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Review Article

REVIEW ARTICLE

Perspectives for clinical use of engineered human host defense antimicrobial peptides

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One sentence summary: Naturally occurring human antimicrobial peptides represent a potential source of new and safe antimicrobial drugs to treat infections with unmet medical needs.
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ABSTRACT

Infectious diseases caused by bacteria, viruses or fungi are among the leading causes of death worldwide. The emergence of drug-resistance mechanisms, especially among bacteria, threatens the efficacy of all current antimicrobial agents, some of them already ineffective. As a result, there is an urgent need for new antimicrobial drugs. Host defense antimicrobial peptides (HDPs) are natural occurring and well-conserved peptides of innate immunity, broadly active against Gram-negative and Gram-positive bacteria, viruses and fungi. They also are able to exert immunomodulatory and adjuvant functions by acting as chemotactic for immune cells, and inducing cytokines and chemokines secretion. Moreover, they show low propensity to elicit microbial adaptation, probably because of their non-specific mechanism of action, and are able to neutralize exotoxins and endotoxins. HDPs have the potential to be a great source of novel antimicrobial agents. The goal of this review is to provide an overview of the advances made in the development of human defensins as well as the cathelicidin LL-37 and their derivatives as antimicrobial agents against bacteria, viruses and fungi for clinical use.

Keywords: defensins; cathelicidin; antibacterial; antiviral; antifungal; infectious diseases

INTRODUCTION

Host defense antimicrobial peptides (HDPs) are key components of the innate immunity system of multicellular organisms that play an essential role in the control of microbial infections. They exhibit a broad range of antimicrobial properties and a potential ability to avoid the emergence of resistance, often observed with other antimicrobial therapies. More than 2700 HDPs have been described so far from the six Kingdoms (bacteria, archaea, protists, fungi, plants and animals; http://aps.unmc.edu/AP/main.php) (Wang, Li and Wang 2016). They can be classified in four groups: (i) α-helical peptides, (ii) peptides containing β-sheet elements, (iii) peptides combining both α and β structures and (iv) peptides free of α and β structures and unusually rich in particular amino acids such as proline, arginine, tryptophan or histidine (Hancock and Lehrer 1998; Zhang and Sunkara 2014). Among HDPs, the two major classes of mammalian antimicrobial peptides are defensins and cathelicidins, being the linear peptide LL-37 the only member of this family present in humans (Guani-Guerra *et al.* 2010).

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Defensins consist of a combination of an α-helical domain linked to a two-stranded antiparallel β-sheet domains via three disulfide bonds, while LL-37 is a lineal peptide with an amphipatic α-helical structure (Sigurdardottir *et al.* 2006; Wilmes and Sahl 2014). They are all cationic, small (29–42 amino acids) and amphipathic peptides displaying broad-spectrum and direct and indirect antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi and viruses (Guani-Guerra *et al.* 2010; Wilson, Wiens and Smith 2013; Wilmes and Sahl 2014). Defensins can be further subdivided into α-, β- and θ-defensins based on their gene structures and conserved disulfide bridges. The α- and β-defensins are linear peptides widely distributed in vertebrate species, while θ-defensins are cyclic peptides present only in certain non-human primates (Wilson, Wiens and Smith 2013). In humans, six different α-defensins, grouped in myeloid (HNP1-4) and enteric (HD5 and HD6), and 31 β-defensins (hBD) have been described so far, although the most studied at the level of their antimicrobial potential and spectrum are hBD1-4 (Scheetz *et al.* 2002; Schutte *et al.* 2002; Selsted and Ouellette 2005). As for the θ-defensins, their mRNA has been found in humans, but due to the presence of premature stop codons they are not translated. However, when those stop codons are avoided, the produced peptides, called retrocyclines, also present antimicrobial activities (Cole *et al.* 2002; Doss *et al.* 2012; Hooven *et al.* 2012).

Besides their antimicrobial activity, some disadvantages for the use of these HDPs have been observed such as their susceptibility to proteolysis, their low activity in physiological conditions and the high cost of their production. However, their limited availability and the high cost of manufacture have prompted the production of HDPs by different approaches (Bai *et al.* 2013; Luan *et al.* 2014; Maiti *et al.* 2014; Vernieri *et al.* 2014; Tomisawa *et al.* 2015). Consequently, the synthesis and evaluation of HDPs-derivatives, -analogs or -mimetics have already been reported with encouraging results, regarding their antimicrobial activity and feasible synthesis (Sigurdardottir *et al.* 2006; Doss *et al.* 2012; Scudiero *et al.* 2013, 2015; Varney *et al.* 2013; Wood *et al.* 2013; Mathew and Nagaraj 2015a,b; Olli, Nagaraj and Motukupally 2015; Sharma, Mathew and Nagaraj 2015; Tripathi *et al.* 2015). Other additional biological functions of the HDPs have been reported, such as the immunomodulatory and adjuvant functions that can help the control of infection and inflammation and wound-healing activity (Zasloff 2002; Wilmes and Sahl 2014; Mangoni, McDermott and Zasloff 2016).

In this review, we focus on the current state of knowledge regarding human defensins, including the rhesus θ-defensins also called RTD, and the cathelicidin LL-37, in terms of their antimicrobial efficacy and spectrum against bacteria, viruses and fungi, as well as the advances made in their development as antimicrobial agents for clinical use.

DIRECT MECHANISMS OF ACTION

HDPs are components of the ancient and non-specific innate immune system. Because of their cationic and amphipathic nature, many of the mechanisms of action of these antimicrobial peptides are intrinsically associated with their interaction with the anionic microbial membranes. It is noteworthy that the reported potency and mechanisms of action of the HDPs have been obtained in most of the cases under non-physiological conditions, since their activity is drastically reduced by the presence of polyanions and divalent cations found in body fluids (Bowdish *et al.* 2005; Mansour, Pena and Hancock 2014). This observation casts serious doubts on the potential future application of these peptides in their actual formulation. However, an in-depth knowledge of their mechanisms of action, as well as their structure–activity relationship characteristics, may help to improve their pharmacokinetics by the modification of their structures to protect them from host degradation. Below is a summarized overview of the currently accepted antibacterial, antiviral and antifungal mechanisms of action of the main human HDPs.

Antibacterial mechanisms of action

The negative charge generally presented at the bacterial cell surfaces, containing lipopolysacarides or teichoic acids among others acidic polymers, guarantees the accumulation of these cationic and hydrophobic antimicrobial peptides in both Gram-negative and Gram-positive bacteria, independently of their actual target of action. It had been proposed for many years that the permeabilization of the bacterial cell membranes was the sole mechanism of action of these peptides. However, nowadays we know that some of these peptides exert their antimicrobial effects through alternative modes of action against different bacterial targets (Table 1, Fig. 1).

As for the membrane-permeabilizing mechanism, different models of interaction have been proposed (Table 1): (i) Barrel-stave pore model, in which the HDPs form dimers or multimers that cross the membrane forming barrel-like channels (Matsuzaki *et al.* 1991; Ben-Efraim and Shai 1997); (ii) Toroidal pore model, in which the peptide forms a monolayer by connecting the outer and the inner lipid layers in the pore (Mor and Nicolas 1994); (iii) Carpet model, where HDPs form a carpet-like structure covering the outer surface of the membrane acting like detergents disrupting the bacterial membrane (Oren and Shai 1998); (iv) other less frequent models are the sinking-raft and the molecular electroporation models, where the HDPs can either bind and sink into the structure of the membrane or create a sufficient electrostatic potential across the membrane to generate pores (Chan, Prenner and Vogel 2006; Dawson and Liu 2008).

Besides the pore formation in lipid membranes to kill bacteria, human HDPs can interact with additional targets to induce bacterial death. α-Defensin HNP1 has been proposed to target the bacterial membrane in the case of *Escherichia coli*; however, it exerts a different mechanism against *Staphylococcus aureus* through the inhibition of lipid II, a bacterial cell wall precursor (de Leeuw *et al.* 2010). The main mechanism of action of the α-defensin HNP2 consists of the lysis of the bacterial membrane like for HNP3; however, it has also been reported for HNP2 to cause aggregation and fusion of vesicles as alternative mechanisms of actions (Ericksen *et al.* 2005; Pridmore, Rodger and Sanderson 2016). HNP4, the less abundant α-defensin in the azurophilic granules of neutrophils, has proven to be 100 times more potent against *E. coli* than HNP1-3 targeting as well as the bacterial membrane (Wilde *et al.* 1989; Ericksen *et al.* 2005). In the same way, the α-defensin HD5, which is more efficient killing Gram-positive than Gram-negative bacteria, also presents as mechanism of action the permeabilization of the bacterial cell membrane. This subject is cause of considerable controversy, since the destabilization of model membranes has not been observed (de Leeuw *et al.* 2010). In a recent work, Mathew and Nagaraj proposed the ability of HD5 to strongly bind DNA as the basis of its mechanism of action, suggesting that this interaction may inhibit essential processes associated with DNA replication, transcription or translation of important genes (Mathew

Table 1. Direct antibacterial activity of HDPs.

HDPs	Target bacteria	Mechanisms of action	References
α-defensins			
HNP1	*Clostridium difficile, Escherichia coli, Enterobacter aerogenes, Salmonella typhimurium, Pseudomonas aeruginosa, Stenotrophomonas maltophilia, Enterococcus faecalis, En. faecium, Staphylococcus aureus, Staphylococcus epidermidis, Bacillus subtilis, Bacillus cereus, Lysteria monocytogenes, Mycobacterium avium, M. intracellulare*	Inhibit cell wall synthesis by binding to lipid II, membrane lysis	Ganz *et al.* (1985), Ogata *et al.* (1992), Turner *et al.* (1998), Wilmes *et al.* (2011), Varney *et al.* (2013), Furci *et al.* (2015)
HNP2	*E. coli, Enterob aerogenes, P. aeruginosa, S. aureus, B. cereus, M. avium, M. intracellulare*	Membrane disruption, aggregation and fusion of vesicles	Ganz *et al.* (1985), Ogata *et al.* (1992), Ericksen *et al.* (2005), Pridmore *et al.* (2016)
HNP3	*E. coli, Enterob aerogenes, P. aeruginosa, S. aureus, B. cereus, M. avium, M. Intracellulare*	Membrane disruption, pore formation	Ganz *et al.* (1985), Ogata *et al.* (1992), Ericksen *et al.* (2005)
HNP4	*E. coli, Enterob aerogenes, En. faecalis, S. aureus, B. cereus*	Alters membrane permeabilization	Wilde *et al.* (1989), Ericksen *et al.* (2005)
HD5	*C. difficile, E. coli, Sa. typhimurium, En. faecalis*	Membrane lysis, DNA interaction	Salzman *et al.* (2003), Furci *et al.* (2015), Mathew and Nagaraj (2015)
HD6	*Sa. typhimurium, Yersinia enterocolitica, Bifidobacterium adolescentes*	Nanonets formation, enviroment-dependent bactericidal activity	Chu *et al.* (2012), Schroeder *et al.* (2015)
β-defensins			
hBD1	*E. coli, Klebsiella pneumoniae, Salmonella enterica* serovar Typhi, *P. aeruginosa, S. aureus, Bifidobacterium* spp., *Lactobacillus* spp.	Block epithelial invasion, membrane lysis	Schroder (1999), Kraemer *et al.* (2011), Schroeder *et al.* (2011), Maiti *et al.* (2014)
hBD2	*E. coli, P. aeruginosa, Acinetobacter baumannii, En. faecalis, En. faecium, S. aureus*	Membrane lysis, disrupt sites of virulence factors	Sahl *et al.* (2005), Routsias *et al.* (2010), Kandaswamy *et al.* (2013), Maiti *et al.* (2014)
hBD3	*E. coli, K. pneumoniae, P. aeruginosa, S. maltophilia, A. baumannii, En. faecium, S. aureus, Streptococcus pneumoniae, Streptococcus pyogenes*	Inhibit cell wall synthesis by binding to lipid II, membrane lysis	Sahl *et al.* (2005), Maisetta *et al.* (2006), Pazgier *et al.* (2006), Sass *et al.* (2010), Wilmes *et al.* (2011)
hBD4	*P. aeruginosa, A. baumannii, S. aureus, S. pneumoniae*	Membrane lysis	Schneider *et al.* (2005), Supp *et al.* (2009)
θ-defensins			
Retrocyclins	*E. coli, P. aeruginosa, S. aureus, L. monocytogenes*	Peptide-induced permeabilization/impairment of the cytoplasmic membrane	Cole *et al.* (2002), Beringer *et al.* (2016)
RTD	*E. coli, P. aeruginosa, S. aureus*	Membrane lysis	Tran *et al.* (2002), Tongaonkar *et al.* (2011), Tai *et al.* (2015), Beringer *et al.* (2016)
Cathelicidins			
LL-37	*E. coli, K. pneumoniae, Sa. typhimurium, Proteus vulgaris, Haemophilus influenzae, Yersinia pestis, P. aeruginosa, S. maltophilia, A. baumannii, Enterococcus* spp., *Staphylococcus* spp., *Streptococcus* spp., *Bacillus* spp., *L. monocytogenes, Lactobacillus acidophilus, Propionibacterium acnes, Helicobacter pylori, Neisseria gonorrhoeae*	Pores formation in bacterial membranes, interfering DNA transcription	Xhindoli *et al.* (2016)

and Nagaraj 2015). Finally, the α-defensin HD6 shows a characteristic mechanism of action in both Gram-negative and Gram-positive bacteria (Schroeder *et al.* 2015). It has been showed that HD6 forms nanonets to capture these bacteria and to block physical contact with the epithelial cells, and therefore preventing bacterial invasion (Chu *et al.* 2012).

As for the β-defensins, hBD1 has been proposed to act like a barrier protecting epithelial cells being infected by commensal bacteria (Schroeder *et al.* 2011). The antibacterial activity of hBD1 seems to be lower than with other defensins; however, after reduction of its disulphide bridges, hBD1 becomes a more potent antibacterial agent (Schroeder *et al.* 2011). Schroeder *et al.* found that in human skin epidermis reduced hBD1 is co-localized with thioredoxin, an ubiquitously expressed oxidoreductase, supporting the hypothesis that thioredoxin may act as a physiological mediator catalyzing reduction of hBD1 in human epithelia (Schroeder *et al.* 2011). Moreover, another new antimicrobial activity of hBD1 is the induction of neutrophils to extrude extracellular traps that capture *S. aureus* in response to the presence of *S. aureus*-derived toxin (Kraemer *et al.* 2011). hBD2 shares with most of HDPs the common mechanism of action, binding to the negatively charged cytoplasmic membranes,

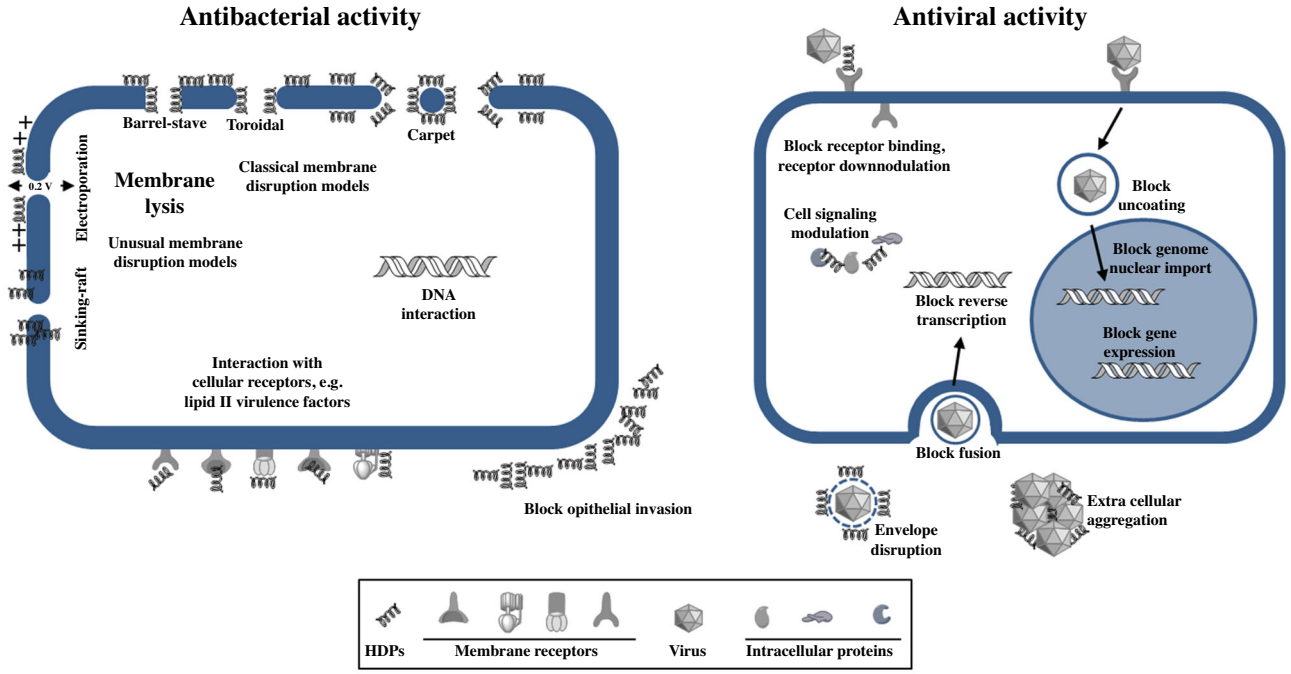

Figure 1. Mechanisms of action of human defensins and cathelicidins.

leading to a leakage of intracellular components and finally death (Sahl *et al.* 2005). Another proposed mechanism of antimicrobial activity for hBD2, observed in *Enterococcus faecalis*, is the disruption at discrete foci, near nascent septal sites, of membrane-localized micro domains of secretion and sorting (Kandaswamy *et al.* 2013). Regarding hBD3, two mechanisms of action have been described, the destabilization of the bacterial membrane and, as in the case of HNP1, the inhibition of lipid II leading to the perturbation of the biosynthesis machinery of the cell wall (Maisetta *et al.* 2006; Sass *et al.* 2010). For hBD4, the only mechanism of action described is the interaction with the bacterial membrane and the subsequent alteration of its integrity leading to bacterial death (Supp *et al.* 2009). In studies carried out with *S. aureus, E. coli* and *Pseudomonas aeruginosa*, the θ-defensins, retrocyclines and RTD showed the same mechanism of action, peptide-induced permeabilization and impairment of the cytoplasmic membrane as these microorganisms (Tongaonkar *et al.* 2011; Tai *et al.* 2015; Beringer *et al.* 2016). Finally, the main direct mechanisms of action proposed for the cathelicidin LL-37 have been the bacterial membrane disruption following the toroidal pore model previously commented on and the probable interference with internal targets such as DNA (Xhindoli *et al.* 2015).

Antiviral mechanisms of action

The antiviral mechanisms of action showed by the HDPs are extremely variable and affect all the steps in the virus life cycle (Wilson, Wiens and Smith 2013) (Fig. 1). Being cationic and amphipathic peptides, the first interference is with the negatively charged lipid bilayer of encapsulated viruses. However, not all enveloped viruses are susceptible to these HDPs and their spectrum of activity can be extremely variable (Table 2). Only α-defensin HNP1 against herpes simplex virus type 1 (HSV-1) and β-defensin hBD2 against respiratory syncytial virus (RSV) have proven to act directly on the viral envelope to deactivate viruses (Daher, Selsted and Lehrer 1986; Kota *et al.* 2008). In other cases, like HNP1or hBD2 and hBD3 against human immunodeficiency virus type 1 (HIV-1), although the binding association has been demonstrated, whether this interaction alters in some way the viral envelope has not been determined (Quinones-Mateu *et al.* 2003; Seidel *et al.* 2010).

α- and θ-defensins HNP1-3 and HD5, RTD, retrocyclins and cathelicidin LL-37 show lectin-like activities; therefore, they are able to disrupt viral glycoprotein involved in binding and fusion of the viruses to host cells (Wang *et al.* 2003; Wilson, Wiens and Smith 2013). HSV infection is blocked by the activity of α-, β- and θ-defensins HNP1-4, HD5, HD6, hBD3, retrocyclins, RTD and cathelicidin LL-37, mainly through the inhibition of the viral-binding and -penetration processes targeting HSV glycoprotein B (gB) and heparan sulphate (Hazrati *et al.* 2006). Defensins HNP1-4, hBD2-3, retrocyclines and LL-37 can neutralize HIV-1 infection by blocking the viral life cycle at different steps. LL-37 has proven to block HIV-1 replication by inhibiting the reverse transcriptase activity (Bergman *et al.* 2007; Wong *et al.* 2011). The mentioned lectin nature of retrocyclins confers on them the ability to prevent HIV-1 entry into host cells, by blocking fusion through the binding to the viral gp120 and the cellular CD4 (cluster of differentiation 4) receptor (Munk *et al.* 2003; Gallo *et al.* 2006; Penberthy *et al.* 2011). hBD2 and hBD3 inhibit HIV-1 infection in three main ways: direct inactivation of virions, modulation of the CXCR4 co-receptor and inhibiting viral replication (Quinones-Mateu *et al.* 2003; Sun *et al.* 2005; Weinberg, Quinones-Mateu and Lederman 2006). On the other hand, all HNP α-defensins, especially HNP1, can act in very different ways in the HIV-1 virus life cycle to block the infection. Their ability to bind the CD4 receptor and the HIV envelope protein gp120 inducing the downregulation of CD4 and CXCR4 co-receptor has been described. Moreover, recently it has been reported that the HNP1 binding to targets is necessary but not sufficient for blocking HIV-1 fusion, and an additional step, as for example defensin oligomerization, must occur to inhibit virus fusion (Demirkhanyan *et al.* 2012). There are other

Table 2. Direct antiviral activity of HDPs.

HDPs	Target virus[a]	Mechanisms of action	References
α-defensins			
HNP1	CMV, HIV-1, HSV-1/HSV-2, IAV, VSV, AAV, HAdV, HPV, PyV	Envelope disruption, block receptor binding, receptor downmodulation, extracellular aggregation, block fusion, block uncoating, block genome nuclear import, cell signaling modulation, block gene expression	Daher, Selsted and Lehrer (1986), Virella-Lowell *et al.* (2000), Buck *et al.* (2006), Hazrati *et al.* (2006), Salvatore *et al.* (2007), Dugan *et al.* (2008), Smith and Nemerow (2008), Doss *et al.* (2009), Smith *et al.* (2010), Wei *et al.* (2010), Demirkhanyan *et al.* (2012), Wilson, Wiens and Smith (2013)
HNP2	HIV-1, HSV-1/HSV-2, IAV, AAV, HPV	Block receptor binding, receptor downmodulation, extracellular aggregation, block fusion, cell signaling modulation	Daher, Selsted and Lehrer (1986), Virella-Lowell *et al.* (2000), Buck *et al.* (2006), Hazrati *et al.* (2006), Salvatore *et al.* (2007), Doss *et al.* (2009)
HNP3	HIV-1, HSV-1/HSV-2, IAV, HPV	Block receptor binding, receptor downmodulation, extracellular aggregation, block fusion, cell signaling modulation	Buck *et al.* (2006), Hazrati *et al.* (2006), Salvatore *et al.* (2007), Doss *et al.* (2009)
HNP4	HIV-1, HSV-1/HSV-2, IAV, HPV	Block receptor binding, receptor downmodulation, cell signaling modulation	Wu *et al.* (2005), Hazrati *et al.* (2006), Salvatore *et al.* (2007), Doss *et al.* (2009)
HD5	HSV-1/HSV-2, IAV, HAdV, HPV, PyV	Block receptor binding, induce viral aggregation, block uncoating, block genome nuclear import, DNA interaction, block gene expression	Buck *et al.* (2006), Hazrati *et al.* (2006), Dugan *et al.* (2008), Smith and Nemerow (2008), Doss *et al.* (2009), Smith *et al.* (2010), Mathew and Nagaraj (2015)
HD6	HSV-1/HSV-2, IAV	Block receptor binding	Hazrati *et al.* (2006), Doss *et al.* (2009)
β-defensins			
hBD1	IAV, PyV	Block fusion	Doss *et al.* (2009)
hBD2	HIV-1, HPIV-3, IAV, RSV, VZV, PyV	Envelope disruption, receptor downmodulation, block fusion, cell signaling modulation, block reverse transcription	Quinones-Mateu *et al.* (2003), Doss *et al.* (2009), Crack *et al.* (2012), Wilson, Wiens and Smith (2013)
hBD3	HIV-1, HSV-1/HSV-2, IAV, VV	Envelope disruption, block receptor binding, receptor downmodulation, cell signaling modulation, block reverse transcription	Quinones-Mateu *et al.* (2003), Leikina *et al.* (2005), Howell, Streib and Leung (2007), Wilson, Wiens and Smith (2013)
hBD4	No antiviral activity reported	–	–
θ-defensins			
Retrocyclins	HSV-1/HSV-2, HIV-1, IAV	Block receptor binding, block fusion, induce viral aggregation	Yasin *et al.* (2004), Doss *et al.* (2009), Lehrer, Cole and Selsted (2012), Gwyer Findlay, Currie and Davidson (2013)
RTD	HSV-1/HSV-2	Block receptor binding	Yasin *et al.* (2004), Lehrer, Cole and Selsted (2012), Gwyer Findlay, Currie and Davidson (2013)
Cathelicidins			
LL-37	RSV, IAV, VV, HSV-1, HAdV19, HIV-1, VZV	Envelope disruption, block receptor binding, block reverse transcription	Wong *et al.* (2011), Crack *et al.* (2012), Vandamme *et al.* (2012), Tripathi *et al.* (2013), Currie *et al.* (2016)

[a]Viruses: CMV, cytomegalovirus; HIV, human inmunodeficiency virus; HSV, herpes simplex virus; IAV, influenza A virus; VSV, vesicular stomatitis virus; AAV, adeno-associated virus; HAdV, human adenovirus; HPV, human papillomavirus; PyV, polyoma virus; HPIV, human parainfluenza virus; RSV, respiratory syncytial virus; VV, vaccinia virus; VZV, varicella zoster virus.

mechanisms of action described for HNP1 to block HIV-1 infection, such as the blockage of internalization steps, the inhibition of gene expression, reverse transcription and integration by interfering protein kinase C (Hazrati *et al.* 2006; Salvatore *et al.* 2007; Doss *et al.* 2009; Contreras *et al.* 2012).

Influenza A virus (IAV) has been reported to be susceptible to the antiviral activity of α-, β- and θ-defensins HNP1-4, HD5-6 and hBD1-3, retrocyclins and cathelicidin LL-37 (Tripathi *et al.* 2013; Wilson, Wiens and Smith 2013). LL-37 has been proposed to block IAV infection by disrupting the viral membrane, while α-defensins act either inhibiting the activity of the enzyme protein kinase C, necessary for IAV endosomal escape and the nuclear entry and viral trafficking, or inducing viral aggregation (Gwyer Findlay, Currie and Davidson 2013; Tripathi *et al.* 2013). Inhibition of the virus fusion has been proposed as the mechanism of action for retrocyclines and hBDs to neutralize IAV infection (Leikina *et al.* 2005).

Other mechanisms of action showed by HNP1-4, HD5 and LL-37 against the non-enveloped viruses, such as human papillomavirus (HPV) and human adenovirus (HAdV), have been

reported. These peptides block the viral endosomal escape by the stabilization of the viral capsid (Buck *et al.* 2006; Smith and Nemerow 2008). Moreover, HD5 and HNP1 have been proposed to induce aggregation of BK virus particles, preventing BK virus infection through inhibition of viral attachment to host cells (Dugan *et al.* 2008; Wilson, Wiens and Smith 2013). RSV is another important virus, in terms of frequency and severity of disease, for which no optimal specific treatment exists. hBD2 and LL-37 are able to neutralize RSV through the destabilization of the membrane of the virion envelope and entry inhibition, although in this case the specific mechanism of action remains undetermined (Kota *et al.* 2008; Currie *et al.* 2013).

Antifungal mechanisms of action

The antifungal HDPs mechanisms of action have also been widely studied (Table 3). The α- and β-defensins have showed antifungal activity against different species of *Candida* such as *Candida albicans*, *C. krusei*, *C. tropicalis* and *C. parapsilosis* (Schroder and Harder 1999; Edgerton *et al.* 2000; Joly *et al.* 2004; Meyer *et al.* 2004; Lu *et al.* 2006; Vylkova *et al.* 2006, 2007; Shi *et al.* 2009; Song *et al.* 2009; Schroeder *et al.* 2011; Rizzo, Losacco and Carratelli 2013; Gacser *et al.* 2014).

The fungicidal mechanism of β-defensins has been shown to be a multifaceted process. hBD2 and hBD3 bind to Ssa1/2 proteins in the surface of *C. albicans*, causing membrane permeabilization (Vylkova *et al.* 2006, 2007; Krishnakumari, Rangaraj and Nagaraj 2009). In a second step, hBD2 and hBD3 act on the energy metabolism by causing depletion of ATP and leading to cell death (Vylkova *et al.* 2007). Alike hBD2 and hBD3, hBD1 has also been reported to cause the membrane destabilization of *C. albicans* leading to cell death (Krishnakumari, Rangaraj and Nagaraj 2009). Moreover, hBD3 can elevate the β-1,3-exoglucanase (Xog1) activity, resulting in reduced *C. albicans* adherence to abiotic surfaces (Chang *et al.* 2012).

The fungicidal mechanism of action of α-defensins has been studied to a lesser extent than that of β-defensins. HNP-1, unlike HBD2 and HBD3, does not require *C. albicans* Ssa1/2 proteins for its antifungal activity but acts in the same way on the energy metabolism, causing depletion of intracellular ATP and increasing extracellular ATP concentrations to kill *C. albicans* (Edgerton *et al.* 2000; Vylkova *et al.* 2006). On the other hand, HD6 has been reported to block *C. albicans* adhesion to human intestinal epithelial cells and to suppress the invasion into these epithelial cells and biofilm formation (Chairatana, Chiang and Nolan 2016).

Caco-2 cells, oral and bronchial epithelial cells and *Pneumocystis* cells have been shown to produce basal levels of hBD2 upon induction by the presence of fungi providing a first line of protection against *C. albicans*, *C. krusei*, *C. tropicalis*, *C. parapsilosis* and *Aspergillus fumigatus* (Meyer *et al.* 2004; Alekseeva *et al.* 2009; Sun *et al.* 2012; Gacser *et al.* 2014). In esophagus and in engineered human oral mucosa tissues, *C. albicans* induce a massive upregulation of hBD2 and hBD3 but just a slightly increased expression of hBD-1 in the esophagus (Kiehne *et al.* 2005; Steubesand *et al.* 2009; Rouabhia *et al.* 2011). Also, the expression of hBD2 and hBD3 by HeLa epithelial cells has been associated with the immune response effect of the probiotic strain *Lactobacillus crispatus* against *C. albicans* (Rizzo, Losacco and Carratelli 2013). Similarly, secretion of α-defensins HNP1-3 is significantly increased in whole blood and in esophagus after exposure to *C. albicans* (Kiehne *et al.* 2005; Gacser *et al.* 2014).

Cathelicidin LL-37 also shows antifungal activity against different *Candida* spp. (den Hertog *et al.* 2005; Lopez-Garcia *et al.* 2005; Ordonez *et al.* 2014; Durnas *et al.* 2016). Therefore, LL-37 can kill *C. albicans* rapidly by binding and destabilizing the cell membrane (den Hertog *et al.* 2005; Ordonez *et al.* 2014). It is suggested that LL-37 accumulate at high concentration near its target in the cell membrane of *C. albicans*, thus stimulating its killing (den Hertog *et al.* 2005). Recently, Durnás *et al.* demonstrated that LL-37 causes extensive surface changes in the *C. albicans* cell membrane, leading to cell death (Tsai *et al.* 2014; Durnas *et al.* 2016). After LL-37 action, *C. albicans* cell membrane is fragmented into a series of adjacent vesicle-like structures of ~100 nm, with exposed inner and outer leaflets (den Hertog *et al.* 2005). Moreover, functional properties of the cytoplasmic membrane of *C. albicans* are affected, leading to the segregation of lipids and proteins, the release of vital components and the subsequent cell death (den

Table 3. Direct antifungal activity of HDPs.

HDP	Target fungi	Mechanisms of action	References
α-defensins			
HNP1	*Candida albicans*	Depletion of ATP	Edgerton *et al.* (2000), Vylkova *et al.* (2006)
HD6	*C. albicans*	Block adhesion to host cells, suppression of biofilm formation	Chairatana, Chiang and Nolan (2016)
β-defensins			
hBD1	*C. albicans*	Membrane permeabilization	Vylkova *et al.* (2006), Vylkova *et al.* (2007), Krishnakumari, Rangaraj and Nagaraj (2009)
hBD2	*C. albicans*	Binding to Ssa1/2, membrane permeabilization, depletion of ATP	Vylkova *et al.* (2006), Vylkova *et al.* (2007), Krishnakumari, Rangaraj and Nagaraj (2009), Chang *et al.* (2012)
hBD3	*C. albicans*	Binding to Ssa1/2 and Xog1, membrane permeabilization, depletion of ATP	den Hertog *et al.* (2005), Tsai *et al.* (2011), Chang *et al.* (2012), Ordonez *et al.* (2014), Tsai *et al.* (2014), Scarsini *et al.* (2015), Durnas *et al.* (2016)
Cathelicidins			
LL-37	*C. albicans*, *C. parapsilosis*	Membrane lysis, genes and proteins modulation, block adhesion to host, binding to Xog1	Sigurdardottir *et al.* (2006)

Hertog *et al.* 2005, 2006). Additional mechanisms of action have been described for LL-37, when small concentration of LL-37 has been used to not affect the membrane permeability and to render *C. albicans* vulnerable to the immune response (Ordonez *et al.* 2014). Thus, Tsai *et al.* demonstrated that sub-lethal concentrations of LL-37 modulated the expression of genes involved in a variety of functions, including transport, regulation of biological processes, response to stress or chemical stimulus and pathogenesis (Tsai *et al.* 2014).

As for the human defensins, LL-37 prevents the adhesion of *C. albicans* to abiotic and biotic surfaces, blocking the formation of biofilm on medical grade silicone and polystyrene (Tsai *et al.* 2011a,b; Scarsini *et al.* 2015). Also, LL-37 reduces the infectivity of *C. albicans* by inhibiting its adhesion to oral epidermoid and urinary bladder cells of female BALB/c mice (Tsai *et al.* 2011b). This inhibitory effect on cell adhesion is mediated by its preferential binding to mannans, the main component of the *C. albicans* cell wall, and partially by its ability to bind chitin or glucans, which underlie the mannans layer (Tsai *et al.* 2011b). In addition, the β-1,3-exoglucanase Xog1, responsible for *C. albicans* cell wall construction and maintenance, has been identified as a LL-37 receptor (Chang *et al.* 2012). Thus, the interaction between Xog1 and LL-37 reduces Xog1 activity, interfering with cell wall remodeling and consequently impairs *C. albicans* adhesion (Tsai *et al.* 2011; Chang *et al.* 2012).

HOST-DIRECTED ANTIMICROBIAL ACTIVITY

In addition to their ability to exert direct antimicrobial activity over a broad spectrum of pathogens, human HDPs have the capacity to modulate the immune response to control infection and inflammation. Defensins as well as cathelicidin LL-37 act by regulating the production of inflammatory messengers, enhancing phagocytosis and intracellular killing of bacteria, stimulate antigen presentation and cell proliferation, induce and inhibit cytokines release and are potent chemotactic agents (Arnett and Seveau 2011; Yeung, Gellatly and Hancock 2011). Thus, hBD1 and hBD2 act as chemotactic factors for memory T cells and immature dendritic cells by direct binding to the chemokine receptor CCR6, whilst α-defensin HNP1-3 act by attracting monocytes, immature dendritic cells, and $CD4^+$ and $CD8^+$ T cells (Territo *et al.* 1989; Yang *et al.* 1999; Yang *et al.* 2000). LL-37 has been described as inhibiting the activation of mitogen-activated protein kinases and Akt signal pathways, thus attenuating *Staphylococcus aureus* LTA-induced inflammatory effects by decreasing the pro-inflammatory cytokines levels *in vitro* (Ruan *et al.* 2013). Moreover, LL-37 has shown its capacity to reduce endotoxin immunotoxicity through its binding to anionic molecules such as LPS (Cirioni *et al.* 2006).

The growing interest generated by the immunomodulatory activities of these HDPs has led to the recent development of the so-called innate defense regulators (IDRs). IDRs are small and synthetic peptides derivate from natural HDPs, which conserve their immunomodulatory activity and lack direct antimicrobial activity (Scott *et al.* 2007; Nijnik *et al.* 2010). One of these regulators is IDR-1, a synthetic IDR constituted by 13 amino acids (KSRIVPAIPVSLL-NH_2) that present protective activity against Gram-negative and Gram-positive bacteria entirely mediated by its immunomodulatory properties (Scott *et al.* 2007; Hou *et al.* 2013). IDR-1 has proven to have a protective effect in murine models of infection by methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *Enterococcus* spp. (VRE) and *Salmonella enterica* serovar *typhimurium* (Scott *et al.* 2007; Hou *et al.* 2013). This peptide was effective when administered locally and systemically, 48 h before to 6 h post-bacterial infection, enhancing the levels of monocyte chemokines by reducing the pro-inflammatory cytokines response, without obvious toxicities (Scott *et al.* 2007). In the same way, an *in vivo* MRSA pneumonia study demonstrated the capacity of IDR-1 and LL-37 to restore pulmonary function while inhibiting inflammation and decreasing the release of inflammatory cytokines (Hou *et al.* 2013). More recently, the novel IDR-1002 demonstrated higher *in vitro* and *in vivo* activities controlling bacterial infections compared to IDR-1(Nijnik *et al.* 2010), with significant reductions in bacterial loads, in invasive *S. aureus* and *Escherichia coli* infection models, with IDR-1002 at a dose of 200 μg (Nijnik *et al.* 2010). IDR-1018, the most advanced IDR synthetic peptide, and IDR-HH2 have also shown immunomodulatory activities in animal models of infection by multidrug-resistant (MDR) *Mycobacterium tuberculosis*, *E. coli*, MRSA and HSV (Wieczorek *et al.* 2010; Steinstraesser *et al.* 2012; Rivas-Santiago *et al.* 2013a; Mansour, de la Fuente-Nunez and Hancock 2015). Moreover, the immunomodulatory activity of these IDRs has been also applied as co-adjuvant to improve the effectiveness of pertussis vaccine or as adjunctive therapy to treat malaria (Achtman *et al.* 2012; Polewicz *et al.* 2013). IDR-1018 has proven also a potent anti-biofilm activity, both preventing biofilm formation and on preformed biofilm, versus *P. aeruginosa*, *E. coli*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, MRSA, *Sa. typhimurium* and *Burkholderia cenocepacia* (Mansour, de la Fuente-Nunez and Hancock 2015; Wang *et al.* 2015b). The predicted mechanism of activity would involve the degradation of stress-induced nucleotides (p)ppGpp involved in biofilm formation and maintenance (de la Fuente-Nunez *et al.* 2014).

Other immunoregulator is SGX94 (susquetide), a fully synthetic 5-amino acid peptide, analog of IDR-1, which targets the protein sequestome-1 (also called p62), a multifunctional protein involved in several cellular signaling pathways like autophagy, and in the activation of the nuclear factors kappa-B and erythroid 2-related factor 2 (Komatsu and Ichimura 2010; White 2012; North *et al.* 2016). This peptide has demonstrated improvement of survival in murine models of infection, either by local and systemic infections and administered both prophylactically or therapeutically. Without a direct bactericidal activity, SGX94 was able to promote bacterial clearance of antibiotic-susceptible and antibiotic-resistant Gram-positive and Gram-negative bacteria (North *et al.* 2016).

ANTIMICROBIAL ACTIVITY OF HDPs ANALOGS

Novel peptides have been designed based on the knowledge generated from the structure–activity relationship studies of natural HDPs to improve their antimicrobial activity, to differentiate their direct antimicrobial activity from their immunomodulatory effects and to decrease susceptibility to proteolytic hydrolysis by the cellular proteases. Smaller truncated peptides containing the minimal domain for activity, chimeric constructions as well as *de novo* designed sequences, bearing structural features that are crucial for the activity of natural peptides, have been developed and reported. Some of these analogs are active against bacteria, viruses and fungi (Tables 4, 5 and 6, respectively); thus, in this section, the different peptides are classified in the defensins and the cathelicidin LL-37 analogs.

Table 4. Direct antibacterial activity of HDP derivatives and analogs.

Original peptide/s	Peptide sequences	Target bacteria	References
α-defensins			
HNP1	AYRIPAIAGERRYGTIYQGRLWAF-$CONH_2$ IAGERRYGTIYQGRLWAF-$CONH_2$ IAAERRYATIYQARLWAF-$CONH_2$	*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus*	Varkey and Nagaraj (2005)
HD6	AFTHRRSYSTEYRYGTTVRGIRHRFL AFTHC^{2}RRSYSTEYSYGTC^{2}TV-NH_2 AFTRC^{2}RRSYSTEYSYGTC^{2}TV-NH_2 AFTRRRSYSTEYSYGTTV-NH_2 AFT*rrr*SYSTEYSYGTTV-NH_2 Myr-AFTRRRSYSTEYSYGTTV-NH_2 Myr-AFT*rrr*SYSTEYSYGTTV-NH_2	*E. coli, Salmonella enterica, P. aeruginosa, S. aureus*	Mathew and Nagaraj (2015)
HD5	ATCYCRGRCATRESLSGVCISGRLYRLCCR ATYRTGRATRESLSGVEISGRLYRLR ATYRTGRATRESLSGVEISGRLYRLR Myr-ATYRTGRATRESLSGVEISGRLYRLR Myr-ATYRTGRATRESLSGVEISGRLYRLR Laur-ATYRTGRATRESLSGVEISGRLYRLR Laur-ATYRTGRATRESLSGVEISGRLYRLR	*E. coli, P. aeruginosa, S. aureus, Bacillus subtilis*	Mathew and Nagaraj (2015), Wang *et al.* (2015)
β-defensins			
hBD1/hBD3	HYNCVSSGGQCLYSACPIFTKIQGTCYRGKAKCCK KYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRRKK HYNCVSSGGQCLYSACPIFTKIQGTCYRGKRKCCRRKK KYYCRVRGGRCAVLSCPIFTKIQGTCSTRGRKCCRRKK KYYCRVRGGRCAVLSCLPKEEQIGKCSTRGAKCCK	*E. coli, P. aeruginosa, Enterococcus faecalis*	Scudiero *et al.* (2010)
hBD1/hBD 3	CPIFTKIQGTC———GG———-RRKK	*E. coli, P. aeruginosa, En. faecalis*	Scudiero *et al.* (2015)
hBD1/2/3	AC^{3}PIFTKIQGT—YRGKAK—C^{6}K FC^{3}PRRYKQIGT—GLPGTK—C^{6}K SC^{3}LPKEEQIGK—STRGRK—C^{6}RRKK	*E. coli, S. aureus*	Krishnakumari, Singh and Nagaraj (2006)
hBD4	ELDRIC1GYGTARRKKC1R ELDRIGYGTARC1RKKC1R ELDRIC1GYGTARC1RKKR ELC1DRIC2GYGTARC1RKKC2R ELC1C2DRIGC3YTARC2RKKRSC1C3L KRSQEYRIGRC1PNTYAC1LKR ELDRIGYGTARRKKR ELDRIUGYGTARURKKUR LDRIUGYGTARURKKUR ELD*d*RIGYGTA*dRd*RKKR Myr-ELDRIGYGTARRKKR Myr-ELD*d*RIGYGTA*dRd*RKKR	*E. coli, P. aeruginosa, S. aureus*	Sharma and Nagaraj (2012), Sharma, Mathew and Nagaraj (2015)
θ-defensins			
Retrocyclin/hBD1	AC^{1}PIFTKIQGTYRGKAKRIGRRIC^1	*E. coli, P. aeruginosa, Burkholderia cepacia, S. aureus, Staphylococcus epidermidis, Corynebacterium amycolatum*	Olli, Nagaraj and Motukupally (2015)
Retrocyclins	GICRCICGKGICRCICGR CICRCIC*ha*CICRCI CIIRI$^{(L)}$P$^{(D)}$PIIIRCICILRL$^{(L)}$P$^{(D)}$PLILRCI	*S. aureus, Gardnerella vaginalis*	Lamers *et al.* (2011), Doss *et al.* (2012), Hooven *et al.* (2012)
Cathelicidins			
LL-37	IGKKFKRIVKRIKKWLRKL GKEFKRIVQRIKDFLRNLVPR KSKEKIGKEFKRIVQRIKDFLRNLVPRTES KRIVQRIKDFLRNLVPRTES KRIVQRIKDFLR	*E. coli, Salmonella typhimurium, P. aeruginosa, Acinetobacter baumannii, S. aureus, S. epidermidis, B. subtilis*	Sigurdardottir *et al.* (2006), Nan *et al.* (2012), Feng *et al.* (2013), Jacob *et al.* (2013)

Defensins analogs

Different α-defensin analogs have been generated and evaluated *in vitro*. Derivatives of HNP1 without cysteines have been proved to maintain certain antibacterial activity (lethal concentrations ranging 1–23 μM), although at significantly higher concentrations than those observed with the natural peptide (0.8–1 μM), against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Varkey and Nagaraj 2005). The insertions of two basic residues, such as arginine, at both the N- and the C-terminal regions of HNP2 have showed to increase its polarity and cationic charge, and it has been reported to significantly enhance the antifungal activity against *Candida albicans*. In contrast, the addition of anionic residues, such as aspartic acid, at the N- and C-terminal fragments renders a totally inactive

Table 5. Direct antiviral activity of HDP derivatives and analogs.

Original peptide/s	Peptide sequences	Target virus[a]	References
α-defensins			
HD5	ATCYCRTGRCATRESLSGVCRISGRLYRLCCR	HSV-2, HIV-1	Wang *et al.* (2013)
β-defensins			
hBD1/hBD3	HYNCVSSGGQCLYSACPIFTKIQGTCYRGKAKCCK KYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRRKK HYNCVSSGGQCLYSACPIFTKIQGTCYRGKRKCCRRKK KYYCRVRGGRCAVLSCPIFTKIQGTCSTRGRKCCRRKK KYYCRVRGGRCAVLSCLPKEEQIGKCSTRGAKCCK	HSV-1	Scudiero *et al.* (2010)
hBD1/hBD3	KYYCRVRGGRCAVLSCPIFTKIQGTCSTRGRKCCRRKK	HSV-1	Scudiero *et al.* (2013)
hBD1/hBD3	CPIFTKIQGTC———GG———-RRKK	HSV-1	Scudiero *et al.* (2015)
θ-defensins			
Retrocyclins	GICRCICGKGICRCICGR ICRLIL$^{(D)}$PPLRLIC	IAV, HIV	Doss *et al.* (2009), Doss *et al.* (2012), Wood *et al.* (2013)
Cathelicidins			
LL-37	GIKEFKRIVQRIKDFLRNLV FKRIVQRIKDFLR GIKEFKRIVQRIKDFLRNLV KIGKEFKRIVQRIKDFLRNLVP	IAV, RSV, HIV	Wang, Watson and Buckheit (2008), Currie *et al.* (2013), Tripathi *et al.* (2015)

[a]Viruses: HSV, herpes simplex virus; HIV, human inmunodeficiency virus; IAV, influenza A virus; RSV, respiratory syncytial virus.

Table 6. Direct antifungal activity of HDPs derivatives and analogs.

Original peptide/s	Peptide sequences	Target fungi	References
α-defensins			
HNP2	RRC^{1}YC^{2}RIPAC^{3}IAGERRYGTC^{2}IYQGRLWAFC^{3}C^{1}RR	*Candida albicans*	Raj, Antonyraj and Karunakaran (2000)
hBD1	AC^{1}PIFTKIQGTYRGRAKC^{1}R AC^{1}P^{D}IFTK^{D}IQGTY^{D}RG^{D}RAKC^{1}R	*C. albicans*	Olli, Rangaraj and Nagaraj (2013)
β-defensins			
hBD3	SC^{1}LPKEEQIGKSTRGRKC^{1}RRKK SC^{1}LPKEEQ^{D}IGKST^{D}RG^{D}RKC^{1D}R ^{D}RKR GKCSTRGRKCCRRKK	*C. albicans*	Olli, Rangaraj and Nagaraj (2013), Lim *et al.* (2016)
hBD4	ELC^{1}C^{2}DRIGC^{3}YTARC^{2}RKKRSC^{1}C^{3}L	*C. albicans*	Sharma and Nagaraj (2012)
Cathelicidins			
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIK LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNL GKEFKRIVQRIKDFLRNLVPR	*C. albicans*, *C. parapsilosis*	Sigurdardottir *et al.* (2006)

molecule (Raj, Antonyraj and Karunakaran 2000). Lineal analogs of HD5 and HD6 have also demonstrated an improved antibacterial activity against Gram-negative and Gram-positive bacteria, with the same concentrations range (2.5–12.5 μM) of the original peptides (Mathew and Nagaraj 2015a,b; Wang *et al.* 2015). Mathew and Nagaraj demonstrated that the antimicrobial activity of α-defensin could be improved by the fatty acylation of the linear peptides, and that amino acids replacements and covalent modifications in selected regions of HD6 could generate antimicrobial peptides from the apparently inactive HD6 (Mathew and Nagaraj 2015a,b).

Regarding the β-defensins, several analogs of hBD1 and hBD3 have also showed promising results (Krishnakumari, Singh and Nagaraj 2006; Scudiero *et al.* 2010, 2013). Krishnakumari *et al.* generated shorter peptides with a single disulfide bridge which had *in vitro* antimicrobial activity at the same range than their parental defensins (MIC, 15–23 μM) against *E. coli* and *S. aureus* (Sahl *et al.* 2005; Krishnakumari, Singh and Nagaraj 2006). Another set of hBD1 and hBD3 analogs were generated by Scudiero *et al.* comprising different domain of these defensins (Scudiero *et al.* 2010). These derivatives showed improved antibacterial activity against *Enterococcus faecalis*, *E. coli* and *P. aeruginosa* with MICs values in the range of 2.5–12.5 μM even at high NaCl concentrations, when it is well known that most of these HDPs are salt-sensitive (Scudiero *et al.* 2010, 2013). These new hBD1/3 derivatives also showed enhanced activity against HSV-1, inhibiting its infectivity at concentrations of 50 μM and, again, some of them showed these antimicrobial activities in presence of high NaCl concentrations (Scudiero *et al.* 2010, 2013). Scudiero *et al.* also proposed the internal region of hBD1 and the C-terminal region of hBD3 as being responsible for the antimicrobial activity of these peptides and so they designed an analog

combining these two domains (Scudiero *et al.* 2015). This novel peptide maintained the same antimicrobial activity against *P. aeruginosa*, *E. coli* and *En. faecalis* as well as against HSV-1, enhancing the stability in serum with respect to the parent defensins (Scudiero *et al.* 2015).

The antimicrobial activity of hBD4 analogs has also been investigated, synthesizing shorter sequences spanning the N-terminal region of hBD4 and containing 1–3 disulfide bridges (Sharma and Nagaraj 2012). These new peptides showed enhanced antimicrobial activity against *E. coli*, *P. aeruginosa* and *S. aureus* compared to hBD4, which was higher as they kept the three disulfide bridges and decrease with a high concentration of NaCl. A new batch of derivatives was designed subsequently in an attempt to obtain antimicrobial active peptides from linearized segments of the N-terminal region of hBD4 (Sharma, Mathew and Nagaraj 2015). They showed that the introduction of α-aminoisobutyric acid (a helix-inducing amino acid) instead of cysteine did not favor antibacterial activity, while it improved antifungal activity (Sharma, Mathew and Nagaraj 2015). They found antibacterial activity when substituted L-arginine with D-arginine in the linear inactive peptide and activity was increased in the fatty acylated peptides (Sharma, Mathew and Nagaraj 2015).

C-terminal analogs of human hBD1 and hBD3, wherein lysines were selectively replaced by L- and D-arginines and L-isoleucine substituted with its D-enantiomer, exhibited antifungal activities by impairing *C. albicans* membrane permeability (Olli, Rangaraj and Nagaraj 2013). When hBD3 was shortened to only 15 amino acid at the C-terminal, higher activity against *C. albicans* was observed (Lim *et al.* 2016). In contrast, single disulfide peptides spanning the C-terminal regions of hBD1, hBD2 and hBD3, called Phd1, Phd2 and Phd3, respectively, showed lower antifungal activity than their respective precursors against *C. albicans* (Krishnakumari, Rangaraj and Nagaraj 2009). Other analogs of hBD3 have been synthetized with native disulfide linkages, with non-native linkages or with replacement of all cysteine residues by aminobutyric acid creating linear peptides. Any of these peptides improved the antifungal activity of hBD3 against *C. albicans* (Hoover *et al.* 2003). The introduction of three disulfide bridges in the N-terminal segment of hBD4 enhanced its antifungal activity by entering rapidly inside *C. albicans* without disrupting the membrane integrity (Sharma and Nagaraj 2012).

The C-terminal region of hBD1 containing a single disulfide bridge was responsible for the antibacterial activity of the peptide and was combined with the RIGRRIC region of retrocyclin in order to improve its antimicrobial activity (Olli, Nagaraj and Motukupally 2015). This chimeric defensin presented antibacterial activity at concentrations under 5 μM against wild-type strains of *E. coli*, *P. aeruginosa* and *S. aureus* and against MDR clinical strains of *Corynebacterium amycolatum*, *S. aureus*, *S. epidermidis* and *P. aeruginosa* (Olli, Nagaraj and Motukupally 2015). This hydrid peptide also showed antimicrobial activity with or without the disulfide bridge and, furthermore, it retained the activity in the presence of serum and NaCl, and it was not hemolytic *in vitro*. Moreover, it had valuable pharmacokinetics characteristics for the development of new therapeutic molecules (Olli, Nagaraj and Motukupally 2015).

Retrocylins, θ-defensins, have also been used to design and synthesize new and more potent antimicrobial peptides. The retrocyclin analog RC-101 has showed significant activity against clinical isolates of *S. aureus* at concentrations as low as 5 μM (Lamers *et al.* 2011). As an antiviral agent, it has demonstrated exerting significantly more potent antiviral activity against IAV and HIV than retrocyclin-1, even presenting a very conservative modification in respect to this one (Arg $\rightarrow$ Lys) (Owen *et al.* 2004; Doss *et al.* 2009). RC-101 also proved to be active against the enfuvirtide-resistant HIV mutants V38A and V38+N126K, which highlighted its potential clinical importance as a fusion inhibitor (Wood *et al.* 2013). In fact, RC-101 is being developed as an intravaginal microbicide to prevent sexually transmitted HIV-1 and bacterial vaginosis by *Gardnerella vaginalis* (Hooven *et al.* 2012; Eade *et al.* 2013).

Other examples of retrocyclin derivatives are hapivirins and diprovirins, cyclic peptides that incorporate key cationic and hydrophobic residues substitutions and synthetic amino acids (Doss *et al.* 2012). These peptides were designed with the purpose of simplifying the structure of retrocyclins, making them easier to synthesize while improving or at least maintaining their antiviral activity against IAV. As a result, they obtained some variants of each derivative with great anti-IAV potency, showing significant reductions of viral load at concentrations ranging 0.4–3 μg mL^{-1}, in the same range as retrocyclin 2 or RC-101 (Doss *et al.* 2012). Those derivatives that showed the most potent antiviral activity were also tested for their potential antibacterial and antifungal activity. They found that diprovirins caused a much stronger inhibition of *S. aureus* and *C. albicans* growth than hapivirins, with *in vitro* activity concentrations between 1.25 and 20 μg mL^{-1}, which highlighted their potential broad-spectrum activity (Doss *et al.* 2012).

Cathelicidin LL-37 analogs

A number of shorter cathelicidin LL-37 variants have been generated during the last few years in order to improve its antimicrobial activity and reduce its toxicity (Wang, Watson and Buckheit 2008; Feng *et al.* 2013; Jacob *et al.* 2013; Tripathi *et al.* 2015). The 21-amino acid fragment GKE displayed similar or higher antimicrobial activity against *E. coli*, *P. aeruginosa*, *S. aureus, C. albicans* and *C. parapsilosis* than the whole LL-37 at concentrations ranging 0.5–100 μM (Sigurdardottir *et al.* 2006). GKE and LL-37 showed antimicrobial activity against *E. coli* independently of the physiological concentration of NaCl ($\pm$150 mM) and the presence of serum (99%) (Sigurdardottir *et al.* 2006). Unlike LL-37, GKE was less toxic, showing a diminished hemolytic activity and lesser DNA-damage induction (Sigurdardottir *et al.* 2006). KR-12 (residues 18–29) is the shorter fragment of LL-37 that has showed antimicrobial activity against for example, MDR *Acinetobacter baumannii* strains (Wang, Watson and Buckheit 2008; Feng *et al.* 2013; Tripathi *et al.* 2015). A series of KR-12 analogs have been designed showing improvements regarding its antimicrobial and antiendotoxic activities. Some of these analogs presented MICs of 4 μM against MRSA isolates (Jacob *et al.* 2013). KS-30 and KR-20, other shorter fragments of LL-37, were also reported to exert antibacterial activity against MDR *A. baumannii* with MICs of 8 and 16 μg mL^{-1}, respectively, and also to prevent biofilm formation (Feng *et al.* 2013). Nan *et al.* generated another batch of analogs consisting in a series of Lys- and/or Leu-substituted derivatives based on the internal region 13–31 of LL-37 (IG-19) (Nan *et al.* 2012). They found some of these IG-19 variants to present great *in vitro* antibacterial activity against both Gram-negative and Gram-positive bacteria, with MICs ranging 2–8 μM against *E. coli*, *P. aeruginosa*, *Sa. typhimurium*, *Bacillus subtilis*, *S. epidermidis* and *S. aureus* (Nan *et al.* 2012).

Smaller derivatives of LL-37 have also showed antiviral activity, essentially against IAV, HIV and RSV (Wang, Watson and

Buckheit 2008; Currie et al. 2013; Tripathi et al. 2013, 2015). The variant GI-20 presented greater anti-HIV activity than LL-37 with less toxicity, thus showing an improved therapeutic index (Wang, Watson and Buckheit 2008). This variant GI-20 also showed a notably anti-IAV activity (Tripathi et al. 2015); the same authors have reported that both LL-37 and the fragment GI-20 had similar neutralizing activity against IAV but, surprisingly, against the influenza A(H1N1)pdm09 strain, LL-37 reduced its activity while GI-20 retained it (Tripathi et al. 2015). RSV has been also showed to be susceptible to a central fragment of LL-37 (amino acids 12–33); this central fragment exerts an anti-RSV activity similar to that of the parental peptide at concentrations ranging 1–25 μg mL^{-1} (Currie et al. 2013).

Similarly for defensins, a number of shorter variants have been generated during the last years in order to improve LL-37 antifungal activity. Truncated variants LL-25 and LL-31 have presented better antifungal activity than LL-37 due to their longer accumulation in the cell membrane of *C. albicans* (den Hertog et al. 2006; Rapala-Kozik et al. 2015). LL-25 also lacks the immunomodulatory properties of LL-37 (Rapala-Kozik et al. 2015).

CLINICAL AND EXPERIMENTAL THERAPEUTIC STUDIES

Because of their small size and relatively simple structure, many HDPs have been produced and investigated *in vivo* in animal models of infection with promising results. In addition to their features as a novel generation of therapeutic agents, HDPs have also showed their utility as adjuvants agents in combination with classical antimicrobial drugs, neutralizing endotoxins and reducing mortality in animal model of infections (Zasloff 2002; Gordon, Romanowski and McDermott 2005; Mygind et al. 2005). However, only very few of them have progressed to early clinical development and to the market (Zhang and Falla 2006). A few cationic peptides have been tested in clinical-efficacy trials, for indications such as skin infections and nasal carriers by MRSA (LTX-109), *Clostridium difficile* infections (LFF551 and surotomycin), oral candidiasis (PAC113), impetigo and diabetic foot ulcers and infections (Pexiganan), oral mucositis and ventilator-associated pneumonia (Iseganan), sepsis (Neuprex), catheter-associated infections (Omiganan), dental caries, targeting *Streptococcus mutans* (C16G2, a synthetic antimicrobial targeted peptide) or wound healing, among others (Zasloff 2002; Hancock and Sahl 2006; Eckert, Sullivan and Shi 2012; Thakur, Qureshi and Kumar 2012; Fox 2013; Mangoni, McDermott and Zasloff 2016).

Three major factors still prevent their clinical development and widespread use: stability, toxicity and cost (van 't Hof et al. 2001). Some of these HDPs are salt-sensitive or serum-sensitive, which reduce significantly their antimicrobial activity and represent a serious problem for their systemic and topical uses. HDPs are relatively susceptible to proteolytic degradation. Upon systemic administration they are cleared quickly due to proteolytic degradation showing poor pharmacokinetics, all of which restrict their potential application (van 't Hof et al. 2001). Thus, a number of different strategies have been applied to circumvent their reduced half-life, such as different ways of administration or chemical modifications in the peptides: introduction of D-amino acids, amidation at the N-terminus, design of peptidomimetics and introduction of synthetic aminoacids or peptic cyclation (Wiley and Rich 1993; Adessi and Soto 2002; Chongsiriwatana and Barron 2010).

This section is mainly devoted to providing a review of the *in vivo* direct-antimicrobial activity in animal models of infection of defensins and cathelicidin LL-37 and also a subsection for the revision of peptides used to treat infections indirectly by modulating the immune response.

Experimental *in vivo* defensins studies

Antibacterial studies

As experimental therapeutics, HDPs have been used in a number of animal models of infection. The efficacy of bacterium-derived recombinant human β-defensins hBD1-2 was tested in a murine model of *Salmonella* infection (Maiti et al. 2014). Mice of all treatment groups were challenged intraperitoneally (i.p.) with an inoculum of *Salmonella enterica* serotype *typhi* containing 10^3 CFU, followed by i.p. administration of hBD1 (100 μg), hBD2 (100 μg) or a combination of hBD1/2 (2:1, 66 μg of hBD1 and 33 μg of hBD2; 1:1, 50 μg each). Mice mortality was monitored during 10 days to evaluate the efficacies of these therapeutic molecules. The authors concluded that the administration of these defensins, both alone and in combination, increased mice survival and decreased *Sa. typhimurium* load in peritoneal fluid, liver and spleen (Maiti et al. 2014). Similar results were reported when the hBD1-2 combination was expressed and extracted from tobacco plants (Patro et al. 2015).

Another study has evaluated the therapeutic efficacy of RTD-1 in a murine *P. aeruginosa* chronic lung infection model (Beringer et al. 2016); nebulized treatment with RTD-1 (167 μg kg^{-1}) resulting in a significant decreased in the *P. aeruginosa* load in lungs, as well as airway leucocytes and weight loss compared with untreated controls (Beringer et al. 2016). RTD-1 was also evaluated in two animal models of peritoneal infection. An *Escherichia coli* murine peritonitis model was induced by a single i.p. injection of 8×10^8 CFU of log-phase *E. coli* K12 strain, and mice were treated immediately with a single subcutaneous injection of RTD-1 (5 mg kg^{-1}), and monitored for 28 days (Schaal et al. 2012); RTD-1 significantly increased mice survival in 30% after 22 days of bacterial challenge (Schaal et al. 2012). In the same work, they performed a cecal-ligation/puncture (CLP) induced sepsis model, in which polymicrobial peritonitis is induced (Eskandari et al. 1992), and 4 h following CLP surgery each animal received normal saline (controls) or 5 mg kg^{-1} of RTD-1 intravenously (i.v.); moreover, a separate group of mice was treated with RTD-1 (5 mg kg^{-1}, i.v.), but the single administration was delayed 24 h after CLP surgery; in both experiments, animals were evaluated daily for 28 days (Schaal et al. 2012). A single dose of RTD-1 significantly improved survival (80%) of mice infected i.p. with *E. coli*. Moreover, a single intravenous dose of RTD-1 administered 4 h after CLP surgery resulted in long-term survival (90%) by day 22 post-surgery (Schaal et al. 2012). They also found that escalating doses of RTD-1 from 0.2 to 3.0 mg kg^{-1} over 14 days administered i.v. to two chimpanzees resulted in no adverse clinical effects, no detection of anti-RTD-1 antibodies, and normal metabolic panels and blood counts during the 60 days of the study (Schaal et al. 2012).

Antiviral studies

A recombinant mouse β-defensin 3 proved also *in vivo* antiviral activity in a murine model of infection with a mouse-adapted IAV (Jiang et al. 2012). Mice were challenged intranasally with a dose of 10× the lethal dose 50 (LD_{50}) and were assigned to different treatment regimens. Therapy began 12 h after infection and was performed once a day for 3 weeks. The most effective regimen improved mice survival to 83.3% when the peptide was

administered i.v. at a concentration of 10 mg kg^{-1} per day 3 weeks post-virus challenge (Jiang *et al.* 2012).

HD5 and its derivatives have been also evaluated in a lethal murine model of HSV-2 (Wang *et al.* 2013). All the mice were pretreated with progesterone subcutaneously (2 mg) and 5 days later each mouse was inoculated with a lethal dose of HSV-2 (10^5 PFU). Prior to or after viral inoculation, mice received an intravaginal instillation of the peptide HD5 or E21R-HD5 (HD5 derivative carrying arginine instead of glutamic acid at position 21) solutions (10 mg mL^{-1}) as a prophylactic or therapeutic administration, while control mice received PBS alone (Wang *et al.* 2013). Both HD5 and E21R-HD5 showed strong anti-HSV-2 activity either as prophylactic or therapeutic use, increasing the survival rates (60% and 70%, respectively) compared to non-treated controls (10%) (Wang *et al.* 2013).

Experimental *in vivo* cathelicidin studies

Antibacterial studies

Cathelicidin LL-37 showed promising therapeutic effects in an experimental model of progressive pulmonary tuberculosis in mice infected by endotracheal instillation with 2.5×10^5 CFU of *Mycobacterium tuberculosis*, and treated by intratracheal instillation with LL-37 60 days post-bacterial challenge three times a week at dose of 1 mg kg^{-1} (Rivas-Santiago *et al.* 2013). LL-37 generated significant 3–10-fold reductions of *M. tuberculosis* load in lungs, both against a drug-susceptible strain and a MDR strain, after 28–30 days of treatment.

In another study, a single systemic administration of LL-37 (1 mg kg^{-1}) showed protection against lethal bacteremia in three different sepsis rat models by *E. coli*, two rat models of intraperitoneal infection and a CLP model, without differences among those treated with LL-37 or polymixin B (1 mg kg^{-1}) (Cirioni *et al.* 2006).

Antiviral studies

In a recent work by Currie *et al.*, they demonstrated the antiviral activity of LL-37 in a murine model of pulmonary RSV infection (Currie *et al.* 2016). Mice received 5.6×10^5 PFU of RSV by intranasal delivery and were treated intranasally with 10 μg per mouse of LL-37 in three different groups, either administered 1 h before infection, at the same time of the infection or 1 h post-infection, and further with a daily dose of 10 μg per mouse until day 6 post-infection. Concomitant delivery of LL-37 showed significant protective effects on weight loss and viral load while neither pretreatment nor delayed application of the peptide achieved protective effects (Currie *et al.* 2016). These results may be explained because of the necessity of the pulmonary co-localization of LL-37 with RSV to exert its direct antiviral activity. Barlow *et al.* demonstrated that LL-37 (nebulized 1 day prior to and for 7 days post-infection with 10x LD_{50}) provided significant protection against a lethal infection with influenza A(H1N1)/Puerto Rico/8/1934 by improving the animal survival rate and avoiding the weight loss at the same level as the well characterized influenza antiviral zanamivir (Barlow *et al.* 2011).

Experimental *in vivo* studies based on HDPs immunoregulatory activities

Many HDPs have been evaluated in animal models of infection, and they have demonstrated improvement in infection outcomes, the recruitment of immune cells to the site of the infection or to influence the secretion of pro-inflammatory and anti-inflammatory cytokines by the modulation of the immune system (Zasloff 2002; Scott *et al.* 2007; Steinstraesser *et al.* 2008).

IDR-1 was evaluated in MRSA and VRE animal models of infection with satisfactory results in both cases (Scott *et al.* 2007; Hou *et al.* 2013). The peptide was effective reducing bacterial counts and mortality at concentrations ranging from 8 to 24 mg kg^{-1}, and using different routes of administration in the thigh and intraperitoneal models (Scott *et al.* 2007). In this case, the immunomodulatory activity of IDR-1 was related to the recruitment of monocytes and macrophages to the site of the infection, and also with the decrease of pro-inflammatory (TNF-α and IL-6) and the increase of anti-inflammatory (IL-10) cytokines, a perfect combination to control infections and inflammation at the same time (Scott *et al.* 2007). In case of the intratracheal-induced MRSA murine pneumonia model, IDR-1 and LL-37 also showed a decrease in the production of TNF-α and IL-6 in bronchoalveolar lavage fluid and serum, and they could trigger the recruitment of lymphocytes to the site of the infection (Hou *et al.* 2013). Moreover, both peptides showed a significant amelioration of the induced pneumonia according to the histopathology data, with the best result obtained at concentration of 0.8 mg kg^{-1} for LL-37 and 0.6 mg kg^{-1} for IDR-1 (Hou *et al.* 2013). Another IDR peptide, IDR-1002, has shown an enhanced chemokine induction activity compared to IDR-1 that was confirmed in invasive *S. aureus* and *E. coli* infection models (Nijnik *et al.* 2010). The i.p. administration of IDR-1002 at concentrations ranging from 20 to 100 μg mL^{-1} demonstrated stronger chemokine induction and protection than IDR-1, enhancing the recruitment of neutrophils and monocytes, although without increasing the levels of pro-inflammatory cytokines IL-6 and TNF-α (Nijnik *et al.* 2010).

The immunomodulatory activities of IDR-1018 have been demonstrated to be effective reducing soft tissue infections and disrupting bacterial biofilm formation (Achtman *et al.* 2012; Rivas-Santiago *et al.* 2013; Mansour, de la Fuente-Nunez and Hancock 2015). It has proven together with IDR-HH2 to be effective reducing bacterial loads in a murine pneumonia model by *M. tuberculosis* leading to a reduction in lung inflammation (Rivas-Santiago *et al.* 2013). It has also been effective in the treatment of *S. aureus* infections in several animal models like the murine model of implant infection or the murine and porcine wound healing models (Zasloff 2002; Steinstraesser *et al.* 2012; Choe *et al.* 2015). The mechanisms of action of IDR-1018 involve the downregulation of inflammatory cytokine expression (e.g. TNF-α) and upregulation of genes involved in the erythropoiesis, as well as reduction of pro-inflammatory mediators such as nitric oxide synthase or the regulation of chemokines and cytokines production by the modulation of macrophages and neutrophils differentiation and activation (Achtman *et al.* 2012; Choe *et al.* 2015).

As previously mentioned, the immunomodulatory activity of the synthetic peptide SGX94 has been evaluated in different animal models of infection and also in a Phase 1 and Phase 2 clinical studies (North *et al.* 2016). Its safety and effectivity in clearing bacterial infections has been assayed in mice and rats, in models of peritoneal, skin, gastrointestinal and lung infections, against methicillin-sensitive *S. aureus*, MRSA, *P. aeruginosa* and antibiotic-resistant *Burcholderia pseudomallei* (North *et al.* 2016). Despite the absence of direct antibacterial activity, SGX94 showed significant broad-spectrum antibacterial activity, sustained even in the absence of a continued exposure to the peptide (North *et al.* 2016). Concentrations ranging 5 to 50 mg kg^{-1} were found to be efficacious controlling the infections whether administered therapeutically or prophylactically (North *et al.*

2016). Importantly, and as it was previously described for IDR-1, the activity of SGX94 was not dependent on immunosuppression (Scott *et al.* 2007). In clinical trials, Phase 1 and Phase 2 studies showed that SGX94 was involved in the modulation of pro- and anti-inflammatory cytokine levels, reducing the damage produced as a consequence of innate immune activation and, consequently, the number of registered infections was reduced in patients treated with SGX94 (Kudrimoti *et al.* 2016; North *et al.* 2016).

To date, *in vivo* studies of infection to evaluate the therapeutic efficacy of IG-19, a peptide derived from the LL-37, to control bacterial or virus infections have not been carried out. However, the immunomodulatory properties of this peptide in a murine model of collagen-induced arthritis have been demonstrated (Chow *et al.* 2014); mice injected subcutaneously with IG-19 at 6 mg kg^{-1} per mouse every 48 h for 5 weeks did not show any significant adverse effect. More importantly, IG-19 showed a significant reduction on pro-inflammatory cytokine levels in serum, which together with its direct antibacterial activity makes it a candidate for future studies to evaluate its potential effect to control infections *in vivo* (Chow *et al.* 2014).

PRODUCTION OF HDPs FOR BIOLOGICAL AND CLINICAL APPLICATIONS

The fascinating characteristics of these HDPs may prompt the development of a novel generation of antimicrobial drugs in the near future, with wider spectrum of activity, novel targets and mechanisms of action and less propensity for emergence of microbial resistances. However, there are some obstacles that should be first overcome regarding the optimization of their pharmacokinetic characteristics. Moreover, a great obstacle for a wider clinical and commercial application of these HDPs and their derivatives relies on the ability to find a scalable cost-efficient production method. The high cost of production of these antimicrobial peptides is due to several factors, such as the complexity of their secondary structure and also for the lack of suitable expression system (Table 7).

The study of the structure and the antimicrobial activity of these HDPs require large amounts of correctly folded peptides, which are difficult to obtain from natural sources because of the low-yield concentrations. Previous studies have reported the chemical synthesis of α-defensins which is feasible but very costly (Dawson *et al.* 2000; Raj *et al.* 2000). The chemical synthesis of defensins has been performed so far using Boc chemistry for chain assembly and anhydrous hydrogen fluoride (HF) for deprotection and cleavage. However, this methodology is inefficient to generate great amounts of the peptide, and it is impractical for many laboratories due to the safety hazards and restrictions for HF (Wu *et al.* 2004). Wu *et al.* reported the chemical synthesis of α-defensins HNP4, HD5 and HD6 using directly crude peptides which showed an overall yield of 10%–16% with high purity (Wu *et al.* 2004). More recently, Vernieri *et al.* described a new methodology based on the use of the fluoromethyloxycarbonyl chloride (Fmoc) strategy for an optimized solid-phase synthesis of HD5 (Vernieri *et al.* 2014). As a result, they obtained a correctly folded HD5, highly pure (> 95%) and with an overall yield of 15 mg per run.

Another approach that has been attempted in many occasions has been the use of recombinant techniques (Pazgier and Lubkowski 2006; Wang *et al.* 2010; Chapnik *et al.* 2012). The most commonly used host cell has been *Escherichia coli* mainly because it grows easily, fast and is inexpensive, and also because it is a well-established expression system (Pazgier and Lubkowski 2006; Tomisawa *et al.* 2015). Disadvantages of the use of these recombinant strategies to produce HDPs are their potential toxicity to the host and their susceptibility to proteolytic degradation (Li 2009). This degradation could be prevented by expressing these peptides as fusion proteins in *E. coli*; however, there will always be a necessary extra step to remove the fusion protein tag either by enzymatic or chemical cleavage, with the potential risk of unfavorable degradation of the recombinant peptides (Huang *et al.* 2006; Xu *et al.* 2006). The peptide yield obtained by this methodology is highly variable with some authors reporting productivities of 1.70–2.68 g L^{-1} and other reaching concentrations around 100 mg L^{-1} with 74.7% purity (Xu *et al.* 2006; Wang *et al.* 2010). The formation of inclusion bodies as a novel way to prevent degradation and improve productivity has also been assayed with promising results compared to the conventional method (Tomisawa *et al.* 2015).

Other host cells that have been used to improve the stability of the recombinant protein and the yield biosynthesis of these peptides are *Saccharomyces cerevisiae*, *Pichia pastoris*, a methylotrophic yeast, and *Chlorella ellipsoidea*. *Pichia pastoris*, an unicellular algae, has proven to be an effective system for expression of recombinant proteins, including HD5, hBD2, sheep BD1 or porcine BD2 among others plants and invertebrate defensins but with very variable yield of the purified peptides, ranging from 1 to 383.7 mg L^{-1} depending on each case (Hsu *et al.* 2009; Zhao and Cao 2012; Peng *et al.* 2014). Disadvantages of the yeast systems are the low stability of these peptides at 28°C and the proteolytic activity in the fermentation medium (Cipakova and Hostinova 2005). As well as with the yeast, the use of *C. ellipsoidea*, apart from the fact that it presents a high growth rate and easy control production, can be used together with the fodder in cattle industry, enabling the direct use of centrifugation and

Table 7. Comparison of recombinant expression systems.

	Plants	Mammalian cells	Bacteria	Yeast/algae	Baculovirus infected insects
Production cost	Low	High	Low	Medium	High
Production speed	Low	Medium	Very high	High	Medium
Folding	Medium	Optimal	Poor	Poor	Medium
Glycosilation	Medium	Optimal	Poor	Poor	Medium
Expression level	>10 g kg^{-1}	<10 mg L^{-1}	10–30 g L^{-1}	<1 g L^{-1}	1–5 g L^{-1}
Protein yield	Very high	High	Medium	High	Very high
Scale-up-capacity	Very high	Very low	High	High	Medium
Storage	Easy	Difficult	Medium	Medium	Difficult
Government regulation	Difficult	Easy	Medium	Medium	Difficult

resulting cell-free medium without further peptide purification (Damaso *et al.* 2003).

The baculovirus-infected insect cell system is the first choice for production of those cytosolic proteins that are unable to be produced in prokaryotic hosts, but for production of secreted mammalian proteins its use is more limited (Unger and Peleg 2012). There are two main limitations to the use of this platform; first of all the high cost, as expensive as mammalian cell culture, and secondly the requirement of viral transduction and all the extra steps associated with the use of viral stocks (Jarvis 2009). Notwithstanding, there have been some works reporting the production of defensins using this baculovirus-insect platform, the results of which suggest that the use of this platform could be considered as an option for high efficient production of recombinant defensins (Cai *et al.* 2004; Fukushima *et al.* 2013).

In general, mammalian cells are the preferred system to produce these recombinant peptides, mostly because their ability to produce proteins similar to those naturally occurring in human, and because the CHO cell line still represents the major mammalian host for protein production (Zhu 2012). Also, for this cell line, and that is something that has not changed since the first recombinant product approval, the preferred culture system is large-scale stirred tank bioreactors (Hacker, De Jesus and Wurm 2009). With the techniques employed nowadays, the improvements in media composition, and bioprocess developments, volumetric productivity can be achieved up to 1–5 g L^{-1} (Wurm 2004).

Transgenic plants as bioreactors have also been subject of study, because they are easy and cheap to grow and are able to express foreign proteins at high levels. Thus, they are a promising biofactory systems for HDPs. Advantages for the use of plant-based systems include their low costs of production compared to microbial and mammalian cell culture systems, minimum risks of product contamination with endotoxins or human pathogens and the mass-scale production of recombinant proteins, among others.

Several vaccine antigens and therapeutic proteins have been expressed in plants, specifically by integrating them in their chloroplast genomes (Arlen *et al.* 2007, 2008; Farran *et al.* 2008; Lee *et al.* 2011). An advantage of the use of chloroplast, for example in the case of the tobacco plant, is the possibility of scaling up the production to up to 40 metric tons of biomass $acre^{-1}$ per year, leading to potential yields up to 2 kg of peptides, enough for preclinical or clinical studies (Lee *et al.* 2011). An alternative to the chloroplasts is the use of seeds as natural warehouse for proteins. Rice seeds have been considered good bio-factories due to their high yields (up to 2 metric tons $acre^{-1}$ per year) and their easy handle (Stoger *et al.* 2005). Thus, the production of cecropin A, a linear and cationic antimicrobial peptide from insects, has been recently reported using rice seeds as storage (Bundo *et al.* 2014). These authors showed that the use of rice endosperm for production and accumulation of the peptide had no impact on seeds viability and presented an accumulation ranging from 10 to 100 ng per seed (Bundo *et al.* 2014). As an additional advantage of this approach, the cecropin A produced in these plants conferred them resistance against bacterial and fungal pathogens, which has additional implications for plant protection (Bundo *et al.* 2014; Merlin *et al.* 2014). In summary, plants are promising bio-factories systems for HDPs, they have successfully been used for the production of different proteins for therapeutic applications, and the results obtained indicate that they can sustain the production of HDPs and potentially turn it into an optimized large-scale purification method for this aim (Merlin *et al.* 2014).

CONCLUDING REMARKS

HDPs involve a wide group of potent and broad-spectrum antimicrobial molecules that present a number of attractive properties. Other than their broad-spectrum and direct activity against Gram-negative and Gram-positive bacteria (mostly hBD and HNP), virus (HD and HNP) and fungi (mostly hBD and LL-37), HDPs can exert immunomodulatory, wound healing and adjuvant functions. They can control infection and inflammation, present chemotactic signals for immune cells, by inducing cytokines and chemokines secretion and can neutralize exotoxins and endotoxins. In this way, IDRs, defensins derivatives lacking direct antimicrobial activity but able to induct immune response of the host, have emerged as potential new tools to fight against infections. In any case, these new strategies also present the advantage of generating low propensity to microbial adaptation, probably because of their non-specific mechanisms of action. This became a key factor considering the current emergency alert regarding the lack of therapeutic alternatives for MDR bacteria and viruses. Thus, the potential clinical use of HDPs and their derivatives represents a promising strategy for the treatment of infections caused by MDR bacteria and viruses. This fact is clearly reflected in the exponential growth in the number of research studies for the discovering of HDPs in recent years and in the interest of several companies worldwide on their development.

Unfortunately, some disadvantages including stability, susceptibility to proteolysis, low activity in physiological conditions, and high cost of production must be circumvented before these fascinating peptides can be introduced in the clinical practice. The highest *in vitro* direct antimicrobial activities of these HDPs are only achieved, in most of the cases, in absence of serum, which generates doubts as to whether their activity *in vivo* follows a microbial-directed or a host-directed mechanism. It seems mandatory to understand the structure-based activity of these peptides, to be able to isolate the function in charge of their reported direct antimicrobial activities and to design new derivatives with improved pharmacokinetics parameters. In the same way, the wide number of different physiological functions exerted by these HDPs has to be fully characterized to prevent future adverse side effects of their use as antimicrobial drugs. Some of these issues have been already improved and continuous work is ongoing to solve those still unsatisfactory issues with the aim of making HDPs viable therapeutic alternatives in the near future.

Although showing very low stability due to the presence of L-amino acids in their structure, IDR peptides show great potential because their activity is directed to the launch of a state of protection rather than acting directly on pathogens. Also, other advantages of the use of IDRs are the probable reduction in the emergence of resistance and, in addition, their potential use together with common antimicrobial drugs because of the observed additive effect when used in combination.

With the probability that these HDPs reach the market, it would be desirable not to repeat the same mistake we have made in the past with traditional antimicrobial drugs, that is to say, we must make a better use of these new generation of antimicrobial drugs to prevent the admittedly unlikely possibility of the emergence of resistant microorganisms due to the permanent exposure to HDPs.

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