Cathelicidins – Nature's Attempt at Combinatorial Chemistry

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Abstract: Cathelicidins are a family of diverse antimicrobial peptides found in granules of mammalian neutrophils. Cathelicidins are active against a broad range of microbes in different environments. Aside from their antimicrobial activity, cathelicidins possess other biological properties including cytotoxic activity towards mammalian cells. Several studies have shown that the amino acid sequence of cathelicidins can be modified to temper undesired properties, such as hemolytic and cytotoxic activity, and at the same time maintain antimicrobial activity. These properties make cathelicidins ideal templates in combinatorial chemistry for designing de novo antimicrobial peptides for therapeutic use. However, one of the major challenges will be to screen these peptides in experimentally relevant models that reflect the environments in which the peptides should be therapeutically active.

INTRODUCTION

Antimicrobial peptides (AMPs) are important effector molecules in host defense of eukaryotes from insects to humans [1]. The importance of AMPs for host defense is now becoming evident. Experiments in mice have demonstrated that targeted disruption of the gene for an AMP [2] or defects in generation of active AMPs [3] leads to reduced bacterial clearance. Two major human skin disorders, psoriasis and atopic dermatitis, are both characterized by a defective skin barrier and increased risk of skin infections. However, patients with atopic dermatitis have much higher incidence of skin infections than patients with psoriasis and this difference is now linked to differences in the levels of AMPs in these two disorders [4,5].

Cathelicidins are a family of very diverse antimicrobial peptides found in mammalian neutrophils, and they constitute a major part of the AMPs released from these cells in some species. All cathelicidins share a conserved N-terminal domain (cathelin) of approximately 100 amino acids. The antimicrobial peptide portion resides in the C-terminus and becomes active following proteolytic cleavage from the cathelin propiece Fig. (**1**). For all cathelicidins, the mature AMPs generated by proteolytic activation are between 12-100 residues. They are active against a broad spectrum of bacteria, and some fungi and enveloped viruses. While the N-terminal cathelin part is very conserved between different species, the C-terminal AMPs are quite diverse.

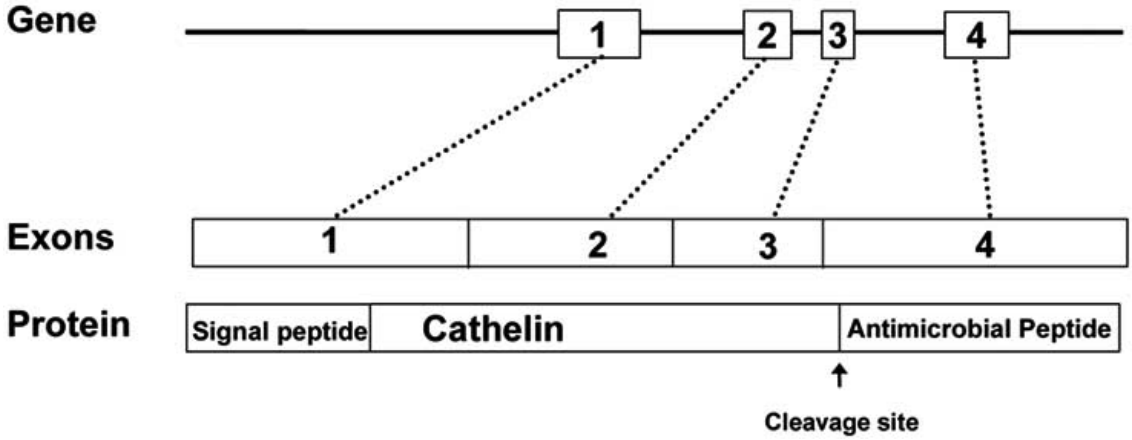

Fig. (1). Structure of the cathelicidins.

Cathelicidins consist of a conserved signal peptide and the "cathelin" prosequence encoded by the first three conserved exons and of the diverse the C-terminal mature peptides. The peptides must be cleaved of the holoprotein in order to become active. Because of the variable cleavage sites (encoded by the variable fourth exon) cathelicidins are processed by different enzymes.

The primary structure is reflected in the genomic organization of the cathelicidin genes. Cathelicidin genes are composed of 4 exons and 3 introns Fig. (**1**). There is great

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homology inter- and intra-species in the first three exons, which encompass the cathelin domain but no homology in the fourth exon encoding the antimicrobial domain and the cleavage site between the cathelin portion and the antimicrobial domain [6-11]. It has been argued that the different members of the cathelicidin family have evolved by duplication from a common ancestor gene that encodes the cathelin portion [12].

Despite the fact that cathelicidins are located in neutrophils and become active during the same inflammatory and infectious processes in mammals, the active antimicrobial peptides are remarkably diverse. This probably reflects the rapid evolution of AMPs to combat the species-relevant microbes in a variety of different circumstances. Thus, cathelicidins can be viewed as Nature's attempt of varying the sequences of AMPs to achieve an effective host defense - a theme that has been further elaborated in adaptive immunity.

This review will describe the known chemical structures and biology of several different cathelicidins.

ACTIVATION OF ANTIMICROBIAL ACTIVITY

Cathelicidins are synthesized as preproproteins. Following cleavage of the signal peptide, cathelicidins are stored as proproteins in neutrophil peroxidase-negative granules [13;14]. The cathelicidin proproteins are not antimicrobially active [15,16] and can be considered probiotics. The antimicrobial activity is unleashed following proteolytic cleavage of the AMP from the cathelin domain. Neutrophil cathelicidins are proteolytically processed to generate active antimicrobial peptides only after degranulation into the phagolysosome [17] or to the exterior when neutrophils migrate into the tissues at sites of inflammation [17-19]. This proteolytic process is catalyzed by proteases from azurophil granules. Due to differences in the cleavage sites, different proteases are responsible for this process. For example, the human cathelicidin hCAP-18 is processed to generate LL-37 by proteinase 3 [19] while porcine and bovine cathelicidins are processed by neutrophil elastase [15,18].

hCAP-18 is also expressed at various epithelial sites including the skin [20], squamous epithelia [21], epididymis [22], sweat glands [23] and salivary glands [24]. The epithelia-derived hCAP-18 is processed differently from the neutrophil-derived protein. hCAP-18 produced in the epididymis and present in seminal plasma is processed to generate an active AMP (ALL-38) in the vagina following sexual intercourse. The acid vaginal milieu activates the prostate-derived protease gastricsin, which processes hCAP-18 [25]. Sweat has been demonstrated to contain proteases that process the active peptide LL-37 and generate truncated peptides with greater antimicrobial activity [26].

It has recently been demonstrated that the cathelin domain of the human cathelicidin, hCAP-18, also possesses antimicrobial activity following release of the antimicrobial peptide LL-37 [16]. The cathelin domain exerted antimicrobial activity towards gram-positive bacteria not susceptible to LL-37. Thus, the cleavage generated two antimicrobial molecules with complementary activity [16].

PRIMARY AND SECONDARY STRUCTURE OF THE CATHELICIDINS

All antimicrobial peptides including the cathelicidins are "membrane active" (for recent reviews of the membrane actions of AMPs see [27] or [28]). Most AMPs are lytic and destabilize the membrane by forming pores (barrel-stave mechanism) as described for defensins [29] or by a more detergent-like effect (carpet mechanism) as described for LL-37 [30]. Other peptides such as PR-39 are not lytic but cross the bacterial membranes and are believed to kill bacteria by interacting with intracellular targets [31]. All cathelicidins are positively charged and hydrophobic. The positive charge is believed to facilitate the initial interaction with the negatively charged phospholipids in bacterial membranes, and the hydrophobic properties are important for the subsequent insertion into the bacterial membrane.

As mentioned above, the cathelicidins encompass an extremely diverse family of peptides 12-100 amino acids in length. Based on structures the AMPs can roughly be divided into three groups:

1. ***α-helical peptides*** – peptides forming an amphipathic α-helix. This group contains the peptides such as PMAP-36, LL-37, CRAMP, CAP-18, BMAP-27, and SMAP-29.
2. ***Peptides enriched one or two amino acids*** – This group of very different peptides is represented by Bac5, Bac7, prophenins, indolicidin, and PR-39. Indolicidin is very rich in tryptophan and proline [32], prophenins are rich in proline and phenylalanines, while Bac5 and Bac7 are proline/arginine-rich peptides.
3. ***Loop peptides*** – peptides with β-sheet structures that form loops formed by either one or two disulfide bridges. The three-dimensional structure of the peptides in this group resembles the defensins and they have, together with the newly described θ-defensins, been named minidefensins [33]. Cathelicidin peptides from this group include the dodecaptides and the protegrins.

An example of the structure of a peptide from each group is shown in Fig (**2**).

α-Helical Peptides

While ovine, bovine and porcine neutrophils contain a variety of cathelicidins (for recent reviews see [34,35]) other species such as human, rabbit and mouse contain only one member of the cathelicidin family. When only one cathelicidin is present, the chemical structure is always an α-helix. When more than one cathelicidin is present at least one of these is always an α-helical peptide, demonstrating the importance of this molecular motif in host defense. The α-helical cathelicidins are active against a variety of gram-negative and gram-positive bacteria [36-39]. However, some of these peptides also exhibit cytotoxic activity towards mammalian cells, as has been reported for the human peptide LL-37 [40]. Many α-helical cathelicidins can also bind and neutralize the biological effects of LPS [41,42].

There are important variations in the antimicrobial actions and cytotoxic effects of different α-helical peptides.

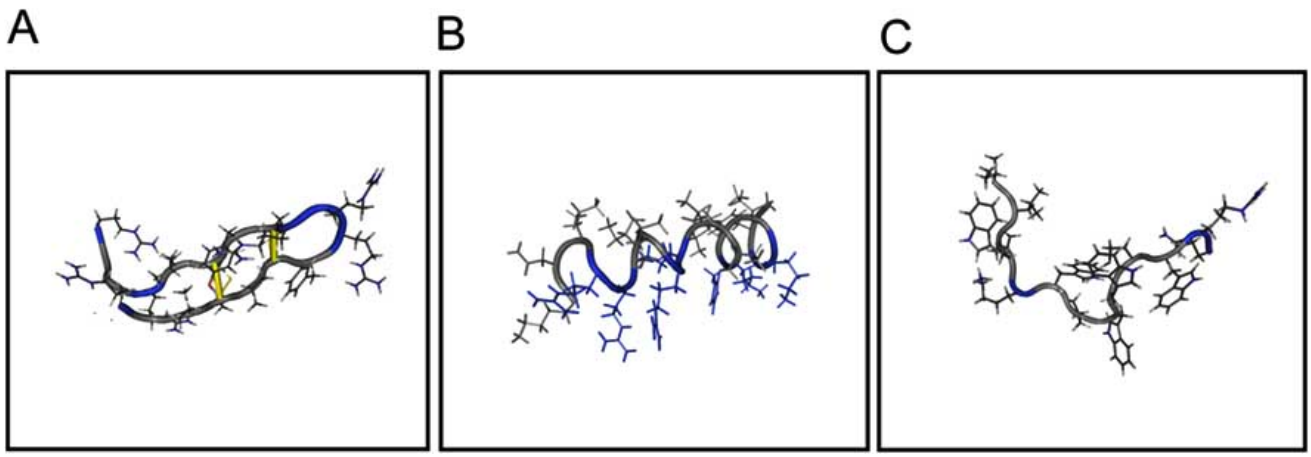

Fig. (2). Solution structure of three cathelicidins.

A. Solution structure of the "loop peptide" protegrin-1. Sequence: VTLDQIKDPLDITCNEVQGVRGGRLCYCRRRFCVCVGRG. The peptide has disulfide bridges between C_1-C_4 and C_2-C_3. The solution structure was obtained by homonuclear magnetic resonance spectroscopy [95].

B. Solution structure of the A-helical peptide ovispirin-1. Sequence:

KNLRRIIRKIIHIIKKYG

The solution structure was obtained by nuclear magnetic resonance [44].

C. Solution structure of indolicidin bound to dodecylphosphocholine micelles. Indolicidin is rich in tryptophan and proline, and represents the cathelicidins enriched in one or two amino acids. Sequence: ILPWKWPWWPWRRP

The solution structure was obtained by nuclear magnetic resonance spectroscopy [96].

Hydrophobic residues are shown in blue.

By studying peptides derived from frog magainins, it was found that increased cationic charge is associated with increased activity against Gram-negative bacteria, and that increased hydrophobicity is associated with increased activity against Gram-positive bacteria but also with cytotoxicity against mammalian cells [43]. Specific studies of cathelicidin-derived α-helical peptides have also shown that especially the cytotoxic effect of the peptides is linked to hydrophobicity [37]. Removing the C-terminal residues of the naturally occurring peptides BMAP-27 and BMAP-28 reduced the cytotoxic effect without affecting the antimicrobial activity. Simply exchanging hydrophobic residues in the hydrophobic tails with hydrophilic residues also greatly reduced cytotoxic activity, but these substitutions also changed (but not necessarily diminished) the antimicrobial activity of these peptides [37]. For the synthetic α-helical cathelicidin-derived peptide, ovispirin-1, a change of a single residue has been shown to greatly decrease the cytotoxic activity but without inducing major change in the antibacterial activity [44]. Thus, small substitutions can alter the activity of α-helical peptides, and indeed the different activities of the cathelicidin α-helical peptides illustrate this fact [37,44].

Peptides Enriched One or Two Amino Acids

Despite the differences in molecular structure, peptides enriched in one or two amino acids generally display a broad spectrum of antimicrobial activity [45-48]. However, proline/arginine-rich peptides apparently differ in their mode of action. While Bac5 and Bac7 rapidly permeabilize bacterial membranes [49], PR-39 is believed to exert its antibacterial activity by binding to intracellular targets [31]. Proline/arginine-rich peptides also exhibit protective effects against endotoxin [50]. As with α-helical peptides, truncated forms of certain proline/arginine rich peptides exhibit enhanced antimicrobial activity [51]. The tryptophan/proline-rich peptide indolicidin (13 residues) permeabilizes bacteria [52] but bacterial killing is probably also mediated by interaction with intracellular targets [53]. Indolicidin has anti-HIV-1 activity [54]. Entrapment of indolicidin in liposomes greatly reduced cytotoxicity and allowed administration of high and therapeutical doses of the peptide in animals with fungal infection [55]. The mode of action of the prophenins (rich in prolines and phenylalanines) is not known.

Loop Peptides

Protegrins resemble defensins with their β-sheet-like structure, but protegrins are approximately only half the size of defensins and contain only two disulfide bridges instead of the canonical three found in defensins. The mode of action of protegrins is similar to that of defensins, i.e. they kill the bacteria by forming voltage- and charge-dependent pores in the bacterial membranes [56]. Protegrins are remarkably antimicrobial towards a variety of organisms [57-60] including activity against HIV-1 [61]. The protegrin structure enables a wide range of amino acid substitutions and alterations without loss of the antimicrobial activity

[56,62]. The dodecapeptides are, as the name implies, twelve-residue peptides found in ovine and bovine neutrophils [63,64]. Their β-sheet structure is stabilized by only one disulfide-bridge [64]. Despite the small size, their three-dimensional structure still resembles that of defensins [65]. The broad antimicrobial activity of dodecapeptides is independent of their disulfide bridge and can be improved by small modifications in the amino acid sequence [65].

In summary, many studies have demonstrated that structurally disparate cathelicidins can be modified to decrease or abolish undesired effects like cytotoxicity and hemolysis without diminishing the antimicrobial activity.

ADDITIONAL BIOLOGICAL EFFECTS OF THE CATHELICIDINS

It is important to note that some cathelicidins have biological activities apart from the antimicrobial activity. Peptides such as LL-37 and PR-39 possess chemotactic activity [66,67], and both of these peptides stimulate angiogenesis [68,69]. Both these activities of LL-37 are presumably mediated by binding to the formyl peptide receptor-like 1 (FPRL1). No specific receptors have been identified for PR-39. Both PR-39 and LL-37 have important functions in wound healing [70,71]. Additionally, LL-37 has also been demonstrated to activate the innate immune response [72] and influence the differentiation of dendritic cells [73].

It was recently shown that when LL-37 was processed in human sweat, the truncated forms that were generated were more active in bacterial killing than the parent peptide. Nonetheless, these truncated peptides had diminished immunostimulatory activity as measured by IL-8 release from keratinocytes [26]. These studies suggest that certain modifications of cathelicidins can maintain and even increase the antibacterial activity while reducing other potentially harmful biological effects. These non-antimicrobial effects are important considerations when screening for peptide-based therapeutics.

RESISTANCE AND INACTIVATION OF CATHELICIDINS

Increasing resistance worldwide against conventional antibiotics is an escalating problem in clinical medicine. Certain species of bacteria have evolved effective mechanisms to combat a wide variety of AMPs: for example, direct inactivation of the peptide by proteolysis [74,75], by efflux pumps [76] or by changing the properties of the membranes [28,77]. However, the most appealing feature about designing a drug from AMPs is the relative lack of resistance towards naturally occurring AMPs both *in vitro* and *in vivo*. For example, when methicillin-resistant *Staphyloccus aureus* was subcultured at sub-inhibitory concentrations of a conventional antibiotic, norfloxacin, for nine successive passages the MIC (minimal inhibitory concentration) increased 320-fold. The same experiment performed with a cathelicidin-analog IB-367 resulted only in a two-fold increase in MIC [56]. Several features probably contribute to this lack of resistance:

a) Rapid irreversible damage to bacteria. It has been postulated that one reason for the lack of resistance is the rapid and irreversible damage of bacterial membranes caused by AMPs [78]. Such damage is difficult to fix. This is in contrast to conventional antibiotics, which kill bacteria slowly and where cell morphology is intact. This enables resistance to develop.

b) Multiplicity of peptides. Upon colonizing the body, pathogens encounter a large variety of AMPs. It is now becoming evident that AMPs have multiple targets in the microbes and different AMPs have different targets (see [28] for extensive review). It has therefore been argued that AMPs kill bacteria by a "multi-hit process" [79]. Mutation of multiple targets is unlikely, and mutation of only a single target will not render bacteria resistant to multiple AMPs *in vivo*. In many ways bacterial infection shares common features with another type of cellular invasion: growth of cancer cells. In cancer chemotherapy e.g. in Hodgkin's Lymphoma, it has long been acknowledged that monotherapy frequently leads to resistance by the cancer cells. Thus multi-drug regimens have been successfully developed [80]. In fighting a cellular invasion caused by bacteria it is noteworthy that the body fights cellular invasion of bacteria with a wide array of antimicrobial substances present at the same time, i.e. by natural polytherapy.

STRATEGIES FOR DESIGNING NEW DRUGS FROM CATHELICIDINS BY COMBINATORIAL CHEMISTRY

Since most species generate many AMPs it is an important question whether additional levels of a single AMP or AMP-analog will make any difference during infection *in vivo* where the levels of endogenous AMPs are already high. Studies with overexpression in mice of the human cathelicidin hCAP-18/LL-37 in the lung [81,82] and human defensin-5 in the intestine [83] demonstrated that increased amounts of a single AMP increases bacterial clearance. LL-37 binds and neutralizes the effects of LPS [41], and mice overexpressing hCAP/LL-37 had increased survival rates following exposure to LPS [81]. Thus, both the antimicrobial and LPS-neutralizing effects of AMPs are found in vivo by local overexpression of a single AMP. These studies demonstrate that additional levels of a single AMP may be beneficial in a therapeutic setting.

Due to their presence in neutrophils, cathelicidins are delivered to the local site of infection and therefore act as local antibiotics. The cathelicidins and other AMPs are antimicrobially effective at micromolar concentrations. To obtain therapeutic concentrations of cathelicidins it will probably be most feasible to design AMPs as topical antibiotics. Most screening schemes are designed to find compounds with a high therapeutic index: high antimicrobial activity and low cytotoxicity. As elucidated above, apparently subtle changes in the sequence of the cathelicidins can impart great influence on both the cytotoxicity and antimicrobial effect and spectrum for all different forms of this family of peptides. Hence, it should be feasible to design peptides with desired antimicrobial activity and little or no cytotoxicity or other side-effects. Though relevant antimicrobial activity and low toxicity are

necessary for an antimicrobial drug, additional biological aspects must be taken into consideration if a drug should be successfully designed from AMPs and these aspects should be reflected in the screening strategy and testing of suitable peptides.

In using combinatorial chemistry and high throughput screening to design local antibiotics based on cathelicidins two separate but equally important goals must be achieved:

1) Design of a peptide with appropriate antimicrobial actions and low toxicity

2) Design of a peptide that will be effective in the appropriate local infectious environment

Design of a Peptide(mix) with Appropriate Antimicrobial Actions and Low Toxicity

In Nature, individual cathelicidins and other AMPs hardly ever act alone, but act together with other AMPs present in the local environment. Several studies address synergy in bacterial killing between different AMPs [84,85]. Cathelicidins with very different molecular structures, for example the porcine loop-peptide protegrin and the human α-helical LL-37, have synergistic antibacterial activity. Since pigs also have cathelicidins with the α-helical motif, including PMAP-36 [38] and PMAP-37 [86], and these peptides are released from the neutrophils along with protegrins, the synergistic effect might be important for the antimicrobial activity generated by cathelicidins in *vivo*.

When designing drugs from cathelicidins or other AMPs, it might therefore be advantageous to aim for a mix of two (or three) peptides with synergistic effects rather than modifying a single peptide for optimal killing. Design of a combination therapy of synergistic AMPs could offer some advantages: A) The total concentration of peptide to obtain a therapeutically relevant concentration could be lowered. One of the challenges in designing drugs from AMPs would be to generate therapeutic concentrations at the target sites. B) A combination of peptides would have a broader spectrum of antimicrobial activity. C) Multiple types of antimicrobial peptides would decrease the chance of microbial resistance. A mixture of structurally different peptides could be designed to increase the number of potential targets in the bacteria. Additionally, a combination of structurally diverse AMPs could be designed to minimize the risk of inactivation by a single resistance mechanism.

While much is known about the synergistic effect of bacterial killing by AMPs it is largely unknown if there are synergistic effects in the cytotoxic activity of AMPs as well. This question will need to be addressed in future studies.

Making a multi-drug cocktail is more laborious and expensive than designing and testing a single compound. However, no AMP or AMP-analogue has so far been developed as a successful drug. Drugs developed from AMPs will likely be used in circumstances where conventional antibiotics fail, e.g. in infections with multidrug-resistant bacteria either used as a salvage therapy or in a combination with conventional antibiotics. Designing a cocktail with synergistic activity could lower the total amount of peptide needed in a drug. This would in turn potentially lower the production cost (but alas not the development costs). A multi-peptide cocktail may significantly lower the risk of development of resistance. The difference between a multi-drug cocktail and single peptide could therefore mean the difference between a usable drug and just an interesting biological compound.

Design of a Peptide that will be Effective in the Appropriate Local Environment

It is conceivable that exposure to different microbial environments gives rise to significant species-related differences in the AMPs. But it remains an intriguing question why some mammals have so many different cathelicidins stored in neutrophils. Bovine neutrophils contain a great number and variety of structurally different cathelicidins together with several defensins. Neutrophil-derived cathelicidins will have to combat this variety of microbes in very diverse local environments. As one example, the conditions in the pH-neutral airway liquid fluid will be very different from the dry and acid environment of the skin. Hence, the structural diversity of the cathelicidins might therefore not only enable these peptides to effectively combat a great variety of microbes but also to preserve antimicrobial activity in very different environments.

Examination of the antimicrobial activity of AMPs in *local* environments has yieldedresults that are very different from the those found in the laboratory. The antibacterial activity of pure lysozyme (muramidase) is limited to *Micrococcus luteus* [87] (originally termed *M. lysodeikticus* by A. Fleming). Thus, lysozyme would seem unsuitable as a template for further drug development. However, as discussed above AMPs seldom if at all act alone *in vivo*. In determining the main antibacterial components of airway fluid one group found that lysozyme was one of the most important cationic antimicrobial substances present in airway fluid [88]. This is a somewhat surprising finding, given the aforementioned *in vitro* studies of lysozyme's antibacterial activity. However, lysozyme has been shown to act synergistically with several human AMPs [84]. The pronounced activity of lysozyme in airway fluid is probably due its interaction with other antimicrobial factors present that augment the inherent antibacterial activity of lysozyme. Conversely, local components or anatomical structures may inhibit the function of AMPs. The antibacterial activity of the human cathelicidin LL-37 is inhibited by serum [40] likely due to adsorbance by lipoproteins [89]. Bacteria might even in some instances modify the environment and cause inactivation of AMPs. For example in connective tissues bacterial proteases degrade proteoglycans resulting in the release of glycosaminoglycans. The released anionic glycosaminoglycans bind and inactivate the cationic AMPs [90]. Hence, the local environment can either augment or abolish the antibacterial activity of a given AMP *in vivo*.

One of the major challenges in designing a drug from AMPs will be to test or screen a potential peptide (or mix of peptides) in a biologically relevant model. AMPs will interact with other molecules in the local environment and the activity of the putative peptides should therefore be tested at conditions closely resembling the environment where the AMP is purported to be therapeutically active. For instance, if a peptide is intended for use as a mucosal antibiotic then it should as a minimum be tested in the

relevant mucosal fluid. Since the sequence of AMPs can be modified without loss of antimicrobial activity it should consequently also be possible to design antimicrobial active peptides without undesired interactions (i.e. inactivation) with the local environment of the infectious site.

Even though the local environment at mucosal sites may inhibit AMPs, designing a drug for *specific* mucosal sites may offer advantages. The presence of antimicrobial components in various body fluids is beginning to be elucidated [91,92], thus peptides could be designed to act synergistically with other AMPs present at the mucosal site, where the drug is going to be applied. In designing agents for oral mucositis it could therefore be advantageous to design peptides that act synergistically with AMPs present in saliva, e.g histatins [93]. It is becoming increasingly clear that the properties of the specific local milieu must be incorporated into the design and screening strategy for the development of therapeutically active AMPs.

Probiotics

Lessons learned from proteolysis and activation experiments with cathelicidins teach us that these AMPs could be designed as probiotics. All cathelicidins are stored as inactive proproteins in neutrophil granules. Also the epithelially expressed human cathelicidin, LL-37/hCAP-18, is produced as the inactive proprotein at epithelial sites such as epididymis and is present as the proprotein in seminal plasma [22]. The antimicrobial active peptide, ALL-38 is generated form hCAP-18 in seminal plasma after exposure to the vaginal milieu by a proteolytic process [25]. Accordingly it could be possible to design a proprotein with a suitable site, where the antimicrobial action would be unleashed by cleavage at the site of infection. In the case of the human cathelicidin, the proprotein is not antimicrobial but both the antimicrobial peptide and the cathelin part display antimicrobial activity following cleavage [16]. Thus, probiotics might be able to generate two molecules with distinctive antimicrobial activity. Another possibility would be to design probiotics, where one of the sequences released by cleavage could act as a target sequence directing the molecule either towards the microbe or to the relevant anatomical location.

In the frog *Xenopus laevis*, certain AMPs (magainins) are synthesized as a large proprotein, where subsequent cleavage generates several AMPs [94]. If infections occur in a proteolytic environment it should be possible to synthesize AMPs as larger proproteins, which are subsequently cleaved to active smaller peptides at the site of infection. Synthesizing AMPs as proproteins may offer some advantages for the half life and stability of the peptides locally *in vivo*.

CONCLUDING REMARKS

The biology of the cathelicidins demonstrates to what extent Nature has varied the structures of AMPs to achieve an effective host defense in mammals. The variation in the natural design of these peptides has provided a host defense effective in very different environments and towards an abundance of diverse microbes. In that sense cathelicidins are Nature's attempt at combinatorial chemistry. This structurally diverse family of peptides may provide useful templates for design of new antimicrobial agents active in appropriate environment towards clinical relevant microbes. Several studies have demonstrated how the properties of these peptides can be altered without any affecting the antimicrobial activity. Combinatorial chemistry and high-throughput screening offer powerful techniques for generating and screening novel AMPs from existing templates. One of the major challenges will now be to apply these techniques to design and screen peptides for activity in the appropriate infectious environment.

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