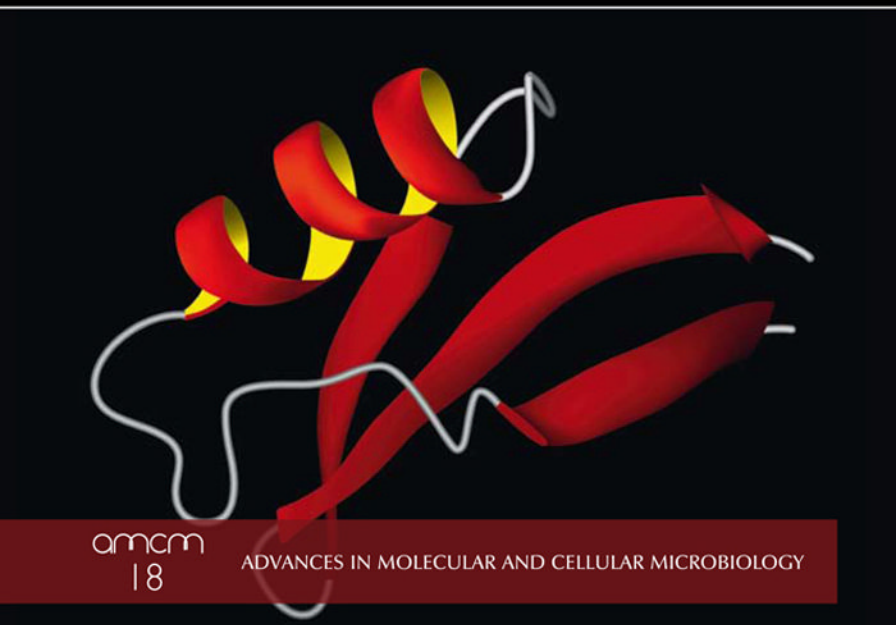
Antimicrobial Peptides

Discovery, Design and Novel Therapeutic Strategies

Edited by Guangshun Wang



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Edited by

Guangshun Wang, PhD

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Advances in Molecular and Cellular Microbiology

Through the application of molecular and cellular microbiology we now recognize the diversity and dominance of microbial life forms that exist in all environments on our planet. These microbes have many important planetary roles, but for humans a major problem is their ability to colonize our tissues and cause disease. The same techniques of molecular and cellular microbiology have been applied to the problems of human and animal infection since the 1990s and have proved to be immensely powerful tools in elucidating how microorganisms cause human pathology. This series has the aim of providing information on the advances that have been made in the application of molecular and cellular microbiology to specific organisms and the diseases they cause. The series is edited by researchers active in the application of molecular and cellular microbiology to human disease states. Each volume focuses on a particular aspect of infectious disease and will enable graduate students and researchers to keep up with the rapidly diversifying literature in current microbiological research.

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Preface

The significance and benefits of naturally occurring antimicrobial peptides to human health have recently begun to be appreciated. As effector molecules of the innate immune system, they are capable of rapidly eliminating invading pathogens to keep us healthy; as signalling molecules, they elegantly modulate the adaptive immune system to trigger other physiological actions. The purpose of this book is to provide a comprehensive account on current antimicrobial peptide research in two major directions. The first direction delineates the classic path for peptide development, ranging through identification, design, structure and mode of action studies. The second direction describes novel strategies for developing peptide therapeutics based on our knowledge of host defence antimicrobial peptides discovered in living organisms.

The book commences with a vivid and inspiring introduction contributed by Professor Michael Zasloff, a distinguished forerunner in the field. Part I provides an overview of nomenclature, classification and bioinformatic analysis of antimicrobial peptides from bacteria, plants and animals. Subsequently, lantibiotics from bacteria and cyclotides from plants are presented. These unique peptide templates with multiple sulfur-mediated bridges are resistant to proteases, rendering them attractive for engineering new compounds for food preservation, pest control and curing diseases. Part II discusses database-aided peptide prediction and design methods, synthetic combinatorial libraries and peptide mimetics that expand the conformational space of natural antimicrobial peptides. These approaches also allow for the optimization of the desired properties and therapeutic index of the peptide analogues or mimicries. An in-depth understanding of the mode of action of these peptides is essential for drug development. As a consequence, Part III covers the biophysical and structural characterization of antimicrobial peptides and their complexes. While many peptides (e.g. magainin and protegrin-1) target bacterial membranes, apidaecin, buforin II, PR-39 and others can cross bacterial membranes and associate with internal targets such as heat-shock proteins and nucleic acids. Structural determination sheds light on how such peptides recognize membrane or non-membrane targets at atomic resolution, and provides the basis for structure-based peptide design. Finally, Part IV focuses on novel strategies for developing peptide-based therapies. These include liberating LL-37 from the inactive bound state in infected lungs, enhancing the expression of human cathelicidin and human β-defensin-2 by vitamin D, and stimulating immune responses to bacterial invasion by applying peptide analogues that may or may not kill bacteria directly.

Antimicrobial Peptides: Discovery, Design and Novel Therapeutic Strategies has been written, and anonymously reviewed, by an enthusiastic group of recognized investigators in the field.

As the editor, I thank all of the authors and reviewers for their contributions. In particular, I am grateful to Professor Zasloff for his excellent introduction and Professors Richard M. Epand, Amram Mor and Donnatella Barra, who recruited the anonymous reviewers for the chapters written by the editor and his co-authors. This book is directed to graduate students, postdoctoral fellows, research faculty, principal investigators, educators, clinicians and all others who are interested in the education, research, development and applications of antimicrobial peptides.

Guangshun Wang, PhD

Introduction

Michael Zasloff

I have been asked by Dr Wang to provide an introduction to this wonderful book on antimicrobial peptides that he and our colleagues have assembled. Dr Wang asked me to explain how I entered the field, to describe my experiences in the area of therapeutic development and to express my thoughts regarding the 'big questions' or perhaps 'the grand opportunities' that remain in our field.

To this day I remain amazed when I examine the cornea of a human eye and see a clear epithelial surface, which I know to be a fragile cellular layer exposed continuously to microbes of unimaginable diversity. Why does the cornea look so healthy? Why does the body not need to call great numbers of phagocytes to defend the surface, which is under continuous microbial assault? What protects that surface?

Most of the epithelial surfaces of the body exhibit the almost mysterious, incomprehensible 'health' that is so evident in the eye. An endoscopic view of the lumenal wall of a healthy descending colon or rectum reveals a transparent single-celled layer with blood vessels visible beneath, without redness, swelling or signs of inflammation, despite the direct physical contact between that intestinal wall and faecal bacteria. Or consider the mouth. If we are healthy and we should bite our tongue, the wound heals over the course of a day or so, within an environment far from sterile, populated by an incomprehensible diversity of bacteria, fungi and viruses. How could such a wound heal were the principal antimicrobial defences phagocytic cells attracted to the site (as proposed by Metchnikoff)?

My first moment of amazement regarding the 'mystery' of our harmonious relationship with the microbes around us came when I saw, as a resident at the Children's Hospital in Boston, a newborn infant with cystic fibrosis. The infant was not yet 3 days old. The child had been born with meconium ileus, and cystic fibrosis was correctly suspected to be the associated medical condition. What amazed me was that *Staphylococcus aureus* and *Pseudomonas aeruginosa* were cultured from the upper respiratory tract of that newborn, before the onset of overt bronchial disease. The prevailing hypothesis at the time was that these organisms infested the airways of individuals with cystic fibrosis because the poorly draining highly viscous bronchial mucous secretions presented a favourable niche for these specific microbes. But clearly this explanation made no sense in the case of this newborn. My assumption was that a mechanism of antimicrobial defence existed that protected the bronchial epithelium, but was yet to be discovered.

Some years later, while at the National Institutes of Health and involved in studies that required the use of the oocytes of the African clawed frog, I was struck one day that the surgical wounds that I created on these animals in the course of removing their ovaries healed without

evidence of inflammation or infection. This 'ah-ha' moment was followed by a realization that the wound on the abdomen of the frog could only heal if a potent antimicrobial mechanism existed in the skin of that animal, since these aquatic frogs were healing without difficulty in tanks that were densely populated with microbes.

Within several months I discovered the presence of antimicrobial peptides in the skin of *Xenopus laevis*, the first being magainin. Some years earlier Hans Boman and colleagues had described the remarkable cecropins in the silk-moth pupa, and Bob Lehrer and colleagues had characterized the defensins in mammalian phagocytic cells. With my observation, three clear examples of the existence of antimicrobial peptides in animals had been described, each operating in a different physiological context.

With the help of many colleagues I began to search the tissues of every animal I could study, especially those tissues that seemed to be protected by 'mysterious' antimicrobial defences. In addition, I wanted to better understand how antimicrobial peptides were used in humans to maintain health, and how their failure to function properly might cause disease. At about the same time I founded Magainin Pharmaceuticals. As a physician scientist, I was thrilled by the opportunity to have the chance to bring a discovery made at the bench to the clinic.

In the beginning, most of us were amazed by the antimicrobial properties of these naturally occurring, simple, short amphipathic peptides. They could rapidly kill practically every species of bacteria and many species of fungi, inactivate viruses and lyse protozoa. I can recall a dramatic moment when, following the addition of a solution of magainin into the abdominal cavity of a mouse filled with Ehrlich ascites tumour cells, I withdrew some ascites and could see that the peptide had killed most of the tumour cells within minutes of administration. Furthermore, the simplicity of the design of the α-helical peptides and the simplicity of the Merrifield method of peptide synthesis fostered an explosion of structure–activity studies. In addition, studies of animals and plants led to the discovery of an enormous diversity of naturally occurring antimicrobial peptides.

An understanding of the mechanism of action of antimicrobial peptides has evolved over time. As amphipathic, cationic peptides, it was apparent from the start that antimicrobial peptides targeted the membranes of the microbes they killed, a conclusion consistent with studies utilizing model membranes. Precisely how a particular peptide strikes the fatal blow in a particular microbial target remains the subject of continued and active investigation.

We continue to learn more about how antimicrobial peptides participate in defence against microbes and permit multicellular organisms to live in balance with microbes. In general, most healthy epithelial surfaces exposed to microbes express an array of antimicrobial peptides, and a détente of sorts is maintained between the microbes that utilize that surface as a niche and the epithelium that is being inhabited. Upon injury to the epithelium, batteries of different antimicrobial peptides are generally launched. Of great interest are the details of the scenarios that characterize the responses of various organs to specific microbes and the settings in which they fail to unfold normally. I believe that the existence of microbial sensors, such as the Toll-related receptors, permits this system of defence to mould an antimicrobial response that is far more microbe-specific than might have been otherwise imagined.

We have come to learn that antimicrobial peptides, initially discovered on the basis of their antibiotic activity, exhibit other biological properties in the context of injury and infection that promote recovery and healing. The cathelicidins can stimulate epithelial growth and promote angiogenesis. Several of the mammalian defensins and cathelicidins can attract cells of the immune system such as macrophages, neutrophils and dendritic cells. In the context of injury, as the process of repair unfolds in time, batteries of antimicrobial peptides are successively expressed, protecting the healing surface in a temporally appropriate context and calling into the healing environment immune cells that complement the process. The relatively low potency of the chemokine activity of most antimicrobial peptides (micromolar) ensures that only those cells within close range of the site of injury will be called to action, as opposed to the more systemic signalling that occurs with the release of classical cytokines.

The discovery that antimicrobial peptides are inducible has opened up the possibility of new therapeutic opportunities. The remarkable discovery that the human cathelicidin gene is induced by vitamin D, and the association of certain bacterial and viral illnesses with vitamin D deficiency, has vast implications with respect to public health. Clinical studies suggesting that vitamin D supplementation can reduce the incidence of tuberculosis, upper respiratory infections and influenza A have been reported and many other trials are underway. The discovery that simple nutrients and metabolites, such as butyrate and isoleucine, can induce epithelial antimicrobial peptides is being evaluated in studies using the oral administration of these substances in the treatment of bacterial and viral dysentery in humans.

Abnormal regulation of the expression of antimicrobial peptides has been linked in recent years to several human diseases. Conditions such as eczema and Crohn's disease are associated with the depressed expression of specific antimicrobial peptides. In diseases such as cystic fibrosis, the milieu (the ionic strength of the airway surface fluid) in which the antimicrobial peptide is normally designed to operate is disturbed. In these conditions the failure of antimicrobial peptides to protect the epithelial barrier forces the body to support the defence of the barrier by mounting a secondary inflammatory response that creates the havoc of disease. In diseases such as psoriasis, however, an unexplained over-expression of epithelial antimicrobial peptides appears to excite the inflammatory arm of the adaptive immune system, creating the skin lesions that characterize this disease.

Although humans have one cathelicidin gene, we have numerous copies of the defensin family locus, resulting in differing levels of expression of both the α- and β-defensins between individuals. How these genetic differences play out in health and disease is of great interest.

The development of antimicrobial peptides as human therapeutics remains a challenge. Early on it became clear that peptides such as magainin, although active in the test tube, exhibit a poor therapeutic index when evaluated in the setting of an infected animal. Most of the antimicrobial peptides discovered in nature seem to have this characteristic. Unfortunately, we do not know how to re-engineer a peptide to improve its pharmacological activity in such a way that would make it a more effective therapeutic. Certain naturally occurring molecules that exhibit the efficacy and safety in animals expected for a therapeutic – such as plectasin from a fungal organism – have, however, been discovered. Surely the insights molecules such as these will provide will help advance the development of antimicrobial peptides as drugs. In addition, remarkable non-peptide antimicrobial molecules have been created that mimic the activity of antimicrobial peptides. These synthetic molecules exhibit an attractive therapeutic index, and can be synthesized inexpensively. In other words, it is very likely that antimicrobial peptides, be they of natural or synthetic origin, will surely join the armamentarium of anti-infective agents in the coming years.

Many of the contributors to this book have been responsible for establishing the foundations of what we now know about antimicrobial peptides. I hope the knowledge that is shared here in this book will stimulate others to make new discoveries and further advance this exciting and important field.

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1 A Database View of Naturally Occurring Antimicrobial Peptides: Nomenclature, Classification and Amino Acid Sequence Analysis‡

Guangshun Wang,* Xia Li and Michael Zasloff

Abstract

The nomenclature and classification of naturally occurring antimicrobial peptides (AMPs) are complex and have not been fully standardized. The Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php) is a useful tool that facilitates peptide naming and classification. The names of AMPs are normally derived from peptide properties, source species or a combination of both. In the database, AMPs are classified based on source organisms (protozoa, bacteria, archaea, fungi, plants and animals), biological activities (antibacterial, antifungal, antiviral, antiparasitic, spermicidal and insecticidal), peptide features (charge, length, hydrophobic residue content, chemical modification and three-dimensional structure), binding targets (membranes and non-membranes) and mechanisms of action of the peptides. This database also enables bioinformatic analysis of AMPs that reveals amino acid composition signatures as well as frequently occurring residues.

All organisms possess specialized defence systems tailored for survival in a variety of environments. Antimicrobial peptides (AMPs) bridge this diversity by functioning as key components of defence systems. In invertebrates, AMPs are the major defence molecules of innate immunity. In vertebrates, AMPs serve not only as effectors in innate immunity, but also as modulators for adaptive immune systems (Shai, 2002; Tossi and Sandri, 2002; Zasloff, 2002; Boman, 2003; Brogden *et al*., 2005; Zanetti, 2005; Hancock and Sahl, 2006; Amiche *et al*., 2008; Conlon, 2008). The identification of cecropins, magainins and defensins in insects, amphibians and humans in the 1980s (Steiner *et al*., 1981; Selsted *et al*., 1985; Giovannini *et al*., 1987; Zasloff, 1987) stimulated research on AMPs and led to the isolation and characterization of hundreds of new peptides. Meanwhile, the global problem of antibiotic resistance further fuelled this research field with a hope of identifying new therapeutics.

The rapid increase in the number of AMPs demands a more efficient data registration and management method. In the past several years, 13 databases have been built for AMPs (Table 1.1). These databases facilitate information management, annotation, retrieval and peptide analysis. This chapter focuses on peptide nomenclature, classification and sequence analysis based on the

‡ Chapter editor: Donatella Barra.

* Corresponding author.

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Table 1.1. Chronological listing of major databases dedicated to AMPs.

Year[a]	Database	Web site	Content
2002	AMSDb	http://www.bbcm.univ.trieste.it/~tossi/amsdb.html	Plant/animal AMPs
2002	SAPD	http://oma.terkko.helsinki.fi:8080/~SAPD	Synthetic AMPs
2004	Peptaibol	http://www.cryst.bbk.ac.uk/peptaibol/home.shtml	Fungal AMPs
2004	APD	http://aps.unmc.edu/AP	Natural AMPs
2004	ANTIMIC	Not active	Natural AMPs
2006	PenBase	http://penbase.immunaqua.com	Shrimp AMPs
2006	Cybase	http://research1t.imb.uq.edu.au/cybase	Cyclotides
2007	BACTIBASE	http://bactibase.pfba-lab-tun.org/main.php	Bacteriocins
2007	Defensins	http://defensins.bii.a-star.edu.sg	Defensins
2007	AMPer	http://marray.cmdr.ubc.ca/cgi-bin/amp.pl	Plant/animal AMPs
2008	RAPD	http://faculty.ist.unomaha.edu/chen/rapd/index.php	Recombinant AMPs
2009	PhytAMP	http://phytamp.pfba-lab-tun.org/main.php	Plant AMPs
2010	CAMP	http://www.bicnirrh.res.in/antimicrobial	All AMPs

[a] The year when the database was described in a published article. Some databases were built even earlier (see text).

updated version of the Antimicrobial Peptide Database (APD), which contains AMPs with fewer than 100 amino acid residues (Wang and Wang, 2004; Wang *et al*., 2009).

1.1 Database Scope and Overview

The major databases for AMPs shown in Table 1.1 can be classified into three categories: (i) general databases for natural AMPs (AMSDb, ANTIMIC, APD and CAMP); (ii) specialized databases for natural AMPs (Peptaibol, PenBase, Cybase, BACTIBASE, Defensins and PhytAMP); and (iii) specialized databases for non-natural AMPs (SAPD and RAPD). In the following paragraphs, we briefly describe the functions of each database in a chronological order.

The first version of the Antimicrobial Sequences Database (AMSDb) (approximately 300 entries) was built by an undergraduate as a part of thesis work and placed online in 1997 (Alex Tossi, personal communication, 2006). The information was extracted from Swiss-Prot and kept in the same data format. According to the AMSDb, the number of AMPs nearly doubled between 1994 and 2001. As of 2004, this database hosted 895 peptides from eukaryotes, including both precursor and mature peptide sequences. Among them, 278 entries contained the word 'precursor'. For some AMPs such as human LL-37, both the precursor protein and mature peptide were registered. The database had also collected 45 antimicrobial proteins. In addition, this database tabulated the AMP literature (1997–2004) alphabetically based on the last name of the first author. Only AMPs from prokaryotes were excluded (Tossi and Sandri, 2002). In 2002, a database for synthetic antibiotic peptides (SAPD) was also established (Wade and Englund, 2002). Since registration is needed, SAPD is not a freely accessible tool.

In 2004, three additional databases were simultaneously reported in the database issue of the journal *Nucleic Acids Research*. The Peptaibol database, originally created in 1997, contains 307 peptides isolated from soil fungi (Whitmore and Wallace, 2004). As the 'peptaibol' name implies, such peptides (<20 residues) are rich in non-standard amino acids such as α-aminoisobutyric acid, isovaleric acid, ethyl norvaline and the imino acid hydroxyproline, which are represented by the non-amino acid letters U, J, Z and O, respectively. This is an important feature, as other databases have collected peptides with the standard 20 amino acids or their derivatives. Also published are two general databases (APD and ANTIMIC), which cover AMPs from a variety of biological sources. These two databases, both created by graduate students as part of their master degree

theses, contain registered information as well as some useful tools for AMP research. Unfortunately, ANTIMIC (Brahmachary *et al*., 2004) became inaccessible several years ago. The first version of the APD (Wang and Wang, 2004) collected mature AMPs primarily from natural sources, ranging from protozoa to bacteria, archaea, fungi, plants and animals, including humans. Gene-encoded AMPs that are post-translationally modified are also collected, as are a few AMPs synthesized by multienzyme systems. Some synthetic peptides of particular interest are included, too. At present, the following polypeptides are not collected: (i) propeptides or precursors of AMPs; (ii) genome-predicted or isolated peptides, the antimicrobial activities of which have not been experimentally validated; (iii) peptides that have been found to be inactive against microbes; and (iv) antimicrobial proteins with greater than 100 amino acid residues. These boundaries merely reflect the current status of the database, rather than set restrictions for future database expansion. The first version of the APD collected 525 mature and active peptides. Among them, 498 were annotated to have antibacterial activities, 155 antifungal activities, 28 antiviral activities and 18 anticancer activities. The APD provides interactive interfaces for peptide search, prediction and design. It also provides statistical data for a selected group of peptides or all peptides in the database. The database has since been updated and expanded significantly. By March 2010, when this chapter was written, there were 1528 peptide entries in the APD.

Subsequently, several other databases, mostly for special types of AMPs, were built. While PenBase is dedicated to shrimp AMPs (Gueguen *et al*., 2006), Cybase is a specialized database for cyclic polypeptides from bacteria, plants and animals (Mulvenna *et al*., 2006). Some cyclic polypeptides such as plant cyclotides have been found to be antimicrobial or human immunodeficiency virus (HIV) inhibitory (Chapter 3). In 2007, AMPer (Fjell *et al*., 2007), BACTIBASE (Hammami *et al*., 2007) and Defensins Knowledgebase (Seebah *et al*., 2007) were also established. AMPer is an AMP prediction tool developed based on the peptides collected in AMSDb and Swiss-Prot. After a recent update, BACTIBASE now hosts 177 bacteriocins. The Defensins database provides detailed information for various defensins as well as related literature, including patents. In 2008, a specialized database for recombinant AMPs (RAPD) was also built to document peptide expression, host, carrier, cleavage method and yield (Li and Chen, 2008). In 2009, another specialized database was established for plant AMPs (PhytAMP) (Hammami *et al*., 2009). Currently, PhytAMP contains 271 peptide entries. In January 2010, CAMP appeared (Thomas *et al*., 2010). This database has collected 1192 peptides with known activities, 1589 peptides from patents and 1084 predicted AMPs, the antimicrobial activities of which have not been validated. The database also incorporates a few prediction tools. For raw data and additional information, users can consult the original articles, Swiss-Prot, the Protein Data Bank and PubMed.

1.2 Nomenclature of Antimicrobial Peptides

Peptide name search is a common feature of all the databases listed in Table 1.1. A unique aspect of the name search of the updated APD (Wang *et al*., 2009) is that it allows for multiple-word searches, thereby increasing search accuracy and flexibility. Up to three search words can be entered into the search boxes. The output decreases with increases in the number of search words. For example, we found 179 AMPs when 'defensin' was searched; 12 entries appeared when both 'defensin' and 'human' were searched; and six human α-defensins appeared when 'defensin', 'human' and 'alpha' were all searched. Note that other Greek letters such as β, γ and δ are represented in the APD as beta, gamma and delta, respectively. A study of the AMP names collected in the APD reveals a complex picture. Various methods have been employed to name a newly identified peptide. These methods fall into three categories: (i) peptide-based method; (ii) source-based method; and (iii) source and peptide combined method.

1.2.1 Peptide-based method

AMPs are named based on a variety of peptide properties. First, the name magainin is derived from the Hebrew for shield and defensin was derived from 'defence', implying the functional role of this family of peptides. Secondly, many AMPs have obtained their names from the amino acid sequence: human histatins are rich in histidine residues; PR-39 is a 39-residue AMP rich in proline and arginine residues; and LL-37 is a 37-residue cathelicidin starting with two leucine residues. Other synonyms for LL-37 (most common) used in the literature and collected into the database are LL37, FALL-39 (old) and leucine, leucine-37 (rare). For plant cyclotides and cyclic dodecapeptide, 'cyclo' or 'cyclic' means polypeptide circularization. Thirdly, the word cathelicidin was coined from the well-conserved 'cathelin' domain of the precursor proteins (Zanetti, 2005). Therefore, cathelicidins represent a family of AMPs that share the common cathelin domain. In this book, hCAP-18 (18-kDa human antimicrobial protein) refers only to the precursor protein of human cathelicidin. Fourthly, peptide targets have also found their way into AMP names. While bacteriocins kill bacteria, AFP1 stands for antifungal protein 1. Sometimes both the structure and activity of the peptide are implicated in the name. For example, in the name of τ-defensin, τ reflects the cyclic, cysteine-bridged structure and defensin the activity.

1.2.2 Source-based method

The most common approach is to derive the peptide name from the name of a source species. Usually, either the genus or species name is taken. For example, sesquin is derived from *Vigna sesquipedalis* and palicourein is taken from *Palicourea condensata*. Sometimes, a combination of the scientific name is adopted. For instance, Hyfl E is derived from *Hybanthus floribundus* E. In other cases, the peptide name is based on the common name of an organism (e.g. termicin from termites). Abbreviations of animal names are used to name homologous AMPs. The name of bovine β defensin-1 (bBD-1) is analogous to human β defensin-1 (hBD-1). Other animal source abbreviations include p (pigs, e.g. PMAP-36), e (equine, e.g. e-CATH-1), s (sheep, e.g. SMAP-29) and oa (ovine, e.g. OaBac5). The sex of an organism is also implied in the case of insect andropin (male specific). Sometimes the names of organs or tissues are also used. Examples are human neutrophil peptide-1 (HNP-1), liver-expressed AMP-2 (LEAP-2), dermcidin from skin and human salvic from salivary glands.

1.2.3 Source and peptide combined method

In many cases, source organisms and peptide features have been combined to assign a unique name. The APD has collected 27 plant AMPs that were named by using the first letters of the scientific names of the species followed by AMP. For instance, Ib-AMP was abbreviated from *Impatiens balsamina* AMP. When multiple similar peptides are found, they may be named by appending numbers (e.g. Ib-AMP1 to Ib-AMP4). When available, the database gives both full and abbreviated names. In addition, the peptide part can also represent peptide family or peptide activity. While So-D1 was abbreviated from *Spinacia oleracea* defensin 1 (peptide family), Ee-CBP originated from *Euonymus europaeus* chitin-binding protein (activity).

The chaotic situation with the AMP nomenclature has spurred efforts in the field to achieve consistency. Based on peptide features and source species names, PenBase proposed a nomenclature for shrimp AMPs (Gueguen *et al*., 2006). For example, the recommended AMP name '*litvan* PEN3-1' is composed of three parts:

- *Litvan* was abbreviated from the species name *Litopenaeus vannamei*.
- Based on the conserved amino acid pattern, it was assigned to penaeidin subgroup 3 (PEN3).
- The number '1' means that it is the first AMP found in that species.

Both Amiche *et al*. (2008) and Conlon (2008) proposed a consistent nomenclature for amphibian AMPs. This led to the re-naming of some peptides (Amiche *et al*., 2008). To facilitate the transition from the old names to the recommended names, the APD has also collected both old and new names, with the preferred name appearing first. It is suggested that the name of the first published AMP in this genus takes priority. Orthologous peptides from the same genus and different species can be distinguished by capitalizing the first letter of the species name. Two capitalized letters should be used if the same letter has been used by another species (Conlon, 2008).

The nomenclature of AMPs is further complicated by the discovery of antimicrobial activities of polypeptides, which were initially identified and named based on other biological functions or medical significances. For example, plant-isolated kalata B1 was used as a uterotonic agent to facilitate childbirth in Congo. Its name is derived from a native medicine, *kalata-kalata* (Gran, 1973). The discovery of γ-core in cysteine-containing AMPs such as defensins led to testing the antimicrobial activity of brazzein (Young and Yeaman, 2004), which is a well-known sweet-tasting protein (Hellekant and Danilova, 2005). The peptide name is derived from the African plant *Pentadiplandra brazzeana*. It has been demonstrated that some neuropeptides and hormones exhibit antimicrobial activity and can participate in host defence as well (Brogden *et al*., 2005). These peptides were initially named in a different biological context. Likewise, chemokines such as CXCL14 also display wide-spectrum antibacterial activity (Maerki *et al*., 2009). Chemokines have their own naming rules and are grouped based on the number and arrangement of conserved N-terminal cysteine motifs: C, CC, CXC and CX3C, where 'X' is a non-conserved residue (Cole *et al*., 2001). Interestingly, some known AMPs such as human defensins and cathelicidin LL-37 are known to have chemotactic effects (Murakami *et al*., 2004; Taylor *et al*., 2008). Thus, chemotaxis is an important property of AMPs that links the innate and adaptive immune systems (Zasloff, 2002).

1.3 Annotation and Classification of Antimicrobial Peptides

1.3.1 Source organisms and peptide family classification

Source organisms and their taxonomy

AMPs in the APD are classified based on their source organisms. The classic five-kingdom system proposed by Robert H. Whittaker in 1969 has been adopted. The five kingdoms are Prokaryotae (bacteria and archaea), Protista (protozoa and algae), Fungi (fungi), Plantae (plants) and Animalia (animals). This classification was enabled in the database by appending key words such as 'bacteria', 'plants' or 'animals' behind peptide names. For example, partial information in the 'name' field for peptide entry AP01228 is represented as: '*Microcin V (MccV, (old name) Colicin V, ColV; class 2a microcins, bacteriocins, Gram-negative bacteria)*'.

The above text indicates that microcin V (abbreviated as MccV), once called colicin V (or ColV), is a class IIa microcin (one type of bacteriocin) synthesized by a Gram-negative bacterium. This format allows users to retrieve AMPs from a specific kingdom. The number of peptides in different kingdoms is given in Fig. 1.1. These numbers were obtained by database query using search words such as 'plants' and 'fungii' in the name field. Note that the use of 'plants' and 'fungii' for search gave more accurate results, since occasionally the names of some AMPs contain 'plant' or 'fungi'. Human AMPs are annotated as an independent group, currently with 54 entries isolated from saliva, skin, eyes, liver and other organs. Furthermore, prokaryotic AMPs have been split into two groups: bacteria and archaea. This separation enables a calculation of AMPs from the three domains proposed by Carl R. Woese in the 1970s: bacteria, archaea and eukarya (Fig. 1.1A). There are 118, two and 1369 peptides from these domains, respectively. We conclude that AMPs have been identified in all life domains or kingdoms.

Figure 1.1A shows that 71.3% of peptides in the APD originate from animals. The diversity of animal AMPs requires further

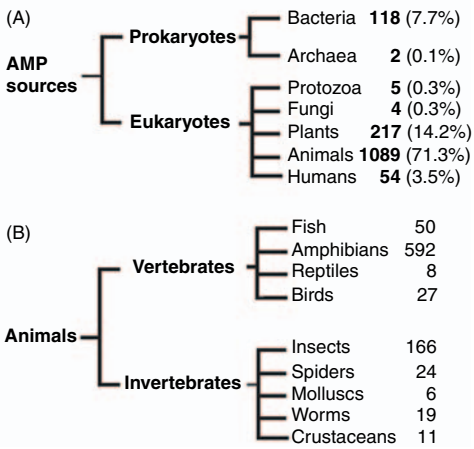

Fig. 1.1. (A) The number of antimicrobial peptides from a variety of kingdoms. (B) The number of AMPs from selected animal families. Data from the Antimicrobial Peptide Database analysed in February 2010. (Total AMP number: 1528.)

classification. Broadly, they belong to either invertebrates or vertebrates. For invertebrates, source names such as insects, spiders, molluscs, worms and crustaceans are included behind the peptide names; for vertebrates, fish, amphibians, reptiles, birds and mammals are used as additional classifiers. The number of AMPs from select animal groups currently annotated in the database is provided in Fig. 1.1B. In particular, amphibian peptides account for 38.7% of total AMPs, representing a rich source of AMP discovery. Likewise, the APD has collected 166 AMPs from insects.

The source organism for an AMP is not an issue as long as the organism under investigation is 'pure'. However, problems may occur when the organism is so large that a microbial species or parasite also lives within it. For example, cecropin P1 was initially thought to be isolated from pigs. Later, it was found that this peptide originated from a large round worm (nematode) living in pigs (Andersson *et al*., 2003). The origin of an AMP can be more complex in the parasite–host system. For example, a parasitic tick generates AMPs in its gut by enzyme digestion of the haemoglobin protein from cattle or rabbit blood (Fogaça *et al*., 1999; Nakajima *et al*., 2003). In these cases, it is proper to use the host–parasite system to represent the source species of the AMP.

Peptide family classification in different kingdoms

Peptide family classification is enabled, and can be searched, in the name field of the APD. Bacterial AMPs have a general name of bacteriocins. Initially, those isolated from Gram-positive bacteria were divided into four families based on the original classification scheme of Klaenhammer (1993). A fifth group was proposed by Kemperman *et al*. (2003). Class I peptides are extensively modified after translation and are referred to as lantibiotics. Examples are nisins, ericins and lacticins. Lantibiotics are complex and have been further classified (see Chapter 2). Peptides in class II are composed of normal amino acids without chemical modifications. Class III bacteriocins are peptides conjugated to lipid or other moieties, which have not been isolated to date. Class IV contains large bacteriocins. In the current APD, there are no members of class III or IV bacteriocins from Gram-positive bacteria. Finally, class V contains circular AMPs where the N- and C-termini of the polypeptide chain are connected by a peptide bond. Enterocin AS-48, gassericin A, circularicin A and closticin 574 are circular bacteriocins.

Cotter *et al*. (2005a) have simplified the classification scheme for bacteriocins from Gram-positive bacteria. In this approach, classes I and II (above) are maintained. While the original class III is removed, class IV is re-assigned as a new class III for bacteriolysins larger than 10 kDa. In addition, there are subclasses in class II. While pediocin-like peptide bacteriocins (e.g. leucocin A and divercin V41) are placed in class IIa, class IIb contains bacteriocins with two independent polypeptide chains. Plantaricin JK and lactocin 705 are examples of class IIb members. The above circular class V bacteriocins are re-assigned as class IIc. The remaining linear non-pediocin peptides are combined into class IId (e.g. entericin Q and MR10). We have adopted this modified approach (summarized in Table 1.2) because

Table 1.2. Classification of bacteriocins.

Gram-positive bacteria	Definition	Gram-negative bacteria	Definition
Class I	Lantibiotics	Class I	Microcins <5 kDa
Class II	Non-lantibiotics	Class II	Microcins 5–10 kDa
IIa	Pediocin-like peptides	IIa	S–S bond-containing
IIb	Two-chain peptides	IIb	Linear
IIc	Circular peptides	Class III	Colicins >10 kDa
IId	Non-pediocin-like peptides		
Class III	Large proteins >10 kDa		

it is similar to that proposed for Gram-negative bacteria below.

Recently, bacteriocins from Gram-negative bacteria have also been classified (Table 1.2). Small peptides (<10 kDa), referred to as microcins, were further classified into two groups. Class I microcins are small (<5 kDa) and their backbones are extensively modified after translation. Class II microcins are larger (5–10 kDa). These are further separated into class IIa and class IIb. Class IIa peptides contain disulfide bonds, while class IIb microcins are linear peptides with a C-terminal chemical modification (Duquesne *et al.*, 2007). To be complete, we have assigned large bacteriocins (i.e. colicins >10 kDa) as class III. The APD has adopted class 1, class 2 and class 3, rather than class I, class II and class III, to improve database search accuracy. At present, there are three class 1 microcins and six class 2 microcins in the APD.

Based on sequence similarity and cysteine motifs (Egorov *et al.*, 2005), plant AMPs have been classified into seven families (Table 1.3). Cyclotides are treated as a new group. The number of peptides and examples in each family are given in Table 1.3. Plant AMPs are discussed in Chapter 3.

In animals, the classification of AMP families is more complex and we only highlight a few here. In amphibians, magainins and dermaseptins are well known. In addition, the following AMP families are well established: brevinin-1, brevinin-2, esculentin-1, esculentin-2, japonicin-1, japonicin-2, nigrocin-2, palustrin-1, palustrin-2, ranacyclin, ranatuerin-1, ranatuerin-2 and temporin (Conlon, 2008; Conlon et al., 2009). The APD has collected 99 brevinins, 70 temporins, 42 ranatuerins and 20 esculentins.

In insects, the well-known families are linear cecropins, disulfide-linked defensins (e.g. sapecin, SmD1, heliomicin, termicin, royalisin and gallerimycin) and Pro-rich peptides (e.g. drosocin, apidaecin IA, abaecin, lebocins and pyrrhocoricin) (Bulet and Stocklin, 2005).

In marine invertebrates, Otero-González *et al.* (2010) have described AMPs from different phyla such as porifera, cnidaria (e.g. aurelin), mollusca (e.g. MGD-1, mytilins, Cv-Def and Cg-Def), annelida (e.g. arenicins), arthropoda (e.g. penaedins, arasins, tachyplesins, polyphemusins, big defensins and tachystatins), echinodermata and chordata (tunicates) (e.g. clavanins).

Table 1.3. Classification of plant AMPs.

Group	Plant AMPs	Number	Examples
1	Defensins	44	So-D1
2	Thionins	10	Tu-AMP1, Cp-thionin II
3	Lipid-transfer proteins	0	
4	Hevein-like peptides	4	Ee-CBP, WAMP-1
5	Knottin-like peptides	0	
6	Glycine-rich peptides	1	Pg-AMP1
7	MBP-1 homologues	1	MBP-1
8	Cyclotides	126	Circulin A, kalata B1

In mammals, including humans, the major AMP families are defensins, cathelicidins and histatins (Zanetti, 2005). In the APD, there are 121 defensins and 53 cathelicidins from animals. In addition, there are 12 human defensins and two human cathelicidin peptides (LL-37 and ALL-38) encoded by the same cathelicidin gene (detailed in Chapter 9). Other well-known human AMPs are dermcidin, LEAP-1 (hepcidin) and granulysin. It is evident that the classification schemes for AMPs from a variety of biological sources are still in the early stages due to incomplete information.

1.3.2 Classification based on biological activities

Activity spectrum of AMPs

The APD also divides natural AMPs into subgroups based on antimicrobial activities. Some peptides have a narrow activity spectrum, while others have a wider spectrum. Both defensins and cathelicidins are wide-spectrum AMPs (Zasloff, 2002; Zanetti, 2005). Bactericocins are characterized by narrow-spectrum activity as well as very low minimal inhibitory concentrations at nanomolar levels (antimicrobial activity is usually represented in micromoles). At present, the APD allows searching for AMPs with the following biological activities: antibacterial, antifungal, antiviral, antitumoral, antiparasital, spermicidal and insecticidal. These fields can be searched individually, allowing users to retrieve a list of AMPs with the desired properties (Table 1.4). For instance, the database has collected 1180 peptides with antibacterial activity. Among these, 206 peptides display activity against only Gram-positive bacteria and 108 peptides show activity against only Gram-negative bacteria. Antiparasital, spermicidal and insecticidal peptides are searched by entering keys ZZP, ZZS and ZZI, respectively, in the name field. The numbers of peptides with different activities are listed in Table 1.4. In addition, 175 AMPs display a cytotoxic effect on human cells (e.g. red blood cells). These activities can also be searched in combinations. For example, there are at least 388 peptides with both antibacterial and antifungal activities, but only 41 peptides with both antifungal and antiviral activities. Likewise, 40 peptides have been found to have antibacterial, antiviral and antifungal effects. However, merely 12 peptides show antibacterial, antiviral, antifungal and anticancer activities. Because of the partial overlap in activity spectrum, some AMPs can be included in different groups.

Table 1.4. Peptide classification in the Antimicrobial Peptide Database based on biological activity.

Peptide activity	Search icon/key	Number of peptides
Antibacterial	Icon	1180
Antifungal	Icon	451
Antiviral	Icon	98
Antitumoral	Icon	101
Antiparasitic	ZZP	16
Spermicidal	ZZS	8
Insecticidal	ZZI	16
Haemolytic	Icon	175

It is pertinent to point out that the antimicrobial activity evaluated using laboratory bacterial strains may not be biologically relevant in real cases. In addition, for quantitative structure–activity studies, one should be cautious in utilizing the antimicrobial activity data obtained from different laboratories evaluated under different conditions (e.g. strains, media and methods).

Synergistic effects of AMPs

A set of AMPs from one specific species can be obtained by searching the database using the scientific name in the 'AMP source species' field. A search using '*Odorrana grahami*' returned 26 AMPs. In the original proteomic study (Li *et al*., 2007), 107 peptides were detected from the skin of a single frog *O. grahami*. Many of these peptides have not yet been registered into the APD due to a lack of activity data. Likewise, we found 26, 27 and 54 peptides when *Viola odorata* (an Australian plant), *Bos taurus* (cow) and *Homo sapiens* (humans) were queried, respectively. These numbers should be regarded as minima for two reasons. First, scientific

names may be absent in the original articles. Secondly, some peptides are not included due to a lack of activity data.

Scientists have been curious as to why frogs generate such a large number of peptides. One possibility is that multiple peptides are created for different functions that are not yet fully understood. Another possibility is that the shear multiplicity of environmental microbes requires a variety of host defence peptides. Furthermore, some peptides can work together to achieve optimal protection. Significantly, some peptides are ineffective when evaluated alone, but become antibacterial when evaluated in combination (Li *et al*., 2007). Amphibian AMPs that show a synergistic effect include magainin 2 and PGLa (Matsuzaki *et al*., 1998; Strandberg *et al*., 2009); magainin 2 and cecropin A (Cirioni *et al*., 2008); and temporins A, B and L (Rosenfeld *et al*., 2006). Note that temporins A, B and L were found to show a synergistic effect in both antimicrobial and antiendotoxin (lipopolysaccharide) activities. Some bacteriocins such as enterocin L50 (Cintas *et al*., 1998) and lichenicidin (Begley *et al*., 2009) comprise two independent peptide chains, which display higher activity when combined in a 1:1 ratio. Peptide synergistic information can now be searched through the 'additional information' field by using 'synerg' or via the name field by entering the 'JJsn' key. By March 2010, the APD had collected 15 such synergistic pairs.

1.3.3 Classification based on peptide characteristics

Peptide charge, length and hydrophobic residue content

In the database search interface of the APD, there are search icons for peptide charge, length and percentage of hydrophobic residues. Based on the charge, AMPs can be classified into cationic and anionic peptides. Of a total of 1528 AMPs, 1355 peptides have a net positive charge, 95 have a net charge of zero and 78 have a net negative charge. For a particular AMP, the net charge can vary slightly depending on the nature of chemical modification. For example, the charge increases by 1 when the C-terminus of the peptide is amidated. Evidently, the majority (88.6%) are cationic AMPs with an average net charge of +4.4 per peptide. Figure 1.2 shows the number of AMPs as a function of net charge. The distribution for all AMPs (Fig. 1.2A) is relatively smooth with a peak at +3. For bacterial AMPs (Fig. 1.2B), two peaks exist at +2 and +4, while the peaks shift slightly to 0 and +2 for plant AMPs (Fig. 1.2C). The amphibian AMPs appear to have a more clustered net charge distribution, with the majority in the range of +1 to +4 (Fig. 1.2D). There are a few outliers not depicted in these plots. Oncorhyncin II and OaBac11 (Anderson and Yu, 2003; Fernandes *et al*., 2004) are the most positive peptides with a net charge of +30. In amphibians, the most positively charged AMP is buforin II with a net charge of +13. Current research is primarily focused on cationic AMPs, with a favourable assumption that they can selectively target negatively charged bacterial membranes. The identification of anionic peptides, however, has further expanded the AMP spectrum. The two most negatively charged peptides contain a net charge of –6. While the anionic peptide SAAP (sequence DDDDDD) requires Zn^{2+} to be antibacterial (Brogden *et al*., 1996), naegleriapore B is a pore-forming peptide from a parasitic protozoon (Herbst *et al*., 2002). Anionic AMPs have also been found in other sources such as amphibians and humans (Schittek *et al*., 2001; Lai *et al*., 2002). Anionic dermcidin acts using a different mechanism from cationic LL-37 (Senyürek *et al*., 2009).

AMPs can also be classified based on their length. In the APD and this book, peptides are defined as containing fewer than 100 amino acid residues. The amino acid sequence of an AMP, in the single-letter amino acid code (e.g. C, G and R), can be entered in part or full into the search box of the 'sequence' field. This is the most accurate method of discovering whether a particular peptide has been registered in the APD. The majority of AMPs (98.4%) are listed with a complete amino acid sequence. Peptides with

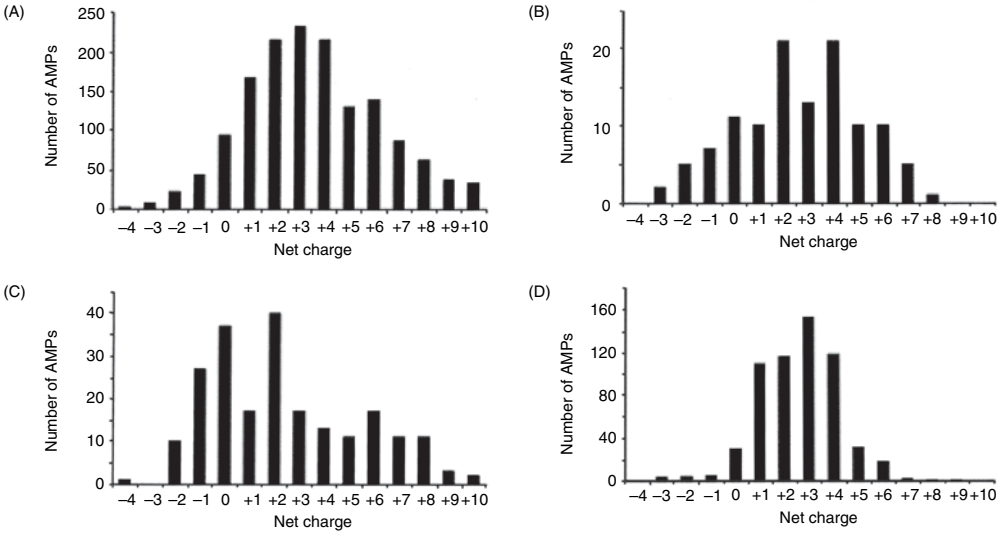

Fig. 1.2. Distribution of antimicrobial peptides in the Antimicrobial Peptide Database versus net charge from (A) all sources, (B) bacteria, (C) plants and (D) amphibians. The total number of peptides in each kingdom is provided in Fig. 1.1.

incomplete amino acid sequences can be searched in the name field by using 'BWQ'. The database also allows searching for AMPs in a defined length range, such as 11–20 or 21–30 amino acids. The number of AMPs as a function of peptide length is plotted in Fig. 1.3. When all of the 1528 AMPs in the database (Fig. 1.3A) are analysed, the distribution plot gives a peak at 30 (i.e. 21–30 residues). In both bacteria (Fig. 1.3B) and plants (Fig. 1.3C), most of the AMPs have 21–50 residues, whereas 97.3% of amphibian AMPs have 11–40 residues (Fig. 1.3D). The shortest peptides collected in the database contain only five residues, whereas the longest peptides contain fewer than 100 residues due to the peptide definition.

AMPs can also be classified based on their hydrophobic residue content. A distribution of the AMPs in the database versus hydrophobic residue content (hydrophobic residues divided by total residues) is presented in Fig. 1.4. In the APD, the following residues are defined as hydrophobic: isoleucine, valine, leucine, phenylalanine, cysteine, methionine, alanine and tryptophan. This was obtained by expanding the original set of hydrophobic residues defined by Kyte and Doolittle (1982). For all AMPs, the peak is located at 41–50% (labelled as 50%) (Fig. 1.4A). Out of 118 bacterial AMPs (Fig. 1.4B), 56 have a hydrophobic residue content between 31 and 40%, and 110 out of 217 plant AMPs (Fig. 1.4C) are within the hydrophobic residue content of 31–40%. Nearly all amphibian AMPs (Fig. 1.4D) have a hydrophobic content between 41 and 70%. The higher hydrophobic content in this animal family is related to a high content of alanine residues. Three AMPs from bacteria show hydrophobic contents even greater than 80%. They are gramicidins that form transmembrane ionic pores. These unusual AMPs differ from the majority of the peptides collected in the APD in that they are synthesized by a non-ribosome multienzyme system.

Chemical modifications of AMPs

Antimicrobial peptides can also be classified based on the types of chemical modifications. This is the case with bacteriocins from Gram-positive bacteria (Table 1.2). Post-translational modifications play a critical

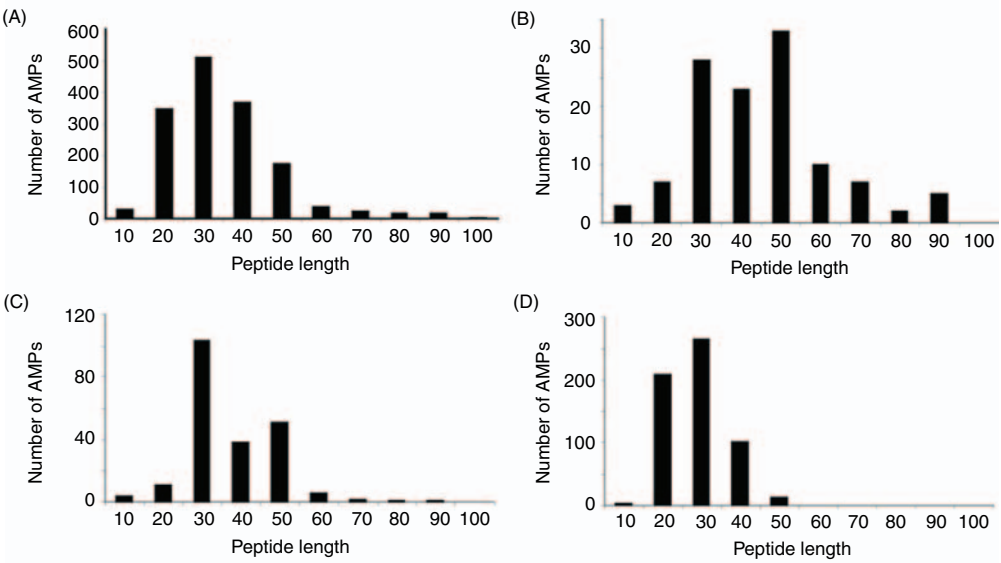

Fig. 1.3. Distribution of antimicrobial peptides in the Antimicrobial Peptide Database versus peptide length from (A) all sources, (B) bacteria, (C) plants and (D) amphibians. The total number of peptides in each kingdom is provided in Fig. 1.1. Each column represents the number of the peptides in a range defined between the two adjacent numbers. For example, '20' means '11–20'.

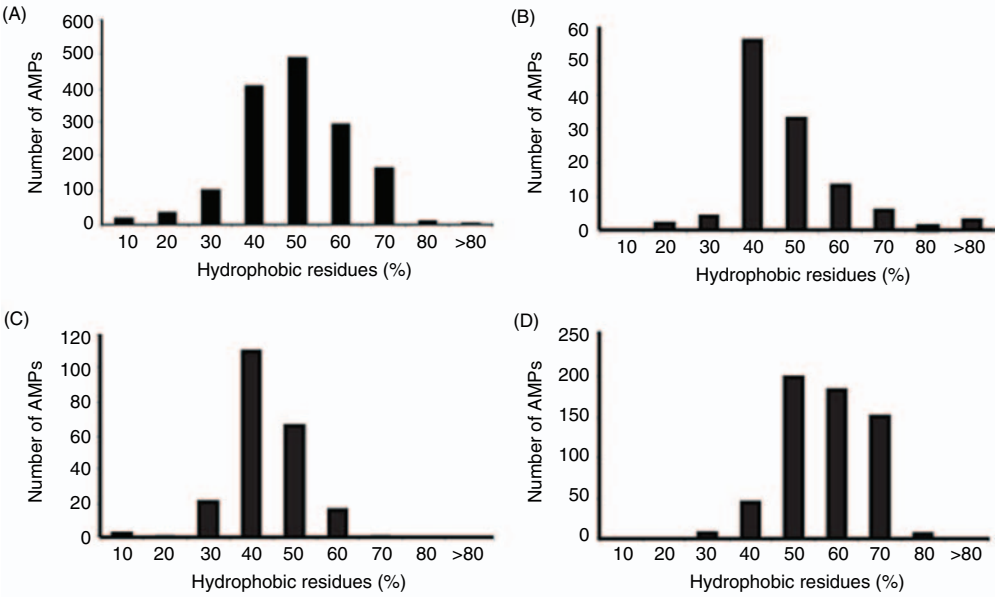

Fig. 1.4. Distribution of antimicrobial peptides in the Antimicrobial Peptide Database versus hydrophobic residue (%) from (A) all sources, (B) bacteria, (C) plants and (D) amphibians. The total number of peptides in each kingdom is provided in Fig. 1.1. Each column represents the number of the peptides in a range defined between the two adjacent numbers. For example, '20' means '11–20'.

role in modulating antimicrobial activity or other biological functions of the peptides. For example, both aurein 1.2 (unpublished data) and anoplin (Dos Santos Cabrera *et al*., 2008) have been found to lose their activities after removal of C-terminal amidation. However, not all amidated peptides display increased activity (Dennison *et al*., 2009). Amidation can also improve peptide stability. Joanne *et al*. (2009) proposed that amidation might be involved in GraS/GraR (glycopeptide resistance-associated protein S/R)-mediated AMP sensing at the bacterial surface. Enterocin AS-48 is the first circular bacteriocin identified and an excellent candidate as a food preservative. Recent investigation of its linear form revealed that circularization is required for peptide structure rather than bactericidal activity (Montalbán-López *et al*., 2008). Wilson-Stanford *et al*. (2009) found that oxidation of lantibiotics such as nisin A disrupted antibacterial activity. It was proposed that the oxidized form of sulfur is unable to bind to lipid II of the cell wall. Although one may wonder whether the oxidation occurs *in vivo* or during purification, it is good practice to prevent oxidation if it inactivates the peptide. Attachment of a bacterial receptor-recognizable moiety to the peptide allows the peptide to be smuggled into the bacterium via the 'Trojan horse' trick. Both microcin C7 (MccC7) and microcin E492 possess such a ferric-binding moiety, which is also important for antibacterial activity. MccC7 is cleaved within the cell and the resulting peptide targets tRNA synthetase and inhibits protein synthesis (Roush *et al*., 2008).

Table 1.5 provides an expanded list of the search keys for chemical modifications with peptide examples. When XX was searched in the name field, 465 peptides (30.5%) appeared, indicating that post-translational modifications are common for AMPs. Many linear peptides (272 in the APD) are C-terminally amidated (XXA). In natural AMPs, the amidation group is derived from a glycine residue. However, 139 peptides (9%) have been found to have a circular structure (XXC) where the N- and C-termini of the peptide are covalently linked by forming a peptide bond (Table 1.5). Circular AMPs have been found in bacteria, plants and animals. Not all peptides are circulated in the same way, however. Microcin J25 was originally thought to form a head-to-tail peptide bond. It is now established that a

Table 1.5. Database search keys for AMP chemical modifications.[a]

Search key	Chemical modification	Number of peptides	Examples
XXA	C-terminal amidation	272	Aurein 1.2, indolicidin
XXB	C-terminal iron-binding moieties	4	MccE492, MccH47
XXC	Polypeptide chain cyclization between the N- and C-termini	139	Cyclotides; class IIc bacteriocins
XXD	D-amino acids	7	Bombinin H4; lactocin S
XXE	N-terminal acetylation	8	Alpha-MSH, paenibacillin
XXF	Carboxylic-acid-containing unit	1	Hymenoptaecin
XXG	Glycosylation (e.g. Thr)	7	Drosocin, heliocin
XXH	Halogenation (F, Cl, Br)	1	Styelin D
XXK	Hydroxylation (e.g. Lys, Arg, Tyr)	1	Styelin D
XXO	Oxidation (e.g. Trp, Cys)	8	Piscidin 4, chrombacin
XXP	Phosphorylation (e.g. Ser)	2	Human histatin 1
XXQ	N-terminally cyclic glutamate	12	Gomesin, Ib-AMP1, heliocin
XXS	Sulfation	1	Chrombacin
XXT	Thioether bridge formation	22	Lantibiotics, e.g. nisin A, mersacidin, lacticin 3147

[a] Some of the keys were originally released in Wang *et al.* (2009). A key search can be performed in the name field.

ring structure is realized between the backbone amide of residue glycine 1 and the side chain of glutamic acid 8 (Rosengren *et al*., 2003). In addition, some amino acid residues also form a ring structure. N-terminal glutamine residues can spontaneously cyclize to become pyroglutamates. This N-terminal blocking makes peptide sequencing by Edman degradation ineffective. The APD has collected 12 such peptides (XXQ in Table 1.5). They have been isolated from plants, spiders, insects, amphibians and reptiles, revealing another general modifying mechanism in nature.

D-amino acids were first identified in natural AMPs from amphibians (Simmaco *et al*., 2009). They have also been found in bacteriocins (Cotter *et al*., 2005b). The APD lists seven D-amino-acid-containing peptides (Table 1.5). However, the identification and characterization of D-amino acids requires elegant techniques. Kawai *et al*. (2004) reported that gassericin A and reutericin 6 are cyclic bacteriocins with an identical amino acid sequence differing only in the number of D-alanines. A recent revisit of these two AMPs demonstrated that they are identical and there are no D-amino acids (Arakawa *et al*., 2010). This work indicates the importance of purifying natural peptides to homogeneity before a full biochemical and biophysical characterization is made.

Some AMPs may be chemically modified in multiple ways. For instance, the sequence of styelin D from the sea squirt is extensively modified, including halogenation (XXH) of tryptophan 2 and hydroxylation (XXK) of arginine, lysine and tyrosine residues (detailed in the database). Such modifications might be essential for the peptide to remain active even at high salt concentrations. Indeed, the native peptide is more active than a synthetic unmodified peptide (Taylor *et al*., 2000). The current version of the APD allows a simultaneous search for three types of chemical modifications (Table 1.5) in the name field. When XXA (amidation), XXP (phosphorylation) and XXS (sulfation) were searched, only chrombacin appeared.

Understanding the mechanism of chemical modification of natural AMPs may provide unique tools for peptide engineering. Cotter *et al*. (2005b) found an enzyme that converts a dehydrated L-serine to D-alanine. Such enzymes may be harnessed to incorporate D-amino acids into bacterially expressed polypeptides. The discovery of the broad substrate specificity of the nisin modification enzymes (Rink *et al*., 2005) may open the door to enzyme-mediated introduction of thioether rings into a peptide template for required biological activity or structural stability.

Three-dimensional structures of antimicrobial peptides

Protein structures have been classified into α, β, αβ and α+β families (Murzin *et al*., 1995). Proteins in the α family are composed of primarily α-helices, while those in the β family consist of primarily β-strands. In the αβ family, α-helices and β-strands are interspersed. However, the α-helices and β-strands in the α+β family are largely segregated. We have adopted this classification scheme for AMPs with two modifications. First, the αβ family contains all AMPs that have both α and β structures, regardless of whether they are segregated. Secondly, different from the scheme of Murzin *et al*. (1995), we define a non-αβ family that includes all AMPs that form neither α nor β structures. Thus, we classify AMP structures into four families: α, β, αβ and non-αβ. Representatives from the four structural families are provided in Fig. 1.5. Magainin, an α-helical AMP (Fig. 1.5A), is a typical member of the α family. Lactoferricin B (Fig. 1.5B), with a pair of β-strands, is used as a representative of the β family. Heliomicin, with both α-helices and β-strands (e.g. in Fig. 1.5C), is a member of the αβ family. Finally, indolicidin, with neither α nor β structures (Fig. 1.5D), is a representative member in the non-αβ family. These four types of peptide structures (α, β, αβ and non-αβ) can be searched in the 'structure' search field by choosing 'helix', 'beta', 'combined helix and beta' and 'rich in amino acids', respectively. Although the word 'rich' includes all peptides rich in certain amino acids, they may or may not form a non-αβ structure. When the terms 'rich' and 'NMR' (nuclear

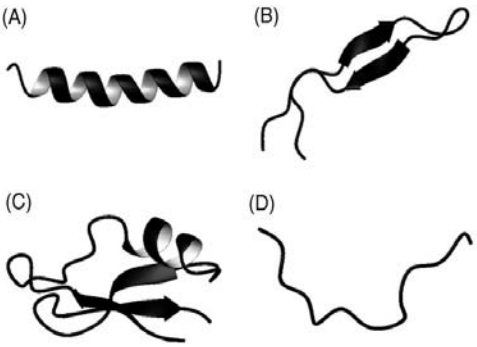

Fig. 1.5. Representative structures of antimicrobial peptides in the Antimicrobial Peptide Database (APD): (A) α-helix (frog magainin; PDB ID: 2MAG); (B) β-sheet (bovine lactoferricin B; PDB ID: 1LFC); (C) αβ with both α-helices and β-sheet (insect heliomicin; PDB ID: 1I2U); (D) non-αβ structure (bovine indolicidin; PDB ID: 1G89). Adapted from the face page of the APD website (http://aps.unmc.edu/AP).

magnetic resonance) are searched simultaneously, only AMPs with non-αβ three-dimensional structures are found. Table 1.6 gives the statistics for the four types of NMR structures of AMPs from different sources. It is evident that all known AMP structures of plant origin contain β-strands, while helical structures are common in peptides from other sources. A further discussion of the three-dimensional structure of AMPs is provided in Chapter 9.

Table 1.6. Nuclear magnetic resonance (NMR) structural statistics of AMPs collected in the Antimicrobial Peptide Database.[a]

Kingdom	α	β	αβ	Non-αβ	Total
Bacteria	13	4	2	0	19
Plants	0	12	19	0	31
Animals	69	24	17	3	113
Humans	4	2	3	0	9

[a] Information in the table can be obtained by performing a multiple-choice search. By entering 'bacteria' into the name field and selecting 'NMR' in the structural method field and 'helix' in the structure field, one can obtain 13 bacterial AMPs with known helical structures determined by NMR.

1.3.4 Peptide-binding targets and mechanisms of action

Broadly, AMPs can be classified into membrane targeting and non-membrane targeting. It is assumed that many membrane-targeting AMPs disrupt bacterial membranes by three major mechanisms: carpet, barrel-stave and toroidal models (Ludtke *et al*., 1996; Shai, 2002). These models are depicted in Fig. 5.1 (Chapter 5). However, there are other possible models (Chapter 7). In addition, AMPs associate with other components on the cell surface. SMAP-29 (sheep myeloid antimicrobial peptide-29) and hRNase 7 may bind to an OprI (outer membrane protein I) of Gram-negative *Pseudomonas aeruginosa* for activity (Lin *et al*., 2010). While nisin A is known to bind lipid II and forms pores in the inner membranes of Gram-positive bacteria, short nisins may work by blocking cell-wall synthesis due to the deprivation of lipid II (Hasper *et al*., 2006). For some plant AMPs such as Cy-AMP1, chitin-binding ability is critical for antifungal activity (Yokoyama *et al*., 2009). Some defensins have been shown to bind specifically to carbohydrate moieties of glycoprotein 41 (gp41) of HIV-1 and CD4 of T-cells to inhibit viral entry into human cells (Gallo *et al*., 2006). In contrast, some amphibian AMPs are proposed to inhibit HIV-1 infection by disrupting the viral envelope (VanCompernolle *et al*., 2005). Therefore, a more inclusive term is cell-surface-binding peptides.

Non-membrane-targeting peptides include all other AMPs that interfere with cell function or survival by binding to intracellular targets. Broadly, these peptides may be termed cell internal-target-binding peptides. While proline-rich peptides are known to interact with heat-shock proteins, some histone-generated AMPs are capable of binding to nucleic acids (Chapter 8). Table 1.7 lists the keys for searching AMP binding partners. It should be emphasized that an illustration of the binding of an AMP to a particular molecule *in vitro* does not necessarily mean that the molecule is exactly the target *in vivo* that causes microbial death. Recently, we started registering the information on

Table 1.7. Keys for the search of binding partners or targets of AMPs.[a]

Key	Binding partner	Number of peptides	Examples
BBBh2o	Oligomers in water	6	hBD-3; LL-37
BBBm	Oligomers in membranes	1	Protegrin-1
BBIl	Metal ions (e.g. Zn^{2+})	12	Histatin 5, hepcidin 25
BBW	Bacterial cell wall precursors (e.g. lipid II)	3	Mersacidin, lactocin S
BBL	Lipopolysaccharides	26	Temporin L, LL-37
BBr	Bacterial cell surface receptor (protein)	1	SMAP-29, hRNase 7
BBMm	Inner membranes (e.g. lipid bilayers)	48	Magainins, LL-37
BBN	Nucleic acids (DNA/RNA)	5	Buforin II, indolicidin
BBP	Proteins (inside cells)	16	Drosocin, pyrrhocoricin
BBS	Carbohydrates	21	HNP1, AFP1, Ac-AMP2, RTD-1

[a] Some of the keys were originally published in Wang *et al*. (2009). A key search can be conducted in the name field.

possible mechanisms of action into the database. This is a poorly defined zone because the mechanisms of action of AMPs are, in general, not well understood.

1.4 Amino Acid Sequence Analysis of Antimicrobial Peptides

The annotation and classification of AMPs into a variety of families with a common feature enables sequence analysis by bioinformatic tools. For example, we have calculated the amino acid compositions of AMPs from different kingdoms using the database tool. Figure 1.6 shows the average amino acid content (composition signature) for all AMPs from bacteria, plants and animals. The types of abundant amino acids (approximately 10% or greater) differ. We refer to these abundant amino acid residues as frequently occurring residues (Wang *et al*., 2009). In 118 bacterial AMPs, residues alanine and glycine are frequently occurring residues (Fig. 1.6A). Residues cysteine and glycine are abundant in 217 plant AMPs (Fig. 1.6B), while residues leucine, glycine and lysine occur frequently in 1089 AMPs from animals (Fig. 1.6C). In addition, there are some amino acids that are rarely used. For example, methionine residues have the lowest percentage in known plant AMPs.

Figure 1.7A presents the average amino acid content of 548 peptides from frogs. Residues leucine, alanine, glycine and lysine

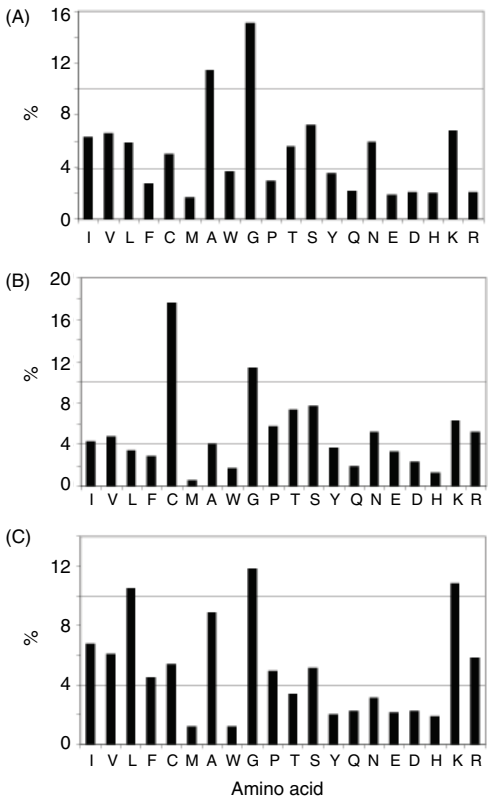

Fig. 1.6. Average amino acid percentages of antimicrobial peptides from (A) bacteria, (B) plants and (C) animals. The numbers of peptides analysed from bacteria, plants and animals were 118, 217 and 1089, respectively. Frequently occurring residues are defined as those that have a percentage of approximately 10% or greater.

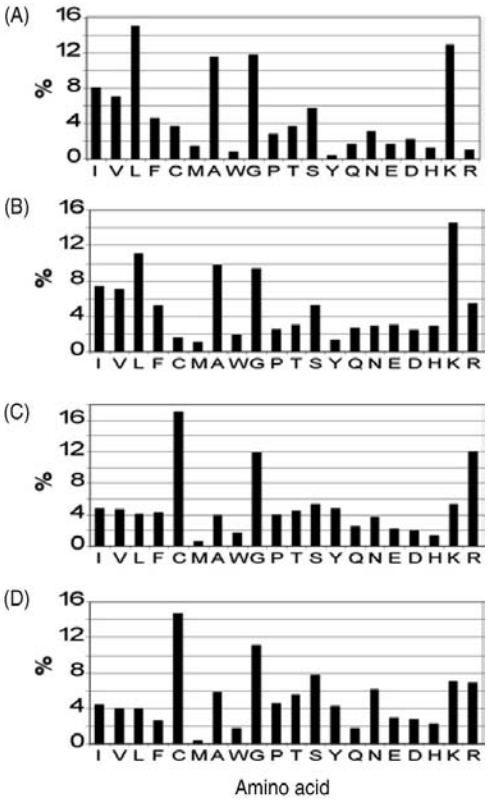

Fig. 1.7. Average amino acid percentages of (A) 548 frog antimicrobial peptides, (B) 243 helical peptides, (C) 81 AMPs with β structures and (D) 49 AMPs with an αβ fold as found for β-defensins collected in the Antimicrobial Peptide Database.

occur frequently. This list of residues may contain structural information. As many amphibian AMPs such as magainins and aureins are known to associate with bacterial membranes and adopt amphipathic α-helices (Haney *et al*., 2009), we hypothesize that the same set of abundant residues also occurs in AMPs with known helical structures. A statistical analysis of 241 AMPs annotated as 'Helix' in the APD (based on NMR and circular dichroism) reveals that residues leucine, alanine, glycine and lysine are indeed abundant (Fig. 1.7B). In contrast, the abundant residues in 81 AMPs with known β structures are cysteine, glycine and arginine (Fig. 1.7C). The typical members of the β-fold family are human α-defensins with three β-strands. These abundant residues are important in terms of both the structure and activity of these peptides. While cysteine residues form critical disulfide bonds that maintain the defensin fold, the glycine residue endows flexibility to the peptide chain and is an integral part of the γ-core motif (Young and Yeaman, 2004). Arginines are essential for antibacterial and antiviral activities. A similar amino acid composition signature was obtained (Fig. 1.7D) when the 49 members of the αβ-fold family were analysed. The typical examples of this family are human β-defensins, which comprise one α-helix and three β-strands. Different from the β-fold family, where arginine residues are more abundant than lysine residues, β-defensins have an approximate 1:1 arginine to lysine ratio (compare Fig. 1.7C and Fig. 1.7D). The essential role of arginine in α-defensins has been substantiated by Zou *et al*. (2007). They found that the effect of arginine to lysine mutations on antibacterial activity is more pronounced for α-defensins than for β-defensins.

We also analysed a set of peptides with relatively well-conserved structures. While the frequently occurring residues in 21 bacterial lantibiotics were cysteine, threonine and serine (Fig. 1.8A), they were cysteine and glycine in 126 plant cyclotides (Fig. 1.8B). Interestingly, the most abundant residues in these two families of peptides were identical (cysteine, glycine, threonine and serine) and their amino acid composition signatures (overall distribution patterns) were more or less similar. Although cysteine residues are common in these two peptide families, their structural roles are entirely different. In lantibiotics, they form several three-membered ring structures that confer stability to the peptides. Such thioether rings are normally formed between cysteine residues and dehydrated serine or threonine, making them abundant in lantibiotics. In contrast, cysteine residues in cyclotides are known to form disulfide bonds, also conferring stability to the peptide structure. As a consequence, these natural templates are attractive for developing a new generation of therapeutic agents.

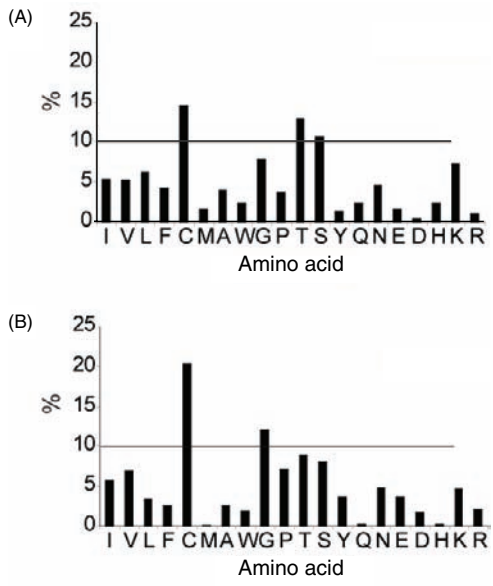

Fig. 1.8. Average amino acid percentages of (A) 21 lantibiotics from Gram-positive bacteria and (B) 126 cyclotides from plants collected in the Antimicrobial Peptide Database (March 2010).

1.5 Concluding Remarks

In summary, the APD is a useful tool for the naming, classification and analysis of naturally occurring AMPs. By including synonyms, including old and new names, the likelihood of finding a peptide in the database increases. This practice also facilitates the transition to and adoption of newly suggested names. We should emphasize that the classification issue of AMPs is not fully resolved due to incomplete information as well as the diversity of the peptides. Therefore, accuracy will improve with the refinement of the classification schemes in the future. Correct classifications of AMPs improve information searches. Our recent updates have led to a substantial increase in both peptide entries and search functions. For newly established search functions currently under annotation, users should treat the search result as 'a minimum' while the annotation is ongoing. Many hypothetical AMPs, predicted or isolated, have not been collected owing to a lack of activity data. This practice is in line with the word 'antimicrobial', at least according to *in vitro* microdilution or diffusion assays (Wiegand *et al*., 2008). The final establishment of a *bona fide* AMP requires *in vivo* data.

The success in database expansion and enhancement has validated the flexibility of our original design. This important feature will enable further growth of this database in the future. For example, how a particular AMP is expressed and regulated in response to bacterial invasion is a fundamental question. Understanding this process promises novel therapeutic strategies. In addition, information such as microbial type, expression cell, translation and post-translational modification enzymes, transport proteins and self-immunity can be entered into the 'additional information' field (e.g. see microcin J25, APD ID: 480). It is also clear that the biological functions of AMPs are not limited to antimicrobial activities, as discussed above. In particular, mammalian cathelicidins and defensins have multiple biological functions, in particular immune modulation. Functions such as chemotaxis, apoptosis, wound healing and tumour metastasis can also be entered into the 'additional information' field of the APD (e.g. see human LL-37, APD ID: 310). Therefore, this field can annotate detailed information regarding the biology of AMPs as host defence molecules. Detailed classification and annotation also enable a more meaningful bioinformatic analysis of AMPs. Such results are useful in guiding peptide prediction and design, which we discuss in Chapter 4.

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2 Lantibiotic-related Research and the Application Thereof

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Abstract

Since the 1925 discovery that strains of *Escherichia coli* can retard the growth of neighbouring bacteria, the study of bacteriocins has continuously evolved. During the intervening period, a large and heterogeneous collection of these antimicrobial peptides has been isolated from a myriad of sources and numerous investigations have been carried out with a view to harnessing their potency. The most thoroughly investigated class of bacteriocins, the lantibiotics, is the main focus of this review. These antimicrobial peptides inhibit many human and animal pathogens. They have been the focus of considerable efforts to maximize the potential of existing lantibiotics, identify new and better lantibiotics from nature and utilize bioengineering-based approaches to further improve upon existing well-characterized compounds.

2.1 Introduction to Bacteriocins

In order for an organism to survive, thrive and proliferate in a particular niche, it is essential that it competes successfully. In the microbial world, many bacteria gain an upper hand by producing antimicrobial compounds that inhibit their competitors. Bacteriocins, which are bacterially produced ribosomally synthesized peptides with antimicrobial spectra that can range from very narrow to extremely broad, are among the most potent examples of bacterially produced antimicrobials (Tagg *et al*., 1976; Jack *et al*., 1995; Riley and Wertz, 2002; Cotter *et al*., 2005b). Indeed, bacteriocins frequently exhibit antimicrobial activity at nanomolar levels; this contrasts with, for example, the cationic antimicrobial peptides produced by eukaryotic cells, which exhibit similar levels of activity only at micromolar concentrations (Nissen-Meyer *et al*., 2009).

The identification of the first bacteriocin, a colicin (i.e. an *Escherichia coli*-associated bacteriocin), occurred in 1925 (Gratia, 1925). This was soon followed by the first report of a bacteriocin produced by a Gram-positive bacterium, when inhibition of *Lactobacillus bulgaricus* by *Streptococcus lactis* (since renamed *Lactococcus lactis*) was noted (Rogers, 1928). Since then, bacteriocins produced by lactococci and other lactic acid bacteria (LAB; i.e. microorganisms with a long history of safe use in food and of considerable industrial importance) have continued to be the focus of much attention (Nissen-Meyer *et al*., 2009). A number of different classification systems exist for LAB bacteriocins, but the simplest, and that used for this review, was proposed by Cotter *et al*. (2005b). This system comprises two classes: (i) class I bacteriocins – the post-translationally modified lantibiotics (Chatterjee *et al*., 2005; Cotter *et al*., 2005a);

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and (ii) class II bacteriocins, which are a heterogeneous group of non-lanthionine (Lan)-containing peptides. As a consequence of their unusual structure, unique mechanisms of action and potential as antimicrobials for food, veterinary and clinical applications, the lantibiotics have received much attention in recent years. They are the focus of this chapter.

2.2 Biosynthesis and Post-translational Modifications

The name lantibiotics is derived from 'Lan-containing antibiotics' and reflects the feature that is shared by all of these peptides: intra-molecular rings formed by the thioether amino acids Lan and methylanthionine (meLan) (Sahl *et al*., 1995). Lantibiotic-associated gene clusters can be located on chromosomes (including transposable elements) or plasmids. In addition to the genes encoding a structural peptide(s) and the post-translational modification machinery that acts thereon, other genes responsible for regulation, transport and immunity are usually also present (Cotter *et al*., 2005b). The following paragraphs summarize the steps involved in lantibiotic biosynthesis.

The lantibiotic prepropeptide contains a leader region, which is eventually cleaved, and a propeptide, which is modified to become the active antimicrobial. The corresponding gene is generically designated *lanA*. Lantibiotics can be further subdivided according to the enzymes that catalyse Lan and meLan formation. Type I prepropeptides such as the prototypic lantibiotic nisin A undergo dehydration as a result of the catalytic activity of the NisB protein (generically referred to as LanB enzymes) (Karakas Sen *et al*., 1999). As a result, specific serine and threonine residues are dehydrated to become the unique amino acids 2,3-dehydroalanine (Dha) and 2,3-dehydrobutyrine (Dhb), respectively. Some or all of these new residues are then the subject of a LanC-catalysed reaction. This results in their interaction with the thiol groups of cysteines within the peptide to form Lan and meLan from Dha and Dhb, respectively. Insight into the role of LanC was first provided by Meyer *et al*. (1995) who, while working on the lantibiotic Pep5, showed that with the removal of the *pepC* gene, the peptide produced contained dehydrated residues but not Lan or meLan bridges. In addition to being essential for converting the peptide to an active form, the Lan and meLan structures are also believed to contribute to protease resistance (Kluskens *et al*., 2005). Unlike the type I peptides, which are modified by two separate modification enzymes, type II peptides such as lacticin 481 and mersacidin are modified by a single protein referred to as LanM (Willey and van der Donk, 2007). Another variation on this theme arises as a consequence of the existence of two peptide lantibiotics (e.g. lacticin 3147) that require the combined activity of two Lan- and meLan-containing peptides for optimal activity. In most cases, the production of lantibiotics of this kind relies on the presence of two LanM proteins, each of which is responsible for the dehydration and cyclization of its respective subunit (McAuliffe *et al*., 2000; Lawton *et al*., 2007b).

A third category of peptides, type III, is not described in this review as those identified to date, while possessing Lan and meLan structures, lack antimicrobial activity (Willey and van der Donk, 2007). Yet another category, modified by a novel group of lantibiotic synthetases designated LanL, has recently been discovered (Goto *et al*., 2010). It remains to be established if LanL-modified peptides possess antimicrobial activity, and thus it has been suggested that the term 'lantipeptides' be used to describe Lan-containing peptides that lack antimicrobial activity (Goto *et al*., 2010). With respect to the lantibiotics, in addition to the LanB, LanC and LanM enzymes, a number of other enzymes have been associated with the modification of specific peptides (Kupke and Gotz, 1997; Peschel *et al*., 1997; Majer *et al*., 2002; Cotter *et al*., 2005c).

After modification, the peptide is transported via an ABC transport system to the cell surface (Fath and Kolter, 1993) and the leader sequence is proteolytically

removed – although not necessarily in that order – leading to the production of the active lantibiotic (Havarstein *et al*., 1995). For type I lantibiotics, these individual steps are carried out independently by a transporter (designated LanT) (Qiao and Saris, 1996) and a protease (LanP) (Ye *et al*., 1995; Siezen, 1996); in the case of type II lantibiotics, both activities are carried out by one enzyme (also designated LanT) (Rince *et al*., 1994; Chen *et al*., 1999; Altena *et al*., 2000). To ensure that the active antimicrobial does not target the producing cells, immunity proteins are produced. Two immunity mechanisms have been described. One relies on a single immunity protein, LanI, while the second involves a multicomponent ABC-transporter system referred to as LanEFG. Indeed, some lantibiotic producers utilize two such mechanisms (Draper *et al*., 2008).

The remaining group of lantibiotic-associated proteins to which we have yet to refer is the regulators. Initial studies in this area focused on the regulation of nisin and its related peptide subtilin by two-component signal transduction systems, which consist of a histidine kinase (LanK) and a response regulator (LanR) (Klein *et al*., 1993; Engelke *et al*., 1994; Stock *et al*., 2000; Kleerebezem, 2004). Examination of the genetic determinants of a subtilin producer revealed the presence of the *spaK* and *spaR* genes. Using a strategy of gene deletion, it was shown that mutants lacking these genes were no longer capable of lantibiotic production (Klein *et al*., 1993) A similar approach also revealed the importance of the corresponding nisin genes (Engelke *et al*., 1994). In both cases, it has been established that the associated antimicrobial peptides also function as pheromones as part of a LanRK-mediated quorum sensing system, which facilitates autoregulation (Kleerebezem, 2004). While two-component systems play a major role in the production of a number of other lantibiotics, such as mersacidin (Guder *et al*., 2002), other quite different regulators have also been noted (McAuliffe *et al*., 2001).

2.3 Classification

A scheme to classify lantibiotics was first proposed by Jung in 1991. This scheme grouped the peptides into two distinct categories. The first class, type A, contained the elongated, flexible, amphipathic peptides. These peptides had a net positive charge and were thought to function through the permeabilization of the cell membrane. The second class, type B, contained globular proteins with a net negative or neutral charge. These proteins were thought to function by inhibiting sensitive cells through the formation of complexes with specific membrane components. While some of this information continues to be pertinent, the use of this system has been made more difficult by investigations that have established that some lantibiotics act in multiple different ways, coupled with the identification of new and more diverse lantibiotics. This issue has been addressed by approaches that reflect the genes and proteins involved in lantibiotic production.

Most recently, this has involved the fusion of two compatible approaches (Piper *et al*., 2009a) in which peptides are classified on the basis of the sequence of the lantibiotic propeptide (Cotter *et al*., 2005a) or the composition of the associated modification/transport systems (Pag and Sahl, 2002; Willey and van der Donk, 2007). Using this combined approach, lantibiotics can be subdivided into the three types (i.e. types I, II and III, referred to above) and 12 subgroups. The subclassification of the type I and II lantibiotics into 12 subgroups is based on the alignment of the unmodified amino acid sequence of the structural peptide. In each case, homology results in the classification of the lantibiotic in question, together with the eponymous member of the group (i.e. planosporicin, nisin, epidermin, streptin, Pep5, lacticin 481, mersacidin, LtnA2, cytolysin, lactocin S, cinnamycin and sublancin; Fig. 2.1). Thus, for example, the mersacidin subgroup includes RumB, plantaricin C, mersacidin, michiganin,

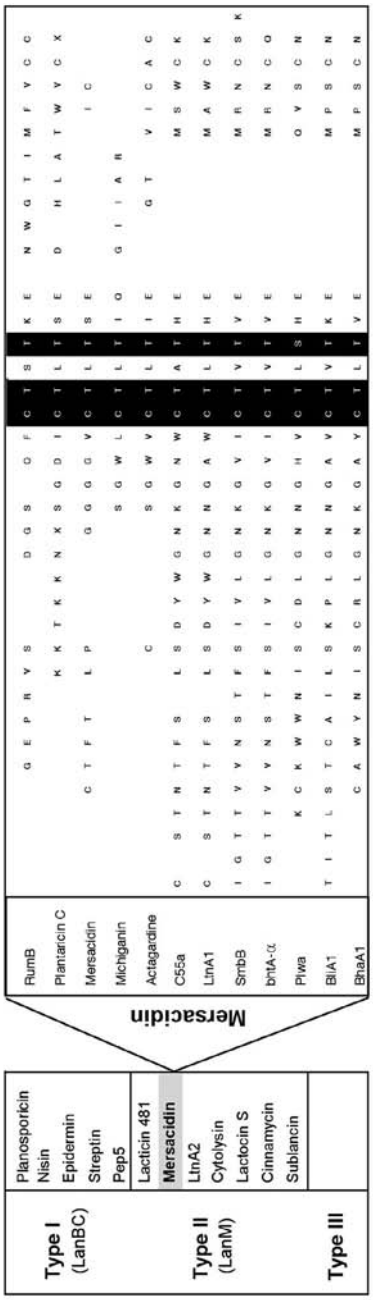

Fig. 2.1. Lantibiotic classification. Lantibiotics can be divided into type I, II and III peptides. The type I and II peptides can be further divided into 12 groups on the basis of the sequence of the lantibiotic propeptide. In one representative case, the mersacidin-like peptides, each of the corresponding propeptides is listed and aligned. Amino acids that are 100% conserved are highlighted in black.

actagardine, C55a, LtnA1, SmbB, bhtA-α, Plwa, BliA1 and BhaA1, all of which are modified by LanM enzymes (i.e. are of type II) and contain a number of residues that are conserved across the group. It should be noted that problems can arise when trying to add newly purified lantibiotics for which a structure, but not gene sequences, is available to this system. Thus, while it is apparent from its unusual structure and modifications that microbisporicin represents a 13th lantibiotic subclass (Castiglione *et al*., 2008), it will not be possible to satisfactorily incorporate it into the system until the associated biosynthetic genes have been identified and the *lanA* gene sequenced.

2.4 Lantibiotic Subgroups: Biology, Structure and Mode of Action

2.4.1 Nisin-like lantibiotics

Nisin A is the most thoroughly characterized lantibiotic. This 34 amino acid peptide contains three dehydrated amino acids and five thioether rings (Fig. 2.2) (Gross and Morell, 1971; Buchman *et al*., 1988). Nisin A is the eponymous member of the nisin-like lantibiotics, which also include nisin Z, nisin F, nisin U, nisin U2, nisin Q, subtilin, ericin S and ericin A. The mechanism of action of nisin A (and nisin Z, which differs from nisin A by one amino acid) has been investigated in depth and it is likely that all related peptides function in a broadly similar manner. In addition to a long-standing appreciation of its ability to form pores, it was revealed in the mid-1990s that nisin binds to lipid II, an essential precursor of peptidoglycan, and thus also inhibits cell wall synthesis (Brotz *et al*., 1998; Breukink *et al*., 1999). Structural analysis of the lipid II–nisin complex has revealed that the N-terminal region of the nisin peptide is responsible for lipid II binding (Hsu *et al*., 2004). The C-terminal end of the peptide, which is linked to the N-terminus by a three amino acid 'hinge' region, is the pore-forming domain (Breukink *et al*., 1997; Wiedemann *et al*., 2001). The efficiency of pore formation is greatly enhanced by lipid

II binding (Breukink *et al.*, 1999). A third mode of action has also been revealed. It is now apparent that nisin can induce the autolysis of susceptible staphylococcal strains as a consequence of the release of two cell wall hydrolysing cationic enzymes during normal autolysis of dividing cells (Hasper *et al.*, 2004).

2.4.2 Epidermin-like lantibiotics

Epidermin is produced by *Staphylococcus epidermidis* and is encoded by a structural gene, *epiA*, which is located on a 54 kb plasmid (Schnell *et al.*, 1992). The active lantibiotic is a 21 amino acid peptide that contains four rings, including three (me)Lans

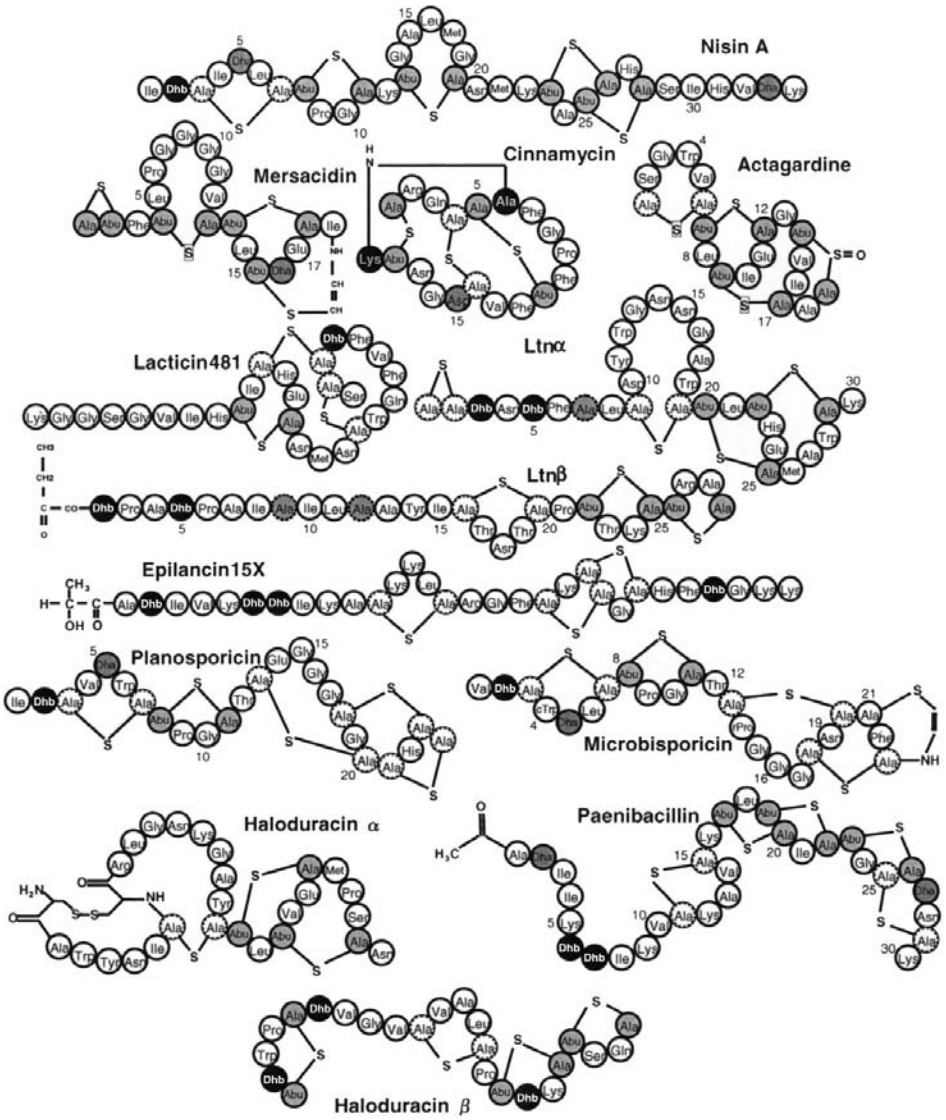

Fig. 2.2. Some representative lantibiotic structures. Modified residues are shaded or dashed. Ala-S-Ala, lanthionine (dashed); Abu-S-Ala, β-methyllanthionine (light grey); Ala, D-alanine (grey dashed); Dha, dehydroalanine (dark grey); Dhb, dehydrobutyrine (black, white text); Asp, β-hydroxy-aspartate (grey, white text); Lys-NH-Ala, lysinoalanine (black, grey text).

and a 2-aminovinyl-D-cysteine (Allgaier *et al*., 1986). After epidermin, gallidermin is the most extensively studied epidermin-like lantibiotic. The two peptides are structural analogues that differ by only one residue (at position 6) (Kellner *et al*., 1988). Nuclear magnetic resonance (NMR) spectroscopy studies into the structure of gallidermin have revealed that the rigid N-terminally located rings A and B are connected to the C and D rings at the C-terminal end of the peptide by a somewhat flexible region spanning residues A12 to G15 (Ottenwalder *et al*., 1995). A comparison of the structures of epidermin and nisin shows high homology in the N-terminal, lipid II-binding ends of the peptides (Hsu *et al*., 2004). Their C-terminal ends, however, differ considerably, thereby explaining the inability of epidermin to form pores in certain targets (Bonelli *et al*., 2006).

2.4.3 Planosporicin- and streptin-like lantibiotics

In addition to the nisin- and epidermin-like peptides, two other peptides – planosporicin and streptin – resemble the former with respect to their N-terminal domains. Otherwise, they differ quite considerably from these and from one another. As a result both are the eponymous (and sole) members of two lantibiotic subclasses. Planosporicin, produced by *Planomonospora* species, contains both Lan and meLan residues, which generate five intramolecular thioether bridges (Fig. 2.2) (Castiglione *et al*., 2007; Maffioli *et al*., 2009). Planosporicin functions primarily through the inhibition of peptidoglycan synthesis but, unlike nisin and epidermin, does not bind to the D-Ala-D-Ala motif of lipid II (Castiglione *et al*., 2007). Streptin is a *Streptococcus pyogenes*-associated lantibiotic, two major forms of which have been purified. Streptin 1 is the fully mature 23 amino acid peptide, while streptin 2 has three additional residues at the N-terminal end (TPY) (Karaya *et al*., 2001; Wescombe and Tagg, 2003).

2.4.4 Pep5-like peptides

Pep5 is a tricyclic lantibiotic consisting of 34 amino acids (including three Lan bridges: one meLan and two Lan) (Bierbaum *et al*., 1994, 1996). It is both screw-shaped and highly cationic, the latter property being due to the presence of six lysine and two arginine residues in the mature peptide (Pag *et al*., 1999). It is produced by *Staph. epidermidis* strain 5 (Kaletta *et al*., 1989), with the associated genes located on the 20 kb plasmid pED503 (Ersfeld-Dressen *et al*., 1984; Meyer *et al*., 1995).

Pep5 also has another less common feature: an N-terminal 2-oxobutyryl group that is thought to be formed through the non-enzymatic hydrolysis of N-terminal Dhb residues (Xie and van der Donk, 2004). Pep5 exerts its mode of action by forming voltage-dependent pores in the cytoplasmic membrane of sensitive cells (Sahl *et al*., 1987; Kordel *et al*., 1989). These pores cause the leakage of essential metabolites and ATP out of the cell, resulting in the cessation of cellular metabolic processes and ultimately causing cell death. Pep5 can also induce the autolysis of staphylococci through the activation of cell wall hydrolysing enzymes (Bierbaum and Sahl, 1985, 1987).

2.4.5 Lacticin 481-like lantibiotics

The type II lacticin 481-like subgroup is the largest lantibiotic subgroup. Its associated peptides are notable by virtue of lacking post-translational modifications other than the common Dha, Dhb, Lan and meLan residues (Dufour *et al*., 2007). The *Lactococcus lactis* subspecies *lactis* CNRZ481-produced eponymous member of this group, lacticin 481, is 27 amino acids in length and contains two Lans, one meLan and one Dhb residue (Fig. 2.2) (Piard *et al*., 1993; van den Hooven *et al*., 1996). Interestingly, although lacticin 481 is quite similar to the related variacin (five amino acid differences), variacin appears to have a much broader target-cell spectrum of activity (Pridmore *et al*., 1996). Studies with another member of this group,

nukacin ISK-1, indicate it to be bacteriostatic and incapable of pore formation (Asaduzzaman *et al*., 2009), whereas the related streptococcin SA-FF2 peptide causes the formation of short-lived pores in target cells (Jack *et al*., 1994).

2.4.6 Mersacidin-like lantibiotics

Mersacidin is a small lantibiotic produced by *Bacillus* species (Chatterjee *et al*., 1992). The biosynthetic gene cluster (12.3 kb in size) consists of ten genes and is located on the bacterial chromosome. This hydrophobic and neutral peptide contains three meLans and a single 2-aminovinyl-2-methylcysteine corresponding to four intramolecular rings (Fig. 2.2) (Chatterjee *et al*., 1992). Mersacidin does not form pores in the cell membrane of sensitive cells, but does inhibit cell wall synthesis through binding with lipid II (Brotz *et al*., 1995). An NMR study carried out by Hsu *et al*. (2003) showed that mersacidin can change its conformation depending on whether it is in the presence of lipid II. In addition to one-peptide lantibiotics such as mersacidin, the mersacidin-like peptides also contain the A1 peptide of a number of two-peptide lantibiotics: lacticin 3147 (i.e. Ltnα and Ltnβ; Fig. 2.2) (Ryan *et al*., 1999b), staphylococcin C55 (Navaratna *et al*., 1998), plantaricin W (Holo *et al*., 2001), BHT (Hyink *et al*., 2005), Smb (Yonezawa and Kuramitsu, 2005), lichenicidin (Begley *et al*., 2009; Dischinger *et al*., 2009) and haloduracin (Fig. 2.2) (McClerren *et al*., 2006; Lawton *et al*., 2007a). These two-peptide lantibiotics only exhibit optimal activity when the A1 component is combined with its A2 counterpart. These conserved A2 peptides are referred to as the LtnA2-like peptides (see next section).

2.4.7 LtnA2-like peptides

Lacticin 3147, produced by *L. lactis* subspecies *lactis* DPC3147, is the most extensively studied two-peptide lantibiotic (Ryan *et al*., 1996). The genes responsible for the production of the lantibiotic, and of the associated immunity proteins, are encoded on a 60.2 kb conjugative plasmid, pMRCO1, which contains ten open reading frames (Dougherty *et al*., 1998). The mechanism of action of lacticin 3147, which is also lipid II mediated, depends on the presence of both components (i.e. Ltnα and Ltnβ, derived from the LtnA1 and LtnA2 propeptides, respectively). More specifically, Ltnα first binds to lipid II in sensitive cells. This binding is thought to lead to a conformational change that produces a high-affinity binding site for the Ltnβ peptide. Cell death occurs through the permeabilization of the cell membrane (Wiedemann *et al*., 2006), leading to the efflux of potassium ions and phosphate and resulting in hydrolysis of cellular ATP. The related haloduracin peptide has recently been shown to function in a similar way (Oman and van der Donk, 2009).

2.4.8 Other type II lantibiotics

Other type II subgroups include the cinnamycin-like, sublancin-like, lactocin S-like and cytolysin-like subgroups. While there are a number of cinnamycin-like peptides, sublancin, lactocin S and cytolysin are the sole members of their respective subgroups. Cinnamycin is a tetracyclic lantibiotic produced by *Streptoverticillium griseoverticillatum*. It is a 19 amino acid peptide and contains the unusual residues lysinoalanine and 3-hydroxyaspartic acid (Fig. 2.2). In addition to its antimicrobial activity, cinnamycin and related peptides have other potentially useful pharmaceutical properties, including the inhibition of phospholipase A_2 and angiotensin-converting enzyme (Fredenhagen *et al*., 1990; Kaletta *et al*., 1991; Hosoda *et al*., 1996). Sublancin, produced from *Bacillus subtilis* 168, is a 37 amino acid peptide that contains one meLan and two disulfide bridges. The presence of the stabilizing Lan bridges along with relatively weak disulfide bridges suggests a conformational uniqueness that could confer a selective advantage (Paik *et al*., 1998). Lactocin S, produced from *Lactobacillus sake* L45, is a 37 amino acid lantibiotic (Mortvedt *et al*., 1991; Skaugen *et al*., 1997). This lantibiotic is noteworthy as a consequence of

containing D-alanine residues (Skaugen *et al*., 1994). Among lantibiotics, only lacticin 3147 shares this trait. At a neutral pH, lactocin S exhibits a net neutral charge (Rawlinson *et al*., 2002). Finally, cytolysin is a two-peptide lantibiotic ($CylL_L$ and $CylL_S$) produced by enterococci. It is unique by virtue of its ability to target both eukaryotic and prokaryotic cells (Booth *et al*., 1996).

2.5 Current and Future Use of Lantibiotics for Food Applications

The most prominent event in the majority of food fermentations is the conversion of sugars to lactic acid by LAB. In addition to lactic acid, LAB can produce other metabolites with antimicrobial activity such as hydrogen peroxide, diacetyl, acetoin and bacteriocins, including a number of lantibiotics such as nisin A. Highlights in the industrial application of nisin A as a food preservative include its initial use by the food industry in 1953 and its approval by the World Health Organization, European Union and US Food and Drug Administration in 1969, 1983 and 1988, respectively. Currently, nisin A is approved for use in over 48 countries worldwide (Delves-Broughton *et al*., 1996; Cotter *et al*., 2005b; Deegan *et al*., 2006). Nisin can be added to a food in number of ways. These include the direct application of the peptide, in a highly purified form if necessary, as an 'additive' (being one of only two authorized natural food antimicrobials, the other being the anti-mould additive natamycin); the introduction of a nisin-producing bacteria, as a starter or an adjunct culture, to a fermented food (and the subsequent production of nisin *in situ*); and using the producer to make a food-grade fermentate, which can be dried to make a powdered 'ingredient' that can be incorporated into either fermented or non-fermented foods. In the case of nisin, all three alternatives are employed, for example 'Additive' Nisaplin (Danisco, Copenhagen, Denmark), Nisin-producing cultures (from culture providers such as Chr. Hansen, Horsholm, Denmark, and CSK, Leeuwarden, The Netherlands) or 'ingredient' DURAFresh (Kerry Bio-Science, Co. Kerry, Ireland).

Other than nisin, lacticin 3147 and lacticin 481 are the two most extensively studied LAB lantibiotics. Both exhibit traits that suggest they have commercial value, and a few selected examples of potential applications are presented here. In the case of lacticin 3147, it has been established that lactococcal producers of lacticin 3147 can be employed as starter cultures for the manufacture of Cheddar cheese (Ryan *et al*., 1996). Lacticin 3147 producers successfully reduce the pH of milk to 5.2, while also generating sufficient quantities of the lantibiotic to control the adventitious non-starter LAB (NSLAB) over a 6-month ripening period (Ryan *et al*., 1996). This is significant as NSLAB can be the cause of flavour defects and calcium lactate formation (Thomas and Crow, 1983). The use of lacticin 481-producing strains as adjunct cultures in cheese production has also been mooted (O'Sullivan *et al*., 2003). This lantibiotic, which is produced by *L. lactis* strains (Piard *et al*., 1992), demonstrates a higher ability to lyse sensitive lactococci than lacticin 3147 when used in combination with the starter culture *Lactococcus* HP. The associated benefits are the release of intracellular enzymes, thereby speeding up the ripening process, and a reduction in the numbers of NSLAB (O'Sullivan *et al*., 2003). Studies investigating the use of a lacticin 3147-based powder as a biopreservative have also yielded interesting results (Morgan *et al*., 2001). Like nisin, a fermentate containing lacticin 3147 provides an alternative means of introducing the lantibiotic into food. Incorporation of a whey powder (10%), which was fermented with a lacticin 3147 producer, was found to bring about a 99.9% reduction in *Listeria monocytogenes* in natural yoghurt and an 85% reduction in pathogen numbers in a cottage cheese sample within a 2-h time frame. It has also been established that an 80% reduction in *Bacillus cereus* numbers occurs within 3 h when 1% powder is added to soup. For a comprehensive review on the use of bacteriocins as biological agents for food safety, see Deegan *et al*. (2006).

2.6 Lantibiotics and Their Medical Applications

The possibility of using lantibiotics to control or treat multidrug-resistant forms of pathogens such as *Staphylococcus aureus*, *Enterococcus* species and *Clostridium difficile* has gained increased attention in recent years due to a number of positive results obtained by researchers in the field (for a comprehensive review, see Piper *et al*., 2009a). *In vitro*, many lantibiotics, including lacticin 3147, mutacin B-Ny266, nisin and mutacin 1140, show activity against clinical targets such as methicillin-resistant *Staph. aureus* (MRSA), vancomycin-resistant *Enterococcus faecalis* (VRE), penicillin-resistant *Pneumococcus*, *Propionibacterium acne*, *Streptococcus mutans*, *Strep. pyogenes*, *Strep. pneumoniae*, *C. difficile, Listeria* and *Bacillus* species (Severina *et al*., 1998; Galvin *et al*., 1999; Mota-Meira *et al*., 2000; Brumfitt *et al*., 2002; Rea *et al*., 2007; Ghobrial *et al*., 2009; Piper *et al*., 2009b). It is also interesting that both Pep5 and epidermin successfully inhibit the adhesion of staphylococcal cells to the surfaces of catheters (Fontana *et al*., 2006). It is important to note, however, that these represent just a selection of the studies that have highlighted the efficacy of lantibiotics against Gram-positive clinical pathogens. It is anticipated that the number of studies in this area will continue to increase as a consequence of the further investigation of existing lantibiotic peptides and the continued identification of new forms of these antimicrobials. Two recent examples of note are the two-peptide lantibiotic lichenicidin, which exhibits antimicrobial activity against MRSA and VRE strains (Begley *et al*., 2009), and microbisporicin, which is active against MRSA, VRE and clinical streptococci (Castiglione *et al*., 2008).

While the *in vitro* success of a chemotherapeutic agent does not always correspond to *in vivo* efficacy, a number of studies have indicated that this may not be a major failing of lantibiotics. It has been revealed that mutacin B-Ny266 can be as active as vancomycin against MRSA *in vivo* (Mota-Meira *et al*., 2005), mersacidin can be employed to eradicate a nasal MRSA infection (Kruszewska *et al*., 2004) and nisin F, both alone and when used in combination with lysozyme and lactoferrin, can successfully treat respiratory tract MRSA infections in mice (De Kwaadsteniet *et al*., 2009). Trials investigating the use of lantibiotics to control the microorganisms responsible for dental plaque, halitosis, 'strep' throat (Hillman, 2002; Burton *et al*., 2006; Dierksen *et al*., 2007) and even bovine mastitis (Ryan *et al*., 1999a; Twomey *et al*., 2000) have all been successful.

2.7 Engineering of Lantibiotics

Lantibiotics are gene encoded. Advantage can be taken of this trait to engineer novel variants of the parent peptide. Such variants have been used to study structure–function relationships and, in some cases, engineering strategies have led to the generation of peptides with enhanced antimicrobial activity. Lantibiotic engineering can take place *in vivo* (i.e. by manipulating the original producing strain or expressing the genes heterologously in an alternative host) or *in vitro* (i.e. by harnessing the activity of purified forms of the individual components of biosynthetic machinery outside of a host). The application of engineering to lantibiotic research commenced in 1992. Although initial nisin-focused investigations did not lead to the production of variants with enhanced activity (Kuipers *et al*., 1992), they clearly demonstrated the power of this technology. During the same year, the extreme consequences of making single, deliberate amino acid changes were demonstrated when it was established that a single residue change in subtilin resulted in a 57-fold increase in its biological and chemical stability (Liu and Hansen, 1992). This technology has continued to be applied, and in 2006 the first alanine-scanning mutagenesis of a lantibiotic, lacticin 3147, was completed (Cotter *et al*., 2006a). In this study, alanine (or glycine in cases where an alanine was already present) was introduced in place of the 59 amino acids, in turn, and the impact on the antimicrobial activity of the associated producing strain was

quantified. The data generated highlighted specific areas of the peptides in which subsequent site-specific mutagenesis approaches might be beneficial. This strategy was taken a step further when site-saturation mutagenesis was employed to engineer both nukacin ISK-1 (Islam *et al*., 2009) and mersacidin (Appleyard *et al*., 2009). Both studies provided an in-depth insight into the structure–function relationships within the respective peptides. In the case of nukacin ISK-1, two variants displaying a twofold increase in specific activity were identified (Islam *et al*., 2009).

The use of engineering to study or improve nisin has also continued at pace. Since the 1990s, this lantibiotic has been the subject of a number of engineering-based strategies, which have employed site-directed, site-saturation and random mutagenesis. While various different regions of the peptide have been engineered, the N-terminal and 'hinge' regions have received the greatest attention. The benefits of manipulating the hinge (consisting of Asn20-Met21-Lys22) have been particularly notable (Yuan *et al*., 2004; Field *et al*., 2008). Yuan *et al*. (2004) employed a site-directed approach whereby either positively or negatively charged amino acids were introduced into the hinge. These studies demonstrated that specific changes (i.e. N20K and M21K) increased the activity of the peptide against Gram-negative bacteria such as *Shigella*, *Pseudomonas* and *Salmonella* species. In the case of Field *et al*. (2008), screening of a bank of random mutagenized nisin variants revealed that a K22T variant displayed enhanced activity against the mastitic pathogen *Streptococcus agalactiae*. This prompted the use of site-saturation mutagenesis for each of the individual hinge residues that, when coupled with a larger selection of target strains, led to the identification of a number of peptides with enhanced activity against *Strep. agalactiae*, *Staph. aureus* and *L. monocytogenes*. Yet another study, focusing on rings A and B at the N-terminal end of nisin A, showed that the various activities of nisin A can be altered by changing the amino acid arrangement in this region of the peptide (Rink *et al*., 2007). Two mutants, designated KFI and KSI (the letters indicate the amino acids present at positions 4, 5 and 6), displayed increased antimicrobial activity against a number of bacteria such as *Leuconostoc mesenteroides*, *Lactobacillus johnsonii* and *Lactococcus lactis*. KFI, and another variant, VFG, also inhibited the outgrowth of *B. subtilis* 168 spores more effectively than the wild type.

It should be noted that these and other (bio)engineering-related strategies have also been used for a variety of other purposes, such as increasing lantibiotic production (Cotter *et al*., 2006b; Heinzmann *et al*., 2006), introducing Lans into class II bacteriocins (Majchrzykiewicz *et al*., 2010) and even post-translational modification of other bioactive peptides (Kuipers *et al*., 2004; Kluskens *et al*., 2009; Rink *et al*., 2010). In addition to these approaches, the ever-improving ability of chemists to generate lantibiotic-like peptides through synthetic chemistry (Cobb and Vederas, 2007; Arnusch *et al*., 2008; Ross *et al*., 2010) is particularly exciting and has already facilitated the creation of potent nisin–vancomycin hybrids (Arnusch *et al*., 2008).

2.8 Screening for New Lantibiotics

While scientists are continuing with their efforts to further improve known lantibiotics, there is still considerable merit attached to identifying new peptides. It has also become apparent in recent years that bioinformatics can be a very useful means of screening for such novel lantibiotics. The availability of databases, including both general (NCBI) and dedicated (BAGEL and BACTIBASE) systems (see Chapter 1, Table 1.1, for more) (de Jong *et al*., 2006; Hammami *et al*., 2007, 2010), has facilitated the use of *in silico* approaches to lantibiotic screening. The two-peptide lantibiotic lichenicidin (Begley *et al*., 2009) was identified using such an approach. Here, the highly conserved nature of the *lanM* gene was exploited to screen the ever-increasing number of bacterial genome sequences that are publicly available. Initial screening revealed 89 *lanM* genes, of which 61 had not previously been associated with lantibiotic production. One of the potential

novel lantibiotic producers identified, *Bacillus licheniformis* ATCC 14580, was selected and from it lichenicidin was isolated (Begley *et al*., 2009). A similar approach was previously employed to identify another two-peptide lantibiotic, haloduracin, which is produced by *Bacillus halodurans* C-125 (McClerren *et al*., 2006; Lawton *et al*., 2007a). Bioinformatics has also been of considerable use when designing engineered lantibiotics and novel Lan-containing peptides. A study by Rink *et al*. (2005) used bioinformatics to predict the impact of flanking amino acids on the dehydration of serine and threonine residues, and subsequent Lan and meLan formation, in lantibiotic peptides. An *in silico* comparison of known lantibiotics found that the majority of modified serines and threonines were flanked by hydrophobic residues. *In silico* models predicted the likely impact of specific residues on modification when located adjacent to hydroxyl-amino acid residues. The subsequent creation, and investigation, of these peptides validated this theory.

The discovery of the lantibiotics microbisporicin and planosporicin was achieved using a more traditional screening method (Castiglione *et al*., 2007, 2008). This method involved 120,000 broth extracts obtained by fermenting 40,000 actinomycetes. The microbial products were screened to assess their activity against *Staph. aureus* before selecting those that retained activity following exposure to a β-lactamase cocktail or D-alanyl-D-alanine affinity resin (i.e. they were neither β-lactams nor vancomycin-like glycopeptides). Those that retained antimicrobial activity after these steps were selected for further investigation. This strategy yielded 35 lantibiotics, of which five showed little or no similarity to any known lantibiotics.

2.9 Concluding Remarks and Perspectives

The lantibiotic class of bacteriocins has the potential to be employed in a wide variety of ways. As outlined above, the lantibiotics have shown great promise as chemotherapeutics that can target the multidrug-resistant Gram-positive clinical pathogens, including the particularly problematic pathogens MRSA, VRE and *C. difficile*. A number of these peptides are particularly attractive as a consequence of research that has established that they have mechanisms of action and target binding sites that are distinct from those of non-lantibiotics. This, coupled with their high potency and generally non-cytotoxic nature, could lead to these compounds having clinical applications in the future. The possibility of engineering new and improved lantibiotics, producing novel chemotherapeutics through the fusion of lantibiotics with antibiotics, introducing lantibiotic-associated modifications into non-lantibiotics and chemically synthesizing new lantibiotic-like peptides – as well as the ongoing use of traditional and *in silico* strategies to find novel compounds – all bring this potential to a new level. In addition to these new markets, it should not be forgotten that a lantibiotic, nisin, has been successfully employed by the food industry for over a half century. Other lantibiotics have the potential to be similarly employed and, as a consequence of the limited activity of nisin against certain target strains and species, together with its poor activity at neutral pH, there are obvious niche-markets that these can fill. The commercial potential of lantibiotics and lantibiotic-related technology and the cutting-edge fundamental science that underpins lantibiotic research will ensure that these peptides continue to attract great attention in the coming years.

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3 Antimicrobial Peptides in Plants

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Abstract

This chapter provides an overview of plant antimicrobial peptides. It mainly focuses on one particular class of plant defence peptides, namely the cyclotides, which have been discovered over the last decade in plants from the *Rubiaceae*, *Violaceae* and *Cucurbitaceae* families. Cyclotides have a head-to-tail cyclized peptide backbone and a cystine knot motif formed from their six conserved cysteine residues, which makes them exceptionally stable. This chapter describes their isolation and characterization, structure and biosynthesis, and applications. The structural stability of cyclotides makes them excellent scaffolds for the engineering of novel therapeutic proteins. Advances in methods for the production of cyclotides and their potential clinical applications are also described.

3.1 Introduction to Plant Antimicrobial Peptides

Unlike animals, plants lack the ability to evade the attack of predators or pathogens through movement. With the 'fight or flight' defence response not available to them, plants have evolved an impressive array of defence molecules to confer protection once physical barriers have been compromised. This chapter describes a subset of this defence arsenal, namely peptide-based molecules produced by plants as antimicrobial agents directed against fungi, bacteria or viruses. Peptides that protect against attack by herbivores such as insects have been reviewed elsewhere (Ryan, 2000; Kessler and Baldwin, 2002; Gruber *et al*., 2007a; Ryan *et al*., 2007; Howe and Jander, 2008; Craik, 2009). Many antimicrobial peptides (AMPs) from plants were originally discovered through screening programmes directed at a variety of pharmaceutical activities. For a broader perspective on the pharmaceutical activities of plant natural products, readers are referred to other reviews (O'Keefe, 2001; da Rocha Pitta and Galdino, 2010).

To set the parameters of this chapter, we will define a peptide as a structure consisting of <100 amino acid residues, and we will not cover the literature for proteins of >100 amino acids. A search for the term 'antimicrobial plant peptide' in the UniProtKB database (Bairoch *et al*., 2005; UniProt Consortium, 2010) yields >400 sequences, but many of these have >100 residues and are thus not the subject of this chapter. AMPs isolated from endophytes (Yu *et al*., 2010) living in symbiosis with plants are also not covered here. In summary, we focus on peptides of <100 amino acids produced endogenously by plants to protect themselves against bacteria, viruses or fungi.

Most plant AMPs act by compromising the structural integrity of the most prominent target in microbes, their outer membrane, which has dramatic consequences for the

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microbe. This mechanism of action is achieved by either non-specific targeting of microbial membrane lipids or through targeting specific macromolecular components in the membrane. As shown in Table 3.1, many plant AMPs are positively charged, which is thought to play an important role in their interaction with membrane lipid head groups. In addition to their positively charged primary structure, plant AMPs display the typical range of secondary structure motifs seen in proteins, (i.e. α-helices, β-strands, β-turns and loops). As well as having well-defined secondary structures, they typically have well-defined tertiary structures, often as a result of spatial restraints from disulfide bonds or in some cases via cyclization of the peptide backbone. Both of these restraint types are combined in the cyclotide class of peptides (Craik *et al*., 1999), which incorporate three disulfide bonds and a cyclized peptide backbone, conferring remarkable structural stability to these molecules. Cyclotides are a major focus of studies in our laboratory and hence we devote a substantial section of this chapter to them.

Most classical AMPs from plants can be divided into four main classes: the lipid-transfer peptides, the thionins, the plant defensins (previously named γ-thionins) and the hevein/knottin-type chitin-binding peptides (Broekaert *et al*., 1997). Additional miscellaneous plant AMPs do not fit into these categories. PhytAMP is a curated online database of plant AMPs that focuses on AMPs shown experimentally to be expressed (Hammami *et al*., 2009). As of February 2010, this database contains just over 270 sequences. In addition to the peptide families noted above, PhytAMP recognizes the cyclotides, snakins, β-barrelins and impatiens peptides as plant AMPs. With the exception of cyclotides, which are documented in a dedicated database, CyBase (Mulvenna *et al*., 2006a; Wang *et al*., 2008a), these miscellaneous peptide families have only a few members and BLAST searches against the UniProtKB database return only a few (<30) protein hits from the plant kingdom. Several other databases also contain information about plant AMPs. These include the Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php) (Wang and Wang, 2004) and the AMSDb (Antimicrobial Sequences Database) from the Anti-infective Peptides Laboratory of Allesandro Tossi at the University of Trieste (http://www.bbcm.univ.trieste.it/~tossi/pag1.htm). Table 3.1 gives an overview of the largest families of AMPs from plants, indicating the size, charge and number of disulfide bonds, as well as information on secondary structures.

3.1.1 Lipid-transfer proteins

Non-specific lipid-transfer proteins (LTPs) from plants typically contain >90 amino acids. They are the largest peptides considered in this chapter, falling just below our arbitrary cut-off of 100 amino acids. They have an overall positive charge and, as their name suggests, they bind lipids in a

Table 3.1. Overview of structural features of AMPs from plants.

Peptide class	Size (residues)	Charge[a]	Number of disulfide bonds	Secondary structure[b]	No. entries in UniProtKB[c]
Lipid-transfer proteins	90–100	+8	4	h	940
Thionins	45–47	+7 or +10	3 or 4	h, s	266
Defensins	40–50	+6	4	h, s	418
Chitin-binding peptides					
Hevein-like	~50	–1	4	h, l, s	545
Knottins	~30	+3	3	h, l, s	
Cyclotides	28–37	–2 to +3	3	h, l, s	172

[a] Charge of side chains at neutral pH; values are for prototypic examples from each family.
[b] h, helix; l, loop or turn; s, sheet.
[c] Number of entries in UniProtKB based on a search with the relevant peptide class keywords (e.g. 'thionin') and the word 'plant'.

non-specific manner, without a preference for a particular type of lipid. *In vitro*, these peptides have been shown to transfer lipids from one membrane to another. In an early example, transfer of radioactively labelled phosphatidylcholine from artificial vesicles to chloroplasts from spinach was shown to be mediated by an LTP isolated from spinach leaves (Miquel *et al*., 1988). Similarly, electron paramagnetic resonance and fluorescence experiments have been used to show that an LTP from maize seeds was able to shuttle lipids containing spin labels from artificial vesicles to membranes from human erythrocytes and fluorescently labelled lipids from artificial vesicles to bovine chromaffin granules, respectively (Geldwerth *et al*., 1991). In that study, negatively charged lipids with phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatic acid head groups were found to be transferred to the target membrane at similar rates; thus, a direct charge interaction between positively charged LTP and negatively charged lipid head groups appears to be important. This lipid-transfer activity has been implicated in plant cell development (Nieuwland *et al*., 2005), but generally a variety of bioactivities, including defence against pathogens, cuticle synthesis and plant growth and development are linked with LTPs (Yeats and Rose, 2008). Membrane targeting of LTPs from plants has been shown to inhibit some bacterial (Molina *et al*., 1993) and fungal (Terras *et al*., 1992) plant pathogens *in vitro*. Terras *et al*. (1992) showed that increasing the ionic strength of the assay medium abrogates antifungal activity, indicating a charge-dependent mechanism of action, most likely involving interactions with the charged head groups of lipids in the fungal membrane.

The first crystal structure of a plant LTP was for an example from *Zea mays* in complex with palmitate (Shin *et al*., 1995). The solution structures of LTPs from *Triticum aestivum* (wheat) (Gincel *et al*., 1994), *Hordeum vulgare* (barley) (Heinemann *et al*., 1996) and *Z. mays* were early examples solved by nuclear magnetic resonance (NMR) and are shown in Fig. 3.1A (Gomar *et al*., 1996). The structures of LTP from barley in complex with palmitate-coenzyme A (Lerche *et al*., 1997) and palmitate (Lerche and Poulsen, 1998) were also solved by NMR. The global fold of LTPs reveals a bundle of four helices that cage lipid chains in the hydrophobic core formed between them (Fig. 3.1B and Fig. 3.1C). Only minor changes in the C-terminal region of LTPs have been reported when

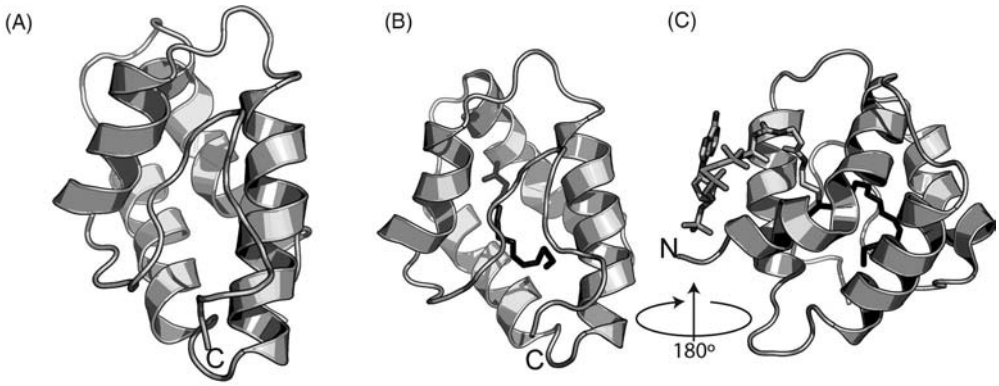


Fig. 3.1. Structural features of lipid-transfer proteins (LTPs). (A) The apo (lipid-free) form of LTP from *Zea mays* comprises four α-helices (Protein Data Bank (PDB) access code: 1afh). (B) Palmitate (black, stick representation, carboxyl group on top) is deeply buried in the cage formed by the four helices of LTP from barley (PDB: 1be2). (C) Palmitate-coenzyme A (CoA) conjugate binds to barley LTP in a similar manner; the lipid (black) is buried inside the peptide, whereas CoA is located on the surface of the peptide (PDB: 1jtb). Note that these are two complexes solved for the same LTP; for sake of clarity, C is rotated by 180° in the view shown.

binding to lipids (Lerche *et al*., 1997; Lerche and Poulsen, 1998).

In general, plant AMPs are of much interest, not only for the control of plant diseases (Montesinos, 2007), but also due to their ability to attack human pathogens, including *Candida* and *Aspergillus* species (Thevissen *et al*., 2007). These properties, together with the growing problem of resistance to conventional antibiotics (Hancock and Sahl, 2006), make plant AMPs interesting as novel human therapeutic leads. However, as far as LTPs are concerned, such applications need to be evaluated carefully because some plant LTPs have been identified as allergens in food (Zuidmeer and van Ree, 2007). For example, studies have reported that allergy to hazelnut (Flinterman *et al*., 2008) and wheat (Inomata, 2009) may be associated with these peptides.

3.1.2 Thionins

Thionins comprise 45–47 residues. They are divided into five classes depending on the number of disulfide bonds, number of basic residues and overall charge. Type I thionins have four disulfide bonds and are highly basic, with a charge of +10. Type II thionins also have four disulfide bonds, but have a reduced basic character with a charge of +7. Type III and IV thionins both have three disulfide bonds. Type III thionins have a charge of +7, whereas type IV thionins are neutral. Type V thionins appear to be truncated variants of other thionins (Bohlmann and Apel, 1991; Stec, 2006). In general, it appears that antimicrobial activity is correlated with the overall charge: the higher the positive charge, the higher the activity (Stec, 2006).

Thionins adopt an overall shape similar to the letter L (Stec, 2006). The structure consists of a β-strand followed by two α-helices (helix-turn-helix motif) and a second β-strand completing a double-stranded β-sheet. Several structures have been found to accommodate lipid moieties in the groove formed by the two α-helices and two β-sheets (Fig. 3.2) (Stec *et al*., 1995; Debreczeni *et al*., 2003).

The first two thionins to be discovered were identified (Balls *et al*., 1942a,b) and isolated (Fisher *et al*., 1968) from *T. aestivum* (wheat) germ. Their names, α- and β-purothionins, reflect that they are wheat peptides (Greek: *puro* = wheat) and that they have a high sulfur content (Greek: *thio* = sulfur). Mixtures of α- and β-purothionins, as well as purified peptides, were tested against a range of phytopathogenic bacteria. Activity was shown against *Pseudomonas solanacearum*, *Xanthomonas phaseoli*, *Corynebacterium michiganense* and others, but not against *Pseudomonas savastanoi* (Fernandez de Caleya *et al*., 1972). Tests with purified purothionins showed that α-purothionin was more active against *X. phaseoli* than β-purothionin, which in turn was more active against *P. solanacearum* (Fernandez de Caleya *et al*., 1972). These complementary activities of α- and β-purothionins exemplify how the diversity of AMPs in a single plant confers resistance to a diverse spectrum of pathogens.

The antimicrobial activity of thionins is thought to be enabled via an interaction with the head groups of lipids in membranes and has been studied in detail. Hughes *et al*. (2000) showed that the presence of negatively charged phosphatidylserine in artificial lipid mixtures resulted in the formation of membrane pores acting as ion channels. However, NMR and infrared spectroscopic evidence exists that in the absence of negatively charged lipids, thionins are also capable of binding both neutral lipids and those with positive charges (Richard *et al*., 2002, 2005). This broad binding ability may explain their broad antibacterial spectrum.

Overall, thionins are a widely studied class of plant defence peptides. They potentially have applications as drug leads, reflecting their presence as active compounds in traditional medicines from plants used since prehistoric times (Stec, 2006). Their activities against phytopathogenic microbes have also led to their incorporation in transgenic plants to increase or confer resistance to plant pests (Carmona *et al*., 1993; Chan *et al*., 2005).

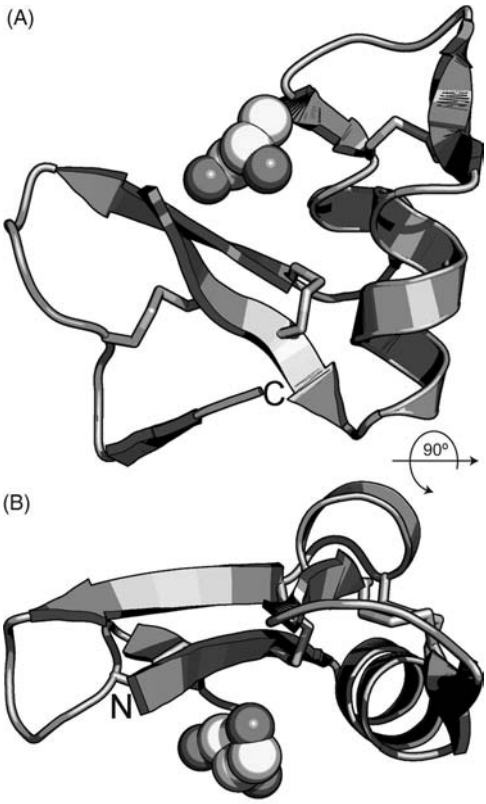


Fig. 3.2. The structure of type III β-purothionin from *Triticum aestivum* determined by X-ray crystallography (Stec *et al.*, 1995). (A) Two extended β-sheets are stabilized by two disulfide bonds (stick representation), one between the sheets and a second linking the first β-strand and the C-terminus. A short third β-strand is located at the C-terminus. A single disulfide bond links the α-helices. The structure shows the complex of the thionin with glycerol (sphere representation) in the groove between the helices and sheet. (B) For an overview, the perspective is rotated by 90° (top view of A).

3.1.3 Plant defensins

Plant defensins are basic cysteine-rich peptides of 45–54 residues (Thomma *et al.*, 2002; Lay and Anderson, 2005; Pelegrini and Franco, 2005). They are primarily recognized as potent antifungal agents and exert their activity at the membrane of fungi, typically inhibiting fungal growth (Lay and Anderson, 2005). They resemble thionins in their basic charge, cysteine content and size. This resemblance resulted in their original assignment as γ-thionins (Colilla *et al.*, 1990; Mendez *et al.*, 1990). This terminology was abandoned when it became apparent that they shared more characteristics of their primary and tertiary structures with insect and mammalian defensins than with other classes of thionins (Terras *et al.*, 1995). Plant defensins typically have a well-defined three-dimensional structure that comprises a triple-stranded β-sheet and an α-helix between the first and second β-strand. The overall structure is maintained by four disulfide bonds. Two are formed between the helix and the third β-sheet, while one tethers the C- and N-terminus for Rs-AFP1 (*Raphanus sativus* (radish) antifungal protein 1) (Fant *et al.*, 1998) and Psd1 (*Pisum sativum* (pea) defensin 1) (Almeida *et al.*, 2002). The remaining disulfide bond links the loop between the first β-strand and helix and the C-terminal end of the second β-strand.

In contrast to LTPs and thionins, which non-specifically target membranes, many defensins, including Dm-AMP1 from *Dahlia merckii* (dahlia) (Thevissen *et al.*, 2000a,b, 2003), Rs-AFP2 (Thevissen *et al.*, 2004) and Psd1 (Lobo *et al.*, 2007), have specific molecular targets in the plasma membrane of fungi.

Dm-AMP1 is active against the filamentous fungus *Neurospora crassa* and the unicellular fungus *Saccharomyces cerevisiae*. Binding of Dm-AMP1 to both fungi is saturable, indicating that a specific target with a finite concentration in the membrane exists (Thevissen *et al.*, 2000b). Dm-AMP1 binding competes with that of highly homologous peptides, but not with other membrane-active defensins of lower homology. In *S. cerevisiae* it has been reported that sphingolipids are determinants of sensitivity towards Dm-AMP1 (Thevissen *et al.*, 2000b). Evidence for this comes from the fact that fungal strains of *S. cerevisiae* lacking the gene that encodes the enzyme required in the final step in the synthesis of the sphingolipid mannose-(inositol-phosphate)$_2$-ceramide show increased resistance against Dm-AMP1 (Thevissen *et al.*, 2000a). The mechanism of action probably involves the

direct interaction of Dm-AMP1 with sphingolipids, as sensitivity to Dm-AMP1 is not altered by the disruption of glycosyl phosphatidylinositol-anchored peptides, which may be stabilized in the membrane by sphingolipids. Enhancement of binding in the presence of the fungal sterol ergosterol also suggests the involvement of lipid rafts in the interaction of Dm-AMP1 with membranes (Thevissen *et al*., 2003).

Rs-AFP2 is inactive against *S. cerevisiae*, but is highly active against *Candida albicans* and *Pichia pastoris*. The lack of glucosylceramide, a type of glucosphingolipid, in resistant species is believed to be the functional difference between sensitive and resistant species. Following the strategy exemplified in the Dm-AMP1 example, sensitivity to Rs-AFP2 has been mapped to glucosylceramide; *C. albicans* and *P. pastoris* strains lacking the gene coding the enzyme in the final step of glucosylceramide synthesis are resistant to the peptide (Thevissen *et al*., 2004). The interaction of Rs-AFP2 and glucosylceramide is independent of sterol content of the membrane and selective for glucosylceramide from fungi, as plant and mammalian glucosylceramides do not interact with Rs-AFP2 (Thevissen *et al*., 2004). The fact that sterol content does not have a role in this interaction indicates a mechanism of action different from that of Dm-AMP1 (Thevissen *et al*., 2004). Recently, it has been shown that Rs-AFP2 induces the generation of reactive oxygen species (ROS) in *C. albicans* (Aerts *et al*., 2007). This effect was linked to the antifungal activity as the presence of ascorbic acid, a scavenger of ROS, inhibited production of ROS and abolished antifungal activity.

It was recently shown that defensin Psd1, from *P. sativum*, may interact with the cell-cycle-related protein cyclin F from *N. crassa* (Lobo *et al*., 2007). Identification of cyclin F as the molecular target was achieved in two steps. First, potential binding targets were identified from a yeast two-hybrid system. The initial screen identified nine potential protein targets, including eight proteins localized in the nucleus and one in the plasma membrane. Cyclin F was confirmed as a target in a glutathione *S*-transferase-tagged pull-down experiment. In an experiment with rat retinal neuroblasts (a model system to study progression through the cell cycle), it was shown that Psd1 inhibition of cyclin F blocks transition from the S to G2 phase during the cell cycle (Lobo *et al*., 2007). The localization of Psd1 to the fungal nucleus may occur as a result of an interaction of Psd1 with the fungal membrane, with subsequent internalization. The interaction of Psd1 with artificial membranes was recently studied by NMR (de Medeiros *et al*., 2010). Changes in chemical shifts, an indicator for changes in the chemical environment, were monitored for Psd1 in solution in the presence and absence of lipids. The interactions observed were then mapped on to the three-dimensional structure of Psd1 (Fig. 3.3) (Almeida *et al*., 2002).

Plant defensins are attractive candidates in the search for novel antimicrobial compounds (Thomma *et al*., 2003; Thevissen *et al*., 2007; Carvalho and Gomes, 2009). They have been shown to be readily expressed as recombinant peptides in common expression systems, even allowing isotopic labelling as demonstrated, for example, for Psd1 expression in *P. pastoris* (de Medeiros *et al*., 2010). Plant defensins have also been used in transgenic plants to increase resistance to phytopathogens, as reviewed by Lay and Anderson (2005).

3.1.4 Chitin-binding peptides

The fungal membrane is predominantly (>90%) composed of polysaccharides (Latge, 2007), including chitin, a polymer of β1,4-linked *N*-acetylglucosamine (Fig. 3.4A). Chitin and its parent carbohydrate moiety, monomeric *N*-acetylglucosamine, are targeted by chitin-binding peptides, a subclass of carbohydrate-binding lectins.

Hevein, isolated from the latex of *Hevea brasiliensis* (rubber tree), was the first cysteine-rich chitin binding protein from plants shown to inhibit fungi *in vitro* (Parijs *et al*., 1991); however, the activity was only modest. Two homologues of hevein, Pn-AMP1 and Pn-AMP2, were isolated from *Ipomoea nil* (Japanese morning glory) and

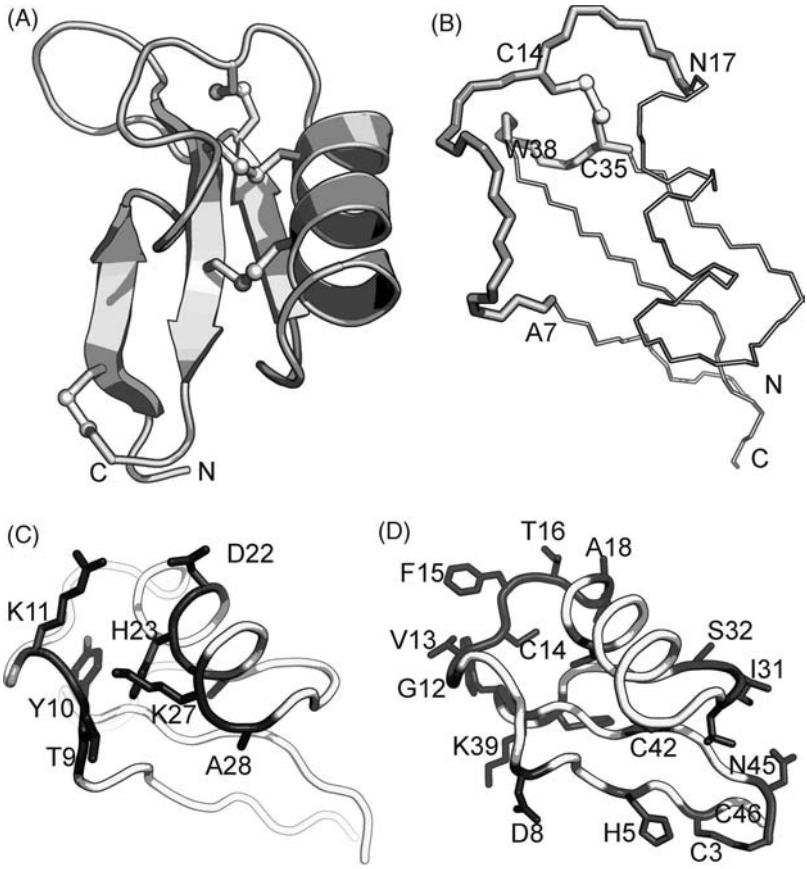


Fig. 3.3. Structure of Rs-AFP1 (*Raphanus sativus* antifungal protein 1; Protein Data Bank (PDB) access code: 1ayj) and active residues of Psd1 (*Pisum sativum* defensin 1; PDB: 1jkz). (A) Rs-AFP2 adopts the typical fold of plant defensins. The N- and C-termini are in close proximity due to a disulfide bond (ball and stick representation) from C3 to C51. (B) Backbone trace of Psd1. Residues of loop 1 (A7–N17) and turn 3 (H36–W38) and C35 are highlighted by stick representation. (C) Residues that experience chemical shift perturbation in the presence of phosphatidylcholine vesicles are shaded. (D) In the presence of 10% monohexosylceramide in the phosphatidylcholine vesicles, the distribution of residues affected by chemical shift perturbations changes significantly. Loop 1 is affected from G12 to N17, as is W38 in turn 3 and the neighbouring K39 (N17, L30 and W38 are not labelled for clarity).

showed much higher activity to a set of fungi *in vitro*. The mode of action is most likely disruption of the fungal cell membrane; it was shown using fluorescently labelled peptide and confocal microscopy, in conjunction with electron microscopy, that Pn-AMP1 accumulates in the septa of fungal hyphae (Koo *et al*., 1998).

Antifungal activity has also been observed for two small peptides, Ac-AMP1 and Ac-AMP2, from *Amaranthus caudatus* (pendant amaranth) (Broekaert *et al*., 1992). They also have some activity against Gram-positive bacteria, although to a lesser extent. Their amino acid sequence bears some resemblance to other chitin-binding peptides but they lack the C-terminal domain, which includes two cysteine residues (Broekaert *et al*., 1997). Both peptides show marked affinity to chitin at neutral pH. In other recent evidence demonstrating the importance of chitin binding, mutations in the chitin-binding protein Cy-AMP1 (*Cycas revoluta*, cycad) that decrease affinity for chitin also

decrease antimicrobial activity against fungi but not Gram-positive and Gram-negative bacteria (Yokoyama *et al*., 2009). This indicates that binding to chitin is essential for the antimicrobial activity of chitin-binding peptides. However, the detailed mechanism of membrane disruption is still unknown.

Figure 3.4 shows the structure of a mutant of Ac-AMP2, incorporating two non-natural amino acids in complex with chitotriose, that was solved in solution using NMR (Chavez *et al*., 2005). In the synthetic peptide, phenylalanine in position 18 and tyrosine in position 20 were both substituted with 4-fluorophenylalanine. The complex reveals the interaction of the aromatic side chain of residues 18 and 20 with the rings of the central *N*-acetyl glucosamine residue and

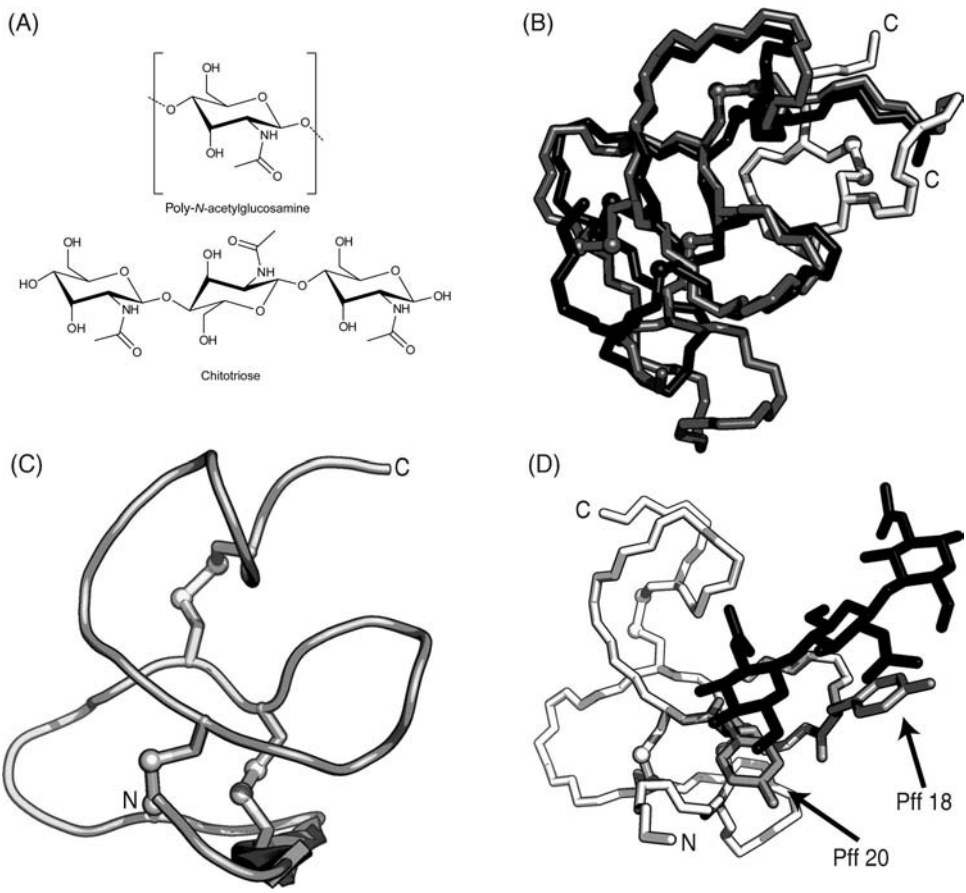


Fig. 3.4. Chitin and chitin-binding peptides. (A) β1,4-linked *N*-acetylglucosamine (top) is the building block of chitin. Chitotriose (bottom) is a defined oligomer of *N*-acetylglucosamine. (B) An overlay of the solution structures of hevein (grey, Protein Data Bank (PDB) access code: 1hev) (Andersen *et al*., 1993) and Ac-AMP2 (*Amaranthus caudatus*; dark grey, PDB: 1mmc) (Martins *et al*., 1996) reveals their structural similarity. The C-terminus of hevein is highlighted in light grey to clarify the extension of hevein relative to Ac-AMP2. The C-termini are labelled. (C) Solution structure of Ac-AMP2. (D) Solution structure of a synthetic Ac-AMP2 analogue (in the same orientation as in C in complex with chitotriose (dark grey)). The synthetic peptide incorporates two non-natural *para*-fluorophenylalanine (Pff) residues in positions 18 and 20 (arrows) to stabilize the complex with the carbohydrate through aromatic interactions. The rings of the side chains of residues 18 and 20 are aligned with the plane of the carbohydrate rings (PDB: 1znt).

the residue on the non-reducing end, respectively, a common recognition motif in carbohydrate binding (Jimenez-Barbero *et al*., 2006).

In general, the broad importance of chitin-binding proteins arises from the ubiquitous importance of their targets. Complex carbohydrates are essential components of cell surfaces and glycoproteins in many organisms, including pathogens and humans (Gabius *et al*., 2004). Lectins with high specificity for certain carbohydrate motifs, including *N*-acetylglucosamine and its oligomers, are valuable research tools and have potential as therapeutics (Gabius *et al*., 2004; André *et al*., 2009). For example, lectins, including chitin-binding lectins, have been investigated for their ability to inhibit human immunodeficiency virus (HIV) (Balzarini, 2006) and cancer cells (Liu *et al*., 2010). These studies have mainly focused on proteins, but the results may encourage further research into chitin-binding peptides to exploit their therapeutic and pharmaceutical advantages over proteins (da Rocha Pitta and Galdino, 2010). Chitin-binding ability is linked to plant protection against herbivore insects. The insect gut is lined with the peritrophic membrane, which contains chitin, and chitin-binding lectins have been shown to be toxic to insects (Hegedus *et al*., 2009).

3.1.5 Miscellaneous AMPs from plants

In addition to the major classes of plant AMPs noted above, there are several peptides that do not fall into large families, but none the less have important activities or novel structures. For example, a series of AMPs that are expressed as a single precursor peptide and processed into their final form occur in *Impatiens balsamina* (rose balsam) (Tailor *et al*., 1997). These relatively short peptides, comprising approximately 20 residues, are highly homologous and are stabilized by two disulfide bonds to form well-defined structures (Patel *et al*., 1998). As can be seen in Fig. 3.5, the Ib-AMPs are expressed as precursors that contain six highly homologous repeats. Ib-AMP1 is present in three copies (1a, 1b and 1c), whereas the other peptides appear only as single copies. The precursor protein comprises a signal peptide and seven propeptide regions of approximately 28 residues located before each mature peptide region.

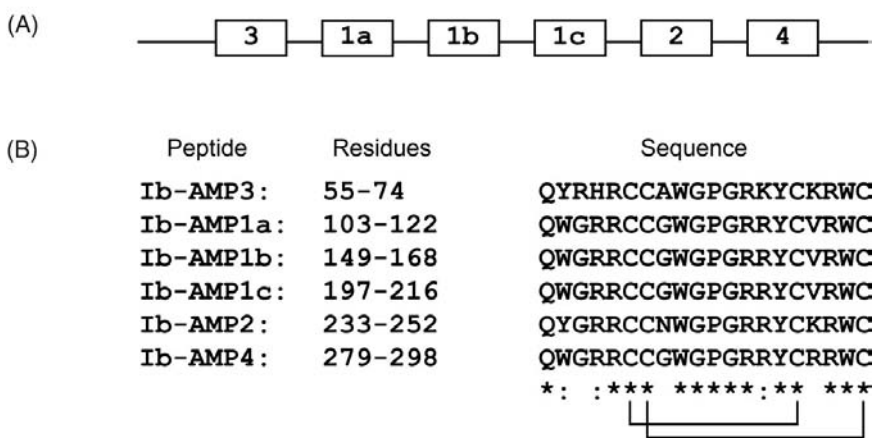


Fig. 3.5. Organization of Ib-AMP precursor (from *Impatiens balsamina*) and mature AMPs. (A) The precursor contains six repeats encoding four individual peptides. Ib-AMP1 is present in three identical copies (a–c). The mature peptide regions are preceded by an acidic propeptide region that is proteolytically cleaved during the maturation process. (B) The sequence alignment of the mature Ib-AMPs reveals the high homology and identity of the three copies of Ib-AMP1. The disulfide connectivity is indicated. '*' indicates identical residues and ':' indicates highly similar residues.

MiAMP1 (*Macadamia integrifolia* AMP1), as its name suggests, was originally isolated from macadamia nuts and has antifungal activity (Marcus *et al*., 1997). It has three disulfide bonds and a three-dimensional structure that is unique among plant AMPs (Fig. 3.6A) (McManus *et al*., 1999). The structure comprises two classic Greek key motifs (Hutchinson and Thornton, 1993) made up of two β-sheets consisting of four strands. Each β-sheet has three strands as part of one Greek key motif, whereas the last strand belongs to the other Greek key motif (Fig. 3.6B). The arrangement of the two Greek key motifs forms a global structure referred to as a Greek key β-barrel (Fig. 3.6B), and the name β-barrelin has been proposed to designate this fold (McManus *et al*., 1999). So far, no other AMPs presenting this fold have been identified. The overall structure has twofold axis symmetry, and the structural similarity led to the discovery of mild sequence homology between the fragments of the first and second Greek key motifs (Fig. 3.6C).

3.1.6 Non-ribosomal antimicrobial peptides

Cyclopeptide alkaloids differ from all of the other peptides mentioned so far in this chapter in that they originate from non-ribosomal synthesis. They contain a number of non-coding amino acids and an organic moiety, which facilitates cyclization. Evidence of antimicrobial activity is only available for a few compounds. A review by Tan and Zhou (2006) gives a comprehensive list of cyclopeptide alkaloids and their biological activity. The molecular mechanism at the heart of their antimicrobial activity is unknown, although these compounds are of much interest due to their diverse biological activities (Joullié and Richard, 2004; Tan and Zhou, 2006). A report by Morel *et al*. (2005) highlights some of the structure–activity relationships encountered in this class of peptides. The composition, stereochemical configuration and arrangement of the residues have a great influence on activity (Fig. 3.7).

3.2 Cyclotides

Cyclotides are a structurally fascinating family of plant cyclic proteins. They have a wide range of activities, including against plant pests and pathogens. Cyclotides first came to notice in the early 1970s (Gran, 1970, 1973a) when a peptide was identified as the active component of a traditional medicine used in Africa. A decoction of an African herb, *Oldenlandia affinis* – the vernacular name of which is 'kalata kalata' – is used to accelerate contractions during childbirth. The active ingredient was found to be a 29 amino acid peptide, which was named kalata B1 (Gran, 1973a). This peptide attracted further interest because of its unusual physical properties: it can resist high temperatures for an extended period of time (Gran *et al*., 2000) and is impervious to the action of chemical chaotropes and proteases (Colgrave and Craik, 2004).

Kalata B1 features two post-translational modifications that are at the origin of its stability: a cyclized head-to-tail backbone and compact cystine knot. Together, these are termed the cyclic cystine knot motif (Fig. 3.8) (Saether *et al*., 1995; Rosengren *et al*., 2003). This interesting protein architecture is the signature motif of the cyclotide protein family (Craik *et al*., 1999).

Cyclotides are a major focus of investigation in our laboratory. Strategies to isolate cyclotides have been established and have led to a broad estimate of the population of the family at about 50,000 peptides (Gruber *et al*., 2008). Cyclotides are active against various pests and pathogens and have activities beneficial to human health (Daly *et al*., 2009; Henriques and Craik, 2010), including anti-HIV activity (Gustafson *et al*., 2004). Cyclotides are also interesting in the protein-engineering field, as the stability of the cyclotide scaffold can be exploited to enhance the bioactivity of peptide drugs (Henriques and Craik, 2010).

3.2.1 Diversity of plant cyclotides

Cyclotides are widespread in the plant kingdom and are present in high abundance

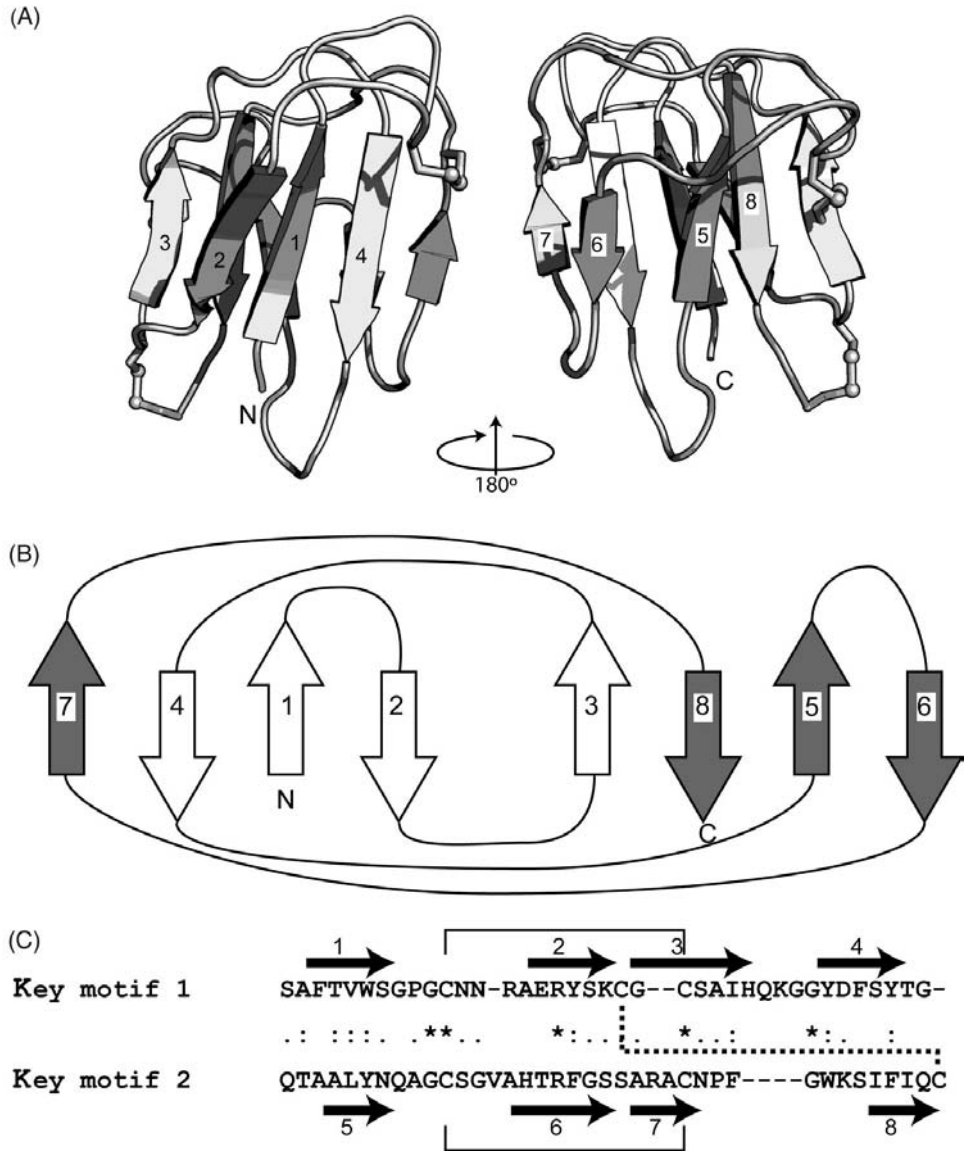


Fig. 3.6. Structural features of MiAMP1 (*Macadamia integrifolia* AMP1; Protein Data Bank access code: 1c01). (A) Three-dimensional structure. The disulfide bonds are shown in a ball and stick representation with N- and C-termini indicated. The Greek key motifs are in light grey (motif 1) and dark grey (motif 2). The representations are rotated by 180° for clarity. (B) Schematic showing the two β-sheets in MiAMP1 and the β-strand 'swap' between Greek key motif 1 (white filling) and motif 2 (grey filling). (C) An alignment of Greek key motif 1 (residues 1–38) and motif 2 (residues 39–76) reveals a weak sequence identity, but high similarity in the arrangement of the β-strands (arrows). Similarity: '*', identical; ':', highly similar; '.', similar. The symmetrical disulfide bonds within the motifs are indicated by solid lines on the top and bottom, respectively, and the disulfide bond tethering the two motifs together is indicated as a dashed line between the sequences.

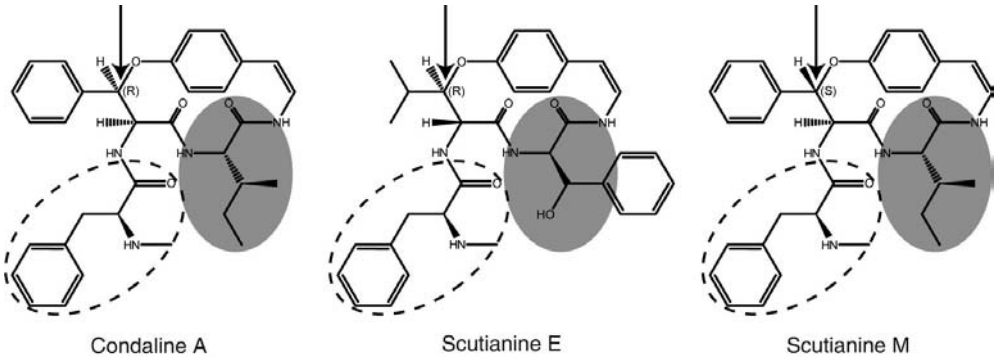


Fig. 3.7. Cyclopeptide alkaloids from plants. The arrows indicate the stereochemistry at position 3, which differs in scutianine M (right) compared with condaline A (left) and scutianine E (middle). The stereocentre occurs as a result of a cross-link to the β-carbon of phenylalanine or valine, respectively. Condaline A and scutianine M both have an isoleucine residue (grey highlight), whereas scutianine E features the unusual amino acid β-phenylserine at this position. The dashed outline highlights *N*-methyl phenylalanine. The breadth of the activity spectrum and activity decreases from left to right (Morel *et al.*, 2005).

in at least two plant families, the *Violaceae* (violet) and *Rubiaceae* (coffee) families (Gruber *et al*., 2008). Most plants express non-overlapping suites of different cyclotides, but some individual cyclotides have been discovered in multiple plants. For instance kalata S, also known as varv A (Göransson *et al*., 1999), has been isolated from six different *Violaceae* species (*Viola odorata*, *Viola tricolor*, *Viola arvensis*, *Viola baoshanensis*, *Viola yedoensis* and *Viola biflora*) and one *Rubiaceae* species (*O. affinis*). Thirty-four cyclotides have been isolated from the *Rubiaceae* family and 142 cyclotides from the *Violaceae* family. Two cyclic proteins, MCoTI-I and MCoTI-II, having the same structural topology but with unrelated sequences to other cyclotides, are found in the *Cucurbitaceae* family (*Momordica cochinchinensis*) (Hernandez *et al*., 2000). Because of their plant origin and cyclic cystine knot motif we classify them as cyclotides, but they are also referred to as cyclic knottins (Heitz *et al*., 2001).

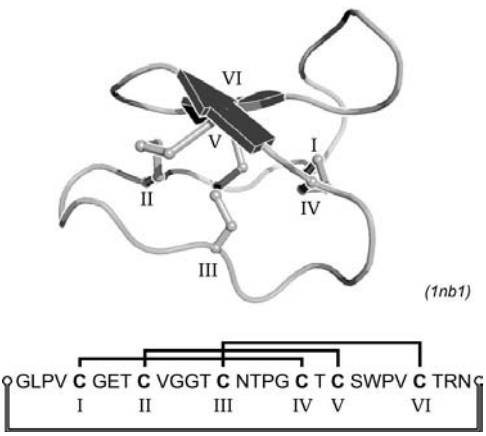


Fig. 3.8. The three-dimensional structure and sequence of the prototypical cyclotide kalata B1. Cyclotides are characterized by a cyclic cystine knot motif, which includes a head-to-tail cyclic backbone and knotted arrangement of three cystines. The cystine side chains are represented in a ball and stick format in the structure and the disulfide connectivities are shown by black thick lines above the sequence. The half-cystines are numbered using Roman numerals. Kalata B1 has a small β-sheet, represented by arrows on the structure.

CyBase is a database established to catalogue the sequences and structures of naturally occurring and engineered cyclic proteins, including cyclotides (Wang *et al*., 2008a). It currently provides information on 158 different sequences of wild-type cyclotides. Figure 3.9 shows a screenshot of a web page from this database.

An inspection of all the sequences currently in CyBase shows that cyclotides range from 27 to 37 amino acids in size. They

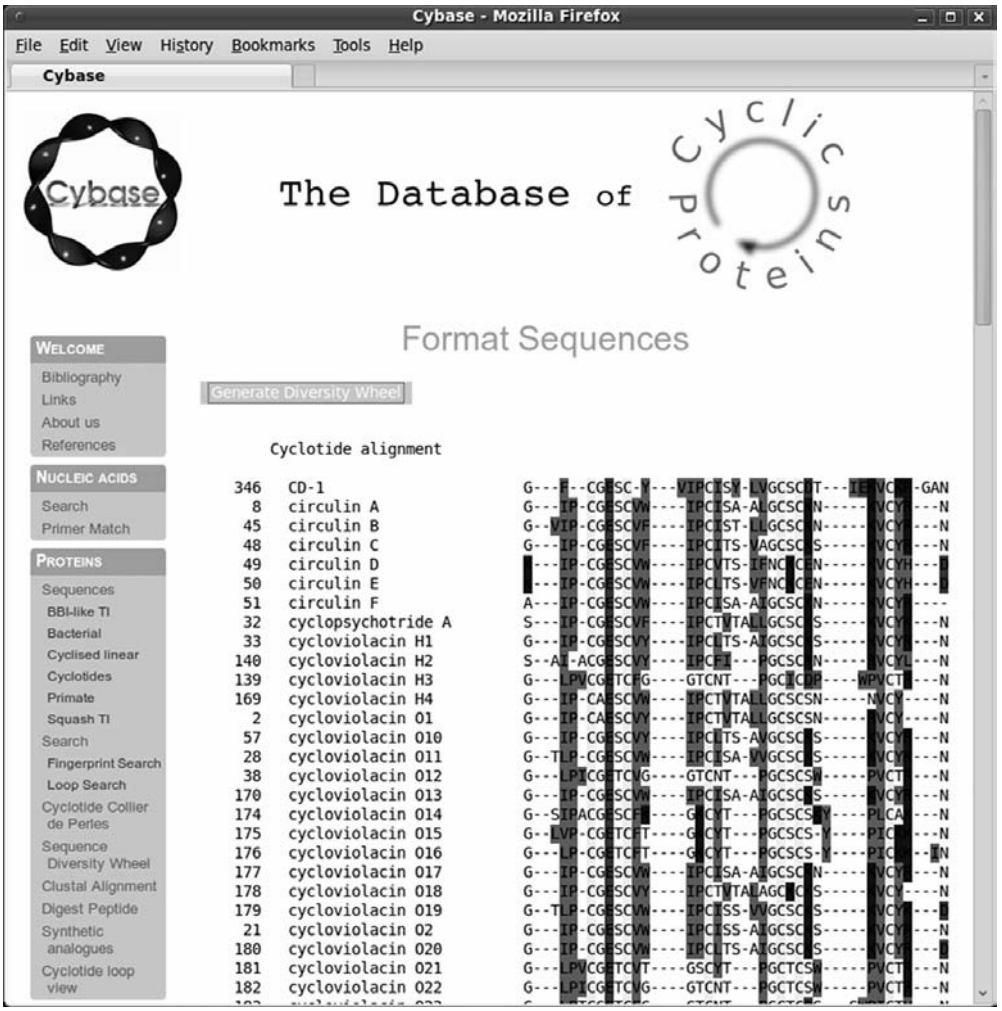


Fig. 3.9. Alignment of cyclotide sequences carried out with CyBase. This database incorporates specific tools for the study of cyclic proteins, including the alignment of cyclotide sequences, as shown here. The cysteine residues of the cyclotide cystine knot motif are aligned and gaps are inserted into the middle of the inter-cysteine regions. The vertical menu on the left provides access to search pages, lists and tools of cyclic protein and nucleic acid sequences.

invariably contain six cysteines forming the three disulfide bonds that constitute their characteristic cystine knot motif, as shown in an overlay of structures in Fig. 3.10 (Craik *et al*., 1999). The inter-cysteine regions are referred to as loops and have different ranges of amino acid lengths and sequence variability (Fig. 3.11). The loops with the greatest sequence diversity are loops 3, 5 and 6. Loop 3 is particularly interesting because its conformation is relatively conserved (Fig. 3.10) despite a high level of sequence variability (Fig. 3.11). Loop 6 accommodates the longest loop sequence seen so far in cyclotides (i.e. ten amino acids). By contrast, loops 1 and 4 are short and conserved in length, with three and one amino acids, respectively. Some positions seem to be crucial for cyclotide folding, including the cysteines and a glutamate in the second

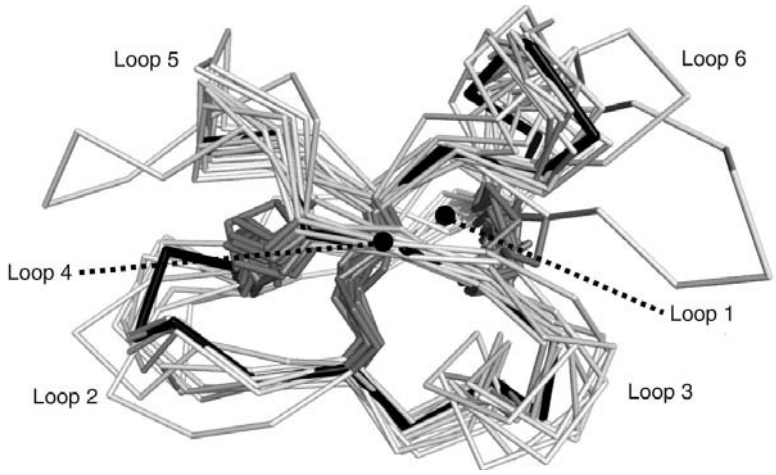


Fig. 3.10. Overlay of cyclotide structures. Cystine side chains are represented in dark grey and the cyclotide backbones are in light grey, except for the backbone of kalata B1, which is in black. The cystine knot motif is highly conserved and the cystine side chain positions align well. The conformations of loops 1, 2, 3 and 4 are highly conserved, whereas loops 5 and 6 are more variable. The structures shown are: circulin A (Protein Data Bank access code: 1bh4), circulin B (2eri), cycloviolacin O1 (1nbj), cycloviolacin O2 (2kcg), cycloviolacin O14 (2gj0), kalata B1 (1nb1), kalata B2 (1pt4), kalata B7 (2jwm), kalata B8 (2b38), palicourein (1r1f), tricyclon A (1yp8), varv peptide F (1k7g), vhl-1 (1za8) and vhr 1 (1vb8).

position of loop 1, which forms hydrogen bonds with the backbone amides of the first two positions in loop 5 (Rosengren *et al*., 2003; Göransson *et al*., 2009). The penultimate position of loop 5 is also important as it is often occupied by a *cis*-proline, which confers a conceptual twist in loop 5, leading to the analogy of a Möbius strip (Craik *et al*., 1999). Depending on the presence or absence of this *cis*-proline, cyclotides have been divided into Möbius and bracelet structural subfamilies (Craik *et al*., 1999). The impact of sequence variations on cyclotide activities is discussed in Section 3.2.4.

Methods that detect cyclotide peptide expression in plants (see Section 3.2.2) and the isolation of transcripts using molecular biology techniques have facilitated an increase in knowledge on cyclotide diversity (Simonsen *et al*., 2005; Gruber *et al*., 2008). Screening has been carried out in many tissues from hundreds of plant species (Simonsen *et al*., 2005; Gruber *et al*., 2008) and it is clear that cyclotides are present in all parts of plants. Cyclotides appear to be expressed in all species from the *Violaceae* family (Simonsen *et al*., 2005; Burman *et al*., 2010), but their occurrence in *Rubiaceae* species is more sparse, with <10% of examined species of this family expressing cyclotides (Gruber *et al*., 2008). The *Violaceae* and *Rubiaceae* belong to the monophyletic groups – rosids and asterids, respectively – which are both part of the eudicot class. The rosids and asterids are thought to have diverged 100–150 million years ago (Yang *et al*., 1999). To explain the gaps in cyclotide expression in the *Rubiaceae* and also the occurrence of cyclotides in both the *Violaceae* and *Rubiaceae*, it has been hypothesized that the current cyclotide distribution results from convergent evolution in different plant families (Gruber *et al*., 2008). This hypothesis is supported by the fact that the enzymes that appear to be responsible for the cyclization process are ubiquitous in plants (discussed in Section 3.2.3). In support of the convergent evolution hypothesis, cyclotide-like sequences have been detected in the *Poaceae* family (grass), which includes important cereal crop species such as wheat, maize and rice (Mulvenna *et al*., 2006b).

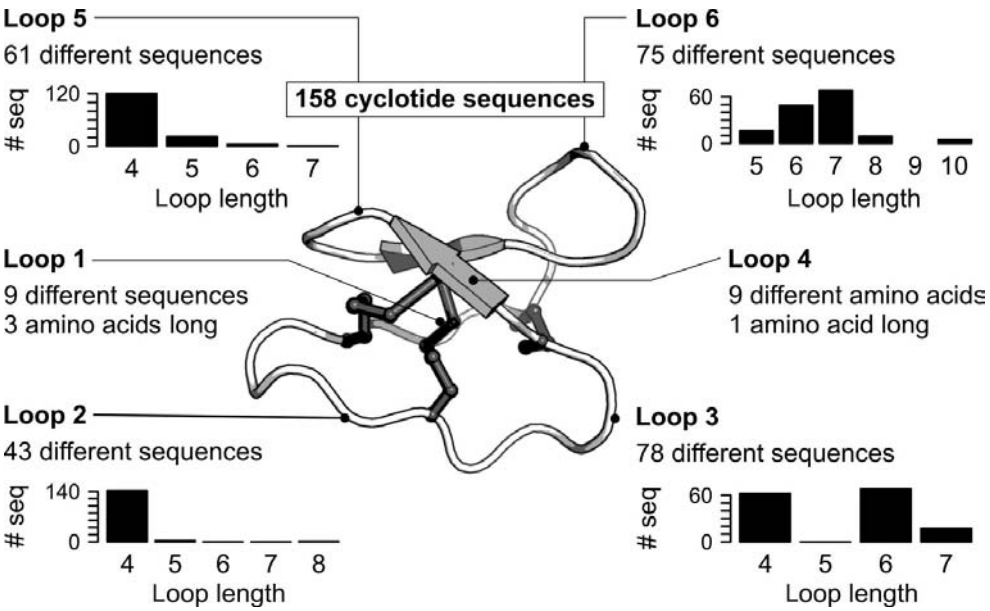


Fig. 3.11. Sequence variability of cyclotide loops. Each loop is identified on the three-dimensional structure at the centre of the figure. The number of different sequences is indicated. For loops 2, 3, 5 and 6, a bar graph represents the number of loops with a given length. For a given loop length, the loops having identical sequences are counted independently. Therefore, the total number of sequences represented in the graph is 158, which is the total number of cyclotide sequences in CyBase. Loops 1 and 4 do not vary in length, and their length and number of different sequences are provided as text.

3.2.2 Detection and isolation of cyclotides

A recently developed rapid cyclotide screening strategy (Gruber *et al*., 2008) has facilitated cyclotide discovery. First, different parts of a target plant are ground and incubated in dichloromethane/methanol. Water is then added to separate the methanol layer, which is fractionated and tested for peptide content, with a focus on peaks displaying similar hydrophobicity and mass to known cyclotides. Fractions that contain peptides in the mass range of 2.5–4.0 kDa are selected, and then reduced and alkylated to detect mass shifts corresponding to the reduction of the three cyclotide cystines into six alkylated cysteines. Cyclotides detected in initial screening can be subsequently characterized using a well-established protocol (Colgrave and Craik, 2004; Trabi and Craik, 2004; Colgrave *et al*., 2005) and sequenced by nanospray tandem mass spectrometry.

The isolation of transcripts having similarities with known cyclotides is an alternative strategy that has also been widely employed (Simonsen *et al*., 2005; Herrmann *et al*., 2008; Trabi *et al*., 2009; Zhang *et al*., 2009; Burman *et al*., 2010), even though it is not yet possible to predict if the translated peptide will be post-translationally modified to include a cyclic cystine knot motif. Nevertheless, sequencing of cyclotide cDNAs has revealed a conserved organization of the regions of cyclotide precursors, and this conservation has provided insights into cyclotide biosynthesis (Jennings *et al*., 2001).

3.2.3 Cyclotide biosynthesis

Cyclotides are gene products and their cDNA transcripts reveal a conserved organization of their precursors (Jennings *et al*., 2001). This comprises a signal sequence that directs the precursor to the endoplasmic reticulum, a

pro-region and one or several repeats, each comprising an N-terminal repeat, a cyclotide domain and a C-terminal region (CTR) (Kaas and Craik, 2010). The structure of the *O. affinis* kalata B2 precursor is shown in Fig. 3.12.

An asparagine endopeptidase (AEP) has been shown to be involved in the backbone cyclization of cyclotides (Saska *et al*., 2007; Gillon *et al*., 2008). This enzyme cleaves the peptide bond after a conserved asparagine that occupies the last position of the cyclotide domain. It has been postulated that before cleavage occurs, the first positions of the CTR could bind in pockets on the surface of AEP (Gillon *et al*., 2008). After cleavage and the release of the CTR, the first positions of the cyclotide domain, which have similar sequences to the first positions of the CTR, occupy the pockets. The N- and C-termini of the peptide would then be in close proximity and could be ligated by AEP, therefore cyclizing the protein (Gillon *et al*., 2008).

Twenty-three of the 158 cyclotide sequences currently recorded in CyBase have an aspartate at the position usually occupied by the conserved asparagine. AEP has reduced catalytic activity at an aspartate residue (Müntz *et al*., 2002) and, consequently, other enzymes might be involved in cyclotide backbone cyclization. Interestingly, during a small-scale expressed sequence tag project, a transcript coding for an asparaginase was isolated from *O. affinis* (Qin *et al*., 2010). Asparaginases catalyse the hydrolysis of asparagine into aspartate. Therefore, the unusual aspartate could have been formed by post-translational modification of the conserved asparagine after cyclization (Qin *et al*., 2010).

A protein disulfide isomerase (PDI) from *O. affinis* has been shown to interact with a linear kalata B1 precursor protein and improve its oxidative folding (Gruber *et al*., 2007b). In the absence of the PDI, a stable intermediate in the folding pathway of kalata B1 *in vitro* does not lead directly to the native product and requires disulfide shuffling for eventual formation of the cystine knot (Daly *et al*., 2003). Thus, PDI activity *in vivo* might

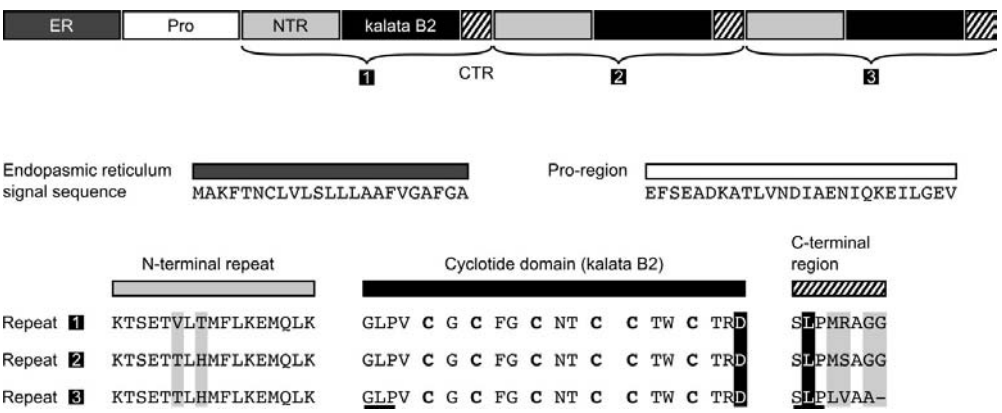


Fig. 3.12. *Oldenlandia affinis* kalata B2 precursor. The precursor comprises an endoplasmic reticulum (ER) signal sequence (dark grey background), a pro-region (white background) and three N-terminal repeats (NTRs, light grey background), three kalata B2 domains (black background) and three C-terminal regions (CTRs, diagonal stripes). An NTR, a domain and a CTR consecutive in the sequence form a repeat unit. At the bottom of the figure, the three repeats in the kalata B2 precursor are aligned and the positions presenting sequence variability are on a grey background. Whereas the NTR is highly conserved between repeats, the CTR is more variable. The three first positions of the CTR (underlined in the last repeat) have been identified as important for the enzymatic cyclization process and are homologous to the first three amino acids of the mature domain (also underlined). The last position of the domain is usually an asparagine but sometimes an aspartate, as is the case for kalata B2. This last position and the second position of the CTR are crucial for enzymatic cyclization to occur and are marked with a black background.

be important to prevent a significant number of cyclotides being trapped into intermediates with non-native disulfide bonds. Moreover, similarities between hydrophobic-solvent-assisted folding and folding using *O. affinis* PDI suggest that the chaperone activity of PDI might also improve the folding of cyclotides (Gruber *et al*., 2007b). Cyclotides have a hydrophobic patch on their surface that might interact with a hydrophobic domain of the PDI. The cyclotide hydrophobic patch has also been shown to be important for the binding of cyclotides to dodecylphosphocholine micelles, used as model membranes (Shenkarev *et al*., 2006, 2008; Wang *et al*., 2009).

3.2.4 Biological activities of cyclotides

Cyclotides first came to notice for their uterotonic activity employed in a native medicine application (Gran, 1970), but they have a range of other bioactivities including haemolytic (Daly *et al*., 1999), cardiotoxic (Gran, 1973b), antitumour (Herrmann *et al*., 2008), antifungal (Tam *et al*., 1999), anthelmintic (Colgrave *et al*., 2009), anti-HIV (Gustafson *et al*., 1994, 2004; Daly *et al*., 2006; Wang *et al*., 2008b) and, reportedly, antibacterial activities (Tam *et al*., 1999), as well as inhibition of trypsin (Hernandez *et al*., 2000) and neurotensin (Witherup *et al*., 1994). In this section, we focus on their purported antimicrobial properties. First, however, we give a brief overview of their pesticidal activities, because we believe that this host-defence activity is the primary biological function of cyclotides.

Pesticidal activity

The natural function of cyclotides appears to be to defend their host plants from insect pests (Jennings *et al*., 2001, 2005; Gruber *et al*., 2007a; Craik, 2009; Daly *et al*., 2009). Kalata B1 is a potent inhibitor of the growth and development of *Helicoverpa* species (Jennings *et al*., 2001), moths whose larvae feed on a wide array of plants, including a range of agricultural plants (maize and cotton). *Helicoverpa* are among the most polyphagous and cosmopolitan pests.

Cyclotides also have potent activity against *Haemonchus contortus* and *Trichostrongylus colubriformis*, two economically important gastrointestinal nematode parasites of livestock. This shows that the pesticidal properties of cyclotides are not specific to *Helicoverpa* (Colgrave *et al*., 2008). Cyclotides also have molluscicidal activity against *Pomacea canaliculata*, a serious pest of rice in South-east Asia, with a comparable potency to a commercial pesticide (Plan *et al*., 2008). *P. canaliculata* causes billions of dollars worth of damage on rice plantations each year (Sin, 2003) and there may be a role for cyclotides as a novel class of molluscicidal agents.

Cyclotides seem to act by a mechanism that affects cell membrane integrity. Upon ingestion of kalata B1, disruption of the gut membrane of caterpillars has been observed by light scanning microscopy and transmission electron microscopy (Barbeta *et al*., 2008). The integrity of a hydrophobic patch on the surface of the kalata B1 structure was found to be important not only for insecticidal activity, but also for membrane-binding affinity, as revealed by alanine-scanning mutagenesis (Simonsen *et al*., 2008; Huang *et al*., 2009). This suggests that the membrane-binding and insecticidal activities of cyclotides are correlated.

Anti-HIV activity

Initially two cyclotides, circulin A and circulin B, were discovered in the course of screening for anti-HIV natural products in a drug discovery programme at the US National Cancer Institute (Gustafson *et al*., 1994). In the anti-HIV assay, plant extracts were added to cultured T cells and subsequently exposed to infectious virus. The number of surviving cells was quantified after 6 days of incubation and compared with uninfected cells and untreated cells (Gustafson *et al*., 2004). With this protocol, compounds that inhibit early steps of the HIV infection could be detected. In the primary anti-HIV screen, extracts from *Chassalia parvifolia* were found to be active,

and circulin A and circulin B were identified as the bioactive compounds (Gustafson *et al*., 2004). Following this study, several other native cyclotides were reported to have anti-HIV activity (Daly *et al*., 2004, 2006; Gustafson *et al*., 2004; Chen *et al*., 2005; Ireland *et al*., 2008; Wang *et al*., 2008b).

The anti-HIV activity of cyclotides is interesting, but the mechanism of action is still unclear. It has been proposed that cyclotides could prevent HIV from interacting or fusing with host cells (Henriques and Craik, 2010). For efficient viral infection, viruses must deliver their genomes into the interior of target cells. Before virus entry into the cell, the HIV infection process is initiated by HIV receptor recognition at the surface of host cells, followed by fusion of the viral and host cell membranes (Cooley and Lewin, 2003). The cytoprotective effect of cyclotides has been reported to be associated with a decreased level of infectious virions (Gustafson *et al*., 1994), whereas no effect on HIV reverse transcriptase activity was detected (Gustafson *et al*., 2004). Such observations support the hypothesis that cyclotides exert their effect before the entry of the virus into the cell, inhibiting membrane targeting and/or membrane fusion.

Cyclotide bioactivities seem to correlate with membrane-binding properties, as supported by biophysical studies with model membranes (Shenkarev *et al*., 2006, 2008; Huang *et al*., 2009; Wang *et al*., 2009) and by cell membrane disruption observed in the insecticidal studies referred to above (Barbeta *et al*., 2008). As a hydrophobic patch has been found to be crucial for the interactions with membranes (Huang *et al*., 2009), it is reasonable to propose that cyclotides modulate their protective effect by a membrane-binding mechanism inhibiting HIV entry into the cell (Henriques and Craik, 2010). Specifically, anti-HIV efficiency shows a correlation with the extent of a hydrophobic patch on the surface of cyclotides (Ireland *et al*., 2008). Nevertheless, at this stage it is premature to hypothesize that cyclotides exert their effects by interacting with either the HIV membrane or the host cell membrane, or with both. Investigation of direct 'virucidal' activity would give more insights in the antiviral mode of action of cyclotides.

The natural cyclotides tested so far for their anti-HIV activity have a low *in vitro* therapeutic index (i.e. the ratio of their therapeutic effects to toxic effects) (Gustafson *et al*., 1994), which has limited their progression to the clinic as candidates for anti-HIV therapeutics. Nevertheless, other natural cyclotides have been shown to possess higher therapeutic indices (i.e. 44 for cycloviolacin Y5) (Wang *et al*., 2008b), thus reviving the possibility of cyclotide-based anti-HIV drugs. The possibility of decreasing the toxic effects of cyclotides by a single mutation (Simonsen *et al*., 2008; Huang *et al*., 2009), together with a more complete understanding of the mechanism of action, might accelerate the process.

Antibacterial and antifungal activity

Cyclotides have distinct hydrophobic and hydrophilic patches (Fig. 3.13), which resemble the amphipathic nature of AMPs. These properties led Tam *et al*. (1999) to hypothesize that cyclotides might exhibit antimicrobial activity. Four cyclotides were tested against selected bacteria and fungi, and selective antimicrobial activity was reported, as summarized in Table 3.2. Although low micromolar minimal inhibitory concentrations (MICs) were reported for some microbes (e.g. the MIC against *Staphylococcus aureus* was 0.26 μM in the absence of salt), the antimicrobial activity was ablated in the presence of physiological salt concentrations. Salt-dependent activity is often seen for AMPs, as high salt levels can affect electrostatic interactions with bacterial membranes (Bals *et al*., 1998; Wei *et al*., 2007). Currently, is not possible to determine if an increase in antimicrobial activity correlates with increased electrostatic attractions between cyclotides and bacterial membranes, as the effect of salt concentration on cyclotide membrane affinity has not been reported. Nevertheless, a lack of activity at physiological conditions brings into question the antimicrobial value of the cyclotides tested, probably accounting for the fact that since the study in 1999, no follow-up has been

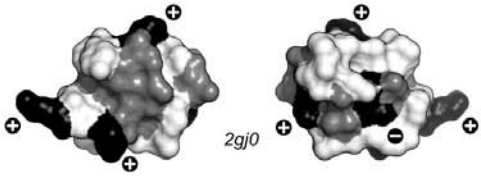
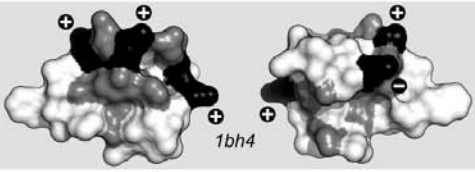
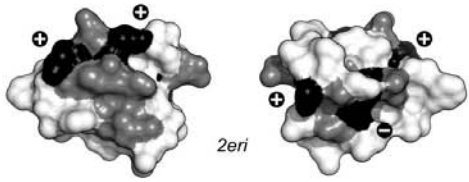
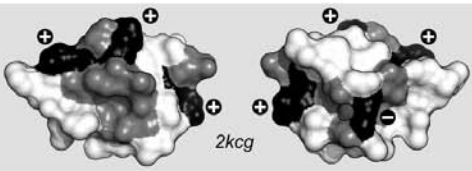
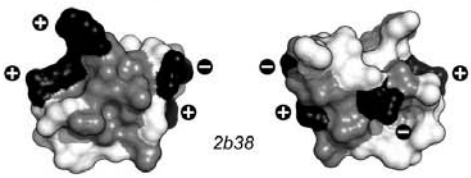
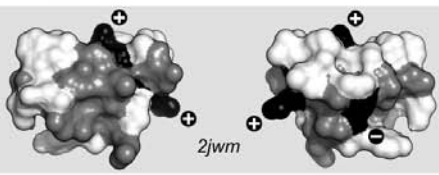
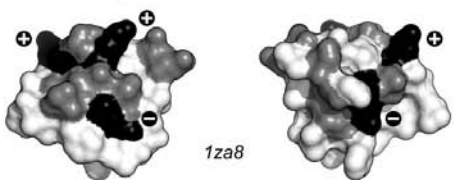
Name	Class	Charge on surface	# +	# –	Charge
Cycloviolacin O14	Möbius	 *2gj0*	4	1	+3
Circulin A	Bracelet	 *1bh4*	3	1	+2
Circulin B	Bracelet	 *2eri*	3	1	+2
Cycloviolacin O2	Bracelet	 *2kcg*	3	1	+2
Kalata B8	Bracelet	 *2b38*	3	2	+1
Kalata B7	Möbius	 *2jwm*	2	1	+1
Vhl-1	Bracelet	 *1za8*	2	2	0

Fig. 3.13. Surface characteristics and charges of wild-type cyclotides with known structure. The surfaces of all known wild-type cyclotides are represented by two views that differ by a rotation of 180° along the vertical axis. Structures located below each other have similar orientations (alignment of the cystine knot). The surfaces are darkened according to the properties of the amino acid under the surface: hydrophobic (white), hydrophilic but not charged (grey) and charged (black). The charge of the amino acids is shown on the surface. The number of positive and negative charges is given in columns 4 and 5, respectively, and the total charge of the cyclotide is provided in the last column. Protein Database numbers are shown between the structures.

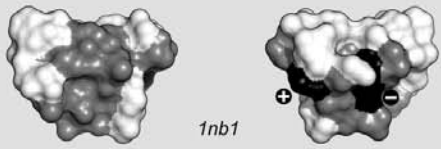
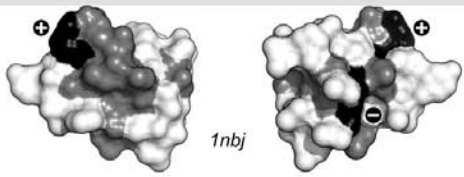
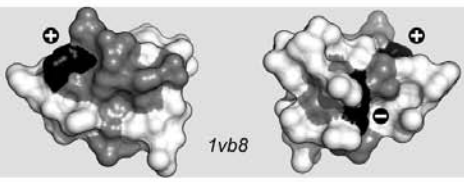
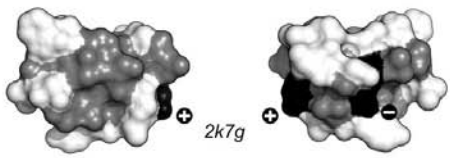
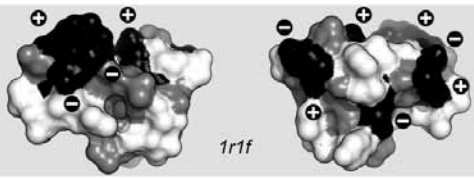
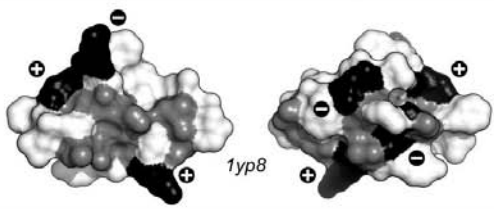
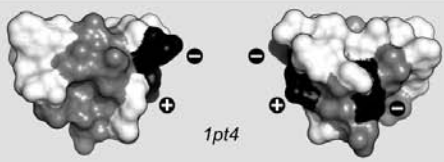
Name	Class	Charge on surface	# +	# –	Charge
Kalata B1	Möbius	 *1nb1*	1	1	0
Cycloviolacin O1	Bracelet	 *1nbj*	1	1	0
Vhr 1	Bracelet	 *1vb8*	1	1	0
Varv peptide F	Möbius	 *2k7g*	1	1	0
Palicourein	Bracelet	 *1r1f*	4	5	–1
Tricyclon A	Bracelet	 *1yp8*	2	3	–1
Kalata B2	Möbius	 *1pt4*	1	2	–1

Fig. 3.13. *Continued.*

Table 3.2. Antimicrobial activity measured for cyclotides under conditions with (100 mM NaCl) and without salt. Comparison of results obtained in two independent studies; adapted from its original form reported by Henriques and Craik (2010).

	Activity reported by Tam *et al.* (MIC (μM))[a]								Activity reported by Gran *et al.*[b]					
	Circulin A		Circulin B		Cyclopsychotride		Kalata B1		Kalata B1		Kalata B2		Kalata B7	
Organism	Salt	No salt	Salt	No salt	Salt	No salt	Salt	No salt	Salt	No salt	Salt	No salt	Salt	No salt
Gram-negative														
Escherichia coli	>500	>500	>500	0.41	>500	1.55	>500[c]	>500	✓	✓	×	×	✓	✓
Pseudomonas aeruginosa	>500	>500	48.0	25.5	50.2	13.5	>500	>500						
Proteus vulgaris	>500	54.6	>500	6.80	>500	13.2	>500	>500						
Klebsiella oxytoca	>500	>500	15.6	8.20	13.2	5.80	>500	54.8						
Haemophilus influenzae									×	×	×	×	×	×
Gram-positive														
Staphylococcus aureus	>500	0.19	>500	13.5	>500	39.0	>500	0.26	×	×	×	×	×	×
Micrococcus luteus	>500	>500	>500	>500	>500	48.0	>500	40.4						
Fungi														
Candida kefyr	>500	18.6	>500	29.0	48.0	14.0	>500	21.4						
Candida tropicalis	>500	19.4	>500	>500	>500	56.5	>500	>500						
Candida albicans	>500	>500	>500	>500	>500	>500	>500	>500						

[a] Minimum inhibitory concentrations (MICs) were determined after testing seven concentrations of peptide in a radial diffusion assay with underlay gel containing 1% agarose and 10 mM phosphate buffer without salt (no salt) or with 100 mM NaCl (salt); 5 μl of each concentration was incubated at 37°C for 3 h +16–24 h. The bacteria clear zones were measured and MICs were determined from the dose–response curves (Tam *et al.*, 1999).

[b] The antibiotic effect on *Escherichia coli* was tested after addition of 10 μl of 5 mg ml^{-1} peptide stock solution to agar plates; peptides were incubated at 37°C for 3 days. Antibacterial effects on *Staphylococcus aureus* and *Haemophilus influenzae* were evaluated by adding peptide directly to the growth medium. Peptide concentrations of 2, 4, 8 or 16 mg l^{-1} were incorporated and plates were inoculated with either *S. aureus* or *H. influenzae* and incubated at 37°C for 1 day (Gran *et al.*, 2008).

[c] Grey shading highlights contradictory results between the two independent studies.

published apart from one recent report (Gran *et al*., 2008). The results obtained by the only two reports on the antibacterial activities of cyclotides are compared in Table 3.2.

Kalata B1 is the only cyclotide that is common to both studies and contradictory results were obtained. Tam *et al*. (1999) reported that kalata B1 is inactive against *Escherichia coli* and active against *Staph. aureus*, whereas Gran *et al*. (2008) reported the opposite. In these two studies, activity was assessed using different experimental set-ups. Tam *et al*. incubated the bacterial strains with different concentrations of kalata B1 and determined the MIC in a radial diffusion assay, and concluded that kalata B1 is inactive against *E. coli* (MIC >500 μM in the presence or absence of salt). On the other hand, Gran *et al*. concluded that kalata B1 was active against *E. coli* based on the effect of 10 μl of 5 mg ml^{-1} kalata B1 (approximately 1700 μM) added to the agar plate. The very high concentration used in the Gran *et al*. study might explain the difference of activity observed for *E. coli* between the two studies.

Regarding the results obtained against *Staph. aureus*, Tam *et al*. concluded that kalata B1 was active against *Staph. aureus* as the MIC was found to be 0.26 μM in conditions without salt, but inactive in conditions with salt (MIC >500 μM). In the study by Gran *et al*., a lack of activity against *Staph. aureus* was concluded based on incorporation of kalata B1 into the bacterial growth medium, whereby 16 mg ml^{-1} of kalata B1 (approximately 5.5 μM) did not affect bacterial growth. The reasons for the differences in the two studies are not fully understood.

Classic antibacterial peptides selectively target negatively charged bacterial membranes over neutral mammalian cells and kill microbes through a membrane disruption mechanism (Yeaman and Yount, 2003). An amphipathic structure, with well-defined and distinct hydrophobic and positively charged patches, has been identified as a determinant for bacterial membrane targeting by antibacterial peptides (Dathe *et al*., 1997). The most frequent global charge identified in classical antibacterial peptides is +5 or +6, emphasizing the importance of the net positive charge for antimicrobial activity (Giangaspero *et al*., 2001). Although cyclotides have distinct hydrophobic and hydrophilic patches, most are not highly positively charged; the total charge is close to zero and most of the currently known cyclotides have a charge between –1 and +2 (see Figs 3.13 and 3.14). In addition, kalata B1 does not have a preference for negatively charged membranes at physiological ionic strength (Huang *et al*., 2009). Overall, cyclotides have a poor profile as antibacterial peptides.

Some cyclotides have a global charge of +3 (Fig. 3.14), but they have not yet been tested for their antimicrobial properties. The limited studies on the antibacterial activity of cyclotides make it difficult to judge the potential applications of native cyclotides as antimicrobial drugs; however, many more cyclotides wait to be discovered and new examples might reveal different characteristics, including higher charge and potentially stronger antimicrobial activities.

The cyclotides so far reported have revealed the remarkable plasticity of the cyclotide framework. Their tolerance for substitution suggests that cyclotides can be used as a scaffold and engineered to include a foreign sequence with a desired activity (Craik *et al*., 2006a). As already mentioned,

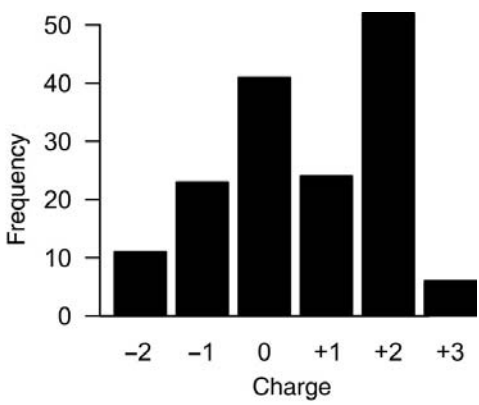


Fig. 3.14. Total charge of cyclotides based on 158 cyclotide sequences in CyBase. Arginines and lysines are positively charged, while aspartates and glutamates are negatively charged. Histidines are taken as neutral.

the bioactivities of cyclotides seem to broadly correlate with membrane-binding affinity. Whereas the disruption of the hydrophobic patch in kalata B1 leads to loss of function and a decrease in membrane-binding properties (Huang *et al*., 2009, 2010), the insertion of positive charges in selected locations increases bioactivity and membrane leakage properties (Huang *et al*., 2010). These properties suggest that cyclotides can be modulated so that toxic properties can be eliminated and antimicrobial activity improved. In this sense, cyclotide frameworks – with their circular structure and high resistance to thermal, enzymatic and chemical degradation – are a potential new class of antibiotics that could be designed to have both high stability and membrane-disrupting properties.

3.2.5 Cyclotide engineering and mass production

Despite being rapidly degraded by proteases *in vivo* and having generally low stability, peptides have generated much interest in drug design because they bind to their molecular targets with high affinity and specificity. The cyclotide cyclic cystine knot motif confers high resistance to different kinds of denaturants and can potentially overcome the stability and degradation problems of peptides (Craik *et al*., 2006a, 2009; Henriques and Craik, 2010). The fact that a range of loop sizes is tolerated by the cyclic cystine knot framework suggests that cyclotides are potentially amenable to substitution by a range of foreign epitopes in protein engineering and drug design studies (Craik *et al*., 2006b). Indeed, cyclotides may be thought of as a natural combinatorial library that is expressed on an ultra-stable structural scaffold (Craik *et al*., 2001). As explained previously, loops 2, 3, 5 and 6 are the most naturally variable and are the preferential sites for grafting biologically active epitopes. Because there is a degree of cooperatively between sequences in the different loops of the cyclotide scaffold (Daly *et al*., 2006; Gunasekera *et al*., 2009), not all grafted peptides can be successfully folded and thus optimization is sometimes required to find the most appropriate combination of grafted sequences and sites (Gunasekera *et al*., 2008). Studies investigating the tolerance of the cyclotide framework to substitution and grafting have been recently reviewed (Henriques and Craik, 2010).

The next step towards the development of cyclotides as pharmaceuticals will be to discover or design examples that have high potencies and selectivities for proven therapeutic targets. Assuming that such results are forthcoming in the near future, it will then be necessary to achieve high-yield production for scale-up testing. Unfortunately, the plant-based production of engineered cyclotides is problematic as transgenic plants have so far only produced low yields (Saska *et al*., 2007; Gillon *et al*., 2008). The most probable explanation for the low yields is that the transgenic plants lack optimized enzymes required for the correct processing of cyclotides. At least three alternative cyclotide production strategies have been attempted, including solid-phase synthetic chemistry, production in bacteria and plant cell culture.

Solid-phase peptide synthesis is a classical and straightforward method to produce peptides, but it has a high cost that is incompatible with mass production. A biomimetic strategy based on chemoenzymatic cyclization by an immobilized protease has been developed to complement this method (Thongyoo *et al*., 2007), and has shown improved yields over the usual native chemical ligation/cyclization reaction. More recently, Austin *et al*. (2009) developed an ingenious strategy to biosynthesize a cyclotide-based library in *E. coli* cells. *E. coli* lacks the required enzymes to cyclize proteins, but an alternative approach based on a modified protein-splicing unit, or intein, was put into practice and directly employed inside living *E. coli* cells (Fig. 3.15). This method generally produced good yields of cyclic proteins.

Plant cell-culture technology is a promising strategy because cyclotides can be directly cultivated in cells from cyclotide-producing plants (Dörnenburg, 2009). This should overcome the limitations of cyclotide

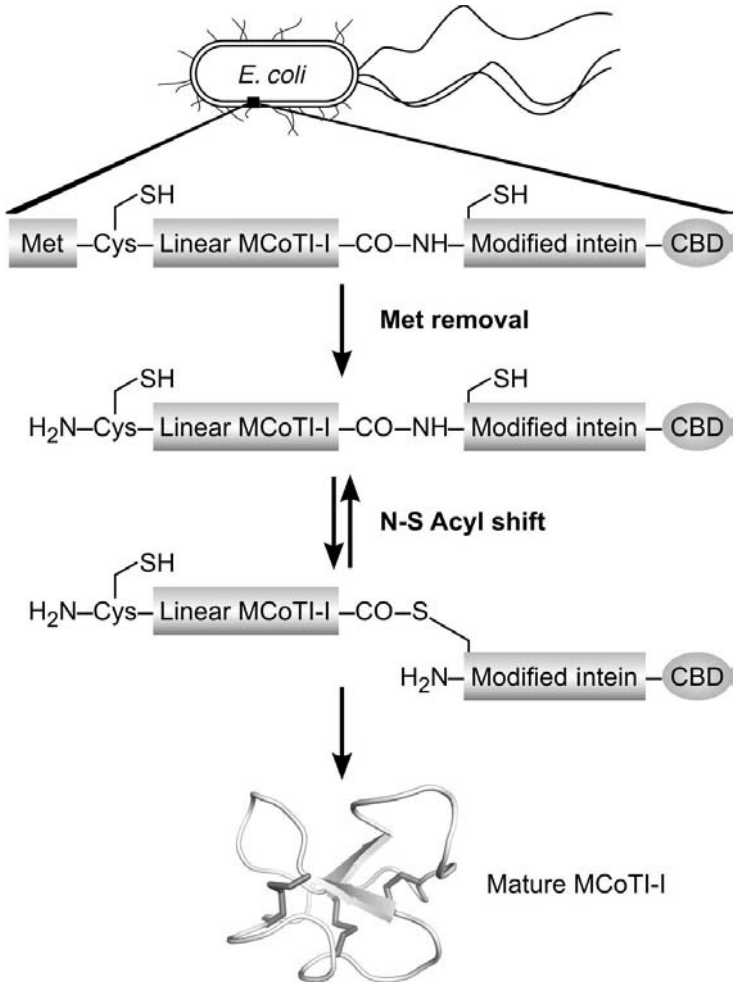


Fig. 3.15. The cyclization strategy used by Austin *et al*. (2009) to produce cyclotides in *Escherichia coli*. An intein was used as a protein-splicing unit and the last step of cyclization was performed by an intramolecular native chemical ligation reaction. A cellulose-binding domain (CBD) was fused at the C-terminal end of the intein region. Figure adapted from Austin *et al*. (2009). MCoTI-I, *Momordica cochinchinensis* trypsin inhibitor I.

production in model plants such as tobacco. Figure 3.16 illustrates cyclotide production using this approach.

3.3 Concluding Remarks and Perspectives

As is clear from this chapter, plants produce a variety of structurally diverse peptides that they use for defence purposes against microbes and other pests. Studies in our laboratory are focused primarily on one class of these molecules, the cyclotides, the principal natural function of which is thought to be as insecticidal agents. In addition to this presumed native function, however, cyclotides have been reported to have a variety of antimicrobial properties. Most extensively studied have been their antiviral properties, with a large number of cyclotides showing significant activity against HIV. Although they have promising potencies, their therapeutic index is not at

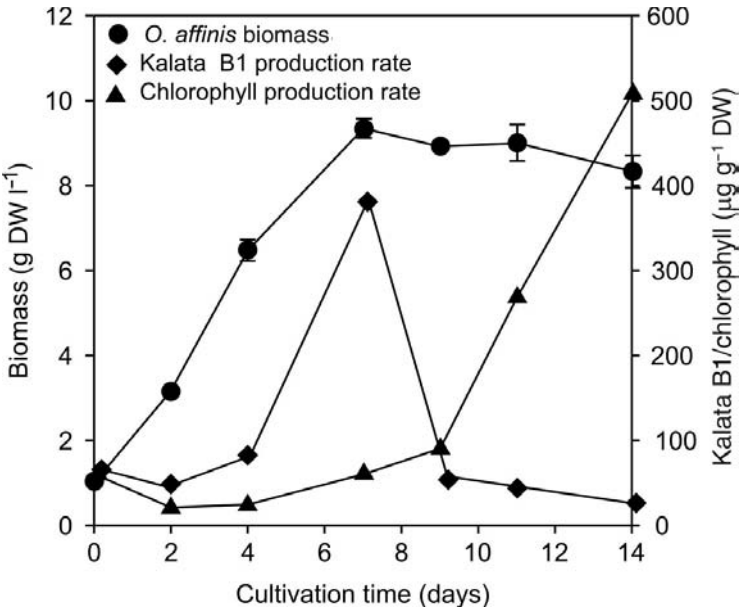


Fig. 3.16. *Oldenlandia affinis* cell culture growth and production kinetics of the batch cultivation process. Kalata B1 accumulation is maximized after 7 days, corresponding to the beginning of the stationary phase. DW, dry weight. Figure adapted from Dörnenburg (2009).

present sufficiently high to justify their clinical use as antiviral agents in humans. Nevertheless, with a large number of cyclotides predicted to be discovered over the coming years, it is possible that natural examples with better therapeutic indices may be found. Furthermore, with several routes to their synthesis available, the design of synthetic analogues with improved potency and reduced toxicity is a promising area for future investigation.

Cyclotides have been widely reported in the review literature to have antibacterial activity. However, as we have indicated here, a careful examination of the literature shows that only two original research papers have reported this activity and to an extent they present conflicting results. Thus, there is a question about the significance and relevance of the reported antibacterial activity of cyclotides. This is an area that needs further investigation and, in particular, pathogens relevant to plants should be examined. The studies so far have focused on human pathogenic bacteria, rather than on bacteria that might be typically encountered by plants.

One of the major unsolved problems in cyclotide research is the mechanistic basis of their action. They have an impressive array of biological activities and it would be simplistic to imagine that all of these activities can be accounted for by a single mechanism. However, if there is a common theme, then membrane binding appears to be a central player. Although it is equivocally established that cyclotides bind to membranes and some aspects of their binding have been delineated, the way in which they form pores in membranes remains a puzzle. Another mystery is how plants themselves are unaffected by the membrane-disrupting properties of cyclotides. Cyclotides are probably stored inside membrane-containing organelles within plants, yet they do not harm the producing plants. Thus, the systematic study of the interactions of cyclotides with a range of different plant membranes is an important area of investigation.

Finally, it will be of interest to determine why individual plants produce so many cyclotides – in some cases, up to 100 cyclotides per plant. The variation from one

cyclotide to another within a producing plant is often not particularly large and raises the question of why a plant would devote a significant amount of its energy resources to producing a suite of similar peptides. Perhaps some of the questions raised here are related, and the pore-forming ability of cyclotides might involve a mix-and-match interaction between different cyclotides. At this stage this is speculation, but it is an interesting area for future studies. The studies of membrane binding of cyclotides reported so far show that purified individual cyclotides can certainly disrupt membranes, but we do not yet know how mixtures of cyclotides, as occurs naturally in plants, might affect membranes.

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4 Database-aided Prediction and Design of Novel Antimicrobial Peptides‡

Guangshun Wang

Abstract

The isolation and characterization of potent antimicrobial peptides from natural sources has opened a new avenue to the development of future antimicrobials. To further expand the peptide space, new antimicrobial peptides can be predicted or designed based on natural peptide templates. By using the knowledge derived from mature peptides, propeptides, or both (i.e. precursor proteins), a variety of peptide prediction tools have been developed. Furthermore, bacteriocins are predicted under the genomic context by considering not only the peptide itself, but also the accompanying genes for its expression, processing and export. This chapter also discusses database-aided peptide design based on database screening, sequence shuffling, grammar-based design and *de novo* design. Finally, major strategies for improving peptide selectivity and stability are highlighted.

The majority of the known antimicrobial peptides (AMPs) were isolated from natural sources by using classic chromatographic methods. Recently, genomic and proteomic methods have also been applied to peptide identification in a more efficient manner (Conlon *et al*., 2004; Li *et al*., 2007). As the isolation and characterization of new AMPs is time-consuming and labour-intensive, other strategies have been used to expand the peptide repository. Based on common features in the AMP expression and processing machinery, bioinformatic approaches have predicted additional AMPs not yet isolated from natural sources (Schutte *et al*., 2002; De Jong *et al*., 2006; Fjell *et al*., 2007; Torrent *et al*., 2009). This chapter summarizes database-aided peptide prediction and design. Because the Antimicrobial Peptide Database (APD) (Wang and Wang, 2004) contains a well-registered set of mature peptides, it has motivated the development of prediction programs by other laboratories (Nagarajan *et al*., 2006; Lata *et al*., 2007, 2010). The output of these programs is 'yes' (antibacterial) or 'no' (not antibacterial). For a more quantitative structure–activity relationship (SAR) evaluation, a clean activity dataset for AMPs is required. By 'clean', we mean that the antimicrobial activity data should be obtained under the same conditions (e.g. the same laboratory, bacterial assay method, strain, plate-reading method, data processing). Some laboratories have compiled or generated such activity datasets for a large number of AMPs against the same target bacterial strain. These datasets have then been utilized to train the programs to predict the activity of new candidate sequences (Cherkasov *et al*., 2009; Juretić *et al*., 2009). The establishment of the method for rapid synthesis of small peptides in laboratories laid a solid foundation for designing new peptides and testing their

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efficacies (Merrifield *et al*., 1995). The earlier work contained the germ for modern large-scale peptide screening and design. The APD also fostered research efforts in peptide design (Loose *et al*., 2006; Duval *et al*., 2009; Wang *et al*., 2009, 2010). In this chapter, we describe these new developments ranging from peptide prediction to design. These methods expand the peptide sequence space of natural AMPs. Subsequent chapters further expand the peptide space by describing combinatorial peptide libraries (Chapter 5) and novel chemical mimics of natural AMPs (Chapter 6).

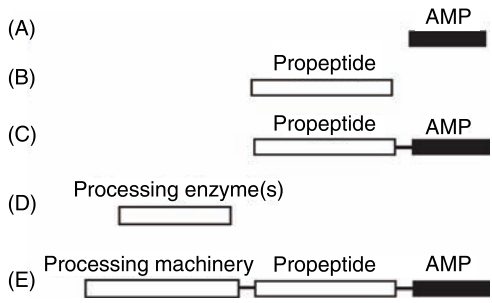

Fig. 4.1. Information used in antimicrobial peptide prediction. (A) Methods in category A involve only mature peptides. (B) Methods in category B utilize the high similarity of propeptide sequences. (C) Methods in category C use information from both propeptides and mature peptides. (D) Methods in category D take advantage of the sequence similarity of processing enzymes of lantibiotics. (E) BAGEL provides a more sophisticated approach by including all useful information in predicting potential AMPs in the bacterial genome.

4.1 Database-aided Antimicrobial Peptide Prediction

The prediction of AMPs is a non-trivial problem due to diversity in sequence, structure and function. The establishment of databases (Table 1.1, Chapter 1), however, has spurred research activities in this direction. For example, the well-registered AMPs in the APD (Wang *et al*., 2009) have been utilized as a known positive dataset to train and test a variety of prediction algorithms and approaches. The similarity between the trained peptides and the unknown constitutes the basis for prediction. The AMP prediction methods fall into five categories depending on the amount of information used in the computer programs. The first method (Fig. 4.1A) involves only the amino acid sequence information of mature peptides. The second method (Fig. 4.1B) utilizes the amino acid sequences of propeptides and mature AMPs. The third method (Fig. 4.1C) uses both propeptides and mature peptides. For bacteriocins, the method involves either using processing enzymes (Fig. 4.1D) or using context information related to the AMP expression and processing (Fig. 4.1E).

4.1.1 Prediction based on mature peptides

Knowledge-based prediction

The prediction protocol in the APD is based on simple calculations of peptide length, charge, hydrophobic residues content and amino acid composition (Fig. 4.1A). If the calculated properties of an input sequence match with those of any known AMPs in the database, the program will inform the user that 'Your input sequence has been found in our database'. Next, a prediction is made based on the amino acid composition that determines the possible class of the peptide candidate (β-sheet, residue-rich or α-helix). The amino acid percentages of AMPs from bacteria, plants and animals are listed in Table 4.1. If the properties of the peptide are out of the defined ranges in the database, it will be judged as being less likely to be antimicrobial. In addition, the prediction interface can also perform sequence alignment to identify AMPs with similar sequences (Wang and Wang, 2004). This interface will be upgraded in the next release.

The Fourier transformation method

Based on the mature peptides in the APD (Wang and Wang, 2004), Nagarajan *et al*. (2006) derived an indexing method for peptide properties such as hydrophobicity, charge, polarity, cysteine content and amino acid distribution. Fourier transformation

Table 4.1. Amino acid properties useful for AMP prediction and design.

AA[a]	AA[b]	Residue mass	BacP (%)[c]	PlaP (%)[d]	AniP (%)[e]	ΔG^{o}_{tf} (kcal mol^{-1})[f]	PV[g]	AASI[h]
I	Ile	113.16	6.25	4.29	6.72	2.45	**0.198**[i]	1.97
V	Val	99.13	6.52	4.7	5.97	1.66	**0.200**	2.37
L	Leu	113.16	5.8	3.31	**10.43**	2.31	0.246	1.74
F	Phe	147.18	2.66	2.82	4.5	2.43	0.246	1.53
C	Cys	103.14	5.00	**17.96**	5.28	2.09	**0.165**	1.73
M	Met	131.20	1.61	0.51	1.18	1.67	0.265	2.50
A	Ala	71.08	**11.35**	3.9	8.86	0.42	0.307	1.89
W	Trp	186.21	3.64	1.61	1.27	3.06	**0.172**	2.00
Y	Tyr	163.18	3.49	3.64	1.99	1.31	**0.185**	2.01
G	Gly	57.05	**15.04**	**11.29**	**11.89**	0.0	0.265	**2.67**
P	Pro	97.12	2.92	5.81	4.94	0.98	0.327	0.22
T	Thr	101.11	5.57	7.43	3.39	0.35	0.242	2.18
S	Ser	87.08	7.18	7.64	5.15	–0.05	0.281	2.14
Q	Gln	128.13	2.13	1.91	2.29	–0.30	0.248	**3.05**
N	Asn	114.10	5.88	5.18	3.13	–0.82	0.240	2.33
E	Glu	129.12	1.86	3.39	2.12	–0.87	0.449	**3.14**
D	Asp	115.09	2.03	2.34	2.23	–1.05	0.479	**3.13**
H	His	137.14	1.99	1.21	1.97	0.18	0.202	**3.00**
K	Lys	128.17	6.77	6.15	**10.86**	–1.35	**0.111**	2.28
R	Arg	156.19	2.05	5.27	5.84	–1.37	**0.106**	1.91

[a] Amino acid in the single-letter code.
[b] Amino acid in the three-letter code.
[c,d,e] Amino acid percentage of AMPs from bacteria (BacP), plants (PlaP) and animals (AniP), respectively (Wang *et al.*, 2009). Slight variations in these values are anticipated with increases in the number of AMPs in the database (Wang *et al.*, 2009).
[f] Hydrophobic parameter, as represented by the transfer of free energy of an amino acid side chain from the octanol phase to water (Fauchere and Pliska, 1983). An application of this was illustrated in Wang *et al.* (2005).
[g] Bactericidal propensity value (PV) (Torrent *et al.*, 2009).
[h] Amino acid selectivity index (AASI) (Juretić *et al.*, 2009).
[i] Some outstanding values are in bold.

analysis revealed a distinct peak in the power spectrum of peptide sequences. This finding forms the basis for identifying potential AMPs by scanning the protein sequences derived from the genomic data. As a test, this Fourier transformation filter found three positive hits from 10,000 randomly generated sequences of 16 residues each. The antimicrobial activities of those peptides have not yet been evaluated experimentally.

Machine-learning methods

Also based on the positive AMP dataset of the APD (Wang and Wang, 2004), Lata *et al.* (2007) applied machine-learning algorithms to AMP classification and prediction. Neural networks handle information in a way similar to the human brain. The artificial neural network (ANN) is a powerful machine-learning method that is insensitive to noise and correlated inputs. The ANN can be trained using carefully registered known AMP data and perform predictions based on the extracted rules. The support vector machine (SVM) approach includes a set of related supervised learning methods used for classification and regression. In conjugation with peptide terminal sequence analysis, the authors reported an accuracy of 88% for ANN and 92% for SVM. A recent update of the APD (Wang *et al.*, 2009) led to the improvement of this prediction program. By including new peptide entries and source information annotated in the APD, the overall prediction accuracy reached 98.95%

(Lata *et al*., 2010). We made some test runs of the program (www.imtech.res.in/raghava/antibp2/submit.html) using the newly registered AMPs, which are not likely to be part of the training set. When 'SVM' and 'full sequence composition' were selected, the program gave no prediction for AMPs with <15 residues. Among 17 AMPs with >15 residues, 12 were correctly predicted as antibacterial (i.e. 71% accuracy).

AMP sequence motifs

Based on a manually compiled list of disulfide-containing AMPs such as defensins from plants and animals, Yount and Yeaman (2004) identified a well-conserved 'GXC' sequence motif. This motif is common in defensins, ranging from plants to bacteria, fungi and animals. In the three-dimensional structures of peptides, this sequence motif forms a 'γ-core'. Using this signature model, the authors were able to identify previously unidentified AMPs such as brazzein and charybdotoxin that exerted direct antimicrobial activity against bacteria and *Candida albicans*. This motif does not apply to the cysteine-bridged Rana box at the C-termini of some amphibian AMPs (Conlon *et al*., 2004).

Structural motif-based prediction has also been performed at the gene level. Based on the conserved six-cysteine β-defensin structural motif, Schutte *et al*. (2002) were able to identify 28 new human and 43 new mouse β-defensin genes in five syntenic chromosomal regions by combining the HMMER and BLAST analysis tools. HMMER (http://hmmer.janelia.org/) is a bioinformatics tool based on the hidden Markov models. These newly discovered genes were missed in previous annotations of human and mouse genomes. Future studies will elucidate when, where and why particular defensins are expressed or silenced.

4.1.2 Prediction based on highly conserved propeptide sequences

The sequence alignment of cathelicidin precursor proteins has led to an interesting discovery. While the amino acid sequences for the mature AMP region are highly variable, the peptide sequences prior to the mature peptide portion (propeptide) are remarkably similar. This observation laid the basis for identifying novel cathelicidins (Fig. 4.1B). In analogy to swine PR-39, a human version sequence was initially predicted as FALL-39 (Agerberth *et al*., 1995). Subsequently, Sørensen *et al*. (2001, 2003) isolated LL-37 from human neutrophils and ALL-38 from the reproductive system. In this case, the experimentally elucidated protease cleavage sites on the precursor protein differed slightly from those predicted. This example illustrates that the precursor sequence of each AMP is also useful for AMP prediction. Indeed, the precursors of ranid AMPs also possess a common and highly conserved pro-region, usually acidic and terminated at a typical processing signal lysine/arginine. This finding has been utilized as a basis for identifying AMPs on a large scale in amphibians (Li *et al*., 2007).

4.1.3 Prediction based on both propeptides and mature peptides

In the AMPer prediction model, Fjell *et al*. (2007) considered both propeptide sequences and mature peptides (Fig. 4.1C). Data from the Antimicrobial Sequences Database (AMSDb) (Tossi and Sandri, 2002) were validated and expanded as a training dataset. The basic idea was to classify the peptides in the database into multiple groups (or clusters) and then calculate the property profiles as a basis for subsequent prediction. Fjell *et al*. (2007) identified 146 clusters for mature peptides and 40 clusters for propeptides (186 clusters in total). The prediction was accomplished by matching the profiles of the unknowns with those of the known 186 clusters. The mutual overlap between the matched pairs should be >90%. In this manner, they identified an additional 229 mature peptides from the 230,133 peptides collected in the Swiss-Prot database. Most of these peptides could be associated with

known activity data in the literature. This tool achieved a high prediction accuracy of 99%.

4.1.4 Prediction based on processing enzymes

It has been illustrated that sequence similarities among polypeptide modification enzymes such as LanM are useful in identifying novel lantibiotics. Because this approach does not utilize polypeptide information of either AMPs or their precursors, it can be regarded as a fourth method (Fig. 4.1D). Both haloduracin and lichenicidin were identified in the genomes using this approach (McClerren *et al*., 2006; Begley *et al*., 2009).

4.1.5 Genomic context-based bacteriocin prediction

A possible reason for missing AMPs during gene annotations is the small size of their open reading frames (ORFs). De Jong *et al*. (2006) developed BAGEL (http://bioinformatics.biol.rug.nl/websoftware/bagel) to detect AMPs in a bacterial genome. This program incorporates a few published ORF prediction tools, including Glimmer/RBSfinder, Zcurve and GeneMark, for the detection of small ORFs for AMPs. In addition, the program also considers the existence of gene clusters that encode proteins for processing, modification, transport, regulation and/or immunity of bacteriocins. Finally, the program compares the candidate with those in the knowledge-based bacteriocin database generated by the authors. Because this approach is of an integrated nature and differs from all the other methods discussed above, we shall name it as 'genomic context-based AMP prediction' (Fig. 4.1E). Using BAGEL, the authors found one additional possible bacteriocin from the genomic sequences of both *Streptococcus pneumoniae* TIGR4 and R6. This online tool has also been used to identify putative bacteriocin genes (Majchrzykiewicz *et al*., 2010).

4.2 Database-aided Peptide Design and Improvement

4.2.1 Database screening for HIV-inhibitory antimicrobial peptides

Natural AMPs show inhibitory activities against human immunodeficiency virus (HIV). Examples are insect melittin and cecropin, amphibian AMPs, mammalian cathelicidins, defensins and plant cyclotides (Wachinger *et al*., 1998; VanCompernolle *et al*., 2005; Jenssen *et al*., 2006; Lehrer, 2007; Ireland *et al*., 2008). Because effective HIV vaccines are not yet available, there is a need to develop topical microbicides that prevent the sexual transmission of HIV (Turpin, 2002; Buckheit *et al*., 2010). Currently, <5% of the AMPs in the APD are known to be HIV inhibitory. We have hypothesized that a large number of AMPs in our database (Wang and Wang, 2004), with a variety of sequences, contain useful templates for developing novel agents that may prevent HIV transmission. To test this hypothesis, we recently conducted a database-based peptide screen against HIV type 1 (HIV-1). In total, 30 natural peptides were selected by considering peptide length, charge, cysteine content, toxicity to mammalian cells and uniqueness of the candidate sequences (Wang *et al*., 2010). The well-accepted and highly standardized cytopathic effects inhibition assay in CEM-SS cells was utilized to evaluate the efficacy and toxicity of candidate peptides. The therapeutic index (TI) is defined as the ratio of TC_{50} to EC_{50}, where EC_{50} is the concentration required for 50% inhibition of viral replication and TC_{50} is the concentration required for a 50% reduction in cell viability. We found that 11 peptides displayed an EC_{50} of <10 μM. These include an uperin 7.1 mutant, temporin-PTa, clavanin B, ponericin L2, spinigerin, piscidin 3, brevinin-2-related, maculatin 1.3, ascaphin-8, melectin and temporin-LTc. Of these peptides, frog temporin-PTa, temporin-LTc, insect ponericin L2 and spinigerin had a TI of >10.

For longer peptides such as human cathelicidin LL-37 and bovine BMAP-27, we also identified the major HIV-inhibitory regions (Wang *et al*., 2008). In the case of

LL-37, FK-13 was identified as the smallest HIV-inhibitory region; KR-12, which differs from FK-13 by just one N-terminal phenylalanine (Table 4.2), turned out to be inactive. Among the series of peptides tested, the LL-37-derived GI-20 displayed one of the highest TIs. Likewise, BMAP-18, the N-terminal 18 residues of BMAP-27, was found to have a TI of >20 (Table 4.2). We are now developing anti-HIV microbicides based on these peptide templates.

We found excellent overlap between the minimal antibacterial region (Li *et al*., 2006a) and the smallest anti-HIV region of human LL-37 identified experimentally (Wang *et al*., 2008). Torrent *et al*. (2009) developed a theoretical method to spot active regions in antimicrobial proteins. A bactericidal propensity index value (PV) was calculated for each amino acid (Table 4.1). The PVs for residues arginine, lysine, cysteine, tryptophan, tyrosine and isoleucine are ≤0.2 and are favoured in AMPs. A potentially active region should have a low PV value on average. This method achieved a prediction accuracy of 85%.

4.2.2 Sequence shuffling is a primitive combinatorial approach

Sequence reversal influences the activity of AMPs (Fig. 4.2B). Merrifield *et al*. (1995) found that the hybrid peptide became less antimicrobial after sequence reversal. In another case, sequence reversal did not cause a selective loss in haemolytic activity (Subbalakshmi *et al*., 2001). We found that the LL-37-derived core AMP (FK-13) was active against both *Escherichia coli* and HIV. After sequence reversal, retro-FK13 retained its bactericidal activity against *E. coli* (Li *et al*., 2006b), but lost its HIV-inhibitory activity (Table 4.2). These examples indicate that the sequence order of AMPs determines the activity spectrum. Sequence reversal, however, is only a special case of sequence shuffling or rearrangement (Fig. 4.2B), since the latter can produce numerous new sequences (i.e. a miniature combinatorial library).

To evaluate the effect of sequence shuffling on anti-HIV-1 activity, we created some new peptides based on an aurein 1.2 analogue, where phenylalanine 13 of aurein 1.2 was mutated to tryptophan 13 (sequence: GLFDIIKKIAESW). The peptides were generated by rearranging the 13 amino acid residues of peptide 1 and by modelling known helical AMPs. These peptides were subjected to HIV-1 inhibition evaluation as described above. Among the eight peptides synthesized, two displayed reduced TIs, three showed little variation and two showed improved TIs. Hence, sequence shuffling provides an approach for generating better HIV-inhibitory peptides (Wang *et al*., 2010).

Table 4.2. Select HIV-1-inhibitory peptides identified from the APD. Adapted from Wang *et al*. (2008, 2010).

Name	Peptide sequence	EC_{50} (μM)[a]	TC_{50} (μM)[a]	TI[a]
AZT[b]	–	0.009	>0.5	>55.6
KR-12	KRIVQRIKDFLR-NH_2	>63.5	>63.5	–
FK-13	FKRIVQRIKDFLR-NH_2	3.4	10.4	3.1
Retro-FK13	RLFDKIRQVIRKF-NH_2	>58.1	33.7	<0.58
GF-17	GFKRIVQRIKDFLRNLV-NH_2	0.98	8.9	9.1
GI-20	GIKEFKRIVQRIKDFLRNLV-NH_2	1.08	22.7	21
BMAP-18	GRFKRFRKKFKKLFKKIS	0.35	8.45	24.1
GLK-19	GLKKLLGKLLKKLGKLLLK	>47.5	25.1	<0.53
GLR-19	GLRRLLGRLLRRLGRLLLR	4.4	25.7	5.8
DRS S9	GLRSKIWLWVLLMIWQESNKFKKM	31.6	>32.9	>1.04
DRS S9r3	GLRSRIWLWVLLMIWQESNRFKRM	1.25	>32.1	>25.7

[a] EC_{50}, the concentration required for 50% inhibition of viral replication; TC_{50}, the concentration required for a 50% reduction in cell viability; TI, therapeutic index.

[b] Azidothymidine (AZT; also known as zidovudine) is a Food and Drug Association-approved anti-HIV drug that is used to prevent HIV transmission from infected pregnant women to their children.

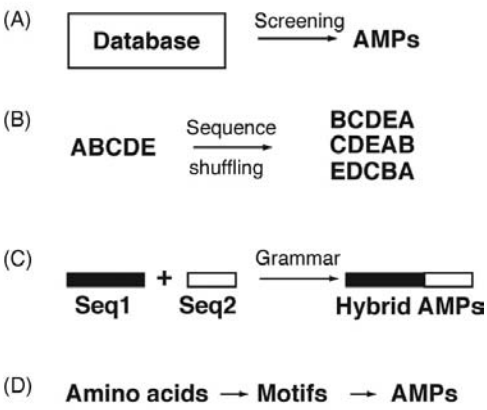

Fig. 4.2. Methods for antimicrobial peptide design based on databases such as the APD. These include (A) database screening, (B) peptide sequence shuffling, (C) grammar-based or modular design and (D) *de novo* design from amino acids to motifs and from motifs to peptides.

A large-scale operation of sequence shuffling is to construct a combinatorial library, where the amino acid residues at all or selected positions can be varied and optimized (Chapter 5). Monroc *et al*. (2006b) demonstrated that the TIs of cyclic peptides against plant pathogenic bacteria were improved using a combinatorial library approach. Recently, advances have also been made in computer-aided screening and identification of short-peptide antibiotics (Cherkasov *et al*., 2009). This was made feasible as a consequence of technical innovations in large-scale peptide synthesis using the SpotArrays, as well as activity evaluation based on luminescence assays. It was necessary to build biased peptide libraries that were rich in residues tryptophan, arginine and lysine and did not contain glutamic acid, aspartic acid, cysteine and proline, because active candidates were absent in 200 randomly synthesized peptides. The ANN approach and quantitative SAR with 44 descriptors were combined and trained using 1400 peptides with measured activities against *Pseudomonas aeruginosa*. Remarkably, the protocol successfully predicted 94% of the most active candidates from the 100,000 peptides *in silico*. It is interesting to note that highly active peptides were tryptophan-rich (discussed in Chapter 9), whereas poorly active candidates contained no or only one tryptophan. This approach nicely illustrates the feasibility of discovering novel AMPs from a computer-generated virtual library.

4.2.3 The hybrid approach and grammar-based peptide design

A classic approach for generating new templates is the hybrid method. In this approach, a new AMP is obtained by combining the parts of amino acid sequences from two different AMPs (Fig. 4.2C). Merrifield *et al*. (1995) synthesized various peptide hybrids based on a cecropin and a melittin to help elucidate the SARs of AMPs. Their studies uncovered the modular nature of AMPs. A large-scale hybrid version is described below.

Loose *et al*. (2006) derived a linguistic model based on the original 525 natural AMPs collected in the APD (Wang and Wang, 2004). In the linguistic model, natural peptide sequences are treated as sentences and the amino acids are regarded as words. From the 525 AMPs, the authors identified 684 regular grammars with the aid of the Teiresias pattern-discovery tool. These 'grammars' are in essence the simple rules that define the AMP 'language'. Each 'grammar' consists of a string of ten amino acids. The authors generated a library of synthetic peptides with 20 amino acids each by combining two grammars (or building blocks) in each case. To find new candidates, the authors selected a subset of peptides that are dissimilar to natural templates. Using this approach they identified D28 and D51, which showed antibacterial activity against both Gram-positive and Gram-negative bacteria. Although it is difficult to predict the success rate of this approach, the 'grammar' approach is likely to generate AMPs that are not found in nature.

4.2.4 *De novo* peptide design

In the reductionist method, few amino acids are utilized in peptide design: two for helical

peptides and five for β-sheet proteins (Villain *et al*., 2000). A prototype helix design only involved lysine and leucine, which represent positively charged and hydrophobic components, respectively. Such peptides are referred to as LK peptides. Among a series of peptides constructed, 14- or 15-residue peptides in the amphipathic helix pattern were found to be most active against bacteria (Blondelle and Houghten, 1992). Shorter peptides are inactive and longer peptides tend to be haemolytic. Wang *et al*. (2009) found that a 12-residue LK peptide was inactive. Kang *et al*. (2008) were able to obtain a highly active LK-peptide with merely 11 residues only after including a tryptophan residue, an excellent membrane anchor (discussed in Chapter 9). Monroc *et al*. (2006a) revealed that the linear form of *de novo*-designed peptides of 4–10 residues displayed no antimicrobial activity. However, cyclization enhanced the hydrophobicity of the peptides and rendered them antibacterial. These results agree with the fact that the shortest helical peptides collected in the APD are of 10–12 residues.

Wang *et al*. (2009) found that glycine, leucine and lysine are the three most frequently occurring residues of AMPs from animals (AniP in Table 4.1), including frogs. This sheds light on the biological significance of the earlier choice of leucine and lysine in peptide design, above. Using these three residues, we designed a GLK peptide in three steps (Fig. 4.2D). In brief, these three residues can form different sequence motifs such as GLK and LGK, which can in turn be combined into multiple peptides. The number of AMPs containing a specific sequence motif is searchable in the APD. For example, 64 peptides in the APD contain the LGK sequence. When highly used motifs are chosen, the likelihood of the peptide being antimicrobial increases. By following the amphipathic pattern, the number of peptides can be further reduced. We found that GLK-19, a 19-residue peptide consisting of only glycine, leucine and lysine (Table 4.2), was more active against *E. coli* K12 than human LL-37 (Wang *et al*., 2009). The inclusion of a low glycine content may improve peptide selectivity, as peptides consisting only of leucine and lysine are known to be cytotoxic to human cells (Braunstein *et al*., 2004). Recently, Juretić *et al*. (2009) arrived at an amino acid selectivity index based on TIs and amino acid occurrences in peptides (Table 4.1). Adepantin-1 showed the highest TI. The seven glycine residues in adepantin-1 (sequence GIGKHVGKALKGLKGLLKGLGES) could be important for peptide selectivity. Interestingly, this peptide is also rich in glycine, leucine and lysine residues (17 out of 23).

In both the 'grammar'-based (Fig. 4.2C) and frequently occurring amino acid-based (Fig. 4.2D) approaches, amphipathic segregation is along the peptide backbone (Fig. 4.3A). Based on the APD, Duval *et al*. (2009) designed a two-segment amphipathic structure (Fig. 4.3B). The peptide was found to be active against both Gram-positive and Gram-negative bacteria, indicating the feasibility of an alternative peptide design as previously demonstrated by Glukhov *et al*. (2008). It is of outstanding interest to note that dermaseptin S9 (DRS S9 in Table 4.2), a natural AMP from the South American hylid frog (Lequin *et al*., 2006), possesses the amphipathic structure depicted in Fig. 4.3B.

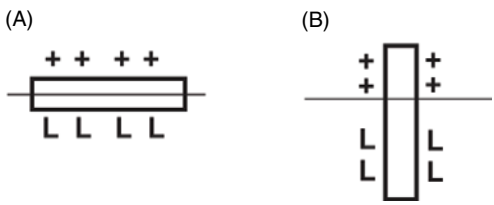

Fig. 4.3. Amphipathic structures can be designed in (A) the classic way or (B) the two-segment mode, where + and L represent hydrophilic and hydrophobic moieties of the design, respectively. The amphipathic nature would be blurred if an end-on view is displayed for model B.

4.2.5 Database-aided enhancement of peptide anti-HIV activity

Figure 4.4A shows the contents of five groups of amino acid residues in antibacterial, antifungal, antiviral and anticancer peptides, and AMPs with toxic effects on mammalian cells. While the average contents of the negatively charged residues DE (3.8–4.9%), the GP group (15–16%) and the polar residues TSYQN (15–17.5%) were similar in different peptide activity groups, the contents of the hydrophobic residues IVLFCMAW (41–50%) and the positive residues HKR (17.5–20.3%) varied by up to 10%. In particular, peptides toxic to mammalian cells possessed the highest hydrophobic content, whereas antifungal peptides had the lowest hydrophobic content. Coincidently, the content of positively charged residues was highest for antifungal peptides, probably complementing the corresponding low hydrophobic content (Fig. 4.4A). A further investigation revealed that the average percentages of lysine and arginine residues in the five groups of peptides differ. On average, antiviral peptides in the APD possessed the highest arginine content (solid columns, Fig. 4.4B). We decided to put this observation to use. When HIV-1 inhibitory activity was evaluated, GLK-19 was found to be inactive. However, the peptide (GLR-19) became HIV inhibitory (Table 4.2) after the lysine residues of GLK-19 were substituted with arginine. Likewise, while frog DRS S9 showed a poor HIV-inhibitory activity, an arginine mutant (DRS S9r3) displayed a high TI of approximately 26 (Table 4.2) (Wang *et al*., 2010). Currently, we are investigating which arginine is most important and the mechanism of inhibition. Because of limited examples, it is too early to state that such mutations are a general strategy for improving the efficacy of HIV-inhibitory peptides. Other factors such as peptide sequence, viral strains and molecular targets could also play a role.

4.3 Strategies for Improving Cell Selectivity of AMPs

Toxic side effects of chemotherapeutic agents on healthy human cells are undesirable and must be minimized. As noted above, AMPs with toxic effects on mammalian cells possess the highest hydrophobic content among the five AMP groups (Fig. 4.4A). This database finding concurs with the conclusion from SAR studies of AMPs (Tossi *et al*., 2000; Chen *et al*., 2005; Li *et al*., 2006a; Mowery *et al*., 2009) and points at the direction for improving peptide selectivity. According to Fig. 4.4A, it is necessary to adjust the hydrophobic content of the peptide to <50% in order to reduce cytotoxicity to human cells. Ahmad *et al*. (2009) showed that substituting large hydrophobic residues (e.g. leucine or isoleucine) with small alanine residues reduced the cytotoxicity of BMAP-28 on mammalian cells. Alternatively, the peptide hydrophobic content can be reduced by deleting a hydrophobic segment from the intact AMP sequence. Skerlavaj *et al*. (1996) found that removal of the hydrophobic tail of BMAP-27 decreased peptide cytotoxicity. By removing both the N- and C-terminal segments from human cathelicidin LL-37, Wang (2008) identified KR-12, the smallest antibacterial fragment, with 12 residues. KR-12 displays low toxicity to human cells (Table 4.2), while its parent peptide LL-37 is highly toxic.

Incorporation of peptoids also improves cell selectivity of the peptide (Song *et al*., 2005; Zhu *et al*., 2007). The side chain of peptoids is shifted from the α-carbon position to its backbone nitrogen position. The absence of the amide proton in the peptoid disrupts hydrogen bonding, leading to a less helical structure. Therefore, peptoid-containing peptides have a reduced affinity for membranes of zwitterionic lipids. Song *et al*. (2005) found that incorporation of Nala (alanine peptoid) onto the hydrophobic surface of the peptide is more effective than incorporation onto the hydrophilic face in improving cell selectivity of the peptide. For a model helical peptide KLW, incorporation of two Nala residues at positions 9 and 13 improved peptide selectivity. Zhu *et al*. (2007) utilized a different approach by replacing proline residues in tritrpticin and indolicidin with Nlys (lysine peptoid). Both peptides became more selective after engineering.

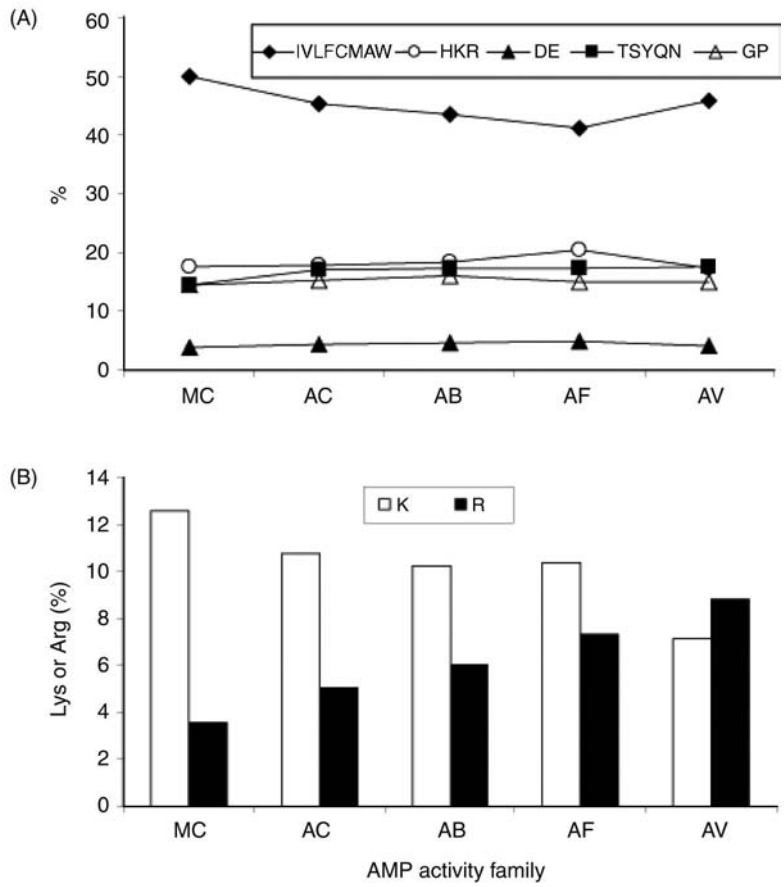

Fig. 4.4. (A) Average contents of a group of amino acids in AMPs with different activities. Hydrophobic residues include isoleucine, valine, leucine, phenylalanine, alanine, methionine, cysteine and tryptophan; positive residues are histidine, lysine and arginine; negative residues are glutamic acid and aspartic acid; polar residues comprise serine, threonine, asparagine, glutamine and tyrosine; and the GP group contains glycine and proline residues. (B) Average content of lysine (K) and arginine (R) in the five groups of AMPs. AB, antibacterial; AC, anticancer; AF, antifungal; AV, antiviral; MC, toxic to mammalian cells.

Peptide hydrophobicity can also be scaled down by partial incorporation of D-amino acids (Sharon *et al*., 1999; Chen *et al*., 2005). By combining D-amino acid incorporation with disulfide-bond linked cyclization, Oren and Shai (2000) found that the engineered LK peptide (i.e. leucine- and lysine-based peptide) adopted a helical structure with improved cell selectivity. Li *et al*. (2006a) identified a major antimicrobial region corresponding to residues 17–32 of human LL-37 (GF-17) (Table 4.2). When D-amino acids were introduced at residues 20, 24 and 28 of GF-17 (numbered as in LL-37), we obtained a peptide that retained toxicity to *E. coli* K12, but displayed no toxic effect to human cells. The structural basis for peptide selectivity improvement by D-amino acid incorporation is described in Chapter 9. Li *et al*. (2006a) proposed that a decrease in hydrophobicity is a unified approach to improving cell selectivity of membrane-targeting AMPs.

4.4 Strategies for Improving Peptide Stability to Proteases

Another hurdle in developing AMP-based therapeutics is the short lifetime of these peptides *in vivo*, probably due to degradation by endogenous proteases from either bacteria or testing animals. The diverse structural scaffolds of natural AMPs collected in the APD are remarkable and inspiring (see the face page of the APD). In general, polypeptides with a folded structure are more resistant to protease cleavage. For example, disruption of disulfide bonds or the key salt bridge of human α-defensin 5 (HD-5) leads to rapid degradation (Tanabe *et al*., 2007; Rajabi *et al*., 2008). In addition, N-terminal acetylation, C-terminal amidation, introduction of D-amino acids or non-standard amino acids and peptide cyclization are all known post-translational modification strategies in natural peptides (Table 1.5, Chapter 1). These strategies can be applied to stability enhancement of AMPs (see below) as well as other natural peptides (Craik and Adams, 2008).

In analogy to circular AMPs in nature, investigators have designed different chemical strategies to connect the ends of the peptides to achieve higher stability. Dathe *et al*. (2004) achieved cyclization of an arginine- and tryptophan-rich peptide RRWWRF-NH_2 by HAPyU chemistry (Ehrlich *et al*., 1996). They found that the cyclized peptide had higher antimicrobial activity and cell selectivity than its linear counterpart. Another group produced cyclization by disulfide bond formation. While the cyclic melittin analogue displayed increased antibacterial activity but decreased haemolytic activity, cyclic magainin 2 had a marked decrease in both antibacterial and haemolytic activity (Unger *et al*., 2001). Therefore, the effect of cyclization on the properties of linear peptides is peptide dependent.

Fluorine is incorporated into 20% of pharmaceuticals on the market (Muller *et al*., 2007). Fluorinated amino acids have been introduced into AMPs to improve protease stability without disrupting the original peptide structure (Niemz and Tirrell, 2001; Meng and Kumar, 2007). An increase in size or hydrophobicity due to fluorination has been proposed as the basis for enhanced peptide stability (Lee *et al*., 2004; Gottler *et al*., 2008).

Finally, incorporation of D-amino acids provides another useful approach for improving peptide stability. One of the early demonstrations was the synthesis of all-D-peptide analogues by Merrifield *et al* (1995). In addition, partial incorporation of D-amino acids may improve peptide stability. Using helical peptides as models, Shai and colleagues introduced D-amino acids at every two to three residues to improve peptide selectivity (Pouny and Shai, 1992; Sharon *et al*., 1999). Similarly, Hong *et al*. (1999) found that incorporation of D-amino acids at the terminal regions did not disrupt the antibacterial activity of the peptide, but improved peptide stability in serum. Significantly, Braunstein *et al*. (2004) demonstrated that injection of a D,L-peptide into mice cured animals infected with bacteria. Of note, promising results have also been obtained using peptide mimics (see Chapter 6).

4.5 Concluding Remarks

It is evident that the creation of the APD with a clean and well-registered dataset has facilitated database-based peptide prediction. The classification of AMPs into a variety of groups in the database (Wang *et al*., 2009) constitutes a useful step and was used to improve the accuracy of antibacterial peptide prediction (Lata *et al*., 2010). By grouping AMPs into more than 140 clusters, AMPer (Fjell *et al*., 2007) has achieved a relatively high accuracy in AMP prediction. In addition, the APD provides a set of useful templates for peptide design. The choice of a starting peptide template is not always trivial. When feasible, database screening, and preferably virtual screening *in silico* followed by activity evaluation, is a useful approach. Sequence shuffling, including sequence reversal, generates a miniature combinatorial library of peptides. Combining peptide building blocks (i.e. the grammar-based approach) is an amplified version of the traditional hybrid approach. In addition, building novel

peptides using a minimal number of amino acids, such as leucine and lysine, is the classic *de novo* approach. Coincidentally, these residues overlap with part of the frequently occurring residues identified from the database analysis of hundreds of amphibian AMPs, thereby endowing biological significance to these residues utilized for *de novo* peptide design. The desired properties of AMPs can also be improved based on three-dimensional structures (Chapter 9). Continuing peptide design work not only deepens our knowledge of AMPs but also enriches the peptide space, biologically and chemically. For future therapeutic use of AMPs, it is important to achieve cell selectivity and *in vivo* stability, and to reduce production costs. It is noteworthy that T-20, an HIV fusion inhibitor, has been produced in multi-tons by a combination of solid-phase and solution-phase methodologies (Fuzeon; Roche), indicating that chemical synthesis offers a practical approach for large-scale peptide production. In addition, peptides can be produced by bacterial expression (see Chapter 9). With the discovery of novel AMPs with interesting polypeptide scaffolds as well as the development of new tools by exploiting natural post-translational enzyme modification systems (Chapters 2–3), we have reason to believe that the peptide production issue can be resolved in the future. All of these are promising advances in overcoming hurdles on the way to the development of AMP-based therapies.

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5 Discovery of Novel Antimicrobial Peptides Using Combinatorial Chemistry and High-throughput Screening

William C. Wimley

Abstract

The field of antimicrobial peptide (AMP) research has now spanned three decades, in which hundreds of AMPs have been discovered, designed or engineered. Yet despite a vast literature, obvious structure–function relationships are rare and this has created a bottleneck in the discovery of novel AMPs. Instead of rigorous structure–function principles, AMP activity may be best addressed using the physical chemistry concept of 'interfacial activity', a concept that does not help one predict or engineer AMP activity. In this chapter, I address one method of circumventing this engineering bottleneck: combinatorial chemistry and high-throughput screening. Combinatorial methods are first discussed from the perspective of library synthesis techniques, for both indexed and non-indexed methods. This is followed by a discussion of available high-throughput screening techniques. Finally, I address the accomplishments to date generated using combinatorial chemistry and high-throughput screening and future directions the field may take. Combinatorial chemistry and high-throughput screening are powerful and effective tools for discovering novel AMPs. The future of this field holds great promise.

5.1 The Interfacial Activity Model of AMP Activity

Antimicrobial peptides (AMPs) exert their biological activity by first acting on microbial membranes (Steiner *et al*., 1981; White *et al*., 1995), sometimes followed by additional effects on the microbial cell. Yet, despite decades of intense study, compelling structure–function relationships for AMPs are rarely found and evidence for stable, well-defined transmembrane pores (Fig. 5.1) is rarely observed. Recent literature suggests that physical impact on membranes, such as lipid domain formation (Chapter 7), is not dependent on specific amino acid sequences or on specific three-dimensional peptide structures (Hilpert *et al*., 2005, 2006; Jin *et al*., 2005; Mowery *et al*., 2007; Rausch *et al*., 2007). Instead it depends on 'interfacial activity', which has been referred to as 'the ability of a molecule to bind to a membrane, partition into the membrane–water interface and to alter the packing and organization of the lipids' (Rathinakumar and Wimley, 2008; Rathinakumar *et al*., 2009). Interfacial activity (Fig. 5.1) is derived from the appropriate balance of interactions between and among peptides, water and membrane lipids. These interactions depend more on the amino acid composition of a peptide and on its physical chemical properties than on its exact sequence or secondary/tertiary structure (Rathinakumar and Wimley, 2008; Rathinakumar *et al*., 2009; Chapter 7). In support of this idea, Hilpert *et al*. (2006) have shown that a high

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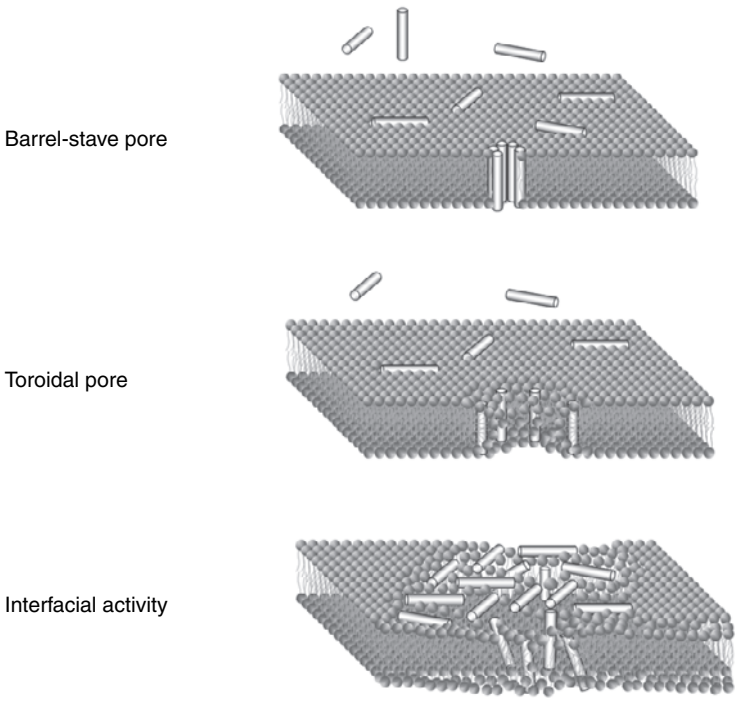

Fig. 5.1. Some schematic models of antimicrobial peptide activity. The literature contains numerous mechanistic models to explain the action of antimicrobial peptides on lipid bilayers. The barrel-stave and toroidal pore models, while commonly referenced, do not explain the experimentally observed actions of most antimicrobial peptides. The interfacial activity model (Rathinakumar and Wimley, 2008) can explain the actions of most AMPs. Most importantly, the interfacial activity model can be used as a basis for designing compositionally varied combinatorial peptide libraries.

percentage of random versions of a potent AMP retain good activity and some even have improved activity.

Although AMPs are known to adopt a variety of three-dimensional structures in lipid bilayers, the rational design of AMPs based on structure-specific ideas is rare (Chapter 9). However, design based on the principle of interfacial activity is not yet possible because the physical chemical basis of interfacial activity has not been parameterized. In light of these considerations, the use of combinatorial chemistry and high-throughput screening is an especially attractive approach to discovering novel AMPs. To design a library, one can use the principle of interfacial activity by requiring libraries to contain peptides that adhere to common attributes of known AMPs (e.g. peptide length, amino acid composition, charge, hydrophobicity; see Chapter 1). This would allow the design of rational libraries from which the most active sequences can readily be determined by high-throughput screening. In this chapter, I explore the design, selection and screening of combinatorial peptide libraries toward the efficient discovery of novel AMPs.

5.2 Combinatorial Chemistry Methods

5.2.1 Overview of library synthesis

Almost as soon as the Merrifield method of solid-phase peptide synthesis became widely known, combinatorial peptide synthesis was envisioned as a natural extension of the chemistry. The power of combinatorial peptide synthesis is derived, in part, from

the fact that the chemistry required to make a library is no more complex than to perform a non-combinatorial synthesis. No novel reaction protocols, equipment or protection strategies are needed. Combinatorial peptide chemistry is thus directly accessible to researchers with a moderate chemistry knowledge. In this section, I describe some of the more commonly used synthetic approaches to combinatorial peptide library synthesis.

5.2.2 Non-indexed methods

In any high-throughput screen, one must be able to select for active compounds and then identify the active molecule or molecules exactly. Combinatorial peptide libraries can be separated into two broad categories: (i) *indexed* libraries, discussed in detail below, which use spatial or chemical indexing that allows active sequences to be known during the course of the screen; and (ii) *non-indexed* libraries, in which indirect analytical methods, such as Edman sequencing, mass spectrometry or iterative deconvolution, must be used to identify active sequences after they have been selected in the screen. Here, I discuss some commonly used non-indexed combinatorial library methods.

Houghten and colleagues were some of the earliest users of combinatorial chemistry and high-throughput screening to identify novel AMPs (Blondelle *et al*., 1996). They used several simple, partially indexed library design methods in which the peptides screened initially were random mixtures of peptides containing fixed residues at some positions and random mixtures of amino acids at others. The relative abundance of each amino acid in the varied position was controlled by adjusting amino acid concentrations according to their relative reaction rates. Identification of active peptides was accomplished by positional scanning or iterative deconvolution. When necessary, mixtures with activity were further subdivided by iterative deconvolution based on rounds of additional synthesis and testing until single active sequences were obtained. While this approach requires a large amount of peptide synthesis for deconvolution, and risks the possibility that peptides in the mixture will act synergistically or antagonistically, it is simple and effective, as shown by Houghton and colleagues, and can successfully lead to the discovery of novel potent AMPs.

Perhaps an even more powerful approach to non-indexed combinatorial peptide libraries is the one-bead:one-sequence method, also known as split and recombine libraries. In a one-bead:one-sequence library, a researcher takes advantage of the discrete nature of solid-phase synthesis resins, which are typically in the form of polymer microbeads, often made from polystyrene and sometimes with grafted polyethylene glycol. A synthesis slurry of resin beads can be combined into a single vessel when common chemistry is performed; it can be split into separate vessels for the addition of separate amino acids when a combinatorial site is reached. Recombination of the resin beads into one vessel allows for randomization before the next split. Each bead thus has a unique history of amino acid additions, and contains only peptides of a single sequence. Spatial separation of the beads is required for screening. Typical one-bead:one-sequence libraries have 50,000–1,000,000 beads g^{-1} of resin or 0.2–2.5 nmol of peptide per bead. Table 5.1 shows the statistics for some commonly used library beads. One-bead:one-sequence libraries are spatially segregated but are usually not indexed. Screening is performed on individual beads and the positive sequences are identified *post facto* using chemical sequencing, mass spectrometry or deconvolution.

AMP discovery requires that peptides tested are free in solution, a fact that places limitations on the types of combinatorial libraries that can be used. For example, classical phage display or selectide-type peptide libraries (Lam *et al*., 2003), designed to identify peptide sequences that bind to particular targets, must be modified to allow peptides to be released. For one-bead:one-sequence libraries, an orthogonal approach to synthesis and release must be employed. Typically, researchers use a photolabile or

Table 5.1. Some available synthesis microbeads for one-bead:one-sequence libraries.[a]

TentaGel polystyrene with grafted polyethylene glycol	Median diameter (μm)	Typical loading (mmol g^{-1})	Bead density (beads g^{-1})	Peptide per bead (nmol per bead)	Conc. (μM) (measured in 100 μl)
	90	0.2	1×10^6	0.2	1
	300	0.2	8×10^4	2.5	10

[a] TentaGel beads (Rapp-Polymere) are commonly used for one-bead:one-sequence libraries. Library size, which is inversely proportional to the amount of peptide per bead, ranges from 10^4 to 10^6 beads g^{-1}, an easy bench-top synthesis scale. In my experience, photolabile linkers allow for release of only a portion of the peptide on the bead; the last column reflects the actual measured release of peptide into 100 μl of buffer.

other non-acid-labile linker that can be cleaved independently from peptide chemistry and side-chain deprotection, which is generally acid dependent. In my work, I have used a UV-cleavable photolabile linker that allows for the peptide-bead linker to be cleaved when the bead is dry, followed by physical segregation of the beads, extraction of peptide into buffer and screening.

5.2.3 Indexed methods

When the individual members of a library can be identified directly, either by their spatial location or by a coded 'tag' associated with them, the library is considered an 'indexed' library. Indexing combinatorial libraries is a powerful method, which has the advantage of not requiring tedious deconvolution or expensive chemical sequencing. An example of an indexed combinatorial library is the SPOT synthesis method (Frank, 2002) and its variants, in which synthesis is done on spatially indexed spots on a cellulose sheet, followed by cutting of the 'spots' and cleavage into individual samples. SPOT synthesis can be performed with technology as simple as a pipetting robot, as described by Hancock and colleagues (Hilpert *et al*., 2005). Similarly, multi-pin libraries (Maeji *et al*., 1990) are carried out using multiple pins as solid supports with multiple reaction chambers controlling the chemistry. The size of SPOT libraries and pin libraries depends on a laboratory's investment into the synthetic technology, but libraries with thousands to hundreds of thousands of members are readily achievable.

5.3 High-throughput Screening

In the research and development department of a large commercial pharmaceutical enterprise, 'high-throughput screening' means testing millions of compounds rapidly in massive parallel, automated, robotic applications. On the other hand, in a small academic laboratory, it may mean screening only a few thousand peptides by mostly manual techniques. In either case, high-throughput screening can be defined by the common characteristic of performing selection experiments on molecular libraries at a pace that is orders of magnitude faster than achievable using the traditional approach of single sequence design, synthesis, purification and characterization (Fig. 5.2). As discussed above, combinatorial peptide library synthesis is not, in itself, technically challenging. The real challenge lies in the design of the high-throughput screen needed to efficiently select the most active members from a library. In the following sections, I explore the various approaches that can be used to select for AMP sequences in peptide libraries.

5.3.1 Biological assays

The most direct selection method for antimicrobial activity is to screen libraries using living microbes. Broth dilution assays (Wiegand *et al*., 2008) test for the ability of a peptide to inhibit the growth of microbes in a rich nutrient broth. Generally a peptide and a microbe inoculum are incubated overnight in media. Given the long incubation and short doubling time for most

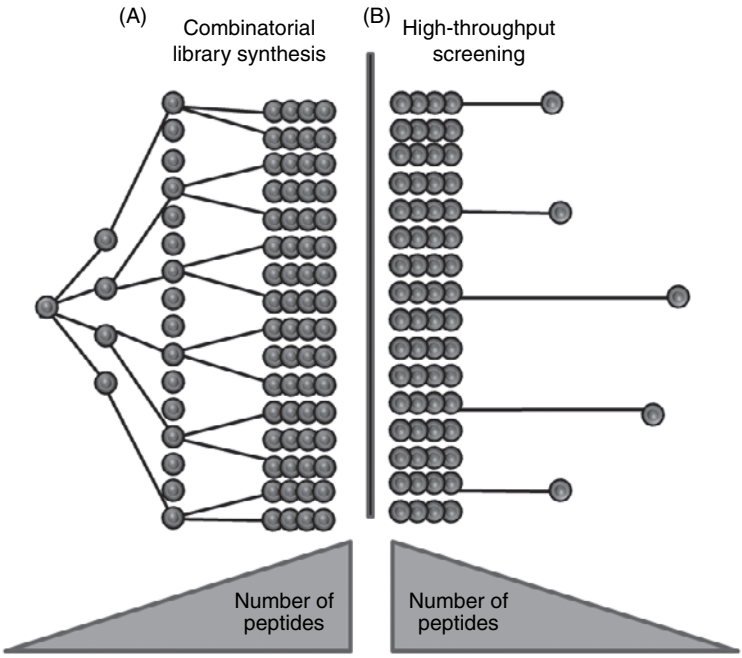

Fig. 5.2. Combinatorial library synthesis and high-throughput screening. Library design and the high-throughput screening must be balanced. (A) Library size increases very rapidly with its complexity. The larger the library, the greater the amount of sequence space that can be explored. (B) High-throughput screening must be able to identify a small number of highly active library members in a reasonable amount of time and effort. Screens with higher throughput and better discrimination allow a researcher to design more complex libraries.

microbes, expansion of the inoculum to an opaque, stationary phase will occur unless completely inhibited by the peptide. In practice, intermediate results are usually not observed (Rausch *et al*., 2007; Rathinakumar and Wimley, 2008). Activity can be detected photometrically, but even visual inspection will suffice in this experiment because of the binary nature of the result. Broth dilution screening is readily performed in a multiwell plate format and requires a minimum of automation, robotics or specialized detection methods. Figure 5.3 shows an example of a broth dilution screen in a 96-well plate format. For more rapid screening, increased sensitivity and automated throughput, one can couple a broth dilution assay with a secondary detection method using real-time growth curves or by using engineered enzyme activity (Hilpert *et al*., 2005).

A second classical antimicrobial activity experiment that can be adapted to high throughput is the agar diffusion assay (Wiegand *et al*., 2008), in which peptides are spotted on a thin layer of nutrient agar seeded with bacteria. Peptides will diffuse into the agar and active peptides will create a clearance zone where they inhibit bacterial growth. The clearance area is proportional to the potency of the peptide. Examples of agar diffusion experiments are shown in Fig. 5.4. An advantage of agar diffusion is that the results are semi-quantitative. The most potent members of a library can readily be identified from the diameter of the spots. In one-bead:one-sequence libraries, another possible advantage of agar diffusion is that peptides do not need to be extracted from the beads for the assay as they do in a broth dilution assay. As shown in Fig. 5.4, scattering beads

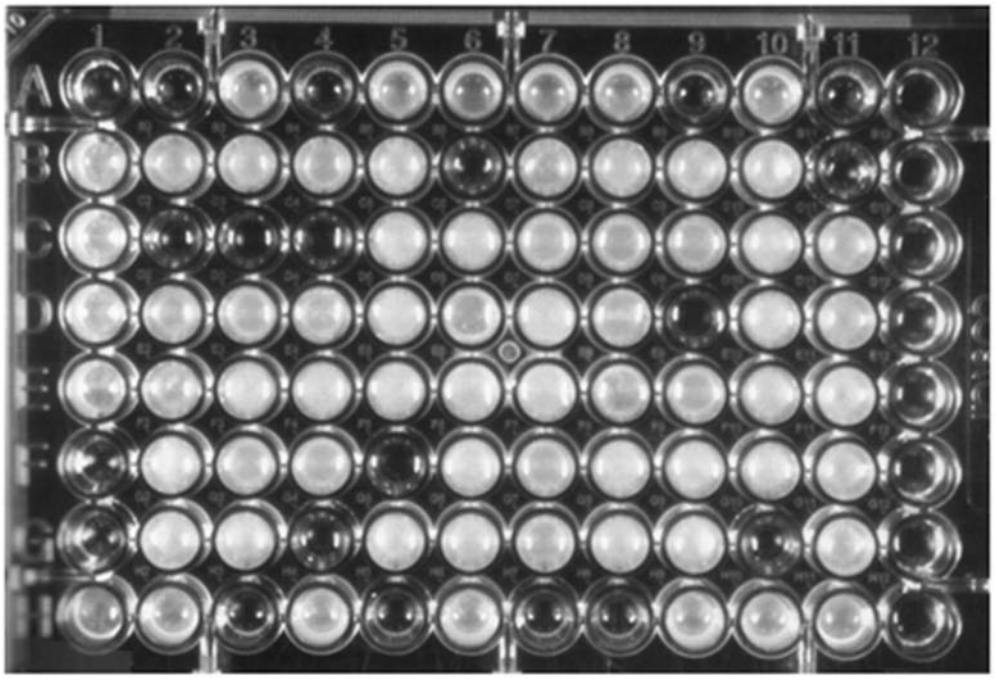

Fig. 5.3. Broth dilution assay as a high-throughput screen. In this example of a 96-well screen, each well contains a 2.2 μM concentration of one member of a combinatorial peptide library, rich growth medium and an inoculum of 10^3 *Escherichia coli* bacteria. After overnight incubation the wells are either opaque, indicating stationary-phase growth, or they are transparent, indicating no growth (i.e. the bacteria have been sterilized by the peptide). This is a very stringent high-throughput screen, which requires no sophisticated equipment to perform. Wells C11–H11 have no added peptides and wells C12–H12 have no added bacteria.

with dry-cleaved photolabile linker on to the agar layer is sufficient to obtain good antimicrobial activity because many of the peptides will spontaneously diffuse from the beads into the agar and inhibit growth. In my experience, not all peptides diffuse from the beads into the agar at a useful rate, but this may actually be advantageous because the slowly diffusing peptides are the least soluble, and therefore also the least desirable AMPs.

Recently, an intriguing 'whole animal' high-throughput antimicrobial screen was described (Moy *et al*., 2009). In this screen, *Candida elegans* nematodes in small nutrient chambers were used to test compounds simultaneously for antimicrobial activity and toxicity towards the nematode.

5.3.2 Selection of broad-spectrum peptide antibiotics

Identifying peptides with potent antimicrobial activity against one species of microbe is surprisingly easy. For example, I and others have designed biased libraries in which more than a quarter of all members have potent antimicrobial activity (Rausch *et al*., 2007; Rathinakumar and Wimley, 2008; Rathinakumar *et al*., 2009). A large proportion of random sequence variants of known AMPs have also been shown to have good activity (Hilpert *et al*., 2006) as well as many engineered variants of natural antimicrobial activity. These results arise from biased libraries and biased sequences, which are rich in aromatics and hydrophobic and

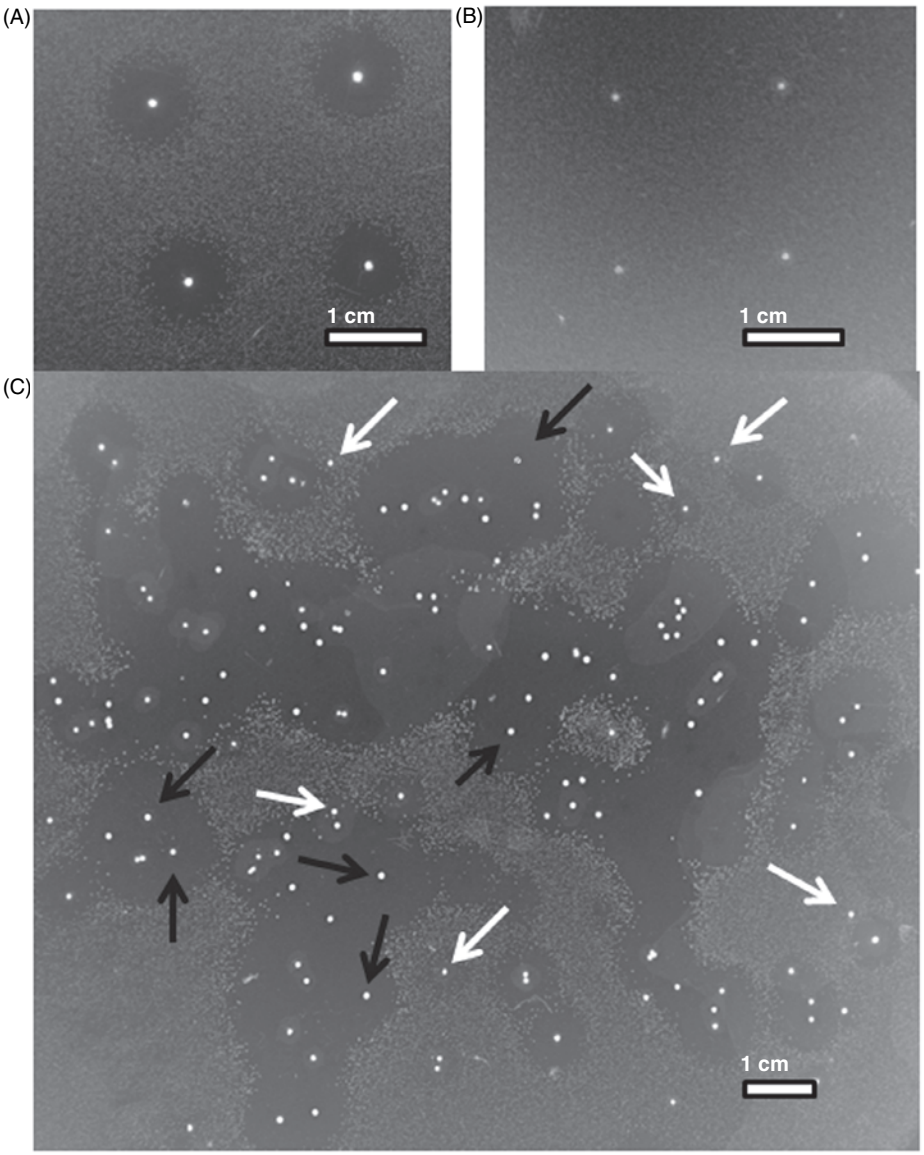

Fig. 5.4. Agar diffusion assay for high-throughput screening of one-bead:one-sequence libraries. (A,B) For controls, the bovine AMP indolicidin was linked to synthesized resin microbeads using a photolabile linker. Indolicidin beads were placed on a thin layer of nutrient agar seeded with *Escherichia coli* bacteria. (A) The photolabile linker is cleaved with UV light and the released peptides create a large clearance zone where no bacteria grow. (B) The linker is not cleaved and no clearance zone is created. (C) Beads from a combinatorial peptide library described elsewhere (Rathinakumar and Wimley, 2008) are scattered on to an agar diffusion plate. This is the same library screened by broth dilution in Fig. 5.3. Some beads (black arrows) create large zones of clearance, while others (white arrows) are less active.

cationic residues and, as a result, contain many antimicrobial members.

Broad-spectrum antimicrobial activity, defined as activity against multiple classes of microbes, is desirable, but is rarer and more difficult to select than species-specific activity. Selecting directly for broad-spectrum activity using biological screens can be achieved if a single library member is screened in parallel against microbes from multiple classes. This approach requires a complex experiment that reduces the throughput of a screen. However, it has the advantage over other screens that broad-spectrum peptides are identified directly and unambiguously. To test the effectiveness of this approach, I have performed parallel multispecies screening using a sample of a one-bead:one-sequence library described in the literature (Rathinakumar and Wimley, 2008; Rathinakumar *et al*., 2009). We screened in parallel for activity against *Escherichia coli* (a Gram-negative bacterium), *Staphylococcus aureus* (a Gram-positive bacterium) and *Cryptococcus neoformans* (a fungus). We found species-specific antimicrobial activity to be surprisingly common, while overlapping (i.e. broad-spectrum) activity was quite rare. Tables 5.2 and 5.3 give information on these experiments.

Biological assays have been used successfully to select AMPs from combinatorial libraries. However, the single-species nature of most experiments and the difficulty in performing multispecies experiments with high throughput are problems that have not yet been overcome. The next section describes lipid-vesicle-based high-throughput screens, which are simpler to perform and have been shown to select specifically for peptides with broad-spectrum activity and low toxicity.

5.3.3 Non-biological assays

Countless studies in the literature show that AMPs interact with and perturb lipid bilayer membranes *in vitro* as well as *in vivo*. A typical *in vitro* experiment is performed using unilamellar lipid vesicles with an entrapped marker for assaying membrane permeability (Rausch and Wimley, 2001). Experimental lipid compositions vary widely between laboratories, but mixtures of anionic and zwitterionic lipids are often used to

Table 5.2. Abundance of broad-spectrum AMPs in a combinatorial library.[a]

	Number of peptides	Percentage
Total peptides screened	1040	100
Active peptides	175	16.8
Combination of microbes sterilized		
Only *Escherichia coli*	82	7.9
At least *E. coli*	115	11.1
Only *Staphylococcus aureus*	54	5.2
At least *S. aureus*	79	7.6
Only *Cryptococcus neoformans*	3	0.3
At least *C. neoformans*	12	1.2
Only *E. coli* and *S. aureus*	27	2.6
Only *E. coli* and *C. neoformans*	2	0.2
Only *S. aureus* and *C. neoformans*	3	0.3
All three organisms	4	0.4

[a] Spectrum of antimicrobial activity in a one-bead:one-sequence combinatorial peptide library described elsewhere (Rathinakumar and Wimley, 2008; Rathinakumar *et al.*, 2009). Individual peptides were extracted from beads and the solution was divided into three 96-well plates, where the peptide concentration in each well was about 2 μM. Wells were seeded with *E. coli* (a Gram-negative bacterium), *S. aureus* (a Gram-positive bacterium) or *C. neoformans* (a fungus). Sterilization assays showed that there were many highly active peptides in the library (17%), but that only a small fraction (0.4%) had potent broad-spectrum activity against all three classes of microorganism.

Table 5.3. Example data table for AMPs. Data were selected from a 16,384-member combinatorial library using high-throughput screening (Rathinakumar and Wimley, 2008; Rathinakumar *et al.*, 2009). To assess the results of a selection process, one must have data for many aspects of biological activity, as shown here. Experiments are described in detail in recent publications (Rausch *et al.*, 2005, 2007; Rathinakumar and Wimley, 2008; Rathinakumar *et al.*, 2009).

	Minimum sterilizing concentration (μM)				Haemolysis (%)		Cytotoxicity (%)	Leakage (%)				
	Escherichia coli	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Cryptococcus neoformans*	Human RBC	Sheep RBC	HEK293 cells	Liposome	*E. coli*	*S. aureus*	*C. neoformans*	HEK293 cells
Peptide[a]												
RRGWVLDLVLYYGRR	1.9	2.9	2.4	9.0	25	7	15	72	61	22	3	1
RRGWVLALVLYYGRR	4.9	1.8	2.4	8.5	41	4	6	67	6	65	1	7
RRGWVLALVLRYGRR	1.9	1.4	2.1	3.6	28	8	8	65	91	52	12	6
RRGWVLALYLRYGRR	2.1	1.5	1.6	4.1	23	6	2	93	94	47	16	2
RRGWVLRLALYAY	1.5	3.3	1.3	2.7	11	6	2	25	89	71	33	3
RRGWALRLVLAY	1.9	2.9	2.4	2.6	18	6	3	39	80	76	32	5
RRGWRLVLALVY	1.4	2.4	1.7	5.4	28	5	2	69	100	72	1	7
WYLTLTLGYGRR	3.1	2.8	2.9	6.2	43	6	1	79	17	84	10	14
WALRLVLYY	3.8	2.8	1.9	6.1	12	4	3	50	24	97	9	4
WVLVLRLGY	1.7	3.6	2.9	10.6	26	8	9	55	16	89	4	3
Control compounds												
RRGWALRLVLAY (D-Leu$_{10}$)[b]	9.7	8.8	>10	6.5	4	10	4	1	10	ND	0	1
RRGWALRLVLAY (All D)[c]	1.3	2.8	2.0	6.5	12	1	ND	4	93	24	2	1
Indolicidin[d]	1.2	2.9	5.3	4.7	14	19	2	24	14	23	7	4
Melittin[e]	1.5	1.3	0.9	1.1	100	93	93	98	100	100	100	100
PMSD[f]	>15	>15	>15	>15	2	4	4	ND	0	3	3	ND
Ampicillin	0.4	0.1	4.4	>15	ND	ND	ND	ND	0	0	0	ND
Triton-X	ND	ND	ND	ND	100	100	100	100	ND	ND	ND	ND

[a] All novel peptides from the library are nine, 12 or 15 residues in length. The library has the form {RRG}WOLOLOLOY{RRG}-amide, where the {RRG} terminal basic cassettes are randomly present or absent and the O residues can be one of the following amino acids: NDTRGAVY. The W, L and C-terminal Y residues are fixed.

[b] The peptide RRGWALRLVLAY, in which the C-terminal-most L-leucine is replaced with a D-leucine.

[c] The peptide RRGWALRLVLAY, in which the entire sequence is composed of D-amino acids.

[d] The bovine neutrophil AMP indolicidin has the sequence ILPWKWPWWPWRR-amide.

[e] The honeybee venom peptide melittin is a 22-residue, α-helical pore-forming peptide that potently permeabilizes all lipid bilayer membranes, synthetic or biological. In all assays, 5 μM melittin had the same permeabilizing effect as membrane dissolution with detergents (e.g. Triton X-100).

[f] PMSD is a sequence that contributes to a membrane-permeabilizing β-barrel in the context of the whole multimeric perfringolysin O protein toxin. However, the membrane-spanning sequence alone is inactive and is used for a control peptide.

Abbreviations: HEK293, human embryonic kidney cell line 293; PMSD, perfringolysin membrane-spanning domain; ND, no data; RBC, red blood cell.

mimic microbial membranes, while phosphatidylcholine and/or phosphatidylcholine/cholesterol are used to mimic mammalian membranes. Because cationic/hydrophobic AMPs with good interfacial activity are expected to perturb any lipid bilayer membrane to which they bind well enough, the exact anionic lipid content is probably not a crucial factor in vesicle-based characterization of AMPs.

We have developed a vesicle-based high-throughput screen to select for potent vesicle-permeabilizing peptides from combinatorial libraries (Rausch and Wimley, 2001; Rausch *et al*., 2005; Rathinakumar and Wimley, 2008; Rathinakumar *et al*., 2009). The high-throughput screen utilizes large unilamellar vesicles with the lanthanide metal terbium (III) (Tb^{3+}) trapped inside and the aromatic chelator dipicolinic acid (DPA) added to the external solution. The Tb^{3+}–DPA complex, which forms only when the membranes have been permeabilized, is highly fluorescent and can be quantitated. Assays are performed in the 96-well plate format and fluorescence is used to rate permeabilization. Figure 5.5 shows high-throughput screening using this method. By adjusting the concentration of lipid vesicles added to each well, the stringency of the assay can be adjusted to suit the library being studied. Figure 5.6 shows permeabilization data for a combinatorial library screened by this method.

When performing high-throughput screens for AMPs, it is important to also select *against* peptides that have poor solubility. In the vesicle-based screen described above, we also used a 'premixing' step in which library

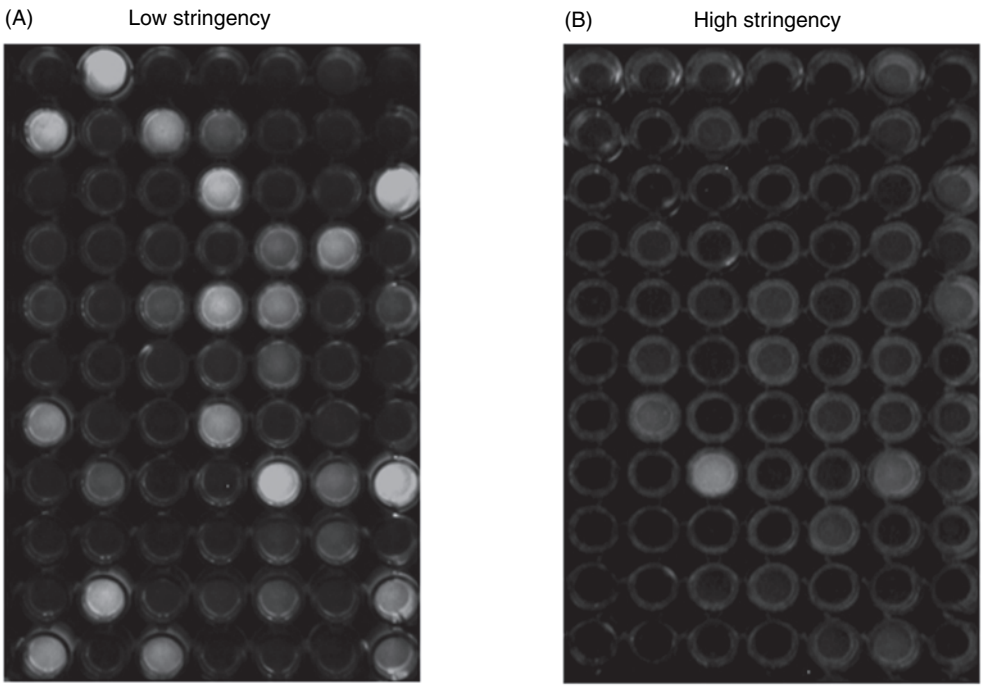

Fig. 5.5. A vesicle-based high-throughput screen for bilayer-permeabilizing peptides. Large unilamellar vesicles containing entrapped terbium (III) (Tb^{3+}) and external dipicolinic acid (DPA) are added to each well, which also contains about 10 μM of one peptide from a combinatorial library. Permeabilization of the vesicles results in Tb^{3+}–DPA complex formation, which is highly fluorescent. These plates were photographed under UV light. (A) A screen with a lower lipid concentration gives high sensitivity, but lower stringency. (B) A screen conducted with a higher lipid concentration used for lower sensitivity and higher stringency screening. In practice, stringency is altered to provide the desired number of positive peptides (Rausch *et al*., 2005).

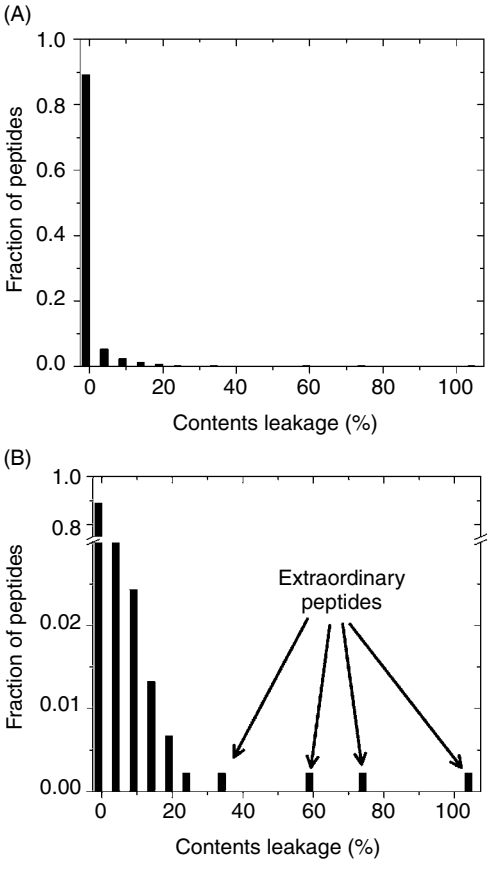

Fig. 5.6. Example statistics for a high-throughput screen using a one-bead:one-sequence library. Several thousand library members were screened using a high-stringency, vesicle-based screen as shown in Fig. 5.5. (A) The majority of sequences have little or no membrane-permeabilizing activity. (B) An altered *y*-axis shows that a few single peptides have exceptional activity. These exceptional peptides have potent, broad-spectrum antimicrobial activity.

peptides were added to buffer and incubated for several hours before the addition of the vesicle test solution. Insoluble peptides will precipitate and will be inactive. By using these two orthogonal screening steps, we select only for peptides that are soluble and have good activity. In one library tested, we found that two-thirds of all vesicle-active peptides are also *insoluble*. The remaining active peptides were highly soluble in water and thus amenable to experimentation and perhaps development into therapeutics.

5.4 Accomplishments

Combinatorial methods have successfully led to the discovery of novel peptides with potent antimicrobial activity. For example, Houghten and colleagues found novel AMPs using a synthesis-intensive iterative deconvolution method (Blondelle *et al*., 1996) by screening against individual organisms, including fungi. Peptides as short as six residues, with a classical composition of basic and aromatic amino acids, were found, some with potent antifungal activity, others with broad-spectrum antimicrobial activity. Similarly, Hancock and colleagues used indexed synthesis on cellulose sheets to explore the activity of a peptide library designed around the sequence of the AMP Bac2A (Hilpert *et al*., 2005). They found specific sequences with improved broad-spectrum activity over the parent sequence.

In recent years, we have used one-bead:one-sequence libraries and orthogonal high-throughput lipid vesicle-based screens to select soluble, pore-forming peptides from libraries designed to explore narrow regions of sequence space. In these experiments, peptides were selected based on their ability to permeabilize lipid vesicles composed of 90% zwitterionic phosphatidylcholine and 10% anionic phosphatidylglycerol at peptide:lipid ratios of 1:50 to 1:200. Using libraries of 10,000–16,000 members, about 0.1% of each library had potent membrane permeabilizing activity and was also water soluble. Importantly, we showed that each of the sequences selected in the vesicle-based screen had potent broad-spectrum activity, being able to sterilize Gram-positive and Gram-negative bacteria as well as fungi at peptides concentrations of <10 μM. At the same time, active peptides showed only moderate haemolytic or cytotoxic effects on mammalian cells.

5.4.1 Beyond high-throughput screening

Identification of potential AMPs using high-throughput screening is only the first step in the pipeline towards therapeutic AMPs. First, selected peptides must be tested for broad-

spectrum activity against multiple classes of microbes. Peptides must then be tested for their lytic or toxic effects on mammalian cells. Experimentally, these latter effects are quantitated by measuring lysis of mammalian red blood cells, lysis of mammalian nucleated cells or cytotoxicity against living mammalian cells. To exemplify these critical experiments, a complete example dataset is shown in Table 5.3. This gives the characteristics of peptides selected from the combinatorial library, described elsewhere (Rathinakumar and Wimley, 2008; Rathinakumar *et al*., 2009), that we have used throughout this chapter as an example. A vesicle-based high-throughput screen (Rausch *et al*., 2005) was used to identify ten peptides from the 16,384-member library. As shown in Table 5.3, these ten peptides have potent broad-spectrum activity against microbes, and only minimal lytic or toxic effects against red blood cells and cultured human cells.

5.5 Future Directions

Combinatorial chemistry and high-throughput screening are powerful and effective tools for discovering novel AMPs. The future holds great promise as advances in technology drive increases in throughput. However, throughput is probably not the critical factor in the discovery of therapeutically useful AMPs. Even libraries of <10,000 members contain potent broad-spectrum AMPs that can be identified with manual screening (Rausch *et al*., 2005; Rathinakumar and Wimley, 2008). Perhaps more importantly, improvements in our understanding of AMP mechanisms of action will drive improvements in library design, leading to more effective peptides. We can also look forward in the near future to orthogonal high-throughput screens that select in parallel for (or against) other factors that determine the true therapeutic effectiveness of a potential antibiotic drug. For example, screens could include selection based on membrane permeabilization, bioactivity, solubility, bioavailability, pharmacokinetics, cytotoxicity, susceptibility to acquired resistance and other factors. We may envision an orthogonal high-throughput pipeline strategy for the development of novel AMPs that uses high-throughput screening to select active peptides from rationally designed libraries, followed by focused bioactivity, formulation and toxicity assays to select the most promising candidates for clinical testing.

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6 Chemical Mimics with Systemic Efficacy

Amram Mor

Abstract

Host-derived cationic antimicrobial peptides (AMPs) and their synthetic variants are widely regarded as a potential source of future therapeutic agents against a broad range of pathogens. This is due to a remarkable set of advantageous properties, ranging from molecular simplicity that readily allows for time- and cost-efficient establishment of structure–activity relationships, to an essentially non-specific multitargeted mode of action, which significantly limits the capacity of pathogens to develop efficient resistance mechanisms. Nevertheless, difficult challenges need to be overcome as we move towards their eventual use in therapeutics, including improved bioavailability, toxicity and production costs. To address these issues, an increasingly rich variety of strategies for improving AMP properties are currently available for designing chemical mimics that reproduce the most critical biophysical characteristics of AMPs. In this chapter, I review the main strategies for the *de novo* design of AMP mimics that have generated systemically active lead compounds and showed promise for therapeutic development. Conceptually, the known designs can be distinguished on the basis of backbone rigidity. Thus, after a brief discussion of recent advances on representative approaches used to generate relatively stiff antimicrobial structures, this chapter focuses on the acyl-lysyl approach, which allows the design of structurally bendable molecules that none the less can selectively target a variety of microbial cells.

6.1 Introduction

After over two decades of intensive worldwide efforts, the ubiquitous occurrence of host defence peptides (HDPs) and their critical roles as major immunity effectors are now well established. It is widely believed that this peptide-based defence system may be useful in fighting the emergence and spread of multidrug resistance to available chemotherapeutic drugs. Clearly, various fine details of their mechanism of action are still poorly understood. However, structure–activity relationship studies have helped to reveal new strategies for the specific targeting of pathogens and highlighted the crucial role of physicochemical parameters such as amphipathy, supramolecular organization and conformational flexibility in interactions with target cells.

The antimicrobial properties of HDPs were initially investigated for their role in providing the first line of defence against a wide range of invading pathogens (Boman and Hultmark, 1987; Bevins and Zasloff, 1990; Hoffmann, 1995; Nicolas and Mor, 1995; Hancock and Lehrer, 1998; Bulet *et al*., 2004). Many were found to exhibit activity against a broad spectrum of bacteria, fungi, protozoa and enveloped viruses (Andreu and Rivas, 1998; Ganz, 1999; Mor, 2009). Due to their unique mode of action, antimicrobial peptides (AMPs) have demonstrated excellent potential in treating very common

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as well as sometimes fatal illnesses that may not be cured by conventional antibiotics. All the evidence indicates that, in contrast to common antibiotics, the antibacterial action of these peptides follows a multiple-hit scheme with several potential targets, including the cytoplasmic membrane, cell division and cell-wall and macromolecule synthesis (Shai, 2002; Zasloff, 2002; Brogden, 2005), making it difficult for the pathogen to acquire resistance.

The therapeutic potential of AMPs became evident very early after their discovery. As therapeutic agents, AMPs present various a priori advantages over antibiotics, including molecular simplicity, broad-spectrum activity (Tossi *et al*., 2000; Radzishevsky *et al*., 2005; Jenssen *et al*., 2006), potentially low levels of induced resistance (Perron *et al*., 2006) and concomitant broad anti-inflammatory activities (McInturff *et al*., 2005; Guarna *et al*., 2006; Easton *et al*., 2009). Nevertheless, as potential drug candidates, HDPs can present a variety of shortcomings that need to be addressed, particularly towards development as systemic therapeutic agents. Namely, antimicrobial activity of many HDPs is significantly reduced in the presence of salts and divalent cations (Goldman *et al*., 1997; Bals *et al*., 1998; Minahk and Morero, 2003) and is susceptible to pH changes (Lee *et al*., 1997; Rydlo *et al*., 2006) and plasma components (Oh *et al*., 1999; Rozek *et al*., 2003; Radzishevsky *et al*., 2005). HDPs can also suffer from poor pharmacokinetic properties and systemic toxicity, as well as high production costs (Bradshaw, 2003; Yeaman and Yount, 2003; Gordon *et al*., 2005; Marr *et al*., 2006).

Another concern for therapeutic uses of AMPs pertains to the emergence of extreme resistance phenomena to the host defence system. In addition to their ability to develop resistance mechanisms such as efflux pumps, secreted proteases and alterations of the cell surface, bacteria may also sense AMPs via a variety of two-component sensor/regulator systems (e.g. PhoPQ, PmrAB). These regulate specific gene expression leading to greater stability of the outer membrane and adapting bacteria for survival (Gunn, 2008; Otto, 2009). As some AMPs have been found to activate one or more two-component sensor/regulator systems, concern was voiced that the general use of AMPs might provoke the evolution of resistance that will compromise our natural defences against infection. In that sense, AMP mimics that retain antibacterial activity but lack the ability to activate PhoQ or its orthologues would represent preferable therapeutics.

A rich range of strategies have been put forward in the attempt to alleviate one or more of these shortcomings. These have included the use of peptides composed of D-amino acids (Bessalle *et al*., 1990; Wade *et al*., 1990; Shai and Oren, 1996), combinatorial libraries (Eichler and Houghten, 1995; Blondelle *et al*., 1996; Blondelle and Lohner, 2000) and a wealth of sequence templates or minimalistic approaches (Tossi *et al*., 1997, 2000; Epand *et al*., 2003; Jing *et al*., 2003; Dartois *et al*., 2005; Deslouches *et al*., 2005). These modification strategies have yielded several peptide versions with systemic antibacterial activities; to name a few examples, a 24-mer peptide termed WLBU2, which is based solely on cationic (arginine) and hydrophobic (valine and tryptophan) residues (Deslouches *et al*., 2005), and cyclic D,L-hexapeptides composed of lysine and tryptophan only (Dartois *et al*., 2005). Most recently, a dimeric proline-rich peptide (A3-APO) was designed to attack both the bacterial membrane and the Enterobacteriaceae-specific domain of the heat-shock protein DnaK in order to reduce toxicity (Szabo *et al*., 2010). Although A3-APO was bactericidal to *Escherichia coli* at a relatively high concentration (30 $\mu g\ ml^{-1}$) *in vitro*, the designer peptide was effective against systemic *E. coli* infections in different mouse models after multiple dosing of 10 mg kg^{-1}, whereas the maximal tolerated dose (MTD) was >20 mg kg^{-1}.

Another set of strategies has opted for the *de novo* design of peptide-like constructs that mimic HDP structure or function (representative structures are shown in Fig. 6.1). While differing with respect to their prime philosophies, these strategies share a common rationale, which is based on the idea that reproduction of the critical biophysical characteristics in unnatural oligomers should

presumably be sufficient to endow activity and, by the same token, circumvent the limitations associated with peptide pharmaceuticals (Latham, 1999; Adessi and Soto, 2002; Patch and Barron, 2002). Three representative strategies that have generated systemically active compounds are briefly discussed below. Additional interesting mimic systems have been recently reviewed (Epand *et al*., 2008a; Lai *et al*., 2008; Som *et al*., 2008; Van Bambeke *et al*., 2008; Rotem and Mor, 2009; Tew *et al*., 2010).

6.2 *De Novo*-designed Rigid Peptidomimetics

6.2.1 Hairpin-shaped peptides

The β-hairpin fold of the HDP protegrin-1 has been used as a starting point for designing stabilized peptidomimetics with a looped sequence linked to a D-proline–L-proline template, which helps to stabilize conformations within the macro-cycle (reviewed in Robinson *et al*., 2008). Following iterative rounds of peptidomimetic library synthesis and screening, two variants emerged (POL7001 and POL7080). These have specific antipseudomonal activity (minimum inhibitory concentrations (MICs) in the nanomolar range), while other Gram-negative and Gram-positive bacteria are largely insensitive. Unlike the parent peptide, however, these peptidomimetics do not act by a membranelytic mechanism, consistent with the nanomolar range of active concentrations. It is proposed that they interfere with outer membrane biogenesis by inhibiting lipopolysaccharide (LPS) transport. The peptidomimetics have been evaluated in a mouse septicaemia model after inoculation with *Pseudomonas aeruginosa* and demonstrated substantial activity with calculated median effective doses in the range of 0.25–0.55 mg kg^{-1} (Srinivas *et al*., 2010).

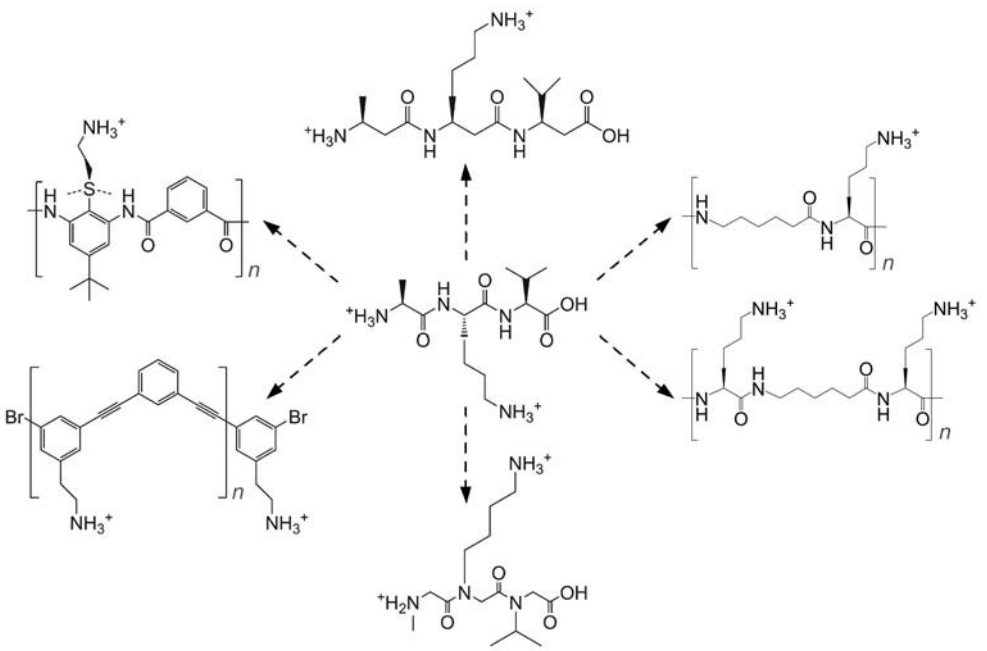

Fig. 6.1. Chemical formulae of representative building blocks used for the *de novo* design of AMP mimics. The α-peptide sequence (centre) can be replaced with β-peptide (top), peptoid (bottom), arylamide (upper left), phenylene ethynylene (lower left), aminoacyl-lysyl (upper right) or lysyl-aminoacyl-lysyl (lower right) subunits.

6.2.2 Peptoids

Poly-*N*-substituted glycines (peptoids) are distinguished from peptides by the fact that the side chains are attached to the backbone amide nitrogen instead of the α-carbon (Zuckermann *et al*., 1992). Recognized attributes of peptoids include protease resistance (Miller *et al*., 1994) and prevention of both backbone chirality and intrachain hydrogen bonding. Instead, peptoids can be driven to form stable helical structures via a periodic incorporation of bulky α-chiral side chains (Armand *et al*., 1998; Kirshenbaum *et al*., 1998; Wu *et al*., 2001, 2003; Seo *et al*., 2010).

Certain peptoid variants exhibit broad-spectrum antibacterial activity and low mammalian cytotoxicity. For example, a 12-residue compound referred to as 1-pro6 displays potent antibacterial activity (MIC values of 3.1 and 1.6 μM against *E. coli* and *Bacillus subtilis*) and low haemolytic activity (median lethal concentration (LC_{50}) of >110 μM against human red blood cells (RBCs)) (Chongsiriwatana *et al*., 2008). The most recent designs (binaphthyl-based peptoids 2a and 2b) have shown promising antibacterial potencies against a panel of Gram-positive pathogens (MIC_{50} in the μg ml^{-1} range). The peptoids were rapidly bactericidal with a concentration-dependent profile, and induced resistance was slow to develop *in vitro*. The authors concluded that these compounds may act by more than one mode of action, including inhibition of cell-wall cross-linking and cell-membrane disruption (Bremner *et al*., 2010). A non-membrane lytic mechanism was also reported in a recent study using soft X-ray tomography, showing that peptoid treatment suppresses the formation of a pathogenic hyphal phenotype of yeast cells such as *Candida albicans* and changes both cell and organelle morphology (Uchida *et al*., 2009). It has also been reported that *in vivo* potency is maintained both systemically and topically, using the mouse nasal decolonization model (Bremner *et al*., 2010).

6.2.3 Arylamides

Aided by molecular dynamics simulations, a class of facially amphiphilic arylamide oligomers has been generated based on alternating 1,3-phenylene diamine units connected by an isophthalic acid. The resulting conformationally restrained backbone oligomers displayed a typical membrane-active mode of action (reviewed in Scott *et al*., 2008; Tew *et al*., 2010). Oligomeric variants using hydrophobic and hydrophilic side chains and/or switching benzene with pyrimidine have yielded several compounds with potent antimicrobial activities *in vitro* (Tew *et al*., 2002; Liu *et al*., 2004; Tang *et al*., 2006). Non-peptide analogous amphiphiles with a strictly hydrocarbon backbone, termed phenylene ethynylene oligomers, have been designed to mimic structural attributes of the HDP magainin (Tew *et al*., 2006). The most active compound, meta-phenylene ethynylene (mPE), composed of three phenyl rings, showed potent antimicrobial activity against a large panel of pathogenic bacteria with a MIC of 0.4–14 μM and 50% haemotoxicity at 153 μM.

The mode of action of these oligomers has been investigated using various biophysical techniques, including Langmuir films at the air–water interface (Arnt and Tew, 2002, 2003), grazing incidence X-ray diffraction (Ishitsuka *et al*., 2006), small-angle X-ray scattering, sum frequency generation vibrational spectroscopy and dye-leakage experiments with model membranes (Chen *et al*., 2006; Yang *et al*., 2007). Phenylene ethynylenes were described as efficient hole-punching membrane-active antimicrobials (Yang *et al*., 2008). Using lipid-knockout mutant strains of *E. coli*, which drastically out-survived the wild-type parent strain, it was concluded that bacterial membrane permeation depends on the presence of high concentrations of phosphatidylethanolamine, owing to its role in inducing negative intrinsic curvature within bacterial membranes. As often observed with HDPs, mPE was also able to tightly bind additional anionic biomolecules such as DNA and LPS, as assessed by its ability to retard DNA migration in gel electrophoresis and to inhibit LPS-mediated activation of macrophages (Beckloff *et al*., 2007). Moreover, using selective-pressure-type experiments under conditions that led to the emergence of resistance to conventional antibiotics, bacterial resistance to these chemical mimics was not observed.

Unfortunately, few *in vivo* data exist in the literature to reflect the possible therapeutic potential of these amphiphiles. While, the MTD of mPE in mice was found to be 10 mg kg^{-1} (Tew *et al*., 2006), suggesting a potential therapeutic window, to my knowledge no efficacy studies have been published since. In contrast, short arylamide foldamers were recently reported to exhibit systemic efficacy *in vivo* (Choi *et al*., 2009). The most potent variants reduced staphylococcal load to various extents upon the systemic administration of 1–10 mg kg^{-1}, while MTD was found at 20 mg kg^{-1}.

Collectively, these studies seem to imply that rigidifying the conformation of the peptidomimetic oligomers may lead to improved antibacterial activity and selectivity, and consequently suggest potential adverse effects of molecular flexibility in these respects.

6.3 Acyl-lysyl Oligomers

6.3.1 Design

Oligomers of acylated lysines (OAKs) were originally designed to test the necessity for a stable fold in AMPs (Radzishevsky *et al*., 2007a,b). To promote the lack of a stable secondary structure, acyl bridges were intercalated between cationic amino acid residues. The lack of hydrogen-bonding opportunities in such constructs is expected to limit the formation of stable folds because of the rotational freedom of the carbon atoms within short-acyl chains (the particular case of long acyls will be discussed further below). On the other hand, the OAK molecule is provided with a chance to reveal antimicrobial activity as it is composed of cationic and hydrophobic residues; both properties have long been suspected to represent essential requirements for antimicrobial activity. This method therefore enables a fold-independent systematic tool for a gradual control of molecular hydrophobicity (i.e. by changing acyl chain lengths) and charge (by changing the nature, position or number of cationic residues).

An OAK library including >150 sequences composed of either α (aminoacyl-lysyl) or β (lysyl-aminoacyl-lysyl) subunits has been produced and characterized (Radzishevsky *et al*., 2007a,b, 2008; Livne *et al*., 2009). Analysis of the data obtained from this library enabled pertinent conclusions to be drawn as to the properties required for the potency and selectivity of OAKs, as detailed in the following paragraphs.

6.3.2 Structure–activity relationships

Figure 6.2 depicts a plot of the charge (Q) and hydrophobicity (H) of the OAKs tested. It also highlights relationships that exist between HQ values and selective sequences – sequences that combine low haemolysis ($LC_{50} \geq 100$ μM, as assessed against human erythrocytes) with high antibacterial activity (MIC ≤ 3.1 μM, as assessed against representatives of Gram-negative and Gram-positive bacteria, *E. coli* and *Staphylococcus aureus*, respectively). Table 6.1 shows the biophysical properties of the most active sequences (highlighted in Fig. 6.2).

One of the main trends that emerged from these data was that active sequences presented a bell-shape behaviour with an optimal window of HQ values per tested variable (Radzishevsky *et al*., 2008; Livne *et al*., 2009). As shown in Fig. 6.2, selective sequences also displayed a specific (relatively narrow) window of HQ values for each activity tested. Both the relative positions and slopes are indicative of distinct HQ requirements for selective antibacterial activities. Thus, for *E. coli*, hydrophobicity requirements were quite strict (i.e. H = 45–50), while charge levels were distributed over a large band of values (Q = 6–9). These values were strikingly different for *S. aureus*, both in tolerating a wider range of hydrophobicity values (H = 40–50) and in requiring lower charge values (Q = 3–5). As detailed further below, selective antiplasmodial activity seems to require a different yet distinct window of HQ values. This trend may therefore provide a useful guideline in the search for new and improved OAK sequences.

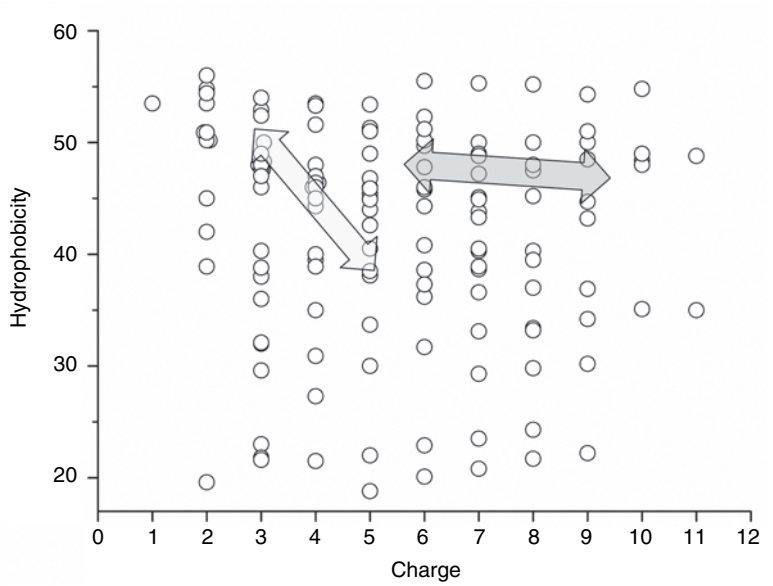

Fig. 6.2. A charge (Q) and hydrophobicity (H) map of representative oligomers of an acylated lysine (OAK) library. Plotted are the HQ values of each of >150 OAK sequences. Details are found elsewhere (Radzishevsky *et al.*, 2008; Livne *et al.*, 2009). The distributions of the most selective OAKs (listed in Table 6.1), defined as sequences that combine low haemolysis (median lethal concentration ≥100 μM) with high antibacterial activity (minimum inhibitory concentration ≤3.1 μM), are highlighted. The light grey and dark grey arrows represent the active window against *Staphylococcus aureus* and *Escherichia coli*, respectively.

Table 6.1. List of the most selective oligomers of acylated lysines (OAKs; highlighted in Fig. 6.2).

OAK designation [a]	OAK sequence[a]	Q[b]	H[b]
Active against Gram-positive bacteria			
*$C_{12(\omega7)}$K-β_{12}[c]	$C_{12(\omega7)}$K-K$C_{12}K_a$	3	50
C_8–2β_{12}	C_8-KC_{12}K-K$C_{12}K_a$	4	45
NC_{12}–2β_{12}	NC_{12}-KC_{12}K-K$C_{12}K_a$	5	38.5
Active against Gram-negative bacteria			
C_{12}K-5α_8	C_{12}K-C_8K-C_8K-C_8K-C_8K-C_8K_a	6	49.7
C_{12}K-6α_8	C_{12}K-C_8K-C_8K-C_8K-C_8K-C_8K-C_8K_a	7	50
C_{12}K-7α_8	C_{12}K-C_8K-C_8K-C_8K-C_8K-C_8K-C_8K-C_8K_a	8	47.5
C_{12}K-8α_8	C_{12}K-C_8K-C_8K-C_8K-C_8K-C_8K-C_8K-C_8K-C_8K_a	9	48.5
*C_{12}K-3β_{10}	C_{12}K-KC_{10}K-KC_{10}K-K$C_{10}K_a$	7	44.9
C_{12}K-4β_{10}	C_{12}K-KC_{10}K-KC_{10}K-KC_{10}K-K$C_{10}K_a$	9	44.7

[a] For N-terminal acyls: C_8, octanoyl; C_{10}, decanoyl; C_{12}, dodecanoyl; NC_{12}, aminododecanoyl; $C_{12(\omega7)}$, dodecenoyl (all intercalating residues are aminoacyl derivatives); a, amide.

[b] Q, charge; H, estimated hydrophobicity (percentage of acetonitrile eluent) as determined by reversed-phase high-performance liquid chromatography using a C_{18} column.

[c] Asterisks indicate OAK sequences that displayed systemic antibacterial efficacy using the mouse thigh-infection model.

A number of additional sequences displayed potent antimicrobial properties, namely when hydrophobicity exceeded the optimal HQ window. This often led to self-assembly in solution and consequently to haemotoxicity (Radzishevsky *et al*., 2008; Livne *et al*., 2009). Moreover, close inspection of the data shows that self-assembly phenomena can often explain why sequences in the neighbourhood of active compounds (herein referred to as the 'active HQ window') are inactive *de facto*. This is not to say that all types of aggregates are necessarily inactive, especially considering that an aggregated compound can be inactive against one microorganism but active against another. For example, aggregated OAKs that were inactive against bacteria displayed potent antiplasmodial properties (Radzishevsky *et al*., 2007a), highlighting the importance of aggregate interactions with bacterial cell-wall components. An inverse example is represented by the fact that aggregated OAKs can be made to exhibit potent antibacterial activity, namely by weakening the hydrophobic forces at play (Sarig *et al*., 2008), as further discussed below.

The biophysical properties of OAKs are very much dictated by the nature of the N-terminal acyl. Thus, the presence of an N-terminal acyl essentially determined the molecular hydrophobicity of the whole series, regardless of the charge or type of subunits. In contrast, in OAKs that lacked an N-terminal acyl, the hydrophobicity value readily increased with increasing backbone length. In this regard, potency clearly required the presence of an N-terminal acyl, where dodecanoic acid most often represented the optimal choice (Radzishevsky *et al*., 2007a,b, 2008; Livne *et al*., 2009).

Comparing OAKs with short versus long acyls provided additional insight into the structure–activity relationships. For instance, antibacterial activity could be highly dissociated from both stable conformation and haemolytic activity (Radzishevsky *et al*., 2007b, 2008). Thus, butyl- and octyl-based OAKs were consistently devoid of haemolytic activity and displayed circular dichroism (CD) profiles that reflected random structures, despite interaction with liposomes and other hydrophobic media known to induce secondary structures. In contrast, dodecanoyl-based OAKs were often haemolytic, highly aggregated and displayed CD profiles of stable folds reminiscent of supramolecular organizations. Support for this notion was provided by various experiments that attempted to affect the aggregate properties, either by increasing backbone charge (Radzishevsky *et al*., 2008; Livne *et al*., 2009) or using analogous unsaturated (less hydrophobic) acyls (Sarig *et al*., 2008, 2010). This led to 'normalization' of the CD profile and the simultaneous loss of haemolytic activity.

The correlations existing between aggregated OAKs and haemolytic activity provide strong support to the hypothesis underlying OAK design, proposing a link between stable flds (i.e. rigid structures) and haemolytic properties of AMPs (Radzishevsky *et al*., 2007b). It is stipulated that rigid structures are more inclined to perturb the RBC membrane architecture (provided they are hydrophobic enough), whereas OAK interactions with RBCs are inefficient in perturbing the membrane – precisely because of their high backbone flexibility. This logic, however, does allow the very same OAKs to inflict a range of damage upon bacterial membranes (Epand *et al*., 2008b, 2009; Livne *et al*., 2009; Zaknoon *et al*., 2009; Sarig *et al*., 2010) due to the inherent differences in membrane composition, and possibly even promotes OAK internalization to the cytoplasm in some cases (Rotem *et al*., 2008; Held-Kuznetsov *et al*., 2009). Moreover, the available data support the view that the intercalated acyls can suppress the formation of stable molecular folds as long as the acyl chains are not too long (i.e. <12 carbons). According to this view, dodecanoyl residues are able to create amphipathic rigid structures by bending or collapsing on themselves, unlike short acyls. In that case only, the dodecanoyl-based OAKs gain the opportunity to stabilize in aqueous solutions through self-assembly, whereas their haemolytic activity reflects the ability to disassemble and reorganize within the RBC membrane, much like amphipathic conventional AMPs (Feder *et al*., 2000; Avrahami *et al*., 2001; Radzishevsky *et al*., 2005).

6.3.3 Antibacterial properties

As illustrated in Fig. 6.2, the OAK library includes a number of OAK sequences that potently affect bacterial viability *in vitro*. Thus, sequences such as the octamer derivative $C_{12}K\text{-}7\alpha_8$ (Radzishevsky *et al*., 2007b) and its hexamer analogue $C_{12}K\text{-}5\alpha_8$ (Rotem *et al*., 2008) displayed significant growth inhibitory activity against a large panel of bacterial species, but were most potent against the Gram-negative species. In contrast, $NC_{12}\text{-}2\beta_{12}$ (Zaknoon *et al*., 2009) and $C_{12(\omega7)}K\text{-}\beta_{12}$ (Sarig *et al*., 2010) were rather active against Gram-positive species, while $C_{16(\omega7)}K\text{-}\beta_{12}$ (Sarig *et al*., 2008 and unpublished data) and $C_{12}K\text{-}3\beta_{10}$ (Livne *et al*., 2009) displayed essentially non-selective broad-spectrum cytotoxicity. Another non-selective broad-spectrum OAK worth mentioning, $C_{12}K\text{-}2\beta_{12}$, was recently evaluated for its antibacterial effect on cultures of *Helicobacter pylori*, a gastric pathogen that has developed resistance to virtually all current antibiotics. The OAK exhibited MIC and minimum bactericidal concentrations of 6–26 μM and 15–90 μM, respectively, across six different *H. pylori* strains (G27, 26695, J99, 7.13, SS1 and HPAG1). *H. pylori* was completely killed after 6–8 h of incubation in liquid cultures containing two times the minimum bactericidal concentration of $C_{12}K\text{-}2\beta_{12}$. The OAK demonstrated low haemolytic activity and high stability, with efficacy maintained after incubation at extreme temperatures (4–95°C) and low pH, although reduced killing kinetics were observed at pH 4.5 (Makobongo *et al*., 2009). Thus, while these results suggest that OAKs may be a valuable resource for the treatment of various bacterial infections, they also evidenced a broad range of time periods required to affect bacterial viability. These significant differences, observed even between highly analogous sequences, suggest the occurrence of mechanistic differences.

Various mechanistic studies performed with the most active sequences have substantiated the idea that OAKs can indeed exert a number of distinct antibacterial modes of action. Thus, the octamer derivative $C_{12}K\text{-}7\alpha_8$ exerts bactericidal activity through membrane disruption, as evidenced by its ability to rapidly (within minutes) induce membrane depolarization and leakage of cytoplasmic solutes in correlation with time–kill curves (Radzishevsky *et al*., 2007b). However, when compared against the very same bacteria, the hexameric version $C_{12}K\text{-}5\alpha_8$ was unable to disrupt the plasma membrane and the bactericidal rates were much slower (Rotem *et al*., 2008). It was suggested that $C_{12}K\text{-}5\alpha_8$ exerts its antibacterial effect after translocating into the cytoplasm, where the OAK inhibits biosynthesis of macromolecules through a direct (probably non-specific) interaction with DNA. With the aim of understanding the mechanisms involved, the interaction of this pair of analogues with model phospholipid membranes was investigated by a number of complementary methodologies, including surface plasmon resonance, isothermal titration calorimetry, differential scanning calorimetry and nuclear magnetic resonance (Epand *et al*., 2008b; Rotem *et al*., 2008). The collective data indicated that only $C_{12}K\text{-}7\alpha_8$ exhibited a high capacity to induce clustering of anionic lipids (Epand *et al*., 2008b). This clustering effect is believed to lead to the lateral segregation of domains rich in anionic and zwitterionic lipids, producing phase-boundary defects that ultimately breach the permeability barrier of the cell membrane. The data also suggest that the hexamer's aptitude to undergoing internalization is linked to its lower binding affinities for cell-wall components (e.g. LPS) and cytoplasmic membrane phospholipids.

Moreover, comparing the mechanism of action of $C_{16}K\text{-}\beta_{12}$ with that of $C_{16(\omega7)}K\text{-}\beta_{12}$ or $C_{12(\omega7)}K\text{-}\beta_{12}$ has further accentuated the notion that tiny differences can lead to drastic mechanistic changes. As mentioned above, various studies of AMPs (Avrahami *et al*., 2001; Rotem *et al*., 2006) and OAKs (Radzishevsky *et al*., 2008) have evidenced strong relationships between hydrophobicity, aggregation and haemotoxicity (Javadpour and Barkley, 1997; Radzishevsky *et al*., 2005). Thus, excess hydrophobicity and consequent

self-assembly in aqueous media appear to be responsible for poor antibacterial performance and potential toxicity, as reflected by haemolysis. This issue was initially addressed by attempting to manipulate a highly aggregated short sequence ($C_{16}K$-β_{12}) that exhibited both antibacterial and haemolytic properties. Substitution of the N-terminal acyl with its unsaturated counterpart ($C_{16(\omega7)}K$-β_{12}) significantly altered the OAK's supramolecular organization, even though the critical aggregation concentration (CAC) did not change (Sarig *et al*., 2008). Thus, although antibacterial performance did improve, haemolytic activity remained high enough to compromise its potential use in systemic therapy. Extending this study, the N-terminal acyl was replaced with the less hydrophobic shorter acyl, dodecanoyl (Sarig *et al*., 2010). As a result, the $C_{12(\omega7)}K$-β_{12} CAC became significantly higher than that of $C_{12}K$-β_{12} (CACs of approximately 50 and 10 μM, respectively). Reversed-phase high-performance liquid chromatography analysis of these compounds confirmed that the acyl substitution was accompanied by reduced hydrophobicity, as reflected by the elution time (50% and 54% acetonitrile eluent, respectively). As anticipated, disassembly was responsible for reducing toxicity to mammalian cells as assessed with erythrocytes and primary fibroblasts. Yet $C_{12(\omega7)}K$-β_{12} displayed potent growth inhibitory activity against Gram-positive bacteria (MIC 2.5–5 μg ml^{-1}), while Gram-negative bacteria were generally less susceptible. Furthermore, the introduction of the double bond also drastically affected the antibacterial mechanism of action. Thus, unlike $C_{12}K$-β_{12}, which displayed classically rapid membrane disruptive bactericidal activity, the unsaturated analogue $C_{12(\omega7)}K$-β_{12} exhibited a strict bacteriostatic mode of action (Sarig *et al*., 2010). In addition, recent unpublished data indicate that the more hydrophobic unsaturated analogue $C_{16(\omega7)}K$-β_{12} reverted back to the bactericidal mode of action (causing 99% death within 2 h) as assessed against multiple strains of different bacterial species. Interestingly, however, $C_{16(\omega7)}K$-β_{12} did not disrupt the bacterial membrane(s). Cell death was apparently caused by the OAK's ability to interfere with DNA functions in a similar manner to that of $C_{12}K$-$7\alpha_8$ (Rotem *et al*., 2008), as evidenced by both the direct interaction with DNA under live conditions and the resulting inhibition of biosynthesis.

There is a growing list of conventional AMPs such as PR-39 (Boman *et al*., 1993), tPMP-1 and HNP-1 (Xiong *et al*., 1999), buforin II (Park *et al*., 2000) and pyrrhocoricin (Kragol *et al*., 2001) that are proposed to exert antibacterial activity through interactions with intracellular targets (Nicolas, 2009). Combined with the OAK findings, the collective data show that small differences (such as a single charge, backbone length or even a single double bond) are required for an antibacterial compound to switch from one mechanism to another. Further studies are, however, needed to validate this hypothesis, including investigation into the mechanism of action of a compound against a large panel of bacterial species in order to determine the possible generalization of this phenomenon. The vast majority of mechanistic insights published in the literature are based on investigations that have used a single strain of a single bacterial species, which prohibits conclusions on whether the compound always uses a particular mechanism, or whether it uses distinct mechanisms on distinct strains or species. It is also possible that an antibacterial compound can use multiple or combined mechanisms. This is often touted in the literature, but has yet to be convincingly demonstrated experimentally. Stretching this idea further, the data support the notion that simultaneous treatment of infections with a cocktail of compounds acting by distinct mechanisms may represent a more efficient treatment approach. Nature seems to use this strategy via the concomitant production and delivery of multiple closely related HDPs (Levy *et al*., 1994; Mor *et al*., 1994).

Various preliminary *in vivo* investigations performed so far with four OAK sequences have revealed a fair ability of OAK-based compounds to affect bacterial viability in mice and moreover highlighted

some still ill-understood sequence dependencies. Thus, although exerting distinct bactericidal mechanisms of action *in vitro*, the analogues $C_{12}K\text{-}7\alpha_8$ and $C_{12}K\text{-}5\alpha_8$ have shown remarkable efficacy in peritonitis–sepsis models, where both OAKs have increased the survival of infected mice by up to 100% after single- or multiple-dose intraperitoneal administration (Radzishevsky *et al*., 2007b; Rotem *et al*., 2008). In another unpublished study, $C_{12}K\text{-}7\alpha_8$ was examined in a *P. aeruginosa* pneumonia infection model when administered directly to the lungs by inhalation. At 25 μg per mouse, the OAK substantially reduced the lung bacterial population by up to 2 logs (similarly to tobramycin) as compared to inoculated vehicle controls. MTD studies indicated that prolonged exposure to high dose levels of $C_{12}K\text{-}7\alpha_8$ (up to 100 μg in the respiratory tract) did not cause overt toxicity in the mice. This study indicates that $C_{12}K\text{-}7\alpha_8$ has potential for use as an antimicrobial agent in the treatment of severe lung infections caused by Gram-negative pathogens. However, when tested with the thigh-infection model, both $C_{12}K\text{-}7\alpha_8$ and $C_{12}K\text{-}5\alpha_8$ failed to significantly reduce bacterial load, suggesting a poor potential for systemic efficacy, probably due to poor tissue-penetration capacity. In contrast, $C_{12}K\text{-}3\beta_{10}$ and $C_{12(\omega7)}K\text{-}\beta_{12}$, which exerted *in vitro* bactericidal and bacteriostatic modes of action, respectively, were both efficient in the thigh-infection model (Livne *et al*., 2009; Sarig *et al*., 2010).

Typically, OAK MTDs in mice upon intravenous and intraperitoneal administrations were approximately 5 and 10 mg kg^{-1}, respectively. The subcutaneous route of administration is much better tolerated (at least up to 20 mg kg^{-1}), although efficacy doses are >tenfold and sometimes >100-fold lower (unpublished data).

Collectively, the current data raise interesting questions pertaining to the relationship between the OAK sequence and *in vivo* efficacy. Namely, are β-OAKs more suitable for systemic development than α-OAKs? If so, why? Is it a function of small-molecule pharmacokinetics? Or pharmacodynamics? We will attempt to address these questions in future studies.

6.3.4 Antiparasitic properties

A variety of *in vitro* and *in vivo* antiparasite investigations of AMPs have suggested that these notorious antibacterial compounds may represent a powerful tool for developing new drugs to fight parasites in the host. Human parasites are responsible for millions of deaths around the world every year. Of particular concern is the causative agent of human malaria, *Plasmodium falciparum*, of which a large number of strains are drug resistant (Bruce-Chwatt, 1982). In addition to known resistance to the classical chloroquine-based drugs, there have been reports of field strains of *P. falciparum* with resistance to artemisinins, more recently introduced antimalarial drugs (Krishna *et al*., 2006). There is a clear need for new antimalarial agents.

HDPs such as magainins and cecropins were reported to display antiparasitic activities over 20 years ago (Boman *et al*., 1989; Gwadz *et al*., 1989). Since then, antiparasitic activities have been reported for numerous additional AMPs (Rivas *et al*., 2008). In addition to the naturally occurring antiparasite peptides and their synthetic derivatives, recent studies suggest that potent antiparasitic properties can be generated from *de novo*-designed AMP mimics (Mor, 2009). Various OAK sequences have been shown to inhibit the growth of different plasmodial strains in culture (half the maximal inhibitory concentration (IC_{50}) of 0.08–0.14 μM). Certain sequences displayed highly selective antiparasitic activity, where the ratio of LC_{50} (haemolysis) to IC_{50} (parasite growth inhibition) was >10,000 for the most selective OAK, $3\alpha_{12}$ (Radzishevsky *et al*., 2007a). The available data suggest that the OAK does not exert its antiplasmodial action by lysis of infected RBCs, as is the case with various HDPs. Rather, it seems that it is able to cross the erythrocyte plasma membrane and target internal components such as the mitochondrion or nucleic acids of the parasite. As described above, OAKs have

shown these abilities in other cell types (Rotem *et al*., 2008; Held-Kuznetsov *et al*., 2009).

The most recent attempts to generate improved antiplasmodial OAKs have revealed additional octanoyl-based OAKs that inhibit parasite growth at sub-micromolar concentrations (mean IC_{50} 0.3 ± 0.1 μM) (unpublished data). Both the ring and trophozoite stages of the parasite developmental cycle were sensitive to the most potent OAK, which moreover exhibited no haemolytic activity at least up to 150 μM (<0.4% haemolysis). Interestingly, the most potent non-haemolytic OAKs all localized near the *S. aureus* HQ window (Fig. 6.2), but contained distinct sequences (i.e. did not display antibacterial activity). Preliminary *in vivo* data from acute toxicity, pharmacokinetic and efficacy studies provide evidence for the potential of OAK sequences generated during this study to be considered suitable candidates for development as systemic antimalarials. Namely, upon daily intraperitoneal administration (4 × ≥25 mg kg^{-1} day^{-1}) to *Plasmodium vinckei*-infected mice, the OAK displayed high blood concentrations (approximately 100 μM) that were sustained for at least several hours. It also demonstrated the ability to eliminate parasitaemia, albeit not without chronic toxicity. Nevertheless, the encouraging results obtained in this study regarding the selectivity and pharmacokinetic properties of some compounds justify further investigation, with the aim of delivering antimalarial analogues with high safety.

Although antiparasitic properties have not yet been investigated as thoroughly as antibacterial activities, an increasing amount of experimental evidence supports the view that the antiparasitic activity of AMPs also emanates from interactions with multiple targets. Remarkably, at least a few peptides exhibit *in vitro* high potency and selectivity factors of several orders of magnitude, and appear to be endowed with the formidable ability to cross a number of membrane systems before reaching their target(s) within the intracellular parasite. While differences in membrane composition are likely to contribute to this selectivity, the molecular basis for these observations remains largely unclear. None the less, these studies suggest that efficient antimalarial therapeutic drugs can be engineered based on the physicochemical attributes imbedded in the molecular formulae of AMPs.

6.4 Concluding Remarks and Perspectives

The fact that most HDPs and their synthetic mimics essentially act by non-specific mechanisms has long created serious doubts as to their ability to exert sufficient selectivity to be considered ideal candidates for drug development, particularly for systemic therapies. In that respect, an increasing number of recent studies have provided a variety of counterarguments that significantly contribute to dissipating this doubt.

Through distinct approaches for designing chemical mimics of HDPs, at least three major achievements have been realized: simplifying the pharmacophore composition, increasing its robustness and miniaturizing its size. These achievements should significantly enhance the efficiency of future structure–activity relationship studies in better defining the active principles, which will in turn drive the discovery of new and improved compounds.

Beyond their capacity to affect microbial viability directly, various AMPs have been proposed to significantly modulate host immune responses. Another unmet challenge to be addressed in the future is therefore the effect of these chemical mimics on the host immune system and its contribution to the observed *in vivo* efficacies.

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7 Biophysical Analysis of Membrane-targeting Antimicrobial Peptides: Membrane Properties and the Design of Peptides Specifically Targeting Gram-negative Bacteria

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Abstract

Gram-negative bacteria are more resistant to many antimicrobial agents than Gram-positive bacteria. This is because drugs must pass through an additional barrier of the outer membrane in order to access the cytoplasmic membrane or the interior of the cell. In spite of this fact, the presence of the outer membrane also provides an alternative target for the action of antimicrobial agents. In addition, the cytoplasmic membranes of Gram-negative bacteria are generally more enriched with the phospholipid phosphatidylethanolamine than those of Gram-positive bacteria. In this chapter, we outline novel strategies for using the interaction of antimicrobial agents with components of the membranes of Gram-negative bacteria in order to design agents that have a higher toxicity for these bacteria.

7.1 Differences in Chemical Composition and Molecular Organization of Gram-positive and Gram-negative Bacteria

Most bacteria are routinely classified according to the criterion of whether they are stained by Gram stain. This phenomenological criterion divides bacteria into two general groups – Gram-positive and Gram-negative – that also differ in the nature of their cell membrane(s). Gram-negative bacteria are characterized by having two membranes: an inner cytoplasmic membrane surrounding the cell and an outer membrane (Fig. 7.1A). The outer membrane is unique in its properties and composition. It is quite permeable to small molecules with a molecular mass of ≤700 Da. This is because of the presence of β-barrel proteins called porins, in which the centre of the β-barrel is an aqueous pore extending through the outer membrane. A simplified diagram depicting the differences in outer membrane organization between Gram-positive and Gram-negative bacteria is shown in Fig. 7.1. Peptide molecules covering part of the surface of the membrane are shown as red ovals.

When a peptide tries to gain entry into a Gram-negative bacterium membrane it first traverses the sugar chains in the O-antigen

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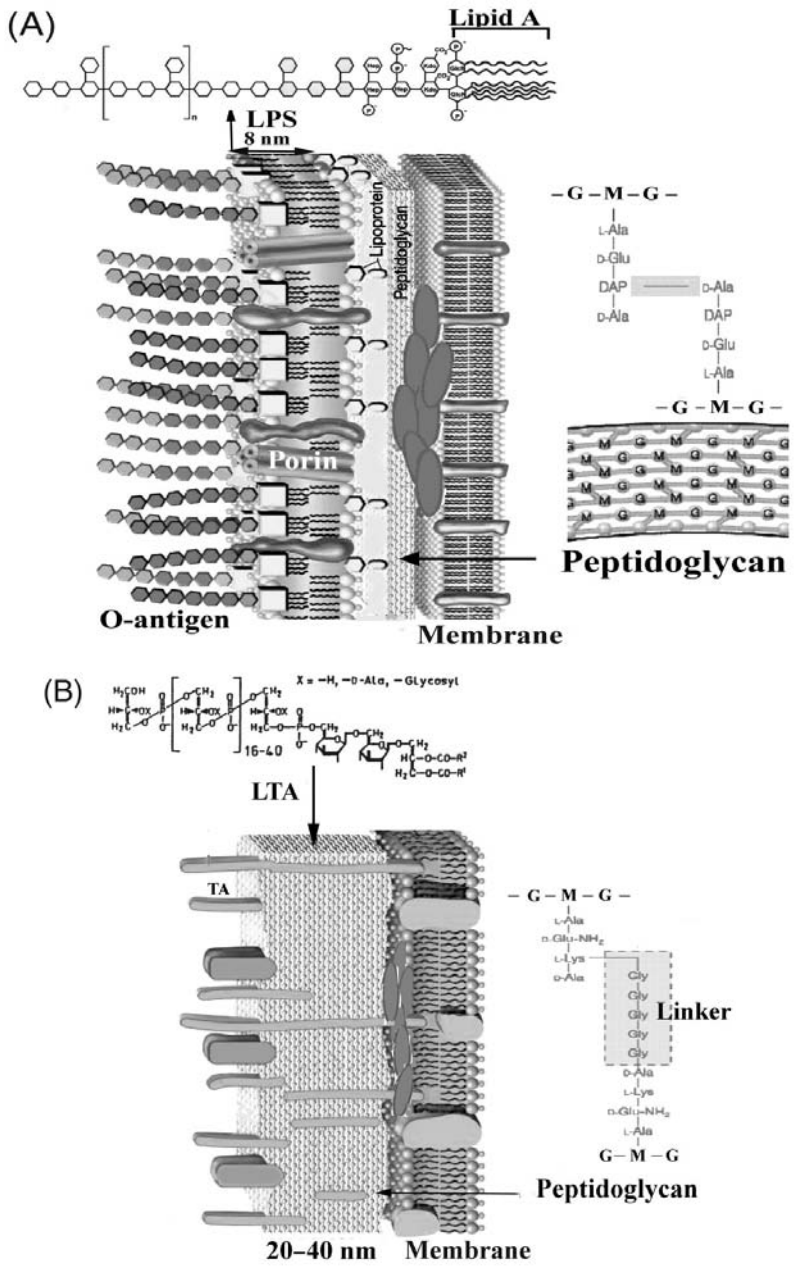

Fig. 7.1. Schematic diagram of the architecture of the membranes of Gram-negative and Gram-positive bacteria (not to scale). (A) Gram-negative bacteria (from left to right): O-antigen depicted as hexagons on the outer monolayer of the outer membrane; porins traversing the lipopolysaccharide (LPS) layer are depicted as cylinders; the LPS layer is followed by lipoproteins; the peptidoglycan layer is shown as dots; ovals represent peptides; the inner membrane is presented with transmembrane proteins. The complete LPS structure is shown on top and the peptidoglycan structure to the right. G, *N*-acetylglucosamine; M, *N*-acetylmuramic acid; DAP, diaminopipelic acid. (B) Gram-positive bacteria (from left to right): teichoic acid (TA) and lipoteichoic acid (LTA) as long, thin structures reaching through the peptidoglycan layer, which is depicted as dots; peptides are ovals; the membrane is shown with transmembrane proteins. The LTA structure is shown on top and the peptidoglycan structure to the right.

layer (Fig. 7.1A). If it has a molecular mass larger than approximately 700 Da then it crosses the lipopolysaccharide (LPS) layer, which is about 8 nm thick. If it does not bind completely to the phosphate moieties of the lipid A portion in the LPS molecule then it will continue its journey through a number of lipoproteins and a peptidoglycan layer. In Gram-negative bacteria, the peptidoglycan layer comprises only 10% of the dry weight of the cell and it is composed of a glycan backbone of alternating repeating sugar units of *N*-acetylglucosamine (G) and *N*-acetylmuramic acid (M). These are cross-linked directly by four amino acids (two L- and two D-amino acids) on each layer, thus forming a rigid mesh that allows facile passage of peptides. The peptide molecules accumulate in the periplasmic space, achieving high concentrations that can be many fold the bulk concentration of peptide. From there they are attracted electrostatically to the outer leaflet of the inner membrane. At each step the peptide molecules are exposed to a number of proteins that participate in specific biosynthetic and other functions of the bacteria.

In trying to gain entry into a Gram-positive bacterium, a peptide encounters teichoic acid covalently bound to a sugar backbone and lipoteichoic acid covalently bound to the membrane (Fig. 7.1B). It gains direct access to the peptidoglycan layer, which is 50% of the dry weight of the cell and contains a number of associated proteins. The peptidoglycan layer in Gram-positive bacteria differs from the one in Gram-negative bacteria in the linkage between the amino acids. This is not direct, but is rather done through an amino acid bridge, which varies within species as a result of adaptation. This makes the peptidoglycan layer wider and more accessible to the peptide, which can easily traverse it to reach the outer leaflet of the cytoplasmic membrane, in spite of the fact that this layer is 20–40 nm in length. Gram-positive bacteria have little or no periplasm; consequently, the cationic peptides accumulate directly on the anionic lipids by electrostatic attraction, reaching threshold concentrations for membrane disruption. Given the number of anionic components that cationic antimicrobial peptides (AMPs) are exposed to on their journey towards the cytoplasmic membrane, and the number of other components they come in contact with, they emerge as truly amazing agents.

In addition to the differences described above, bacterial species vary greatly in the phospholipid composition of their membranes. In general, the membranes of Gram-negative bacteria have a far higher phosphatidylethanolamine (PE) content than those of Gram-positive bacteria, although there are some exceptions.

7.2 Role of Bacterial Membrane Lipid Composition in Antimicrobial Sensitivity

Some antimicrobial agents target specific lipid components of bacterial membranes. An example of this is the AMP nisin, which binds specifically to lipid II (Breukink *et al*., 1999). Duramycin and cinnamycin are two other AMPs of the same lantibiotic class as nisin that specifically target PE (Clejan *et al*., 1989). In addition to drugs that target specific lipids, less specific interactions of antimicrobials with membrane lipids, such as electrostatic or hydrogen-bonding interactions, can also influence the potency and mechanism of action of antimicrobial agents. This can be particularly important because the lipid composition of bacterial membranes varies widely, resulting in certain bacterial species being more susceptible to some antimicrobial agents than others. For example, the selective toxicity of two isomeric α/β-peptides is dependent on the lipid phase preference of the lipids in the bacterial membrane (Epand *et al*., 2005). The toxicity of a cationic steroid compound has been shown to be dependent on the PE content of the membrane (Epand *et al*., 2007). Additionally, the toxicity of certain oligo-acyl-lysines is most potent with bacteria that contain both anionic and either zwitterionic or uncharged lipid in their membrane, so that these agents can promote the clustering of anionic lipid (Epand, R.M. *et al*., 2008). The

Table 7.1. Phosphatidylethanolamine content and CSA-8 minimum inhibitory concentration for several species of bacteria.

Bacteria	Minimum inhibitory concentration ($\mu g\ ml^{-1}$)	Phosphatidylethanolamine content (%)
Gram-negative bacteria		
Proteus mirabilis	500	80
Escherichia coli	36	80
Pseudomonas aeruginosa	20	60
Caulobacter crescentus	5	~0
Gram-positive bacteria		
Bacillus polymyxa	35	60
Bacillus anthracis	20	43
Bacillus cereus	50	43
Bacillus subtilis	0.5	12
Staphylococcus aureus	2	~0
Streptococcus pyogenes	0.5	~0

variation in PE content for several species of bacteria is shown in Table 7.1.

Table 7.1 also shows some exceptions to the general rule. For example, the Gram-negative bacterium *Caulobacter crescentus* has a low PE content while three related Gram-positive bacteria, *Bacillus polymyxa*, *Bacillus anthracis* and *Bacillus cereus*, have a higher content of PE. A variable PE content can also be found in the genus *Clostridium*.

The relationship of PE content to the antimicrobial action of the cationic sterol compound CSA-8 illustrates the importance of membrane phospholipid content to the potency of this antimicrobial agent (Epand *et al*., 2007). This conclusion was further confirmed using a mutant form of *Escherichia coli* that was unable to synthesize PE (Epand *et al*., 2007). This study demonstrates that the lipid composition of the bacterial membrane can be more important in determining sensitivity to an antimicrobial agent than the presence or absence of an outer membrane. This is an example of the more common situation in which Gram-positive bacteria are more sensitive to the action of an antimicrobial agent than Gram-negative bacteria. In the next paragraphs, we describe other cases in which antimicrobial agents can be more toxic to bacteria with a high PE content.

7.3 Antimicrobial Agents that Target the Outer Membrane of Gram-negative Bacteria

In addition to damage to the cytoplasmic membrane that may lead to toxicity, one must also consider a role for the outer membrane of Gram-negative bacteria. There are several ways in which the outer membrane can affect bacterial viability. The outer membrane and cell wall provide structural rigidity to protect the cell against damage caused, for example, by osmotic stress. Several classical antibiotics act by affecting the synthesis of cell wall or outer membrane components.

Another role of the outer membrane can be to act as a barrier for the passage of antimicrobial agents to the plasma membrane. The interaction of negatively charged LPS of the outer membrane with cationic antimicrobial agents can prevent the toxicity of these agents by inhibiting access to the cell membrane. An interesting example of this is the blockage of penetration of the small cationic peptides, the temporins (Mangoni *et al*., 2008). This study showed the dependence of the barrier function of the outer membrane on the length of the polysaccharide chain of LPS. In addition, synergistic action between pairs of temporin molecules was demonstrated.

Recently, a novel mechanism of microbial toxicity has been proposed in which antimicrobial agents act by blocking the passage of polar molecules across the outer membrane. In this manner, the agent can inhibit growth of the organism without accessing or damaging the plasma membrane. Elucidation of this mechanism was facilitated by the availability of a strain of *E. coli*, ML-35p, specially constructed to simultaneously monitor the passage of chromogenic substrates across the inner and outer membranes (Lehrer *et al*., 1988). An example of an antimicrobial agent that blocks the flux of small molecules across the outer membrane of *E. coli* was a flexible sequence-random polymer containing cationic and lipophilic subunits, which acts as a functional mimic of host-defence peptides (Epand, R.F. *et al*., 2008). At low concentrations the polymer permeabilizes the outer and inner membranes; at higher polymer concentrations, however, permeabilization of the outer membrane is progressively diminished, while the inner membrane remains unaffected. A similar mechanism has also been proposed to explain the mechanism of the action of a miniature oligo-acyl-lysine against Gram-negative bacteria (Epand *et al*., 2009a).

7.4 Antimicrobial Agents that Promote Clustering of Anionic Lipids

7.4.1 Evidence for lipid clustering

The ability of certain antimicrobial agents to cluster anionic lipids has been demonstrated by several methods using mixtures of lipids corresponding to those found in bacterial membranes. The results of these analyses allow the prediction of which bacterial species will be most susceptible to the action of a particular antimicrobial agent based on the lipid composition of the bacterial membrane.

We have used differential scanning calorimetry (DSC) to assess anionic lipid clustering (Epand, 2007). The advantages of this method are that it has broad applicability and does not require the introduction of probes that can perturb the system. The basis of the method is that one chooses two lipids that form a homogeneous mixture, giving rise to a single-component phase transition. This places a limit on the lipids that can be used in that they must be miscible and have a gel to liquid crystalline-phase transition temperature >0°C and below the temperature of denaturation or membrane dissociation of the antimicrobial agent. For the purpose of testing for the ability of a compound to cluster anionic lipid in the presence of zwitterionic lipids, we have frequently used mixtures of tetraoleoyl-cardiolipin (TOCL) and 1-palmitoyl-2-oleoyl-PE (POPE). PE and cardiolipin (CL) are both major lipid components of bacterial membranes and both have headgroups with hydrogen-bonding capabilities. PE is zwitterionic, while CL is anionic. Cationic antimicrobial agents would be expected to bind preferentially to the anionic CL component of this mixture. If the preference for interacting with CL over PE is great enough, then the cationic agent will promote phase separation between the two lipids. In some, but not all, cases the CL-rich domain can be observed at a lower temperature since TOCL has a phase transition below 0°C. However, there are cases in which the transition of the domain enriched in CL is not observed because it is shifted or broadened as a result of binding the cationic agent. POPE in pure form has a transition at 25°C and, since a less cationic antimicrobial agent will bind to the domain enriched in this lipid, the transition temperature of the membrane domain enriched in POPE will be higher than that of the initial pure lipid mixture and closer to that of pure POPE. In general, the transition should be reversible on heating and cooling. However, in some cases the cationic agents interact more rapidly with the lipid in the liquid crystalline state and therefore larger temperature separations of high and low melting components are observed in cooling scans compared with heating scans.

The evidence from DSC can be further tested using spectroscopic methods. Nuclear magnetic resonance (NMR) has provided evidence for lateral phase separation. A useful NMR method for membrane samples is magic angle spinning NMR (MAS/NMR). This version of solid-state NMR allows one to

acquire well-resolved spectra using high molecular weight membrane samples. A convenient isotope to use for the study of membranes is ^{31}P. There are few phosphates, usually only one, in most phospholipids. The paramagnetic isotope of phosphorous, ^{31}P, has a natural abundance of almost 100% and ^{31}P has a spin quantum number of ½, resulting in it not being broadened by nuclear-quadrupole coupling. There have been two applications of ^{31}P MAS/NMR to test the formation of anionic lipid clusters with antimicrobial agents (Epand, R.M. *et al*., 2008; Epand *et al*., 2009b). Even in lipid mixtures that appear to mix homogeneously, ^{31}P MAS/NMR still allows the separate identification of each component of the mixture if they have sufficiently different chemical shifts. Although the chemical shift of each lipid component will be towards each other as a result of the presence of the other lipid in the environment, the two peaks are still resolvable (Epand, R.M. *et al*., 2008; Epand *et al*., 2009b). If clustering of anionic lipid occurs, the isotropic chemical shift of the zwitterionic component will be more towards that of the pure zwitterionic component. The line width of each peak is a second independent parameter that can also be used to test for demixing. The line width of the anionic component increases as a result of slower motion after binding the cationic antimicrobial agent, while that of the zwitterionic component changes from its value in the lipid mixture to that found for the pure zwitterionic component. This analysis has also been performed as a function of temperature to show the generality of the result when both components are in the liquid crystalline phase.

Another NMR approach makes use of ^{2}H-NMR to measure order parameters of deuterated lipids (Jean-Francois *et al*., 2008). Anionic and zwitterionic lipid components can be separately studied by deuteration of one of the components of the pair. The results suggest that the zwitterionic component experiences two different environments that are in slow exchange. One of the components resembles that of the pure zwitterionic component. These results are also in accord with the clustering of anionic lipid to produce a domain enriched in zwitterionic lipid. Deuteration can also be used to distinguish one lipid from another in a mixture using Fourier transform infrared spectroscopy (Arouri *et al*., 2009). Deuteration is performed to separate the absorption frequencies of the deuterated and protonated components. The CH_2 stretching vibrations are sensitive to the conformation of the lipid acyl chains, and can be used to follow the changes in the *trans* to *gauche* ratio. For the protonated component, the frequencies of the symmetric and antisymmetric CH_2 stretching vibrations are at approximately 2850 cm^{-1} and 2929 cm^{-1}, respectively. With this method, the antimicrobial cationic peptide RRWWRF has been shown to cause segregation of anionic and zwitterionic lipids only in the gel phase with dimyristoyl-phosphatidylcholine (DMPC)/dipalmitoyl-phosphatidylglycerol (DPPG) mixtures, while with dipalmitoyl-phosphatidylethanolamine (DPEE)/DPPG – a lipid mixture more representative of bacterial membranes – phase separation was observed in both liquid and solid phases (Arouri *et al*., 2009).

All of the above methods have their advantages and limitations. However, they are all indirect with regard to specifying the morphological properties of the samples. Some imaging methods have also been applied that give information about the size and properties of the domains. A recent study using atomic force microscopy in combination with polarized fluorescence microscopy showed the formation of domains in model membranes using a small cationic AMP, PFWRIRIRR-amide (Oreopoulos *et al*., 2010). This study showed the time evolution of the formation of these domains. In addition, freeze-fracture electron microscopy has provided evidence for the formation of domain structures with this same cationic AMP (Epand, R.M. *et al*., 2010). There is thus direct evidence from imaging studies, for the peptide PFWRIRIRR-amide, that membrane domains are formed.

7.4.2 Bacterial species specificity of toxicity

The ability of certain antimicrobial agents to cluster anionic lipids is of particular interest

because it explains the selective toxicity of these agents towards certain bacterial species. If an antimicrobial agent has greater affinity for one lipid species over another, this is sufficient to promote segregation of lipid components. This can happen even with a mixture of two lipids with different anionic headgroups, as we have shown with cationic amphipathic helical peptides that can segregate CL from phosphatidylglycerol (PG) (Epand, R.F. *et al*., 2010); however, this phenomenon is not associated with the specificity of toxicity for different bacterial species. In contrast, when an anionic lipid is clustered away from lipids that are zwitterionic or have no overall charge, the result is a bacteriostatic action. The reason why these two different kinds of lipid clustering have different consequences for bacterial toxicity is not known, but may reflect differences in the size of the domains that are formed or the nature of the phase boundary between domains.

The sequestering of anionic lipids away from ones with no overall charge will occur only in bacterial species that contain significant amounts of zwitterionic or uncharged lipids. All bacterial species contain large fractions of anionic lipids, generally in the form of PG and CL. However, their content of zwitterionic or uncharged lipids varies widely, from close to 0% to about 85%. The most common zwitterionic lipid is PE. In addition, some bacteria have species of aminoacyl-PG, most of which are zwitterionic, as well as zwitterionic lysyl-CL. Uncharged glycosyl-diacylglycerols may also be present. However, few bacterial species have a large amount of this lipid as a free polar lipid component of the plasma membrane, an exception being *Streptococcus pyogenes* in which some of the membrane lipids are free glycosyl-diglycerides (Shaw, 1970; Rosch *et al*., 2007). It should also be pointed out that glycosyl-diglycerides are also components of lipoteichoic acid (LTA), a major component of the cell wall of Gram-positive bacteria. However, this glycosyl-diglyceride is not usually free as a phospholipid component of the cell membrane, but rather is a covalently linked moiety of the cell wall.

Unlike mammalian membranes, which have very low amounts of cationic lipids, the principal one being sphingosine, bacteria can have large amounts of cationic lipids. The separation of cationic and anionic lipids by cationic antimicrobial agents would not be very efficient because the cationic lipid would prevent the cationic peptide from binding to the membrane. A cationic lipid that is frequently found in bacterial membranes is lysyl-PG. It has been shown that the presence of cationic lipids is a factor in increasing the resistance of bacteria to cationic antimicrobial agents (Peschel *et al*., 2001; Camargo *et al*., 2008). However, at least in the case of *Staphylococcus aureus*, most of this lipid is found on the cytoplasmic surface of the plasma membrane. Resistance to antimicrobial agents occurs in those strains of *S. aureus* in which the lipid is translocated to the cell exterior by a mechanism facilitated by a membrane protein (Ernst *et al*., 2009; Mishra *et al*., 2009). Although the membrane-sidedness of lysyl-PG in *S. aureus* has been determined, in general there is little information about the sidedness of membrane lipids in bacteria.

We will now summarize the ratio of the minimal inhibitory concentration (MIC) against *S. aureus* and *E. coli*. These two bacterial species represent a species with a low content of neutral and zwitterionic lipids (*S. aureus*) and another with a high content of the zwitterionic lipid PE (*E. coli*; see Table 7.1). We have chosen these two species as representative because they are commonly used and there is more information about MICs with these bacteria. We recognize that there is a potential complication with *S. aureus* because some strains contain lysine-PG, but since this lipid is generally on the cytoplasmic side of the membrane, we assume it will not greatly affect the measured values of the MIC. Thus, *S. aureus* represents a bacteria that cannot segregate anionic from zwitterionic or charged lipids, while *E. coli* can segregate these two types of lipids. An antimicrobial agent that acts largely by the lipid clustering mechanism will thus be more toxic to *E. coli* than to *S. aureus* and hence have a high ratio of *S. aureus* MIC to *E. coli* MIC. These two bacteria also differ in that *E. coli* is a Gram-negative bacterium with an outer membrane, while *S. aureus* is a Gram-positive bacterium.

Another factor lowering the ratio of *S. aureus* MIC to *E. coli* MIC may be a result of the outer membrane of *E. coli* not allowing passage of the antimicrobial agent.

The MSI peptides are examples of cationic amphipathic helical AMPs (Table 7.2). They are representative of a large group of AMPs. MSI-103 and MSI-469 have identical sequences, but MSI-469 also has an N-terminal octyl group. The results indicate that the octyl group does not have a major effect on antimicrobial activity. MSI-843 and MSI-1254 are also *N*-octyl peptides, the difference between them being that MSI-843 has ornithine as its cationic residue, while MSI-1254 has diaminobutyric acid. We have shown that MSI-1254 does not cross the outer membrane of *E. coli* (Epand, R.F. *et al*., 2010). This explains the relatively high MIC for this bacteria among the MSI peptides and also the low ratio of 1 for the MICs against the two different bacteria. The ratio of MIC *S. aureus*/MIC *E. coli* is >1 for all of the other MSI peptides.

KR-12, GF-17 (Table 4.2, Chapter 4) and GF-17 D3 are fragments of the human cathelicidin peptide LL-37. KR-12 is the shortest fragment of LL-37 possessing antimicrobial activity (Wang, 2008). It is capable of clustering anionic lipids and shows specificity for *E. coli* relative to *S. aureus* (Table 7.2). The longer LL-37 fragment, GF-17, does not have this specificity in microbial toxicity. This is because GF-17 is more potent against *S. aureus* and not because it is less potent against *E. coli*. The lack of selective toxicity suggests that GF-17 is not acting principally by a charge cluster mechanism. This is supported by our findings that GF-17, but not KR-12, can promote the release of entrapped dye from liposomes (Epand *et al*., 2009b). Interestingly, when three D-amino acid residues are introduced at different locations in GF-17,

Table 7.2. Specificity of antimicrobial agents and charge.

Agent	Charge[a]	Charge/ residue	MIC[b] (μM) for *Escherichia coli*	Ratio of *Staphylococcus aureus* MIC to *E. coli* MIC	Reference
MSI-78	10	0.45	2.5	2	Epand, R.F. *et al*. (2010)
MSI-103	7	0.33	0.8	15.6	Epand, R.F. *et al*. (2010)
MSI-469	6	0.29	1.5	4	Epand, R.F. *et al*. (2010)
MSI-843	6	0.6	2	4	Epand, R.F. *et al*. (2010)
MSI-1254	6	0.6	7.6	1	Epand, R.F. *et al*. (2010)
KR-12	6	0.5	66	>4	Epand *et al*. (2009b)
GF-17	6	0.35	14	0.5	Epand *et al*. (2009b)
GF-17 D3	6	0.35	32	>8	Epand *et al*. (2009b)
PR-9	5	0.56	10	32[c]	Epand, R.M. *et al*. (2010)
RR-9	5	0.56	80	64[c]	Epand, R.M. *et al*. (2010)
PI-9	5	0.56	3.5	8[c]	Epand, R.M. *et al*. (2010)
Cateslytin	5	0.33	30	>3	Radek *et al*. (2008)
$C_{12}K\text{-}7\alpha_8$	8	1[d]	3.1	16	Rotem *et al*. (2008)
$C_{12(\omega7)}K\text{-}\beta_{12}$	3	1[d]	>50	<0.1	Epand *et al*. (2009a)
Magainin 2	3.5	0.15	40	>1	Epand, R.F. *et al*. (2010)

[a] Estimated for pH 7 using + ½ for histidine.
[b] MIC, minimum inhibitory concentration.
[c] Ratio of Gram-positive bacteria without phosphatidylethanolamine (*S. aureus*) to Gram-positive bacteria with phosphatidylethanolamine (*Bacillus megaterium*).
[d] Not a peptide, but an oligo-acyl-lysine.

the new peptide, GF-17 D3, acquires bacterial species specificity of its antimicrobial action. This substitution destroys the amphipathic helix and GF-17 D3 no longer promotes dye release from liposomes and consequently loses its toxicity against *S. aureus*. We have suggested that, in general, an increase in the conformational flexibility of a peptide facilitates the charge cluster mechanism (Epand and Epand, 2009). This is in accord with the observation that the charge cluster mechanism is more important for GF-17 D3 than for GF-17. NMR studies also show that the amphipathic helix of GF-17 D3 is largely destroyed (Li *et al*., 2006).

The peptides PR-9, RR-9 and PI-9 are all cationic nonapeptides. The three have been shown by DSC to cluster anionic lipids (Epand, R.M. *et al*., 2010) and the resulting domains that are formed with PR-9 have been imaged by freeze-fracture electron microscopy (Epand, R.M. *et al*., 2010) as well as by atomic force microscopy and polarized total internal reflection fluorescence microscopy (Oreopoulos *et al*., 2010). From circular dichroism (Epand, R.M. *et al*., 2010) and NMR (Zorko *et al*., 2009) measurements, as well as the short length of the peptides, it is likely that they are conformationally flexible even when bound to a membrane. These peptides were shown to bind to the LPS layer in Gram-negative bacteria. Therefore, the charge cluster mechanism was assessed in Gram-positive bacteria containing mostly anionic lipids in their cytoplasmic membrane versus those containing large amounts of PE or uncharged lipids (Table 7.2). The ratio of the MICs for two bacterial genera within the same species was found to be dramatically high, reflecting the absence of an outer membrane.

Cateslytin has been shown by ^{2}H-NMR to cluster anionic lipids (Jean-Francois *et al*., 2008) and this peptide also exhibits specificity in its antimicrobial action.

C_{12}K-7α_8 is an oligo-acyl-lysine, a large group of antimicrobial agents, many of which exhibit significant selective toxicity towards certain bacteria (Livne *et al*., 2009; Rotem and Mor, 2009; Zaknoon *et al*., 2009; Sarig *et al*., 2010). C_{12}K-7α_8 has been shown to cluster anionic lipids (Epand, R.M. *et al*., 2008) and it has a very high ratio of *S. aureus* MIC/*E. coli* MIC (Table 7.2). In contrast, a shorter oligo-acyl-lysine, $C_{12(\omega7)}$K-β_{12}, does not cluster anionic lipids and has a low ratio of *S. aureus* MIC/*E. coli* MIC because it deposits on the LPS layer of Gram-negative bacteria (Epand *et al*., 2009a).

7.4.3 Putative mechanism of action

It would be expected that the recruitment of anionic lipids to a region of the bacterial membrane would result in the concentration of negative charge in a domain to which cationic peptides would congregate, possibly causing the formation of a pore. However, the pore that is formed is not very large. Antimicrobial agents that cause clustering do not cause leakage of dyes from membrane mimetic liposomes of bacterial cytoplasmic membranes and are not toxic to *S. aureus*. However, they may breach the membrane barrier to a limited extent, causing slow leakage of contents and/or depolarization of the membrane. Evidence for this comes from studies of C_{12}K-7α_8, one of the best examples of an antimicrobial agent causing clustering. It has been shown with C_{12}K-7α_8 that, in the presence of EDTA added to allow the dye $DiSC_3(5)$ to reach the inner membrane, the cytoplasmic membrane of *E. coli* is depolarized (Radzishevsky *et al*., 2007) and there is even some slow influx across the cytoplasmic membrane of *E. coli* ML-35 of small organic molecules (Rotem *et al*., 2008). This breach of the membrane barrier may be sufficient to cause the bactericidal effect.

Furthermore, in addition to concentrating the cationic antimicrobial agent, the domain of anionic lipids would be surrounded by an interface with the rest of the membrane that would be under line tension and be less stable. Of course, it is likely that there are always domains in bacterial membranes (Matsumoto *et al*., 2006) that have phase boundary defects. However, those that form in the presence of lipid-clustering antimicrobial agents form very rapidly (Oreopoulos *et al*., 2010) and the bacteria would not have time to compensate for this rearrangement.

In addition to this mechanism, there can also be damage to the bacteria as a result of the redistribution of lipids in the membrane. This may disrupt existing natural domains in the membrane or decrease the available anionic lipid required for specific protein functions.

7.4.4 Properties of antimicrobial agents that favour clustering

Some antimicrobial agents are more effective than others in promoting the clustering of anionic lipids. A sufficient number and density of positive charges are required. By density of positive charges, we are referring to the net number of positive charges divided by the total number of residues. Magainin 2 is not effective in clustering anionic lipids, although a very similar peptide, MSI-78, can induce clustering (Epand, R.F. *et al*., 2010). We suggest that the difference between these two peptides is a consequence of the much greater density of positive charges on MSI-78 compared with magainin.

Another important property is the ability to pass the outer membrane of Gram-negative bacteria. This allows for a wider range of bacterial species to be tested for the correlation between *in vitro* clustering of anionic lipids and increased toxicity to bacteria that are rich in both anionic and zwitterionic lipids, as are most Gram-negative bacteria that have a high content of PE (with some exceptions, such as *C. crescentus*). More hydrophobic compounds, such as the oligo-acyl-lysines, appear more capable of crossing the outer membrane than short, arginine-rich peptides (Epand, R.M. *et al*., 2010).

Conformational flexibility is another property associated with an increased ability to cluster anionic lipids. This has been discussed above in comparing the antimicrobial activity of GF-17 with GF-17 D3.

7.4.5 Sources of energy to account for clustering and entropy of demixing

Many cationic AMPs are not structured in solution, but acquire an α-helical conformation in the presence of anionic lipids. This transformation provides –0.4 kcal mol^{-1} residue of free energy. If the process of helix formation were to be coupled with lipid phase segregation, the energy provided by helix formation could contribute to overcoming the unfavourable energy of mixing required to segregate the lipid components. In addition, insertion of aromatic residues or an acyl chain of a lipopeptide can also supply the necessary free energy for cluster formation and stabilization. In general, the formation of any kind of non-covalent bonding, either within the peptide or lipid or between the lipid and peptide as a consequence of the interaction of the peptide with lipid, could be coupled with the unfavourable energy of lipid clustering. Non-covalent interactions could include an increase in the number of hydrogen bonds in addition to those accounted for by helix formation or salt bridges, such as those between arginine residues and the phosphate groups in the associated lipids. The relative importance of these various forces will vary from one system to another.

7.5 Concluding Remarks and Perspectives

The mechanism of clustering anionic lipids is a component of the mechanism of action of many antimicrobial agents, including oligo-acyl-lysines, some amphipathic helical peptides and small, arginine and lysine-rich peptides. This phenomenon is more important for antimicrobial agents that have a high number and density of positive charges, that are conformationally flexible and that are sufficiently hydrophobic to penetrate the outer membrane of Gram-negative bacteria. Several factors contribute to the importance of this mechanism in the design of agents for clinical application. Optimizing the potency and effectiveness of agents that promote this phenomenon will allow targeting of antimicrobial agents to specific strains of bacteria. In addition, it may provide a mechanism of bactericidal action that does not involve the lysis and disruption

of bacteria and may therefore have fewer toxic side effects when applied to humans, allowing for faster clearance of bacteria by macrophages.

Further studies are required to fully elucidate how lipid clustering leads to bacteriostatic effects, as well as to enhance our understanding of the relationship between membrane domains normally present in bacteria and those that are formed as a result of the interaction of an antimicrobial agent with a bacterial membrane. In addition, there has been interest in cationic antimicrobial agents that can function as anticancer agents. The role of clustering of exposed phosphatidylserine in cancer cells by antimicrobial agents should be investigated.

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8 Non-membrane Targets of Antimicrobial Peptides: Novel Therapeutic Opportunities?

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Abstract

The emergence and rapid spread of multiresistant bacteria necessitates every effort to develop new classes of antibiotics with novel targets and modes of action. One potential source of novel antibiotics is the cationic antimicrobial peptides (AMPs), which constitute an important component of the innate immune system in a variety of organisms. Most AMPs exert their activity by interacting with bacterial membranes, thus perturbing their permeability. However, an increasing number of peptides are being described that translocate across the bacterial membranes and act on intracellular targets in bacteria. These non-membrane-active AMPs have been shown to bind and inactivate intracellular biopolymers such as nucleic acids and proteins without destroying or remaining attached to the bacterial membranes. As such, they have emerged as viable candidates for the treatment of human infections. In this chapter, we focus on the six well-characterized, non-membrane-active AMPs (buforin II, PR-39, indolicidin, apidaecin, drosocin and pyrrhocoricin) and discuss whether binding of these peptides to their intracellular targets correlates with bacterial cell death. The potential exploitation of these peptides as human therapeutics is also discussed.

8.1 Introduction

Of worldwide concern is the increasing development of bacterial and fungal strains that are resistant to currently available antimicrobial drugs. This worsening situation has spurred herculean efforts to develop new classes of antibiotics with novel targets and modes of action (Makovitzki *et al*., 2006). One potential source of novel antibiotics is the cationic antimicrobial peptides (AMPs), which are produced by all species of life and play a key role in primary host defence against infection by pathogenic micro-organisms (Boman, 1995; Hancock and Scott, 2000; Zasloff, 2002). AMPs are derived through the proteolysis of precursor proteins/peptides that are encoded by the host genome and synthesized on ribosomes. These defence peptides are short (10–50 amino acids) and contain an overall positive charge (in general, +2 to +9) and a substantial proportion (>30%) of hydrophobic residues (Hancock and Sahl, 2006). These properties permit AMPs to fold into amphipathic α-helix and/or β-sheet structures upon contact with the negatively charged bacterial membranes. These structures can then insert themselves into the membranes of infectious particles and create pores by 'barrel-stave', 'carpet' or 'toroidal-pore' mechanisms (see Fig. 5.1, Chapter 5), which results in the

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dissipation of transmembrane potential and subsequent cell death (Oren and Shai, 1998; Shai, 2002; Huang *et al*., 2004).

The targeting of bacterial membranes by AMPs is mainly composed of two steps: the initial binding of the peptides to the cell surface and subsequent membrane permeabilization (Huang, 2000, 2006). The mechanism by which AMPs cause bacterial cell death usually does not involve binding to specific receptors on the cell membrane, but is rather a non-specific interaction with membrane phospholipids (Yeaman and Yount, 2003). The surface of bacterial cells is composed of negatively charged components, such as lipopolysaccharide and teichoic acids, and an electrostatic interaction between the cationic AMPs and the negatively charged bacterial cell surface plays an important role in their antibacterial activity (Matsuzaki, 1999). Although the formation of ion channels and transmembrane pores and extensive membrane rupture eventually leads to the lysis of bacterial cells (Steiner *et al*., 1988; Wimley *et al*., 1994; Ludtke *et al*., 1995), not all AMPs are thought to exert their major action on bacterial membranes. It has been shown that the ability of various AMPs to depolarize the cytoplasmic membrane potential of *Escherichia coli* varies widely, with certain peptides being unable to cause depolarization at the minimal inhibitory concentration (MIC), while others cause maximal depolarization below the MIC (Wu *et al*., 1999). For all of the peptides, representing most of the structural classes, there is no correlation between the concentrations leading to complete membrane permeabilization and the MIC.

In fact, an increasing number of peptides are being described that translocate across bacterial membranes and act on intracellular targets in bacterial cells. They may inhibit protein or cell-wall synthesis, interact with DNA or RNA or inhibit some sort of enzymatic activity (Cudic and Otvos, 2002; Markossian *et al*., 2004; Brogden, 2005; Otvos, 2005; Cho *et al*., 2009). Most of these non-membrane-active peptides, except for buforin II, which contains a single proline residue in a mid-chain position, over-represent proline and adopt a non-α-helical extended structure (Table 8.1). Proline is an amino acid that creates a rigid bend in the peptide backbone since the carboxyl and amino groups of this backbone are covalently bonded. Thus, proline is generally accepted as a helix breaker (Williamson, 1994). The influence of proline residues on the structure and activity of α-helical peptides has been demonstrated by a systematic structure–activity relationship study using peptide analogues without proline, or with one or two proline residues (Zhang *et al*., 1999). The percentage helicity of the peptides and their ability to permeate the cytoplasmic membrane of bacteria decrease as a function of the number of proline residues incorporated, suggesting the existence of intracellular targets. This does not completely

Table 8.1. Amino acid sequences of select non-membrane-active AMPs.

Peptide	Amino acid sequence[a]	Intracellular targets in bacteria	Mode of action
Buforin II	TRSSRAGLQF**P**VGRVHRLLRK	Nucleic acids (DNA and RNA)	Inhibition of transcription/ translation
PR-39	RRR**P**R**PP**YL**P**R**P**R**PPP**FF**PP**RL**PP**RI**PP** GF**PP**RF**PP**RF**P**	Nucleic acids?[b]	Inhibition of cell division
Indolicidin	IL**P**WKW**P**WW**P**WRR-NH_2	DNA	Inhibition of replication leading to filamentation
Apidaecin 1a	GNNR**P**VYI**P**Q**P**R**PP**HRRI	DnaK	Inhibition of DnaK-assisted protein folding
Drosocin	GK**P**R**P**YS**P**R**P**T*SH**P**R**P**IRV	DnaK	Inhibition of DnaK-assisted protein folding
Pyrrhocoricin	VDKGSYL**P**R**P**T***PP**R**P**IYNRN	DnaK	Inhibition of DnaK-assisted protein folding

[a] Proline residues are in bold and *O*-glycosylated threonine residues are indicated with asterisks.
[b] Interaction of PR-39 with nucleic acids has not been directly confirmed.

exclude some type of interaction with the bacterial membrane, and supports a model in which penetration across the membrane is followed by binding to and inhibition of functional intracellular end molecules (Otvos, 2002). In this chapter, we focus on the six well-characterized, non-membrane-active AMPs (buforin II, PR-39, indolicidin, apidaecin, drosocin and pyrrhocoricin) and discuss whether binding of these peptides to their intracellular targets correlates with bacterial cell death. The potential exploitation of these peptides as human therapeutics is also reviewed.

8.2 Buforin II: α-Helical AMP with Single Proline Residue Binding to Nucleic Acids

Buforins are α-helical AMPs isolated from the stomach tissue of the Asian toad *Bufo bufo gargarizans*. They display a broad spectrum of antimicrobial activity against bacteria and fungi (Park *et al*., 1996). Buforin I is a 39-amino acid peptide with complete sequence identity to the N-terminal region of histone H2A, which specifies the protein's DNA-binding activity. Buforin II is a 21-residue peptide that is produced from buforin I by treatment with the endoproteinase Lys-C. Buforin II represents an interesting example of an α-helical AMP because it employs a unique mechanism of activity that does not involve membrane perturbation (Haney *et al*., 2009).

The structure of buforin II was determined using nuclear magnetic resonance (NMR) spectroscopy and restrained molecular dynamics (Yi *et al*., 1996). Buforin II adopts a helix-hinge-helix structure in 50% trifluoroethanol; the N-terminal extended α-helix (residues 5–10) and the C-terminal α-helix (residues 12–21) are separated by a proline residue at amino acid position 11. The four N-terminal residues are relatively unstructured. Interestingly, the Pro11 residue distorts the C-terminal helix in such a way as to maximize the amphipathic nature of the C-terminal helix. This originally led researchers to postulate that buforin II interacts with bacterial membranes in a similar fashion to other amphiphatic α-helical AMPs. However, the mechanism of antimicrobial action of buforin II proved to be different from those of AMPs that function by membrane permeabilization. Using fluorescein isothiocyanate-labelled buforin II and gel-retardation experiments, it was revealed that buforin II kills bacteria without cell lysis and has a strong affinity for DNA and RNA *in vitro* (Park *et al*., 1998). Kobayashi *et al*. investigated the interaction of buforin II with phospholipid membranes and compared these results with those of similar experiments with magainin 2 (Kobayashi *et al*., 2000). These researchers used equipotent tryptophan-substituted peptides to fluorometrically monitor peptide–lipid interactions. Control circular dichroism (CD) studies showed that, like magainin 2, buforin II binds selectively to liposomes composed of acidic phospholipids. However, the fluorometric experiments revealed that, in contrast to magainin 2, buforin II translocates across liposome membranes efficiently without inducing significant membrane permeabilization or lipid flip-flop. Furthermore, the Pro11 residue, which induces a kink in the buforin II α-helix, is the key structural feature required for buforin II's unique properties.

A subsequent study by Kobayashi *et al*. (2004) revealed that buforin II crosses lipid bilayers in a manner similar to that of magainin 2 – via the transient formation of a peptide–lipid supramolecular complex (toroidal) pore. However, the presence of Pro11 distorts the helical structure of buforin II, concentrating five basic amino acid residues in a limited amphipathic region (Arg5–Lys21); this structure destabilizes the pore by enhanced electrostatic repulsion and enables efficient translocation of buforin II into the cell. The importance of the Pro11 residue was also demonstrated in a systematic structure–activity relationship study (Park *et al*., 2000). In this study, antimicrobial potencies, secondary structures and mechanisms of bacterial killing action were assessed for a series of structurally altered synthetic buforin II analogues. The results revealed that the proline hinge (Pro11) is a key structural factor for the cell-penetrating property without permanent membrane association, while the cell-penetrating efficiency, which depends on α-helical content, is a critical factor for determining the

antimicrobial potency of buforin II. Indeed, these experiments showed that only a single amino acid substitution at the Pro11 position changes buforin II into a membrane-active magainin-like peptide. Conversely, insertion of a proline hinge region (Arg5–Gly11) into the N-terminal helix of magainin 2 switches this AMP from a membrane-permeabilizing peptide to a cell-penetrating one.

Because buforin II was shown to bind nucleic acids *in vitro*, it has been hypothesized that buforin II kills bacteria by interacting with their nucleic acids after translocation across the cell membrane (Park *et al*., 1998). Although the proposed mechanism is quite intriguing, many questions remain to be answered. The connection between nucleic acid binding and antimicrobial activity has not been demonstrated directly, and it is unclear whether buforin II and nucleic acids interact in a specific manner or whether they only bind to each other because of their opposite net charges. Uyterhoeven *et al*. (2008) recently characterized the nucleic acid binding properties of buforin II using molecular modelling and a fluorescent intercalator displacement assay. These researchers observed that, in addition to non-specific electrostatic attractions between a cationic peptide and nucleic acids, specific side chains (Arg2 and Arg20) of buforin II form interactions with DNA that are stronger than the non-specific electrostatic ones. They also showed that disruption of the buforin II–DNA interactions by substituting basic residues of buforin II with alanine generally decreases the antimicrobial activity of buforin II. Moreover, we recently found that buforin II dose-dependently inhibits the transcription and translation of the *lacZ*α gene of pUC19 *in vitro* (unpublished data, 2010). In addition, when *E. coli* harbouring pUC19 is treated with buforin II at concentrations below the MIC, the amount of lacZ expressed, which is determined by β-galactosidase assay, decreases as the concentration of buforin II increases, suggesting that buforin II inhibits transcription or translation in bacteria. Taken together, these results support the assertion that buforin II kills bacteria by translocation into the cell and inhibition of transcription or translation through interaction with nucleic acids, although it does not preclude buforin II from having another as yet unidentified intracellular target.

With its primarily intracellular target inhibition and ability to move between various modes of action, buforin II derivatives are ideal candidates for antimicrobial drug development. However, poor protease stability of short AMPs such as buforin II severely limits their therapeutic value. Recently, Meng and Kumar (2007) reported that incorporation of hexafluoroleucine at selected sites (Leu18 and Leu19) of buforin II results in a simultaneous increase in potency and resistance to protease degradation. This observation suggests that fluorination may be an important strategy for increasing the metabolic stability of buforin II. In addition to antimicrobial activity, buforin II and buforin IIb – a synthetic analogue of buforin II that contains a proline hinge between the two α-helices and a model α-helical sequence at the C-terminus (3×RLLR) – selectively targets cancer cells through interaction with the cell-surface gangliosides. Buforin IIb then traverses cancer cell membranes without damaging them and induces mitochondria-dependent apoptosis (Lee *et al*., 2008). Buforin IIb also displays powerful cytotoxic activity when injected into solid tumours in p53-deficient mice (Cho *et al*., 2009). These results suggest that buforin IIb may be developed into a novel therapeutic agent for the treatment of cancers.

8.3 PR-39 and Indolicidin: Mammalian Proline-rich Peptides Inhibiting Macromolecule Synthesis and Cell Division

PR-39 is a 39-amino acid cathelicidin-derived AMP isolated from porcine small intestine and neutrophil azurophilic granules. It is rich in proline (49%) and arginine (24%) (Agerberth *et al*., 1993; Shi *et al*., 1994). Within its primary sequence, PR-39 is characterized by seven repeats of Xaa-Pro-Pro-Xaa. CD and Fourier-transform infrared spectroscopy performed with PR-39, either in aqueous solution or in the presence of lipid vesicles, have suggested that they adopt a disordered left-handed polyproline II helix structure

because of the proline residues disposed consecutively in the linear peptide chain (Cabiaux *et al*., 1994). PR-39 has antibacterial activity against several strains of both Gram-positive and Gram-negative bacteria (Bevins, 1994). The mechanism of PR-39 bactericidal activity has been investigated by isotope incorporation experiments (Boman *et al*., 1993). In contrast to the killing by membrane disruption seen with most other AMPs, Boman and colleagues found that bacteria are killed by PR-39 through a mechanism that stops protein and DNA synthesis. PR-39 requires a lag period of about 8 min to penetrate the outer membrane of wild-type *E. coli*, while subsequent killing is quite fast. Kinetic data suggest induced proteolysis in the target bacteria may be important for bactericidal activity. Consistent with this suggestion is the finding that actively growing cells are killed more rapidly than non-growing cells. In addition, Shi *et al*. (1996a) found from a scanning electron micrographic study that PR-39 and its N-terminal 1–26 fragment, PR-26, induce filamentation of *Salmonella typhimurium* without forming pores on the bacterial outer membrane. Cells exposed to these peptides have an extremely elongated morphology, which indicates that the peptide-treated cells are unable to undergo cell division.

Unlike with buforin IIb and other cell-penetrating AMPs, the translocation of PR-39 into the bacterial cell and its subsequent interaction with intracellular target molecules such as nucleic acids have not yet been confirmed. In addition, a study on the secondary structure and membrane interaction of PR-39 showed that its secondary structure is not altered upon incubation of the peptide with negatively charged vesicles and that nearly all of the added peptide is membrane bound (Cabiaux *et al*., 1994). Based on these results, Shi *et al*. (1996a) suggested that PR-39 and PR-26 bind to molecules anchored on the outer membrane of Gram-negative bacteria and that these molecules are necessary for formation of the septum. However, multiple cellular targets, including proteins containing PR-39-binding domains, have been identified in eukaryotic cells, and PR-39 has been shown to enter eukaryotic cells and regulate many biological processes in which these targets are directly involved (Gennaro *et al*., 2002; Lehrer and Ganz, 2002; Zanetti, 2004; Sang and Blecha, 2009). Taken together, it is not clear whether bacterial cell filamentation caused by PR-39 and PR-26 is due to the blocking of DNA replication through DNA binding or the inhibition of membrane proteins involved in septum formation.

In addition to antimicrobial properties, PR-39 exerts multiple and diverse actions on mammalian cells. PR-39 has been found to induce syndecan expression in mesenchymal cells, and to influence cell motility and metastatic potential in wound repair (Gallo *et al*., 1994). The peptide binds to the cytosolic component of NADPH oxidase complex protein p47phox (Shi *et al*., 1996b) and a signalling adaptor protein p130Cas (Chan and Gallo, 1998), suggesting an important role for this peptide in inflammation. PR-39 also plays a critical role in limiting cardiac injury through induction of angiogenesis (Li *et al*., 2000; Bao *et al*., 2001; Gaczynska *et al*., 2003) and inhibition of apoptosis in hypoxic endothelial cells (Ramanathan *et al*., 2004; Wu *et al*., 2004). These results underscore the therapeutic potential of PR-39 in a number of diseases, including inflammation, ischemia–reperfusion injury and heart diseases.

Indolicidin is isolated from the cytoplasmic granules of bovine neutrophils. It has a unique composition consisting of 39% tryptophan and 23% proline, and the native peptide is amidated at the C-terminus (Selsted *et al*., 1992). Indolicidin is the smallest of the known naturally occurring linear AMPs, contains the highest percentage of tryptophan of any known protein and consists of only six different amino acids. Owing to the presence of tryptophan residues interspersed with proline residues throughout the sequence, it probably assumes a structure distinct from the well-described α-helical and β-structured peptides. Solution structures of indolicidin in different environments have been determined by fluorescence, CD and NMR spectroscopy. However, the proposed structures of indolicidin on lipid membranes have been somewhat controversial. It has been suggested that indolicidin adopts a polyproline helix structure (Falla *et al*., 1996) or a turn structure (Ladokhin *et al*., 1997, 1999). However, Rozek *et al*. (2000) showed that indolicidin adopts a wedge-shaped structure with hydrophobic

tryptophan residues in the trough, flanked by two positively charged regions (see Fig. 9.6A, Chapter 9), which appears ideal for intercalation between the lipid molecules of the lipid bilayer. Moreover, a study by Hsu *et al.* (2005) revealed that indolicidin adopts multiple conformations in aqueous solutions and membrane-mimicking environments, suggesting that the structural plasticity accounts for indolicidin's effects.

Indolicidin exhibits significant activity against a wide range of targets, including bacteria, fungi, protozoa and human immunodeficiency virus (HIV)-1 (Zanetti *et al*., 2002; Chan *et al*., 2006). Like other membrane-permeabilizing peptides, the antimicrobial action of indolicidin was thought to increase the permeability of the cytoplasmic membranes of target microorganisms. However, the complete mode of action of this peptide has not been fully elucidated and is still under debate. Although indolicidin binding to bacterial membranes results in membrane permeabilization (Falla *et al*., 1996; Zhao *et al*., 2001; Schibli *et al*., 2002; Nan *et al*., 2009) or thinning (Shaw *et al*., 2006; Hsu and Yip, 2007), it does not cause cell lysis at concentrations four times the MIC of the peptide (Subbalakshmi and Sitaram, 1998; Wu *et al*., 1999). Thus, it is conceivable that indolicidin uses its membrane-affinity property to enter the cytoplasm and exerts its antibacterial activity by attacking other targets, similarly to PR-39. In fact, Subbalakshmi and Sitaram (1998) demonstrated that indolicidin induces filamentation in *E. coli* cells as a result of preferential inhibition of DNA synthesis, rather than RNA and protein synthesis. Moreover, Hsu *et al*. (2005) confirmed that indolicidin binds DNA in gel-retardation and fluorescence quenching experiments. These researchers also demonstrated that indolicidin prefers to bind duplex DNA rather than single-stranded DNA, using surface plasmon resonance. It is thought that indolicidin prefers to bind strongly to phosphate groups via its positively charged amino acids, and the tryptophan residues stack between the nucleotide bases or deoxyriboses in each strand of the DNA duplex. A subsequent study revealed that indolicidin interferes with the formation of the catalytic HIV-1 integrase–DNA complex by directly binding to DNA and not to integrase (Marchand *et al*., 2006). Indolicidin also interferes with topoisomerase-I-mediated DNA relaxation without unwinding DNA, suggesting that indolicidin may inhibit a large variety of DNA processing enzymes through DNA binding. Taken together, indolicidin is thought to cross the membranes into the cytoplasm at concentrations above the MIC but below the minimal lytic concentration, and kills bacteria by multiple actions at the level of DNA.

The short sequence and broad spectrum of activity are features that make indolicidin appealing as a putative anti-infective agent. Although indolicidin is an effective AMP, various attempts have been made to find derivatives that are more antibacterial and less cytotoxic. Omiganan pentahydrochloride, a synthetic analogue of indolicidin, is currently under clinical development for the prevention of catheter-related local and bloodstream infections as well as for the treatment of acne and rosacea (Melo *et al*., 2006; Vaara, 2009). It has been reported that indolicidin binds abasic-site-containing DNA (Marchand *et al*., 2006). Abasic sites are considered to be the most frequent DNA lesions in mammalian cells, with an estimated frequency of 10,000 events per day per cell (Boiteux and Guillet, 2004). Therefore, further investigations on the potential interference of indolicidin with DNA repair mechanisms are needed to use this peptide in human therapeutics.

8.4 Apidaecin, Drosocin and Pyrrhocoricin: Short, Insect Proline-rich Peptides Binding to Heat-shock Protein DnaK

Apidaecin, drosocin and pyrrhocoricin are short, proline-rich AMPs isolated from insects (*Apis mellifera*, *Drosophila melanogaster* and *Pyrrhocoris apterus*, respectively). They consist of 18–20 amino acid residues (Casteels *et al*., 1989; Bulet *et al*., 1993; Cociancich *et al*., 1994). The names of these peptides reflect their origin rather than a subdivision among the individual sequences. Apidaecin, drosocin and pyrrhocoricin are remarkably similar in amino acid composition and sequence motif pattern (Li *et al*., 2006). In addition to being

overrepresented in the sequences, proline residues are frequently associated in doublets and triplets with basic residues (arginine or histidine). The Pro-Arg-Pro/Pro-His-Pro motifs are either evenly distributed along the entire peptide sequence or are concentrated in certain subdomains (Bulet and Stocklin, 2005). Drosocin and pyrrhocoricin also contain a glycosylated threonine in the mid-chain position, although the presence of sugar is not always necessary for antimicrobial activity (Otvos, 2002). The presence of sugar increases the *in vitro* antibacterial activity of drosocin (Bulet *et al*., 1996), but decreases that of pyrrhocoricin (Hoffmann *et al*., 1999). The solution conformations of these peptides have been determined by CD and two-dimensional NMR spectroscopy. Drosocin and pyrrhocoricin adopt random coil structures in aqueous solution, and remain largely unstructured in 50% trifluoroethanol; there are, however, clear indications of the presence of small populations with elements of local structure, most probably turns (McManus *et al*., 1999; Otvos *et al*., 2000a). In addition, apidaecin appears to form an ordered oligomer in a membrane-mimicking environment (Dutta *et al*., 2008).

Apidaecin, drosocin and pyrrhocoricin are generally active against Gram-negative bacteria, while only a few Gram-positive species are affected (Bulet and Stocklin, 2005). The most unique feature of their mode of action is a complete lack of membrane permeabilization activity. Casteels and Tempst (1994) showed that the addition of apidaecin does not cause any unmasking of the β-galactosidase activity of *E. coli* ML-35, a strain with a phenotype allowing an easy calorimetric evaluation of the kinetics of inner-membrane permeabilization, and the sensitivity of apidaecin-resistant mutant strains to pore-forming AMPs is undiminished. Moreover, unlike most AMPs that kill bacteria in a non-stereospecific fashion (Oren and Shai, 1998), the all-D enantiomers of apidaecin, drosocin and pyrrhocoricin are totally inactive against strains susceptible to the all-L isomers, suggesting that the antibacterial effects of these peptides could involve stereoselective recognition of a chiral cellular target, most likely a protein (Casteels and Tempst, 1994; Bulet *et al*., 1996; Otvos *et al*., 2000a). Although the above-mentioned non-membrane-active AMPs, such as buforin II, PR-39 and indolicidin, act on intracellular components such as nucleic acids, they do not appear to have a specific protein target.

By using a biological assay to selectively measure chiral interactions with bacterial targets, Castle *et al*. (1999) observed that all-D enantiomers of apidaecin are rapidly associated with *E. coli* cells, but are then almost entirely recovered by exhaustive washing, whereas the all-L apidaecins and all-L and all-D magainins are not. This implies that apidaecins likely permeate and traverse the outer membrane in a non-specific fashion, followed by an apparently irreversible process due to sequentially stable binding to a periplasmic or inner-membrane component, or through further translocation. These researchers also showed that apidaecin uptake in *E. coli* is energy-driven, irreversible and can be partially competed by proline in a stereospecific manner. Moreover, failure of certain apidaecin mutants to kill cells after entering the cytosol implies the existence of another downstream target. Based on these results, they proposed a permease/transporter-mediated mechanism for the mode of action of apidaecin on *E. coli*. The proposed mechanism involves an initial, non-specific binding of the peptide with an outer membrane component and self-promoted uptake in the periplasmic space, followed by interaction with an inner membrane-bound transporter/receptor protein allowing translocation into the cytosol. In the final step, the peptide in the cytosol meets one or more macromolecular targets, the inhibition of which results in bacterial cell death.

Putative target proteins of apidaecin, drosocin and pyrrhocoricin in the bacterial cell have been identified by mass spectroscopy, Western blotting and fluorescence polarization (Otvos *et al*., 2000b). Otvos *et al*. found that these peptides specifically bind to the *E. coli* heat-shock protein DnaK, which functions co-translationally and post-translationally to promote protein folding and inhibit the formation of toxic protein aggregates of misfolded proteins (Bukau and Horwich, 1998; Hartl and Hayer-Hartl, 2002; Slepenkov and Witt, 2002), but not to the homologous DnaK fragment of *Staphylococcus aureus* (a species not susceptible to the peptides) or the

human equivalent Hsp70. These peptides also interact with lipopolysaccharide, which could be responsible for their initial binding to the bacterial surface, and in a non-specific manner with the bacterial chaperonin GroEL. In addition, an inactive pyrrhocoricin analogue, made of all-D amino acids, does not interact with DnaK. This suggests that inhibition of DnaK's function by these peptides might be responsible for bacterial killing.

A subsequent study by Kragol *et al*. (2001) revealed that pyrrhocoricin and drosocin affect DnaK's two major functions: ATPase activity and refolding of misfolded proteins. In this study, they found that biologically active L-pyrrhocoricin diminishes the ATPase activity of recombinant DnaK, while the inactive D-pyrrhocoricin analogue and membrane-active AMPs such as cecropin A or magainin 2 fail to inhibit ATPase activity of DnaK. In addition, both alkaline phosphatase and β-galactosidase activities (reflecting DnaK's function of refolding misfolded proteins) of live bacteria are reduced upon incubation with L-pyrrhocoricin or drosocin. In contrast, D-pyrrhocoricin, magainin 2 or buforin II have only a negligible effect. According to fluorescence polarization and dot-blot analysis of synthetic DnaK fragments and labelled pyrrhocoricin analogues, the peptide binds to the hinge region around the C-terminal helices D and E of DnaK, located just above the conventional substrate-binding site. Structure–activity relationship studies of pyrrhocoricin identified that the N-terminal half (residues 2–10) is responsible for inhibition of the ATPase activity of DnaK, while the C-terminal half (residues 11–20) is responsible for membrane penetration (Kragol *et al*., 2002). Overall, these data suggest that the binding of pyrrhocoricin and drosocin to DnaK prevents the frequent opening and closing of the multihelical lid over the peptide-binding pocket of DnaK, permanently closes the cavity and inhibits chaperone-assisted protein folding. The sequence of the D-E helix is remarkably dissimilar in various bacterial and mammalian DnaK proteins. This may explain the selectivity and relatively restricted spectrum of activity of these peptides and their lack of toxicity to eukaryotic cells. In fact, Bikker *et al*. (2006) demonstrated that the activity spectrum of drosocin can be 100% correlated with the homology to *E. coli* DnaK at the D-E helix region in the 31 studied bacterial species. However, there is some disagreement as to the specific binding site of DnaK. Chesnokova *et al*. (2004) studied the chemistry of pyrrhocoricin binding to DnaK using complementary pre-state kinetic and single-turnover ATPase assays, and showed that pyrrhocoricin binds to and stimulates the ATPase activity of both wild-type and lidless variants of DnaK. In addition, a study of CD curves by Zhou *et al*. (2008) showed that obvious spectral alteration is detected when apidaecin is added to lidless DnaK, while no alteration is observed when it is added to DnaK D-E helix fragments. Thus, these researchers suggested that pyrrhocoricin binds primarily to the conventional substrate-binding site of DnaK, much like other substrates, and effectively decreases the cellular concentration of DnaK by competing with natural substrates.

Ideally, a protein present only in bacteria that carriers a significant function, but is absent or non-homologous in human cells, may form the basis for the rational design of species-specific antibacterial peptides and peptidomimetics (Casteels and Tempst, 1994). DnaK shares only 50% sequence homology with eukaryotic Hsp70 (Bardwell and Craig, 1984), with some well-conserved N-terminal regions and some less conserved C-terminal ones (Karlin and Brocchieri, 1998). Therefore, the variable sequence domain of DnaK appears to offer a sensible target for antimicrobial drug development, and apidaecin, drosocin and pyrrhocoricin are an excellent starting point for these species-specific drug development efforts. It is conceivable that drugs that act on a specific target protein likely have the disadvantage of an easier selection of drug-resistant strains through mutations at the target level, similarly to conventional antibiotics, which generally act by binding to a specific target (Gennaro *et al*., 2002). However, Cassone *et al*. (2009) recently showed that induced resistance to the designer proline-rich peptide dimer A3-APO does not involve changes in the intracellular target DnaK, thus dispelling the concern of target modification and arguing for focused research for novel bacterial targets.

8.5 Concluding Remarks and Perspectives

The emergence and rapid horizontal spread of antibiotic-resistant traits in bacteria of human and veterinary clinical significance has been a driving force in the search for new classes of antibiotics (Coates *et al*., 2002). AMPs have been regarded as a potential solution to serious worldwide problems caused by infectious diseases (Mookherjee and Hancock, 2007). The potential value of AMPs for clinical purposes includes their use as single antimicrobial agents, synergistic agents to existing antibiotics, immunostimulatory agents and endotoxin-neutralizing agents (Gordon *et al*., 2005). The non-membrane-active AMPs discussed in this review display many of the desirable features of a novel antibiotic. As with any new class of antimicrobial therapeutics, a central issue is whether resistance can be provoked. Unlike most AMPs, which kill bacteria via subsequent cytoplasmic membrane disruption, non-membrane-active AMPs translocate across the bacterial membranes and act on intracellular targets in bacteria. The fact that these peptides have multiple targets, as well as their fundamental interaction with the bacterial membrane, means the chances of resistance by target modification are slim, as this would require the complete alteration of the membrane or bypassing of several biochemical pathways (Marr *et al*., 2006). Buforin II, PR-39 and indolicidin, which target nucleic acids, have the potential to be developed into more efficient, broad-spectrum antimicrobials; apidaecin, drosocin and pyrrhocoricin, which target the variable sequence domain of DnaK, are an excellent starting point for species-specific drug development efforts. In addition to their direct antibacterial effects, some of these non-membrane-active AMPs, such as buforin II and PR-39, exert multiple and diverse actions on mammalian cells, suggesting their therapeutic potential in a number of human diseases, including cancer and inflammation. Moreover, peptides such as buforin II and indolicidin, which at the same time cross the eukaryotic membrane and bind DNA, may be used as a gene-delivery vehicle in the treatment of a variety of genetic and acquired diseases.

Despite the fact that non-membrane-active peptides show great potential as novel antibiotics, a number of issues must be solved before these peptides can be developed as human therapeutics. Although these peptides exhibit significant *in vitro* activity against bacteria, they are cleaved *in vivo* by endogenous mammalian proteases, severely reducing their therapeutic value. In particular, chymotrypsin-like enzymes attack proteins at basic residues, which are an obligate feature of AMPs. In this regard, there are strategies for protecting peptides from proteases, including liposomal incorporation and chemical modification (McPhee *et al*., 2005). Advances in peptide engineering, including physical (structural), chemical or composition modifications of AMPs, will likely contribute to the development of peptide antibiotics and improve their efficacy. In fact, the designer proline-rich peptide dimer A3-APO kills β-lactam- and fluoroquinolone-resistant clinical isolates even in the presence of serum proteases and is effective against systemic models of infection *in vivo*, showing clear potential for therapeutic utility (Szabo *et al*., 2010). The next step in the development of new antibiotics is the large-scale production of pharmacologically pure AMPs in a cost-effective manner. Novozymes Inc. has reported, using a proprietary fungal-based system, recombinant production of the peptide plectasin at the scale and purity required for therapeutic administration (Mygind *et al*., 2005). Jang *et al*. (2009) recently developed a novel translationally coupled, two-cistron expression system for the production of recombinant AMPs in their natural forms. Using this system, they produced approximately 100 mg of several potent AMPs from 1 l of *E. coli* culture. These efforts may lead to a universal cost-effective solution for the mass production of AMPs, and AMPs may soon fulfil their promise as alternatives to conventional antibiotics for the treatment of human infections.

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9 Structural Studies of Antimicrobial Peptides Provide Insight into Their Mechanisms of Action‡

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Abstract

This chapter reviews structural studies of antimicrobial peptides (AMPs), with a focus on human defensins and cathelicidins. Also discussed are the major steps for structural determination of AMPs by nuclear magnetic resonance spectroscopy, including bacterial expression and purification, sample preparations, data collection, sequential signal assignments, structure calculations, validation and coordinate deposition. A variety of three-dimensional structures (α-helices, β-strands, αβ-fold and non-αβ structures) have been discovered for AMPs, which can induce similar biophysical consequences in membranes such as positive curvature or lipid domain formation. Therefore, it is the amphipathic surface, not polypeptide backbone scaffolds, that is essential for the antimicrobial activity of AMPs. A proper presentation of side chains (e.g. cationic and hydrophobic) on the surface of AMPs determines not only membrane perturbation potential, but also other biological functions. Reduction in hydrophobicity is a fundamental strategy to improve peptide selectivity. Structures of AMPs in complex with membranes or non-membrane targets also form the foundation for engineering a new generation of antimicrobials that will supplement or replace traditional resistant antibiotics.

9.1 Human Defensins and Cathelicidins

In mammals, defensins and cathelicidins are the two major families of antimicrobial peptides (AMPs) (Zasloff, 2002; Zanetti, 2005). Six α-defensins have been found in humans: four from neutrophils (HNP-1, -2, -3 and -4) and two from intestinal Paneth cells (HD-5 and -6) (Lehrer *et al*., 2009). Note that HNP-1, -2 and -3 have essentially identical sequences and only differ at the N-terminal residue (Table 9.1). Even such a small sequence difference influences the antifungal activity of these peptides. While HNP-1 and -2 are active against *Candida albicans*, HNP-3, with an additional aspartic acid residue at the N-terminus, is inactive (Raj *et al*., 2000). The antimicrobial and chemotactic activities of HNP-4 are somehow comparable to other neutrophil defensins. While HD-5 is highly bactericidal, the antibacterial activity of HD-6 has yet to be observed (Szyk *et al*., 2006). Table 9.1 also contains four well-known human β-defensins (hBD-1, -2, -3 and -4). Salts appear to affect the antimicrobial activities of defensins, indicating the importance of electrostatic interactions in bacterial membrane binding (Pazgier *et al*., 2007). A third family of defensins is small (18-residue minidefensins) and in circular form (θ-defensins). They are expressed in non-

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Table 9.1. Primary structures of human cathelicidins and defensins.

APD ID[a]	Name	AA sequence	No. AA residues	Net charge	Hydrophobic % (PBP)[b]
176	Human α-defensin-1 (HNP-1)	ACYCRIPACIAGERRYGTCIYQG RLWAFCC	30	+3	53 (1.07)
177	Human α-defensin-2 (HNP-2)	CYCRIPACIAGERRYGTCIYQGR LWAFCC	29	+3	51 (1.17)
178	Human α-defensin-3 (HNP-3)	DCYCRIPACIAGERRYGTCIYQG RLWAFCC	30	+2	50 (1.42)
179	Human α-defensin-4 (HNP-4)	VCSCRLVFCRRTELRVGNCLIGG VSFTYCCTRV	33	+4	51 (1.40)
180	Human α-defensin-5 (HD-5)	ATCYCRTGRCATRESLSGVCEIS GRLYRLCCR	32	+4	40 (2.60)
181	Human α-defensin-6 (HD-6)	AFTCHCRRSCYSTEYSYGTCTV MGINHRFCCL	32	+4	40 (1.71)
451	Human β-defensin-1 (hBD-1)	DHYNCVSSGGQCLYSACPIFTKI QGTCYRGKAKCCK	36	+5	36 (1.30)
524	Human β-defensin-2 (hBD-2)	GIGDPVTCLKSGAICHPVFCPRR YKQIGTCGLPGTKCCKKP	41	+7	36 (0.90)
283	Human β-defensin-3 (hBD-3)	GIINTLQKYYCRVRGGRCAVLSCL PKEEQIGKCSTRGRKCCRRKK	45	+11	33 (2.87)
675	Human β-defensin-4 (hBD-4)	FELDRICGYGTARCRKKCRSQEY RIGRCPNTYACCLRKWDESLL NRTKP	49	+7	32 (3.35)
310	Cathelicidin LL-37	LLGDFFRKSKEKIGKEFKRIVQRIK DFLRNLVPRTES	37	+6	35 (2.99)
624	Cathelicidin ALL-38	ALLGDFFRKSKEKIGKEFKRIVQRI KDFLRNLVPRTES	38	+6	36 (2.87)

[a] Additional information for each peptide can be found in the APD (http://aps.unmc.edu/AP/main.html) by entering the APD ID number in the search interface (Wang *et al.*, 2009).

[b] Hydrophobic % is calculated as the ratio between the numbers of hydrophobic residues and total residues. Abbreviations: AA, amino acid; APD, Antimicrobial Peptide Database; HNP, human α-defensin; PBP, protein-binding potential (Boman, 2003).

human primates, but not in humans due to the stop codon in the pseudogenes (Tran *et al.*, 2002). Although the exact disulfide bond (S–S) pattern varies, all defensins possess multiple such bonds. In human α-defensins (29–33 residues), the three S–S bonds are Cys^{I}–Cys^{VI}, Cys^{II}–Cys^{IV} and Cys^{III}–C^{V}; in human β-defensins (36–49 residues), they are Cys^{I}–Cys^{V}, Cys^{II}–Cys^{IV} and Cys^{III}–Cys^{VI}. The S–S bond connections in rhesus monkey θ-defensins are Cys^{I}–Cys^{VI}, Cys^{II}–Cys^{V} and Cys^{III}–Cys^{IV}. The cysteines in these peptides are labelled from Cys^{I} to Cys^{VI} according to their order in the sequence. The S–S bridges, as well as the circular polypeptide chain in the case of θ-defensins, confer stability to the three-dimensional (3D) structure of defensins. In addition to the known antibacterial activity, human defensins possess other functional roles in host defence such as anti-human inflammatory virus (HIV), antitoxin, chemotaxis and immune-modulation properties (Lehrer *et al.*, 2009).

The precursor proteins of the cathelicidin family share a well-conserved N-terminal 'cathelin' domain while the sequences at the C-terminus are highly variable, encoding a plethora of AMPs (Zanetti, 2005). Several cathelicidins exist in animals such as sheep, cows and pigs. For example, PMAP-23, protegrin-1 and PR-39 are all pig cathelicidins that form α-helix, β-hairpin and extended structures in membrane environments, respectively. In contrast, only one cathelicidin gene has been found in humans. The precursor of human cathelicidin is an 18 kDa

human cationic antimicrobial protein (hCAP-18). Depending on the expression sites, hCAP-18 can be cleaved into different forms of active peptides. In neutrophils, the peptide, released by proteinase 3, contains 37 residues and starts with two leucines, and is thereby referred to as LL-37 (Gudmundsson *et al*., 1995). In human production systems, the released peptide is ALL-38. LL-37 and ALL-38 (Table 9.1) have similar antimicrobial activities (Sørensen *et al*., 2003). In addition, LL-37 can be cleaved into short active peptides in human skin or sweat, indicating a regulatory role of proteases in host defence (Yamasaki *et al*., 2006). Of note, these LL-37 fragments display greater antimicrobial activity in the presence of physiological salts such as carbonate (Dorschner *et al*., 2006). Therefore, it is important to perform antibacterial assays under physiological conditions. Of these protease products of hCAP-18, LL-37 has been best studied. This molecule protects humans from infectious diseases (Nizet *et al*., 2001; Pütsep *et al*., 2002), cystic fibrosis (Bucki *et al*., 2007) and sepsis by neutralizing lipopolysaccharides (LPS or endotoxin) (Cirioni *et al*., 2006). LL-37 and its fragments all inhibit HIV-1 infection (Bergman *et al*., 2007; Wang *et al*., 2008), but they may also perform different biological functions. While LL-37 promotes cancer metastasis, an N-terminal fragment (LL-25) inhibits the process (Weber *et al*., 2009). In addition, a C-terminal fragment has been shown to have anticancer activity *in vitro* (Li *et al*., 2006a). It is proposed that overexpressed LL-37 attracts mesenchymal stem cells to tumour microenvironments by directly interacting with formyl peptide receptor-like 1 (FPRL-1). The chemotactic activity is attributed to the N-terminal region of LL-37, and the antibacterial region is mapped to the C-terminus of the peptide (Braff *et al*., 2005). A recent review lists >100 LL-37 fragments discovered by various laboratories (Burton and Steel, 2009). KR-12, the smallest antimicrobial fragment with merely 12 residues (Table 4.2, Chapter 4), has been mapped to residues 18–29 of LL-37 (Wang, 2008c). Evidently, polypeptide fragmentation provides a useful approach for elucidating the functional regions of AMPs. Like human defensins, LL-37 also possesses other biological functions such as immune modulation, angiogenesis, apoptosis and wound healing (Nijnik and Hancock, 2009).

To elucidate the functional roles of defensins and cathelicidins, 3D structures are essential. While circular dichroism (CD) and Fourier transform infrared spectroscopy are low-resolution techniques that give an estimation of secondary structures of proteins in various states, X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy are able to determine the structure of proteins to the atomic resolution. By February 2010, the Antimicrobial Peptide Database (APD) (Wang *et al*., 2009) contained 195 NMR structures and ten crystal structures, indicating that NMR is the major player (Wang, 2006; Haney *et al*., 2009). As a consequence, this chapter focuses on structural studies of AMPs by NMR. An outline of the procedure for NMR structural determination of AMPs is provided in Fig. 9.1. For structural analysis, highly purified AMPs are required. Because cationic AMPs usually associate with anionic bacterial membranes, NMR studies are often conducted in membrane-mimetic systems. Also discussed are NMR methods, major structural types (α-helices, β-sheets and extended structures), peptide–membrane interactions and structure-based peptide design.

9.2 Bacterial Expression and Purification of Antimicrobial Peptides

Most AMPs are relatively short and can readily be synthesized by the solid-phase method (Merrifield *et al*., 1995). In particular, this method enables the incorporation of isotope-labelled amino acids at specific sites. Such peptides are important for solid-state NMR studies. Advances have also been made in the chemical synthesis of defensins (Raj *et al*., 2000; Wu *et al*., 2004; Klüver *et al*., 2006).

For longer peptides or those with multiple disulfide bonds, chemical synthesis is less efficient and bacterial expression shows advantages. In addition, bacterial expression enables uniform labelling of polypeptides

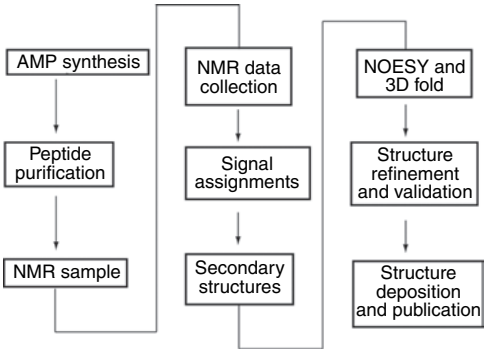

Fig. 9.1. A flow chart for the structural determination of AMPs by NMR spectroscopy. The peptide may be synthesized chemically using the well-established solid-phase method or biologically using bacterial expression systems. In many cases, it is difficult to purify a sufficient amount of peptide from natural organisms for structural studies. NOESY, nuclear Overhauser effect spectroscopy.

at an acceptable cost, facilitating 3D double- and triple-resonance NMR studies of AMPs (Fig. 9.1), especially when two-dimensional (2D) homonuclear NMR experiments do not provide sufficient spectral resolution (e.g. LL-37; see Wang, 2008c). By using recombinant DNA technology, the AMP gene can be inserted into an expression vector (Fig. 9.2) and expressed in bacteria, yeasts or cell-free expression systems (Wang, 2008a). The AMP gene can be chemically synthesized or amplified via polymerase chain reactions using natural DNA as a template. Both the codons in the synthetic AMP gene and DNA oligomers for polymerase chain reaction amplification can be optimized with the aid of computer programs (Puigbo *et al*., 2007; Welch *et al*., 2009). A carrier protein is chosen to attenuate the potential toxicity of AMPs to the expression host and to protect the peptide from being degraded. For a particular case, the carrier may be optimized by comparing peptide expression levels. Other favourable properties of the carriers are increased solubility and easy separation from the recombinant peptide. Some tested carrier proteins are glutathione *S*-transferase, maltose-binding protein, thioredoxin, *Staphylococcus aureus* protein A, immunoglobulin G binding protein, OprF, RepA, baculoviral polyhedron, PaP3.30, small ubiquitin-related modifier (SUMO), PurF and TAF12 (Zhang *et al*., 1998; Kim *et al*., 2008). In the case of LL-37, carriers such as thioredoxin, glutathione *S*-transferase and a family III carbohydrate-binding module (CBM3) have been used for peptide expression in *Escherichia coli* (Table 9.2). The recombinant LL-37 was not separated from thioredoxin by high-performance liquid chromatography (Li *et al*., 2006c), but was separated from CBM3 (Ramos *et al*., 2010). The introduction of the CBM3 carrier simplifies the original protocol (Li *et al*., 2006c) developed for the expression and purification of recombinant LL-37. There are also other LL-37 expression and purification protocols (Yang *et al*., 2004; Moon *et al*., 2006). The bacterial expression systems in Table 9.2 gave peptide yields ranging from 0.3 to 2.6 mg l^{-1} of bacterial culture. By fusing histonin to part of PurF protein and adopting a multimeric expression strategy, Kim *et al*. (2008) obtained 167 mg of the recombinant peptide from 1 l of bacterial culture. It appears that the expression yields of recombinant AMPs vary substantially depending on the peptide and expression systems and purification methods.

Since a purification tag (Fig. 9.2) is attached to the fusion protein, it can be

Table 9.2. Select bacterial expression systems for human LL-37.

Molecular form	Carrier protein	Cleaving agent	Peptide yield (mg l^{-1})	Reference
GS-LL-37	Trx	Thrombin	1.1	Yang *et al*. (2004)
P-LL-37	Trx	Formic acid	1.7	Li *et al*. (2006c)
LL-37	GST	Factor Xa	0.3	Moon *et al*. (2006)
P-LL-37	Trx	Formic acid	2.6	Li *et al*. (2007)
P-LL-37	CBM3	Formic acid	1.0	Ramos *et al*. (2010)

Abbreviations: CBM3, family III carbohydrate-binding module; GST, glutathione *S*-transferase; Trx, thioredoxin.

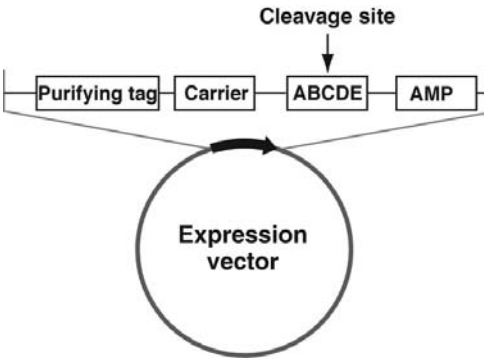

Fig. 9.2. A putative expression vector for AMPs. Several purifying tags and carrier proteins applied to the expression of human LL-37 are listed in Table 9.2. Some commonly used cleavage methods and cleavage site sequences (represented by ABCDE in the figure) are listed in Table 9.3.

rapidly separated from other unwanted molecules by affinity chromatography. The purified fusion protein is next cleaved by a protease or chemical agent to release the recombinant peptide. Select cleaving agents and peptide cleaving sites are listed in Table 9.3. Factor Xa (Moon *et al*., 2006), enterokinase (Bang *et al*., 2010) and thrombin (Li *et al*., 2007) are frequently used proteases. Recently, tobacco etch virus protease (TEV) and SUMO have become popular. A mutant form (Ser219Val) of TEV is a better choice, since it is 100-fold more stable than the wild type (Kapust *et al*., 2001). Another attractive feature of TEV is that it appears to be active even in the presence of detergents. This property is useful for removing purification tags from membrane proteins in detergent-containing buffers (Lundbäck *et al*., 2008). SUMO recognizes the entire protease fold and efficiently cleaves the carrier protein from the peptide; the peptide released possesses no additional residues from cloning. Only polypeptides starting with a proline residue are difficult to cleave (Malakhov *et al*., 2004).

Although chemical cleavage is not as specific as enzyme cleavage, it offers some advantages. Chemical cleavage is cost-effective and suitable for industrial production. The commonly used chemical cleaving agents are cyanogen bromide (Park *et al*., 2009; Seo *et al*., 2009), hydroxylamine (Hu *et al*., 2010) and formic acid (Li *et al*., 2006c; Ramos *et al*., 2010). The cleavage sites for these agents are given in Table 9.3. Due to their small size, chemical agents are able to access the cleavage sites of fusion proteins and release the expressed AMPs. In contrast, poor or no cleavage is sometimes reported when proteases are used for cleavage, probably owing to inaccessibility of the cleavage sites by a bulky protease. In particular in the case of LL-37, its aggregation may hinder the access of the proteases and cause poor cleavage at the site adjacent to the peptide by enzymes. Cleavage may be successful after a formic acid cleavage site is introduced (Table 9.3). Recombinant LL-37 obtained in this manner retains an extra proline residue at the N-terminus of the peptide. It is important to validate that extra residues of recombinant proteins from expression constructs do not substantially alter the biological properties of the expressed polypeptide. We found that the recombinant LL-37 displayed similar antibacterial activity to the native peptide. Therefore, this form of recombinant LL-37 is suitable for structural studies. For NMR studies, isotope-labelled peptides were

Table 9.3. Select enzymes and chemical agents and cleavage sequences.

Protease	Cleavage sequence	Chemical agent	Cleavage site
Thrombin	Arg↓Gly, Lys↓Leu or Lys↓Ala	CNBr	Met↓Xxx
Enterokinase	AspAspAspAsp↓Lys	Formic acid	Asp↓Pro
Factor Xa	IleGluGlyArg↓Xxx	Hydroxylamine	Asp↓Gly
SUMO protease 1	SUMO↓Xxx[a]		
TEV	GluGlnLeuTyrPheGln↓Gly/Ser		

[a] Xxx should not be proline.

Abbreviations: CNBr, cyanogen bromide; SUMO, small ubiquitin-related modifier; TEV, tobacco etch virus protease.

obtained by using a minimal labelling medium, where glucose and ammonium chloride are replaced with ^{13}C- and ^{15}N-labelled molecules (Li *et al*., 2006c; Seo *et al*., 2009). Similarly, defensins have also been expressed and purified in *E. coli* for structure–activity relationship studies (Chen *et al*., 2006; Pazgier and Lubkowski, 2006; Seo *et al*., 2009). It is worth noting that no chemical or enzyme cleavage was needed in a recently reported cistron expression system, where the termination codon of the first cistron (encoding an anionic polypeptide) overlaps with the initiation codon of the second cistron (encoding a cationic AMP). This interesting system may be of universal use as a cost-effective AMP expression system (Jang *et al*., 2009).

9.3 Structural Studies of Membrane-targeting Antimicrobial Peptides

9.3.1 Membrane-mimetic models

Many AMPs function by targeting biological membranes. Due to the complex nature of bacterial membranes, high-resolution NMR studies of membrane proteins are usually performed in membrane-mimetic models. Lipid bilayers are regarded as the closest membrane-mimetic model (Fig. 9.3A). This model is suitable for solid-state NMR studies that provide insight into peptide orientation as well as aggregation state in lipid bilayers (Opella and Marassi, 2004; Mani *et al*., 2006; Resende *et al*., 2009). As the name implies, bicelles (Fig. 9.3B) are a hybrid of bilayers and micelles. The size of a bicelle is determined by the molar ratio between the long- and short-chain lipids. While large bicelles are good for solid-state NMR, smaller bicelles (molar ratio <0.25) are useful for solution NMR studies. Structural determination in this interesting model is gaining momentum (Losonczi and Prestegard, 1998; Opella and Marassi, 2004; Prosser *et al*., 2006; Wu *et al*., 2010). Detergent micelles (Fig. 9.3C), nevertheless, are most commonly used in solution NMR studies. Keifer *et al*. (2004) compared the translational diffusion coefficients of a membrane-targeting peptide in complex with seven different micelles. Due to the small size of the peptide, micelles determined the diffusion rate of the complex. The longer the acyl (alkyl) chain of lipids (detergents), the slower the diffusion of the complex. The rapid tumbling of smaller micelles led to a narrower spectral linewidth. Evidently, small micelles are desirable for high-resolution NMR studies as long as the native structure of the peptide is reserved. Sodium dodecylsulfate (SDS) and dodecylphosphocholine (DPC) are the two most popular micelles (Wüthrich, 1986; Opella and Marassi, 2004), deuterated forms of which can be purchased from commercial sources. Deuteration makes the proton signals of micelles invisible, thereby simplifying the ^{1}H-NMR spectra of the peptide–detergent complex. The two membrane-mimetic agents have identical C12 carbon chains that differ only in their head groups (for chemical structures, see Wang 2008b). While SDS has an anionic sulfate head group, DPC has a zwitterionic phosphatidylcholine head group. These two micelles are used to mimic bacterial membranes and human cell membranes, respectively (Haney and Vogel, 2009).

Because the major anionic lipids in bacterial membranes are phosphatidylglycerols (PGs), high-resolution structures of AMPs in such an environment are of great interest. We have demonstrated structural determination of AMPs in PGs with an acyl chain length ranging from six to ten (Wang *et al*., 2003; Wang, 2008c). We chose dioctanoyl PG (D8PG) because it can stabilize the membrane-bound structures of AMPs at a much lower concentration than dihexanoyl PG. A low concentration of protonated D8PG reduces the intense micelle proton signals, thereby improving the quality of NMR spectra. It is also important to control the pH of the sample to about 5–6. A lower pH can reduce the solubility of the AMP–D8PG complex and a high pH can cause the loss of important amide proton signals (Wang, 2007). For NMR analysis, high temperatures are helpful owing to faster tumbling of the peptide–micelle complexes in solution. Indeed, improved NMR spectra have been reported at 50°C (Park *et al*., 2007; Wang,

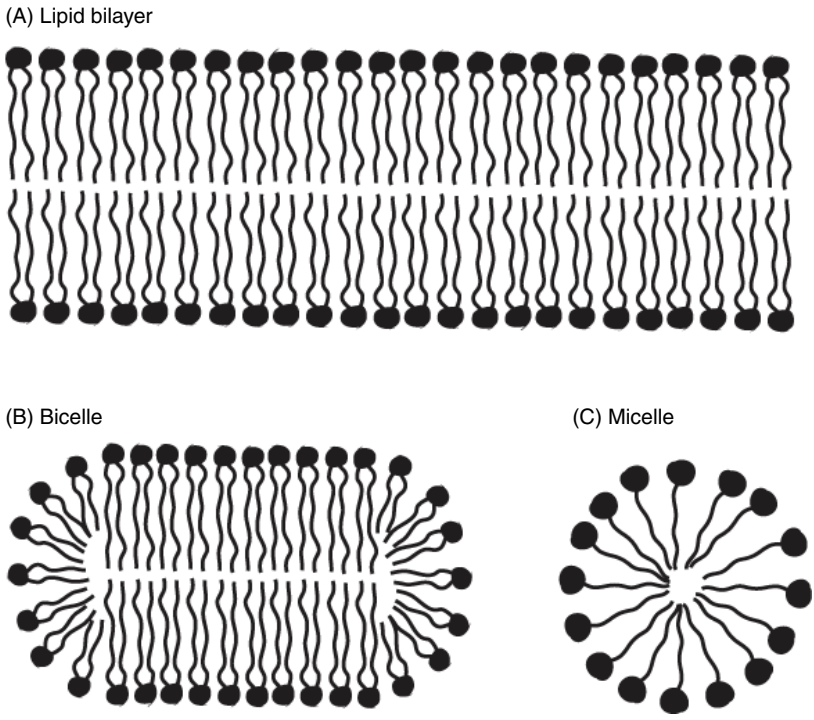

Fig. 9.3. Three membrane-mimetic models for NMR studies. (A) A lipid bilayer is composed of long-chain lipids; (B) a bicelle comprises a mixture of short- and long-chain lipids; and (C) a micelle consists of short-chain lipids (see the text for more details).

2008c). We also suggest the use of the NMR chemical shift standard externally, since anionic compounds such as sodium 2,2-dimethyl-silapentane-5-sulfonate (DSS) may interact with cationic AMPs (Wang *et al.*, 2003). Note that DSS is unable to form a micelle by itself based on translational diffusion measurements by NMR (Keifer *et al.*, 2004).

9.3.2 NMR methods

Two-dimensional NMR methods

NMR spectroscopy was established as a structural tool by Kurt Wüthrich (2002 Nobel Laureate), whose group determined the first protein structure during 1984–1985. The earlier 2D homonuclear NMR work of peptides and proteins is summarized in a classic book by Wüthrich (1986). Two-dimensional NMR methods are well suited for structural studies of AMPs because of their relatively small size (usually <50 residues). The standard set of 2D (^{1}H, ^{1}H) NMR experiments includes total correlation spectroscopy (TOCSY), nuclear Overhauser effect spectroscopy (NOESY) and double-quantum-filtered correlation spectroscopy (DQF-COSY). These NMR experiments allow for sequential signal assignments in two steps (Wüthrich, 1986). First, the amino acid spin systems are identified in the TOCSY spectrum and verified in DQF-COSY. Secondly, the relationships of these spin systems are established based on NOESY spectra. In principle, nuclear Overhauser effect (NOE) cross peaks can be detected when two protons are within 5 Å. Signal assignments constitute a critical step towards structural determination of AMPs (Fig. 9.1).

Natural abundance NMR spectroscopy and its applications

The birth of cryogenic NMR probes increased the signal to noise ratio by several-fold, allowing the recording of 2D heteronuclear correlated spectra at the natural abundance (0.36% for ^{15}N and 1.11% for ^{13}C). High concentrations of peptides, if possible, are always recommended to further improve the signal to noise ratio. I routinely collect (^{1}H, ^{15}N) or (^{1}H, ^{13}C) heteronuclear single-quantum coherence (HSQC) spectra for micelle-bound peptides. The heteronuclear chemical shifts can be assigned based on the known proton resonances achieved from 2D NMR. The ^{15}N and ^{13}C chemical shifts have multiple applications. First, they can be employed to verify ^{1}H chemical shift assignments (Wang, 2006). Secondly, heteronuclear chemical shifts also contain structural information. Statistical analysis of protein NMR chemical shifts has revealed that ^{13}Cα are up- and downfield shifted in β-sheet and α-helical structures, respectively. This means that the ^{13}Cα secondary shifts (differences between the measured and random shifts) are positive in helical regions and negative in β-stranded regions (Wishart and Sykes, 1994). Such empirical relationships enable the identification of secondary structures (α-helices or β-strands) in polypeptides (Fig. 9.1). Since ^{13}C chemical shifts are related to protein dihedral angles, Ad Bax and colleagues have established and improved the TALOS program, which predicts backbone dihedral angles of polypeptides based on ^{1}Hα, ^{13}Cα, ^{13}Cβ, ^{13}C carbonyl and ^{15}N chemical shifts (Shen *et al*., 2009). For unlabelled AMPs, fewer than five chemical shifts have been found to be useful in predicting backbone angles (Wang, 2007). Therefore, the measurement of natural abundance chemical shifts offers a practical approach to obtaining dihedral angles, since it is difficult to measure scalar couplings for micelle-bound peptides. The inclusion of natural abundance chemical-shift-derived backbone angles into structural calculations has improved the structural quality of micelle-bound peptides (Wang *et al*., 2005). Thirdly, natural abundance ^{13}C chemical shifts can be employed to provide dynamic information. The basis of this application is the observed correlation between ^{15}N heteronuclear NOE values and secondary ^{13}Cα shifts (Wang, 2010). A structured region is rigid with positive ^{15}N NOE values and large ^{13}Cα secondary shifts. In contrast, residues in a disordered region display negative ^{15}N NOE and small ^{13}Cα secondary shifts. Therefore, a plot of the secondary ^{13}Cα shifts versus residue number of AMPs can be used to locate rigid or flexible regions. Fourthly, the secondary ^{13}Cα plot may also be applied for structural validation. A poorly defined region may result from insufficient NMR restraints or peptide motion. The poorly defined region may be regarded as truly flexible if the ^{13}Cα secondary shifts are also small in the corresponding region. Lastly, the plot of (^{1}H, ^{13}C) cross-peak intensity as a function of residue number displays a wave pattern. Interestingly, residues with lower peak intensities tend to be hydrophobic. This finding may offer an approach for identifying micelle-binding residues (Wang, 2010).

Heteronuclear 3D NMR studies of isotope-labelled AMPs

For helical peptides or those with difficult sequences, 2D NMR may not provide sufficient spectral resolution (see Fig. 1 in Wang, 2008c). Under these circumstances, 3D heteronuclear NMR methods are needed. The power of 3D NMR results from its high resolution by separating the signals in a crowded 2D spectral region onto multiple 2D planes along the third dimension, allowing structural studies of proteins in the 20–30 kDa range (Bax and Grzesiek, 1993). For small AMPs, an ^{15}N-labelled peptide might be sufficient to resolve the overlapped cross peaks by recording 3D-edited TOCSY and NOESY (e.g. Park *et al*., 2007). In this case, sequential signal assignments can still be achieved using the classic NOE-based method (Wüthrich, 1986). If spectral overlap persists, triple-resonance NMR experiments can be applied to a ^{15}N,^{13}C-labelled peptide. In this method, sequential signal assignments are obtained by utilizing the simultaneous

i and i – 1 types of through-bond connectivities, typically from ^{15}N to $^{13}C_i$ and $^{13}C_{i-1}$ in pairs of experiments such as HNCACB and CBCA(CO)NH. While the HNCACB experiment provides through-bond connectivity information from the backbone amide proton to nitrogen as well as to α and β carbon nuclei from both residues i and i – 1, CBCA(CO)NH only provides connectivities from ^{1}H and ^{15}N to α and β carbon nuclei of residue i – 1. The ^{1}H, ^{13}C and ^{15}N chemical shifts of ^{15}N,^{13}C-labelled LL-37 in complex with SDS or D8PG micelles were assigned in this manner (Wang, 2008c). For more difficult problems, four-dimensional NMR spectra may be recorded as well. With ^{15}N labelling, heteronuclear NOEs and longitudinal and transverse relaxation times (T_1 and T_2) of backbone nitrogen nuclei can be measured to provide site-specific information on peptide dynamics in solution (Kay *et al*., 1989).

NMR structures

The next critical step in structural determination (Fig. 9.1) is to convert NMR measurements into coordinates that allow us to view the folding of a polypeptide chain in space. Since NOE is inversely proportional to distance to the power of six (r^{-6}), NOESY spectra yield a set of distance restraints for structural calculations (assuming the same correlation time for the entire molecule). The current practice is to classify the cross peaks into strong, medium, weak and very weak and then convert them to distance ranges (1.8–2.8, 1.8–3.8, 1.8–5.0 and 1.8–6.0 Å). The common lower boundary (1.8 Å) is the shortest distance for two protons to approach in space during distance geometry or simulated annealing calculations. Thus, different distance sets can be sampled within the distance ranges to generate multiple conformations that all satisfy the boundary conditions. This is why NMR structures determined in this manner are usually reported as an ensemble of structures (10–50). However, the distance restraints obtained from NOESY spectra are incomplete and structural calculations are normally complemented with other NMR restraints such as spin-spin coupling constants and hydrogen bonds. The $^{3}J_{HNH\alpha}$ values are >8 Hz in a β-stranded protein structure and <6 Hz in an α-helical structure (Wüthrich, 1986). Doreleijers *et al*. (2009) showed that more recent software could give better-quality structures. An ensemble of structures is chosen, usually based on the lowest energy. The root mean square deviation (RMSD) is calculated to reflect structural precision. Low RMSD values indicate that the peptide region is well defined by a sufficient number of NMR restraints in that region; high RMSD values suggest motion. High RMSD values may also result from insufficient NMR restraints due to poor spectral quality or overlap. This issue can be resolved by performing additional measurements, such as of ^{15}N NOE values (Wang, 2008c). Prior to coordinate deposition into the Protein Data Bank (Fig. 9.1), the structural quality of the AMP should be validated by viewing the violated distance and angle restraints and structural geometry as represented by a Ramachandran plot, and by using other tools such as PROCHECK. Structural coordinates of shorter peptides (<23 residues) are now required to be deposited with the BioMagResBank (Markley *et al*., 2008).

9.3.3 Three-dimensional structures of antimicrobial peptides

Based on 3D structures, AMPs are classified into four major groups: α-helices, β-sheets, αβ structures and non-αβ structures (i.e. extended or loops) (Chapter 1). In the following discussion, β-sheet-containing AMPs (i.e. β-sheets and αβ structures) are combined under the same umbrella of 'β-sheet structures'.

AMPs with α-helical structures

More than 100 AMPs have been annotated as having helical structures in the APD (Wang *et al*., 2009). When multiple coordinates are available for a particular AMP in the Protein Data Bank, only the high-quality structure is

linked to the APD and discussed herein. A few AMPs are helical even in aqueous solutions, primarily due to the formation of helix-bundle structures. Saposin-like proteins (approximately 80 residues) form pores in bacterial membranes. Such polypeptides occur widely in nature, ranging from protozoan parasites (e.g. *Entamoeba histolytica*) to worms (e.g. *Caenorhabditis elegans*) and mammals (e.g. *Sus scrofa*), including humans. The helix-bundle structure of these antimicrobial proteins is further stabilized by three disulfide bonds. In the structure of caenopore-5 from *C. elegans* (Pro81 *cis* conformer in Fig. 9.4A), the five helices are located between residues 6–16, 24–36, 45–50, 58–66 and 70–78 (Mysliwy *et al*., 2010). In addition, there are two S–S bonds (Cys6-Cys80 and Cys9-Cys74) between helices I and V at the N- and C-termini of the protein; the third S–S bond (Cys35-Cys49) is formed between helices II and III. This AMP plays an essential role in the survival of the worm by eliminating any *E. coli* ingested. Upon association with bacterial membranes, the helix bundle structure may open at a site that has several exposed hydrophobic side chains (arrow in Fig. 9.4A). Despite a similar protein fold, the antibacterial activity of tick microplusin is attributed to Cu^{2+} binding, another antimicrobial mechanism different from pore formation in bacterial membranes (Silva *et al*., 2009). The structures of both caenopore-5 and microplusin were determined by 3D heteronuclear NMR techniques. Another unique AMP that forms a helix bundle structure in water (determined by 2D solution NMR) is distinctin. This amphibian peptide comprises two chains linked by one S–S bond. This S–S bond is critical for structure stability to proteases (Fig. 9.4B) rather than antimicrobial activity (Dalla Serra *et al*., 2008). Recent solid-state NMR studies have revealed that both helices are located on the membrane surface of lipid bilayers, excluding the possibility of pore formation (Resende *et al*., 2009).

In the membrane-bound state, a variety of helical structures have been determined for linear AMPs with <40 residues. Amphibian magainin 2 (Gesell *et al*., 1997), dermadistinctin K (Verly *et al*., 2009), fish pleurocidin (Syvitski *et al*., 2005), insect spinigerin (Landon *et al*., 2006) and human LL-37 (Wang, 2008c) all contain a single helical region with one or both ends disordered. In the high-resolution structure of micelle-bound LL-37 determined by 3D NMR, residues 2–31 form a continuous helical structure, whereas the C-terminal residues 32–37 are disordered (Fig. 9.4C). Such a structure is fully consistent with ^{15}N heteronuclear NOE measurements, supporting that only the C-terminus is flexible. The high-resolution LL-37 structure determined by 3D NMR methods is interesting in several aspects. First, it is the longest continuous helix (with 30 residues) found for AMPs to my knowledge. Secondly, the hydrophobic surface of the amphipathic helix is punctuated by a hydrophilic residue Ser9, leading to segregation of the hydrophobic surface into two regions. Thirdly, there is a helical bend between residues 14–16, and all hydrophobic side chains are located on the concave surface of the curved structure. Interestingly, the corresponding helical-bend residues in homologous primate cathelicidins (Zelezetsky *et al*., 2006) are usually glycines (Wang, 2008c). These data suggest that the LL-37 structure determined in SDS micelles serves as a useful model to understand the activity of homologous cathelicidins. Fourthly, the aromatic–aromatic packing at the N-terminal region of LL-37 is proposed to play a role in peptide aggregation, as well as to confer chemotactic and cytotoxic properties to the peptide.

Some AMPs have been found to adopt a helix-hinge-helix motif (also referred to as a 'helix-break-helix' or 'helix-turn-helix' motif). Examples are insect cecropin A (Holak *et al*., 1988), fish pardaxin 4 (Porcelli *et al*., 2004), spider latarcin 2a (Dubovskii *et al*., 2006), amphibian gaegurin 4 (Chi *et al*., 2007) and dermaseptin B2 (Galanth *et al*., 2009). The linker region of these peptides was proposed to be important for antimicrobial activity, allowing optimal binding of both helices to bacterial membranes (Park *et al*., 2007; Galanth *et al*., 2009). Normally, these AMPs induce positive membrane curvature, leading to toroidal pore formation or micellization of membranes (Haney *et al*., 2010). However,

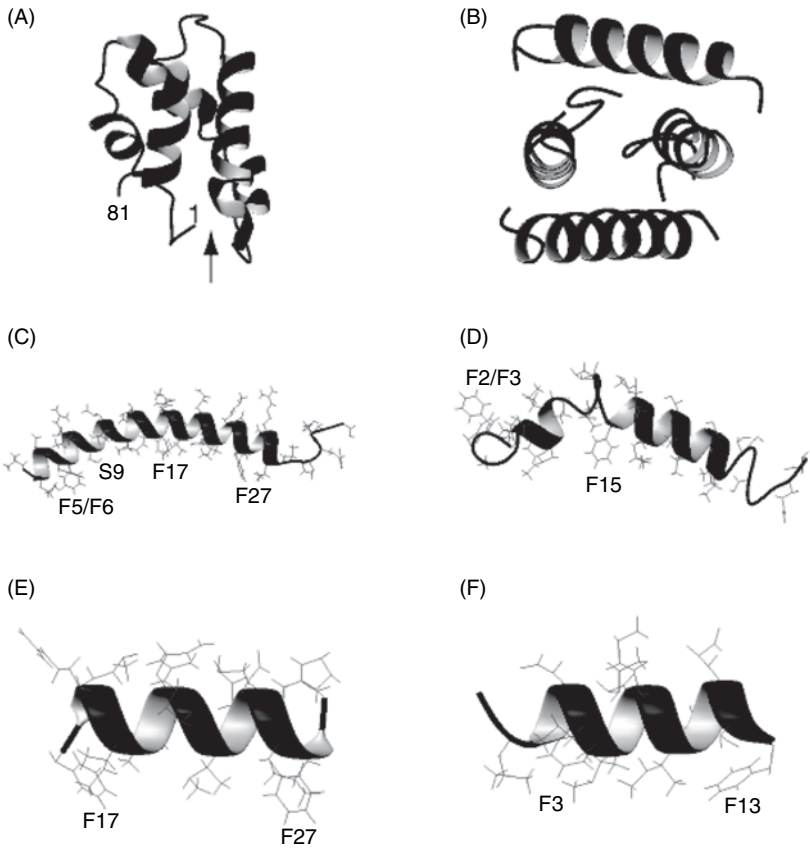

Fig. 9.4. Three-dimensional structures of AMPs from the α-helical family. Depicted are: (A) caenopore-5 from *Caenorhabditis elegans* (Protein Data Bank (PDB) ID: 2JS9) (arrow, exposed hydrophobic side chains); (B) distinctin, a two-chain AMP from frogs (PDB ID: 1XKM); (C) LL-37, a cathelicidin from humans (PDB ID: 2K6O); (D) pardaxin 4 from fish (PDB ID: 1XC0); (E) FK-13, an LL-37 antimicrobial core peptide discovered by NMR (PDB ID: 2FBS); and (F) aurein 1.2 from Australian bell frogs (PDB ID: 1VM5). In the short peptides (panels C–F), phenylalanine residues (labelled) are common and are important for anchoring these AMPs into bacterial membrane. To clearly view the polypeptide fold, the disulfide bonds in S–S-containing peptides are not displayed here, but are described in the text.

pardaxin 4 was found to induce negative curvature in lipid bilayers. The aromatic side chains are not located on the same side in the 3D structure (Fig. 9.4D). In fact, they might not be needed or able to, because the N-terminal short helix is rather hydrophobic and can insert into the membranes. The C-terminal helix is amphipathic and its membrane orientation and mode of action depend on lipid chain length and composition (Porcelli *et al*., 2004). This peptide induces pore formation in phosphocholine vesicles, but vesicle lysis in the presence of anionic PG (Vad *et al*., 2010).

In the helical family, AMPs with longer polypeptide chains tend to display toxicity to mammalian cells (Ciornei *et al*., 2005; Dubovskii *et al*., 2006). This observation laid the foundation for truncating such AMPs to improve peptide selectivity. By using NMR, we have identified a 13-residue antibacterial core peptide (FK-13) corresponding to residues 17–29 of human LL-37 (Li *et al*., 2006a). FK-13 showed a similar antibacterial

activity to LL-37 and adopted a helical structure upon association with micelles (Fig. 9.4E). Subsequently, Phe17 of FK-13 was found to be critical for cytotoxicity to human cells, because removal of this residue led to KR-12 that displayed selective toxicity against *E. coli* (Wang, 2008c). FK-13 is comparable in size to aureins and temporins, the shortest helical peptides from amphibians in the APD. For comparison, the high-quality structure of micelle-bound aurein 1.2 is presented in Fig. 9.4F (Wang *et al*., 2005). Interestingly, we obtained a peptide that shows sequence homology to aurein 1.2 after reversing the sequence of FK-13 (Li *et al*., 2006b). Retro-FK13 (sequence in Table 4.2) showed a higher antibacterial activity than aurein 1.2 owing to additional cationic residues (five versus two). In contrast, aurein 1.2 and the non-toxic bacterial membrane anchor both have two positively charged residues in their membrane-binding region. The antibacterial activity of aurein 1.2 was attributed to a longer and broader hydrophobic surface than that of the membrane anchor. These structure–activity relationship studies have revealed and emphasize the importance of both the hydrophobic surface and cationic side chains of the amphipathic helix in binding to bacterial membranes. The combined consideration of charge and a hydrophobic surface is important, because we have found it difficult to correlate a single structural parameter (e.g. helicity, net charge, hydrophobic surface area, transfer free energy) with antibacterial activity in a series of helical peptides (Wang *et al*., 2005).

AMPs with β-sheet structures

Human defensins have a folded structure in water, enabling crystallization and structural determination by X-ray crystallography (Hill *et al*., 1991; Szyk *et al*., 2006). Human α-defensins share a similar three antiparallel β-strand fold stabilized by three S–S bonds (Fig. 9.5, panels A–E). The disulfide bonds, as well as salt bridges, are critical to maintaining the defensin fold. Using HNP-2 as a model, the structural basis for the Gly-Xaa-Cys motif (Chapter 4) was elucidated. The conserved glycine in the classic β-bulge has the backbone dihedral angles of a D-amino acid. Indeed, the protein fold was retained when Gly16 was changed to a D-amino acid (alanine, glutamic acid, phenylalanine, arginine, threonine, valine or tyrosine), but the fold was disrupted when Gly16 was changed to L-alanine (Xie *et al*., 2005).

Likewise, structures of hBD-1, hBD-2 and hBD-3 have been determined by X-ray diffraction and NMR (Hoover *et al*., 2000, 2001; Bauer *et al*., 2001; Sawai *et al*., 2001; Schibli *et al*., 2002). Human β-defensins contain one N-terminal helix packed on the three β-strands (Fig. 9.5, panels F–H). Using hBD-1 as a model, Pazgier *et al*. (2007) determined the structure of ten mutants by X-ray diffraction. These mutants have a protein fold that is identical to the wild type. It is proposed that the charged residues Arg29, Lys31, Lys33 and Lys36 are critical for antibacterial activity, whereas the N-terminal helical region (residues 1–8), including adjacent residues such as Lys22, Arg29 and Lys33, form the surface for chemotaxis to CCR6-transfected HEK-293 cells. It is more convenient to map the binding sites by NMR. This is because membrane binding to an isotope-labelled protein can cause selective peak shifts of those residues that form the binding surface (this is also the principle for screening potential drug molecules by NMR) (Shuker *et al*., 1996). As an example, the NMR-identified membrane-binding sites on the structure of plant defensin Psd1 are indicated by an arrow in Fig. 9.5I (De Medeiros *et al*., 2010).

X-ray crystallography and NMR provide complementary information under different conditions. While the structural coordinates determined in crystals are normally more accurate, NMR can be applied to the study of peptide dynamics in solution (Skalicky *et al*., 1994), as well as the interaction with bacterial membranes (see below). In addition, NMR also provides insight into the oligomerization of defensins in solution. While a dimer for hBD-1 and octamer for hBD-2 have been found under crystal conditions (due to molecular packing), they are monomers in solution. However, hBD-3 is a dimer in solution. The dimeric structure of hBD-3 is proposed to be important for its greater

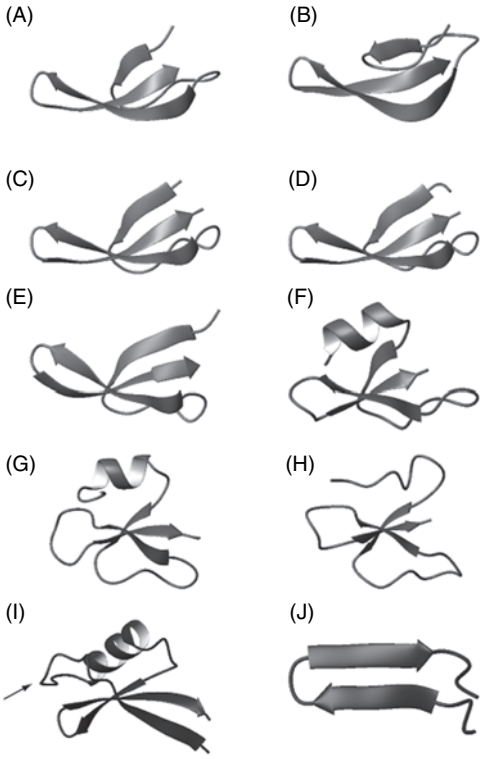

Fig. 9.5. Three-dimensional structures of AMPs from the β-sheet family. Depicted are: (A) HNP-1 (Protein Data Bank (PDB) ID: 3GNY); (B) HNP-3 (PDB ID: 1DFN); (C) HNP-4 (PDB ID: 1ZMM); (D) HD-5 (PDB ID: 1ZMP); (E) HD-6 (PDB ID: 1ZMQ); (F) hBD-1 (PDB ID: 1IJV); (G) hBD-2 (PDB ID: 1FD3); (H) hBD-3 (PDB ID: 1KJ5); (I) Psd1 (PDB ID: 1JKZ) (arrow, membrane-binding site); and (J) RTD-1 (PDB ID: 1HVZ). Structures in panels A–G were determined by X-ray crystallography, while those in panels H–J were determined by NMR spectroscopy. For simplicity, the disulfide bonds are not displayed but are described in the text.

antimicrobial activity than hBD-1 or hBD-2 (Schibli *et al*., 2002). It is not clear whether protein-binding potential (Table 9.1) is related to peptide oligomerization. Another factor that plays a role could be net charge, since hBD-3 has a net charge of +11, higher than that of other human defensins (Table 9.1).

Bacterial membrane composition is another important factor that determines the antimicrobial activity and selectivity of hBD-3 (Böhling *et al*., 2006). Indeed, an increase in positive charge or hydrophobicity of the peptide enhances antimicrobial activity (Klüver *et al*., 2006). Further increases in hydrophobicity make the peptide cytotoxic to human cells. The hydrophobic residue percentages of defensins are given in Table 9.1. Because segments of defensins have been shown to be antibacterial, the entire sequence is not a must. Furthermore, the cysteine residues are not required for antibacterial activity (Hoover *et al*., 2003). However, the fifth cysteine residue is critical for chemotaxis activity (Taylor *et al*., 2008). Recent NMR studies have revealed that, without cysteine residues, the hBD-3 mutant adopts a two-domain helical structure in micelles (Chandrababu *et al*., 2009), which differs entirely from the structure of native hBD-3 (Fig. 9.5H). Therefore, the cysteine residues are essential for stabilizing the defensin folds (Fig. 9.5), which are essential for specific interactions with chiral molecular targets such as proteins. Indeed, disruption of the S–S bonds of HNP1 and HD5 is detrimental to their binding to Zn^{2+}-dependent metallo-protease (bacterial lethal factor) or HIV gp-120 (Wei *et al*., 2009).

Circular θ-defensins such as RTD-1 (Trabi *et al*., 2001) contain two antiparallel β-sheets stabilized by three S–S bonds (Fig. 9.5J). Several linear AMPs adopt a similar β-hairpin structure. These include thanatin (Mandard *et al*., 1998), protegrin-1 (Aumelas *et al*., 1996), tachyplesins (Laederach *et al*., 2002), polyphemusin I (Powers *et al*., 2004), gomesin (Mandard *et al*., 2002) and arenicin-2 (Ovchinnikova *et al*., 2007). Thanatin consists of only one S–S bond. This bridge is essential for specific interaction of the peptide with *E. coli*, but unimportant for non-specific binding to membranes of the Gram-positive bacterium *Micrococcus luteus* (Imamura *et al*., 2008). Other AMPs listed above contain two S–S bonds. The S–S bond distant to the β-turn region is more important than the one close to the turn. Similar to the situation with defensins (Landon *et al*., 2008), the different cationic and hydrophobic side chains present on the same backbone fold (Fig. 9.5J) determine their antimicrobial activity spectrum (Rodziewicz-Motowidło *et al*., 2010). In addition, peptide oligomerization plays a role. While solid-state NMR data

indicated the existence of different oligomers for protegrin-1 in lipid bilayers (Mani *et al*., 2006), molecular simulation led to an octamer model as the active conformation in bacterial membranes (Langham *et al*., 2008).

AMPs with non-αβ structures

Several tryptophan-rich peptides have been found to adopt non-αβ structures (extended, turns or loops). Rozek *et al*. (2000) found that the CD spectra of indolicidin in the presence of membrane-mimicking vesicles or micelles did not resemble those of either α-helical or β-sheet proteins. In DPC micelles, NMR structural determination revealed a unique amphipathic structure, where the middle tryptophan-rich region plays the major role to anchor the peptide into the micelle, while the peptide termini are exposed and sampling a wide conformational space (Fig. 9.6A). In the structure, the aromatic rings of Trp6 and Trp9 pack against Pro7 and Pro10, respectively. It is worth noting that the structure of indolicidin in SDS micelles somewhat differs from that in DPC. Such a difference could be real, as CD spectra of the peptide in lipid vesicles or in micelles also differ (Rozek *et al*., 2000). Likewise, Vogel and colleagues found that tritrpticin adopted an amphipathic turn structure in SDS micelles (Fig. 9.6B), where the three tryptophan residues are clustered, but terminal cationic residues occupy a broader conformational space, implying motions (Schibli *et al*., 1999). Tinoco *et al*. (2002) found a WW+ motif in micelle-bound PW2 (Fig. 9.6C), an AMP (sequence: HPLKQYWWRPSI) obtained from phage display libraries. The WW+ motif was also found in other peptides. It is remarkable that the side chain of Arg9 is well defined in this case and packs against the aromatic ring of Trp8, providing an example for the cation–π interaction. The aromatic ring has π electron clouds on the surface, whereas the arginine side chain donates positive charge (Burghardt *et al*., 2002; Chan *et al*., 2006). As a fourth example, the structure of a tryptophan-rich segment corresponding to residues 4–14 of lactoferrin B2 is presented in Fig. 9.6D (Nguyen *et al*., 2005). In SDS micelles, this peptide also adopted a non-αβ structure with a distorted backbone. In these structures (Fig. 9.6), the six-membered aromatic rings of tryptophan residues tend to point towards the membrane. In the case of indolicidin and lactoferrin, this is supported by fluorescence spectroscopy as well as by 5-doxyl stearic acid spin label experiments. The lactoferrin peptide could be further shortened to a hexamer fragment with a similar antimicrobial activity to the long fragment (Chan *et al*., 2006). All of these NMR structures indicate a unique role for tryptophan in micelle binding. Tryptophan residues have long been recognized as an important membrane anchor, preferring the membrane–water interface (Wang *et al*., 1996; Yau *et al*., 1998; Schibli *et al*., 1999). Arginine residues provide positive charges for recognition of negatively charged bacterial membranes. The importance of these two residues is further verified by the identification of hexapeptides based on combinatorial library screening (Chapter 5). These hexamers are rich in both arginine and tryptophan residues and adopt extended structures in micelles (Rezansoff *et al*., 2005).

Then what is the minimal length for a tryptophan/arginine-rich peptide to be active against bacteria? Strøm *et al*. (2003) answered this question. While several hexapeptides with 50% of each residue were effective against both *E. coli* and *S. aureus*, only one pentamer with three tryptophan residues (WRWRW-amidated) showed good activity against both bacteria. Synthetic tetramers were inactive. It appears that five residues are required for a tryptophan/arginine-rich peptide to be active. (Coincidently, the shortest AMPs collected in the APD also have five residues (sequences: ACSAG, AMVSS and AMVGT). It is not clear whether these earthworm peptides also act by binding to bacterial membranes.) However, the inactive tryptophan/arginine peptides are useful templates to design selective AMPs (for other strategies, see Chapter 4). By attaching three hydrophobic tert-butyl moieties to the tryptophan ring, Haug *et al*. (2008) made an inactive tripeptide RWR bactericidal. Importantly, the tripeptide analogue only associated with anionic membranes, but not

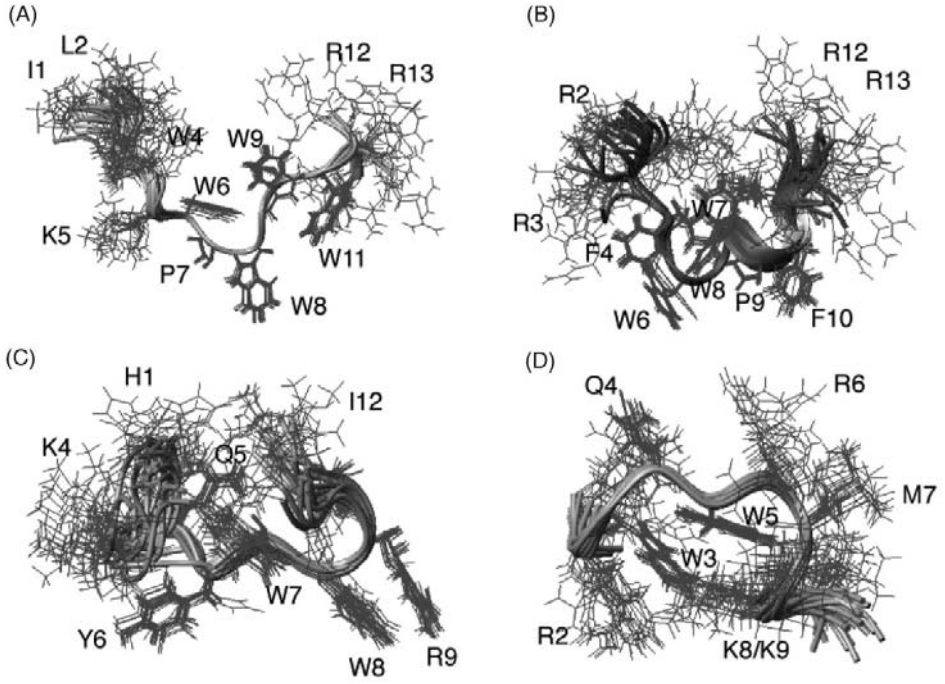

Fig. 9.6. Structural ensembles of AMPs from the non-αβ family. Presented are: (A) 16 structures of bovine indolicidin in dodecylphosphocholine micelles (Protein Data Bank (PDB) ID: 1G89) with the backbone atoms of residues 6–11 superimposed; (B) 19 structures of tritrpticin in sodium dodecylsulfate (SDS) micelles (PDB ID: 1D6X) with the backbone atoms of residues 4–11 superimposed; (C) 19 structures of PW2 in SDS micelles (PDB ID: 1M02) with the backbone atoms of residues 5–9 superimposed; and (D) 20 structures of lactoferrin B2(4–14) in SDS micelles (PDB ID: 1Y5C) with the backbone atoms of residues 3–5 superimposed. Key residues are labelled. All structures were determined by two-dimensional NMR.

with lipid vesicles consisting of phosphocholines. This example indicates the feasibility of engineering peptides.

In summary, membrane-targeting AMPs can adopt various 3D structures, ranging from α-helices to β-sheet, αβ-fold and non-αβ structures (Figs 9.4–9.6). Furthermore, peptides with different 3D structures can induce similar effects, such as positive curvature in membranes (Haney *et al*., 2010) or lipid domain formation (Epand *et al*., 2010). Therefore, it is not the specific type of 3D structure of the peptide, but the amphipathic nature that is critical for membrane targeting. Because of this, we have proposed a model that unifies membrane-targeting AMPs (Wang *et al*., 2005). In this universal model, the membrane perturbation potential of an AMP is determined by a proper display of hydrophobic and cationic side chains on the peptide surface, rather than the structural scaffolds underneath the peptide backbone.

9.3.4 Interactions of antimicrobial peptides with bacterial membranes by NMR spectroscopy

The interactions of human LL-37 with bacterial inner and outer membrane components

HSQC spectra are useful to follow the conformations of human LL-37 in different states. In an HSQC spectrum, the backbone amide proton of each amino acid gives only

one cross peak with a covalently bonded nitrogen nucleus. In water and at an acidic pH (Fig. 9.7A), the (^{1}H, ^{15}N) cross peaks of LL-37 are sharp and clustered in a narrow spectral region of 8.0–8.6 ppm, indicating a randomly coiled structure as previously suggested by CD (Johansson *et al*., 1998). At approximately pH 7, only a few cross peaks remain (Fig. 9.7B), probably due to the formation of elongated aggregates (Li *et al*., 2007) and perhaps structural heterogeneity. Since CD spectra indicate a helical structure at this pH (Johansson *et al*., 1998) and size-exclusion chromatography suggests a tetramer for synthetic LL-37 (Li *et al*., 2007), we propose that LL-37 adopts a four-helix bundle structure at the physiological pH. As only Ser37 is clearly visible, Fig. 9.7B also implies that residues 2–36 of LL-37 participate in tetramer formation. Such a helix bundle could dissociate into monomers when bound to anionic membranes (Oren *et al*., 1999).

LPS is the major outer membrane component of Gram-negative bacteria. For some AMPs, binding to LPS is the first step in interaction with such bacteria. Furthermore, LPS can cause sepsis. In this sense, the binding of AMPs to LPS is a desired property. In the presence of LPS (Fig. 9.7C), the cross peaks for the N-terminal region of ^{15}N-labelled LL-37 disappeared, but the signals for a few C-terminal residues were detected. This NMR spectrum implies that the N-terminal portion of LL-37 is bound to LPS and forms large complexes, leading to line broadening. The observation of intense signals for C-terminal residues 35–37 at nearly the same positions in the spectrum as observed for them in SDS or D8PG micelles indicates that the tail of LL-37 is also flexible in complex with LPS. In the presence of lipid A (portion of LPS, see Fig. 7.1), CD spectra indicated a helical structure (Turner *et al*., 1998). Therefore, the ordered–disordered structural model of LL-37 determined in SDS micelles (Fig. 9.4C) can be applied to the LPS case. Consistent with this model, deletion of a few N-terminal residues from LL-37 reduced LPS binding activity (Kirikae *et al*., 1998).

Next, LL-37 moves towards the inner membrane of bacteria. We utilized D8PG as a model, since it is a PG with shorter acyl chains. The NMR T_1/T_2 ratio-derived correlation times suggest that the LL-37–D8PG complex (6.9 ns at 50°C) tumbles slightly slower than the LL-37/SDS complex (7.0 ns at 25°C). This may explain why the NMR spectra of LL-37 in D8PG were poor at lower temperatures, but became well dispersed at 50°C (Fig. 9.7D). However, all the NMR data collected for LL-37 in the two membrane models are remarkably similar and indicative of similar structures (Wang, 2008c). In addition, we found similar conformations for several other peptides in SDS and D8PG micelles (Wang *et al*., 2003, 2004, 2005; Li *et al*., 2006a). Therefore, the LL-37 structure determined in SDS (Fig. 9.4C) can be applied to the PG case as well. In summary, the high-quality structure of LL-37 provides a basis for understanding its interactions with bacterial outer and inner membranes. While nearly the entire LL-37 is required for peptide–peptide interactions (i.e. oligomerization), only the long amphipathic helix is involved in peptide–membrane interactions (Fig. 9.7).

Atomic insight into peptide–membrane interactions

To provide additional insight into the mechanism of action of LL-37, we also explored the possibility of detecting peptide–lipid interactions directly by NMR. We noted that the amide proton signals of arginine side chains of LL-37-derived fragments tend to overlap with aromatic protons of phenylalanine residues in SDS. In DPC micelles, they can overlap with the backbone amide protons. However, they are well resolved in D8PG micelles (see Wang, 2008b). This unique spectral window for arginine side chains enabled the detection of arginine–PG interactions by solution NMR for the first time (Wang, 2007). A useful strategy is to draw one-dimensional NMR slices at resolved lipid signals. The well-resolved D8PG protons at 2.34 ppm (C2-H) and 5.25 ppm (Hβ) (lipid head group) enabled through space ^{1}H–^{1}H dipolar interactions with the arginine side chain amide protons at 7.3–7.5 ppm (summarized in Fig. 9.8). As

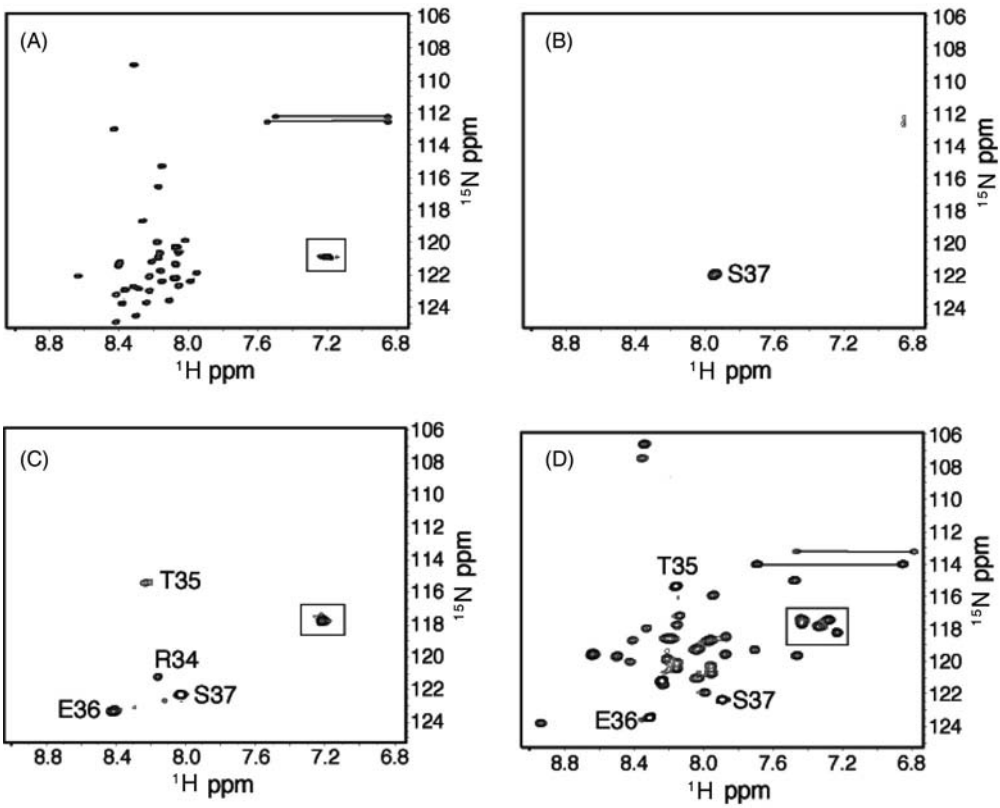

Fig. 9.7. Heteronuclear single-quantum coherence spectra of ^{15}N-labelled recombinant LL-37 in water at: (A) pH 3.6 and (B) pH 6.9; (C) in the presence of lipopolysaccharide (LPS to peptide molar ratio 1:1, pH 5.4, 30°C); and (D) in the presence of D8PG (peptide to D8PG ratio 1:30, pH 5.4, 50°C). The arginine side chain peaks are boxed and asparagine/glutamine side chain signals are connected by lines. Figure adapted from Li *et al.* (2007) and Wang (2008c).

these intermolecular NOE cross peaks showed normal build-up curves with the increase in mixing time (Wang, 2007), they resulted from direct dipolar interactions rather than spin diffusions (Wüthrich, 1986). Thus, such NMR measurements provide direct evidence for the association of cationic peptides with anionic lipids. The arginine–PG interactions observed herein provide the driving force for lipid domain formation in lipid bilayers induced by cationic peptides. For KR-12, the smallest bactericidal fragment of LL-37, the lipid domain formation is supported by solid-state NMR, differential scanning calorimetry and freeze-fracture electron microscopy (Epand *et al.*, 2009, 2010). The clustering of anionic PGs around cationic AMPs can have a global impact on the bacterium, in particular those biological processes that require PGs. Examples are bacterial signal-transduction pathways (Wang *et al.*, 2003) and voltage-dependent potassium channels (Schmidt *et al.*, 2006). Therefore, lipid domain formation will perturb the bacterial membrane physiology, leading to bacterial death (Wang, 2008c).

In addition, intermolecular NOE observations provide evidence for the approximation of hydrophobic side chains of peptides to acyl chains of lipid micelles (Fig. 9.8). For the bacterial membrane anchor, aurein 1.2 and LL-37 fragments (e.g. KR-12 and GI-20, sequences in Table 4.2), intermolecular NOEs were detected from the

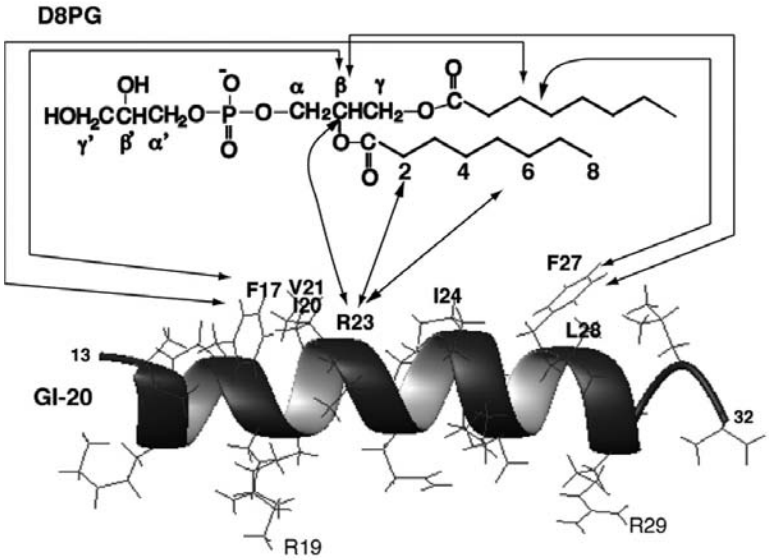

Fig. 9.8. Schematic representation of peptide–lipid interactions according to intermolecular nuclear Overhauser effect cross peaks measured for GI-20 in complex with dioctanoyl phosphatidylglycerol (D8PG) (peptide to D8PG molar ratio 1:5, pH 5.4, 25°C). GI-20 is a synthetic peptide corresponding to residues 13–32 of human LL-37, with the positions between residues Ile13 and Gly14 swapped (sequence: GIKEFKRIVQRIKDFLRNLV-NH_2). NH_2 indicates C-terminal amidation. Note that Phe17 and Phe27 are likely to interact with different lipid molecules due to the excess of D8PG. For a detailed description, as well as the raw NMR data, please refer to Wang (2007a).

peptide aromatic rings to the protons of the lipid head group as well as acyl chains (Wang *et al*., 2003, 2005; Wang, 2007). Similar results were observed for the aromatic rings of Phe5, Phe6, Phe17 and Phe27 on the hydrophobic surface of intact LL-37 in complex with D8PG micelles (Wang, 2008c). We conclude that these peptides associated with the membrane surface. In the case of amphibian aurein 1.2 and human LL-37, our conclusions are in line with solid-state NMR measurements in lipid bilayers (Henzler Wildman *et al*., 2003; Balla *et al*., 2004). Solution NMR, however, provides the long-desired evidence that these amphipathic AMPs indeed associate with bacterial membranes via both electrostatic and hydrophobic interactions. These results for animal AMPs indicate that solution and solid-state NMR studies provide complementary insight into peptide–membrane interactions in the different membrane-mimetic systems depicted in Fig. 9.3.

Interactions of AMPs with specific membrane components

Bacterial AMPs also target bacterial membranes. Hsu *et al*. (2002) have investigated the interactions of nisin Z, a bacteriocin, with cell-wall precursor lipid II by NMR. Using an ^{15}N-labelled peptide, they found selective chemical shift perturbation at the N-terminal region of this lantibiotic, indicating that the N-terminal region, including rings A, B and C, is responsible for nisin recognition. The C-terminal region (rings D and E) appeared not to be involved in the recognition. However, the hinge between these two domains is critical for biological activity. Such an interaction pattern is consistent with mutagenesis studies as well as other NMR measurements such as temperature coefficients. The insertion of the C-terminus into the membrane completes the pore formation process.

In response to pathogenic invasion, plants produce defence proteins that recognize chitin, a polysaccharide in the cell wall of fungi as well as in the exoskeleton of insects. Aboitiz *et al*. (2004) established a structural model for a plant hevein in complex with chitin based on NMR data. The extended binding site of the protein can accommodate at least five *N*-acetylglucosamine units. Plant and insect defensins also recognize sphingolipids in fungal membranes (Thevissen *et al*., 2004). In the case of plant Psd1, De Medeiros *et al*. (2010) identified the binding sites for glucosylceramide (indicated by an arrow in Fig. 9.5I) by NMR based on both chemical shift mapping and relaxation measurements. The binding involves hydrophobic interactions with residues Val13, Phe15, Ala18 and Trp38, as well as hydrogen bonding with Thr16 and Asn17. The recognition of unique fungal sphingolipids is essential for plant and insect defensins to induce subsequent events, release of reactive oxygen species or cell-cycle inhibition that leads to cell death (Aerts *et al*., 2007; Lobo *et al*., 2007).

In summary, AMPs from bacteria, plants and animals can recognize different bacterial membrane components by adopting a variety of 3D structures (Figs 9.4–9.6). These structural data will be invaluable for the future engineering of different molecular devices that specifically target certain microbes based on differences in membrane composition.

9.3.5 Structural basis of peptide selectivity

Due to cytotoxicity to human cells, many AMPs cannot be utilized directly. Select strategies for improving peptide selectivity (or therapeutic index) to reduce peptide hydrophobicity have been discussed in Chapter 4. These strategies can be grouped into two types based on whether there is conformational change in the peptide backbone. First, the peptide can be partially truncated or mutated residue by residue to reduce hydrophobicity without changing the structure of the peptide backbone. A successful example is the identification of selective KR-12 from human LL-37 (Wang, 2008c). Secondly, peptide hydrophobicity can also be diminished by altering the peptide backbone structure. Shai and colleagues have conducted extensive studies of the effect of selective D-amino acid incorporation on peptide structure and activity. Their scheme of incorporating D-amino acids at every two to three residues along the peptide chain has effectively disrupted helical structures of peptides based on CD and NMR studies (Papo and Shai, 2004, and references cited therein). Using this incorporation scheme, we found a non-classical amphipathic structure when residues 20, 24 and 28 of GF-17, an LL-37-derived fragment (sequence in Table 4.2), were replaced by D-amino acids (Li *et al*., 2006a). Backbone distortion caused an out-of-phase packing of hydrophobic side chains in GF-17D3 compared to the in-phase hydrophobic packing in a regular amphipathic helix (see Fig. 9 in Li *et al*., 2006a). We propose that the out-of-phase packing of hydrophobic side chains provides a structural basis for the decrease in peptide hydrophobicity, as reflected by a short retention time on the reversed-phase high-performance liquid chromatography column.

9.4 Structure-based Peptide Engineering

The ultimate goal of peptide engineering is to improve peptide selectivity, efficacy, stability, formulation and delivery. Structure-based design deals with one or more aspects of these properties to improve AMP druggability. For example, CP-11, an indolicidin derivative (sequence ILKKWPWWPWRRK-NH_2), has been determined by 2D NMR to have a U-shaped backbone with the N and C termini close to each other when in complex with micelles. Based on this conformation, Rozek *et al*. (2003) introduced a disulfide bond between the termini of CP-11. While CP-11 and cycloCP-11 showed similar antimicrobial activity, the cyclized form was more stable to trypsin than its parent molecule. Micelle-bound structures of AMPs also guide the

design of non-peptidic compounds. By mimicking the 3D structure of cyclo(RRWWRF), Appelt *et al*. (2007) successfully designed peptide analogues without using the peptide backbone. Based on the novel amphipathic structure determined by Li *et al*. (2006a), Gellman and colleagues succeeded in synthesizing random nylon co-polymers as peptide mimetics (Mowery *et al*., 2009). These studies, along with the synthesis of other antimicrobial peptidomimetics (Chapter 6), illustrates that the peptide backbone is not absolutely required. Rather, a proper arrangement of functional groups into an amphipathic scaffold is important. Structure-based engineering is not limited to membrane-bound AMPs. When a unique target molecule is verified within pathogenic microbes, novel peptides can be rationally designed based on the 3D structure of the AMPs in complex with non-membrane targets such as proteins or DNA. Future structural determination of such complexes can borrow the NMR strategies already established and demonstrated for protein–protein or protein–nucleic complexes (Wang *et al*., 2000; Marintchev *et al*., 2007).

9.5 Concluding Remarks and Perspectives

AMPs can interact with membranes and non-membrane targets. Much research has been focused on the membrane-binding property of AMPs (Chapter 7). In the membrane-bound state, they can adopt a variety of backbone scaffolds (α-helices, β-strands and extended structures). Typical examples in the helical family are magainins, aureins, dermaseptins, temporins and human cathelicidin LL-37. Human defensins are typical members of the β-sheet family. Indolicidin and tritrpticin are representative members of the extended-structure (non-αβ) family. Although these polypeptide backbones fold in different manners, their surfaces share a common amphipathic nature (i.e. a clear segregation of hydrophobic and cationic side chains). Evidently, such a feature is essential for a positively charged amphipathic peptide to associate with negatively charged bacterial membranes. We have illustrated by NMR that the long amphipathic helix of LL-37 is responsible for association with bacterial outer- and inner-membrane components (LPS and PGs) (Fig. 9.7). In addition, we have observed direct phenylalanine–PG and arginine–PG interactions by solution NMR. These data depict a picture that the positively charged peptide is indeed located on the negatively charged membrane surface via both electrostatic and hydrophobic interactions. Such interactions provide the driving forces for the formation of non-lamellar phases or lipid domains. It is useful to restate the membrane perturbation potential model (Wang *et al*., 2005). This model emphasizes the importance of a proper spatial presentation of cationic and hydrophobic side chains on AMP surfaces that determine its ability to perturb the bacterial membranes, as well as the outcome of peptide–membrane interactions that lead to bacterial death. Our investigations of peptide–membrane interactions have deepened and broadened our view on AMPs. In addition to lipid bilayers, AMPs also target specific molecules on the cell surface. While lantibiotics such as nisins are known to bind to cell-wall precursor lipid II, insect and plant defensins specifically recognize unique fungal sphingolipids. In other situations, AMPs also recognize carbohydrates. It is evident that NMR has played an important role in providing atomic insights into a variety of molecular interactions involving cationic AMPs.

Not all AMPs target bacterial membranes and interactions of AMPs with other targets have emerged (for examples, see Chapter 8). A hallmark for AMPs to act on non-chiral bacterial membranes is that the D- and L-enantiomers with symmetric 'mirror images' have essentially identical antimicrobial activity (Wade *et al*., 1990; Wei *et al*., 2009). This does not apply when AMPs target chiral molecules such as proteins. The interactions of AMPs with non-membrane targets are important for many biological functions of human cathelicidin and defensins. Under what conditions is there a correlation between peptide aggregation and biological activity? How does an AMP traverse bacterial membranes and bind to intracellular targets

such as DNA or proteins? What is the structural basis of human defensins HNP1–3 and HD-5, but not HNP-4, HD-6 or β-defensins (Wei *et al.,* 2009), in recognition of HIV envelope protein gp120 or protein toxins? How does LL-37 interact with membrane-bound receptors to promote tumour metastasis, while a LL-37 fragment reverses the process? Considering that there are thousands of such AMPs with various molecular targets, structural biology will continue to play an essential role in elucidating the basis of these ancient molecules in host defence and in regulating immune responses.

Note Added in Proof

During the production of this book, high-level bacterial and yeast expression systems suitable for large-scale production of recombinant LL-37 have also been reported (Krahulec *et al.,* 2010; Liu *et al.,* 2010).

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10 Lung Infection: Shifting the Equilibrium Towards the Free and Active Form of Human LL-37 and the Design of Alternative Antimicrobial Agents

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Abstract

Under disease conditions, human LL-37 and other cationic antimicrobial factors are expressed but inactive due to their binding to anionic polyelectrolytes such as DNA, F-actin or acidic polysaccharides. This chapter discusses the basis for this electrostatic interaction and alternative strategies that liberate LL-37 and other amphipathic cations from the bound state so that they are available to fight invading pathogenic bacteria. The potential of this approach for therapeutic use is discussed.

10.1 Introduction

10.1.1 Electrostatic properties of LL-37

Antimicrobial agents are generally, or perhaps universally, multivalent cationic amphiphiles. The fact that similar bactericidal effects can be achieved against Gram-negative and Gram-positive bacteria by natural peptides such as LL-37 expressed in humans (Burton and Steel, 2009; Bucki *et al*., 2010), steroid derivatives such as squalamine expressed in dogfish sharks and a host of synthetic peptide and non-peptide compounds strongly suggests that general physical chemical features, rather than stereospecific biochemical interactions with a unique bacterial target, define the mechanisms by which these agents exert their biological effect. The necessity for a polycationic surface to direct the antimicrobial agent first to the highly anionic bacterial cell wall and then to negatively charged surfaces in the inner membrane, together with the requirement for appropriate hydrophobic surfaces that disrupt bacterial lipid bilayers, can be satisfied by many peptide and non-peptide structures, and the guiding principles for this polycationic and hydrophobic combination are beginning to be elucidated (Yang *et al*., 2007). The generality of this physico-chemical mechanism is perhaps the main reason why bacteria cannot easily develop resistance to these agents by mutations of single or even multiple genes, but it also places restraints on the environments in which antibacterial functions of cationic amphiphiles can be maintained. In particular, the antibacterial effects of LL-37, which has a net charge of +6 at pH 7 (for the micelle-bound three-dimensional structure, see Fig. 9.4C, Chapter 9), are lost in purulent fluids such as cystic fibrosis (CF) sputum. CF sputum is highly enriched in the linear polyelectrolytes DNA and filamentous

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(F)-actin, glycosaminoglycans and mucins produced by the host, as well as in anionic polyelectrolytes secreted by bacteria such as *Pseudomonas* (Weiner *et al*., 2003; Baranska-Rybak *et al*., 2006; Bergsson *et al*., 2009).

10.1.2 Interaction of polyvalent cations with linear polyelectrolytes

Charged lines and surfaces interact with soluble counterions differently from the interactions of two point charges, and when the linear charge density of a rod-like filament is sufficiently high, more multivalent than univalent counterions can condense on the filament surface. If a sufficient number of polyvalent counterions condense, the electrostatic repulsion between like-charged linear polyanions (DNA or F-actin in the case of CF) is overcome by attractive interactions, and multivalent cations, such as LL-37, induce the formation of bundled filament arrays with counterions trapped inside the bundle. Experimental and theoretical work on polyelectrolyte condensation, developed initially to model the condensation of DNA (Perdue *et al*., 1976; Overman *et al*., 1998; Fuller *et al*., 2007; Devkota *et al*., 2009), shows that multivalent counterions do not bind tightly to the surface of the polyelectrolyte, but maintain mobility along the filament surface. When sufficient counterions condense on the filament, the electrostatic repulsions between filaments that keep them separated become weaker than attractive interactions and the filaments collapse into bundles. Previous studies of F-actin (Tang and Janmey, 1996; Tang *et al*., 1997) and alginate (Bu *et al*., 2004; Donati *et al*., 2006) confirm that the polyelectrolyte behaviour first observed with DNA also applies to these filaments, which also have fixed charge spacing smaller than the Bjerrum length and are therefore classified as strong polyelectrolytes.

The mechanism by which polyelectrolyte filaments can trap multivalent cations such as LL-37 depends on the strength of the electric field attracting the counterion to the filament. The electric field, and therefore the force on an ion, emanating from a point charge (such as a small soluble ion) decays with the square of the distance between charges as: $E_{point} = q/4\pi\varepsilon r^2$. Here, q is the charge on the ion (in this case the cationic antimicrobial peptide (AMP)), ε is the permittivity of water and r is the distance between the two charges. In contrast, the field from a line with linear charge density λ_l (such as F-actin, DNA or alginate) decays only linearly with r as: $E_{line} = \lambda_l/2\pi\varepsilon r$. The field from an infinitely large charged surface (here, the anionic surface of the bacterium) is a constant with respect to r for distances that are small relative to the size of the cell. The simplest consequence of these laws is that for a given number of anionic charges, arranging them as distributed points, lines or surfaces has a large effect on their attraction for counterions, with the greatest attraction for the charged surface. Another result of polyelectrolyte theory is that multivalent counterions are drawn to the filament much more avidly than ions of smaller valence, largely because the entropy loss from sequestering a smaller number of high-valence ions to lower the surface charge of the filaments is less than the same degree of charge neutralization due to condensation of a larger number of lower valence counterions. In addition, because the fixed charges on actin, DNA and other biopolymers are univalent (phosphodiesters or carboxyls), sequestration of multivalent counterions produces regions of positive charge that can lead to filament–filament interactions due to the formation of structures analogous to Wigner lattices (Lenac and Sunjic, 1991). These features of polyelectrolytes have recently been shown to account for unusual structures formed in rings of F-actin bundled by divalent metal ions (Tang *et al*., 2001; Cebers *et al*., 2006), and for the fact that filament bundles stabilized by counterions, such as F-actin bundled by lysozyme (Guaqueta *et al*., 2006), cannot be dissociated by increased monovalent ions.

There are many assumptions – including that the lines and surfaces are infinite and uniform – but at the crudest level, this hierarchy of electrostatic attractions rationalizes why cationic antimicrobial agents selectively attack bacterial membranes without an apparent unique bacterial binding

partner, and why polyelectrolyte filaments such as actin, alginate and DNA can inhibit their functions. Of course, AMPs are not simply cations and hydrophobic moieties. The juxtaposition of cationic charges with spacing suitable for binding lipopolysaccharide (LPS), lipoteichoic acid (LTA) or other anionic targets, and other modifications that optimize lethal effects on bacteria, are essential for the biological function of AMPs.

10.2 Production of Anionic Polyelectrolytes by Host and Microbial Sources

During physiological cell turnover, anionic polyelectrolytes located inside the cell, such as DNA and F-actin, are not accumulated in extracellular compartments due to sufficient phagocytic elimination of cell fragments generated during apoptosis. Bacterial infection rapidly leads to infiltration of white blood cells, mainly neutrophils, which are highly enriched in cytoskeletal proteins. During the course of necrosis, both the anionic cytoskeletal filaments and DNA of the neutrophils that crawled into the extracellular fluid, which contains bacteria, are released. Considering that DNA was first isolated from pus due to its high concentration of neutrophils, prolonged infection may generally lead to neutrophil accumulation and necrosis as well as other host cell death that would release polyelectrolytes from the cytoskeleton and the nucleus into the extracellular space. Here, they can inhibit cationic antibacterial peptides (CAPs) and potentially contribute to biofilm production. This concentration of DNA and F-actin is most evident in sputum collected from CF patients with chronic bacterial infection of the lungs. Large DNA-containing fibres, hundreds of microns long, have been documented in CF sputum (Fig. 10.1A) since at least the 1960s (Chernick and Barbero, 1959; Shak *et al*., 1990). They are also present in other inflammatory sites characterized by pus formation (Fig. 10.1B).

The necrotic death of neutrophils and epithelial cells that releases DNA into the extracellular space also releases F-actin and monomeric actin, which can polymerize and form a mixed network of DNA and actin filaments. F-actin bundles (Fig. 10.1) have been identified as an additional abnormal biopolymer that can affect the viscous properties of lung airway fluid in respiratory disease (Vasconcellos *et al*., 1994; Sheils *et al*., 1996).

Bacteria also produce anionic polysaccharides, in part as extensions from the lipid A moiety of LPS and often as much longer polysaccharides extruded into the space surrounding the bacterium. During infection, bacteria can begin to produce exopolysaccharides – alginate in the case of *Pseudomonas aeruginosa* – and form biofilms. These biofilms contain bacterial DNA (Whitchurch *et al*., 2002) along with other polymers that create a hydrated and charged environment around the bacterial surface, preventing access by cationic antimicrobial agents such as LL-37. Other polyanionic

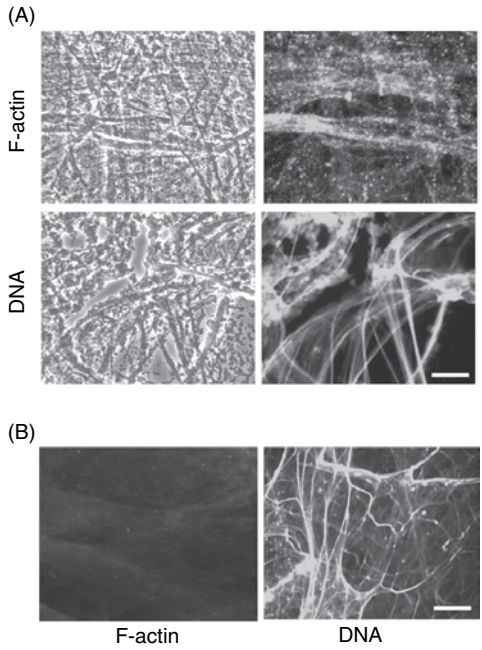

Fig. 10.1. F-actin and DNA bundles in (A) cystic fibrosis sputum and (B) pus samples. F-actin and DNA were visualized by Alexa Fluor phalloidin and YOYO-1 labelling, respectively. The morphology of corresponding cystic fibrosis samples is shown by phase contrast microscopy (black and white images). Scale bars: 200 microns.

bacterial factors that can inhibit LL-37 and other AMPs are bacterial outer-wall constituents, LPS from Gram-negative bacteria such as *P. aeruginosa* and LTA from Gram-positive bacteria, which both appear to be the targets of antimicrobial agents on the bacterial cell wall (Gutsmann *et al*., 2005), but which once released from bacteria are potent inhibitors of AMPs (Bucki and Pastore, 2006). Complex formation by LPS with antibacterial peptides such as cathelicidins has been studied to characterize how the peptides inhibit the endotoxic effects of bacterial LPS (Nagaoka *et al*., 2001). Although systemic concentrations of LPS (endotoxin) are much lower than those of LL-37, in localized chronic infections LPS accumulation may lessen the effect of AMPs on the intact bacteria. Anionic glycosaminoglycans such as chondroitin sulfate, hyaluronic acid and various mucins that function at the surface of epithelial cells as part of a mucosal barrier may also reduce the antibacterial activity of CAPs. However, in the case of mucins and CAPs, which from a functional point of view are both antibacterial, partial inactivation of CAPs in the presence of mucins is compensated for by an increased local CAP concentration at the epithelial surface due to sequestration by these anionic polymers (Felgentreff *et al*., 2006).

10.3 Sequestration and Inactivation of LL-37 by DNA and F-actin

Both bactericidal and bacteriostatic functions of LL-37 are strongly inhibited by DNA and F-actin (Fig. 10.2). These two polymers are highly concentrated at inflammatory sites such as in CF lung airway fluid, where their concentrations can reach up to approximately 20 and 2 mg ml^{-1}, respectively. In the highly purulent sputum characteristic of chronic bacterial infection in the lungs, the expression of antimicrobial factors is often upregulated, but they are not active, presumably due to their sequestration within polyelectrolyte bundles (Weiner *et al*., 2003).

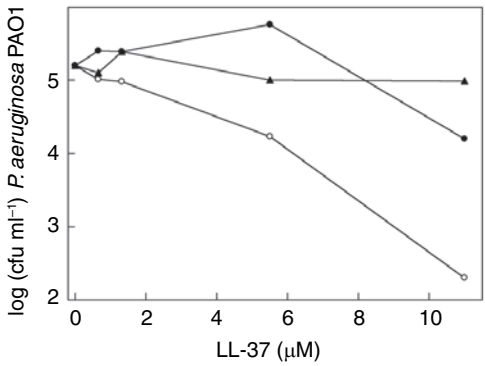

Fig. 10.2. Inhibition of LL-37 function by F-actin and DNA. Growth of *Pseudomonas aeruginosa* PAO1 in a microbroth dilution assay, after incubation with various concentrations of LL-37 alone (open circles) or with the addition of DNA (triangles) or F-actin (filled circles). cfu, colony forming units.

10.4 Releasing LL-37 from Polyelectrolyte Bundles

In the absence of multivalent counterions, the linear polyelectrolytes at sites of infection – including DNA, F-actin, mucin, alginate and the micelles or other aggregates of LPS and LTA from lysed bacteria – would be mutually repulsive and so tend not to aggregate. However, DNA and F-actin, the stiffest polyelectrolytes and therefore the easiest to visualize by light microscopy, are arranged in bundles that are many times larger than the cells from which they come. These aggregates of like-charged polyelectrolytes must be stabilized by strong forces that overcome their electrostatic repulsions, and the multivalent elements of the innate immune system, including LL-37, defensins and lysozyme, are the most abundant such molecules in airway fluid and extracellular sites of infection.

Immunohistochemical assays show that LL-37 is co-localized in bundles of actin and DNA in CF sputum (Bucki *et al*., 2007a). In this scenario LL-37, even when upregulated by the lung epithelium and released from neutrophils, would be trapped within filament bundles and therefore unavailable

to attack bacteria. This hypothesis suggests that strategies that destabilize polyelectrolyte bundles might release LL-37 and thereby enhance endogenous antibacterial function even in the absence of increased LL-37 expression. Polyelectrolyte bundles can be dissociated by several mechanisms. If the counterions are dynamic within the bundles, LL-37 might be released by competitive binding of a stronger polyvalent cation (Purdy Drew *et al*., 2009).

10.4.1 Severing polymers with DNase, gelsolin and alginase

Because the electrostatic effects that sequester multivalent cations to the surface of a highly charged linear polyanion require that the linear polyelectrolyte is sufficiently long as to be approximated as an infinite line, decreasing the filament length to some critical value should abrogate counterion condensation. Experimental tests show that, for actin filaments or DNA, this critical length is in the order of a few hundred nanometres (Tang and Janmey, 1996; Tang *et al*., 1996). This degree of filament shortening can be achieved by depolymerizing DNA with DNase or severing actin filaments with gelsolin, a protein present in both cytoplasm and extracellular fluids and capable of breaking the non-covalent bonds that hold F-actin together (Yin *et al*., 1984). Indeed, release of LL-37 from a pellet and into a supernatant fraction has been observed when CF sputum samples were treated with DNase I, gelsolin, polyaspartate or their combination (Fig. 10.3). These data confirm the hypothesis that LL-37 and other cationic antimicrobial factors are trapped in actin/DNA bundles in CF sputum and can be released by mucolytic agents directed at polyanionic filaments. Additionally, *in vitro* studies show that, by depolymerizing actin with gelsolin or DNA with DNase, antibacterial function is partially restored in CF sputum. Figure 10.4 compares the degree to which addition of gelsolin, DNase and exogenous LL-37 lessen bacterial colony growth from CF sputum. Each of these agents could decrease bacterial outgrowth by approximately 50%, and the combination of DNase I and gelsolin was more effective than either treatment alone. Even high concentrations of LL-37 were not able by themselves to eradicate bacterial growth, but addition of gelsolin significantly increased the function of LL-37. However, upon action of DNase and gelsolin, which disaggregates CAPs–DNA–F-actin bundles, the efficiency of CAP release may still be compromised if CAPs form precipitates with short DNA or F-actin fragments.

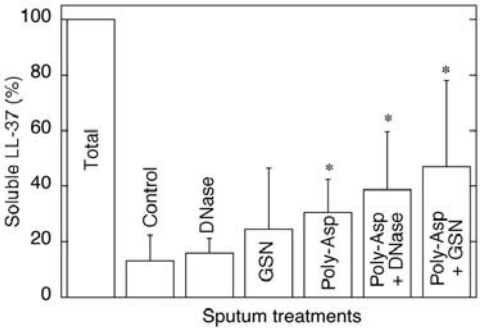

Fig. 10.3. Quantitative immunoblot analysis of LL-37 released from cystic fibrosis sputum as a percentage of the total LL-37 concentration in the whole volume of individual cystic fibrosis sputa. Data represent the means of six different patient samples. Error bars represent standard deviations. **P*<0.05 versus the respective values in supernatant (unpaired *t*-test). GSN, gelsolin; poly-Asp, polyaspartate.

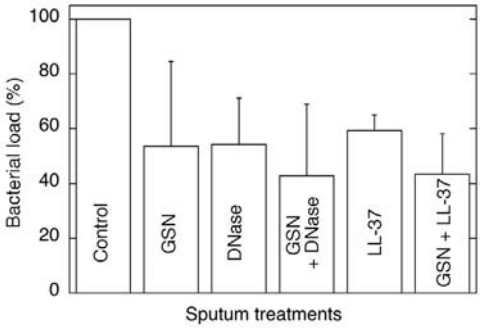

Fig. 10.4. Bacterial load of cystic fibrosis sputa before (control) and after treatment with 0.5 μM gelsolin (GSN) or 100 μg ml^{-1} recombinant human DNase, alone or in combination with GSN, and exogenous LL-37 peptide, alone (3 μM) or in combination with gelsolin (0.5 μM).

Polyelectrolyte networks at the site of infection can also directly affect invasive bacteria. It was recently shown that *P. aeruginosa* growth, in settings that included neutrophils, enhanced formation of biofilms (a pattern of bacterial growth associated with high resistance to antibiotic treatment) by stimulating the expression of alginate by *P. aeruginosa* (Walker *et al*., 2005). Neutrophils are likely to have multiple effects on *P. aeruginosa* biofilm production, some involving the effects of neutrophil-derived hydrogen peroxide on *P. aeruginosa* mucoid conversion. *In vitro*, however, extracts of neutrophils are 94% as potent as intact neutrophils in enhancing biofilm production by *P. aeruginosa*. Of three fractions tested, granular proteins and DNA had little to no effect on biofilm production, whereas purified F-actin had a strong stimulating effect. Depleting actin from whole neutrophil lysates diminished their effect. Therefore, dissolution of bundles with polyanions and depolymerization of actin with gelsolin or DNase might reverse the strong effect of actin and possibly other cellular debris to stimulate *P. aeruginosa* biofilm production.

In addition to its effect on actin, gelsolin may have another beneficial effect in infected fluids. Gelsolin is a strong ligand for LPS and LTA (Bucki *et al*., 2005, 2008a) and can inhibit LPS and LTA cell immunostimulatory effects through inhibition of their binding to Toll-like receptors, after their release from the bacterial cell wall. Bacterial exopolysaccharides such as those produced by mucoid strains of *P. aeruginosa* have been shown to interfere with the antibacterial activity of aminoglycosides and granulocyte-mediated non-opsonic phagocytosis. However, these effects of exopolysaccharides were reversed by pretreatment of the mucoid cells with alginase (Bayer *et al*., 1991; Hatch and Schiller, 1998; Alipour *et al*., 2009), again suggesting that decreasing polyelectrolyte length lessens the inhibition of antibacterial factors.

10.4.2 Destabilizing bundles with small multivalent anions

Theoretical models of polyelectrolyte solutions predict that if bundles of charged filaments are stabilized by polyvalent counterions they will be dissociated by the addition of soluble co-ions. This prediction has been verified by experiments *in vitro* with purified DNA or F-actin (Tang and Janmey, 1996). In the case of actin/DNA bundles in CF sputum, polycationic ligands such as LL-37, histones and lysozyme trapped in the bundles will be released when the polyelectrolyte bundles dissolve, even if the filaments do not depolymerize. Soluble multivalent anions such as polyaspartate and polyglutamate have been shown to both reduce the viscosity of CF sputum (Tang *et al*., 2005) and release LL-37 into the soluble fraction (Fig. 10.3).

The most significant potential of anionic polyaspartate is likely to be its ability to restore, at least partially, the antimicrobial activity of endogenous CAPs within purulent fluids such as CF sputum. This antimicrobial activity is inactivated by a number of factors, including polyelectrolyte filaments (Tang *et al*., 2005). It has been shown that, consistent with the electrostatic hypothesis (Tolker-Nielsen and Hoiby, 2009), the addition of polyaspartate to CF sputum lowered the outgrowth of bacteria from these samples by 30%, while polyaspartate had no direct antibacterial effect when added directly to *P. aeruginosa* PAO1 (Fig. 10.3) (Tang *et al*., 2005). Increased doses of DNase or gelsolin together with polyaspartate may further improve the release of LL-37. These data demonstrate the potential for polyaspartate to lessen bacterial burden through a mechanism that liberates antimicrobial functions already residing within CF sputa. Additionally, polyaspartate effectively prevents and disrupts *P. aeruginosa* biofilm formation. This potentially therapeutic effect of polyaspartate is synergistically enhanced by DNase (Parks *et al*., 2009).

10.5 Designing Antimicrobial Agents Resistant to Inactivation by Polyelectrolytes

In addition to treatments directed at making linear polyelectrolytes less inhibitory to the native antimicrobial agents resident within purulent sputum, alternative antibacterial factors that are more resistant to inactivation

by DNA or F-actin have potential for practical use.

10.5.1 Cationic steroids: minimizing and redistributing charge

AMP activity can be mimicked by cationic steroid antibiotics (CSAs), which were first developed with the intent of reproducing or improving the antibacterial activities of polymyxin B (Ding *et al*., 2004). These antibacterial agents can have potency similar to that of LL-37, but compared to natural antibacterial peptides their net positive charge is usually lower and chemical synthesis provides the opportunity to control their charge distribution. One such synthetic compound, CSA-13, is significantly less sensitive to inactivation by DNA or F-actin compared to LL-37 and shows better efficacy in CF sputum (Bucki *et al*., 2007b). Synthetic CSAs share some structural and functional properties with squalamine, a membrane-active CSA (Moore *et al*., 1993) that was first isolated from tissues of the dogfish shark. Their bactericidal properties are due to membrane disruption and they display a moderate degree of selectivity for prokaryotic over eukaryotic membranes (Salunke *et al*., 2006). Several cholic acid derivates (Bellini *et al*., 1976) such as cholic acid with basic amino acids (Bellini *et al*., 1979; Li *et al*., 1999), guanidine (Savage and Li, 2000) and spermidine (Chen *et al*., 2006) have been characterized as potent antimicrobial agents that are effective against multidrug-resistant bacteria (Schmidt *et al*., 2001) and fungi (Hazra *et al*., 2004). In addition to these steroidal conjugates (Salunke *et al*., 2006), a conjugate of spermine with two dexamethasone moieties displays strong antibacterial activity with low lytic effect on eukaryotic plasma membranes (Fein *et al*., 2009). This molecule also inhibits the inflammatory response induced by bacterial wall products *in vitro*.

10.5.2 Increasing the hydrophobicity of antimicrobial agents

The approach to enhancing the desired bactericidal activity and reducing the haemolytic effect (a measure of peptide toxicity for host cells) of CAPs has been based on rational modifications of existing peptide sequences. Since minor differences in amino acid sequence can produce significant differences in antimicrobial activity (Raj *et al*., 2000), the possibility of strategically varying key amino acids has been explored by many investigators (Travis *et al*., 2000; Hancock and Patrzykat, 2002; Jenssen *et al*., 2006; Saugar *et al*., 2006; Zelezetsky and Tossi, 2006). Sequence modifications offer an enormous number of combinatorial possibilities, including deleting, adding or replacing one or more residues, as well as truncating the peptide or assembling chimeric peptides based on sequences from the same or different species. In addition, conjugating peptides with lipophilic acid (Avrahami and Shai, 2002) or rhodamine B (Bucki *et al*., 2004), incorporating carbonate bond(s) (Lee and Oh, 2000) or peptoid residues (Song *et al*., 2005) and α-amino acid epimerization (Mangoni *et al*., 2006) have been found to change peptide physico-chemical properties and often translate to better biological activity. Enhancing antibacterial potency by manipulating hydrophobicity proves the potential to reduce or reorganize cationic moieties, thereby weakening interactions with anionic polyelectrolytes. However, modifications that increase hydrophobicity are also likely to result in increasing peptide toxicity towards host cells.

10.6 Possible Therapeutic Use

10.6.1 Selective use in some body fluids and not others

Bacterial infection takes place in various areas of the body and CAP activity may vary depending on the local environment factors. Understanding the physiological homeostasis of CAPs in these settings is important to design effective antimicrobial and anti-inflammatory therapies. Generally, CAPs, including human cathelicidin LL-37, have been shown to be active in body fluids with low protein concentrations such as airway surface fluid (Bucki *et al*., 2007a), tears

(Huang *et al*., 2006), sweat (Rieg *et al*., 2006), gastric juice (Leszczynska *et al*., 2009) and saliva (Gutner *et al*., 2009). However, several body fluid components have been identified as potent inhibitors of CAP functions. In blood, most natural and synthetic cationic antibacterial molecules lose their antibacterial activity due to their insertion into plasma lipoproteins and interaction with divalent cations. Accordingly, LL-37 is inactive in the presence of human serum, and its lack of antibacterial activity cannot be explained by proteolytic degradation. LL-37 activity inhibition in human serum is mediated mostly by binding to human apolipoprotein A-I (Wang *et al*., 1998, 2004). Interestingly, the *de novo*-engineered AMP WLBU2 resists the inhibitory action of blood and physiological concentrations of Mg^{2+} and Ca^{2+} (Deslouches *et al*., 2005), suggesting that it is possible to develop antibacterial peptides that will maintain activity in serum. Mucin is another factor, widely present at the surfaces of airway, digestive or urogenital tracts, that can compromise the antibacterial function of LL-37 (Bucki *et al*., 2008b). On the other hand, interaction of CAPs with mucin can increase the concentration of CAPs in the area most subject to microorganism attack, and mucin has been shown to be involved in protecting peptides from proteolysis (Gutner *et al*., 2009). Antimicrobial activity at mucosal sites and ocular surfaces is affected by interactions between different classes of antimicrobial factors (Nagaoka *et al*., 2000; McIntosh *et al*., 2005).

10.6.2 Stimulation of host cells

Considering that bacterial killing by LL-37 occurs primarily by depolarizing and permeabilizing bacterial membranes or by induced autolysis (Ginsburg, 2004), it has been suggested that a similar mechanism of action can be used to target disruption of cancer cells by C-terminal LL-37 fragments (Li *et al*., 2006). In addition to its bacteria-killing ability, LL-37 can also affect host leukocyte functions that are associated with host immune defence. LL-37 affects the life span and inflammatory functions of neutrophils (Barlow *et al*., 2006), the differentiation of dendritic cells that bridge innate with adaptive immune responses (Kandler *et al*., 2006; Skokos and Nussenzweig, 2007) and the chemotactic activities of monocytes, eosinophils and T cells (De *et al*., 2000; Koczulla and Bals, 2003; Khine *et al*., 2006). LL-37 directly activates endothelial cells, which results in the increased proliferation and formation of vessel-like structures in cell culture. Decreased vascularization during wound repair in mice deficient for CRAMP, the murine homologue of LL-37/hCAP-18, shows that cathelicidin-mediated angiogenesis is important for cutaneous wound neovascularization *in vivo* (Koczulla *et al*., 2003). Additionally, LL-37 induces wound healing, proliferation and migration of airway epithelial cells, suggesting that this peptide might be involved in the regulation of tissue homeostasis in the airways (Shaykhiev *et al*., 2005). The regenerative activities of LL-37 support its therapeutic potential to promote wound healing (Tokumaru *et al*., 2005; Carretero *et al*., 2008).

10.7 Conclusion

LL-37 and other cationic antimicrobial factors essential to the innate immune response appear to function largely by physicochemical interactions with bacteria in which electrostatic attraction is a fundamental feature. This mechanism also limits the chemical environments in which these cationic amphiphiles can perform as effective bactericidal agents. Release of polyanionic filaments and other aggregates into the extracellular space in which infection occurs can inactivate the antimicrobial effects of LL-37, even under conditions where it is upregulated and protected against degradation. Multiple strategies directed at disrupting the complex of cationic AMPs with anionic DNA, F-actin, alginate or other polyelectrolytes, or designing novel antimicrobial agents that have less affinity for polyelectrolytes, have the potential to improve antibacterial therapies in respiratory infections and other diseases.

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11 Role of Vitamin D in the Enhancement of Antimicrobial Peptide Gene Expression

John H. White* and Ari J. Bitton

Abstract

1,25-Dihydroxyvitamin D (1,25D), the hormonally active form of vitamin D, was initially identified as a key regulator of calcium homeostasis. 1,25D signals through the nuclear vitamin D receptor, a ligand-regulated transcription factor. More recent studies have revealed that 1,25D regulates a number of physiological processes, including innate immunity. The innate immune system is responsible for rapid, non-specific host responses to pathogenic infection. Unlike adaptive immunity, innate immune responses do not confer lasting protection on the host. 1,25D induces the expression of LL-37 and hBD-2, antimicrobial peptides that serve as vanguards of innate immune responses. Antimicrobial peptides are small proteins with antibiotic properties that can also acts as signalling molecules to modulate specific responses such as wound healing and cytokine induction. This chapter focuses on the gene regulatory activity of 1,25D, with an emphasis on its role in the potentiation of innate immune responses and the promotion of antimicrobial peptide expression.

11.1 Vitamin D

Vitamin D is somewhat of a misnomer in that it is not a true vitamin by classical definition (Crowle *et al*., 1987). A vitamin is an essential substance that is required for proper physiological function, but is not produced in sufficient quantity by the body (Ziegler and Filer, 1996). As detailed below, we can generate physiologically adequate levels of vitamin D from the action of solar ultraviolet (UV) light on the skin. Vitamin D has been best known for its role in calcium homeostasis and bone maintenance. However, there are threads of evidence for a broader spectrum of actions of vitamin D that extend back over millennia. More recently, research has established roles for vitamin D in controlling nervous system function and cell growth and differentiation, and regulating immune system responses. At the molecular level, much of the recent emphasis has refined the focus on vitamin D and its role as a potent modulator of gene expression.

11.2 Early History of Vitamin D

Vitamin D therapy can be traced back to Hippocrates, the father of medicine, who used heliotherapy, or exposure to sunlight, to treat phthisis (tuberculosis (TB)) (Masten, 1935). Nutritional vitamin D therapy arose from the early pharmacology of cod-liver oil

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use. Cod-liver oil was first described as a medicinal agent for the treatment of chronic rheumatism in 1789. Throughout the next century, medical literature documented its effectiveness for treating a number of prevalent conditions such as gout and scrofula, a form of TB that infects the lymph nodes (Guy, 1923). Beginning in the 1820s, studies showed that administering doses of cod-liver oil to afflicted children could cure rickets (Guy, 1923), a nutritional disease characterized by a lack of vitamin D or calcium leading to bone softening and deformity. However, it was not until several decades later that the active compound in cod-liver oil was identified as vitamin D. By 1849, the list of conditions treatable with cod-liver oil had grown to include TB (Grad, 2004; Martineau *et al*., 2007).

Several independent observations in the 19th and early 20th centuries fostered further links between sunlight and cutaneous vitamin D synthesis. This was highlighted by reports such as the following (Guy, 1923; Crowle *et al*., 1987; Grad, 2004; Lin and White, 2004; Martineau *et al*., 2007):

- A relationship was found between the prevalence of rickets and lack of exposure to sunlight.
- UV light therapy was found to cure rickets.
- Feeding UV-irradiated skin to rats was found to have the same protective effects against rickets as did cod-liver oil.
- UV light was also found to be useful in treating cutaneous TB (lupus vulgaris).

These studies support associations established between vitamin D deficiency and the prevalence of certain diseases.

11.3 Vitamin D Deficiency

Today, vitamin D deficiency is regarded as a widespread and completely preventable health concern. Although there is no strict definition, vitamin D deficiency is characterized by low circulating vitamin D metabolite levels, caused by a combination of seasonal variations in sunlight and inadequate dietary consumption. In more temperate climates, this phenomenon is commonly referred to as 'vitamin D winter'. For example, one survey found widespread vitamin D deficiency among female populations across Northern Europe (Andersen *et al*., 2005). Another study found that a significant portion of African-American women living in the USA are seriously vitamin D deficient (Chapuy *et al*., 1997).

While correlations between vitamin D deficiency and disease go back for well over a century, more recent studies have established a direct connection between the two conditions. Since the association between TB and vitamin D deficiency was described over 20 years ago (Davies *et al*., 1985), numerous epidemiological studies have linked vitamin D deficiency to increased rates of cancer, multiple sclerosis, type I diabetes, Crohn's disease and infection (Schwalfenberg, 2007; White, 2008).

11.4 Vitamin D Signalling and Mechanisms of Action

Vitamin D is generated primarily by the photochemical action of UVB radiation (295–320 nm) in the skin (Fig. 11.1), but is also obtained from limited dietary sources such as fish oils and fortified dairy products. Vitamin D refers collectively to closely related molecules (Fig. 11.2) known as vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol) (D'Aldebert *et al*., 2009). Dietary vitamin D_2 is derived from the fungal steroid ergosterol. Vitamin D_3 is found in animal sources such as cod-liver oil and is generated by cutaneous photochemical conversion of the cholesterol metabolite 7-dehydrocholesterol.

Vitamin D undergoes a series of hydroxylation reactions, which are required for both biological activation and deactivation of the molecule. First, 25-hydroxylation is catalysed by the enzymes cytochrome P (CYP)2R1 or CYP27A1, producing 25-hydroxyvitamin D (25D), the major circulating vitamin D metabolite (Jones *et al*., 1998; Cheng *et al*., 2004; Prosser and Jones, 2004; Shinkyo *et al*., 2004; Holick, 2007). This occurs primarily in the liver, but also in the

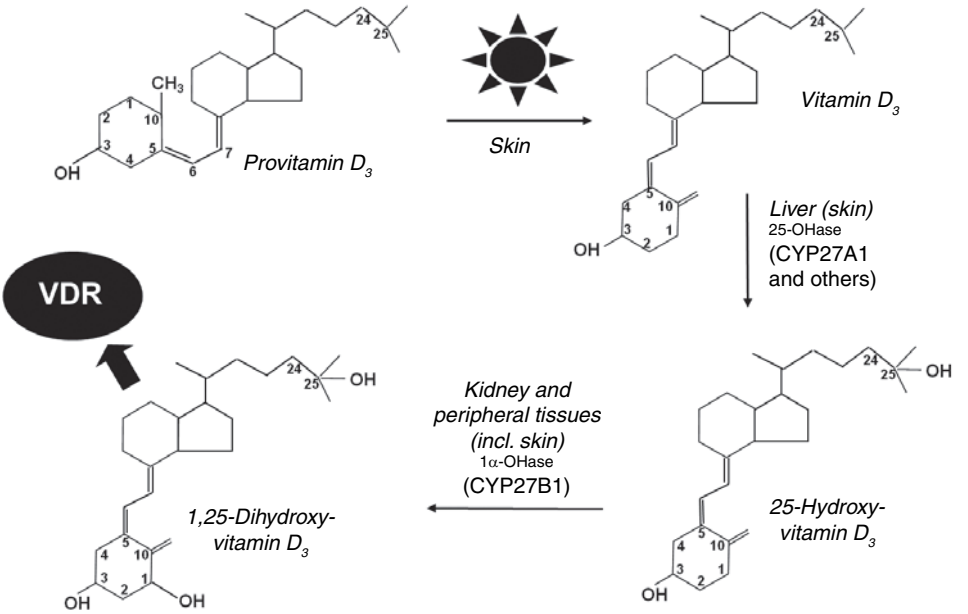

Fig. 11.1. Biosynthesis of 1,25-dihydroxyvitamin D_3. CYP, cytochrome P; VDR, vitamin D receptor.

skin. Then, 1α-hydroxylation of 25D is catalysed by the enzyme CYP27B1, producing hormonally active vitamin D, 1,25-dihydroxyvitamin D (1,25D) (Cheng *et al*., 2004; Prosser and Jones, 2004; Shinkyo *et al*., 2004). Sites of 1α-hydroxylation include the kidneys and peripheral tissues, including several cell types of the immune system. Hormonal 1,25D serves as the molecular ligand of the vitamin D receptor (VDR). Production of 1,25D triggers expression of CYP24, the enzyme that initiates catabolism of 25D or 1,25D via a 24-hydroxylation reaction to generate 24,25-dihydroxyvitamin D or 1,24,25-trihydroxyvitamin D, in a physiological negative-feedback loop. The 24-hydroxylated metabolites undergo further catabolism to calcitroic acid, which is then excreted in urine (Cheng *et al*., 2004; Prosser and Jones, 2004; Shinkyo *et al*., 2004).

Regulation of gene expression by 1,25D occurs via binding to the VDR. The VDR is a nuclear receptor and transcription factor that is part of a family of ligand-regulated transcription factors, which includes receptors for steroid hormones and the hormonal form of vitamin A, among others. Nuclear receptors are composed of highly conserved DNA and ligand-binding domains (Rochel *et al*., 2000; Chawla *et al*., 2001; Cheng *et al*., 2004). Upon ligand binding, the VDR undergoes a conformational change triggering dimerization with a related nuclear retinoid X receptor (RXR). The VDR–RXR heterodimer complex is essential for high-affinity DNA binding with specific DNA sequence motifs known as vitamin D response elements

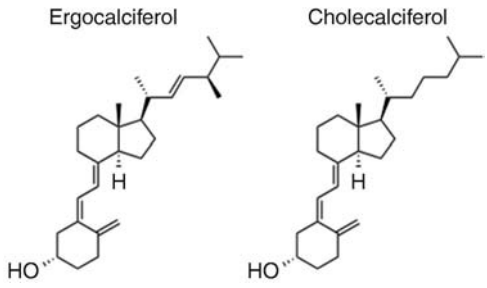

Fig. 11.2. Chemical structures of vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol).

(VDREs), located in the regulatory regions of specific target genes. VDREs are direct repeats (DR) or everted (inverted) repeats (ER) of hexameric nucleotide motifs composed of PuG(G/T)TCA consensus sequences, characterized by their spacing of either three (DR3), six (ER6) or eight (ER8) nucleotides (Lin and White, 2004; Tavera-Mendoza *et al*., 2006). Two copies of cognate DNA sequence motifs reflect the binding of heterodimeric VDR–RXRs in 'heel-to-toe' or 'toes out' orientations (Fig. 11.3) (Rochel *et al*., 2000; Chawla *et al*., 2001; Holick, 2007).

After DNA binding, VDR–RXRs recruit a series of co-regulatory proteins required for the regulation of transcription of adjacent target genes. Regulation of transcription of protein-coding genes in eukaryotes is a biochemically complex process that culminates in recruitment of RNA polymerase II and initiation of transcription. For further information, readers are referred to a series of excellent reviews on the subject (Glass and Rosenfeld, 2000; Dilworth and Chambon, 2001; McKenna and O'Malley, 2002). Recent studies suggest that the VDR can regulate target gene transcription from a considerable distance. For example, work has suggested that a VDRE located as far as 75 kb from the start site can regulate transcription of the gene encoding RANK ligand, a tumour necrosis factor-like ligand secreted by mesenchymal cells (Kim *et al*., 2006).

11.5 Overview of Vitamin D and Immune System Regulation

A substantial body of evidence exists linking vitamin D signalling with regulation of the innate and adaptive arms of the immune system. The VDR is present in most cells of the immune system, including T lymphocytes, neutrophils and antigen-presenting cells such as macrophages and dendritic cells (DCs) (Provvedini *et al*., 1983; Bhalla *et al*., 1984; Brennan *et al*., 1987; Adorini, 2005; Norman, 2006). 1,25D polarizes T-helper (Th) responses towards a more regulatory Th2 phenotype, which is considered to be a key component in its capacity to suppress Th1-driven autoimmune responses. It inhibits DC maturation and T-cell proliferation (van Etten and Mathieu, 2005). Gene expression studies have shown that 1,25D represses the transcription of genes encoding key Th1 cytokines, including interferon-γ and interleukin (IL)-2 (Alroy *et al*., 1995; van Etten and Mathieu, 2005). The net effect of 1,25D signalling leads to a suppression of antigen

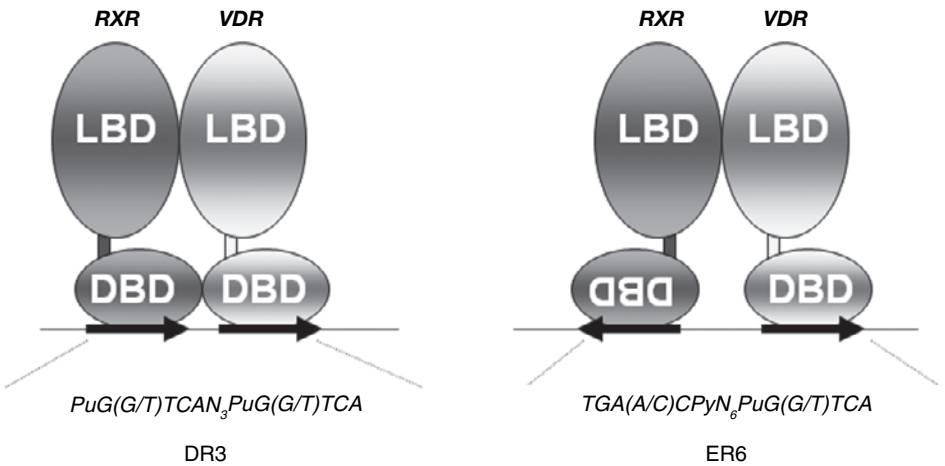

Fig. 11.3. Vitamin D receptor (VDR)–retinoid X receptor (RXR) dimeric binding to vitamin D response elements of the direct repeat (DR; left) or everted repeat (ER; right) type. DBD, DNA-binding domain; LBD, ligand-binding domain.

presentation and activation and recruitment of Th1 cells.

Innate immune responses are found in a wide variety of plant and animal life and represent the front-line defences to pathogenic challenge. The innate immune system defends the host from infection in a non-specific manner but, unlike adaptive immunity, does not confer long-lasting or protective immunity to the host. Molecular evidence for the regulation of innate immune responses by 1,25D has been accumulating for some time. However, it is only in the last 5 years or so that we have begun to fill in many of the details of the central role of vitamin D signalling in innate immune responses in humans.

11.6 Vitamin D as a Regulator of Antimicrobial Peptide Gene Expression

Modern genomics techniques have shifted the focus of vitamin D into fields other than regulation of calcium homeostasis, and have begun to reveal the molecular mechanisms underlying the ancient use of heliotherapy to treat infectious diseases. This new focus has its roots in studies of how 1,25D modulates gene expression. As detailed above, the VDR is a direct regulator of gene transcription with a well-defined binding site (Lin and White, 2004). Therefore, initial large-scale identification of 1,25D target genes began by utilizing a combination of microarrays and genome-wide screens for VDREs, yielding significant results (Akutsu *et al*., 2001; Lin *et al*., 2002; White, 2004; Wang *et al*., 2005). Of particular interest, these screens identified consensus DR3 VDREs located in the promoter region of two genes encoding the antimicrobial peptides (AMPs) defensin β2 (DEFB2/hBD-2) and LL-37 (Wang *et al*., 2004). The 1,25D-dependent binding of the VDR to these elements was confirmed by chromatin immunoprecipitation assays. These assays of VDR binding entail immunoprecipitation of chemically cross-linked complexes of the VDR bound to target DNA sequences, followed by detection of specific DNA sequences by PCR amplification. 1,25D regulation of *DEFB2* transcription was found to be modest (Wang *et al*., 2004). However, it is now clear that 1,25D can enhance *DEFB2* expression with stimulation by other regulators (see below). Remarkably, several studies showed that 1,25D strongly stimulated *LL-37* expression in a number of human cells and tissues (Wang *et al*., 2004; Gombart *et al*., 2005). These findings are highly significant because AMPs represent the vanguards of innate immune responses to infection.

11.7 Antimicrobial Peptides

AMPs are a group of small proteins with intrinsic antibiotic properties that are synthesized and secreted in cells and tissues exposed to environmental pathogens. Much of the effectiveness of AMPs stems from their ability to eradicate a broad spectrum of pathogens, including bacteria, fungi and viruses, without triggering antibiotic resistance (Patil *et al*., 2004; Hancock and Sahl, 2006; Jenssen *et al*., 2006). Additionally, being a natural constituent of host immune systems, AMPs have been discovered to have additional immunoregulatory properties, leading to the term 'immune effector molecules'. These regulatory properties include alteration of gene expression, cytokine induction and modulation of DC responses (Patil *et al*., 2004; Hancock and Sahl, 2006; Jenssen *et al*., 2006; Giuliani *et al*., 2007). As pathogens continue evolving into increasingly antibiotic-resistant strains, AMPs hold promise as a potent method in the control of microbial infection.

Cathelicidins are a family of antimicrobial polypeptides, the precursors of which are characterized by a highly conserved N-terminal cathelin region with a variable C-terminal antimicrobial domain. As a subset of a larger group, the cathelin domain shares sequence homology and function with the cystatin family of cysteine proteinase inhibitors (Zanetti, 2004). Cathelicidins serve as a critical component of the innate immune response, providing rapid defence against infection. While genes encoding for cathelicidins are widely

conserved among vertebrates (Zanetti, 2004; Chang *et al*., 2005; van Dijk *et al*., 2005), the human gene remains the subject of focus.

In humans, a single gene encodes the 18-kDa cathelicidin precursor protein (hCAP-18), widely expressed throughout cells of the immune system. Proteolytic cleavage of the proprotein releases LL-37, also known as human cathelicidin AMP (CAMP) (Fig. 11.4). LL-37 is a linear-chain polypeptide with a net positive charge of +6 due to an excess of basic amino acids (Hancock and Diamond, 2000). The basis of cathelicidin's antimicrobial activity is due to the cationic nature of the peptide. Bacterial cell membranes contain high lipid compositions with a negatively charged surface, thereby creating a selective preference for the positively charged AMP. Upon binding to bacterial cell surfaces, human cathelicidin LL-37 folds into an amphipathic α-helical structure (see Fig. 9.4C, Chapter 9), leading to insertion of the protein and interference with cell membrane integrity. Increasingly, *in vivo* studies have documented a more complete role for human cathelicidin beyond the direct eradication of microbes. Cathelicidins have been shown to mediate a number of immune system responses, including chemotaxis, altering transcription, directing the inflammatory response and promoting wound healing (Hancock and Diamond, 2000; Zanetti, 2004; Chang *et al*., 2005; van Dijk *et al*., 2005).

Defensins represent another family of 1,25D-induced AMPs that contribute to innate immune defence. Similar to cathelicidins, defensins are small polypeptides with cationic properties that are integral to their antimicrobial activity. Found in both vertebrates and invertebrates, defensins are also well recognized for their dual capacity to both eradicate microbes and modulate immune function (Lehrer, 2004; Klotman and Chang, 2006). Structurally, defensins are characterized by a cysteine-rich backbone forming β-pleated sheets stabilized by disulfide bonds (Klotman and Chang, 2006). Classified by their orientation of disulfide bonds to cysteine residues, two subfamilies of defensins exist in humans: α-defensins, which are commonly produced by neutrophils and Paneth cells, and β-defensins, which are secreted by epithelial tissues (for primary and tertiary structures, see Table 9.1 and Fig. 9.5, respectively).

11.8 Regulation of *DEFB2/hBD-2* Expression by 1,25D

1,25D induction of *DEFB2* has been linked with cytokine production during the immune response. As mentioned above, Wang *et al*. (2004) found the induction of *DEFB2* expression by 1,25D alone to be relatively modest (maximum twofold). However, this initial study also showed that fold expression of *DEFB2* could be elevated by the addition of IL-1β to the incubation media. IL-1β is a cytokine and key regulator of inflammatory immune responses. The combined effects of 1,25D and IL-1β on *DEFB2* transcription hinted at crosstalk between the two signalling pathways. Expanding upon these results, Liu *et al*. (2009b) demonstrated the synergistic activity of IL-1β with 1,25D in the potentiation of antimicrobial responses induced by Toll-like receptors (TLRs). TLRs are pattern-recognition receptors that stimulate innate immune responses upon recognition of molecular motifs characteristic of pathogens. More recent research has found that 1,25D stimulates the expression of another pattern-recognition receptor called

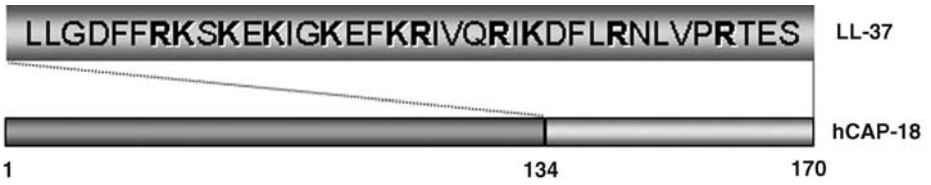

Fig. 11.4. A schematic representation of the precursor protein hCAP-18 and the cleavage product LL-37. Basic amino acids in the LL-37 peptide are noted in bold.

NOD2/CARD15 (Wang *et al*., 2010). NOD2/CARD15 is an intracellular receptor that recognizes lysosomal breakdown products of bacterial peptidoglycan in the form of muramyl dipeptide (MDP). Notably, others have shown that signalling by MDP through NOD2/CARD15 induces expression of *DEFB4* (formerly *DEFB2*/*hBD-2*) (Liu, P.T. *et al*., 2009b). The combination of MDP and 1,25D synergistically induced *DEFB4* expression. These results are of clinical significance because attenuated or defective expression of NOD2/CARD15 or DEFB2/hBD-2 has been associated with the pathogenesis of Crohn's disease (Wang *et al*., 2010). Crohn's disease is a chronic inflammatory bowel disease that arises from a defect in intestinal innate immune responses to bacterial flora.

11.9 1,25D Regulation of *LL-37* is Human and Primate Specific

The induction of antimicrobial gene expression by 1,25D does not appear to be widely conserved among mammals. The VDRE in the human *hBD-2*/*DEFB2* gene is not conserved in the mouse, for example. Moreover, work by Gombart *et al*. (2005) showed that the regulation of *LL-37* expression seen in humans is not conserved in mice. Indeed, a comparison of several mammalian genomes showed that the mechanism in 1,25D induction of *LL-37* is conserved specifically in humans and non-human primates. Conservation of the VDRE in the *LL-37* promoter can be attributed to the presence of the element within a short interspersed element of the Alu subfamily. Alu repeats are a family of transposable DNA elements that are primate and human specific. This specific conservation of the Alu repeat containing the *LL-37* VDRE can be traced to an insertion event dating back to 55–60 million years ago (Gombart *et al*., 2009), prior to the divergence of the primate lineage leading to humans, apes and Old and New World monkeys.

Regulation of the *LL-37* gene by 1,25D is believed to hold a selective advantage given its functional significance in innate immune responses. This significance is highlighted by a number of findings: (i) the VDR is found in most immune cells, including T lymphocytes, neutrophils, DCs and macrophages (Provvedini *et al*., 1983; Bhalla *et al*., 1984; Brennan *et al*., 1987; Adorini, 2005; Norman, 2006); (ii) studies have linked VDR induction of AMP production with TLR signalling in innate immune responses (Stead *et al*., 1990; Brightbill *et al*., 1999; Thoma-Uszynski *et al*., 2001; Liu *et al*., 2006, 2007); and (iii) VDR signalling is important for both the process of pathogen recognition and antimicrobial response during tissue injury (Schauber *et al*., 2007).

TLR pattern-recognition receptors are a family of cell-surface and intracellular receptors. TLRs have long been studied for their roles in responses to microbial infection, and have recently been linked to VDR immunoregulation. TLR2 receptors are cell-surface proteins that recognize bacterial lipopeptides. By 2006, it was established that TLR2 activation of human or murine macrophages directly stimulates antimicrobial activity against TB infection (Liu *et al*., 2006). However, the intermediate signalling pathways leading to AMP production are believed to be different between the two species. In mice, studies showed that antimicrobial activity is dependent upon the induction of nitric oxide synthase (iNOS) and the production of nitric oxide (NO) in infected tissue (Liu *et al*., 2006, 2007). Further work showed that NO-induced antimicrobial activity is mediated through TLR signalling in mice, and that iNOS inhibitors are able to block this induction of antimicrobial activity (Liu *et al*., 2006). Additionally, these studies demonstrated that human macrophages do not depend upon the production of NO for their antimicrobial activity. However, one intriguing study has cast doubts on these initial results and shown that iNOS activity does indeed play a role in host defences to TB infection in human macrophages (Lee *et al*., 2009).

Research has confirmed the induction of AMPs as the basis for TLR-induced antimicrobial activity in human macrophages and linked VDR signalling to AMP production (Liu *et al*., 2007). Using microarrays, studies found that TLR stimulation

upregulated expression of the VDR as well as CYP27B1 in human monocytes (Liu *et al*., 2006, 2007). These findings are highly significant as they reveal that macrophages react to pathogen detection by acquiring the capacity to respond to circulation levels of 25D and convert it into hormonal 1,25D. The functional significance of this discovery was demonstrated by the observation that reduced *Mycobacterium tuberculosis* viability in infected macrophages is dependent on 1,25D-induced production of *LL-37* (Liu *et al*., 2007). Furthermore, *LL-37* expression has recently been linked to the induction of autophagy and the degradation of *M. tuberculosis* in infected human macrophages (Yuk *et al*., 2009). Autophagy plays an important role in the degradation of damaged cellular components and misfolded proteins, as well as in organellar turnover (Baehrecke, 2005). Importantly, a critical role has recently emerged for autophagy as a component of innate immune responses to microbial infection (Deretic, 2009, 2010).

Because of darker pigmentation and reduced cutaneous vitamin D synthesis, African-Americans are known to possess significantly reduced serum 25D concentrations (Stead *et al*., 1990; Nesby-O'Dell *et al*., 2002). Consistent with previous studies, Liu *et al*. (2007) found that serum from African-Americans generally contained lower levels of 25D than that of Caucasians, and induced correspondingly lower levels of LL-37 in TLR2-stimulated macrophages. However, normal physiological function could be restored through proper supplementation of 25D. These studies provide compelling evidence that TLR2-stimulated macrophages produce a substantial AMP response only under conditions of vitamin D sufficiency.

In summary, the above findings are significant for a number of reasons. First, they clearly establish a central role for vitamin D signalling in primary innate immune responses. Secondly, they emphasize the importance of maintaining vitamin D sufficiency for optimal immune system function, and suggest why some ethnicities may be more predisposed to certain infectious diseases. And finally, they signify the importance of regulation of non-renal CYP27B1 expression in immunoregulation by vitamin D.

11.10 Broader Physiological and Pathophysiological Implications of the Regulation of LL-37 Expression by 1,25D

The skin occupies a key position in vitamin D physiology. Epidermal keratinocytes express the hydroxylase enzymes CYP27A1 and CYP27B1 and can thus produce 1,25D (Norman, 1998) in the presence of sufficient UVB irradiation. Additionally, keratinocytes express the VDR, making them responsive to vitamin D inputs. Under conditions of tissue damage, 1,25D regulates a variety of responses in keratinocytes, including changes in cell proliferation, differentiation, cytokine production and gene expression (Dorschner *et al*., 2001; Heilborn *et al*., 2003; Carretero *et al*., 2008; Segaert and Simonart, 2008). Cell-signalling events that underlie wound healing also to lead to a substantial induction of CYP27B1 and subsequently increase LL-37 expression. Moreover, LL-37 induces keratinocyte migration, vital to re-epithelialization of the wound, by transactivating epidermal growth factor receptors responsible for cellular architecture (Dorschner *et al*., 2001; Heilborn *et al*., 2003).

Induction of dermal cathelicidin expression by 1,25D is not always beneficial, as elevated levels of LL-37 have been implicated in several inflammatory skin conditions. Rosacea is a condition characterized by chronic erythema (redness of the skin) in afflicted patients. Notably, rosacea is also characterized by abnormal processing of hCAP-18 peptides, leading to an abundance of truncated cathelicidin peptides in keratinocytes, which contributes to inflammation (Yamasaki *et al*., 2007). Additionally, UV light aggravates rosacea, possibly in part via 1,25D-induced LL-37 expression. Indeed, it has been speculated that azole antimycotics (ketoconazole, itraconazole and metronidazole), used clinically to treat rosacea, may work partly

by blocking CYP450-driven vitamin D metabolism (Yamasaki *et al*., 2007).

Vitamin D analogues are widely used to treat psoriasis, an autoimmune inflammatory skin condition. It is thus paradoxical that LL-37 is overexpressed in psoriasis and may contribute to its pathogenesis. Cathelicidin expression is limited to keratinocytes exposed to injury, stimulating immune responses and promoting wound healing (Dorschner *et al*., 2001; Heilborn *et al*., 2003). During psoriasis, this tightly regulated process is altered, leading to overexpression of the LL-37 peptide and triggering immune responses by activating plasmacytoid DCs (Lande *et al*., 2007). Plasmacytoid DCs are specialized skin cells that sense viral and bacterial DNA through TLR signalling. In particular cases of autoimmune disease, a breakdown in the sensory apparatus occurs allowing for self-DNA to trigger an immune response. In psoriatic skin, Lande *et al*. (2007) established that LL-37 is the mediating factor triggering plasmacytoid DC activation. Lending to the cationic, amphipathic nature of protein, LL-37 directly binds to DNA in plasmacytoid DCs, forming aggregated and condensed structures that are delivered to TLR-9 receptors, thereby activating immune response mechanisms leading to chronic skin inflammation.

VDR-regulated signalling is not limited to dermal epithelial cell types. One recent study showed that both LL-37 and the VDR are expressed in hepatic biliary epithelial cells (D'Aldebert *et al*., 2009). Under normal physiological conditions, the biliary tract maintains a number of defence mechanisms, preserving a microbe-free environment. The authors found that antibacterial activity in the biliary tract is maintained by signalling actions of bile salts through the VDR and a related farnesoid X receptor (FXR). FXR is a member of the nuclear receptor family activated by bile salts. Remarkably, the VDR has also been established as a functional bile acid receptor (Makishima *et al*., 2002). Activation of either the FXR or VDR by bile acids has been found to mediate innate immune function through cathelicidin induction in both hepatic epithelial cells and normal human keratinocytes (D'Aldebert *et al*., 2009, Peric *et al*., 2009). Furthermore, the synergistic enhancement of LL-37 expression is induced by the combination of vitamin D and bile salts, suggesting a novel therapeutic approach to treating inflammatory biliary disease (D'Aldebert *et al*., 2009).

Similarly, VDR regulation of the innate immune response is essential for maintaining lung homeostasis. The epithelial lining of the lungs is constantly exposed to a variety of environmental pathogens, and induction of antimicrobial gene expression is vital in providing a front line of defence against inhaled microbes. 1,25D has been shown to strongly induce *LL-37* expression in Calu-3 cells, a human epithelial airway cell line (Wang *et al*., 2004). Moreover, treatment with 1,25D yielded a significant reduction in the microbial activity of either *Escherichia coli* or *Pseudomonas aeruginosa* in infected Calu-3 cells. *P. aeruginosa* is an opportunistic pathogen associated with the vast majority of respiratory infections in patients afflicted with cystic fibrosis (Speert *et al*., 2002). Notably, patients with cystic fibrosis have also been observed to have low circulating serum 25D levels (Hecker and Aris, 2004). Expanding upon these results, Yim *et al*. (2007) documented the presence of the VDR, and robust induction of cathelicidin, in normal human bronchial epithelial cells. Additionally, this particular study demonstrated the direct contribution of cathelicidin to antimicrobial activity through preincubation of infected normal human bronchial epithelial cells with an anti-LL-37 antibody.

Active vitamin D metabolism has also been established in the endometrium and placenta during gestation. Beginning in the 1980s, studies conducted with rats connected vitamin D deficiency with significantly diminished rates of reproduction (Halloran and DeLuca, 1980; Hickie *et al*., 1983; Kwiecinksi *et al*., 1989). Recently, one intriguing study linked high levels of placental 1α-hydroxylase expression during early gestation with the pleiotropic actions of vitamin D signalling beyond fetal musculoskeletal development (Zehnder *et al*., 2002). Expanding upon these results, another study correlated vitamin D signalling with attenuated immune function during the early

stages of pregnancy in maternal decidual tissue (Evans *et al*., 2006). In upholding an immunosuppressive role, localized synthesis of 1,25D was found to modulate maternal immune function, supporting implantation at the fetal–maternal interface. Furthermore, N. Liu *et al*. (2009) documented the 1α-hydroxylase conversion of 25D to 1,25D, as well as robust cathelicidin induction, in placental human trophoblast cells. However, unlike human macrophages, LL-37 induction in trophoblasts is independent of TLR activation. Taken together, these results clearly demonstrate the broad spectrum of *in vivo* activity for vitamin-D-mediated signalling, moving beyond calcium homeostasis and bone metabolism.

11.11 Therapeutic Role for Vitamin D Analogues

Vitamin D analogues have shown considerable therapeutic promise in treatment of a number of indications. The potential of these analogues stems from their ability to mediate VDR signalling without inducing the calcaemic effects associated with elevated hormonal 1,25D. A vast array of *in vivo* studies have linked treatment with vitamin D signalling to decreased tumour growth in colon (Wali *et al*., 1995), breast (Colston *et al*., 1992), prostate (Colston *et al*., 1997) and pancreatic (Schwartz *et al*., 2008) cancers, and vitamin D analogues have been developed to maximize their anticancer properties while minimizing calcaemic effects. The immunoregulatory properties of 1,25D also serve as a platform for vitamin D analogue development. Psoriasis is one of the few disorders currently treated with vitamin D therapy. Calcipotriol, a vitamin D analogue approved for treatment of psoriasis in both the USA and Europe, stimulates terminal differentiation in keratinocytes (Brown and Slatopolsky, 2008). A number of vitamin D analogues have been approved for topical treatment of psoriasis, without significant calciotropic effects. Additionally, clinical studies have revealed the potential of vitamin D analogues in the treatment of other autoimmune conditions such as type I diabetes and arthritis (Larsson *et al*., 1998; van Etten *et al*., 2003).

Given the central role of 1,25D in stimulating innate immune responses to infection, we speculate that vitamin D analogues may also find a role in drug cocktails designed to combat infectious diseases. Recent findings raise the possibility that vitamin D analogues that strongly induce expression of the NOD2/CARD15–DEFB2/hBD-2 innate immune pathway may be efficacious in combating Crohn's disease (Wang *et al*., 2010). It is noteworthy in this regard that the NOD2/CARD15 pattern-recognition receptor is particularly sensitive to a modified form of MDP produced by mycobacteria (Coulombe *et al*., 2009). Therefore, vitamin D analogues may also find a role as modern-day successors to Hippocrates' heliotherapy in the treatment of TB.

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12 Fine Tuning Host Responses in the Face of Infection: Emerging Roles and Clinical Applications of Host Defence Peptides

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Abstract

Host defence peptides (HDPs) are powerful modulators of human innate immunity, and can modify the outcome of the endogenous host response to infection. The progressive development of pathogen resistance to conventional antimicrobial agents has lead to a new appreciation of HDPs for their ability to fight infection, enhance vaccine responses, limit inflammation and promote wound healing, within the context of human disease. HDPs are a family of cationic, short, amphipathic peptides that include the classical mammalian antimicrobial peptides, cathelicidins and defensins, as well as non-antimicrobial peptides with similar immunomodulatory properties. This chapter reviews our current basic understanding of the anti-infective and immunomodulatory properties of both endogenous HDPs and synthetic derivatives (termed innate defence regulators) with regard to their ability to selectively fine tune the responses of host cells and physiology. The clinical application of these molecules is also discussed, with a focus on past and ongoing clinical trials of HDPs and innate defence regulators as novel therapeutics for infectious and inflammatory diseases.

Infectious diseases have been the primary cause of morbidity and mortality for most of human history. At the end of the 19th century, the average human life expectancy was 25 years (Casanova and Abel, 2005). It was the development of Pasteur's insights into the nature and role of microbes in infections that paved the way for our first medical breakthroughs in hygiene, vaccination and antibiotics. These discoveries led to a rapid acceleration in life expectancy around the world, and infectious causes of mortality have been falling behind the diseases of old age (cardiovascular and neoplastic disease) as the most significant killers facing Western society (WHO, 2008).

Following the introduction of penicillin and the relatively rapid development of other antibiotic classes, we have come to rely on the availability of effective antibiotics to combat infections. However, having now well and truly entered the era of antibiotic resistance, it is necessary to develop novel antimicrobial therapies and strategies to overcome the growing problem of multidrug-resistant bacterial strains. The discovery of naturally occurring antimicrobial peptides (AMPs) provided hope that these could

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become the basis for a new class of antibiotic compounds, although clinical trial results to date have not yet confirmed this potential. Research in this area has increasingly begun to focus on the immunomodulatory properties of these peptides and their applications for human disease, specifically their use as novel anti-infective therapeutics that circumvent antibiotic resistance by selectively enhancing the beneficial aspects of the endogenous host response to pathogens.

12.1 Mammalian Host Defence (Antimicrobial) Peptides

Mammalian peptides with direct antimicrobial activity are generally classified into the two main families of cathelicidins and defensins, although other peptides exist that do not fall into these categories. The defensins are cationic peptides of 18–45 amino acids and are further divided into three subfamilies: the α-, β- and θ-defensins. The α- and β-defensins are genetically distinct, with similar structures dominated by β-strands that differ in the placement of their six conserved cysteine residues that form three intramolecular disulfide bridges (Selsted and Ouellette, 2005). The θ-defensins are cyclic in structure, are only found in non-human primates and have antiviral activity against human immunodeficiency virus-1 (Gallo *et al*., 2006). Defensins are produced by leukocytes in many mammals, including humans, rats and rabbits, but not mice. However, mice, like humans, also produce α-defensins in their intestinal crypts and these include a subclass termed cryptdins (Amid *et al*., 2009). Conversely, mice do not produce leukocyte α-defensins like humans, while bovines are devoid of all α-defensins.

12.1.1 Defensins

The α- and β-defensins are produced as prepropeptides that are cleaved to form propeptides, which in turn require additional processing to form the active peptide. The propiece, which is cleaved to release the active peptide, acts to protect defensins from proteolysis inside cells and protect the cell from autotoxicity related to the strong cationic charge and amphipathicity of the mature peptide (the latter also being managed in part by sequestration into granules). Neutrophil α-defensins are stored within azurophilic granules for release by fusion with cell phagosomes containing ingested microbes; this results in very high local concentrations of defensins and, despite their relatively weak antimicrobial activity, concentrations are sufficient to mediate direct microbicidal activity (Selsted and Ouellette, 2005). Intestinal α-defensins are constitutively expressed and secreted from Paneth cells, with secretion being enhanced by contact with bacterial components such as CpG oligodeoxynucleotides (ODNs). Contact with bacterial components can also stimulate α-defensin secretion by natural killer (NK) cells (Agerberth *et al*., 2000), indicating that, in addition to their intracellular antimicrobial function within neutrophils, such defensins also have important extracellular functions. β-Defensins are produced by a range of tissues, mainly in response to pro-inflammatory cytokines induced by Toll-like receptor (TLR) agonists. For example, β-defensins produced by keratinocytes play important roles in the host defence functions of the skin and in wound healing (Niyonsaba *et al*., 2007).

While the defensins can demonstrate antibacterial activity *in vitro* at low micromolar concentrations, most α- and β-defensins are only weakly effective in the presence of salt concentrations approaching those found *in vivo*; this can be over-ridden by extremely high concentrations of these molecules such as the mg ml^{-1} concentrations found in phagocytic granules or the crypts of the intestine. At least one β-defensin, human β-defensin (hBD)-3, is more cationic than the others, less affected by cation antagonism and probably has meaningful activity at the surface of the skin. Similarly, the θ-defensins are active at these higher salt concentrations, although only when in their cyclic form (Tang *et al*., 1999). This suggests that, while direct antimicrobial activity is important in some physiological niches, where the concentration of peptide is high enough to overcome antagonism by salts, in many other anatomical locations the alternative, immunomodulatory

functions of these peptides probably predominate.

Defensins modulate the functions of many cell types, influencing immune cell recruitment, activation and maturation, wound healing and angiogenesis. For example, human α-defensins 1–3 have been shown to influence the maturation and differentiation of monocyte-derived dendritic cells (DCs), with different outcomes induced by either high or low peptide concentrations (Rodriguez-Garcia *et al*., 2009). Human neutrophil α-defensins 1–3 act as direct chemoattractants for T cells and DCs (Yang *et al*., 1999; Hubert *et al*., 2007), whereas hBD1–4 induce chemotaxis of macrophages and calcium flux in mast cells, while having no effect on T cells or DCs (Soruri *et al*., 2007). α-Defensins also have mitogenic functions, promoting wound healing through the stimulation of fibroblast and epithelial cell proliferation (Murphy *et al*., 1993; Nishimura *et al*., 2004). In addition to affecting mast cell chemotaxis and calcium flux, hBD-3 and hBD-4 have also been shown to induce mast cell degranulation and prostaglandin D_2 production, with mast cell activation being dependent on signalling through the mitogen-activated protein kinase (MAPK) p38 and extracellular signal-regulated kinase (ERK)1/2 pathways (Nishimura *et al*., 2004). As these immunomodulatory functions are generally seen at peptide concentrations lower than those needed for direct antimicrobial activity, and are not inhibited by physiologically relevant salt concentrations (being evident under tissue culture conditions and often in animal models), these immunomodulatory functions are likely to be more broadly physiologically relevant than direct antimicrobial activity.

The *in vivo* significance of defensins in the context of innate immune functions is supported by observations in humans with genetic alterations in defensin production. For example, while defensins are usually induced in response to interferon (IFN)-γ, in those with a particular single nucleotide polymorphism expression of hBD-1 and hBD-3 is constitutive and this confers enhanced protection from candidiasis (Kalus *et al*., 2009). This same single nucleotide polymorphism, together with another associated with hBD-1, also influences susceptibility to Crohn's disease, a microbial-induced inflammatory condition of the intestines (Kocsis *et al*., 2008). Similarly, a predisposition to Crohn's disease has been observed in individuals with a lower copy number of the *hBD-2* gene, and a correspondingly lower expression of *hBD-2* at the mRNA level (Fellermann *et al*., 2006).

12.1.2 Cathelicidins

Cathelicidins are structurally distinct from defensins, containing a characteristic cathelin prodomain and a signal sequence. Despite this related N-terminal region, cathelicidins vary markedly in structure and include α-helical, β-hairpin, β-turn and extended peptides. They are produced by a wide range of species, including mammals, birds and fish, with some species producing up to seven different cathelicidins (Durr *et al*., 2006). Humans have only one cathelicidin, which is synthesized as the proprotein hCAP-18 and cleaved extracellularly to form the active protein known as LL-37. Mice also have a single cathelicidin, known as CRAMP, which has 67% identity to LL-37. Cathelicidins are produced by a range of cell types, including epithelial cells and leukocytes such as neutrophils, monocytes, T cells, B cells and NK cells. They appear in many body locations (e.g. at mucosal and skin surfaces) and fluids (e.g. gastric juices, saliva, semen, sweat, plasma, airway surface liquid and breast milk). Like the defensins, cathelicidins exhibit a broad range of biological activities that include both direct antimicrobial activity and immunomodulatory functions. LL-37 is present in many different tissues and secretions, including sweat and breast milk, and is stored at high concentrations in its proform hCAP-18 in the azurophilic granules of neutrophils. The physiological concentrations of LL-37 are estimated to be up to 1 μM (5 μg ml^{-1}) in airway surface fluid and at mucosal surfaces, and under pathological conditions can increase dramatically, with concentrations up to 300 μM reported in psoriasis (Ong *et al*., 2002; Schaller-Bals *et al*., 2002; Bals and Wilson, 2003). The biological functions of LL-37 have been proposed to

vary depending upon the local conditions; for example, direct antimicrobial activity requires quite high concentrations of the peptide and/or low salt concentrations. Conversely, wound-healing, angiogenic and anti-endotoxic activity and synergistic induction of certain chemokines together with endogenous signalling molecules are all seen at very low (<1 μM) peptide concentrations.

The ability of LL-37 to augment many aspects of the innate immune response has been well established in the literature. For example, LL-37 modulates the production of interleukin (IL)-8 by keratinocytes and epithelial cells (Filewod *et al*., 2009) and inhibits the action of IFN-γ on monocytes, macrophages, DCs and B lymphocytes (Nijnik *et al*., 2009). Studies on human peripheral blood mononuclear cells have shown that LL-37 acts in synergy with immune mediators such as granulocyte–macrophage colony-stimulating factor and IL-1β to enhance the production of cytokines (e.g. IL-6 and -10) and chemokines (e.g. monocyte chemotactic protein-1 and -3). This synergy is mediated through modulation of intracellular signalling pathways, such as via phosphoinositide 3-kinase and MAPK, and subsequent changes in the activation of associated transcription factors (Yu *et al*., 2007). The immune modulation mediated by the cathelicidin leads to a more effective and balanced innate response, with synergistic upregulation of the chemokine response accompanied by anti-endotoxic activity, mediated both by lipopolysaccharide (LPS) binding and modulation of TLR signalling cascades to reduce the production of proinflammatory cytokines such as tumour necrosis factor (TNF)-α. The anti-endotoxic activity of LL-37 is supported by experiments showing that it can protect mice from death after challenge with LPS alone (i.e. any bactericidal activity is irrelevant) and that it can reduce leukocyte responses to LPS *ex vivo* (Mookherjee *et al*., 2007). The mechanism of action is as yet incompletely understood, but several cellular components have been proposed as specific interactors. The roles of surface formyl peptide receptor-like 1 as a direct binding receptor in chemotaxis, of other undetermined G-protein-coupled receptors and of the trans-activated receptors epidermal growth factor receptor and nucleotide scavenging receptor $P2X_7$ in various properties of LL-37 have been described (Tjabringa *et al*., 2003; Tomasinsig *et al*., 2008). Recent studies (Zhang *et al*., 2009) indicate that LL-37 can act as an alternative ligand for the IL-8 receptor (CXCR2) on neutrophils, while Mookherjee *et al*. (2009a) reported a physical and functional interaction between LL-37 and intracellular glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Many other studies have described the signal transduction pathways, transcription factors and effector proteins and pathways involved in peptide activity (Mookherjee *et al*., 2009b).

Studies on cathelicidin activity *in vivo* and *ex vivo* have been undertaken using transgenic mice lacking CRAMP, with notable observations including that the peptide is involved in NK-cell-mediated antitumour responses (Buchau *et al*., 2010), partial protection from bacterial infections (Nizet *et al*., 2001; Braff *et al*., 2005; Iimura *et al*., 2005; Huang *et al*., 2007) and viral infections (Howell *et al*., 2006) and the antimicrobial activity of mast cells (Di Nardo *et al*., 2003). However, these studies have not always discriminated between direct and indirect (modulation of other immune mechanisms) modes of action. Treatment with exogenous CRAMP has also been shown to reduce the severity of colitis induced by dextran sulfate sodium ingestion, with enhanced intestinal mucus production and reduced apoptosis (Tai *et al*., 2007). In addition to the immunomodulatory effects it exerts on mammalian cells, LL-37 is able to prevent biofilm formation by the bacterial pathogen *Pseudomonas aeruginosa* at a concentration far below (at least 1/16th) its minimal inhibitory concentration by specifically modulating bacterial gene expression (Overhage *et al*., 2008).

12.2 The Role of Endogenous Host Defence Peptides in the Response to Infection

The natural human host response to infection cannot be modelled on an agar plate, tissue culture dish or nutrient-broth-filled test tube.

Rather, it is a dynamic and complex process that involves all of the physiological systems and organs necessary for human life. Cationic peptides are responsible for fine tuning immunological and physiological homeostatic parameters in the context of the host response to infectious diseases. In light of the evidence that such peptides have a broad range of biological functions that go well beyond direct antimicrobial activity *in vitro* (Oppenheim and Yang, 2005; Hancock and Sahl, 2006; Mookherjee *et al*., 2006), they are perhaps more accurately termed host defence peptides (HDPs) rather than AMPs (Box 12.1). The creation of synthetic innate defence regulator (IDR) peptides that lack appreciable antimicrobial activity yet show similar physical properties to AMPs and powerful (indirect) anti-infective efficacy mediated through immunomodulatory mechanisms adds further support to this idea.

The human innate immune response to infection is a complex physiological process that orchestrates non-specific responses to infectious threats. Within this dynamic series of events, HDPs play a critical role in coordinating and fine tuning cellular, tissue-specific and global physiological changes that accompany the innate immune response. Considering the critical functions of endogenous HDPs beyond direct antimicrobial killing is essential to an expanded understanding of the important roles played by these molecules within the context of host defence against infection. Although the bulk of HDP research has focused on the direct antimicrobial nature of these molecules, partly due to their location in professional immune cells and the relative ease of measuring such activities, other non-antimicrobial activities have recently gained attention for their role in orchestrating global physiological and biochemical changes that modulate the host response, hence the use of the broader designation HDP when considering activities other than direct actions on microbes.

Immunomodulation by HDPs is often understood to occur at local tissue sites of infection. Differential production of defensins and cathelicidins has been described to occur in epithelial, connective tissue and leukocyte cells against the background of an inflammatory milieu, while local concentrations can also increase rapidly by degranulation of phagocytic cells at the site of infection, suggesting that the immunomodulatory function occurs in a paracrine or autocrine manner. Numerous studies on endogenous HDPs and equivalent synthetic IDR peptides have shown that administering these molecules in infection models, either locally or systemically, results in alterations in global immune function (Table 12.1). Other host-produced peptides, classically grouped together as hormones, play a similar role in the face of infection, allowing the integration of local and systemic host defences with

Box 12.1. Definitions.

Antimicrobial peptides (AMPs): Host-produced or synthetic peptides, and their derivates or mimetics, with typical cationic and amphipathic structural motifs, that mediate direct killing against bacterial cells *in vitro* with a readably measurable minimal inhibitory concentration (MIC). This term is appropriate when antimicrobial activity is being examined.

Host defence peptides (HDPs): Host-produced peptides (and their derivates), with typical cationic and/or amphipathic structural motifs, that exert anti-infective properties *in vivo* through their ability to enhance or modulate the normal host immune response to infectious agents. Innate immunity is the major target but effects on adaptive immunity have been observed. This *in vivo* anti-infective effect can be mediated by one or more of the following: selective immune modulation, modulation of global host physiology or direct antimicrobial activity.

Innate defence regulators (IDRs): Synthetic peptides that exert anti-infective properties *in vivo* by their ability to enhance, complement or modulate the normal host response to infectious agents. This *in vivo* anti-infectious effect is mediated predominantly by their immunomodulatory abilities and includes a subset of activities observed for the HDPs. Structurally, these peptides share features such as cationicity and amphipathicity with HDPs.

Table 12.1. Small cationic peptides that have no antimicrobial activity or activity only in very dilute medium or buffer, but that appear to play a role in the human host response to infection.

HDP family and prototype	Physical properties	Site(s) of production	Role in host response and therapeutic applications	Cellular (immunomodulatory) function
Histatins: histatin 5	24 amino acids, +5 net charge	Parotid and sublingual salivary glands	Play a broad role in maintaining oral cavity homeostasis in the face of oral and periodontal pathogens such as *Porphyromonas gingivalis*. Potent mediators of mucosal wound healing and re-epithelialization. The histatin-derived peptide P-113D has shown efficacy in animal models of *Pseudomonas aeruginosa* sepsis (Cirioni *et al.*, 2004), and has been in Phase II clinical trials (Table 12.2).	Attenuate IL-6- and IL-8-mediated gingival inflammation (Imatani *et al.*, 2000) induced by *P. gingivalis* and prevent LPS-induced apoptosis in gingival fibroblasts (Imatani *et al.*, 2004). Wound healing occurs via an ERK1/2-dependent mechanism (Oudhoff *et al.*, 2008).
Iron-binding peptides: hepcidin	25 amino acids, +2 net charge	Liver	Iron homeostasis is a cornerstone of host defence (Schaible and Kaufmann, 2004). Iron-binding peptides imit the utility of bacterial haemolysins in iron acquisition, while simultaneously activating host antibacterial defences such as phagocytic cells and complement (Ganz, 2009).	Circulating proinflammatory cytokines induce hepatic synthesis (Nemeth *et al.*, 2004). Phagocytic cells produce the peptide following TLR2 or TLR4 activation via STAT1 and NF-κB (Sow *et al.*, 2009), leading to improved innate immune recognition of bacterial pathogens (Wang *et al.*, 2009a). Leads to impaired incorporation of iron into haemoglobin (Rivera *et al.*, 2005) and inflammation-associated anaemia (Nemeth, 2010).
Melanocortins: MSH	α-MSH is 13 amino acids, +1 net charge; γ-MSH is 11 amino acids, +2 net charge	Anterior pituitary gland	Interface endocrine system with the resolution of inflammation. Enhance anti-inflammatory programme in leukocytes and lymphocytes (Sarkar *et al.*, 2003; Yoon *et al.*, 2003; Cooper *et al.*, 2005), without impairing the ability to clear infection. Exhibit significant anti-endotoxin activity (Scholzen, 2003; Yoon *et al.*, 2003) and promote wound healing (Zou *et al.*, 2004; Yoon *et al.*, 2008). The MSH-derived peptide AP214 has shown efficacy in preventing organ failure in animal models of sepsis (Doi *et al.*, 2008) and is in clinical trials (Table 12.2).	Bind to MC receptors found on most human cells, with MC-1R mediating immunomodulation. MC-1R ligation leads to large increases in cellular cAMP via PKA, activation of the MAPK pathway and altered NF-κB responses. In PBMCs, MSH causes downregulation of TNF-α, IL-1β, IFN-γ, IL-6 and IL-8, with simultaneous induction of IL-10 and NO. The immunomodulatory functions of MSH are diverse, cell-type-dependent and well reviewed elsewhere (Böhm *et al.*, 2008).

Table 12.1. *Continued.*

HDP family and prototype	Physical properties	Site(s) of production	Role in host response and therapeutic applications	Cellular (immunomodulatory) function
GI peptides: ghrelin, VIP	Ghrelin is 28 amino acids, +5 net charge; VIP is 28 amino acids, +3 net charge	GI tissue and PNS ganglions that regulate GI function	Regulate energy and nutrition homeostasis, linking metabolism to the endogenous anti-infective response, especially to enteric pathogens (Conlin *et al.*, 2009; Coron *et al.*, 2009). VIP enhances innate immunity to *P. aeruginosa* (Szliter *et al.*, 2007). Ghrelin plays an important role in host defence against pulmonary pathogens by regulating both nutritional state and neutrophil recruitment (Kodama *et al.*, 2008; Kim *et al.*, 2010). Both are in clinical trials for sepsis, where they inhibit toxic inflammation and organ dysfunction (Chorny and Delgado, 2008; Huang *et al.*, 2009; Wang *et al.*, 2009b; Wu *et al.*, 2009), with ghrelin also being investigated in cystic fibrosis (Table 12.2).	Ghrelin acts via the GHSR-1a receptor, in a MAPK phosphatase-1-dependent manner, to inhibit LPS and noradrenaline-mediated proinflammatory cytokine release (Jacob *et al.*, 2010). Acts directly on effector cells to inhibit TNF-α- and LPS-induced release of HMGB1; also enhances their bactericidal function (Chorny and Delgado, 2008; Chorny *et al.*, 2008). VIP-mediated immunomodulation is incredibly complex and dynamic and is well reviewed elsewhere (Delgado *et al.*, 2004). Briefly, VIP binds to the VPAC receptor expressed on nearly every cell (Ceraudo *et al.*, 2008; Lv *et al.*, 2009). Immune-specific effects occur by modulating the function of lymphocytes (Ganea, 1996) and innate immune cells (Szliter *et al.*, 2007) to balance pro- and anti-inflammatory tone.

Abbreviations: cAMP, cyclic AMP; ERK, extracellular signal-regulated kinase; GHSR, growth hormone secretagogue receptor; GI, gastrointestinal; HDP, host defence peptide; HMGB1, high mobility group protein B1; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MC, melanocortin; MC-1R, melanocortin 1 receptor; MSH, melanocyte-stimulating hormone; NF-κB, nuclear factor κB; NO, nitric oxide; PBMC, peripheral blood mononuclear cell; PKA, protein kinase A; PNS, peripheral nervous system; STAT1, signal transducer and activator of transcription 1; TLR, toll-like receptor; TNF, tumour necrosis factor; VPAC, vasoactive intestinal peptide receptor; VIP, vasoactive intestinal peptide.

cardiovascular function, pyrogenic and neuroendocrine responses, iron homeostasis and gastrointestinal motility. These peptides are also summarized in Table 12.1, and are discussed below to further illustrate this concept.

12.2.1 Natriuretic peptides

Natriuretic peptides (NPs) are a family of cationic, ring-shaped autocrine/paracrine peptide hormones secreted by neuronal, cardiac and endothelial cells. They cause discharge of sodium through the urine (natriuresis), as well as having potent vasodilating activities. NPs are implicated in body fluid homeostasis and blood pressure control, playing a role in the regulation of host cardiovascular, haemodynamic and renal function, and are used today as clinical markers in the diagnosis of congestive heart failure (Eleuteri and Di Stefano, 2009). Three members of the NP family are found in humans, namely atrial (or A-type), B-type and C-type, abbreviated as ANP, BNP and CNP, respectively (Potter *et al.*, 2009). Showing high sequence homology across mammalian

species, NPs are secreted as 126–151 amino acid precursors and cleaved into 22–32 amino acid active peptide forms with net positive charges. In addition to their traditional roles, these peptides have now been identified as playing a critical role in fine tuning cardiovascular and innate immune responses in the face of severe infection and sepsis (Mohapatra, 2007; Eleuteri and Di Stefano, 2009; Vesely and de Bold, 2009).

One of the most devastating (and often lethal) complications of sepsis is septic shock, consisting of haemodynamic and cardiovascular collapse leading to renal and multiorgan failure. Consequently, elevated BNP (which has the longest half-life of the NP family members) has been identified as an early clinical marker of sepsis (Rudiger *et al*., 2006; Kandil *et al*., 2008). Surprisingly, elevated BNP in sepsis has been found to precede the onset of septic shock (Vila *et al*., 2008). This was correlated with increased systemic inflammatory cytokines rather than haemodynamic changes, and thus constituted part of the early systemic innate immune response to the infectious agent. This finding was reiterated *in vitro*, with IL-1β and TNF-α inducing BNP production in cardiomyocytes (Vesely and de Bold, 2009), and in animal models, where an ANP/BNP hybrid antagonist was shown to enhance LPS- and IL-1β-induced fever (Miyoshi *et al*., 2006). LPS injection in healthy humans was found to cause rapid and substantial increases in circulating pro-BNP (Vila *et al*., 2008), suggesting that this peptide is part of the early host response to sepsis, perhaps alerting the host physiology to prepare for impending septic shock.

Whereas BNP-directed immunomodulation has largely been studied in the context of cardiac inflammation, the related peptides ANP and CNP have demonstrated immunomodulatory effects on vascular and lung tissue. CNP, which has a net charge of +2, was shown to inhibit leukocyte recruitment and rolling across endothelial surfaces that was driven by IL-1β or histamine in mice, and to inhibit platelet–leukocyte interactions, primarily through the modulation of P-selectin (Scotland *et al*., 2005). Interestingly, ANP, which has a higher net charge of +6, has been found to do just the opposite, increasing mobilization of polymorphonuclear neutrophils and macrophages and enhancing endothelial–leukocyte interactions. ANP is also able to broadly activate immune cells, increasing leukocyte production of bactericidal reactive oxygen species and inducing the maturation and proliferation of T cells and DCs (Potter *et al*., 2009). Conversely, ANP has been shown to have more of an anti-inflammatory/immunosuppressant effect in lung tissue, in both animal models (Wang *et al*., 2009c; Sekino *et al*., 2010) and patient trials (Oda *et al*., 2009). Mechanistically, ANP can enhance the barrier effect of endothelial tight junctions (and thus limit leukocyte recruitment) in response to both inflammatory (LPS) (Irwin *et al*., 2005) and allergic (ovalbumin (OVA)) stimuli (Mohapatra, 2007), probably via the attenuation of LPS- and TNF-α-induced MAPK, ERK1/2, NF-κB and Rho pathway signalling (Xing and Birukova, 2009).

The NPs and other small cationic HDPs that exhibit no or minimal direct antimicrobial activity (i.e. active only in dilute medium or buffer), but play an important role in the endogenous host response to infection, are listed in Table 12.1. These peptides and their IDR derivatives (Box 12.1) have formed the basis of many immunomodulatory peptide agents currently in preclinical or clinical trials (Table 12.2).

12.3 Potential Therapeutic Uses beyond Anti-infective Activity

The range of potential therapeutic applications of HDPs and their synthetic derivatives has been greatly broadened by the discovery of their immunomodulatory properties. Beyond their use as novel anti-infectives against bacteria (Scott *et al*., 2007), viruses (Gallo *et al*., 2006; Falco *et al*., 2009) or fungi (Benincasa *et al*., 2006; Kollef *et al*., 2006), they also have significant potential as adjuvants (Garlapati *et al*., 2009), anti-inflammatories and anti-endotoxic agents (Andra *et al*., 2006) and are showing substantial promise in wound-healing applications (Steinstraesser *et al*., 2008).

Table 12.2. Immunomodulatory host defence peptides, innate defence regulators and their derivatives in clinical trials.

Peptide	Description	Intervention	Most advanced progress	References and trial registries[a]
AP214 (Action Pharma)	Synthetic derivative of the HDP α-MSH (Table 12.1). Parent peptide is fused to a hexalysine sequence at its C-terminus	Sepsis and postsurgical organ failure	Phase II	Doi *et al.* (2008); NCT00903604
CD-NP	Chimeric synthetic NP (37-mer) able to bind to the receptors for all three endogenous NPs. Modified to lack haemodynamic activity	Organ failure	Phase II	Rose (2010); NCT00482937
DiaPep277 (DeveloGen)	HSP60 derivative (24-mer peptide) that induces T-regulatory cells	Autoimmune-mediated diabetes	Phase III	NCT00644501; ISRCTN55429664
EA-230 (Exponential Biotherapies)	Oligopeptide fragment from β-hCG (4-mer, LQGV)	Sepsis	Phase II	van den Berg *et al.* (2009); NA[b]
Ghrelin	Endogenous HDP	Airway inflammation, chronic respiratory infection, cystic fibrosis	Phase II (all)	Table 12.1; JPRN-UMIN000002599; JPRN-UMIN000001598; NCT00763477
Glutoxim/NOV-002 (Pharma BAM/ Novelos)	Hexapeptide with a stabilized disulfide bond (*bis*-(γ-L-glutamyl)-L-cysteinyl-*bis*-glycine disodium salt)	Tuberculosis, myelodysplastic syndromes	Market (Russia); Phase III (N. America)	Sokolova *et al.* (2002); NCT00960726
Heptapeptide-7 (Helix BioMedix)	Peptide fragment (7-mer), derived from the synthetic prototype HB-107 (which is itself derived from cecropin B)	Wound healing, skin regeneration	Phase II	Falla and Zhang (2010); NR
hLF1–11 (AM-Pharma)	Derivative of the cationic AMP human lactoferricin (amino acids 1–11)	Bacteraemia and fungal infections in immuno-compromised haematopoietic stem-cell transplant recipients	Phase I/II Earlier anti-infective trials terminated	van der Does *et al.* (2010); NCT00509938; NCT00509847 (withdrawn); NCT00509834 (terminated)
IMX942 (Inimex)	Immunomodulatory peptide lacking antimicrobial activity, derived from the prototype IDR-1 (which is itself derived from indolicidin)	Nosocomial infections, febrile neutropenia	Phase IA	NA
Iseganan (IB-367) (Ardea Biosciences)	Synthetic protegrin-1 derivative (17 amino acids)	Oral mucositis in radiation therapy patients	Phase III Earlier anti-infective trials terminated	NCT00022373; NCT00118781 (terminated)

Continued

Table 12.2. *Continued.*

Peptide	Description	Intervention	Most advanced progress	References and trial registries[a]
Omiganan (MX-226) (Migenix/BioWest Therapeutics)	Synthetic derivative of indolicidin (12-mer). Retains both antimicrobial and immunomodulatory activity of parent peptide	Topical antiseptic, acne vulgaris, papulopustular rosacea	Phase III	NCT00000435, NCT00027248
OP-145 (OctoPlus)	Synthetic derivative of LL-37 optimized for maximal LPS and LTA binding activity (24-mer)	Chronic bacterial otitis media	Phase II	ISRCTN84220089
Opebacan (Xoma)	21 amino acid peptide derivative of bactericidal/ permeability-increasing protein)	Endotoxemia in haematopoietic stem-cell transplant recipients, burn wounds	Phase II, Phase I	NCT00454155; NCT00462904
PAC-113 (Pacgen Biopharmaceuticals)	Synthetic cationic HDP (12-mer), histatin derivative	Antifungal	Phase IIB	NCT00659971
RDP58 (Genzyme)	Semisynthetic D-amino acid decapeptide derived from HLA class I B2702	Inflammatory bowel disease	Post Phase II	Travis *et al.* (2005); NR[c]
Vasoactive intestinal peptide	Endogenous HDP	Respiratory tract infections, sepsis	Phase I	Table 12.1; NCT00004494

[a] International clinical trial registration number as indexed on the World Health Organization's global trial database (http://apps.who.int/trialsearch/Default.aspx).
[b] Trial is registered, but in a country that lacks publicly searchable trials.
[c] Clinical trial is registered.
Abbreviations: AMP, antimicrobial peptide; hCG, human chorionic gonadotrophin; HLA, human leukocyte antigen; hLF, human lactoferrin; HDP, host defence peptide; HSP, heat-shock protein; IDR-1, immune defence regulator 1; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MSH, melanocyte-stimulating hormone; NA, not available; NP, natriuretic peptide; NR, not registered.

12.3.1 Adjuvant potential

Adjuvants are components of vaccines that are generally not immunogenic themselves, but enhance the immunogenicity of the antigenic components. The inclusion of adjuvants thus enhances the efficacy of vaccines, such that lower doses of antigen are required to achieve effective immune responses and lasting immunological memory. Appropriate adjuvants may also decrease the number of doses required, thus not only reducing the cost of vaccines but also enhancing patient compliance (Nicholls *et al.*, 2010). The development of vaccines to prevent many childhood diseases is frustrated by the limited ability of the immature immune system to respond to non-protein antigens and it would be a great step forward to create an adjuvant that would enable single-dose vaccines that are effective in infants. Additionally, adjuvants that can enhance the mucosal immune response to vaccines, allowing them to be taken orally rather than injected, are also actively sought in the fight against a vast range of diseases.

Human neutrophil defensins have shown adjuvant activity in murine models, enhancing both humoral and cell-mediated antigen-specific immune responses. For example, the inclusion of defensins with ovalbumin for intranasal immunization of mice enabled the enhancement of immunoglobulin (Ig)G antibody responses; despite

the mucosal route of administration, however, IgA responses were not induced (Lillard *et al*., 1999). Additionally, $CD4^+$ T cells isolated from the immunized mice demonstrated enhanced antigen-induced proliferation and increases in the archetypal T-helper (Th)1 cytokine IFN-γ, as well as secretion of the classical Th2 cytokines IL-5, IL-6 and IL-10, demonstrating that defensins are capable of stimulating a balanced immune response. Tani *et al*. (2000) observed similar adjuvant activity when defensins were administered in combination with the model antigen keyhole limpet haemocyanin, showing that IgG1, IgG2a and IgG2b antibody responses were all enhanced by inclusion of the defensins, while *ex vivo* splenocytes from immunized mice produced more IFN-γ and IL-4 and demonstrated greater proliferative responses to antigen. This same study also demonstrated that defensins could be used to boost the immune response to tumour-specific antigens and affect the outcome of a tumour challenge model.

Synthetic IDRs are being investigated as components of vaccine formulations, with promising results. CpG ODNs have been investigated for their adjuvant potential, but cause a significant release of pro-inflammatory cytokines. However, the addition of poly-L-lysine in formulation with CpG ODNs prevents this undesirable inflammatory response while retaining antigen-specific immune responses (Lingnau *et al*., 2002). Similarly, a combination of IDR-HH2 and CpG ODN enhanced antigen-specific antibody responses in mice, including the production of high titres of IgA, IgG1 and IgG2a. This combination also appeared to increase surface markers on DCs and induce cytokine and chemokine responses *ex vivo* (Kindrachuk *et al*., 2009). Formulations including HDPs, CpG ODNs and poly-phosphazene-enhanced antigen-specific cellular and humoral responses in both mice and cattle (Kovacs-Nolan *et al*., 2009a,b,c) and a similar combination showed promise in a neonatal pig model, suggesting applicability of this technology to infant vaccines (Garlapati *et al*., 2009). Additionally, immunization with a formulation of the peptide $KLKL_5KLK$ with deoxyinosine/deoxycytosine, referred to as IC31, has shown the ability to enhance cellular and humoral immune responses in mice, including the activation of DCs and proliferation and differentiation of naive $CD4^+$ T cells (Schellack *et al*., 2006). Hurtado and Peh (2010) recently demonstrated that LL-37 is able to enhance the responses of B cells and DCs to CpG ODNs, and it may well be mimicry of this natural immunomodulatory property that provides the adjuvant activity of IDRs.

12.3.2 Wound-healing activity

LL-37 exhibits angiogenic and wound-healing properties, promoting neovascularization and re-epithelialization in animal models (Koczulla *et al*., 2003; Steinstraesser *et al*., 2006, 2008; Carretero *et al*., 2008). The physiological role of peptides in wound healing is supported by the observations that HDPs such as psoriasin and hBD-2 are more highly expressed in tissues surrounding chronic wounds (Dressel *et al*., 2010; Harder *et al*., 2010) and that the expression of cathelicidins dramatically increases after cutaneous injury (Dorschner *et al*., 2001). Additionally, treatment with exogenous hBD-3 was able to enhance re-epithelialization of wounds in a porcine model (Hirsch *et al*., 2009). Many authors have suggested that the ability of HDPs to enhance wound healing is mediated through direct antimicrobial activity, with the presence of HDPs preventing or limiting infection of the wounds. While antimicrobial activity may well play a role in the wound-healing activity of HDPs, the ability of these molecules to modulate the expression of growth factors and cytokines is likely to be more important.

HDPs are able to stimulate the production of cytokines and growth factors in epithelial cells and keratinocytes. For example, LL-37 stimulates the release of IL-8 from airway epithelial cells by a mechanism that appears to involve transactivation of epidermal growth factor receptor (Tjabringa *et al*., 2003). LL-37 and hBD2–4 are able to induce the secretion of IL-18 from keratinocytes; inhibitor studies have demonstrated

that this involves modulation of p38 and ERK1/2 MAPK pathways, but is independent of JNK (c-Jun N-terminal kinase) (Niyonsaba *et al*., 2005). IL-18 is a pleiotropic cytokine that is classically thought of as an inducer of IFN-γ, but is also able to promote angiogenesis (Park *et al*., 2001). LL-37 and hBD-2–4 also promote the proliferation and migration of keratinocytes and the production of IL-6, IL-10, 10-kDa IFN-γ-induced protein, monocyte chemotactic protein-1, macrophage inflammatory protein (MIP)-3α and RANTES (regulated on activation, normal T-cell expressed and secreted) by these cells (Niyonsaba *et al*., 2007). In a subsequent study, the HDP dermcidin (DCD-1L) stimulated keratinocytes to produce a similar range of cytokines, including TNF-α, IL-8, IFN-inducible protein 10 (IP-10) and MIP-3α (Niyonsaba *et al*., 2009), demonstrating that a wide range of HDPs are able to activate keratinocytes. In an *in vivo* system, hBD-2 has also been found to stimulate chemotaxis of endothelial cells and promote capillary-like tube formation as effectively as vascular endothelial growth factor (Baroni *et al*., 2009). This same HDP also promotes the migration, but not proliferation, of intestinal epithelial cells (Otte *et al*., 2008), as does LL-37 (Otte *et al*., 2009). A synthetic acetylated and amidated 24-mer derivate of LL-37, termed P60.4-Ac (Nell *et al*., 2006), has been shown to inhibit disruption of the respiratory epithelium ultrastructure by bacterial pathogen-associated molecular patterns *in vitro* (Vonk *et al*., 2008). Although some of these activities occur at quite high concentrations of peptides, it has been demonstrated that very low concentrations of peptides synergize with endogenous host molecules such as IL-1β and granulocyte–macrophage colony-stimulating factor, as well as with TLR agonists such as flagellin and poly-IC (Bowdish *et al*., 2005a,b; Filewod *et al*., 2009).

Thus, the range of biological activities of HDPs extends well beyond direct bacterial killing, with an ability to modulate intracellular signalling and gene expression in a wide range of cell types. The downstream effects of such activities are also wide ranging, giving a vast potential for designer synthetic peptide drugs targeting different clinical conditions.

12.4 Recent Clinical Advances in Therapeutic Application of Host Defence Peptides

The introduction of insulin in the 1920s marked the beginning of a new age in pharmaceutical drugs, where endogenous agents (or their mimetics) could be administered with the therapeutic aim of modulating human physiology. These biopharmaceutical agents, typically proteins or peptides and more commonly referred to as 'biologics', have become a mainstay in the treatment of endocrine, autoimmune and other diseases with pathophysiology or dysfunction involving human systems with complex feedback and feedforward regulation. At the forefront of cutting-edge research and development into novel biologics are compounds that act at multiple points within the dynamic regulatory networks underlying disease states, moving beyond the classic one drug–one receptor model of traditional pharmaceuticals.

Peptide-based immunotherapies are still in their infancy, but the use of immune-targeting therapeutics is well established within the practice of Western medicine (Waldmann, 2003). To date, the majority of these biologicals with immunomodulatory action have aimed at the suppression or disruption of normal immune function, rather than its enhancement. As a result, these drugs have become the mainstay of treatments for chronic inflammatory and autoimmune diseases, but show little appreciable value as adjuvants or therapeutics in treating infectious diseases. Such applications are also limited by an inability to selectively target the effector subsets that mediate the necessary components of innate and adaptive immunity (Feldmann and Steinman, 2005), resulting in side effects that can include enhanced susceptibility to infections (Botsios, 2005), significantly high rates of anaphylaxis (Corren

et al., 2009) and, in extreme cases, toxic cytokine storms (Suntharalingam *et al*., 2006).

Immunostimulatory drugs are beginning to make inroads as viable options for the treatment of infectious diseases, often as adjunctive therapy to mainstay antibiotics. A recent Cochrane clinical trial meta-analysis (Del-Rio-Navarro *et al*., 2006), examining the use of 21 different biological immunomodulatory agents in the treatment of pediatric acute respiratory infections, provided fascinating data on the safety and efficacy of these drugs. The Cochrane review found that the use of immunostimulatory drugs reduced the incidence of respiratory tract infections by an average of 40% across the studies, with adverse effects not being significantly different to those associated with placebo groups.

Due to the complexities inherent in studying innate immunity and the corresponding difficulty in selecting appropriate clinical readouts or endpoints in preclinical and Phase I trials, clinical trials of immunomodulatory drugs often evaluate compounds in manners that avoid excessive responses or systemic absorption. For example, RDP58, which is under investigation for the treatment of ulcerative colitis (Travis *et al*., 2005), was structurally modified to prevent both proteolytic degradation and systemic absorption (Holtmann, 2003). Two Phase II trials of RDP58 in a combined cohort of 127 patients found the drug to be well tolerated (adverse effects equivalent to placebo) and efficacious in improving sigmoidoscopy scores in colitis patients receiving medium or high doses of the drug (Travis *et al*., 2005). The pathogenesis of ulcerative colitis is thought to be at least partially due to a dysregulated host response to intestinal microbiota (Xavier and Podolsky, 2007) and, although RDP58 is being investigated for its anti-inflammatory properties, it may also alter host responses to microbiota in a manner similar to HDPs and IDRs.

An exciting area in which the dual anti-infective and anti-inflammatory actions of peptide immunotherapy have potential is the treatment of bacteraemia and septicaemia. While no clinical data are yet publicly available, IMX942 (which is a derivative of IDR-1 and lacks direct antimicrobial activity) has completed Phase I safety trials in chemotherapy patients with febrile neutropenia (Table 12.2). In addition, OP-145, a structural derivate of LL-37, has been reported to show efficacy in clinical trials for the treatment of chronic otitis media in adult patients (Table 12.2). Whether immunomodulatory (immunostimulatory) peptides will become mainstays in the treatment of bacterial antibiotics remains to be seen. However, the successes in clinical trials to date demonstrate significant promise for these peptides in playing, at minimum, an adjunctive role to conventional antibiotic therapy.

Many of the peptides with documented immunomodulatory and antimicrobial activity that have entered clinical trials have done so as topical antimicrobials for infectious, inflammatory or wound-healing applications (Table 12.2). The choice of moving forward with these agents as topical interventions likely reflects difficulties in selecting appropriate systemic biomarkers as safety readouts. Thus, a series of peptides have been tested topically, including omiganan (MX-226), iseganan (IB-367), human lactoferrin (hLF)1–11 and HB-107 (Table 12.2), despite the fact that most of these peptides have demonstrated immunomodulatory activity *in vivo* (Giacometti *et al*., 2003; Lee *et al*., 2004; Falla and Zhang, 2010; van der Does *et al*., 2010). Interestingly, the majority of the peptide (and peptide-derived) drugs under investigation as immunomodulatory anti-infectives are the classical neuroendocrine peptides described in Table 12.1. This is probably because these peptides have been under investigation for decades (albeit in other contexts) and have well-defined receptors and cellular mechanisms. This is in contrast to HDPs, which are relatively new discoveries. Based on these observations, we feel there is a strong possibility that, in the future, immunomodulatory activity will play a substantial role in any clinical benefit demonstrated by HDP- and IDR-based drugs.

12.5 Innate Defence Regulators as Anti-infective Therapeutics

The increased awareness of the natural immunomodulatory functions of HDPs and the role they play in the endogenous anti-infective response has created an exciting new avenue for their application: the use of synthetic peptides that selectively modulate the innate immune response as an anti-infective therapeutic strategy. One of the first synthetic immunomodulatory peptides based on a HDP, immune defence regulator 1 (IDR-1), is able to mediate *in vivo* protection against bacterial challenge in the absence of direct antimicrobial activity (Scott *et al*., 2007). The sequence of IDR-1 is based on the 12 amino acid bovine cathelicidin bactenecin, which has immunomodulatory activities that reflect a subset of those apparent in the 37 amino acid human peptide LL-37 (Bowdish *et al*., 2005a). The functional mechanism of action of IDR-1 in animal infection models appears to involve the concomitant enhancement of chemokine production and moderation of proinflammatory cytokines such as TNF-α, leading to enhanced cellular clearance of bacteria without excessive inflammation. These effects appear to be mediated through modulation of specific intracellular signalling pathways via intracellular binding partners such as sequestosome-1/p62 (Yu *et al*., 2009) or GAPDH, which leads to altered activation states of key transcription factors (Scott *et al*., 2007; Mookherjee *et al*., 2009a). Such activities provide a powerful rationale for designing synthetic peptides with optimized immunomodulatory activities based upon HDPs (e.g. Nijnik *et al*., 2010) and demonstrate that direct antimicrobial activity is not a necessary characteristic for the efficacy of such peptides.

Five immunomodulatory cationic peptides have already demonstrated safety in human clinical trials. Two of these, MX-226 and hLF1–11, were originally developed as AMPs but also demonstrate immunomodulatory activity (van der Does *et al*., 2010). Topical delivery of MX-226 has demonstrated safety and efficacy in Phase II clinical trials that targeted both the inflammatory sequelae of severe acne and a non-infectious inflammatory skin disease, rosacea, demonstrating promise for the treatment of inflammatory conditions independent of antimicrobial activity. Systemic safety has been shown in Phase I clinical trials for hLF1–11, a truncated form of lactoferrin, which was examined in immunocompromised haematopoietic stem-cell transplant recipients. Recent work with hLF1–11 in animal models has also demonstrated its efficacy as a systemic anti-infective through immunomodulatory mechanisms (van der Does *et al*., 2010).

OP-215, a synthetic 24 amino acid derivate of LL-37, has also been shown to be efficacious and safe (compared to placebo) in a randomized, double-blind, placebo-controlled, multicentre Phase II study when applied topically in ear drops to chronic suppurative otitis media patients (F.A.W. Peek *et al*., 2009, unpublished results). Although little information on this peptide is available, it is claimed that it binds LPS and lipoteichoic acid, degrades biofilms and has antimicrobial actions, while its direct chemotactic activity is said to be low. Another peptide, RDP58, which is a cationic peptide derived from the heavy chain of human leukocyte antigen (HLA) class I molecules, has also demonstrated safety in clinical trials (Travis *et al*., 2005). RDP58 reduced the production of the pro-inflammatory cytokines TNF-α, IFN-γ and IL-12, but did not affect the production of several other cytokines including IL-4, -6, -8 and -10. The proposed mechanism of action involves interference with intracellular signalling pathways; however, the specific details and interactors are currently unknown (Travis *et al*., 2005). IMX942, which is based on IDR-1, has completed Phase I safety trials in chemotherapy patients with febrile neutropenia (see www.inimexpharma.com).

While IDR-1/IMX942 and RDP58 are both cationic peptides that modulate innate immunity, they appear to do so via distinct mechanisms. Both peptides suppress the induction of particular proinflammatory cytokines, such as TNF-α, but their effects on other cytokines vary. For example, RDP58 does not affect IL-6 or -10 production, while

the treatment of cells with IDR-1 tends to enhance the production of these two cytokines. The distinct responses to these peptides indicate the value of different design strategies, since RDP58 is based upon HLA class I molecules while IDR-1 is a derivative of a bovine cathelicidin. Importantly, this also indicates that if we can understand the intricacies of immunomodulatory peptide interactions with host cell components and pathways, it may be possible to design peptides with customized immunomodulatory effects to target different diseases.

12.6 Rational Design of Immunomodulatory Peptides

The development and validation of screening techniques is an important aspect of the IDR design process. High-throughput *in vitro* screening of antibacterial activity has been used for the validation and iterative optimization of rationally designed AMPs (Cherkasov *et al*., 2009; Fjell *et al*., 2009). The technical simplicity of *in vitro* testing for direct antibacterial activity has made it possible to collect large datasets using high-throughput screening techniques and use this information to identify particular physical characteristics that correlate with antimicrobial activity. By contrast, the complexity of the interactions between multiple cell types and effector molecules of the immune system mean that it is not possible to thoroughly assess subtle immunomodulatory activities *in vitro*, and simplified markers of this activity must be used. For example, IDR-1 was selected based on markers such as synergistically enhanced release of chemokines from LPS-stimulated human peripheral blood mononuclear cells (Scott *et al*., 2007) and chosen for further analysis after demonstrating an ability to protect mice against bacterial infections. Another possible screen would be the ability to suppress TNF-α induction in response to treatment with TLR agonists (Mookherjee *et al*., 2006). Even with the use of such markers, candidate peptides must ultimately be tested *in vivo*, as isolated cell systems cannot capture the complexity of the innate immune response. Thus, compared to testing of antimicrobial activity, for which there is often a strong correlation between *in vitro* and *in vivo* activity, it is much more labour-intensive and costly to generate the large datasets necessary for applying computational methods to immunomodulatory peptide design.

Despite this constraint, we now have a few precedents where rational peptide design based on structure–function parameters has proven successful. For example, synthetic endotoxin-binding peptides can be optimized for protection *in vivo* (Dankesreiter *et al*., 2000) and a fragment of LL-37 can be optimized for anti-endotoxin activity through appropriate amino acid substitutions (Nagaoka *et al*., 2002). It is also possible to create truncated peptides based on HDPs such as LL-37 that retain either the parent peptide's antimicrobial or immunomodulatory properties (Braff *et al*., 2005; Chapter 9). Taken together, these findings demonstrate that particular domains are necessary for these functions and suggest that it will be possible to identify particular characteristics associated with protection. Other studies have optimized the activities of smaller bactenecin- and indolicidin-like synthetic peptides as components of adjuvant formulations by screening for chemokine-inducing activity followed by *in vivo* analysis (Garlapati *et al*., 2009; Kindrachuk *et al*., 2009; Kovacs-Nolan *et al*., 2009a,c). An iterative design process has also successfully produced new IDRs with greater anti-infective efficacy than IDR-1 (Nijnik *et al*., 2010).

12.7 Limitations and Challenges

All drug candidates must overcome potential toxicities and questions of *in vivo* stability and appropriate delivery routes. Clearly it is possible to design IDRs that protect without substantial *in vitro* or animal-model toxicity (Scott *et al*., 2007; Nijnik *et al*., 2010). However, no systemic toxicology and pharmacokinetic studies have been described

for any peptide and questions still remain regarding potential *in vivo* toxicities. This is particularly important when considering the transition from murine models to use in humans, considering the many differences between the rodent and human immune systems and possible requirements for multiple dosing. While small-animal models provide very valuable information regarding *in vivo* activity, dosing, appropriate formulation and routes of administration, the intricate regulatory networks and feedback loops that make up the mammalian immune system are such that even small differences between hosts can be magnified quite substantially. In cases where severe problems have occurred with immunomodulators (Ponce, 2008), few *in vitro* or *ex vivo* data involving human cells were available and the problems may have been predicted had such experiments been undertaken prior to human trials (Dayan and Wraith, 2008) or with better regulatory oversight (Gottlieb, 2008; Shuchman, 2008). Several peptide-based drugs, and many immunomodulatory drugs of varying compositions, have successfully passed clinical testing and entered the market, demonstrating that it is possible to ensure the safety of such drugs.

The reduction of the *in vivo* stability of peptides by proteolytic processing is also of concern. While small peptides are probably degraded within minutes in many compartments of the host (e.g. blood, where proteases abound), results with IDR-1 indicate that treatments up to 48 h prior to or 6 h after bacterial challenge are protective in animal models (Scott *et al*., 2007). This raises the question of how a peptide that is rapidly degraded can have long-lasting effects and whether enhancing peptide stability is desirable in this context. One possibility is that the peptides prime immune cells; indeed, their ability to readily translocate into immune cells (Lau *et al*., 2005) may protect them from proteolytic degradation. This is consistent with the observation that the peptides are active when delivered by many different routes, possibly indicating that these locally primed cells (or cells containing peptide) migrate to the infection site. Regardless, several methods have been proposed for enhancing the *in vivo* stability and long-term shelf-life of peptides. Additionally, the cost of manufacturing synthetic peptides is very high, so strategies that enhance bioavailability or reduce the size or number of doses required for therapeutic benefit are also important for decreasing the cost of treatment.

One way of enhancing peptide stability is to induce chemical modifications that provide resistance to proteolytic digestion without altering the functional characteristics of the peptide. For example, the use of D-amino acid isomers of peptides is one approach used for RDP58 (Table 12.2), exploiting the specificity of proteases for L-amino acid forms of the peptide. Since the three-dimensional structure of the peptides may be important for their interactions with specific receptors, retro-inverse peptides comprised of D-amino acids in the reversed sequence have been investigated; this preserves the spatial positions of the side chains while maintaining the protease resistance of the D-amino acid forms (Fischer, 2003). Recently, a synthetic peptide, M33, was shown to resist proteolytic degradation when constructed in a tetra-branched form (Pini *et al*., 2009). The peptide demonstrated antibacterial activity against a range of Gram-negative species and protected against endotoxemia in mice. Conversely, creating a cyclic structure – as for defensins – may enhance *in vivo* stability. The use of unnatural amino acid side chains or modified peptide backbones (peptidomimetics) has substantial potential in this regard.

A potential limitation of any novel anti-infective is the development of microbial resistance. Thus, a very important characteristic of IDRs is that there is a lower likelihood of microorganisms developing resistance to them than is the case with direct AMPs (Steinstraesser *et al*., 2009). For the AMPs, it has been argued that their physical action on membranes or their multiple targets in a single cell make resistance unlikely. However, several studies have demonstrated resistance to AMPs mediated by the differential expression of a range of genes, often through blocking uptake (McPhee *et al*., 2003; Kraus and Peschel, 2008;

Ernst *et al*., 2009; Majchrzykiewicz *et al*., 2010; Warner and Levy, 2010). Nevertheless, IDR peptides do not act directly on bacteria, but rather through modulation of the host immune system. Since the innate immune system has co-evolved with pathogenic microbes, subtly augmenting this response should not enhance the selective pressure on bacterial immune evasion mechanisms. Additionally, enhanced innate clearance of microbes mediated by IDRs should avoid the creation of bacterial debris that continues to promote inflammation in the absence of live bacteria (Andra *et al*., 2006), providing yet another advantage over traditional antibiotic therapies; indeed, some of these peptides actually suppress such septic inflammatory responses.

A potentially serious undesirable outcome of treatment with peptides based upon LL-37 is the promotion of tumorigenesis. As discussed previously, LL-37 has demonstrated wound-healing activity, which includes enhanced angiogenesis and proliferation of keratinocytes and epithelial cells. These functions alone suggest the possibility of tumour promotion as a potential side effect of the inappropriate presence of LL-37 and such concerns are compounded by the observation that LL-37 can act as a growth factor for lung cancer cells (von Haussen *et al*., 2008), promote a metastatic phenotype in breast cancer cells (Weber *et al*., 2009) and influence the progression of ovarian tumours (Coffelt *et al*., 2009). However, the very nature of the altered characteristics of cancer cells means that any immunomodulatory molecule could inadvertently become a growth factor for such cells. It is not surprising that LL-37 could be co-opted in such a way, especially if it is a ligand for CXCR2 considering that this receptor is involved in melanoma growth and invasion (Singh *et al*., 2009). However, this limitation is not grounds to assume that certain HDP-based treatments are inherently problematic. Rather, these concerns illustrate that tailored synthetic analogues with some, but not all, of the natural functions of particular HDPs should be considered the optimal design output. Indeed, it seems likely that one can design IDRs with antitumour activity, since some HDPs reduce angiogenesis rather than promote it (Economopoulou *et al*., 2005; Krylov *et al*., 2007), while others promote apoptosis of tumour cells or some have even demonstrated anti-proliferative activity (Suttmann *et al*., 2008). Current research is investigating the feasibility of designing membrane-disrupting peptides that exploit differences in membrane fluidity to permit them to be cytotoxic to tumour cells but not to healthy cells (Mader and Hoskin, 2006; Fadnes *et al*., 2009). Indeed, many of the HDP-based immunotherapies in clinical trials are being explored as cancer therapies (Table 12.2). Therefore, as with any novel immunomodulatory drug, while it is certainly important to remain conscious of the possibility of tumour promotion as a side effect, with the appropriate testing and rational design it is possible to ensure that this scenario never becomes a reality.

12.8 Conclusions

HDPs and their synthetic derivatives have demonstrated significant potential as anti-infective therapeutics and current research promises to reveal crucial mechanistic information that should greatly enhance the development of optimized peptides. These peptides exert powerful actions in the human body, including modulation of innate immunity, suppression of harmful inflammation and promotion of adaptive immunity, and roles in the enhancement of wound healing and resolution of the inflammatory process. New insights and awareness about structure–function activities, cellular binding partners and dynamic functional roles of these peptides will allow the rational design of synthetic peptides targeted for different purposes, not all of which will necessarily be anti-infective. The ability to subtly alter the normal balance between protective innate immune responses, such as chemokine production and concomitant leukocyte recruitment, and the potentially harmful effects of excessive inflammation provides a unique opportunity to promote effective innate immune defences against pathogens.

As the non-antimicrobial anti-infective mechanism of HDPs involves fine tuning the endogenous host response, the use of these agents as biological therapeutics is unlikely to result in the development of microbial resistance. While much research is still required in this area, the potential of this novel approach is enormous and brings hope for providing an alternative strategy in the era of burgeoning antibiotic resistance.

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