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The human cathelicidin LL-37 — A pore-forming antibacterial peptide and host-cell modulator☆

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ABSTRACT

The human cathelicidin hCAP18/LL-37 has become a paradigm for the pleiotropic roles of peptides in host defence. It has a remarkably wide functional repertoire that includes direct antimicrobial activities against various types of microorganisms, the role of 'alarmin' that helps to orchestrate the immune response to infection, the capacity to locally modulate inflammation both enhancing it to aid in combating infection and limiting it to prevent damage to infected tissues, the promotion of angiogenesis and wound healing, and possibly also the elimination of abnormal cells. LL-37 manages to carry out all its reported activities with a small and simple, amphipathic, helical structure. In this review we consider how different aspects of its primary and secondary structures, as well as its marked tendency to form oligomers under physiological solution conditions and then bind to molecular surfaces as such, explain some of its cytotoxic and immunomodulatory effects. We consider its modes of interaction with bacterial membranes and capacity to act as a pore-forming toxin directed by our organism against bacterial cells, contrasting this with the mode of action of related peptides from other species. We also consider its different membrane-dependent effects on our own cells, which underlie many of its other activities in host defence. This article is part of a Special Issue entitled: Pore-Forming Toxins edited by Mauro Dalla Serra and Franco Gambale.

1. Introduction

Cathelicidins are a family of vertebrate host defence peptides (HDPs) defined by the conserved nature of their pro-region rather than to the sequence or structure of the antimicrobial peptides themselves, which are quite variable. It is one of the two principal families of HDPs in mammals, the other one being the defensins [1–3]. The two families differ significantly in the number of expressed peptides (defensins are present in humans as multiple genes, cathelicidin only as one), structure, mode of action and pro-piece, which is much shorter and unstructured in the defensins [4].

Since their discovery in the late '80s, cathelicidins have demonstrated a remarkably wide functional repertoire. They display direct antibiotic activities against bacterial, fungal, viral and parasitic microorganisms. They can act as 'alarmins', helping to orchestrate the immune response to infection. They can modulate inflammation, both enhancing it to aid in combating infection, and limiting it to prevent damage to the host. They can promote wound healing and angiogenesis, and they have also been implicated in elimination of abnormal cells [2,5–9]. Strictly speaking, the term cathelicidin indicates the pro-form [10], while the active HDP is variously indicated by size and sequence features (e.g. human LL-37 starts with two Leu residues and is 37 residues long), by size and provenance (e.g. its bovine orthologue BMAP-34 stands for Bovine Myeloid Antimicrobial Peptide of 34 residues), by provenance and pro-form (e.g. mouse CRAMP stands for Cathelin Related AMP) or by various other features (see [11]). However, it has become customary to refer to the active HDPs as cathelicidins as well.

hCAP18 is the only human cathelicidin. The HDP it releases, LL-37, has been extensively studied since its discovery in 1995, and is a paradigm for the multiple roles of cathelicidin peptides in host defence. As a consequence, the literature is vast and the interested reader is referred

Abbreviations: AFM, atomic force microscopy; AMP, antimicrobial peptide; ATR, attenuated total reflection; BF, biofilm; CD, circular dichroism; CLD, cathelin-like domain; Ch, cholesterol; CREB, cAMP responsive element; D8PG, dioctanoylphosphatidylglycerol; DPC, dodecylphosphocholine; dPG, di-phosphatidylglycerol; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-*rac*-(3-lysyl(1-glycerol)); EGFR, epidermal growth factor receptor; FITC, fluorescein isothiocyanate; FPLC, fast protein liquid chromatography; FPLR, formyl-peptide-like receptor, GPCR, G protein-coupled receptor; hCAP18, human 18 kDa cathelicidin antimicrobial protein; HDP, host defence peptides; LPS, lipopolysaccharide; LTA, lipoteichoic acid; mAb, monoclonal antibody; NET, neutrophil extracellular trap; PAMPs, pathogen associated molecular patterns; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, propidium iodide; SDS, sodium dodecylsulphate; SM, sphingomyelin; SOPC, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; SOPG, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol); SPR, surface plasmon resonance; TLR, toll-like receptor; VDR, vitamin D receptor; VDRE, vitamin D response element; WTA, wall teichoic acid.

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to several comprehensive reviews on different aspects of its structure, mode of action and biological functions [3,7,12–23]. The multiplicity of reported functions is reflected in the adjectives often used to qualify LL-37 — 'multifunctional', 'multifaceted', 'pleiotropic', even '*factotum*'. It manages to carry out all its reported activities with only 37 residues arranged in a simple, linear, amphipathic, helical structure, which spans most of its sequence. In this review our intent is to consider how different aspects of its primary and secondary structures, as well as its marked tendency to form oligomers and bind to other molecules or surfaces as such, may explain some of its cytotoxic and immunomodulatory effects. We consider its modes of interaction with biological membranes and subsequent effects, also contrasting this with the mode of action of its fragments, related peptides from other species and artificial analogues.

2. Discovery of cathelicidins and LL-37

The first papers referring to a cathelicidin (not yet known as such at that time), were by our group in Trieste in 1988 [24,25]. They report on a small, cyclic peptide (first generically referred to as bactenecin, then more specifically as dodecapeptide) isolated from granule extracts of bovine neutrophils. A monoclonal antibody obtained against these extracts was co-reactive to this peptide and a larger, non-bactericidal protein, indicating that it might be produced as an inactive precursor. Two considerably longer, linear and proline-rich antimicrobial peptides, Bac5 and Bac7, as they were also referred to as bactenecins, were subsequently also isolated from a total granule extract of bovine neutrophils. As they had no homology to the dodecapeptide they were thought to be unrelated to it [26,27].

By 1990, using anti-peptide antibodies, it had been determined that bactenecins were synthesized in immature bone marrow cells of the myeloid lineage as prepro-forms, targeted to the so called large granules of bovine neutrophils [28]. Biosynthesis occurred at the myelocyte stage, when large granules are assembled, and was then switched off with further myeloid differentiation. Processing was simple and fast, and the active mature HDPs appeared to be released from the pro-form by a neutral serine protease. It was proposed that the pro-part might contain sequences important for sorting and intracellular transport to granules, and also possess toxicity-neutralizing properties, as the pro-forms were inactive. It was then conclusively shown [29,30] that these peptides were *i*) present in the mature neutrophil granules only as pro-forms from which they were released by proteolytic cleavage, *ii*) that the pro-sequence indeed masked the activity of the mature peptide; *iii*) that this provided a temporal coupling between discharge and their activation, *iv*) that release and concomitant activation of the pro-form in phagosomes were early events after phagocytosis, and *v*) that the serine protease responsible for release was elastase. At about this time, a proline-rich peptide, PR-39, was also reported from pig [31].

An attempt was then made to clone the pro-form of Bac5 via a modified RACE protocol, using degenerate primers based on the sequence of the HDP in a primer extension analysis to complete to the 5′-end, and then using primers from this region to extend back to the 3′-end [32]. This resulted in a series of surprises. The 5′ end was very similar to that of rabbit CAP18, a protein with antimicrobial and LPS-binding activity just then reported, which carried an amphipathic helical peptide at its C-terminus [33], and also had significant sequence identity to pig cathelin, a putative inhibitor of the protease cathepsin L [34]. The RACE strategy led to identifying homologous pro-regions also for the dodecapeptide [35], the Trp-rich bovine AMP indolicidin [36,37] and porcine PR-39 [38], and there soon followed a spate of other porcine and bovine HDPs with quite variable structures, all linked to a cathelin-like domain (CLD) (see [10]), as well as a human homologue.

Three different groups independently reported the human cathelicidin, hCAP18, in 1995. One used PCR probes based on porcine PR-39, and identified the pro-form of a peptide they called FALL-39 [39]. The second went looking for the human equivalent of LPS-binding CAP18, using oligonucleotide probes based on the rabbit sequence [40], while the third directly isolated a 19 kDa protein from the specific granules of human neutrophils and then isolated its cDNA from a chronic myeloid leukaemia library [41].

The human cathelicidin HDP, LL-37, an amphipathic α-helical peptide, had a quite medium-sensitive antimicrobial activity [42] and, unusually, the capacity to adopt this structure also in aqueous solutions at physiological salt concentrations [43]. Most other helical AMPs only do so in the presence of biological membranes. It also had a significant capacity as a chemotactic agent for different types of immune cells, could stimulate release of pro-inflammatory chemokines and cytokines [5], could repress the effects of pathogen associated molecular patterns (PAMPs) such as LPS and LTA [44–46] and could stimulate propagation of some cells involved in healing processes [47,48] — it was a truly pleiotropic host defence peptide. Later studies indicated that these capacities likely depend on its particular structural characteristics.

3. Evolution, expression and features of the pro-region

3.1. Evolution

As mentioned in the previous section, cathelicidin peptides were initially identified as apparently unrelated HDPs, and each was brought into the cathelicidin fold only when the sequence of the pro-region was eventually determined. Given the widely different structures of some of these peptides, and broad species distribution, it sometimes came as quite a surprise. An example are curious helical peptides from the hagfish (*myxini*), a basal vertebrate, containing the unusual modified residue bromo-tryptophan [49]. Some years after their discovery, cloning and sequencing of the cDNA unexpectedly revealed them to be linked to a CLD, suggesting that the cathelicidin family was quite widespread among vertebrates and therefore ancient [50]. Several other members of the family were identified in horse, dog, rodents, birds, fish, amphibians and reptiles [51–57]. It has been suggested that cathelicidins evolved in vertebrates from cystatins, as they share significant structural similarity [58].

In a recent review, 148 database entries for cathelicidins were reported from 31 vertebrate species [7]. We have been keeping track of cathelicidins in protein and annotated or unassembled nucleotide sequence databases and have found evidence for them in 133 vertebrate species to date. They are present in lampreys, another basal vertebrate, numerous fish, amphibian and reptile species, birds, and placental and non-placental mammals (see Fig. 1), confirming their role as ancient components of vertebrate immunity. Analysis of the C-terminal HDP domains, when available, suggests that an orthologue of human LL-37 is present in all placental mammals, and indeed this is often the only cathelicidin present (e.g. in primates, glires and other rodents, as well as carnivores). Such an orthologue has not yet been identified in non-placental mammals or non-mammalian vertebrates.

LL-37 orthologues are relatively easy to identify due to a few characteristic sequence features that are conserved, as indicated in Fig. 2A and B. A conserved pattern of hydrophobic and polar residues should contribute to the formation of a long amphipathic helix in all the putative HDPs. Furthermore, a few segments of the sequence are conserved down to the amino acid level (Fig. 2A, shaded grey). In particular, most orthologues of LL-37 have a proline residue close to the C-terminus, often preceding an arginine, which results in the helix ending with a disordered and a polar tail. In orthologues where this feature is missing, a codon for proline can usually be found at the expected position in the 3′-UTR, following premature stop codons that were introduced during the peptides' evolution (e.g. in cetartiodactyls). Considering over 70 likely orthologous sequences, the length of the putative HDPs varies little, with only few being longer than 40 or shorter than 34 (see Fig. 2C). Furthermore, the conservation of hydrophobic

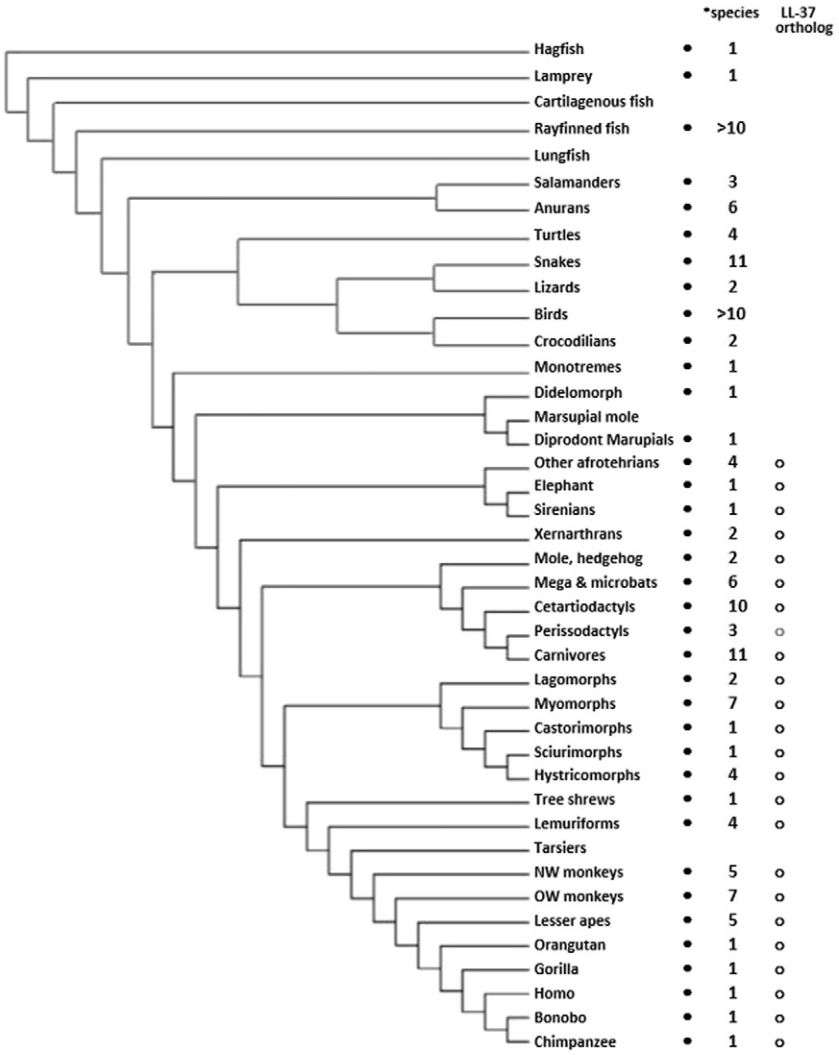

Fig. 1. Vertebrate animal groups in which cathelicidins have been identified, on a cladogram showing phylogenetic relationships in a reverse order from *homo* (according to [261]). Cathelicidin sequences were found in protein or nucleotide sequence databases for 133 species of vertebrates, of which 85 mammalian species and 81 placental mammals. ● indicates that cathelicidins have been identified, and next to it in what number of species within that group. Note that more than one cathelicidin may be present per species. The absence of cathelicidins only indicates that they have not yet been identified. ○ indicates the presence of a cathelicidin that, based on the C-terminal HDP domain, is a likely orthologue of LL-37 (perissodactyls are the least certain assignment).

and polar residues at specific positions suggests that they all adopt a helical conformation with a remarkably well conserved amphipathicity.

On the other hand, the interchange of cationic, anionic and neutral polar residues at certain positions (in particular 11, 15, 18 and 29 by LL-37 numbering) results in a remarkable variation in charge (Fig. 2D, left), with almost half the residues being cationic in a few highly charged orthologues, while these are outnumbered by neutral and anionic residues in a few others with particularly low charge. In any case, the most frequent charge is +6, as in LL-37. This pattern follows and widens that previously observed for the variation of LL-37 orthologues in other primates [59]. All this indicates that LL-37 and its orthologues in eutherians are under positive selection for variation in charge, but purifying selection for size and amphipathicity (to be published).

On helix formation, charged residues cluster on one side of the structure, so that the side-chains of those separated by 3 or 4 positions in the sequence are adjacent to each other and can engage in electrostatic attraction or repulsion. This was early on reported as a determining factor affecting the propensity for helix formation in LL-37, and driving its oligomerization [43]. The attraction/repulsion ratio varies considerably among primate orthologues, with the peptides from human and closely related species (great and lesser apes) having an excess of attractions over repulsions, while those from more distant ones, such as rhesus or presbytis RL-37, have an equal or greater number of repulsions [59]. Extending these considerations to other mammalian orthologues (Fig. 2D, right), the same pattern is evident, with several showing an excess of attractions, and likely an augmented propensity for helix formation, while several others show an excess of repulsions.

From a preliminary extended screening cathelicidins it thus appears that *a*) all placental mammals have an orthologue of LL-37, underlining its importance as an eutherian innate immune effector; *b*) these evolved to have a very wide range of net charges, but a limited range of size and amphipathicity; *c*) some orthologues, and not just in closely related ape species, have a charge distribution more akin to the human peptide (excess of intramolecular electrostatic attractions) while others more like those of monkey RL-37 (excess of intramolecular electrostatic repulsions); and *d*) this likely affects their structuring, oligomerization and modes of interaction with biological membranes, and consequently reflects on their biological functions (see below).

Within the widely dispersed cathelicidin family, which is defined by the conserved pro-region, there is therefore a subfamily of peptides in all eutherian animals that are related by the HDP as well. This type of cathelicidin is invariably present, and quite often is the only one, so it might be considered as the stem eutherian cathelicidin.

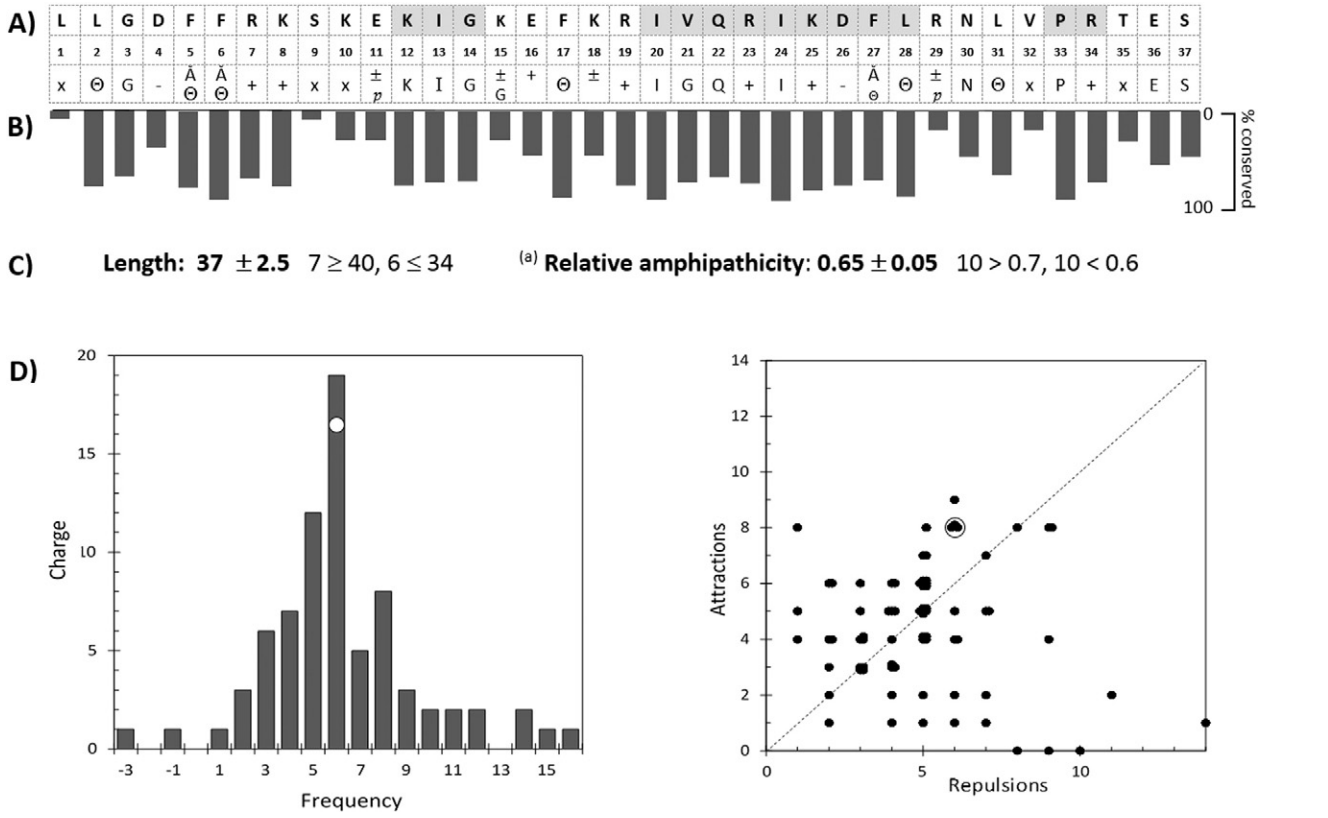

Fig. 2. Primary structural features of LL-37 orthologues. A) Sequence of LL-37, typical sequence features highly conserved in its orthologues are shaded grey; B) conservation of residues (indicated) or residue types, among orthologues (x = variable, Θ = hydrophobic, Å = aromatic, +/− = cationic/anionic, *p* = polar). C) Size and amphipathicity. [(a)]The hydrophobic moment was determined as described previously ([262]), using the CCS scale and considering only the sequence up to the characteristic shaded Pro residue close to the C-terminal. 76 peptides from different placental mammalian species were analysed. The relative amphipathicity was determined by relating this moment to that of a perfectly amphipathic, 18 residue helix. D) Charge distribution (left) and interhelix side-chain attraction/repulsion plot (right). The charge was determined by summing Lys and Arg residues (not His) and then subtracting Glu and Asp residues. Attractions were determined counting Arg/Lys residues spaced $i + 3$ or $i + 4$ apart from Glu/Asp residues, while repulsions counted Arg/Lys residues spaced in that manner from other Arg/Lys, or Glu/Asp spaced in that manner from other Glu/Asp. Charges at the N- and C-terminals were not taken into account. The white circle indicates where LL-37 belongs.

3.2. Expression

hCAP18 is encoded by the *CAMP* gene (for Cathelicidin *AMP*), located at locus p21 of chromosome 3, and is transcribed as a prepro-form with a signal sequence directing it for storage in the granules of some cells, or to be secreted from others. The gene is composed of 4 exons, of which the first three code for the prepro-region and the fourth for the antimicrobial domain (Fig. 3). This arrangement is conserved in vertebrate cathelicidin genes [58], and the human gene maps to a region of conserved synteny with those for cathelicidin genes in pig, bovids, mouse and dog [51,60–63].

The human cathelicidin is widely expressed in numerous types of epithelial cells [64–73] and immune cells (neutrophils, monocytes, macrophages, dendritic cells, NK cells, lymphocytes and mast cells) [13,74–80]. These cells are either in direct contact with the exterior environment or operating at sites of infection, underscoring the importance of LL-37 to host defence. The pattern of expression is quite varied and complex [2,21,81,82], is regulated differently in different cell types, and can be stimulated by both exogenous microbial components or endogenous signal molecules [83]. Different PAMPs such as LPS/LTA or mycobacterial DNA up-regulate its expression in sinus and lung epithelia [82,84], whereas butyrate, a product of intestinal flora, does so in gastrointestinal cells [83,85]. Like other HDPs, induction/release can occur in direct response to stimulation of pattern recognition receptors, such as the toll-like receptors (TLRs) [79,86], or indirectly in response to cytokines released as a consequence of TLR stimulation. PAMP-mediated induction of LL-37 however appears to be secondary to induction by endogenous factors [2].

Vitamin D3 is a potent endogenous inducer of hCAP18 in various cell types [87–92], as the *CAMP* gene promoter sequence contains vitamin D response elements (VDRE) for the vitamin D receptor (VDR), a transcription factor belonging to the steroid/hormone receptor family [88]. This is an evolutionarily recent form of regulation, as it is primate-specific, and likely derives from the exaptation of an ancient Alu short interspersed element [91]. It may be particularly useful to us as diurnal and hairless apes. In human monocytes and macrophages, activation of TLR1/2 by a mycobacterial component activates a signalling cascade that results in the up-regulation of expression for both the VDR and the hydrolase Cyp27B1, which converts the inactive pro-vitamin D to the active form [93]. Expression of hCAP18 also increases, but because it is a downstream target of activated VDR and not as a direct consequence of TLR activation. More generally, there is evidence that vitamin D modulates circulating levels of hCAP18 [92] and that it plays a significant role in enhancing antimicrobial defence at epithelial surfaces, especially for keratinocytes in airways or skin [89].

Another stimulus for cathelicidin expression in humans is endoplasmic reticulum stress signalling following damage to epithelial cells [94]. It is independent of vitamin D-induced VDR activation, and is physiologically useful in the context of the innate immune response to injury and infection, as these can cause ER stress. Induction of cathelicidin by butyrate is also independent of vitamin D induction, and occurs in a cell-specific manner [66]. It mainly concerns colonic epithelia, a district where these short-chain fatty acids are produced by commensal flora, but has no effect on keratinocytes or monocytes. Conversely vitamin D has a strong inducing effect on the latter cells but not on colonic epithelia [83]. hCAP18 induction is also stimulated by the hypoxic conditions

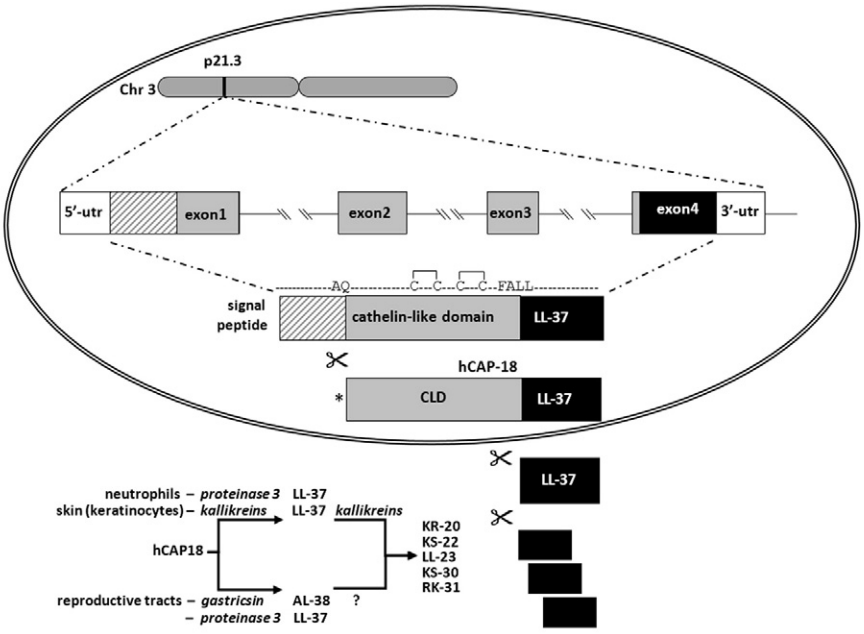

Fig. 3. Schematic representation of the human *CAMP* gene and its products hCAP18 and LL-37. The gene is located on Chr3 p21.3 and its transcript has 4 exons and three introns. Processing to the pro-form hCAP18, with removal of the signal sequence and conversion of N-terminal Gln to pyroglutamic acid (*) occurs before storage in leukocyte granules, or secretion by epithelial cells. The mature HDP, LL-37, is released by proteinase 3, kallikreins or gastricsin, depending on the district, only after extracellular secretion. It is then further hydrolysed by kallikreins in sweat to fragments that can be as, or more, active than the parent HDP. In the reproductive tract, proteolysis is due either to gastricsin from seminal fluid, activated by a drop of pH in the vagina, or by proteinase 3 brought there by granulocytes (adapted from [68,73,100,102,117,263]).

present in some skin regions [95]. These conditions upregulate hypoxia-inducible factor-1α (HIF-1α) that binds to the nuclear translocator receptor HIF-1β to activate hypoxic response elements in target gene regulatory sequences. Along with genes implicated in control of metabolism, angiogenesis, apoptosis and cell stress responses, it increases expression of the *CAMP* gene during infection.

hCAP18 expression can be down-regulated by pathogens such as *Shigella* and *Neisseria*, as well as by some bacterial exotoxins [96–98], and this is connected to bacterial virulence, favouring invasion and subsequent infection. It is reported that induction of LL-37 by some bacterial components acts via a cAMP mediated pathway leading its responsive elements CREB and activator protein-1 (AP-1) to bind to promoter sequences in the cathelicidin gene [99], and that the bacterial exotoxins interfere with this process. Conversely, its expression can be upregulated in some inflammatory and autoimmune diseases [2,23], and it then attracts inflammatory cells and stimulates pro-inflammatory cytokine release, exacerbating these diseases.

3.3. Processing, structure and (uncertain) functions of the pro-form

The *CAMP* gene product is directed either to storage granules or is secreted, and the signal peptide responsible for this trafficking is removed by the signal peptidase [100]. The Gln residue at the N-terminus of the pro-form, which is conserved in cathelicidins from other species, is then converted to pyroglutamic acid [101]. It is not reported how LL-37 is released from the pro-form for cells that do not store it, but this is likely to occur extracellularly. In granulocytes, hCAP18 apparently is not processed intracellularly, but only when released extracellularly and specifically by proteinase-3 at the suitable Ala-Leu cleavage site [102] (see Fig. 3). hCAP18 secreted from eccrine glands and keratinocytes [73,103] is instead processed by kallikrein serine proteases, which first release LL-37 and then continue to produce shorter fragments that initially maintain or increase antimicrobial activity while losing the pro-inflammatory and chemotactic capacities of the parent peptide. This process is modulated by secreted leukoprotease inhibitor/SLPI, whereas this does not affect protease-3 processing of hCAP18 from granulocytes at mucosal epithelial surfaces [102,103]. Processing occurs differently in seminal plasma, where hCAP18 is released from epithelial cells of the epididymis and joins with gastricsin produced by the prostatic gland on seminal vesicles only on ejaculation. Processing to a slightly longer active peptide, AL-38, occurs only after gastricsin is activated by acidification of the vagina, providing another example of temporal control of activation [68].

Remarkably similar 3D structures have been determined for the cathelin-like domain (CLD) of porcine protegrin and human CAP18 (PDB IDs: 4EYC and 1KWI) consistent with the high sequence conservation [104,105]. In both cases the structure of the HDPs has also been determined separately, but how the CLD and HDP relate when still attached in the pro-peptide can only be inferred by modelling [106]. The crystal structure of the human CLD is shown in Fig. 4 together with the NMR structure of the mature peptide in the presence of lipid micelles. In the CLD structure, a concave β-sheet platform, stabilized by two conserved disulphide bridges, nestles a long helical segment at the N-terminus. It adopts a cystatin-like fold despite a significant sequence divergence from cystatins, but lacks structural elements required for cysteine protease inhibition [105]. The structure of LL-37 has been obtained in the presence of both anionic (SDS and D8PG) (PDB ID: 2K6O) and neutral (DPC) micelles [107,108]. In both cases, the helix is bent, possibly due to the curvature of the micelles, with a more-or-less pronounced kink in the region around Gly^{14} (see Fig. 4C) (see below), and adopts an amphipathic conformation with a narrow and well segregated hydrophobic face. The C-terminus is disordered while the N-terminus is more ordered, especially in the presence of the anionic lipids. It is not known if the peptide adopts a helical conformation also when attached to the pro-region, but the same considerations should apply as in bulk solution.

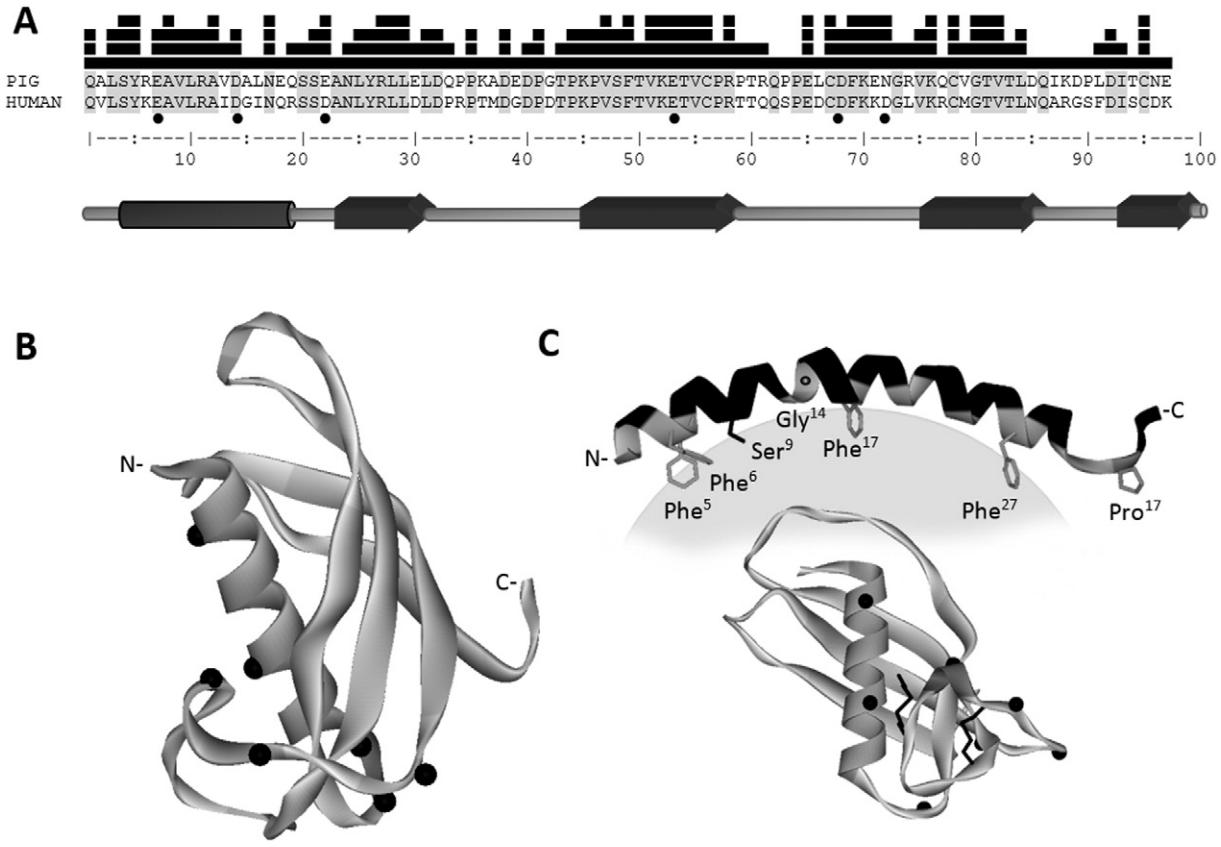

Fig. 4. Structure of the human cathelicidin pro-region and of LL-37. A) Sequence of the hCAP18 and protegrin CLDs (conserved residues are shaded grey). The relative frequency of residues from the human sequence among ~100 eutherian cathelicidin pro-regions, is shown above the alignment (■ <60%, ■■ 60–80%, ■■■ 80–90%, ■■■■ >90%. A topological diagram for the structure is shown below the sequence, indicating the position of the long α-helix and β-strands. B) Structure of the hCAP18 CLD (PDB ID: 4EYC) [105]; C) Structure of LL-37 in the presence of SDS or DOPG micelles (schematically indicated as a grey crescent) (PDB ID: 2K6O) [107]. Another view of the pro-domain with disulphide bridges is shown below this structure. The black spheres indicate the position of conserved anionic residues, corresponding to sequence positions indicated with black dots.

The ample research carried out on LL-37 has resulted in a relatively detailed understanding of its pleiotropic action and of some of the underlying mechanisms, but surprisingly little is known about the function/s of the pro-region [105]. As described in Section 2, it was early proposed that it served to keep the peptides inactive until needed [74,109], but this proposal, while amply accepted, cannot on its own justify the high level of conservation of the CLD. It has also been suggested that the CLD might have its own antimicrobial activity, to complement that of the mature HDP [110]. Another proposal, based on its homology with pig cathelin, was that the CLD acted as a cathepsin inhibitor [111]. However, it was subsequently shown that cathelin's inhibitory activity was likely due to contaminants [112], and while it initially appeared that one bovine cathelicidin inhibited cathepsin [113], a later study on several bovine pro-forms suggested that the activity was too weak to be biologically relevant [114]. Furthermore, as mentioned above, the CLD lacks key structural features necessary for cathepsin inhibition. Uncertainty as to the antibacterial and inhibitory activity of the pro-region is increased by the fact that different studies come to conflicting conclusions. One indicates that the CLD, but not hCAP18, is both antibacterial and a cathepsin inhibitor, while another that hCAP18, but not the CLD is antibacterial, and that neither the CLD or hCAP18 was inhibitory [105,110].

An interesting hypothesis is that the CLD could be a presentation platform for controlled release of the HDP by an appropriate protease, at the right time and in the right place. It has been shown that ProS-PG (the protegrin pro-form) changes its conformation in a pH-dependent manner, and this has led to a model of how this might affect activation/release of PG from its CLD (ProS) [104]. Protegrin is a small β-hairpin peptide, stabilized by two disulphide bonds, so it can associate with and extend the CLD β-sheet. LL-37, on the other hand, as a long helical peptide would be incapable of doing this. The model also proposes that disengagement involves breaking electrostatic interactions, but it has been pointed out that the CLD is almost neutral and so unlikely to engage in strong electrostatic interactions with the HDP region [105]. However, there are a number of relatively well conserved anionic residues in the CLD sequence (indicated by dots in Fig. 4A) that form a strip along one side of its structure (indicated by spheres in Fig. 4B), and which might allow relevant interactions with the HDP domain, so positioning the loop between the CLD and HDP in a manner appropriate for proteolysis.

This hypothesis fits with observations as to what happens to hCAP18 on its release from granulocytes. Studies using a specific mAb directed against hCAP18 have shown that on degranulation and exocytosis a substantial fraction (about a third) remains intact and bound to the external side of the plasma membrane [101]. This suggests a capacity of hCAP18 for membrane interaction that may involve electrostatic attraction and bilayer insertion, possibly mediated by binding to membrane proteins. hCAP18 released into the extracellular medium, as well as LL-37 proteolytically released from it, are rapidly sequestered by plasma apolipoproteins [87,115,116], whereas hCAP18 remaining on the surface of granulocytes accompanies them to the sites of infection. This could confer a spatial specificity to hCAP18 activation by extracellular proteases, concentrating antimicrobial action at the granulocyte surface and allowing inactivation of microbes while minimizing damage to surrounding tissues [101].

A similar scenario presents itself in the reproductive tract during insemination. hCAP18 is constitutively expressed by the epithelium lining the epididymis [69] and concentrates in prostatosomes, small surface-bound organelles that are expelled into seminal plasma where they can fuse with sperm and deliver hCAP18 to it [68]. It then remains

unprocessed on the surface of sperm cells until prostate-derived gastricsin, also present in seminal plasma, is activated by acidic conditions in the vagina [117]. Here hCAP18 would also meet with proteinase-3 released by infiltrating granulocytes. These proteases respectively release active AL-38 and LL-37 to protect the genital tract from infection. This example of spatial and temporal control is quite relevant, as sperm cells are poor in cholesterol [118] and therefore would be particularly susceptible to the cytotoxic effects of LL-37 should it be prematurely released.

4. Mode of action of LL-37 and related peptides

LL-37 is a linear peptide with an excess of basic residues (5 Arg, 6 Lys) over the acidic ones (3 Glu, 2 Asp) conferring a net charge of +6 under physiological conditions (pH 7.0). About 35% of residues are hydrophobic so that it adopts an amphipathic helical structure with a narrow but well-defined hydrophobic sector (see Fig. 4C). All these are essential features for both its oligomerization in aqueous salt solutions, and for peptide–membrane interactions.

4.1. Structure of LL-37 in bulk solution and its relevance to membrane interaction

From the first structural characterization studies it emerged that LL-37 is disordered in aqueous solutions but promptly adopts a helical conformation in the presence of physiological concentrations of certain salt ions, in a manner that is anion, pH and concentration dependent [43]. Interaction with membranes further stabilizes this conformation, as indicated by an increased helical content [43,59]. The capacity of LL-37 to adopt a partially helical structure in the absence of membranes is uncommon among helical AMPs, which usually only adopt this conformation when they insert into the lipid layer of anionic membranes [119]. Folding of LL-37 in physiological solutions obliges it to either oligomerize to shield the hydrophobic surface that forms [43,120,121,122], or to interact with the hydrophobic surface of other molecules present in its environment (e.g. in culture medium or serum) with repercussions on its activity [115,123–125]. It also affects its mode of interaction with biological membranes [122,126–128].

Helical structuring in aqueous salt solutions depends on the formation of a net of intramolecular salt bridges between acidic and basic residues distributed over the whole peptide sequence and spaced $i + 3$ or $i + 4$ apart [43]. For human LL-37, as well as for closely related ape orthologues, attractions prevail over repulsions, whereas they are balanced with or exceeded by repulsions in more distantly related monkey orthologues [59,123]. This pattern seems to recur also in other placental species (see Section 3.1). An excess of intramolecular attractions, together with intermolecular electrostatic and H-bonding attractions [128], drives LL-37 self-association into oligomeric bundles. In this way, the narrow hydrophobic surface of the amphipathic helix is partly shielded from the aqueous environment, and this contributes to oligomerization via the hydrophobic effect. It is likely that all these interactions, intra- and inter-molecular salt-bridging, H-bond formation and hydrophobic effect need to act in a sequential and concerted manner, which in turn requires a precise sequence arrangement, for structuring and oligomerization to occur. Primate orthologues such as *rhesus* or *presbytis* RL-37, with a less favourable salt-bridging arrangement, do not adopt a helical conformation or oligomerize in aqueous salt solutions, but likely form monomeric, globular coils to mask their hydrophobic residues from the aqueous environment [59,122–124].

Structuring and oligomerization reduce the capacity of LL-37 to reach and permeabilize bacterial membranes, as it makes it more rigid and rather 'sticky' by favouring interactions with any molecular surface offering appropriate hydrophobic, electrostatic and H-bonding characteristics. It is most likely for this reason that its antimicrobial activity is notoriously medium dependent. Different studies have thus been undertaken in order to determine: *i*) which factors lead LL-37 to self-assembly; *ii*) how different peptide regions are involved in stabilizing the oligomeric form, *iii*) how many peptide molecules participate in the formation of oligomers and *iv*) if these persist in the presence of bacterial or host-cell membranes.

Johansson et al. first recognized that oligomerization occurred by the concentration dependence of helix formation in CD studies [43], while its pH dependence suggested that salt-bridging was involved. Oren and co-workers then confirmed, through chemical cross-linking experiments, that LL-37 is in equilibrium between the monomeric, dimeric and trimeric forms, depending on peptide concentrations [120], while Li et al. estimated from NMR experiments and FPLC elution times that it could form up to tetramers [121]. Recent studies by our group, in which residue Phe^5 in the LL-37 sequence was substituted with a photoactive 4-benzoyl-L-phenylalanine (a benzophenone analogue) showed that up to covalently linked hexamers can form at higher peptide concentrations [122]. Oligomerization is also indicated by the relative intensities of the double minima that are typical in the CD spectra of α-helices. In lone helices the minimum at ~208 nm tends to dominate over that at 222 nm, whereas in stacked helices the opposite occurs (i.e. $\theta^{208}/\theta^{222}$ goes from >1 to ≤1) [129]. LL-37 spectra in aqueous salt solutions show the latter behaviour [39,43,130].

The involvement of different peptide regions in the formation of the oligomeric state of LL-37 has been extensively investigated [13]. Oren et al. suggested that N-terminal residues are important for oligomerization [120]. Our group confirmed the role of the N-terminal region in initiating dimerization by using parallel and antiparallel covalent dimers of LL-37 in which cysteine residues had been introduced at the N- or C-termini [122]. The N-terminally linked parallel dimer showed a particularly high propensity for helical structuring, even in aqueous solution without salts, suggesting that dimerization starts in this region. By systematically replacing Phe residues in positions 5, 17 and 27 of LL-37 with the fluorescent and IR probe p-cyanophenylalanine [128], we were then able to show that on oligomerization, Phe residues in the central and the C-terminal regions contributed as much, if not more, to the associated form of the peptide as the Phe residues in the N-terminal region.

The oligomeric form of LL-37 is reported to persist in the presence of neutral membranes [120,126], whereas there are conflicting reports on its behaviour in the presence of anionic membranes. Oren et al. suggested that it reverts to a monomeric form in the presence of artificial membranes composed of phosphatidylcholine and phosphatidylserine (PC/PS) [120]. Li et al., using NMR spectroscopy, also observed disaggregation in the presence of D8PG micelles [121]. However, extensive amyloid-like fibrils have been observed by microscopy in the presence of SOPC/SOPG liposomes, suggesting a high level of aggregation occurring with time [128]. Photo-cross-linking studies carried out in the presence of negatively charged PG/dPG liposomes or neutral PC/SM/Ch liposomes showed that LL-37 interacts with both membrane types in an oligomeric form, although the extent of oligomerization decreased with respect to aqueous salt solutions, and more for anionic than neutral membranes [122]. Membrane oligomerization is also confirmed by the $\theta^{208}/\theta^{222}$ ratio in CD spectra, (which tends to be ≤1 in the presence of both types of membranes) and Surface Plasmon Resonance (SPR) that indicate a significant level of LL-37 aggregation on membrane surfaces at higher peptide concentrations [128]. Conversely, rhesus macaque *mmu*RL-37 shows little helix formation in CD studies with neutral membranes, a $\theta^{208}/\theta^{222}$ ratio >1 in the presence of anionic ones, and SPR sensorgrams consistent with monomeric membrane insertion (See Fig. 5 for a schematic representation of their differing modes of membrane interaction).

4.2. Interaction with and effect on bacterial membranes

The NMR structure of LL-37 in the presence of anionic lipid micelles shows a continuous and relatively well-ordered α-helical conformation over most of the sequence, with a narrow and well segregated

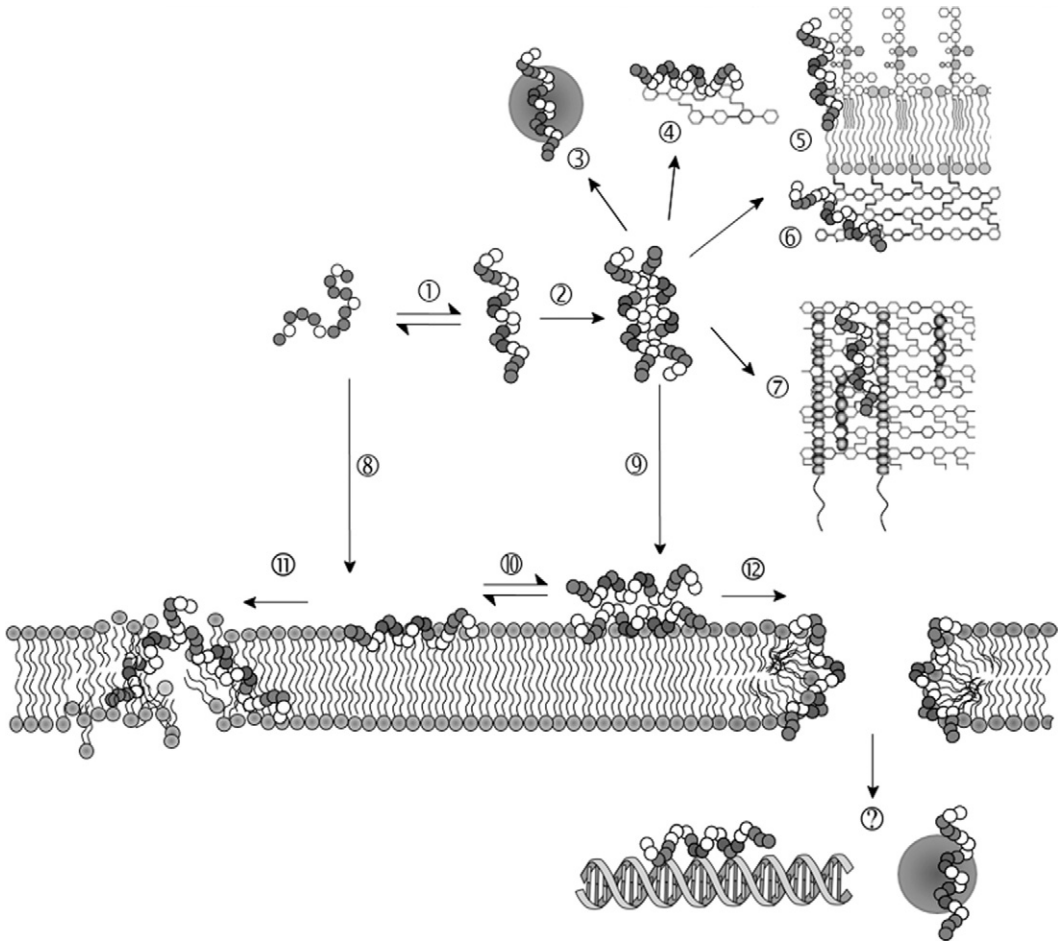

Fig. 5. Antibacterial mode of action of LL-37. In aqueous salt solution, cathelicidin HDPs are in equilibrium between random coil and helical forms (1), with the random coiled form favoured by RL-37 and helical form favoured by LL-37. Structuring obliges the peptide to oligomerize (2) to mask the hydrophobic helix face, or (3) to interact with hydrophobic surfaces of medium or serum components. It may also favour binding with bacterial exopolysaccharides (4), to the outer bacterial cell wall components such as the LPS layer (5) or Gram-negative peptidoglycan layer (6), or the teichoic acids in the Gram-positive peptidoglycan layer (7). These interactions may result in an increased barrier effect towards LL-37 with respect to monomeric RL-37, but may also play a role in halting bacterial growth. Once the peptides translocate to the cytoplasmic membrane surface, RL-37 structures and inserts more deeply into the lipid bilayer as a lone helix (8), while LL-37 binds more on the surface as an oligomer (9), but then may disaggregate to a certain extent (10). Both peptides bind parallel to the membrane surface and RL-37 then rapidly forms small, indeterminate lesions sufficient to allow passage of propidium iodide (∅ 1.6 nm) (11), while LL-37 forms quite sizeable toroidal pores more slowly (12) that eventually allow passage of labelled dextran particles up to 9 nm. These transient pores allow LL-37 to translocate into the bacterium, where it may also interfere with internal targets such as DNA and processes such as transcription.

hydrophobic face and a flexible C-terminus (see Fig. 4C) [107]. The helix is somewhat curved, likely due to the surface curvature of the micelles, with a more-or-less pronounced kink centred around the conserved residues Lys^{12}-Gly^{14} (see Fig. 2), that are preceded and followed by residues that are rather variable in its orthologues. In other primates, positions 15 and 16 are often glycine residues, forming a region of high flexibility [59,107]. In the human peptide, a serine residue at position 9 interrupts the hydrophobic sector into a short N-terminal and a long C-terminal regions (see Fig. 4C), affecting its capacity to completely insert into the membrane lipid layer [128]. In most other primate orthologues Ala or Val replace it, leading to a continuous hydrophobic face. Other relevant sequence positions for peptide–membrane interaction are the four phenylalanine residues spread throughout the sequence, which enhance it. Those in positions 5, 6 and 27 are relatively well conserved in other mammalian orthologues (see Fig. 2). Insertion of these aromatic side-chains into the lipid bilayer has been observed both with NMR and ATR-FTIR spectroscopies and contributes to anchoring the peptide to the membrane [107,128]. Arg^{23}, located at the interface between the hydrophobic and hydrophilic sectors of LL-37, has been shown to interact directly with a phospholipid head-group [128]. It is noteworthy that rhesus RL-37 appears to penetrate more deeply into the bacterial membrane than LL-37, as shown by ATR-FTIR, even though it lacks the central Phe^{17}. The presence of Ser^{9} in LL-37, which interrupts the hydrophobic face, prevents it from penetrating as deeply into the acyl bilayer.

LL-37 was originally identified for its direct antibacterial activity *in vitro*, so that particular attention has been paid to its mechanism for interacting with bacterial membranes and subsequently permeabilising them. As for all cationic HDPs, the initial interaction with the bacterial surface is electrostatic, with lipopolysaccharide (LPS) of the Gram-negative outer membrane or the teichoic acids of the Gram-positive peptidoglycan layer [see Section 4.3 and Fig. 5 (steps 5 & 7)], and with the negatively charged phospholipids that are abundant in the bacterial cytoplasmic membranes. After accumulating on the bacterial surface it then inserts into the lipid layer, a process accompanied by a partial de-aggregation, as indicated in the previous section [Fig. 5 (steps 9&10)].

ATR-FTIR and ^{31}P NMR studies have shown that LL-37 molecules orient parallel to the cytoplasmic membrane surface [127,128,131], positioning themselves at the interface between phospholipid headgroups and acyl region of the bilayer. This suggests that both electrostatic interactions of the peptide's polar sector with the phospholipid headgroups and hydrophobic interactions between its apolar sector and the membrane are required for the subsequent membrane permeabilization mechanism. It was initially suggested that, like other amphipathic helical HDPs, LL-37 acted on the bacterial membrane according to

the so-called 'carpet' model, accumulating to a threshold level over which massive membrane disruption occurred [120,126,132]. More recent studies have observed a positive curvature strain in the membrane bilayer more in agreement with the 'toroidal-pore' model, where peptide molecules associate within the continuous lipid bilayer and cause bending of the surface so that wormhole-like cavities are formed [Fig. 5 (12)] [133,134]. Although transient, this type of lesion can compromise the barrier effect of the membrane, cause a loss of the transmembrane potential and allow leakage of cytoplasmic components, leading to bacterial killing [127,133]. We were able to catch a glimpse of such a pore caused by LL-37 on a supported membrane composed of anionic phospholipids, using atomic force microscopy (AFM) [123]. Conversely, monomeric rhesus RL-37 did not appear to produce discreet pores in the membrane, but resulted in a more widespread and heterogeneous disordering more in agreement with a 'carpet-like' disruptive mechanism [Fig. 5 (11)]. Neutron scattering studies of LL-37 in hydrated stacked membranes also confirmed toroidal pore formation, with an estimate of 5 peptides per pore of ~2–3 nm radius [134], which is a little smaller than our estimate of ~5 nm by AFM.

The oligomeric structure adopted by LL-37 in bulk solution, which partly persists upon membrane interaction, likely favours the 'toroidal' mechanism of membrane permeabilization, as it locally concentrates peptide molecules. The monomeric nature of RL-37 may instead favour its 'carpet'-like mechanism [Fig. 5 (12 vs 11)]. The two peptides have thus been used as prototypes in studies to compare the efficiency of structured/aggregated versus monomeric/unstructured peptides in damaging bacteria [122,123,135]. RL-37 caused a more rapid depolarization and more efficient permeabilization of both *Escherichia coli* and *Staphylococcus aureus* cytoplasmic membranes. ATR-FTIR experiments suggested a deeper insertion of RL-37 into supported PG bilayers and a more disruptive effect on acyl chain order [123,135]. The type of lesion observed by AFM was distinctly different and this was confirmed by *in vitro* flow-cytometric studies with *S. aureus* [135]. Considering the uptake of propidium iodide or FITC-labelled dextran particles of different radius, RL-37 resulted in rapid and extensive membrane permeabilization that however only allowed the smaller PI molecules (≤1.6 nm) to permeate the membrane. LL-37 acted more slowly but eventually resulted in larger lesions, allowing dextran molecules up to 9 nm radius to pass. We interpret this as confirming that oligomeric LL-37 acts via a 'toroidal pore' type mechanism [Fig. 5 (steps 9 & 12)], whereas monomeric RL-37 creates a more heterogeneous and extensive, 'sponge-like', disruption of the membrane more compatible with a 'carpet' type mechanism [Fig. 5 (steps 8 & 11)]. However, we did not observe membrane micellization within the time scale (1 h) of the flow-cytometry experiment, as often proposed for the carpet mechanism, as this would involve the loss of chunks of membrane and allow access to the larger dextran particles. In conclusion, while RL-37 acts via a generalized but less defined disruption of the bacterial membrane, LL-37 acts more like a 'pore-forming toxin' produced by our organism and directed against bacterial cells.

4.3. Interaction with bacterial cell wall components

Lipopolysaccharides are the major components of the Gram-negative outer membrane and are composed of an oligosaccharide region attached to Lipid A. Electrostatic attraction between LL-37 and phosphate groups linked to sugar residues in the inner polysaccharide core of LPS or in the Lipid A head-group is likely an initial binding step [see Fig. 5 (step 5)] before translocation to and insertion into the cytoplasmic membrane. Neville et al. showed that LL-37 is able to efficiently insert into monolayers composed of Lipid A [136]. In support of this interaction, lipid A modifications, such as phosphoethanolamine or glucosamine substitutions that neutralize the charge on phosphate groups, reduce susceptibility to LL-37 [137–140].

CD studies of LL-37 and its orthologues from other primates showed that their structure in bulk solution to some extent affects their interaction with LPS [124]. This interaction enhances the structured/oligomeric form of both human and orang-utan LL-37, while rhesus and presbytis RL-37 interact with LPS as monomeric helices. The RL-37 peptides permeabilized the outer membrane of *E. coli* more rapidly and at lower concentration than the LL-37 peptides [122–124], so that LPS seems to hinder passage of oligomeric peptides more than monomeric ones, with consequences also on the efficiency of cytoplasmic membrane disruption. This conclusion was further supported by testing the covalent (disulphide-bridged) dimeric forms of LL-37 as models for the aggregated form. The efficiency with which covalent dimers (parallel or antiparallel, C- or N-terminally linked) permeabilized the outer membrane of *E. coli* was invariably decreased with respect to LL-37 [122].

Structuring of LL-37 in helical bundles may therefore result in a specific type of interaction with the LPS layer. In this respect, we have very recently reported that inactivation of a gene coding for a kinase responsible for the specific phosphorylation of heptose II in the LPS polysaccharide inner core leads to reduced LL-37 binding to the bacterial cell, whereas other cationic extended or helical cathelicidin HDPs were unaffected [141]. This suggests that specific interactions with LPS inner core may mediate access of LL-37 to the surface of Gram-negative bacteria.

Gram-positive bacteria are enveloped in a thick molecular network composed mainly of peptidoglycan and teichoic acids, which is required to maintain cell integrity and act as a barrier against toxic molecules. Teichoic acids are anionic poly-glycerophosphate molecules either anchored to the cytoplasmic membrane (lipoteichoic acid, LTA) or covalently linked to peptidoglycan strands (wall teichoic acid, WTA). They confer a net negative charge to the bacterial cell surface, which contributes to attract and accumulate cationic HDPs [142]. A common resistance mechanism for Gram-positive bacteria against membranolytic HDPs is to reduce this anionicity and increase the density and rigidity of the cell wall [143]. The former process entails D-alanyl ester formation with LTA or WTA phosphates [144,145]. An *S. aureus* deletion mutant (Δ*ltA*) that lacks the enzyme required for D-alanine insertion (making the peptidoglycan more anionic) is more susceptible to both LL-37 and RL-37 than the *wild-type* strain [122,143]. Conversely, a mutant (*TagO*) lacking WTA altogether (making the proteoglycan layer less anionic) showed a decreased susceptibility. This confirms the relevance of the electrostatic interaction with the constituents of the cell wall in the mode of action of both peptides [Fig. 5 (step 7)]. However, the fact that the concentration of LL-37 needed to inactivate Gram-positive bacteria is four-fold higher than that required against Gram-negative ones, whereas RL-37 tended to be equally active, would suggest that the PG layer impedes the transit of the oligomeric peptide more than that of the monomeric one [59,124,146]. In this respect, the disulphide-linked obligate dimers of LL-37 have an even lower capacity to inhibit the growth of *S. aureus* and permeabilize its membrane [122].

The interaction of LL-37 with exopolysaccharides secreted by many bacteria, also as components of biofilm, also significantly inhibit its antimicrobial activity [147,148][see Fig. 5 (step 4)]. This is not simply due to electrostatic interactions between the negatively charged polysaccharides and the cationic peptide molecules, but seems to involve specific structural features of both interactors, and augments the oligomeric state of LL-37 [149].

4.4. Non-membranolytic effects on bacteria

The antibacterial mechanism of LL-37 is not necessarily restricted to membrane damage. Studies using time-resolved fluorescence microscopy revealed that LL-37 permeabilizes the outer membrane of *E. coli* K-12 within a few minutes, preferentially in the region where cells septate. It then does not immediately cause damage at the cytoplasmic membrane, but first binds to components of the thin peptidoglycan layer and likely interferes with its biosynthesis [(Fig. 5 (step 6)], thus halting growth. Only in a third phase does efficient cytoplasmic membrane permeabilization occur [150]. Interaction with the Gram-

positive *Bacillus subtilis* instead involves slow diffusion of the oligomeric peptide bundles through the PG layer [Fig. 5 (step 7)], followed by a concentration-dependent, cooperative permeabilization of the cytoplasmic membrane [146]. Below the critical lytic concentration, cells still cease to grow, consistent with interference with cell wall biosynthesis, but bacteria can recover if the peptide is washed away. Above this concentration, an initially punctate permeabilization of the cytoplasmic membrane occurs, consistent with pore formation, and growth stops irreversibly.

Once the peptides get to the cytoplasmic membrane, the toroidal pores they form are transient, and on collapse can result in peptide translocation to the cytoplasm. However, it is not clear if these can then inhibit bacterial growth also by interfering with internal targets, such as DNA. LL-37 is capable of forming tight complexes with nucleic acids released from lysed bacterial or host cells, sufficient to protect them from nucleases and deliver them into host cells (see Section 5.2). On the other hand, a toxic concentration (8 μM) of LL-37 that rapidly arrested bacterial growth and affected the shape of *E. coli* did not affect the spatial segregation of DNA-rich and ribosomes-rich regions [151]. LL-37 and nucleic acids are oppositely charged polyionic species, so that electrostatic attractions are bound to occur, but the biological relevance is uncertain [152]. In a recent study, HDPs of different types (proline-rich Bac7, LL-37 and helical BMAP-27) were all found to bind to DNA efficiently in a gel retardation assay. However, in an *in vitro* transcription/translation assay only LL-37 was found to selectively inhibit transcription by a viral polymerase, whereas the Pro-rich Bac7 selectively inhibited translation [153]. This suggests that interactions with nucleic acids, or in any case their effects, may not be completely aspecific.

Another non-lytic mechanism through which LL-37 may defend the host against infections is by countering bio-films (BF), either by causing a weaker attachment of bacteria to each other and/or biological surfaces and thus preventing BF formation, or by helping clear existing biofilm (see below). Furthermore, as mentioned above, LL-37 is able to selectively bind to the exopolysaccharide molecules released from Gram-negative bacteria that contribute to biofilm architecture and are essential for bacterial survival [147–149]. One must however balance this potentially protective effect with the significant reduction in the membrane-directed LL-37 antimicrobial activity caused by exopolysaccharide interactions.

4.5. Effect on host cell membranes

Unlike some other helical HDPs, LL-37 has only a moderate selectivity for bacterial cells and can start to cause membrane permeabilization in host cells at concentrations not much higher than antimicrobial ones. Cytotoxic effects were observed *in vitro* using different cell types such as erythrocytes, lymphocytes and fibroblasts [120,122,124,154]. The membrane of eukaryotic cells is very different in its composition, overall charge and transmembrane potential to that of bacterial cells and its outer leaflet is composed mainly of zwitterionic phosphatidylcholine (PC) and sphingomyelin (SM) phospholipids that do not electrostatically attract cationic HDPs. This, and the varying presence of cholesterol, generally attenuates membrane association of HDPs, reducing their effects. However, cathelicidins of various types and origin do not show a marked selectivity [154–157]. With respect to LL-37, it is likely that its oligomeric helical form in physiological media favours its interaction with host cell membranes. This interaction may be beneficial if it then allows the peptide to modulate cellular activities in different ways (see below). On the other hand it must be carefully controlled or it can lead to membrane lysis and result in necrotic or apoptotic effects.

Different studies using model membranes were performed to better understand the mechanism of interaction of LL-37 with host cell membranes and how it is affected by lipid composition. It was shown by different groups that it inserts less into monolayers or bilayers composed neutral phospholipids (DPPC, DPPE or PC) than into those composed of negatively charged ones (DPPG and PG) [120,135,136,158]. The peptide axis remains parallel to the lipid bilayer and hydrophobic interactions between its apolar residues, and the core of the zwitterionic lipid bilayers is in any case critical for its capacity to affect the membrane [133]. It is not clear why preformation of the helix in bulk solution should favour interaction with host membranes, also increasing cytotoxicity, but it has been independently observed in helical peptides stabilized in different ways. Apart from LL-37, whose structure is stabilized by salt-bridging, it was observed in a designed, artificial AMP stabilized by the presence of helix-favouring aminocyclopentanecarboxylic acid residues [159], which also showed a marked medium dependence of its antimicrobial activity. This was also reported for helical antimicrobial peptides stabilized by the formation of intramolecular lactam bridges between Glu and Lys residues spaced 4 positions apart [160].

Cross-linking studies confirmed that LL-37 interacts with neutral membranes as an oligomer, forming up to tetramers at higher concentrations, and this was confirmed by SPR studies [122]. Altering the propensity to oligomerize alters its ability to interact with and permeabilize neutral membranes, as shown by studies using truncated versions of LL-37. 21-residue fragments taken from the N-terminus, centre or C-terminus of LL-37 are all significantly less haemolytic, suggesting that the full oligomeric peptide is required for efficient interaction with neutral membranes [161,162]. Just removing the first four N-terminal residues, that are key to oligomerization, also significantly reduces cytotoxicity [120]. Conversely, the obligate disulphide linked C-terminal dimer showed significantly higher haemolytic activity and permeabilized monocytes to propidium iodide at much lower concentrations than LL-37. Surprisingly, the N-linked dimer showed a lower toxicity than the *wild-type* peptide, indicating that the hydrophobic N-terminus may play a role in modulating membrane interaction that goes beyond simply mediating oligomerization, and that requires it not to be restrained [122].

Fortunately, the cytotoxic activities of LL-37 are mitigated in human plasma. This is principally due to the non-covalent binding of LL-37 to apolipoprotein AI (apo-AI) [115,116,125], which serves to protect host cells from the toxic effects of LL-37 despite a very high plasma concentration. The K_D is such that at its physiological plasma concentration (~50 μM) apoA-I could scavenge over 90% of LL-37 present at concentrations cytotoxic to host cells (50 μM) [115], leaving a sufficient concentration of unbound peptide for antimicrobial and immunomodulatory effects. It may thus provide a circulating "pool" of LL-37 molecules that can be useful on infection. The residual ability of human peptide to interact with neutral membranes allows it to exert a variety of immunomodulatory activities on different types of host cells (see below). RL-37, which is unstructured in the presence of neutral membranes, and interacts poorly with them in its monomeric form, lacks some of these immunomodulatory activities. A clear example is their different capacity to stimulate fibroblast proliferation [163]. LL-37 is able to activate the P2X7 receptor and induce pore formation, which is involved in the proliferative cell response. The strong helix-forming propensity of LL-37 in aqueous solution is required for this activation, and furthermore its membrane localization appears to enable it to re-establish the pore-forming activity of an otherwise inactive truncated form of P2X7. Conversely, a scrambled form of LL-37 and RL-37, which are unfolded in aqueous solution and do not interact strongly with neutral membranes, were not able to do this.

4.6. Structure/function relationships in LL-37

Self-assembly of LL-37 as a bundle of amphipathic helices appears to be a central feature in enabling it to exert many of its activities. As already mentioned, it requires the formation of a complex net of intra- and intermolecular salt-bridges and H-bonds among its charged and polar side-chains, as well as hydrophobic interactions that allow it to mask the hydrophobic helix face from the polar environment, all of

which are likely to depend on specific evolved sequence characteristics of the peptide. Oligomeric helix formation appears to favour interaction with bacterial and/or host cell components (such as LPS, peptidoglycan, phospholipids and circulating or membrane proteins), and this likely also depends on specific structural characteristics of the molecules it interacts with. These interactions may then lead to structural modifications of both LL-37 and the targeted molecules/surfaces to produce biologically relevant effects.

One must therefore be very wary in interpreting SAR studies of LL-37 based on sequence alterations (mutations or truncations), if these affect or impede oligomerization. Once it disaggregates, an LL-37 variant may switch to quite different types of interactions, leading to different mechanisms of action with different types of outcome that may have little bearing on how the parent peptide acts. An extensive review of reported LL-37 fragments and mutants lists the main functional characteristics of each variant [16], but it is quite challenging to find a clear correlation between the different peptide regions that these fragments cover and the specific activities that have been attributed to native LL-37. Truncation or replacement of key residues can decrease or cancel its ability to oligomerize, and the outcome can be a decrease in a particular activity, if this requires the aggregated peptide state (e.g. binding to host cell membranes and consequent cytotoxic or immunomodulatory effects). Vice-versa, aggregation favours sequestration by medium or serum components, so removing this characteristic can markedly increase other types of activities (e.g. disruption of bacterial membranes). The presence of naturally occurring LL-37's fragments due to enzymatic cleavage at different sites (see Section 3.3), as well as the intent to develop LL-37 as a therapeutic agent, makes it necessary to understand these processes.

Removal of just a few residues from the N-terminus of LL-37 stops self-assembly [120,154,164–166], and markedly reduces toxicity towards host cells, as interaction with their membranes is favoured by this structural feature. Conversely, antibacterial activity is maintained or even increased if truncation does not significantly impact on the overall charge and amphipathicity. The truncated peptides may switch to a mode of membrane interaction and disruption more similar to that of monomeric rhesus RL-37, acting via a carpet mechanism and less affected by sequestering interactions on their way to the bacterial membrane (see Sections 4.1 & 4.2). In this respect, the central region of the LL-37, including residues 11–30, seems to be quite sufficient for this type of antimicrobial activity, given that fragments which include this domain are active against a wide range of microorganisms [164]. The N-terminal region of LL-37, rich in hydrophobic residues, is instead on its own insufficient for antimicrobial activity, as shown by poorly active fragments containing up to the initial 23 amino acids [167]. The fragment GF-17 (residues 17–32) is considered to span the principal antimicrobial region, as it is active against both Gram-positive and Gram-negative bacteria [168], while a shorter fragment spanning residues 18–29 is active only towards Gram-negative bacteria [107]. Both these fragments maintain an amphipathic helical structure in the presence of anionic micelles, as seen by NMR. Furthermore, they have a net charge of +4, which corresponds to the lowest net charge observed among primate orthologues (orangutan LL-37) [59] possibly pointing to a lower charge limit for effective antimicrobial activity in these orthologues.

The central region of LL-37 also appears to be responsible for neutralizing the effects of LPS molecules released at sites of infection, and roughly covers residues 13–32 [45,166,169]. Fragments within this region are able to bind LPS and LTA and to suppress the pro-inflammatory response to these bacterial components via TLR receptors on peripheral blood mononuclear cells (PBMCs). The LPS-binding domain overlaps substantially with that conferring antibacterial activity, which suggests that similar types of interactions are involved, requiring the presence of both cationic and hydrophobic residues. However, subtler interactions with the LPS layer in the outer membrane of Gram-negative bacteria, which affect the behaviour of the parent peptide, may require the full sequence (see Section 4.3).

Attempts to extract SAR data relating to the immunomodulatory effects of LL-37 are more problematic, as these are likely mediated by an initial interaction with host cell membranes (see Section 5.2), which depends on oligomerization. LL-37 stimulates cytokine release from keratinocytes in a manner that requires the whole peptide sequence, by activating G-protein coupled receptors (GPRC) or epidermal growth factor receptors (EGFR) in a non-canonical manner [164]. This is suggested by the fact that its *all-D* enantiomer (which has a different stereochemistry but similar structuring, oligomerization and membrane interaction capacities) is equally active, so interaction with a ligand-binding site is unlikely. Removing a few residues from the N-terminus of LL-37 significantly decreases this activity but does not completely remove it. This suggests that oligomerization favours this process mainly because it shifts the equilibrium more towards the membrane-bound state than for monomeric peptides.

At higher concentrations, orthologues or variants that are unstructured and monomeric in bulk solution will however also partition into neutral membranes. This is clearly evident if one compares observations based on methods sampling the whole peptide population (such as CD or steady-state fluorescence spectroscopies) with those based on methods that can simultaneously sample diverse populations (e.g. membrane bound peptide and free peptide in bulk solution by time-resolved fluorescence) or only probe a region very close to the membrane surface (e.g. ATR-FTIR and SPR). For example, CD and steady-state fluorescence spectra of rhesus RL-37 in the presence of neutral membrane liposomes suggest that it doesn't interact with them, while ATR-FTIR, SPR and time resolved fluorescence, on the other hand, strongly indicate that it does [122,128]. Taken together, these apparently diverging observations indicate that a small part of the peptide must equilibrate into a structured, membrane-bound state while most of it remains unaltered in bulk solution. These observations explain why RL-37 is less haemolytic than LL-37 at lower concentrations, while it has a similar HC_{50} of about 70 μM [170]. The trade-off is that while RL-37 is more efficient and selective in disrupting bacterial membranes, LL-37 can interact with host cell membranes at sufficiently low concentrations to be able to start affecting membrane receptors while not causing substantial membrane damage [163].

5. Pleiotropic roles of LL-37 in host defence

LL-37 contributes to host protection on several different fronts; it has a direct antimicrobial activity; it can act as an 'alarmin', attracting immune cells to the site of infection; it can modulate inflammatory responses, and it can promote cellular proliferation useful to healing processes. It appears that all these processes to some extent depend on, or are affected by, its particular oligomeric form.

5.1. Antibacterial activity of LL-37

LL-37 has a direct antimicrobial activity *in vitro* against a variety of microbial pathogens, including bacteria, fungi, viruses, and parasites [171–174], as has been widely reviewed elsewhere [13,21,175]. Its antibacterial activity covers a broad spectrum of Gram-positive and Gram-negative species, but is more dependent on the medium used for testing its efficacy than other helical cathelicidin peptides (e.g. BMAP-27, BMAP-28, SMAP-29, RL-37 and rabbit CAP-18) [13,42,59,163,176,177]. Several studies have reported rather disappointing *in vitro* MIC values at physiological salt concentrations, in the presence of serum, in the presence of lung surfactant preparations and in the presence of artificial tears [43,178,179,180].

LL-37 is effective *in vitro* against the Gram-negative organisms *Acinetobacter baumanii*, *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Proteus vulgaris*, *Klebsiella pneumoniae* and *Neisseria gonorrhoeae* among others, with activity generally in the 0.5–10 μM range. For Gram-positive bacteria, *in vitro* efficacy is reported against *Staphylococcus* spp., *Enterococcus* spp.,

Streptococcus spp. *Bacillus* spp. *Lactobacillus acidophilus*, *Lysteria monocytogenes* and *Propionibacterium acnes*, among others with an activity range of about 0.5–20 μM. [13,21,39,42,67,97,181–183]. It is, however, difficult to assess the relative activity on different microorganisms deriving from different studies, as these used different medium conditions and/or determination methods (e.g. radial diffusion assay vs broth microdilution assay) that significantly affect results. In studies that tested different bacterial species under the same conditions, the efficacy is generally better against Gram-negative bacteria, especially under more robust conditions (high salt or complete medium) [42,59].

A clear example of how medium can influence the assessment of LL-37's activity concerns its *in vitro* efficacy against common pathogens of the oral cavity. Under hypotonic conditions (e.g. SPB buffer or ~1% tryptic soy broth), antimicrobial activity is in the low to submicromolar range, suggesting that the peptide might be useful as a periodontal therapeutic agent [184,185]. Saliva is hypotonic but rich in carbonate [186], conditions that seem to make bacteria more susceptible [176], but at the same time induce structuring and oligomerization of LL-37 [43], which affects its sensitivity to medium and serum components [170]. Possibly for this reason assays carried out in complete medium, or in the presence of serum, showed a significantly decreased activity [171,185]. It is interesting, in this respect, that other helical cathelicidins or the LL-37 analogue 'pentamide-37', devoid of anionic residues, all of which do not have its propensity for oligomerization, were significantly less affected by these conditions. These considerations apply also for testing in different media [187] or by radial diffusion assays [188].

The MIC values of five different cathelicidins from diverse mammalian species against *Leptospira*, *Borrelia* and *Treponema* evidenced that LL-37 was less active than other cathelicidin peptides [189]. Interestingly, *Leptospira*, which have a Gram-negative like outer membrane with the presence of LPS, are more susceptible than the other two spirochetes that do not have it, further supporting a role for LPS in guiding LL-37 to the surface of Gram-negative bacteria (see Section 4.3).

LL-37 is effective against mycobacteria, and is able to kill *Mycobacterium smegmatis*, *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis* under *in vitro* growth conditions, where it significantly reduced the intracellular survival of the mycobacteria [78]. Killing could be effected either by exogenous administration of LL-37 to infected macrophages, or endogenous stimulation of LL-37 production by exposing the macrophages to vitamin D. In the first case, the peptide may enter cells via endocytosis (see below) and localize in phagosomes with the mycobacteria, thus inactivating them. In the second case, mycobacterial products acting on TLR receptors, (surface bound TLR1/2 and TLR4 or intracellular TLR9) result in up-regulation of the vitamin D receptor (VDR) (see Section 3.2), which when bound to the vitamin promotes LL-37 expression through a VDRE in its promoter region [79,82,93]. How intracellular hCAP18 is processed in this case is however unclear.

It was also demonstrated that LL-37 inhibits the formation of biofilms (BF) by different bacteria. It prevents BF formation in *P. aeruginosa* in multiple ways, including preventing the attachment of bacterial cells, stimulating twitching motility and influencing quorum sensing systems, leading to down-regulation of genes essential for BF development [190,191]. The anti-BF activity does not necessarily parallel the antibacterial activity, as BF formation was prevented at concentrations below those that inhibited growth. Similarly, LL-37 is capable of significantly inhibiting BF formation by *Francisella novicida*, a highly infective lung pathogen, and the opportunistic skin bacterium *Staphylococcus epidermidis*, at sub-toxic concentrations [192,193]. From this, one can conclude that LL-37's antibacterial activity combines interference with bacterial biofilm formation and immuno-modulation of host responses (see next section) with the direct antimicrobial effect, allowing it to contribute to host defence even at concentrations, or under environmental conditions, in which it may not have a direct antibacterial activity.

In the endless battle between host immune effectors and invading pathogens, these have developed a number of countermeasures against the protective role of LL-37. As mentioned in Section 4.3, bacteria have the capacity to alter cell wall components such as LPS or LTA to reduce the electrostatic attraction that guides the peptide to the cytoplasmic membrane. Another strategy commonly used by bacteria is the secretion of proteases. So far, the pathogenic bacteria *Proteus mirabilis*, *Streptococcus pyogenes*, *S. aureus*, *P. aeruginosa*, and *Enterococcus faecalis*, as well as the yeast *Candida albicans*, have all been reported to produce extracellular proteases often capable of degrading both LL-37 and defensins [194].

5.2. Modulatory activities on host cells

Many papers have been published on the capacity of LL-37 to affect host cells in different ways, so much so that it has recently been called a *factotum* peptide [2,21,22,23,44,195,196]. It is reported to have *a*) a chemotactic activity on circulating host immune cells; *b*) the capacity to activate production and secretion of pro-inflammatory signal molecules from epithelial or circulating cells; and *c*) the capacity to modulate the pro-inflammatory activities of microbial components. These effects derive from the capacity to activate or transactivate host cell receptors or to translocate molecular cargo into host cells. That LL-37 does all this may seem surprising, but ultimately derives from its tendency to fold in aqueous salt solution, which then favours both interaction with the neutral membranes of host cells and with extracellular microbial or endogenous molecules.

In essence, this allows several different mechanisms by which LL-37 could affect host cell activities;

i) It can form transient pores or lesions in cellular membranes and allow release of material from the cell;
ii) It can accumulate in membrane regions surrounding receptors and then either interact with their binding sites or with their TM regions and activate them;
iii) It can interact with membrane-anchored factors and cause their release/activation and result in transactivation of receptors;
iv) It can interact with extracellular components, such as microbial LPS or LTA or material released from damaged host cells, and by sequestering them prevent their interaction with TLR receptors thus mitigating their pro-inflammatory effects;
v) It can alternatively deliver such molecules efficiently to intracellular receptors or compartments by endocytosis for a more controlled pro-inflammatory response.

Some of its activities are described below, and are schematically illustrated in Fig. 6.

5.2.1. Release of molecules through membrane permeabilization

Formation of transient pores or lesions in host cell membranes by LL-37 at sub-toxic concentrations, for controlled release of signal molecules, is an unlikely scenario. Several studies involving activation of purinergic receptors by LL-37 have dismissed the possibility that these could be activated by ATP released from cells in this manner [163,197,198]. It has been suggested that release of potassium through pores involving LL-37 could trigger pro-inflammatory events in macrophages, but it is more likely to proceed via activation of purinergic receptor channels themselves [199]. Rather, LL-37 has been reported to lyse apoptotic leukocytes, due to a change in their membrane composition that renders them more susceptible to its cytotoxic effect (resembling LL-37's antimicrobial activity). Release of pro-inflammatory substances from these apoptotic cells may then help to prolong inflammation. However this occurs only at sites poor in serum components, such as the skin or lung epithelia, as this effect, analogously to the antimicrobial activity, is quite serum sensitive [200,201].

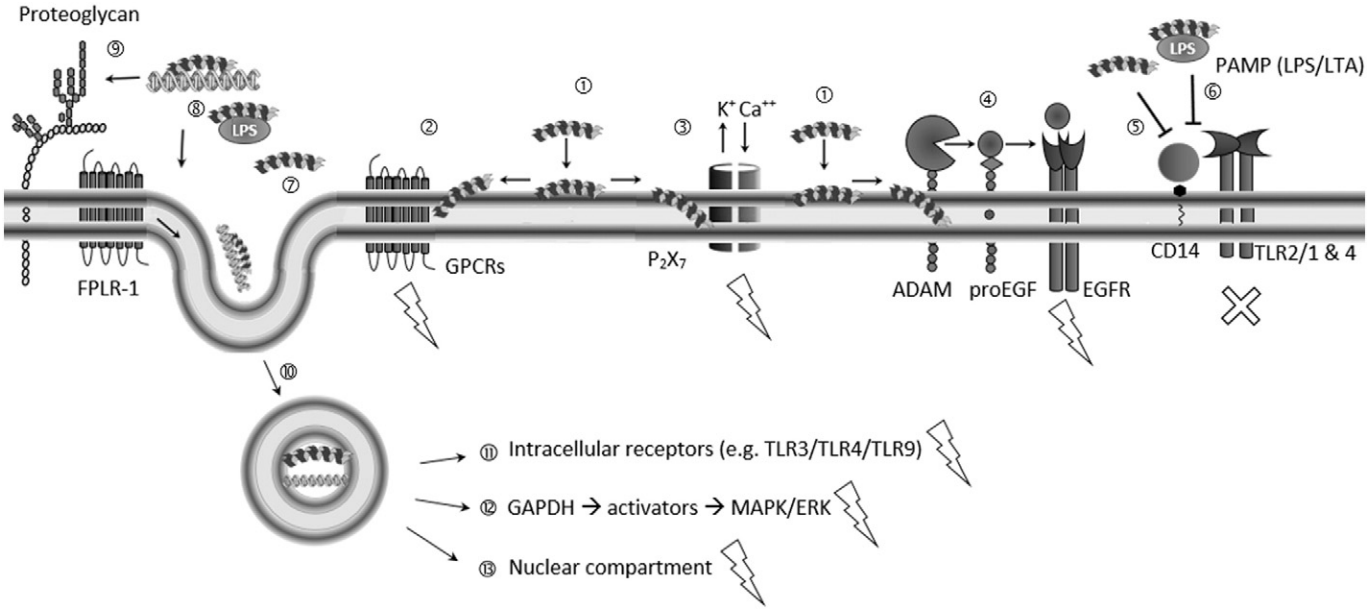

Fig. 6. Schematic representation of some of the activities of LL-37 on host cells. LL-37 interacts with host cell membranes as an oligomer (1), possibly preferring lipid rafts, and likely remains bound to the membrane surface as such (2). It can interact with the TM domains of FPRL-1 receptor and P_2X_7 channel receptor (2)(3) resulting in a non-canonical type of activation. It can also activate membrane proteases to release membrane-anchored growth factors that transactivate the EGFR receptor (4). Interaction with TLR4 or its co-receptor CD14 (5), or binding with PAMPs such as LPS (6), reduces their pro-inflammatory effects. Receptor interactions can also mediate endocytosis of LL-37 aggregates (7)(10). Its binding with extracellular anionic molecules such as dsDNA, dsRNA or LPS, followed by lipid-raft mediated endocytosis assisted by interaction with proteoglycan, FPRL-1 or EGFR, leads to their internalization (8)(9)(10). Endocytosis also allows access for LL-37 to intracellular receptors (11), to proteins such as GAPDH that modulate the activity of signalling pathways (12), or to the nuclear compartment (13), eliciting useful pro-inflammatory responses.

It has recently been reported that LL-37 can facilitate the formation of neutrophil extracellular traps (NETs). Neutrophils and other leucocytes involved in the innate immune system can expel their DNA into the extracellular environment in a controlled manner, forming very wide regions of entrapment that can contribute to the inactivation of bacteria, viruses and multicellular eukaryotic parasites [202]. Pathogens counteract by producing nucleases that degrade the DNA lattice backbone, or by inducing immune-modulatory molecules such as IL-10 that dampen NET production. LL-37 appears to have both a stimulatory and protective role with respect to these structures. It appears to both permeabilize the nuclear membrane, which is deficient in cholesterol and sphingomyelin, thus facilitating the release of DNA [203,204], and then bind to the DNA in NETs, protecting it from staphylococcal DNAses [205].

5.2.2. Receptor activation

LL-37 exerts a plethora of effects that are mediated through the activation of several different types of receptors, expressed on different cell types in different physiological and pathological contexts [21,23]. These include GPCRs such as the formyl peptide receptor-like 1 (FPRL-1), tyrosine kinase receptors such as the epidermal growth factor receptor (EGFR), toll-like receptors (TLR) and the purinergic channel receptor P_2X_7R. However, in what ways LL-37 exerts its action on these receptors is not yet well understood. It has been suggested that the peptide acts in a non-canonical manner by first interacting with and accumulating in the membrane, possibly preferring the cholesterol- and sphingomyelin-rich lipid rafts where receptors are located [Fig. 6 (step 1)] [206], and then interacting with their transmembrane domain [Fig. 6,(steps 2–4)] rather than with specific ligand-binding sites. This is consistent with its promiscuous and generally low-affinity type of activity [163,207–211], and is confirmed by the fact that for GPCR, EGFR and P_2X_7R activation the all-D enantiomer is as effective as the natural peptide. The enantiomer has a different stereochemistry but similar structuring, oligomerization and membrane interaction capacities [163,164].

Activation of receptors by LL-37 can have a series of different outcomes, depending on the stimulated cell, environment and physio/pathological context. Possibly the most common result is that *i*) *it triggers pro-inflammatory signalling cascades* leading to innate immune responses. However, there are also other types of outcome; for example activation of FPRL-1 in endothelial cells induces their proliferation so *ii*) *it promotes angiogenesis* [47]. By activating FPRL-1 and P_2X_7R in neutrophils *iii*) *it suppresses apoptosis*, thus prolonging their life-span during bacterial infections [210,212]. Conversely, by activating P_2X_7R in epithelial cells *iv*) *it induces apoptosis*, and this also may be a useful innate defence mechanism as it could favour clearance of infected cells from the airway epithelium [210]. Furthermore, by interacting with receptors within lipid rafts *v*) *it can promote receptor-mediated endocytosis*. This has been amply reported, and variously involves different receptors (e.g. EGFR, GPCR or P_2X_7R) and different cell types (e.g. macrophages, epithelial cells, immature and plasmacytoid dendritic cells), and can be a means of internalizing anionic molecular cargo (see below) [213–217].

Interaction of LL-37 with the purinergic P_2X_7 receptor in monocytes leads to the opening of a small channel capable of allowing a potassium flux from the cytoplasm [Fig. 6 (step 3)]. This results in NALP3 inflammasome assembly, caspase-1 activation and release of pro-inflammatory cytokines IL-1β and IL-18 [199,218]. It remains to be determined whether the intrinsic channel characteristics of activated P_2X_7R are sufficient for this flux or, as suggested by some reports, if it recruits other subunits such as pannexin to form a larger channel [219,220]. However, as often seen with other effects of LL-37, its activity on P_2X_7R is hard to fathom. Macrophages respond differently to P_2X_7 activation by agonists [199], and LL-37 exerts an inhibiting effect on these, on two fronts; it inhibits the pro-inflammatory effect of LPS via TLR4 receptors (see below) and also inhibits P_2X_7 activation by ATP. This suppresses inflammasome assembly and prevents caspase-1 mediated pyroptosis [221]. Furthermore, LL-37 appears to internalize into macrophages by P_2X_7R-mediated endocytosis, and this results in induction of the eicosanoid thromboxane A_2 [213,222], via activation of the MAPK/ERK pathway and subsequent COX-3 expression.

Besides activation of surface receptors, LL-37 may also act intracellularly in monocytes. Interaction with a membrane receptor, or the

membrane itself, triggers a vesicle-mediated uptake pathway involving cellular cytoskeletal elements [Fig. 6 (steps 7–10)] allowing its delivery to a key glycolytic enzyme, GAPDH [Fig. 6 (step 12)]. LL-37's interaction with GAPDH interferes with its association with one or more activators of the p38 MAPK signalling pathway, and their release results in activation of this pathway leading to production and release of pro-inflammatory chemokines [223].

5.2.3. Transactivation of receptors

LL-37 transactivates EGFR in airway epithelia by triggering a series of molecular events that likely include binding to the membrane, subsequent activation of a membrane-anchored metalloproteinase (a member of the ADAM family), cleavage by this of a membrane-anchored EGFR ligand which can then bind to EGFR to activate the MAPK/ERK pathway [224] [Fig. 6, (steps 1 & 4)]. It thus acts as an important mediator by which neutrophils regulate epithelial cell activity in the lung. The transactivation of EGFR also has a fundamental role for LL-37-induced keratinocyte migration, an important step in wound healing in skin and corneal epithelial cells [225,226]. LL-37-initiated transactivation of EGFR also appears to be required for the lipid raft-dependent endocytosis of LL-37/LPS aggregates into lung epithelial cells, providing a 'non-classical pro-inflammatory' mechanism [217] (see next section).

5.2.4. Binding to endotoxins

LL-37 shares with some of its orthologues, as well as with other AMPs, the capacity to bind to lipopolysaccharide (LPS) [169,170,227, 228,229,230] and neutralize its biological activities [10]. By interacting with LPS released from bacteria, LL-37 exerts a protective action against endotoxin shock, but this involves a complex mechanism with several points of intervention [230] [Fig. 6, (5,6)]. LL-37 binds directly to LPS and can block its binding to the LPS-binding protein (LBP) that delivers it to the CD14/TLR4 receptor complex expressed on the surface of immune cells. This prevents initiation of pro-inflammatory cytokine production from leukocytes and also protects endothelial cells form apoptosis [231]. A second possibility is that it directly antagonizes binding of LPS to CD14. A third is that it causes LPS particles to disaggregate and that this somehow contributes to LPS detoxification [230,232,233, 234]. Protection of the host may also involve the selective altering by LL-37 of inflammatory gene expression and inhibiting translocation of LPS-induced transcription factors to the nucleus, thereby suppressing the production of cytokines by the immune host cells [235]. LL-37 appears to have a similar role in detoxifying LTA [46].

5.2.5. Endocytosis and transport of polyanions into host cells

Several studies indicate that LL-37 can be internalized into mammalian cells via receptor-mediated endocytosis, also carrying molecular cargo with it, and so access different intracellular receptors and/or compartments [Fig. 6, (steps 7–13)]. It is reported to be actively taken up by epithelial cells via an atypical endocytotic process that requires only some of the elements normally involved in this process [208]. It is subsequently trafficked to the perinuclear region in a process dependent on intact microtubules, where it may stimulate production of IL-8.

LL-37 has the capacity to bind to nucleic acids and other anionic molecules or assemblies, such as LPS, and internalize them via some form of endocytosis [214,217]. For example, it forms large aggregated complexes with "self-DNA" released from damaged keratinocytes following skin injury, and then transfers it into plasmacytoid dendritic cells. There it retains the DNA complex in early endocytotic organelles, delivering it to the intracellular receptor TLR9 [216], thus triggering interferon production [Fig. 6 (8–11)]. This bypasses a safety mechanism for discriminating exogenous DNA from self-nucleic-acids, which otherwise could not access this receptor, providing a signal for injury. However, under pathological conditions such as psoriasis (where LL-37 is overexpressed in skin lesions [71] and self-DNA is heavily released) this leads to a breakdown in innate tolerance to self-DNA and is a principal trigger of the pathogenic IFN response in psoriatic skin.

LL-37 can also translocate dsRNA into cells to meet with the intracellular receptor TLR3 and set off a pro-inflammatory response [236]. The oligomeric form of LL-37 complexed with the ribonucleic acid first binds to FPRL-1, which then traffics it to endosomes. After maturation, the acidic environment of the endosomes dissociates the nucleic acid from LL-37 so that it can be recognized by the receptor [Fig. 6 (steps 8, 10, 11)]. Another example of endocytotic mechanism has been described for delivering plasmid DNA from lysed bacteria to the nuclear compartment of various mammalian cells. In this case it involves caveolae-independent endocytosis at cholesterol-rich raft domains and requires a proteoglycan as receptor [Fig. 6, (steps 9, 10, 13)] [206]. The relevance to host defence of targeting bacterial plasmids to the nuclei of host cells, where they can be expressed, is however unclear.

A further example of LL-37 mediated endocytosis is the specific transport of LPS molecules through lung epithelial cells, providing a segregated epithelial response [217]. Unlike immune cells, polarized epithelial cells tend not to express TLR4 on their surface, but rather it is localized at intracellular compartments, making them hyporesponsive to endotoxin. Selective binding of LL-37 to LPS is followed by raft-dependent and EGRF-mediated endocytosis to the TLR4-containing intracellular compartments. Significantly, this 'non-classical' TLR4 activation occurs only through the basolateral side of these cells, thus avoiding internalization of, and an excessive response to, LPS from environmental microbes on the apical side.

6. Therapeutic potential and possible applications of LL-37 and its derivatives

Host defence peptides are attractive candidates for developing novel antibacterial agents [2,237–239]. LL-37 is especially appealing due to its pleiotropic roles in host defence. However, given the complexity of its immune effects, the development of anti-infective agents based on this peptide may not be facile.

The protective effect of human LL-37 against infection is underlined by both clinical observations and findings from animal models [239–242] and this has increased interest in its medical use. Two main sectors for therapeutic intervention in infectious and inflammatory diseases have been considered:

i) recent advances in the understanding of the control of LL-37 expression (*see* Section 3.2) opens the possibility of boosting immunity by stimulating the endogenous production of LL-37;
ii) the antimicrobial, antisepsis and immunomodulatory properties of LL-37 raises the possibility that it could be directly exploited as an exogenously administered therapeutic agent, or used as a template for the development of artificial analogues with optimized biological activities.

6.1. Induction of the LL-37 expression as a therapeutic strategy

The discovery of molecules such as butyrate and vitamin D3 that induce LL-37 expression has opened new avenues for preventing or treating infections by strengthening this aspect of immune defences [81,83,243]. For example, it has been shown that systemic treatment with butyrate induces its orthologue rCAP18 in the colonic epithelium of rabbits, and promotes elimination of *Shigella* in a shigellosis model [244]. Elucidation of the butyrate-dependent LL-37 up-regulation pathways has suggested that histone-deacetylase inhibitors (HDACs) such as butyrate and trichostatin A could be useful for treatment of gastrointestinal diseases in humans [245]. Vitamin D3, administered or light-induced, boosts LL-37 expression in the skin and circulating leucocytes, and its induction in human macrophages, in response to bacterial stimulation, has been shown to be essential for normal microbicidal activity

of these cells against mycobacteria. This suggests a correlation between vitamin D deficiency and susceptibility to tuberculosis, which might be countered by administering this vitamin [93].

Another approach that has been considered for boosting LL-37 expression is the use of adenoviral vectors. Transfer of the *CAMP* gene into a cystic fibrosis bronchial xenograft model resulted in increased LL-37 production and improved microbicidal activity of the airway fluids [246]. Adenovirus-mediated LL-37 gene transfer has also been found to prevent bacterial infections in skin equivalents [247] and in a rat wound model [248], as well as promoting wound healing in diabetic *ob*/*ob* mice by increasing the re-epithelialization rate and formation of granulation tissue in wounds [249]. Electroporation of a plasmid encoding the *CAMP* gene has been proposed as an alternative to adenoviral delivery. This method induced strong expression predominantly in keratinocytes that are at the origin of re-epithelialization, and in a mouse wound model promoted healing and inhibited bacterial growth, while in a mouse ischemia model it up-regulated angiogenic chemokines and increased blood perfusion [250].

Increased levels of hCAP18, induced by molecules such as butyrate or vitamin D or by gene transfer may however also require a concomitant increase in production of the processing enzymes required to release LL-37 (see Section 3.2). Furthermore, given that higher levels of LL-37 exacerbate some inflammatory autoimmune diseases, its induction would have to be carefully controlled. This warrants a more detailed knowledge of all the issues involved [81].

6.2. Development of LL-37 based molecules as direct antimicrobials and/or immunomodulants

The use of LL-37 as a therapeutic approach for systemic infections and/or chronic infected wound treatment is beginning to be explored in the clinic [22]. As LL-37 combines antimicrobial and anti-biofilm activity against numerous Gram-positive and Gram-negative human pathogens with stimulation of re-epithelialization and angiogenesis, its topical application on skin wounds or bedsores should promote their healing [22]. A clinical study using LL-37 for the treatment of venous leg ulcers, the most prevalent type of chronic wound, is ongoing [251]. Preliminary reports on a limited number of patients are very promising, showing that at doses of 0.5 and 1.6 mg/mL LL-37 markedly decreased the mean ulcer area, with healing constants up to six-fold higher than placebo, and no safety concerns. The success of these studies has also suggested the incorporation of an LL-37 analogue into medical gauze, and it was shown to significantly inhibit bacterial growth [252]. However, this analogue has a tethering modification at the N-terminus that could affect its activities.

LL-37, orthologues such as mCRAMP, or synthetic derivatives have also been tested for the treatment of systemic infections. They showed moderate *in vivo* antimicrobial activities in a *M. tuberculosis* mouse model of infection. Repeated intrapulmonary administration of 1 mg/kg LL-37 during late progressive disease induced by both drug-sensitive and -resistant virulent strains led to a reduction in lung bacilli, although it did not clear pneumonia [253]. A 24 amino acid peptide derivative of LL-37 has shown efficacy against biofilm-forming *P. aeruginosa* in an established rabbit model of sinusitis [254]. However, the *in vivo* ability to eradicate *Pseudomonas* biofilms and decrease bacterial counts was only observed at the higher concentration used (2.5 mg/ml), at which pro-inflammatory and ciliotoxic effects were also observed on sinus mucosa. As mentioned in Section 5.1, anti-biofilm effects may also be exerted by non-bactericidal mechanisms. Dean et al. observed in a simple in-vivo model of infection that LL-37 is able to promote *P. aeruginosa* motility and decrease biofilm formation by altering the rate of twitching as well as by down-regulating the expression of biofilm-related genes [191].

A problem with the use of LL-37, as with all therapeutic peptides, is its degradation by proteases. The all-D enantiomer of LL-37 showed improved protease resistance in-vivo in a model of infection using the wax moth caterpillar, and this correlated with increased survival of *Pseudomonas* infected caterpillars with respect to those treated with the native peptide [191]. Variants of LL-37 fragments have been designed by combining library screening and structure-based design, in which introduction of D-amino acid residues reduced degradation by chymotrypsin. Despite a significant deviation from the sequence and structure of native LL-37, one peptide maintained useful antimicrobial, anti-BF and immunomodulatory activities *in vitro*, suggesting potential therapeutic possibilities [255]. However, it is likely to have quite a different mechanism of action to LL-37 (see Section 4.6).

Apart from its direct antimicrobial activity, LL-37 has shown a potent anti-endotoxin activity suggesting that it, or its functional analogues, could be used as antisepsis agents [256]. The capacity of LL-37 to protect against lethal sepsis has been demonstrated in different rat sepsis models, after intravenous administration of 1 mg/ml peptide. It was shown to have a double action, both decreasing circulating endotoxin and reducing its pro-inflammatory effects [257].

LL-37 may also play a complex role in carcinogenesis and its overexpression has been implicated in the development or progression of breast, ovarian and lung cancers [8], so control of its expression could be therapeutically useful also in this sense. It has, however, been shown to suppress tumorigenesis in gastric cancer, which indicates that it has quite intricate and contradictory functions. Exploiting its capacity to internalize nucleic acids into host cells, it has been co-administered to mice with CpG oligo-deoxynucleotides. These are employed in immunotherapy to enhance the tumour suppressing activity of the host immune cells through stimulation of Toll-like receptor TLR-9. It produced synergistic antitumor effects with CpG against ovarian cancer in mice, and enhanced the proliferation and activation of NK cells [258,259]. In fact, the cathelicidin appears to be required for the antitumour activity of NK cells, and in humans may explain the protective effect of vitamin D in several types of cancer [76].

Accumulated data on the numerous immunomodulatory properties of LL-37 [195,260] thus opens many possibilities for using it or its derivatives to stimulate immunity also by exogenous administration, although its extensive effects within the innate immune network requires a much better understanding of the underlying mechanisms for safe and effective use.

7. Conclusions

The human cathelicidin hCAP-18, and more particularly the HDP it carries, LL-37, has a manifest role in our innate immunity, with a remarkably wide range of functions. Endogenous antimicrobial peptides have accompanied animals throughout their evolution, cathelicidins have accompanied vertebrate animals throughout theirs, and orthologues of LL-37 have accompanied eutherian animals, so that these molecules have had a long time to co-evolve with the host's immune system and the changing threats it has to cope with. This has allowed a relatively simple peptide like LL-37 to find ways of carrying out many, apparently quite diverse, functions in humans. One key element of its structure seems to be the capacity to adopt its helical conformation and simultaneously oligomerize in physiological solutions. This determines how it approaches biological membranes and other molecules, how it interacts with them, and subsequently how it forms toxic pores in bacterial membranes or activates signalling or endocytotic processes in host cells, leading to various types of responses useful to combat infection and promote healing. A better understanding of the interconnected factors that allow it to do all that it does will bring a better, more global understanding of this important and wide-ranging immune response, and favour its therapeutic exploitation. It may also prevent the dire consequences of misuse, should this result in resistance to one or more of its protective activities.

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