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Cathelicidins—a rich seam of antimicrobial peptides waiting for exploitation

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Cathelicidins are a ubiquitous family of host defence antimicrobial peptides in vertebrate animals. Unlike other antimicrobial peptide families, it is defined by a large and relatively well conserved proregion rather than by the mature bioactive peptides themselves, which are highly diverse and conform to at least five different structural types, resulting in distinct modes of action. Cathelicidin-derived host defence peptides have a pleiotropic role in immunity, displaying both a direct antimicrobial activity and the ability to boost other host responses to infection and injury. The presence of a relatively well conserved proregion attached to a vast repertoire of structurally and functionally diverse peptides allows mining the increasing number of vertebrate genomes for lead sequences to potentially useful new anti-infective and/or immunomodulatory agents. This should increase the number of cathelicidin-based peptides entering clinical trials, which has been limited to date, despite considerable efforts in the last 2 decades.

KEYWORDS

cathelicidin, CRAMP, LL-37, PrAMP, host defence peptides, antimicrobial peptides, innate immunity

1 Introduction

Cathelicidins are vertebrate host defence proteins characterised by a large and relatively well conserved proregion associated with a highly variable antimicrobial peptide that becomes active upon proteolytic release (Zanetti et al., 1990; 1995; Scocchi et al., 1992; Mookherjee et al., 2013; Tossi et al., 2017). They form one of the most important and widespread vertebrate host defence peptide (HDP) families and are a prime example of the molecular diversity of antimicrobial peptides. Since their discovery by Romeo and co-workers in the early 1990s (Zanetti et al., 1995), cathelicidin-derived peptides have demonstrated a remarkably broad functional repertoire, with direct antibiotic activities reported against bacterial, fungal, viral and parasitic microorganisms, accompanied by the ability to orchestrate other aspects of the immune response to infection and modulate inflammation (Agier et al., 2015; Hancock et al., 2016; van Harten et al., 2018; Alford et al., 2020).

Abbreviations: AMP, antimicrobial peptide; CLD, cathelin-like domain; DFU, diabetic foot ulcers; hCAP18, human 18 kDa cathelicidin antimicrobial protein; HDP, host defence peptide; LPS, lipopolysaccharide; LTA, lipoteichoic acid; VDRE, vitamin D response element.

Strictly speaking, cathelicidin refers to the proforms (Zanetti et al., 1995), while the HDPs are referred to either by their origin (e.g., CRAMP for Cathelin-Related AMP) or origin and size (e.g., BMAP-28 for Bovine Myeloid Antimicrobial Peptide of 28 residues), or structural features (e.g., LL-37 from the first two sequence residues and size) (Tomasinsig and Zanetti, 2005). However, it has become customary to refer also to the active HDPs as cathelicidins. Over the last 3 decades, these have been intensely studied both for their role in vertebrate host defence and for their potential to develop new anti-infective agents for biomedical or veterinary purposes (Mahlapuu et al., 2016; Alford et al., 2020; Valdez-Miramontes et al., 2021; Dlozi et al., 2022; Zhu et al., 2022).

?A3B2 tlsb -.1pt?>The cathelicidins were discovered when Romeo's group found that several structurally distinct and seemingly unrelated bovine HDPs all appeared to be synthesized in myeloid cells as larger precursors, from which they are proteolytically released. Cloning studies showed they all share a homologous proregion (Zanetti et al., 1993) with significant sequence identity to the porcine protein cathelin (Ritonja et al., 1989), hence the name cathelicidin (Zanetti et al., 1995). This and other research groups shortly added small disulphide-bridged peptides (Romeo et al., 1988; Kokryakov et al., 1993), and linear Trp-rich (Selsted et al., 1992), Pro-rich (Frank et al., 1990) and helical peptides (Tossi et al., 1994; Agerberth et al., 1995) from cow, pig, rabbit, human and other mammals (Zanetti, 2004). Some mammals (especially artiodactyls) were found to have several different cathelicidins, while others (e.g., primates, glires, rodents or carnivores) only one, orthologous to the only human cathelicidin HDP LL-37 (Xhindoli et al., 2016). Cathelicidins were then found to be present in all analysed vertebrates (see Figure 1), and many of their HDPs have been characterized with respect to their structure and antimicrobial and other roles within host defence. They differ significantly in size, sequence, structure, physico-chemical properties and biological functions, so that their relationship is essentially due to the presence of the relatively well conserved cathelin-like domain (CLD) in their proforms.

This review provides a brief overview of the structural and functional features of cathelicidin proforms and HDPs, focusing on conserved aspects of the proregion and of genomic organisation that can facilitate mining of new HDPs with potentially interesting antimicrobial and/or immunomodulating functions. It provides a brief review of these functions, as well as considerations on their capacity to affect host cells, leading to beneficial or cytotoxic effects, and of their potential for the development of therapeutic agents. Many of these aspects have been the subject of several recent comprehensive reviews (Mookherjee et al., 2013; Hancock et al., 2016; Tossi et al., 2017; van Harten et al., 2018; Young-Speirs et al., 2018; Alford et al., 2020).

2 Distribution, expression and structural and functional characteristics of cathelicidins

2.1 Distribution and features of the proregion

Cathelicidins are ancient and widespread components of vertebrate innate immunity (see Figure 1), having been identified in basal vertebrate species (hagfish and lampreys) (Uzzell et al., 2003), all other types of fish (Masso-Silva and Diamond, 2014), amphibians (Hao et al., 2012), reptiles (van Hoek, 2014; van Hoek et al., 2019), birds (Wang et al., 2020; van Dijk et al., 2023) and all mammals (Mookherjee et al., 2013; Tossi et al., 2017; van Dijk et al., 2023). No cathelicidins are as yet reported in invertebrate animals. This implies that the cathelicidin gene family is at least 560 million years old, the estimated age of the latest common ancestor between vertebrates and lamprey/hagfish, according to TimeTree (Kumar et al., 2017).

Comparison of the HDP domains suggests that an ortholog of the human cathelicidin gene (*CAMP*, coding for the cathelicidin hCAP-18 and the HDP LL-37, see Figure 1) is present in all placental mammals, and often the only one present (primates, glires, carnivores and several other orders). For other orders (e.g., bats and perissodactyls) multiple cathelicidins are present and derive from duplication and diversification of the CAMP gene, while cetartiodactyls (ruminants, suids, camelids, hypopotamids and cetaceans) have a repertoir of structurally quite diverse HDPs, suggesting a more complex evolution (Xhindoli et al., 2016; Tossi et al., 2017; Zhu and Gao, 2017).

Cathelicidin genes consist of 4 exons, the first three encoding the preproregion and the fourth the HDP domain preceded by a variable number of proregion residues and the HDP cleavage site (see Figure 2). Despite indications otherwise in recent reviews, the HDP almost never begins in the 3rd exon. Mammalian cathelicidin genes cluster in syntenic chromosomal regions (Zhu, 2008a), and the presence of conserved flanking genes suggests this applies also to other vertebrates (see Table 1 in Section 3). Structural and sequence similarities place the proregion in the same superfamily as cystatins and kininogens, and it has been suggested that they derive from a common precursor (Zhu, 2008a), the cathelicidin gene having gained an additional 4th exon corresponding to the C-terminal HDP domain. The HDPs conform to several quite distinct structural types (Figures 2, 3).

The original cathelicidin gene may have carried a helical peptide, as these are the most common and widespread, and was then duplicated, as most non-mammalian vertebrates, marsupials and monotremes have multiple cathelicidins. Some placental mammalian orders still have only one gene, carrying a helical peptide. Others have multiple helical genes. In cetartiodactyl species diversification appears to be driven by the insertion of new and very diverse HDP sequences after duplication (Zhu and Gao, 2009) so that they present a repertoire of different structural types (see Figure 2), which on the basis of characteristic conformations or representative amino acid residues are indicated as Type-A, -B, -P, -U and - W. The HDP domain in any case appears to be positively selected for variation (Zhu and Gao, 2017), while molecular mechanisms such as gene conversion may instead have acted to maintain a low variation in the proregion (Tomasinsig and Zanetti, 2005; Zanetti, 2005).

The structures of porcine and human CLDs are similar (PDB IDs: 1KWI, 1PFP, 4EYC, see Figure 2) and closely resemble that of cystatin (3GAX), consistent with the conservation of key sequence motifs (Sanchez et al., 2002; Yang et al., 2003; Kolodziejczyk et al., 2010; Pazgier et al., 2013). A long helical segment at the N-terminus nestles into a concave β-sheet platform, stabilized by two conserved disulphide bridges, to

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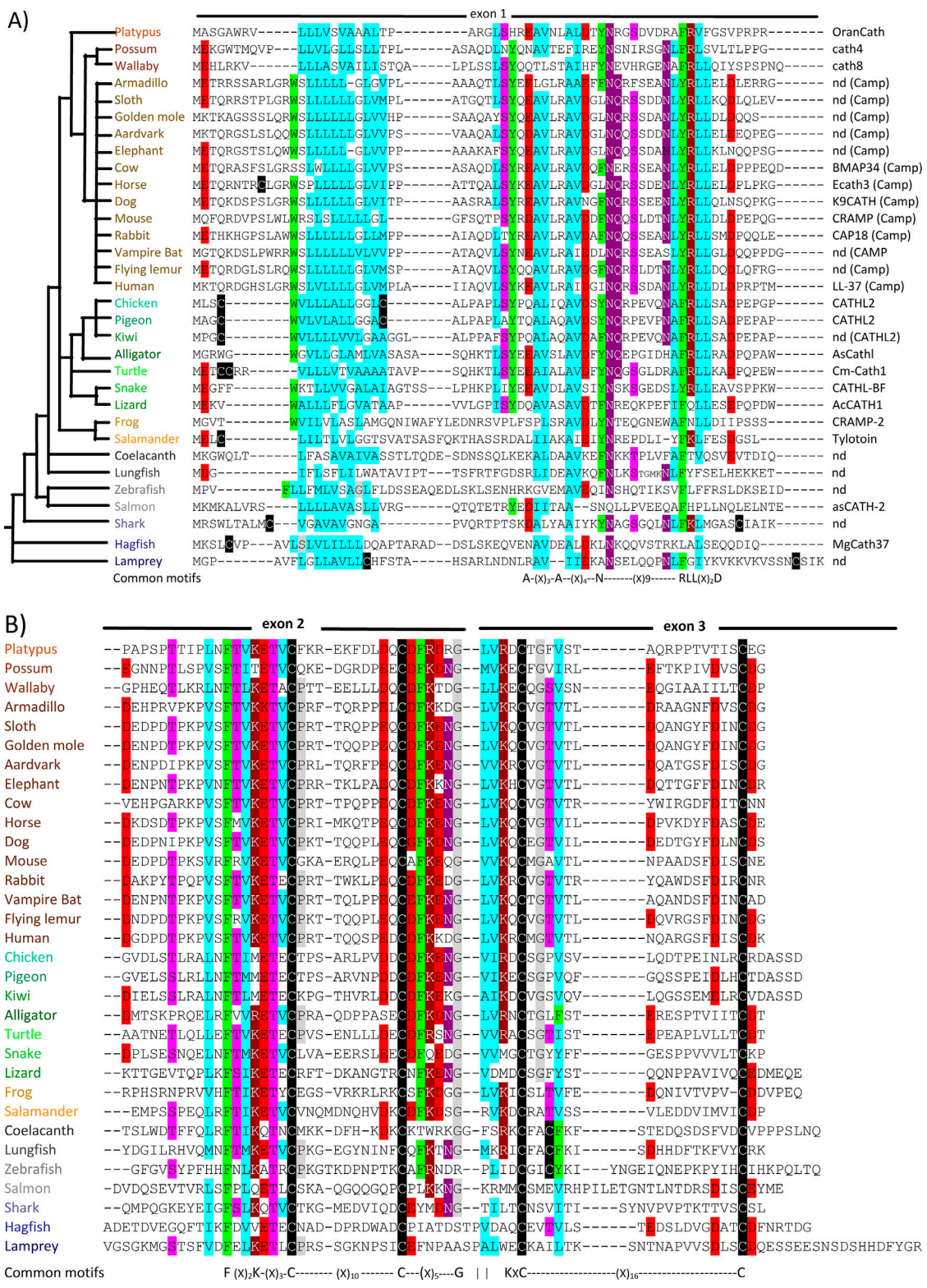

FIGURE 1
(Continued).

which the HDP is attached. The structures of the HDPs were determined separately (see Figure 3), and how these relate to the CLD in the proform has only been inferred by modelling (Sanchez et al., 2002). Although the CLD has a cystatin-like fold, it lacks key sequence elements required for cysteine protease inhibition (Pazgier et al., 2013).

FIGURE 1
(Continued). Examples of cathelicidin sequences selected from throughout the phylogenetic tree (shown top left of panel A) of vertebrate animals. The sequences are divided according to panel **A)** the encoding exon 1, panel **B)** exons 2 and 3 and panel **C)** exon 4 encoding the HDP. Gaps suggested by Clustall Ω are introduced to optimize alignment. Particularly conserved residues are highlighted in colours reflecting their physico-chemical characteristics (■ = Cys; ■ = anionic; ■ = cationic; ■ = polar; ■ = hydrophobic, ■ = aromatic; ■ = Pro, Gly). The cathelicidin name, if this has been assigned, is shown on the right of panel A), otherwise it is indicated as nd (not defined). Motifs useful for identifying cathelicidins (e.g. searching a chromosome or WGS assembly using a browser such as Artemis) are shown below the sequences. For exon 4 (panel C), the sequences of reported HDPs are in bold, underlined with a solid line, while those identified only at the gene level, have the putative HDPs in bold, underlined by a dashed line. For newly identified sequences, determining the mature HDP requires identifying the cleavage site for its release. HDPs were assigned to a structural type based on their primary structure, similarity to known cathelicidin HDPs or evident amphipathic helical structure as evidenced by HeliQuest (Gautier et al., 2008); Type-A[1] are orthologues of the human HDP LL-37, carried by the hCAP18 cathelicidin encoded by the *CAMP* gene. Sequences were obtained from the following groups (species): monotremes (platypus, XP_007655323); marsupials (possum, XP_007499738; tammar wallaby, ACJ76797); xenarthrans (armadillo, XP_004449765; sloth, XP_037684928); afrotherians (golden mole; XP_037684928; aardvark; XP_007949964; elephant, XP_003409939); laurasiatherians (cow, XP_027379316; horse CAA12228; dog, AAR26245); glires (mouse, AAB88303; rabbit, NP_001075774); chiropterans (vampire bat, XP_024421797); dermopterans (flying lemur, XP_00857229); primates (human; NP_004336); fowl (chicken, NP_001020001), neoavian (pigeon, AKN23387); ratite birds (kiwi, PTFC01000132); reptiles (alligator, XP_006037286; turtle, QED55073; lizard, CCI87995; snake, ACI22652); amphibians (frog, XP_018122443; salamander, AHF22104); lobe-finned fish (coelacanth, GAPS01055007; lungfish, XM_044065903); ray-finned fish (zebrafish, NP_001122247; salmon, NP_001117045); cartilaginous fish (shark, GFYY01017650; ray, XP_055520678); jawless fish (hagfish, AF452383, lamprey, XP_032830868. Species names can be obtained from the database entries.

The function(s) of the proregion remains controversial. It was initially hypothesised that it serves to keep the antimicrobial domain inactive until its release into the phagosome or extracellular medium (Scocchi et al., 1992; Sørensen et al., 1997; Zanetti et al., 2002), but it is questionable whether this alone justifies the conservation of the CLD. Proposals that the CLD has complementary antimicrobial activity to the HDP, or that it acts as a cathepsin inhibitor, are weakened by conflicting observations (Zaiou et al., 2003; Zhu, 2008a; Pazgier et al., 2013). In contrast, the hypothesis that the CLD serves as a pH-sensitive platform for the controlled proteolytic release of HDP at the right time and place (Sanchez et al., 2002) fits with the observations 1) that a substantial portion of the secreted human cathelicidin proform hCAP18 (see Figure 1) remains intact and bound to the surface of granulocytes or extracellular vesicles

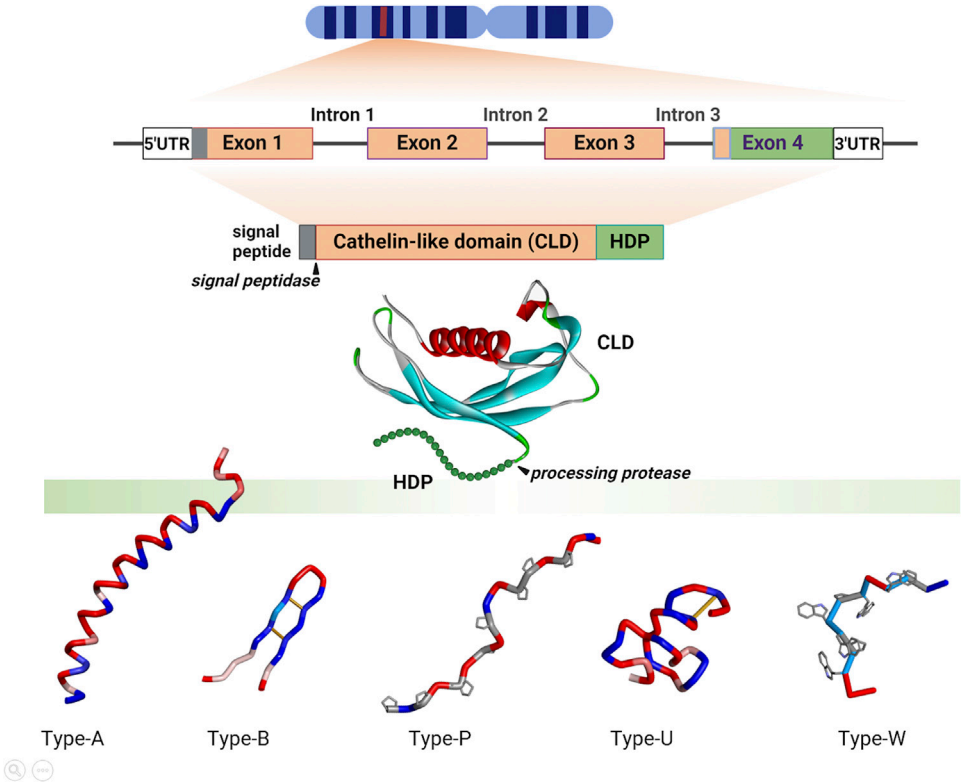

FIGURE 2
Schematic representation of cathelicidin gene, proform and HDP organisation and structures. The gene(s) encoding cathelicidin(s) are located on syntenic chromosomal regions with conserved flanking genes (see below Tables 1, 2). They are organized as four exons and three phase 0 introns, processed to the proform, with removal of the signal sequence by a signal peptidase before storage in granulocytes or secretion by epithelial cells. Mature HDPs of different structural types are released by extracellular or granule proteases (that differ depending on the organism and/or district). Some mammalian species express only one cathelicidin, which is invariably helical, other mammals and most non-mammalian vertebrates express multiple peptides. These can be α-helical (Type-A), disulfide stapled β-harpins (Type-B), Pro-rich peptides with extended conformations (Type-P) or Trp-rich peptides with wedge-shaped conformations (Type-W), or can be intrinsically unstructured (Type-U) as is the case of Gly/Ser-rich peptides found in some fish and reptile species. Structures were adapted from PDB coordinates using Biovia Discovery Studio 2021 [CLD, human cathelicidin pro-region (PDB 4EYC); Type-A, human LL-37 (PDB 2K6O); Type B, porcine protegrin (2MZ6); Type P, bovine Bac7 fragment 1-19 (5HAU); Type U, frog cathelicidin PY (2LR7); Type W, bovine Indolicidin (1G89)]. Created with the assistance of BioRender.com.

(Andersson et al., 2002; Stie et al., 2007), accompanying them to the site of infection and conferring a spatial specificity to HDP activation which concentrates the antimicrobial effect and minimizes cytotoxic effects, and 2) that relatively well conserved anionic residues form a strip on the surface of the CLD that would allow relevant interactions with the cationic HDP domain (Xhindoli et al., 2016). In addition, it would prevent active HDPs from being sequestered by plasma lipoproteins, which would occur if they were released too early (Wang et al., 1998; Sørensen et al., 1999).

2.2 Expression and processing

Cathelicidins are expressed in and secreted by various circulating immune cells or epithelial cells that respectively play an active role in host defence or form barriers against infection (Alford et al., 2020; Valdez-Miramontes et al., 2021). The expression pattern is varied, complex and regulated differently in different cell types and can be stimulated by both exogenous microbial components and endogenous factors, such as vitamin D in primates (Gombart et al., 2005; Lai and Gallo, 2009; Vandamme et al., 2012; van der Does et al., 2012). A similar expression pattern in leukocytes and epithelial cells is observed for bovine cathelicidins, but in this case the presence of several different genes allows differential expression at different sites (Tomasinsig et al., 2010; Kościuczuk et al., 2014; Whelehan et al., 2014). Avian and reptilian cathelicidins also generally derive from heterophils or epithelial cells (Alibardi, 2014; Chen et al., 2017; van Hoek et al., 2019; Wang et al., 2020), and are abundant in snake venom (de Barros et al., 2019). Cathelicidins are widely expressed in amphibian and fish tissues, both constitutively and upregulated by bacterial components during infection (Maier et al., 2008; Hao et al., 2012; Masso-Silva and Diamond, 2014). Manipulating this expression may help reduce the risk of infection in aquaculture environments, due to their direct antimicrobial and immunostimulatory capacities (D'Este et al., 2016).

In human and other mammals, cathelicidin gene products are channelled to storage granules, or secreted as proforms and the active HDP is released by serine proteases acting at appropriate cleavage sites. Elastase has been identified as the operational

TABLE 1 Genomic organisation of cathelicidin and flanking genes in vertebrate animals[1].

Class	Order	[2]N°	[3]Examples of gene organisation	GeneBank ID
Mammals	*Monotremes*	>10	*CATHL CATHL KLHL18 CATHL P15 MAP4 CDC25A CATHL CATHL MYO1G*	NC_041740, NC_052078
	Marsupials	1 - 11	*KLHL18 CATHL CATHL P15 MAP4 CDC25A CATHL CATHL MYO1G*	NW_018344027, NC_077233, NC_045426, NW_020954645
	Primates, Carnivores, Pholidota	1	*KLHL18 MAP4 CDC25A CAMP NME6*	NC_086015, NC_041755, NC_033673, NC_069787, NC_051824, NC_080019
	Rodents, Lagomorphs, Insecti_vores, Culogos, Treeshrews,	2	*KLHL18 P15 MAP4 CDC25A CATHL (CAMP) NME6*	NC_000075, NC_067382, NC_080173, NC_084470, NW_006159616
	[4]*Cetartiodactyls*	4 - 14	*KLHL18 P15 MAP4 CDC25A CATHL5 CATHL6 CATHL2 CATHL3 CATHL4 CATHL1 CATHL7 (CAMP) NME6*	NC_037349, NC_056072, NC_010455, NC_045712, NC_080198, NC_047034
	Perissodactyls	3 - 4	*KLHL18 P15 MAP4 CDC25A CATHL NME6*	NC_009159, NC_052197, NC_080741, NC=060267,
	Afrotherians	2 - 4	*KLHL18 P15 MAP4 CDC25A CATHL CATHL NME6*	NC_087363, NW_006921898, NW_006408642, NW_004443937, NW_022111373
	Xenarthrans	2 - 3	*KLHL18 P15 MAP4 CDC25A CATHL CAMP NME6*	NC_051307, NC_080698
	Chiropterans	2 - 8	*KLHL18 P15 MAP4 CDC25A CATHL NME6*	NC_071394, NC_046300, NC072490 , NC_040909, NW_005871595, NW_026521871
Reptiles & birds	*Neoavian bird orders*	1-4	*KLHL18 CATHL CATHL TBRG4*	Cheng et al., 2015
	Ratite orders	1 (?)	*KLHL18 CATHL TBRG4*	NW_020451127, NC_084664, NC_088099
	Crocodylia	1 - 6	*KLHL18 CATHL CATHL TBRG4 MYO1G CDC25A*	NC_081828, NW_005842192, NW_005842192, NW_005842192, NW_017729004
	Squamata (1)	2 - 7	*KLHL18 CATHL MYO1G TBRG4 CDC25A CATHL CATHL*	NC_085846, NC_088171, NC_056527
	(2)		*KLHL18 CATHL CATHL CDC25A TBRG4 MYO1G*	NC_080542, NW_024098263,
	Testudines	(?) 4	*KLHL18 CATHL CATHL TBRG4 MYO1G CDC25A*	NC_057850, NC_045558, NW_026844119

Organisation of cathelicidin and flanking genes in vertebrate animals (cont.).

Ampibians	*Anura*	2 - 12	*Cath-like KLHL18 CATHL TBRG4 MYO1G CDC25A*	NC_054381; NC_030682; NC_053493, NC_071091
Fish	*Ray-finned fish* (1)	>1	*KLHL18 ASIC1C CDK5 ABCB8 APG9 CATHL OBSCNB GUK1*	NC_059455; NC_044055; NC_049208
	(2)		*KLHL18 CATHL ASIC1C CDK5 ABCB8 APG9*	NC_081191; NC_054537; NC_053158
	Lobe-finned fish (1)	>1	*OBSCN GUK1 CATHL KLHL18 TBRG4*	NC_056731
			OBSCN GUK1 KLHL18 TBRG4 CATHL ABCB8 APG9	NC_088140
	Cartilagenous fish	1 - 2	*KLHL18 TBRG4 CATHL CDK5*	NW_024704743; NC_054472, NC_063335, NC_086097
	Jawless fish	>1	ND	

[1]Gene arrangement for the indicated animals was determined from available genomic data. *Monotremes* (echidna, platypus), *marsupials* (koala, possum, tasmanian devil, wombat); *primates, carnivores*, etc. (chimpanzee, macaque, lemur, loris, dog, pangolin); *glires, etc,* (mouse, rabbit, hedgehog, flying lemur, squirrel); *cetartiodactyls* (cow, sheep, pig, bactrian camel, hippopotamus, bottlenose dolphin); *perissodactyls* (horse, donkey, zebra, rhinoceros); *afrotherians* (african elephant, aardvark, golden mole, tenrec, manatee); *xenarthrans* (sloth, armadillo); *chiropterans* (vampire bat, horseshoe bat, large brown bat, leaf-nosed bat, little brown bat, fruit bat); *neoavian birds* (see reference in table); *ratities* (kiwi, rhea, emu); *crocodylia* (alligator, crocodile); *squamata* (1) *lizards* (2) *snakes*; *testudines* (sea turtle); *anura* (clawed frogs, common frog, common toad, spadefoot toad); *ray-finned fish* (1) *teleosts* (salmon, cod, eel), *holosteans* (gar), (2) *chondrosteans* (paddlefish, sturgeons), *cladistian* (bikirs); *lobe-finned fish* (lungfish, coelacanth); *cartilagenous fish* (ghost shark, great white shark, whale shark, sting ray); *jawless fish* (hagfish, lamprey).

[2]The number of genes is a rough estimate based on currently available data.

[3]▭ Cathelicidin-like protein genes; ▬ possibly linked genes; ▭▬ flanking; ▭/▬ in a nearby chromosomal region; ▭//▬ in a more distant or ▭///▬ very distant chromosomal region. *CATHL* = cathelicidin-like gene; *P15* = cathelicidin related 15 kDa protein gene (aka neutrophil granule protein, NPG). *KLHL18* = kelch-like protein 18; *TBRG4* = transforming growth factor beta regulator 4 (aka *FAST*, kinase domain-containing protein 4); *MYO1G* = unconventional myosin 1 G; *MAP4* = microtubule-associated protein 4; *CDC25A* = cell division cycle 25A phosphatase (aka M-phase inducer phosphatase 1); *NME6* = nucleotide diphosphate kinase 6; *ASIC1C* = acid-sensing ion channel 1C; *ABCB8* = ATP, binding cassette subfamily B member 8; *APG9* = autophagy 9-like protein 1 (aka. *ATG9*); *OBSCNB*, obscurin B; *GUK1* = guanylate kinase 1.

[4]The numbering scheme for cathelicidin genes of cetartiodactyl species is that used in GenBank.

protease in several mammals (Scocchi et al., 1992), while proteinase-3 and kallikrein act in humans (Murakami et al., 2004; Zanetti, 2005; Yamasaki et al., 2006). The putative cleavage sites of avian, reptilian, amphibian and fish cathelicidins suggest that elastase-like proteases are involved (Maier et al., 2008; Gao et al., 2015; Sun et al., 2015; Furlan et al., 2018; van Hoek et al., 2019), but the operational proteases are largely unknown. Processing can be quite complex, and vary in different tissues; human LL-37, when secreted from eccrine glands or keratinocytes, can be further processed to shorter fragments in a manner that modulates its biological activities (Murakami et al., 2004). Furthermore, some cathelicidin HDPs are C-terminally amidated due to the presence of a Gly residue

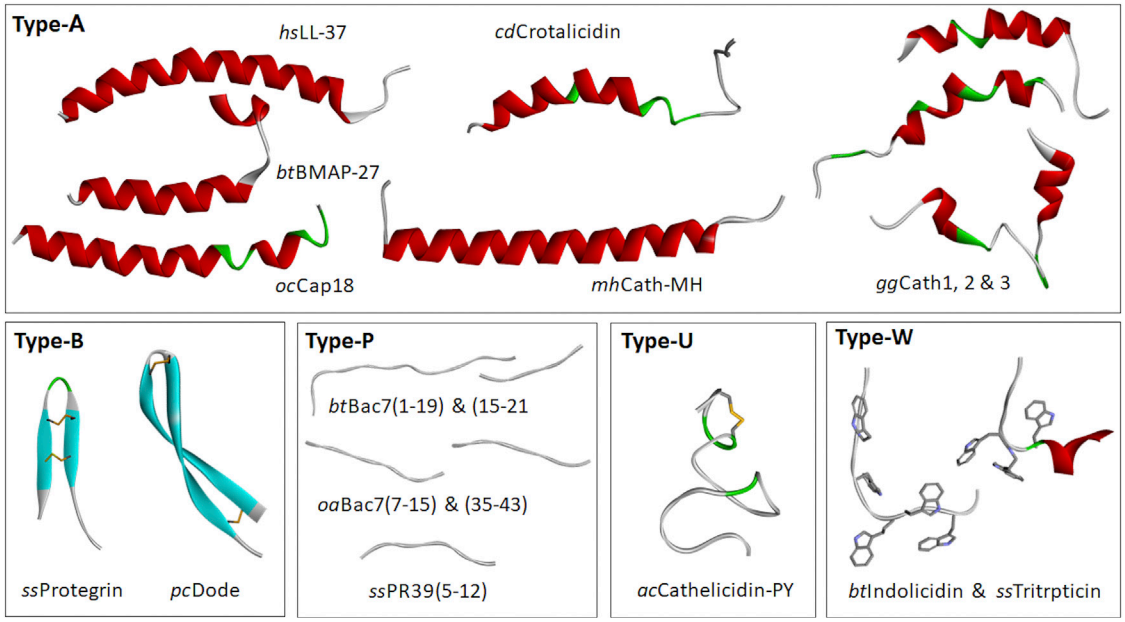

FIGURE 3
Selected cathelicidin HDP structures. These are divided into: Type-A, which display amphipathic, α-helical structures for at least part of their sequence [*Homo sapiens* LL-37, 2K6O; *Bos taurus* (cattle) BMAP27, 2KET; *Oryctolagus cuniculus* (rabbit) Cap18, 1LYP; *Crotalus durissus* terrificus (snake) Crotalicidin, 2MWT; Microhyla heymonsi (frog), CathMH; Gallus gallus (chicken) Fowlicidins 1, 2, and 3, (2AMN, 2GDL and 2HFR)]; Type B, with β-hairpin structures stapled by one or two disulphide bridges [*Sus scrofa* (pig) Protegrin (2MZ6); Physeter cathodon (whale) tandem dodecapeptide PcDode (7OSC)]; Type-P, with proline and arginine-rich extended structures [*Bos taurus* Bac7 (fragments 1-19, 5HAU, bound to bacterial ribosome, 15-21, 4JWD, bound to bacterial DnaK); *Ovis aries* (sheep) Bac7 (fragments 7-15, 4JWE and 35-43, 4JWI, bound to bacterial DnaK); *Sus scrofa* PR39 (fragment 5-12, 4EZO, bound to bacterial DnaK)]; Type-U, which are intrinsically unstructured and include some frog peptides and glycine- and serine-rich peptides from fish and reptiles [*Aquarana catesbeiana* Cathelicidin-PY (2LR7)]; Type-W [*Bos taurus* Indolicidin (1G89) and *Sus scrofa* Tritrpticin (1D6X)]. Structures were prepared using Biovia Discovery Studio 2021.

at the C-terminus or in a C-terminal sequences such as Gly-Lys-Arg or Gly-Arg-Arg, for example, see: (Agerberth et al., 1991; Selsted et al., 1992; Kokryakov et al., 1993; Zanetti et al., 1993; Skerlavaj et al., 1996).

2.3 Structural diversity of cathelicidin HDPs

Figure 3 shows some examples of cathelicidin HDP structures. The most common group is Type-A, with amphipathic helical structures, as found in hagfish, reptiles, amphibians, birds and mammals (see also Figure 1), suggesting that this is the ancestral type (Zhu, 2008a; Zhu and Gao, 2009). This conformation is also commonly found in AMPs unrelated to cathelicidins, and leads to a membranolytic antimicrobial mechanism (Tossi et al., 2000), suggesting convergent evolution of cathelicidin HDPs to this common function.

Cetartiodactyl cathelicidin HDPs show the greatest diversity of structural types (Tomasinsig and Zanetti, 2005; Scocchi et al., 2011). In addition to various Type-A peptides, there are long, Pro- and Arg-rich peptides with extended structures (Type-P), and small, wedge-shaped, Trp-rich peptides (type W) (see Figure 2) (Schibli et al., 1999; Rozek et al., 2000; Tomasinsig and Zanetti, 2005). Another type of cathelicidin HDP peculiar to this mammalian order are small peptides with β-hairpin conformations stapled by one or two disulphide bonds (Type-B). These include the bovid and cetacean dodecapeptides and the porcine protegrin, which exists in several allelic forms (PG-1 to −5) (Choi et al., 2014). Some fish and amphibian cathelicidins also show paired cysteine motifs, but in conjunction with other structural types (e.g., Type-U, see below).

While the majority of cathelicidins from ray-finned fish have sequences similar to that shown for zebrafish in Figure 1, they sometimes bear long, linear peptides that are particularly rich in Gly and Ser residues (Scocchi et al., 2009b). These are found especially in salmonids, and can be divided into two subgroups, with the presence of two cysteine residues in the N-terminal part of the HDP region in *Cath1* type peptides, which is absent in *Cath2* type peptides (Maier et al., 2008). The GS-rich stretch likely remains unstructured even in membrane-like environments, suggesting they are Type-U low complexity, intrinsically disordered sequences (D'Este et al., 2016). GS-rich cathelicidin peptides are also found in some amphibian species. In some cases, cathelicidin HDPs have structures conforming to several structural types. This is the case of the frog peptide Cathelicidin-PY (see Figure 3), which has a very short helical stretch and disulphide bridge within an essentially Type-U structure (Wei et al., 2013).

Cathelicidin HDPs are being identified in an increasing number of non-mammalian species, and it is not always facile to predict the structural type (see Table 1). In some cases, a low cationicity is apparent, which may be an indication that a direct antimicrobial action may not be the principal function.

2.4 Structure-dependent mode of action of cathelicidins

As described above, cathelicidin HDPs essentially conform to the five structural types shown in Figure 3, although some may have features of more than one type. A common aspect is a pronounced cationicity that favours their interaction with bacterial membranes upon proteolytic release from the proform, and many indeed proved to be membrane active, but for some the antimicrobial effect is not primarily based on disruption of the bacterial membrane. The mode of action is summarized schematically in Figure 4, where release and approach to the membrane are shown as step (1).

Type A cathelicidin HDPs are considered primarily membranolytic. Their cationic and amphipathic structure enables efficient interaction with, and insertion into the microbial membrane. The active conformation may form only upon contact with the membrane, where the peptides undergo a transition from an unstructured globule to a helical conformation partly inserted into the lipid bilayer [Figure 4 (2)], whereupon either a detergent-like disruption occurs when a critical concentration is reached (carpet model) (3), and/or cooperative formation of discrete cavities occurs (toroidal pore mechanism) (4) (Tossi et al., 2000). In some cases, peptides can adopt the amphipathic helical conformation in bulk solution, under physiological conditions, leading to oligomerization, so that they approach the membrane as helical bundles (5). These mechanisms may both contribute to bacterial killing, but peptides may favour one mechanism over the other. For example, rhesus RL-37 does not oligomerize and favours the carpet type mechanism (3), whereas the closely related human LL-37 oligomerizes and favours pore formation (4) (Morgera et al., 2009; Xhindoli et al., 2014; 2016). Another consequence of oligomerization is that the preformed helices are susceptible to sequestration by interacting with bacterial membrane or serum components (6), significantly affecting antimicrobial potency and sensitivity to the environment (Tomasinsig et al., 2009; Xhindoli et al., 2014).

With regard to Type-B peptides, the interaction of porcine protegrin with membranes has been extensively studied, and they are reported to act via a membranolytic mechanism (Bolintineanu et al., 2012; Lazaridis et al., 2013; Usachev et al., 2016). They interact

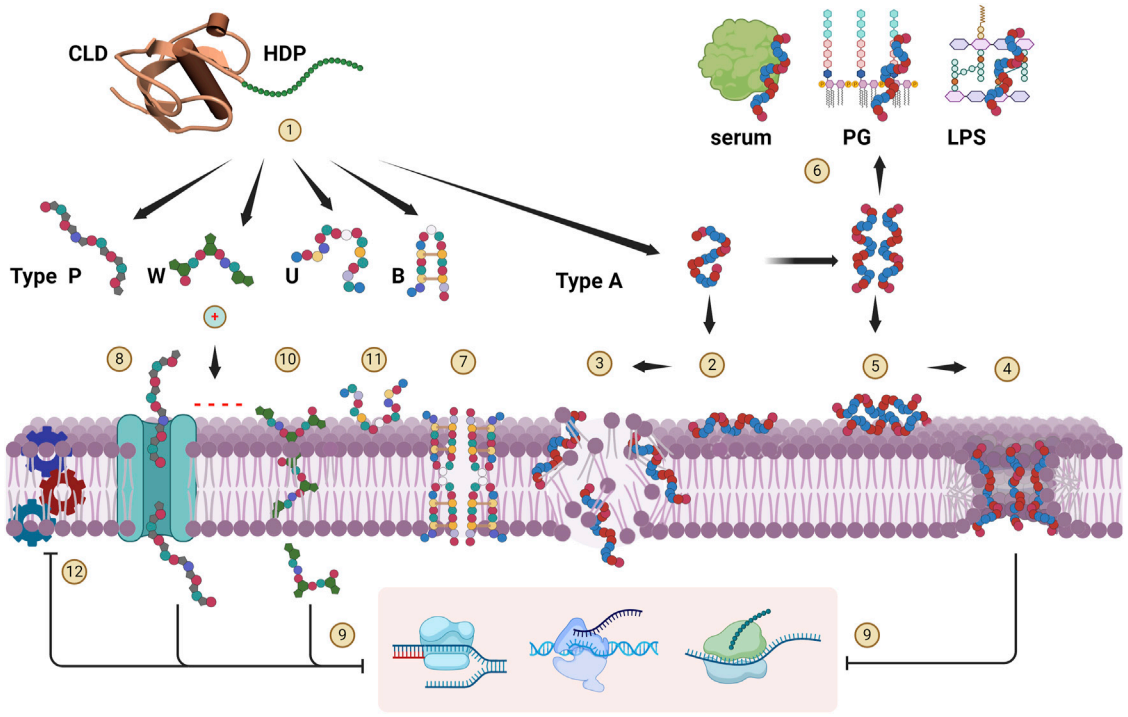

FIGURE 4
Cathelicidin HDP modes of action. Upon release from the CLD (1), cationic HDPs are attracted to the anionic surface of bacterial membranes. Type-A peptides tend to be disordered, and adopt an amphipathic, helical conformation at the membrane surface and insert into it (2). When a critical concentration is reached, they can breach the membrane bilayer in various ways. They can act in a detergent-like manner (carpet mechanism) (3), or form discrete toroidal pores (4). Helical peptides such as human LL-37 can adopt a helical structure already in bulk solution, which promotes aggregation so that they approach the membrane as oligomeric bundles (5), favouring pore formation. They are however more susceptible to sequestration interactions with serum or medium components and bacterial peptidoglycan (PG) components (e.g., LTA) or lipopolysaccharide (LPS) (6), so their antimicrobial activity is quite salt- and medium-sensitive. Type B peptides are reported to penetrate the membrane, oligomerize and form multimeric pores (7). Type P peptides can internalize into some bacteria via specific transport proteins (8) and inhibit cytoplasmic targets (9) (specifically ribosomal subunits) but can also act by membrane lysis at higher concentrations (mechanism 3). Type W peptides are reported to penetrate into the bacterial cytoplasm without compromising the membrane (10), where they then act by selective inhibition of DNA transcription. Inhibition of replication and/or transcription (9) has also been proposed for some Type A peptides once they enter the cell via mechanisms (3) or (4). Intrinsically unstructured type U peptides act on the bacterial surface (11), and although their mechanism of action is not yet known, the antimicrobial activity is quite salt-sensitive and not primarily membranolytic. All HDP types likely saturate the bacterial membrane surface at active concentrations, and inhibition of the membrane protein machinery (12) has been hypothesised to be an important aspect of their antimicrobial action. Created with BioRender.com.

with the membrane as monomers and then oligomerise to form an octameric pore [Figure 4 (7)] (Lazaridis et al., 2013; Usachev et al., 2016). Bovine dodecapeptide may also form S-S-stabilized β-hairpins in solution and dimerize at the bacterial surface (Raj et al., 2000), acting in a similar manner. However, the mode of action is made more complex by the possibility that it forms covalent dimers involving intermolecular disulfide bridge formation (Storici et al., 1996). Interestingly, in this case a parallel or antiparallel covalent dimeric arrangement does not seem to have a dramatic effect on the antimicrobial activity with respect to that of the β-hairpin monomer (Lee et al., 2008).

Type-P cathelicidin HDPs, which belong to the structural group of proline and arginine rich AMPs known as PrAMPs, have a distinct mode of action that relies less on membrane disruption (Scocchi et al., 2011; 2016; Li et al., 2014). Their extended structures do not change significanty upon contact with bacterial membrane surfaces, where they accumulate and then internalize into the cytoplasm also using specific membrane transporters, where they inactivate internal targets [Figure 4 (8) and (9)]. This mechanism has been shown to apply also to unrelated proline-rich peptides from arthropods, another example of convergent evolution of common strutural and functional features (Scocchi et al., 2011; Krizsan et al., 2014). This mechanism is selective with respect to the target bacteria, as only those expressing the transport system are strongly affected (e.g., Gram-negative bacteria such as *E. coli, S.* Typhimurium and *A. baumanii,* but not *P. aeruginosa* or any Gram-positive bacteria), and ii) stereoselective with respect to the peptide itself (Runti et al., 2013; Guida et al., 2015; Scocchi et al., 2016). In contrast to membranolytic peptides, in which the all-D enantiomer works just as well, for Type-P peptides it loses activity. This is likely due to stereoselective requirements for transport and/or for inactivation of the cytoplasmic target (Guida et al., 2015), the bacterial ribosome (Mardirossian et al., 2014; 2018a; Gagnon et al., 2016; Graf et al., 2017). Another bacterial protein that Pro-rich peptides in general bind to, including cathelicidin HDPs, is the chaperone DnaK (Cudic and Otvos Jr, 2002; Scocchi et al., 2009a).

Type-W peptides are so far limited to bovid indolicidins and porcine tritrpticin (see Figure 2) and act by another distinct mechanism. Due to the presence of Trp residues, they have a strong tendency to interact with bacterial membranes which they cross to hit internal targets [Figure 4 (9) and (10)]. They have a wedge-shaped conformation and after partitioning near the membrane-water interface then appear to enter the bacterial cell without significantly compromising membrane integrity, to then selectively inhibit DNA synthesis (Hsu et al., 2005; Chan et al., 2006; Ghosh et al., 2014; Shagaghi et al., 2016; Batista Araujo et al., 2022). It should be considered that indolicidin has 3 Pro residues so may have characteristics of P-type, and that cetacian Type-P peptides are also quite rich in Trp residues, so they may represent a cross between the two structural types and allow a more efficient penetration even in the absence of a suitable transporter (Mardirossian et al., 2018b; Sola et al., 2020).

Type-U peptides are mainly found in non-mammalian vertebrates and have the least well defined mechanism of action, which again appears to be distinct from the others, emphasising that cathelicidin HDPs cover a very broad structural and functional space. They contain Gly and Ser rich sequences (see Figure 1 for examples), which in salmonid fish are usually quite long and sometimes heterogeneous, with cysteine-bridged motifs or other types of flanking domains (Scocchi et al., 2009b). As a result, the mode of action has been studied for rationally selected GS-rich fragments rather than the whole peptide, and it appears that their intrinsically unstructured extended conformations are not strongly affected by membrane interaction (Broekman et al., 2011; D'Este et al., 2016). Moreover, they have a relatively low proportion of hydrophobic residues, so they probably only interact with the surface of membranes and do not insert into them [Figure 4 (11)]. This is consistent with their killing mechanism, which is quite salt sensitive, although the microbicidal mode of action is still unclear. Little is known about the mode of action of anuran GS-rich HDPs, but they probably have similar properties (Hao et al., 2012).

Regardless of the mode of action, all types initially interact with the membrane, and since their active concentrations are in the micromolar range, they completely saturate the bacterial surface (Loffredo et al., 2021), so they are likely to come into contact with vital protein machineries located in the bacterial membranes. This would affect bioenergetics, transport and maintenance of the cell wall. This is known as the *sand-in-the-gearbox* effect (Pag et al., 2008; Vaezi et al., 2014), and could be a relevant component of their killing mechanism [Figure 4 (12)].

2.5 Pleiotropic roles of cathelicidins in host defence

Most cathelicidin HDPs are first tested for their direct antimicrobial activity *in vitro,* even though this is not necessarily their main role in host defence. They often also show a significant ability to influence other aspects of immunity and healing, such as binding and sequestering bacterial components (e.g., LPS or LTA), recruiting or modulating the activities of cellular components of innate and adaptive immunity, and stimulating cell growth in wound healing (Lai and Gallo, 2009; Linde et al., 2013; Hancock et al., 2016; van Harten et al., 2018; Alford et al., 2020). The literature on direct antibiotic and immunomodulatory activities is extensive, partly due to the countless variants that have been developed over the years to investigate structure/function relationships or in an attempt to optimise activity for potential therapeutic applications.

In summary, the direct antibiotic activities of Type-A and -B cathelicidin HDPs, which act via membranolytic mechanisms, tend to be more potent and broad-spectrum *in vitro.* However, outside their physiological context, this is accompanied by appreciable toxicity for eukaryotic cells at their active concentrations, often measured in terms of their haemolytic activity. For this reason, when peptides of these types have been investigated as therapeutic agents, they exhibit appreciable toxicity close to their active concentrations. This also seems to be the case for Type-W peptides. Type-P peptides tend to be significantly less cytotoxic, but have a narrower activity range (Scocchi et al., 2016). Type U peptides are the least well characterized.

It is interesting to note that a certain ability to modulate host cell activities has been found for all structural types. Helical peptides, and in particular LL-37 and mouse CRAMP, have a wealth of reported activities, including attracting immune cells to the site of infection, modulating inflammatory responses via release of cytokines, binding to and inactivating endotoxins, promoting

wound healing, etc (Kahlenberg and Kaplan, 2013; Fabisiak et al., 2016; Xhindoli et al., 2016; Krepel and Wang, 2019). Protegrin analogues (Type-B), indolicidin (Type-W) and Pro-rich (Type-P) cathelicidins also show analogous activities, despite their significant structural diversity (Bowdish et al., 2005; Djanani et al., 2006; Tomasinsig et al., 2006; Kin et al., 2011; Veldhuizen et al., 2014; Zughaier et al., 2014; Gupta et al., 2015; Nakagawa and Gallo, 2015). In general, immunomodulatory activities on host cells are thought to be due to receptor activation, but how cathelicidin HDPs exerts their action is not well understood. It could be that some of them act in a non-canonical manner by accumulating in the membrane surrounding various receptors, possibly favouring the cholesterol and sphingomyelin-rich lipid rafts where receptors often reside, and affecting their transmembrane domains rather than interacting with specific ligand binding sites. This is consistent with a frequently observed promiscuous and generally low-affinity activity, and is supported by the fact that at least for LL-37, activation of some receptors by the all-*D* enantiomer is as effective as the native enantiomer (Xhindoli et al., 2016). Different stereochemistry would not allow a similar interaction with a binding site, whereas the ability for helical structuring, oligomerization and membrane interaction is analogous.

3 Genomic organization and mining for cathelicidins

3.1 Bioinformatic strategies for the identification of novel cathelicidins

The *Vertebrate Genome Project* explicitly aims to generate the reference genomes of all nearly 70,000 extant vertebrate species (Rhie et al., 2021), providing new opportunities for data mining approaches that facilitate the identification of novel cathelicidins while bypassing traditional, labour-intensive isolation techniques. However, the primary sequence diversity among cathelicidin precursors and high variation of the HDP region make this task far from trivial, as with other bioactive peptides (Coelho et al., 2024). Despite conserved aspects of the proregion (see Figure 1), cathelicidins within the same vertebrate class can have up to 80% dissimilarity at the amino acid level. Therefore, although BLAST based methods have proven to be reliable for identifying new members when applied to phylogenetically closely related species (Zhu, 2008b; Ishige et al., 2017; Kim et al., 2017; Lastra et al., 2018; Choi et al., 2022; Kannoth et al., 2023), they may be inadequate in understudied or highly divergent animal groups. Although the reliability of these approaches can be improved by the use of strict orthology inference methods (Castellanos et al., 2023), they are not robust for vertebrate clades in which cathelicidin sequences are as yet poorly represented. The relatively limited number of cathelicidin sequences documented in fish, although coding genes are widely distributed, likely stems from these technical constraints.

In this respect, methods based on Hidden Markov Model (HMM) prove more efficient, as they allow to recognize common structural attributes inherent to all members of a given protein family, which facilitates an appropriate weighting of the evidence provided by conserved sites associated with specific positions, such as those defining the CLD. A benchmark HMM profile for cathelicidins is provided by Pfam entry PF00666, which belongs to the PepSY clan that also includes structurally similar molecules such as cystatins (Kordiš and Turk, 2009). Nonetheless, the cathelicidin HMM profile is based on the alignment of a limited data set of sequences that suffers from a taxonomic bias skewed heavily towards mammals. Consequently, while it has proven to be robust in identifying novel cathelicidins within mammalian and avian species (Cheng et al., 2015; Zhang et al., 2019; Xiao et al., 2020), it was less effective when applied to other vertebrate classes. Indeed, many authentic cathelicidin sequences from fish would escape unequivocal identification using this method, either due to overlap with the HMM associated with cystatins (PF00031), or failure to meet the standard detection threshold for significance. More advanced approaches can iteratively build a new HMM profile from scratch, a process that relies on the initial identification of reliable seed sequences through BLAST searches and may involve the use of the jackhammer tool from the HMMER package (Finn et al., 2011). This sophisticated strategy has recently demonstrated its efficacy and enabled the detection of numerous previously undiscovered cathelicidin genes in frogs (Tang et al., 2024).

Regardless of the chosen strategy, the ability to detect novel sequences strongly depends on the quality of annotation of the selected genomes, which varies greatly depending on the bioinformatics pipeline used and available supporting data. In practice, annotation of cathelicidin loci in many vertebrates lacks precision, with predictions being absent, incomplete and occasionally incorrect. For example, several genuine cathelicidin genes in non-mammalian species are mislabelled either as "kininogen" or as "secreted phosphoprotein 24," which have cathelin-like regions, severely hampering data mining approaches based on keyword searches (Hu et al., 1995; Zhou et al., 2009; Pérez de la Lastra et al., 2021). Furthermore, a correctly identified proregion (exons 1–3) can be linked to an incorrect 4th exon encoding the HDP, as this is the most variable region.

From this perspective, the analysis of *de novo* assembled transcriptomes can be useful. First, it allows the identification of the complete sequence of the protein precursor without uncertainties regarding the correct identification of splicing acceptor and donor sites. Second, it is a concrete indication of the likely biological relevance of the encoded protein, as it distinguishes functional genes from cathelicidin-like pseudogenes that are often reported (Whelehan et al., 2014; Cheng et al., 2015; Zhang et al., 2019; Peel et al., 2021; van Dijk et al., 2023). This approach was initially successfully used to screen Expressed Sequence Tags (EST) datasets (Xiao et al., 2006), and has recently allowed the identification of novel cathelicidins in several animal species (Helbing et al., 2019; Zhong et al., 2020; Kannoth et al., 2023). On the other hand, the sole availability of transcriptome data is limiting, as it depends on the genes being expressed in the tissue that was selected for sequencing, and if similar paralogous genes are present the risk of overlooking some of them due to chimeric assemblies increases.

For genomics studies, information on synteny helps identifying cathelicidin gene clusters in newly released genomes. While significant structural genomic rearrangements are not uncommon in nature, there are indications that the genes flanking the cathelicidin gene clusters are generally well conserved (see below), at least within the same order, and often across higher

taxonomic ranks as well. Therefore, locating the genomic region between these molecular markers, even in the absence of available gene annotations, can help identify novel cathelicidin genes (Cheng et al., 2015; van Hoek et al., 2019), by also exploiting the conserved architecture of all cathelicidin genes which comprises four exons and three phase 0 introns. This approach can leverage a combination of methods based on sequence homology detection and alignment with RNA-seq data to delineate putative exons. However, manual curation remains a crucial aspect for validating these predictive methods and correcting any errors.

3.2 Genomic organization of cathelicidins

The success of bioinformatic methods such as BLASTing of genome or transcriptome databases with known sequences or HMM profiles depends on the availability of a sufficiently representative number of known or inferred sequences. In this respect, InterPro entry PF00666 contains almost 1100 such sequences, which may include homologs from closely related species to the ones being searched. Otherwise, only some or no cathelicidin genes may be found, especially in vertebrate groups where cathelicidins are poorly represented. In these cases, it may be useful to rely on conserved flanking genes to facilitate identification. For example, we have found that in placental mammals the major cathelicidin gene cluster is located between *NME6* and *CDC25A* and/or *MAP4* (see Table 2), which is also confirmed in the literature (Ahn et al., 2022). In birds and reptiles, the indicated flanking genes are *TBRG4* (a.k.a. *FASTK*) and *KLHL18,* respectively (Cheng et al., 2015; van Hoek et al., 2019). Based on this information, it was possible to scan well-annotated vertebrate genomes from different clades to build a picture of syntenic clusters and identify several other linked genes that either surround or are in proximity to cathelicidin gene clusters (Tossi et al., unpublished results), as shown in Table 2.

Among mammals, monotremes exhibit the most diverse organization of cathelicidin-like gene clusters, with two closely spaced clusters flanked on one side by *KLHL18*, suggesting a link to syntenic clusters in non-mammalian vertebrates, and on the other side by *MYO1G*, with *MAP4* and *CDC25A* in between, suggesting a link to syntenic clustering in placental mammals. A third cluster is located on the other side of *KLHL18*. The latter appears to be absent in marsupials, but otherwise the cathelicidin gene organization is similar. In placental mammals, two clusters are present in most orders, the principal one being flanked by *MAP4/CDC25A* on one side and *NME6* on the other. The second cluster contains only the cathelicidin-like protein P15, also known as neutrophil granule protein, which was described some time ago as a divergent member of the cathelicidin family in rabbits. It does not release an antimicrobial HDP but rather synergizes with other immune proteins and binds LPS (Levy, 1996). P15 cathelicidin genes appear to be present in most placental mammals except for primates, carnivores and pangolins (see Supplementary Figure S1) that appear to have one only cathelicidin that is orthologous to the human *CAMP* gene.

Birds appear to have a single cathelicidin cluster flanked by *KLHL18* and *TBRG4*, with the number of genes (1–4) varying according to order and species (Cheng et al., 2015). Reptiles have a more complex arrangement of cathelicidin genes - in general, crocodilians, snakes and turtles have a cathelicidin gene cluster flanked by *KLHL18* on one side, and with *MYO1G, TBRG4* and *CDC25A* at a short distance on the other side. For lizards, this cluster is reduced to one or two genes and a second larger cathelicidin gene cluster is located on the other side of *CDC25A*. Amphibians have a similar cathelicidin gene arrangement, but with the second large cluster on the other side of *KLHL18*.

The cathelicidin gene clusters of fish may be flanked by different genes than in other vertebrates, and determining the arrangement is complicated by the lack of annotations. In Teleostean and Holostean orders of ray-finned fish the cathelicidin gene cluster is located near *KLHL18*, whereas in Chondostrean and Cladistian orders it is located quite far from it and flanked by genes such as *ASIC1C, CDK5, ABCB8, APG9* on one side and *OBSCN* and *GUK1* on the other. In lungfish the cathelicidin gene cluster is flanked by *OBSCN* and *GUK1* on one side and far from *KLHL18* and *TBRG4* on the other. With

TABLE 2 Organization of cathelicidin gene clusters with respect to that of genes that are apparently linked to them.

Group	Cathelicidin gene cluster organization				
Eutherians		NBEAL2; KIF9; KLHL18 (P)		// MAP4; CDC25A © NME6	
Marsupials	OBSCN; GUK1 ///	NBEAL2; KIF9; KLHL18 ©(P)		// MAP4; CDC25A © MYO1G	/// ABCB8; CDK5
Monotremes	OBSCN; GUK1 ///	© NBEAL2, KIF9; KLHL18 ©(P)		// MAP4; CDC25A © MYO1G	/// ABCB8; CDK5
Birds	OBSCN; GUK1 ///	NBEAL2; KIF9; KLHL18 ©	TBRG4; MYO1G	// MAP4; CDC25A	/// ABCB8; CDK5
Reptiles	OBSCN; GUK1 ///	NBEAL2; KIF9; KLHL18 ©	TBRG4; MYO1G	/ MAP4; CDC25A	/// ABCB8; CDK5
Amphibians	OBSCN; GUK1 ///	© / KIF9; KLHL18 © //	TBRG4; MYO1G	/ MAP4; CDC25A	
Lobe-finned fish	OBSCN; GUK1	© // NBEAL2; TBRG4; KLHL18 ©	ABCB8		
Ray-finned fish		OBSCN; GUK1 ©	ABCB8; CDK5		
Cartilaginous fish		KLHL18; KIF9; NBEAL2; TGRB4 ©			/// ABCB8; CDK5
Jawless fish		nd © nd			

©cathelicidin gene cluster; (P) cathelicidin-like P15: /, // and /// roughly indicate the relative distance of flanking genes as explained in footnote 3, Table 1.

coelacanth there is a cathelicidin gene cluster between *KLHL18* and *TBRG4* on one side and *ABCB8* and *APG9* on the other. Cartilaginous fish generally have one or two cathelicidin genes. One type of gene that is always present and close to *KLHL18* and *TBRG4* encodes a rather odd cathelicidin in which the fourth exon consists of only one or two residues and therefore has no HDP domain. This is the only gene present in many sharks, whereas in rays there is a second gene in which the fourth exon encodes a small peptide. It is more distant from *KLHL18* and *TBRG4* and has *CDK5* at some distance on the other side. For jawless fish, there are not enough well-annotated genomes for a comprehensive analysis, but the cathelicidin gene cluster does not seem to be linked to any of the genes found for other vertebrates.

Although the arrangement of cathelicidin genes shown in Table 1 differs somewhat between vertebrates from different orders it suggests a degree of syntenic conservation. Cathelicidin genes are largely located in clusters within a single chromosome, and the same types of linked genes generally occur on the same chromosome. The overall arrangement can be better appreciated in Table 2, which considers the approximate positioning of the cathelicidin gene clusters and possibly linked genes.

4 Therapeutic potential of cathelicidins

Since their discovery, the potential of antimicrobial peptides for therapeutic purposes has been recognised and widely discussed in the literature. After encouraging pre-clinical testing in *in vitro*, *ex vivo* and animal model studies, several AMPs or their synthetic derivatives have been investigated in human clinical trials, including cathelicidin peptides and analogues (Koo and Seo, 2019; Browne et al., 2020; Dijksteel et al., 2021; Moretta et al., 2021). Focusing on trials involving cathelicidin HDPs, despite significant efforts that have been made over the past 2 decades, none have as yet reached clinical use. In fact, some trials are still ongoing while others have been discontinued due to lack of efficacy, unfavourable pharmacokinetic profiles, adverse effects or failure to show improved efficacy with respect to conventional treatments.

Among the first cathelicidins to be developed and tested for potential clinical use were the Type-B peptide iseganan, based on protegrin, and Type-W peptide omiganan based on indolicidin (Toney, 2002; Isaacson, 2003) (see Supplementary Table S1). Both HDPs underwent a complex optimization processes to make them useful for therapeutic purposes, which involved a series of modifications such as residue replacements to increase cationicity or to modulate the hydrophobicity, introduction of non-proteogenic residues or cyclization and C-terminal capping or inverting and/or enantiomerizing the sequence to improve both the stability and activity (Chen et al., 2000; Staubitz et al., 2001; Rozek et al., 2003; Ryge et al., 2004; Ando et al., 2010).

As membrane active molecules, these peptides are therapeutically oriented towards topical use, and iseganan was initially investigated in a clinical trial for preventing oral mucositis in cancer patients undergoing radiation therapy for head and neck cancer, but failed in Phase III due to an apparent lack of efficacy (Giles et al., 2003; Trotti et al., 2004). However, a later study on patients undergoing chemotherapy found it significantly reduces the total oral aerobic bacterial and fungal load in patients undergoing stomatotoxic chemotherapy, showing a clear potential as an oral antimicrobial agent, and should be re-evaluated in the context of increasing resistance to conventional anti-infective agents (Dijksteel et al., 2021; Liang and Sonis, 2024).

Omiganan pentahydrochloride is a 12-mer derivative of indolicidin that demonstrated *in vitro* activity against a significant number of infective clinical isolates, including most ESKAPE pathogens and *Candida spp*. (Sader et al., 2004; Żyrek et al., 2021). It underwent clinical trials for different topical applications including the prevention of catheter-related infections, and treatment of acne, rosacea, atopic dermatitis and papillomavirus-induced genital lesions (Fritsche et al., 2008; Zouboulis et al., 2017; Niemeyer-van der Kolk et al., 2020) and is considered also for vulvovaginal candidiasis and other fungal infections (Rubinchik et al., 2009; Czechowicz et al., 2021; Żyrek et al., 2021). The results from these trials are mostly not yet disclosed or under review so it is not possible at the moment to assess their success.

With regard to Type-A peptides, a lot of interest has been placed on the human cathelicidin HDP LL-37, both as a potential therapeutic agent or as a marker for health or pathological conditions. For example, its levels have been correlated with conditions such as psoriasis, atopic dermatitis and periodontal disease (Kahlenberg and Kaplan, 2013; Lande et al., 2014; Hancock et al., 2016; Turkoglu et al., 2017; Antal et al., 2022; Bhattacharjya et al., 2024). This has prompted numerous clinical studies that are not only evaluating it as a drug for the treatment of pathologic conditions but also investigating its potential role as a biomarker (see Supplementary Table S1). Currently, LL37 is under clinical investigation for the treatment of hard-to-heal venous leg ulcers (HTH VLUs) in the Swedish study LL-37001B (EudraCT: 2012-002100-41). These are amongst the most prevalent type of chronic wounds and affect approximately 1%–3% of the older population in Western countries (Franks et al., 2016). The investigation was prompted by the observation that endogenous LL-37 is present in large quantities in acute wounds, whereas it is absent in chronic wounds, suggesting a critical role of this peptide in the healing process (Heilborn et al., 2003; Fabisiak et al., 2016). Moreover, it was observed that skin-targeted administration of LL-37 in *ex* and *in vivo* models of human acute and chronic wounds improved reepithelization and closure (Carretero et al., 2008; Steinstraesser et al., 2014). Results from phase I/II trials showed that local application of LL-37 twice a week significantly enhanced the healing rate without causing any systemic safety or local tolerability concerns (Grönberg et al., 2014). Subsequently, a phase II multicentric prospective trial (EudraCT: 2018-000536-10) indicated improved healing in relatively large wounds with a negative prognostic factor for healing (Mahlapuu et al., 2021). A recent clinical controlled trial in the USA (NCT04098562) was conducted to assess the effectiveness of a stable, LL-37-containing cream in diabetic foot ulcers (DFUs) and was found to improve the granulation index and healing rate of wounds (Wu et al., 2018; Miranda et al., 2023). These clinical studies support the notion that LL-37 topically-used is safe and well tolerated.

An improvement in the management of wounds may arise from the encapsulation of the HDP in a three-dimensional hydrogel system that allows effective delivery, prolonged stability and

efficient release of the bioactive molecule. The use of dressings based on different types of self-healing multifunctional hydrogel system showed a significantly improved angiogenesis and reduced wound closure times in animal wound models (Hao et al., 2023; Jelodari et al., 2023).

As reported in section 2.2, expression of the single cathelicidin gene (*CAMP*) present in humans and other primates, is upregulated by 1,25-dihydroxyvitamin D3, in addition to its increased expression in response to pathogen exposure and inflammation. In fact, it has been determined that vitamin D response element (VDRE) is localized upstream to the promoter region of the *CAMP* gene, which binds the vitamin D-activated nuclear receptor (VDR), inducing gene expression (Wang et al., 2004; Gombart et al., 2005; 2009; White, 2010). Several clinical studies have suggested a link between vitamin D deficiency, inflammatory pathological conditions (such as urinary tract infections, ulcerative colitis and Crohn's disease) and decreased expression of LL-37 (White, 2018; Deng et al., 2019; Gubatan et al., 2020). These and many other observations suggest that treatment with vitamin D could be a way to increase LL-37 production in contexts in which its levels are insufficient to counteract specific infections and inflammation.

With respect to the use of LL-37 as a biomarker, the peptide was found to represent a T-cell autoantigen in the majority of patients with moderate-to-severe psoriasis and psoriatic arthritis (Lande et al., 2014; Fuentes-Duculan et al., 2017; Frasca et al., 2018). Abnormally high levels of LL-37 are also found in rosacea (Cribier, 2022) where it contributes to its pathophysiology by stimulating cytokine release, angiogenesis, chemotaxis and other pro-inflammatory events. Its presence is due to overexpression and release of the inactive hCAP18 precursor associated with increased levels of the processing enzyme kallikrein 5 from the stratum corneum of epidermidis, suggesting that inhibition of the enzyme might improve the clinical signs of rosacea. A proof of concept pilot study showed the first clinical evidence of the involvement of kallikrein in the pathogenesis of rosacea and its inhibition did correlated with clinical improvement of the disease (Yamasaki et al., 2007; Two et al., 2014; Thibaut de Ménonville et al., 2017).

A problem with the use of Type-A peptides like LL-37 as anti-infective agents is that being membranolytic they are quite cytotoxic and tend to have rather narrow therapeutic windows, even for use as topic agents. Redesigning them to improve this window can however change their mode of action and reduce their multifunctional effects. A great effort has been expended in this direction to develop LL-37 fragments and variants able to maintain or improve antimicrobial activity while reducing toxicity towards eukaryotic cells and susceptibility to degradation by proteases present in mammalian body fluids. Truncation or replacement of residues in the LL-37 can in fact improve its antibacterial capacity, as long as truncation does not significantly alter the overall charge and amphipathic features. For example, central fragments [e.g., LL-37 (7–27) or LL-37 (5–24)] maintain the antibacterial activity against both Gram-positive and Gram-negative bacteria while reducing haemolytic activity with respect to the native peptide (Braff et al., 2005; Thennarasu et al., 2010; Wang et al., 2012; Krishnamoorthy et al., 2023).

The most advanced clinical trial among the truncated variants of LL-37 is the peptide P60.4Ac that was developed from an overlapping synthetic library (Nell et al., 2006). This peptide, derived from the sequence 13-36 of LL-37 with some residue substitutions to improve amphipathicity, was positively tested for ototoxicity in guinea pigs and *in vitro* antimicrobial activity on reference bacterial and fungal strains. Subsequent studies in patients suffering of chronic suppurative otitis media (CSOM) found that the peptide was safe and well-tolerated when used as ototopical drops. A randomized, double blind, placebo-controlled, multicentre phase II[a] study on more than 30 patients showed a successful treatment in 47% of cases vs. 6% in the placebo group (Peek et al., 2020), providing strong support to further develop the peptide for CSOM treatment.

An interesting LL-37 derivative is the peptide 17BIPHE2, based on the short (17–32) fragment, where the native Ile20 and 24 and Leu28 were changed to D-Leu, and Phe17 and 27 to biphenylalanine. Compared to LL-37 and its fragments, this peptide is highly resistant to various proteases present in mammalian body fluids, and displays an increased antimicrobial activity against multidrug-resistant bacteria and a high antibiofilm activity (Wang et al., 2014; Narayana et al., 2019). It was recently shown to be active *in vitro* against *Neisseria gonorrhoeae* and has spermicidal activity on human and mouse sperm via membrane permeabilization. In addition, multiple transcervical injections of the peptide in female mice did not affect the histological features of vagina, cervix and uterus, suggesting its use both as contraceptive and antimicrobial agent against sexually transmitted infections (Lee et al., 2022). Further examples are reported in Supplementary Table S2.

An interesting concept to reduce toxicity is to mimic their endogenous release from the CLD by incorporating a part of it in a pro-drug. In this respect, an artificial pro-form was rationally designed to protect the *D*-enantiomer of the shortened bovine cathelicidin BMAP18, to be released and activated only upon proteolytic cleavage by elastase. In this preclinical study, this prodrug showed a very low cytotoxicity and was correctly converted to *D*-BMAP18 in the presence of cystic fibrosis sputum as a model of a pathologic lung environment, then showing good antimicrobial activity, so it may be a good candidate for aerosol treatment of the CF lung (Degasperi et al., 2022).

Type-P cathelicidin HDPs (PrAMPs) are of significant interest for the development of new antimicrobial compounds due to their potent antimicrobial efficacy (although mainly effective against Gram-negative bacterial species) combined with a lower cytotoxicity than other structural types (Welch et al., 2020). As described in Section 2.4, they display a peculiar mode of action targeting protein synthesis through stalling bacterial ribosomes as their main antimicrobial mechanism (Mardirossian et al., 2014; 2018a; 2018b; Gagnon et al., 2016; Seefeldt et al., 2016), while destabilization of the bacterial membrane is mostly a secondary effect exerted at well above their active concentrations, unless their sequence is modified (Podda et al., 2006; Sola et al., 2020). During the past 3 decades a few Type-P cathelicidin HDPs have undergone extensive structure-activity relationship studies aimed at isolating the pharmacophore sequence by testing numerous fragments and variants, with the aim of reducing synthetic cost and promoting antimicrobial potency through residue substitutions (Panteleev et al., 2018; 2022; Sola et al., 2020; Bolosov et al., 2023; Benincasa et al., 2004; Guida et al., 2015; Mardirossian et al., 2019b). In particular, the application of SPOT-synthesis and of deep mutational scanning protocols have increased the potential of

these studies exponentially, due to the possibility to synthesize large panels of peptides (Lai et al., 2019; Mardirossian et al., 2019a; 2020) or to express peptide variants directly in bacteria (Collins and Hackel, 2024). The antimicrobial potential and low toxicity of some Type-P cathelicidin HDPs, as evidenced by *in vitro* studies, has also been supported by *in vivo* studies in animal models of infection (Benincasa et al., 2010; Di Stasi et al., 2019). However, assessment remains in the pre-clinical phase and data on the pharmaco-kinetics and -dynamics of these compounds are still at the embryonic stage (Benincasa et al., 2015), which, with the lack of experimentation in non-murine animal models, has as yet prevented derivatives of this type of cathelicidin HDP from entering clinical studies.

The potential of Type-P cathelicidin HDPs to fight pathogens is not limited to exploitation of their antimicrobial activity *per se*, but can exploit their capacity to be actively internalized by susceptible bacteria, as well as some eukaryotic cells, as carriers for other types of molecules such as fluorophores or quite large cargo such as PEG, proteins and impermeant PNA, (Sadler et al., 2002; Benincasa et al., 2015; Hansen et al., 2016). They can also be used in this manner to enhance or restore the activity of antibiotics in resistant strains (Gambato et al., 2023).

5 Conclusion

Cathelicidins belong to an ancient, ubiquitous family of vertebrate HDP proforms that carry highly diverse multifunctional bioactive peptides that can be grouped into at least five different structural types. Numerous experimental observations and genetic information indicate that many species possess multiple cathelicidin genes and that the types of derived peptides are structurally diverse. These aspects open up potential access to a large repertoire of bioactive peptides with anti-infective and immunomodulatory activities. The presence of relatively well conserved proregions and a significant degree of syntenic conservation with common flanking genes, facilitates the search for novel cathelicidin sequences in the rapidly growing number of available vertebrate genomes. This process would enable targeted mining approaches and lend itself to machine learning. The features of numerous characterized cathelicidin HDPs can then be used for structure/activity prediction studies aimed at 1) dissecting the functional domains responsible for the pleiotropic activity of some of these peptides; 2) suggesting modifications that could improve their druggability (e.g., stabilized analogues and truncated derivatives that reduce the production costs); 3) exploiting the different mechanisms of action of each structural type – membrane or target specific - to modulate the spectrum of activity, 4) reducing the likelihood of resistance, and 5) improving cytotoxicity and pharmacodynamic profiles. In addition, the particular architecture of cathelicidins, with a conserved proregion that likely keeps the peptide inactive, and avoids sequestration and/or degradation until it reaches the site of infection, could point to a possible delivery method to reduce side effects and not only use them as topical agents. Although the success of anti-infective peptides derived from AMPs in clinical trials has generally been low, concerted efforts in recent years suggest that cathelicidins may be useful both as anti-infective drugs that also aid wound healing (as demonstrated by their application on DFUs) and/or as markers for different conditions.

Author contributions

AT: Conceptualization, Data curation, Writing–original draft, Writing–review and editing, Formal Analysis, Supervision, Validation. MG: Data curation, Writing–original draft, Writing–review and editing, Formal Analysis, Validation. AC: Writing–original draft, Writing–review and editing. SP: Writing–original draft, Writing–review and editing. MM: Writing–review and editing, Writing–original draft. MS: Writing–review and editing, Writing–original draft. DP: Data curation, Writing–review and editing, Formal Analysis, Validation. GM: Writing–review and editing, Data curation, Formal Analysis, Validation. RG: Writing–original draft, Writing–review and editing, Conceptualization.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fddsv.2024.1458057/full#supplementary-material

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