buforin II is one of the most studied AMPs; it has been demonstrated that it crosses the cell membrane and accumulates in the cytoplasm (Park et al., 1998). Other examples include indolicin (interferes with the formation of the cytoplasmic membrane), pleurocidin (inhibits essential nucleic acid and protein synthesis) or mersacidin (alters cell-wall synthesis) (Brotz et al., 1998; Friedrich et al., 2001; Patrzykat et al., 2002; Subbalakshmi and Sitaram, 1998). Although there is detailed information about different AMPs with different modes of action, the clear relationship between their physicochemical properties and activity is not well understood (Melo et al., 2011; Schr, 2010). Nevertheless, there are some changes that have been considered in the design of new AMPs, such as the substitution/introduction of specific amino acid residues into their sequences (like arginine, lysine or tryptophan). Changes in the peptide net charge and a well-defined α-helix structure upon membrane interaction are two major characteristics that can improve peptide biological activity (de la Fuente-Núñez et al., 2015; Hilpert et al., 2005; Irazazabal et al., 2016; Mura et al., 2016). The same specifications have also been used to design synthetic anti-biofilm or host defense peptides, to improve their activity. Both ABPs or HDPs share the same physicochemical properties as other AMPs, but they have different mechanisms of action towards their targets (Hancock et al., 2016; Pletzer and Hancock, 2016). ABPs are often identified as AMPs that have no activity towards planktonic bacteria, but are quite effective against biofilms (Ribeiro et al., 2016). HDPs, unlike the other examples, are described as peptides that have an associated immune activity, but that can also be a conventional AMP or ABP (Hancock et al., 2016). Natural or synthetically designed, these peptides can promote pathogen destruction, not through direct peptide action, but by recruiting immune cells to fulfil the task (Silva et al., 2016). Other targets already described for AMPs include fungi (anti-fungal peptides) and protozoan parasites (anti-protozoan peptides) (Lee et al., 2017; Pimentel-Elardo et al., 2010). The number of such peptides described in the literature has been increasing, either from natural origin, or by synthetic redesign (Gonçalves et al., 2017, 2012b; Menzel et al., 2017). As an example, small fragments that may be efficient in the eradication of *Candida albicans* or *Entamoeba histolytica* were identified using the amino acid sequence of LL-37 (Ordonez et al., 2014; Rico-Mata et al., 2013). An anti-protozoan peptide, $Pv\mathrm{D}_1$, a defensin isolated from *Phaseolus vulgaris* (common bean) seeds, has shown activity towards *Leishmania amazonensis* (do Nascimento et al., 2015). Nowadays, besides these strategies, the conjugation of peptides with different moieties, such as lipids, aromatic groups, metals or nanoparticles, is being tested in order to overcome some limitations in their therapeutic application (Ramesh et al., 2016; Schmidtchen et al., 2014).

2.2. Antiviral peptides

Peptides with different therapeutic activities have been classified according to the target pathogen (bacteria, virus, fungi, *etc.*), sharing some features between them (Vigant et al., 2015). Cationic net charge, high hydrophobicity and a well-defined secondary structure upon interaction with membranes are among the main properties that are important for their activity (Skalickova et al., 2015). In this category, and considering AMPs as the peptides that originate from the innate immune system, as already described above, antiviral peptides (AVPs) appear naturally as peptides that are similar in physicochemical properties to conventional AMPs, but with a different target, namely viruses (Akkarawongsa et al., 2008; Augusto et al., 2017a; Gomes et al., 2017). As a matter of fact, there are AMPs that have dual activity against bacteria and viruses, either directly or by the recruitment of immune cells (Hsieh and Hartshorn, 2016; Skalickova et al., 2015). Considering the fact that patients with severe viral infections often develop later bacterial infection, it would be interesting to have a peptide that could target both pathogens. However, even with the increasing number of peptide drugs entering the pharmaceutical market, an effective multi-target peptide drug has not been developed yet (Badani et al., 2014; Skalickova et al., 2015).

Antiviral peptides are characterized with the same chemical features as AMPs (Fig. 1), and their mechanisms of action have been extensively studied, with the identification of some differences in a broad range of viruses (human immunodeficiency virus - HIV, influenza, dengue or herpes viruses) (Rothan et al., 2014; Skalickova et al., 2015). Examples of AMPs that showed antiviral activity range from lactoferricin, which has activity against herpes simplex virus (HSV), latarcin, with activity towards dengue virus, or the different human defensins that were shown to inhibit HIV infection (Andersen et al., 2004; Rothan et al., 2014; Silva et al., 2014; Wang et al., 2008). In these examples, AVPs act by direct disruption of the viral envelope (in enveloped viruses), inhibition of viral replication (targeting the viral polymerase) or by inhibition of the virus-host cell membrane fusion process (Vigant et al., 2015; Wu et al., 2015). The first mechanism is similar to the direct activity of AMPs that lead to membrane disruption. LL-37 is an example of this, with the addition of the proven host-defense activity that helps the process (Currie et al., 2013; Peter Bergman et al., 2007; Wang et al., 2008). The inhibition of virus replication can also be induced by human neutrophil peptides (HNP) 1 to 4, which are also

classified as AMPs (Hartshorn et al., 2006; Tecle et al., 2007). As a consequence, the virus tends to aggregate, becoming inactive in terms of infection, and then tackled by the immune cells (Hsieh and Hartshorn, 2016).

The most studied mechanism of action of antiviral peptides involves interference in the membrane fusion process, an essential step in infection by enveloped viruses. Antiviral peptides that, by strong interactions, block the activity of the viral fusion protein intermediates that are necessary for viral entry, are considered as a promising class of peptide drugs and have been named fusion inhibitor peptides (Franquelim et al., 2013; Lee et al., 2011). These peptides engage through electrostatic and hydrophobic interactions with the exposed glycoproteins of the fusion process and/or with the membrane lipids (Vigant et al., 2015). Enfuvirtide (T20) is an example of a fusion inhibitor peptide that interacts directly with the fusion proteins, binding also to cell membranes (Matos et al., 2010). C5A, a peptide derived from the protein 5A mastaporan-derived MP7, inhibits the fusion process by targeting membrane lipids (Cheng et al., 2008; Sample et al., 2013; Zhang et al., 2016). The efficiency of fusion inhibitor peptides is directly related to the strength of the interaction and the temporal window of action and, initially, the capacity to diffuse and reach their target (Welsch et al., 2013; Zhang et al., 2016). These dependencies influence their possible therapeutic action. Thus, different strategies have been implemented to overcome the limitations described above, and new ones are being tested, with some examples that will be described later. Conjugation with different moieties, like lipid moieties or PEG, has also been conducted for this kind of peptide, in order to improve their activity, especially with those where the temporal window of action represents a limiting factor (Augusto et al., 2014; Hollmann et al., 2013; Ingallinella et al., 2009; Vilas Boas et al., 2017).

With the aim of inhibiting the initial stage of the assembly of dengue virus, and also a good candidate to target also other related flaviviruses, the peptide inhibitor pep14-23 (Faustino et al., 2015a, 2015b) was designed based on a conserved intrinsically disordered domain of dengue virus capsid protein (Martins et al., 2012). It is intended to block the binding of the viral protein to intracellular lipid droplets (Carvalho et al., 2012; Martins et al., 2012), essential for viral replication (Samsa et al., 2009), as well as to nascent very low density lipoproteins (Faustino et al., 2014). A recently discovered peptide, urumin, isolated from frog skin, exhibited antiviral activity against influenza A virus, by targeting the conserved stalk region of H1 hemagglutinin, which seems crucial for the viral binding to the host cells (Holthausen et al., 2017). Urumin was also able to disrupt influenza virions; however, the underlying mechanism has not been fully determined (Holthausen et al., 2017).

2.3. Cell-penetrating peptides

Cell-penetrating peptides (CPPs) are another class of molecules that share properties with AMPs, namely short amino acid sequence, positive net charge, high content of hydrophobic residues and well-defined secondary structure (Fig. 1) (Bahnsen et al., 2015; Pärn et al., 2015; Rodrigues et al., 2011). The difference between them lies in their activity, with CPPs being able to translocate to the interior of cells, transporting small cargos in the process (Mishra et al., 2011; Rodrigues et al., 2012, 2013, 2015; Skotland et al., 2015). Membrane crossing may occur by different mechanisms, either by direct translocation, or related to endocytosis, directly dependent on the peptide sequence and on the cargo transported (de Figueiredo et al., 2014; Mishra et al., 2011). The cargos already tested include peptides (AMPs, AVPs or HDPs), large proteins, drugs, nucleic acids or nanoparticles (Freire et al., 2017; Gautam et al., 2013; Rodrigues et al., 2011). This major advantage, together with the low toxicity and low production costs, explains the increasing interest in CPPs (Pärn et al., 2015). The translocation features have still not been fully explained, but the peptide amphipathic character is indicated as being responsible for this. The hydrophobic and lipophilic moments of the CPP determine its efficiency, but the balance between these properties is not fully established (de Figueiredo et al., 2014). CPPs, although able to have a direct antimicrobial or antiviral activity, are clearly identified as a new method of intracellular delivery, with this characteristic being considered as a possibility for peptides that have pharmacodynamic limitations in their action (Henriques et al., 2006; Kristensen et al., 2016). Examples of peptides with dual activity are SynB1 or Pep-1-K, AMPs with potent direct antimicrobial activity and the ability to cross cell membranes carrying small molecules (Rousselle et al., 2000; Zhu et al., 2006). Regardless of the promising approach of using CPPs as anti-infective molecules, to the best of our knowledge there are currently no such drugs under clinical trials (Guidotti et al., 2017).

3. Downsides in the therapeutic application of peptides

Despite the intense research in peptide drugs, the number of approved molecules in the antibiotic and antiviral fields has not evolved at the same rate as resistance has emerged (Chen et al., 2005). In fact, the on-going search for new therapeutic molecules is mostly by chemical derivations of the drugs that are already on the market, a strategy that is not showing great advances in overcoming resistance (da Cunha et al., 2016). Nonetheless, it is important to list some already approved peptides that are being used in therapeutics (Table 1). One of the objectives of the research conducted at this level is to identify characteristics that allowed the therapeutic approval of these peptides, and improve them, overcoming the drawbacks that normally limit the therapeutic application of these molecules (Kaspar and Reichert, 2013). Patients' compliance is one of the key factors in converting a product undergoing clinical trials into a commercialized successful drug. Peptides' low bioavailability, limited by their degradation and low epithelial absorption, is the leading difficulty in the therapeutic application of peptides (Hamman et al., 2005). The oral delivery of peptides leads to their degradation by gastric acids, as well as by proteases and peptidases present in the gastro-intestinal (GI) tract. Even if the peptide escapes this degradation route, approximately 80% face another challenge soon afterwards: the intestine's epithelial barrier (Bruno et al., 2013). At the epithelial barrier, peptides have to overcome the mucosal layer, composed of glycoproteins, glycocalyx, mucopolysaccharides, enzymes, electrolytes and water; the brush border membrane with microvilli; and the efflux pumps, which can pump the peptide back into the GI lumen after its absorption (Carino and Mathiowitz, 1999).

In addition, after absorption, peptides face some difficulties, such as the first-pass effect. When absorbed, they enter the hepatic portal system and are likely to be metabolized by the liver, reducing the concentration that actually enters systemic circulation (Bruno et al., 2013). To overcome this limitation, the delivery of the peptides may be carried out through different routes, such as intravenously, subcutaneously or intramuscular. However, classical routes of administration do not guarantee efficient peptide delivery to its site of action. In fact, endogenous proteases may also lead to proteolytic degradation, and chemical modifications can also occur. Sieprawska-Lupa et al. studied the susceptibility of LL-37 to proteolytic degradation by two major proteinases, both produced by *Staphylococcus aureus*, a metalloproteinase (aureolysin) and a glutamylendopeptidase (V8 protease) (Sieprawska-Lupa et al., 2004). They found that aureolysin cleaved and inactivated LL-37 in a time- and concentration-dependent manner, directly affecting the peptide's bactericidal effect (Sieprawska-Lupa et al., 2004). Temperature, pH or high salt concentrations can alter the native structure of the peptide and/or their interaction with membranes, resulting in inactive molecules in circulation. The influence of salt concentration on AMP activity was studied by Turner et al., who reported the effects of NaCl in the minimal inhibitory concentration (MIC) of LL-37 (Turner et al., 1998). In a medium with 100 mM NaCl, some organisms became resistant to the peptide, in comparison to a low-salt medium (Turner et al., 1998).

Table 1
List of peptides FDA-approved to be used in therapeutics, based in their sequence, company, target disease and mechanism of action.

Name	Peptide	Brand/company	Category	Target disease	Mechanism of action	Route of administration
Bacitracin	Mixture of related cyclic peptides	BACiiM/X-GEN Pharmaceuticals	Antimicrobial	Pneumonia and empyema in infants (*staphylococci)*	Interference in cell wall and peptidoglycan synthesis	Topical
Boceprevir	Cyclic peptide	Victrelis/Merck	Antiviral	Hepatitis C virus genotype 1	NS3/4A protease inhibitor	Oral
Dalbavancin	Lipoglycopeptide	Dalvance/Allergan	Antimicrobial	Adult patients with acute bacterial skin and skin structure infection	Interference in cell wall and peptidoglycan synthesis	Intravenous
Daptomycin	Lipopeptide	Cubicin/Cubist Pharmaceuticals, Inc	Antimicrobial	Bacterial infections of skin and underlying tissues	Membrane disruption, and inhibition of proteins, DNA, and RNA synthesis	Intravenous
Enfuvirtide	Small peptide	Fuzeon/Trimeris, Roche	Antiviral	HIV-1	Inhibition of 6HB structure, blocking viral fusion	Subcutaneous
Natural interferon-α or Multiferon	Inteferon α-1	Intron/Roferon-A/Roche	Antiviral	Hepatitis C	Affects cell function/growth and body's natural defences	Subcutaneous
Oritavancin	Glycopeptide	Orbactiv/The Medicines Company	Antimicrobial	Acute bacterial skin and skin structure infection	Cell membrane disruption	Intravenous
Teicoplanin	Glycopeptide	Targocid/Sanofi	Antimicrobial	Several skin, soft tissue, bone, joint, urinary tract, endocarditis and peritonitis associated with continuous ambulatory peritoneal dialysis	Inhibits bacterial cell wall synthesis	Intravenous
Telaprevir	Cyclic peptide	Incivek/Vertex Pharmaceuticals/ Johnson & Johnson	Antiviral	Hepatitis C virus genotype 1	NS3/4A protease inhibitor	Oral
Telavancin	Cyclic lipoglycopeptide	Vibativ/Theravance Biopharma	Antimicrobial	Methicillin-resistant *Staphylococcus aureus* and other Gram-positive infections	Interference in cell wall and peptidoglycan synthesis	Intravenous
Vancomycin	Glycopeptide	Alvanco/Human Pharmacia or Vancocin/Eli-Lilly	Antimicrobial	Gram-positive bacteria	Inhibits proper cell wall synthesis	Intravenous

The search was carried out in the official FDA website (https://www.fda.gov) and in the US clinical trials database (https://clinicaltrials.gov).

Table 2
Databases and webservers available for the improved design of peptides with different activities.

Peptide class	Database	Features	Prediction tools
Antimicrobial	DAMPD[a]	Covers prokaryotes and eukaryotes. Over 1200 peptides sequences. Several tools to compare or calculate peptides' features.	AntiBP2 is a webserver that predicts new AMPs in a protein sequence[b] The source of existing AMPs can also be predicted (frog, fish, insect, mammal, …)
	CAMP R3[c]	Contains information about conserved sequences signatures, patents, structures. Over 8000 AMP sequences.	
	APD3[d]	Over 2800 AMP sequences from 6 kingdoms.	
	BACTIBASE[e]	Dedicated to bacteriocins. Origin, physicochemical and structural information.	
	Defensins knowledgebase[f]	Dedicated to the defensin family of AMPs, with detailed information.	
	Peptaibol Database[g]	Soil fungi antimicrobial and antiviral peptides (unusual amino acids included).	
Antiviral	AVPdb[h]	Over 2500 antiviral peptides targeting 60 viruses. Detailed peptide information concerning the physicochemical properties, structure or origin.	AVPpred allows the prediction of new potent antivirals[i] The algorithm was developed using peptides with experimentally-proven antiviral activity
	HIPdb[j]	HIV inhibiting peptides. Over 900 peptides tested in 35 cell lines.	
Antibiofilm	BaAMP[k]	Data for each AMP include microbial species and the biofilm conditions against which it was tested.	dPABPs is a webserver aiming to help in the prediction and optimization of antibiofilm peptides[l]
Cell penetrating	CPPsite 2.0[m]	Contains around 1700 unique CPP sequences, including linear and cyclic peptides (modified and non-natural residues included) with different cargos.	CPPpred evaluates the input sequence and scores it[n] CellPDD helps in the prediction and optimization of CPP sequences[o]

[a]Seshadri Sundararajan et al. (2012); [b]Lata et al. (2010);[c]Waghu et al. (2016); [d]Seshadri Sundararajan et al. (2012), Waghu et al. (2016), Wang et al. (2016); [e]Hammami et al. (2010); [f]Seebah et al. (2007); [g]Whitmore (2004); [h]Qureshi et al. (2014); [i]Qureshi et al. (2017); [j]Qureshi et al. (2013); [k]Di Luca et al. (2015); [l]Sharma et al. (2016); [m]Agrawal et al. (2016); [n]Holton et al. (2013); [o]Gautam et al. (2013).

Unlike other protein-based drugs, peptides exhibit lower potency due to their short half-life. Antibodies, for instance, which are well established in the pharmaceutical industry, may present elimination half-lives of several days, maintaining a constant concentration in the bloodstream (Tang et al., 2004). Besides, most of the peptides used in clinical practice are for extracellular compartments and, thus, have to compete with biologic products (Fotouhi, 2015). These pitfalls can lead to fast peptide clearance or its accumulation in non-target organs or tissues, implying a higher dosage administration, which may be associated with toxicity and higher production costs (Sachdeva, 2016). In parallel with the pharmacokinetic disadvantages, regulatory agencies are struggling to come up with a global set of guidelines defining the minimum purity for peptide therapeutics, mainly due to two reasons: first, peptide impurities are not easy to avoid; secondly, to produce a higher quality product, with less impurities, the manufacturing costs would increase so that the drug becomes economically unfeasible (Sachdeva, 2016).

4. Different methods for improved peptide design

The design of new peptides or analogues can be a daunting task, considering the number of parameters that must be fulfilled in order to have an ideal candidate. There are three key elements that should be carefully thought through in the design and optimization of peptides: the pathogen, the host and the manufacturing costs. Regardless of the action/target of the peptide, host cell toxicity, biodistribution and protease resistance are important factors to take into account in the drug development process, as described above (Cytryńska and Zdybicka-Barabas, 2015; Kim et al., 2014). Cell, membrane or protein selectivity is also a major point that should be addressed during peptide optimization (Costin et al., 2010; Matsuzaki, 2009). From the therapeutic point of view, the administration of peptides should cause no or minimal side effects. At the industrial level, the production costs must be as low as possible. In order to meet this criterion, peptides with short sequences are preferentially selected (Craik et al., 2013). The design of peptides with few amino acid residues is challenging and should not compromise their activity. Although there is a high supply of peptide when the production cost is low, some have reported that high production levels can cause a decrease in peptide activity/stability due to aggregation in oral formulations (Bak et al., 2015). To overcome such problems, new methodologies should be applied to modify the properties of the peptides.

Many approaches have been developed over the years to generate new peptide sequences, for different targets, based on known peptide databases. Computational design is a very powerful tool that has been refined over time, in which a given peptide/protein sequence can be submitted to one of the available web tools in order to be profoundly analysed regarding its physicochemical properties. Important information is given by these tools, through which new active peptides may be predicted based on the input sequence (Dias et al., 2017; Wang et al., 2011). In many cases, the aim is to optimize a known peptide sequence to be more resistant to proteases or efficient towards the target cell or protein. One of the strategies is the substitution of L- for D-amino acids, to increase protease resistance (Di Grazia et al., 2015). Hydrophobicity is also a parameter that influences the behaviour of peptides with antimicrobial or antiviral activity. The lipid membrane is involved in the function of these two types of peptides in different ways. AMPs first target the membrane, to destroy it, while several AVPs use it as an intermediate to be close to viral proteins upon viral entry. Besides the common amino acid modification to introduce hydrophobicity, the conjugation of sterols or fatty acids is increasing the repertoire of new modifications that positively influence the activity of the peptides (Krishnakumari and Nagaraj, 2015; Pessi, 2015). Covalent attachment of polyethylene glycol (PEG) chains, known as PEGylation, offers increased half-life and improved solubility, without causing an immunogenic reaction in the host (Hamley, 2014). Altogether, these two modifications combined have been successfully used to improve the activity of peptides (Augusto et al., 2014; Pessi et al., 2012; Porotto et al., 2010; Welsch et al., 2013), but also of antibodies (Augusto et al., 2017b; Lacek et al., 2014). Some peptides just need to be protease-resistant to reach their target, while others might need a vehicle to travel to their destination (Pessi, 2015), still others are intrinsically able to permeate membranes, which is the case of CPPs (Kauffman et al., 2015). The latter have been widely used to improve cell delivery, as described above, by their conjugation with nanoparticles, liposomes or biomolecules, such as small interference RNA (siRNA) and essential

proteins, both *in vitro* and *in vivo* (Freire et al., 2017; Jo et al., 2014; Kwon et al., 2015). Peptides are involved in a vast number of applications, and their development using computational techniques and chemical modification approaches will be addressed below.

4.1. In silico approaches to predict and design new peptides

The prediction of new peptide sequences or properties of the already described active peptides by algorithm databases has been gaining interest in the era of "big data" (Table 2). CPPs have been extensively studied as a delivery option for bioactive molecules that are poorly taken up by cells (Raucher and Ryu, 2015). To the best of our knowledge, CPPsite 2.0 is the only online database fully dedicated to experimentally validated CPPs (Agrawal et al., 2016). This website includes access to articles and patents with very detailed information regarding peptide sequence, length and chemical modifications, as well as the cell lines used for CPP evaluation. Many other important parameters are present in the website, which comprises 1699 unique and experimentally validated CPP sequences (Agrawal et al., 2016). CPPsite 2.0 allows the users to create their own dataset to be used by online tools dedicated to the prediction of efficient CPPs. CPPpred (Holton et al., 2013) and CellPDD (Gautam et al., 2013) are the two major and free web servers that have used CPPsite 2.0 as their dataset. CPPpred is a neural network-based *in silico* method, which can predict CPPs ranging from 5 to 30 amino acid residues (Holton et al., 2013). This web server can only evaluate the input sequence, by calculating the probability of the peptide to be cell penetrating. CPPpred scores the input sequence between 0 and 1, in which a score above 0.5 indicates that the peptide has a high chance of being cell penetrating, while a score close to 0 suggests that the peptide is very unlikely to be cell penetrating. The closer the score is to 1, the higher is the CPPpred degree of confidence to classify the peptide as cell penetrating. CPPpred is an efficient tool to predict if a given peptide sequence has the ability to be CPP or not, but it cannot help in the design of new peptides (Holton et al., 2013). In contrast, CellPDD is a web server that offers more tools, in which new peptide sequences can be predicted based on the input sequence (Gautam et al., 2013). The server generates all possible substitution mutants of the query peptide, and classifies them according to the cell penetrating prediction, also giving information about physicochemical properties such as charge, hydrophobicity and other parameters relevant for their activity (Gautam et al., 2013). In the case of larger proteins, their sequences are analysed in terms of possible CPP motifs.

Due to the extensive studies of AMPs, there was a need to create AMP databases, covering peptides from prokaryotes, eukaryotes (Seshadri Sundararajan et al., 2012; Waghu et al., 2016; Wang et al., 2016), bacteria (Hammami et al., 2010), defensins (Seebah et al., 2007) and peptaibols (soil fungi antiviral and antimicrobial peptides) (Table 2) (Whitmore, 2004). The authors of CellPDD and CPPsite 2.0 created AntiBP2, a web server that contains the necessary tools to predict new AMPs based on peptide sequences (Lata et al., 2010). During literature analysis, authors found that certain amino acids are preferred at the C- or N-terminal of AMPs, a key finding for the discrimination of non-antimicrobial peptides. In response to this outcome, Lata et al. (2010) created a more robust model to predict new sequences. In addition, AntiBP2 can also give information about the potential source and family of the input sequence (Lata et al., 2010).

Antibiofilm peptides are a niche inside the AMP category that can target and destroy highly structured and organized populations of bacteria (biofilms). The first manually curated database for this class of peptides was only recently created (Di Luca et al., 2015). Biofilms are being recognized as the principal cause of a wide range of infections and are easily recognized in post-surgical infections on implants (Connaughton et al., 2014) or in chronic infections like cystic fibrosis (Høiby et al., 2010). The need for new antibiotic molecules led to the creation of a web server fully dedicated to the prediction and design of antibiofilm peptides – Design Peptides Against Bacterial Biofilms (dPABPs) (Sharma et al., 2016). 80 ABPs from biofilm-active AMPs database (BaAMP) were used as the positive dataset in the optimization of the implemented model in this web server. The positive dataset includes only peptides that were able to disrupt biofilms from all species tested against, which exclude those shown to be active against only one or two different strains. The aim of dPABBs is to find broad-spectrum peptides active against multiple bacterial species (Table 2).

AVPs are another important group of peptides with therapeutic use (Table 2). There is a need to speed up the discovery of effective new AVPs to treat deadly human viruses, mostly because some viruses create a pandemic cycle worldwide, with the help of globalization. The Antiviral Peptide database (AVPdb) is a manually curated database dedicated to antiviral peptides, targeting 60 clinically important viruses (Qureshi et al., 2014). This database comprises 2683 antiviral peptides experimentally validated, in which 624 are modified peptides. Information regarding sequence, source, targeted virus, and tested cell lines can be found in the database. Besides AVPdb, there is an anti-HIV peptide database that provides experimental records of 981 peptides targeting different steps during the life cycle of HIV (Qureshi et al., 2013). The same authors developed a web server that allows the prediction of potent antivirals, and the design of new peptide analogues (Qureshi et al., 2017). Four individual prediction models for HIV, hepatitis C virus, hepatitis B virus and human herpes virus were created, and a fifth generalist model was implemented for another 26 human viruses. The value of this web server is that it makes it possible to predict potential new antiviral therapeutics with great efficiency.

Clearly, detailed databases have been built to help researchers in the design of active peptides. More importantly, a wide range of tools was developed to predict how likely it is that an input sequence belongs to a known class of biologically active peptides, or not, and which modifications can be made to turn inactive sequences into active ones. However, it is important to mention that *in vitro* optimization does not guarantee increased *in vivo* activity.

4.2. Strategies to improve peptide half-life

Peptides are in general better drug candidates than other chemical compounds, considering properties related to toxicity and activity (Fig. 2). Peptides can be easily eliminated from the body, generally resulting in a safer choice for therapeutic use. During peptide optimization, one of the most important improvements is protease resistance, leading to an increase in the peptide's half-life (Cromm et al., 2016; Heard et al., 2013; Stromstedt et al., 2009; Tugyi et al., 2005; Weinstock et al., 2012). A large number of papers regarding peptide modifications that aim to extend their bioavailability and improve both biodistribution and rate of clearance have appeared in the literature. Strategies include N- and C-modifications, incorporation of non-natural or D-amino acids, cyclization and PEGylation (Gentilucci et al., 2010; Vlieghe et al., 2010; Weinstock et al., 2012). Recently, a manually curated library of experimentally validated peptide half-lives, named PEPlife, was created (Mathur et al., 2016). Sequences and modifications were organized, together with the peptide biological activities, for a total of 2229 peptide entries, divided into linear (1984) and cyclic (245) peptides.

Proteases responsible for peptide degradation are mainly present in the digestive tract and blood plasma, resulting in low peptide bioavailability (Vlieghe et al., 2010). The liver and kidneys are also involved in the fast clearance of peptide metabolites. The proteolytic degradation of peptides can be addressed by protecting their C- and N-terminus, with acetylation or amidation (John et al., 2008; Stromstedt et al., 2009). A relevant strategy for overcoming peptide hydrolysis is to perform modifications on the sequence by substitution of natural L-amino acids for their D-enantiomers, α/β-substituted α-amino acids or even β-amino acids (Gentilucci et al., 2010; Vlieghe et al., 2010). D-Amino acid substitution in a peptide may have implications not only for the peptide's stability, but also for its activity and secondary structure

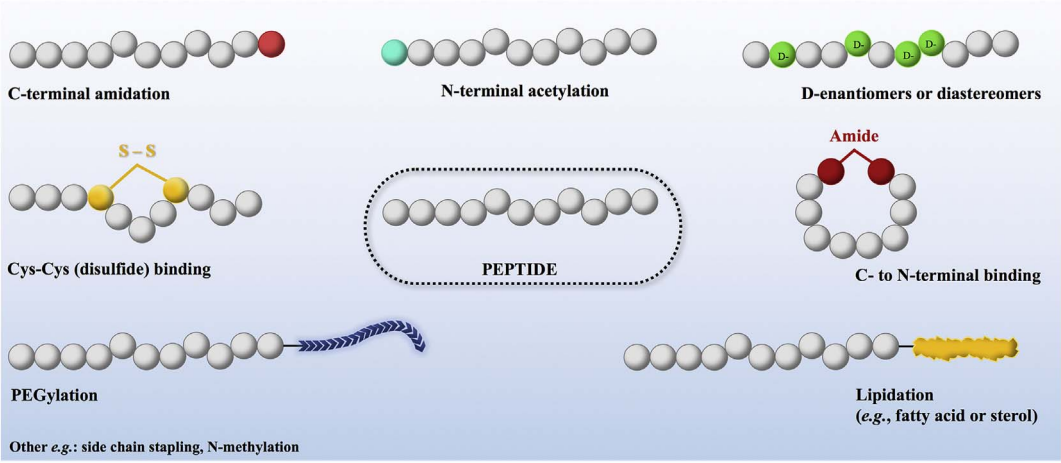

Fig. 2. Different approaches for the improvement of the half-life of peptides.

(Cappiello et al., 2016). A full replacement of the L- by D-amino acid residues within the peptide results in an enantiomer, while partial substitutions in the sequence can produce different diastereomers. Hong et al. (1999) compared several D-diastereomers and a D-enantiomer of an AMP in terms of stability, structure and activity. Interestingly, the substitution for D-amino acids in the middle of the peptide sequence was able to disrupt the α-helical structure of the peptide and abolish its antimicrobial activity, in contrast to the D-enantiomer, where the structural and activity parameters were maintained (Hong et al., 1999). Changes at both C- and N-termini of the peptide did not affect its α-helical conformation or antimicrobial activity, but improved the peptide's stability in the presence of serum (Hong et al., 1999). Membrane binding can also be affected by partial D-amino acid replacement, as it was shown for an AMP extracted from frog skin, whose membrane binding was increased and β-sheet aggregates reduced (Mangoni et al., 2006). Di Grazia et al. (2015) reported that a natural-derived peptide, named esculentin-1a(1-21)NH_2, with two D-amino acids in its sequence, was able to be more stable in serum, less toxic to mammalian cells and more effective against bacterial biofilms, while keeping its high activity against planktonic *P. aeruginosa*. In a different study, de la Fuente-Núñez et al. (2015) showed that the peptides DJK-5 and DJK-6, both composed only of D-enantiomers, destroyed 7 species of preformed biofilms and 30 strains of wild-type and multi-drug-resistant pathogens. The use of non-natural amino acid residues, like fluorinated leucine, in the sequence of antimicrobial peptides was also reported to increase bactericidal activity and protease resistance (Meng and Kumar, 2007). The use of D-amino acids in peptides based on human β-defensins improved their biological activity (Olli et al., 2013). In the antiviral field, cationic peptides with β-amino acids were able to inhibit HSV type 1 infection (Akkarawongsa et al., 2008).

Cyclotides are naturally backbone-cyclized peptides expressed by plants that have enhanced thermal, chemical and enzymatic stability (Gould and Ji, 2011). Efforts have been made to develop cyclic peptides, by bridging cysteine residues (disulfide bond) or by connecting N- and C-terminus *via* an amide bond. Cyclization of bioactive peptides is often associated with improved stability and activity of the molecule (Joo, 2012). In the antiviral battle against dengue virus, a peptide extracted from cone snail venom was found to be a good inhibitor of a protease involved in the virus' replication cycle (Xu et al., 2012). A mutational study showed that the cyclized version of this peptide led to a stable conformation, able to bind and inhibit the viral protease more efficiently (Xu et al., 2012). An AMP named gomesin, isolated from the Brazilian spider *Acanthoscurria gomesiana,* was found to be not only bactericidal and fungicidal, but also cytotoxic against tumour cells (Chan et al., 2013). The authors of this study proved that the cyclization of gomesin improves the stability of the molecule, also enhancing its antitumor activity, without changing its toxic effects on healthy cells.

Arginine residues are one of the major constituents of cell penetrating peptides, and have been used to create polyarginine-based CPPs (Tung and Weissleder, 2003). In particular, a linear peptide made of only nine arginine residues (R_9) was found to be as potent as TAT (a classic HIV-derived CPP) in crossing membranes (Futaki et al., 2001). The positively-charged guanidinium group of arginine is involved in the interaction with the phospholipids of the membranes (Bouchet et al., 2010), a crucial feature for efficient cell penetration. Stiffening the peptide backbone by synthesizing macrocycles was a strategy followed by Traboulsi et al. (2015), in which R_9-based peptides were used as a model to generate new proteolytic stable cyclic CPPs. A hepta-arginine macrocycle was found to be as efficient as the linear R_9.

As previously mentioned, another possible strategy is the PEGylation of peptides and proteins, a well-known chemical approach for overcoming the low bioavailability of molecules in the human body. The attachment of the polyethylene glycol polymer to peptides enables a longer circulation time, in which water solubility and molecule stability are both improved (Hamley, 2014; Mathieu et al., 2017; Thieme et al., 2016; Yin et al., 2016). A synthetic AMP named M33, active against Gram-negative bacteria, was conjugated with PEG at the C-terminus, resulting in a great stability against elastase, a protease from *P. aeruginosa* (Falciani et al., 2014). Some authors reported that antiviral peptides designed to inhibit HIV-1 infection presented decreased antiviral activity upon conjugation with PEG (Falciani et al., 2014). However, this inconvenience was compensated for by increasing peptides' proteolytic resistance and longer half-life time in circulation (Danial et al., 2012).

4.3. Targeting lipid membranes

The membrane, either from the pathogen or the host, is the major intervenient in the mechanism of action of several peptides. In the case of AMPs, the most common aim is to compromise bacterial viability, either by acting directly at the membrane level or by acting on specific intracellular targets. Antiviral peptides may use the membrane of healthy cells as a reservoir to target the viral protein of interest and prevent the infection process (Vigant et al., 2015). Membrane interactions may also induce conformational changes or define the orientational disposition of peptides, facilitating the interaction with the viral target (Avitabile et al., 2016; Faustino et al., 2015a; Melo et al., 2009). There is also the case of CPPs that can target both healthy and non-healthy cells to deliver any type of therapeutic molecule. In the end, chemical composition, as well as peptide structure, will influence biological activity. In the literature, hydrophobicity is a parameter that is often described as undesirable and that leads to peptide aggregation or

formulation issues. However, it is essential to find a balance in the peptide composition in order to allow a good *in vivo* activity and optimal conditions to be used in therapeutic settings. Some authors achieved an increase in peptide hydrophobicity by substituting alanine residues with leucines, known to be more hydrophobic (Chen et al., 2007). Bulky amino acids such as tryptophan and phenylalanine were used as sequence end-tags, to guide peptides to membranes (Pasupuleti et al., 2009), due to their ability to locate among the polar headgroups of phospholipids, close to the lipid-water interface (Chan et al., 2006). There is the case where an increased membrane interaction was obtained by simple substitution of amino acid residues, but cytotoxic and haemolytic effects accompanied the increase in peptide hydrophobicity (Hollmann et al., 2016). To avoid these side effects, new strategies have been applied to increase peptide membrane targeting, without changing the amino acid sequence. The chemical conjugation of sterols or fatty acids to peptides is a recent trend used in the antiviral and antimicrobial fields (Chu-Kung et al., 2010; Pessi, 2015). This approach led to a rise in interest in lipopeptides, either from natural (Eisenstein et al., 2010; Moryl et al., 2015) or non-natural origins (Pessi, 2015).

The derivatization of peptides with fatty acids increases not only membrane affinity, but also their half-life in circulation (Pollaro and Heinis, 2010; Zhang and Bulaj, 2012). Conjugation can be done at the N-terminal, or in the amino acid side-chains of lysine or cysteine residues. The fatty acids that are commonly used for this purpose have a variable number of backbone carbons, including caprylic acid (C8), capric acid (C10), lauric acid (C12), myristic acid (C14), palmitic acid (C16), stearic acid (C18) and eicosanoic acid (C20). An antimicrobial peptide named A2 was conjugated at the N-terminus with fatty acids of different chain lengths (C8–20), and was afterwards tested in terms of biological activity (Húmpola et al., 2017). Authors found that fatty acids up to C14 were the best at increasing the antimicrobial activity of A2, while longer chains promoted toxicity to eukaryotic cells. Lee and Tung (2010) reported that CPPs conjugated to C16 were harmful to cells, in comparison to shorter fatty acid chains (C12–14). A balance between the length of the fatty acid chain and the sequence of the peptide is necessary to optimize the toxicity and activity of the peptide. The short chain of myristic acid, associated with low toxicity, makes it one of the most appropriate fatty acids for use in conjugation to peptide analogues of human defensins. Other authors (Mathew and Nagaraj, 2015a, 2015b) designed and synthesized analogues of human α-defensins 5 and 6 conjugated to myristic acid. They found that N-terminal fatty acylation improved the antimicrobial activity of the analogues, where permeabilization of the membranes was suggested as the mechanism of action. Sharma et al. (2015) took the interesting approach of generating truncated linear versions of the cyclic human β-defensin 4. The new peptides were myristoylated, and were as potent as the original human defensin, exerting its activity by crossing the cell membrane. A recent study revealed the use of myristic acid in CPPs aiming to deliver siRNA across the blood-brain barrier to treat neurodegenerative diseases (Youn et al., 2014).

Apart from fatty acylation, sterol conjugation is a related strategy to increase membrane targeting. While the library of fatty acids reported in the literature comprises different options, the used sterols have been mostly restricted to cholesterol and α-tocopherol (Arnusch et al., 2012; Figueira et al., 2017; Hollmann et al., 2013; Mathieu et al., 2017; Pessi, 2015). Lipid membranes may include different sterols in their composition, and these play important roles such as the maintenance of membrane microfluidity (Dufourc, 2008). In vertebrates, the major sterol is cholesterol (Hannich et al., 2011), while fungi present mainly ergosterol (Hannich et al., 2011). Primitive bacteria present hopanoids, which are developed in extreme environmental conditions (Hannich et al., 2011). Sterols are important not only in the membrane fluidity, but also in the formation of ordered nanodomains, often named lipid rafts, which are considered to be involved in several biological processes, including viral infections (Barman and Nayak, 2007; Carter et al., 2009).

Cholesterol was conjugated to the HIV-1 derived peptide C34, with the purpose of increasing peptide-membrane affinity (Ingallinella et al., 2009). In theory, the concentration of the cholesterol-conjugated peptide would increase at the site where HIV entry takes place. Later, Hollmann et al. proved this hypothesis, suggesting the membrane as a catalyst for the binding between the antiviral peptide and the HIV-1 protein gp41, by placing and concentrating the peptide in specific domains at the membrane (lipid rafts), in a manner that favours its activity (Hollmann et al., 2013). In agreement with this hypothesis, Zhang et al. reported an increase both in the antiviral activity and in the membrane retention of CP32M (anti-HIV fusion peptide) when conjugated with cholesterol (Zhang et al., 2016). The combination of PEGylation and cholesterol in one molecule is a dual strategy aimed at improving membrane targeting to boost antiviral activity. Fusion inhibitor peptides against measles virus and HIV-1 were conjugated with PEG and cholesterol, with a dramatic increase in their antiviral activity and half-life (Figueira et al., 2017; Mathieu et al., 2017; Pessi et al., 2012). The presence of spacers of PEG between the N-terminal of the peptide and the cholesterol molecule allows increased mobility, which apparently favours interaction with the viral protein, reducing spatial restrictions (Augusto et al., 2014). The same trend was also observed for HIV broadly neutralizing antibodies upon PEG and cholesterol conjugation (Lacek et al., 2014), where strong binding to lipid membranes was observed (Augusto et al., 2014; Augusto et al., 2017b). Cholesterol conjugation strategies have also been used in a wide variety of studies to improve the biologic activity of peptides against more deadly viral pathogens, such as Nipah virus (Porotto et al., 2010) and Ebola virus (Higgins et al., 2013). It was also reported in the literature that cholesterol-conjugated peptides can efficiently block endosome-fusing enveloped viruses, such as influenza virus (Lee et al., 2011).

The development of lipopeptide vaccines that promote the immune response of the organism using peptides mimicking viral epitopes is being considered a valid option in vaccine design (Zaman and Toth, 2013). Palmitoylated peptides based on HIV-1 proteins (Nef, Gag and Env) were tested in clinical trials, and a boost in cytotoxic lymphocyte (CTL) levels was observed, which indicates an enhanced response of the immune system when challenged with lipopeptides (Gahéry-Ségard et al., 2003). Additionally, a lipopeptide vaccine based on a human cytomegalovirus CTL epitope was able to induce an antigen-specific CTL response, which killed infected human cells (Diamond et al., 1997). HSV type 2 was also targeted in the development of lipopeptide vaccines, with strong CTL response (Zhang et al., 2009). Recently, Morita et al. described the important role of a new class of MHC class I molecules, which are able to present *N*-myristoylated lipopeptides to CTL (Morita et al., 2016). *N*-myristoylation of proteins or peptides is linked to pathogenesis and CTL ability to sense this post-translational modification, which could indicate the pertinence in vaccine design, since it is unlikely for a virus to mutate the N-terminal without affecting the protein function (Boso et al., 2015; Brenner et al., 2006; Gastaminza et al., 2003). Thus, it would be hard for viruses to evade lipopeptide-specific CTL response (Morita and Sugita, 2016).

5. Future prospects

The therapeutic peptide market is an emerging field that is currently growing. The failure of several small molecules, which led to substantial economic losses in the pharmaceutical industry, and the decrease in innovation concerning classical active ingredients led to investment in peptide-based drugs, and consequently, the appearance of several therapeutic peptides with distinct targets (Lax, 2010). The methods referred to above offer opportunities for improving the functionality of peptides, and for increasing the number of targets considered suitable for drug development. The approval of enfuvirtide, a fusion inhibitor peptide for HIV, is considered the turning point in drug nature policies, since it changed the investors' attitude to biotech in general and to peptides in particular (Otvos, 2008). The FDA approval

rate confirms this progression. While the number of new chemical entities has been stable for about 10 years, the number of approved peptides has been continuously growing, and those already on the market presented an increased volume of sales during this period (Vlieghe et al., 2010). According to Craik et al., the market for protein- and peptide-based drugs represents about 10% of the total pharmaceutical market, and it is increasing (Craik et al., 2013). Numerous scientific publications demonstrate the intense basic research in this field, with thousands of peptides being studied, and 400 to 600 of them are enrolled in preclinical studies (Sachdeva, 2016). Moreover, a large number of peptides are currently in clinical trials, with a high probability that some of these will be approved as pharmaceutical drugs in the near future (Ahrens et al., 2012). Recently, several strategies regarding large-scale production of peptides have been improved, and these are expected to speed up the drug discovery phase. As a result, the entrance of a candidate into clinical phases is faster, in comparison with small organic molecules. Furthermore, peptides do not present as many side effects as classical drugs, which increases the rate of regulatory approval by about 20% (Fox, 2012). In 2012, the FDA launched the antibacterial drug development task force, in order to increase the efficiency of antibiotic development, including clinical trial design (Fox, 2012). Overall, the reduction of time for the development of a new therapeutic product will directly influence the economic value of the final product, making the peptide entity very desirable for the pharmaceutical industry (Lax, 2010). A better understanding of the basic biology involved in different pathogens' infections will allow the identification of drug targets based on protein-protein interactions, which could increase the importance of peptides in the pharmaceutical field even more (Faustino et al., 2014, 2015a; Kovalainen et al., 2015).

Nevertheless, as noted before, peptides also present some limitations that need to be addressed, which are technical hurdles for the development of more effective peptide-based therapeutics. First, the synthesis of peptides must be rethought. Apart from a few exceptions, current strategies rely on expensive coupling reagents, resins and protected amino acids, creating the need for cheaper synthesis and purification methods (Pattabiraman and Bode, 2011). The improvement of pharmacokinetic properties is another challenge for the next generation of peptide-based drugs. A special effort should be made to improve the interaction of peptides with membranes, increasing protease resistance and reducing their clearance (Craik et al., 2013). Another concern to have in mind is the delivery of peptides to their specific target. For a number of years, drug delivery systems appeared just after the drug approval, frequently as a method of outliving the end of the patent and extending it, but the paradigm has changed; nowadays, these delivery systems are being developed as part of a therapeutic formulation (Albericio and Kruger, 2012).

The natural sources from which active peptides can be isolated are virtually unlimited. Thus, the appearance of potent new peptides will not stop, as can be exemplified by the recent case of the first isolated fungal defensin, which showed promising anti-infective therapeutic action (Mygind et al., 2005). In parallel with their growth as therapeutic options, it is expected that in the future peptides will also be used as functional foods and nutraceutics, as vaccines, in diagnostics, in the cosmetic field or even in agriculture to control pests (Kovalainen et al., 2015). With the advances that are arising in this field, the potential of peptide drugs is enormous, and during the next few decades, peptides will probably assume a leading role in modern medicine and pharmaceutics.

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