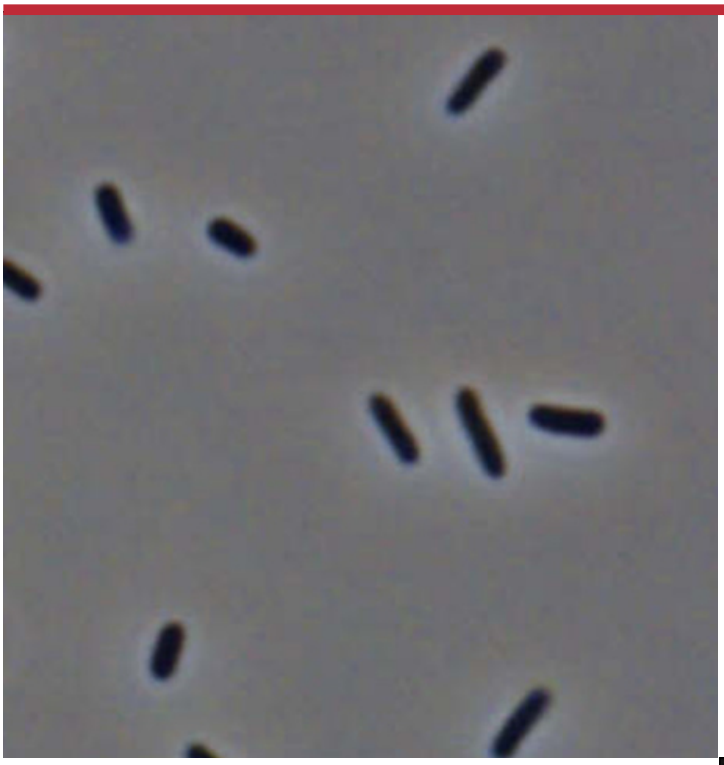
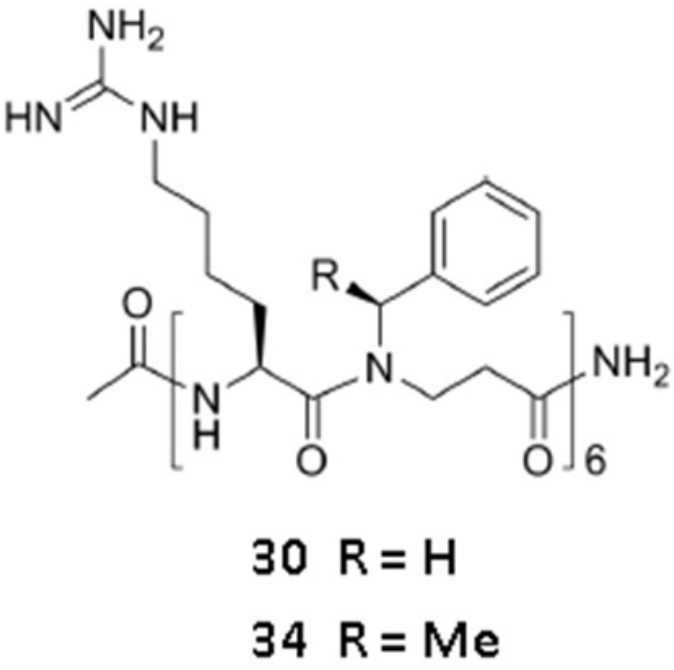
Spectrum and activity of novel antimicrobial peptidomimetics





Line Hein-Kristensen
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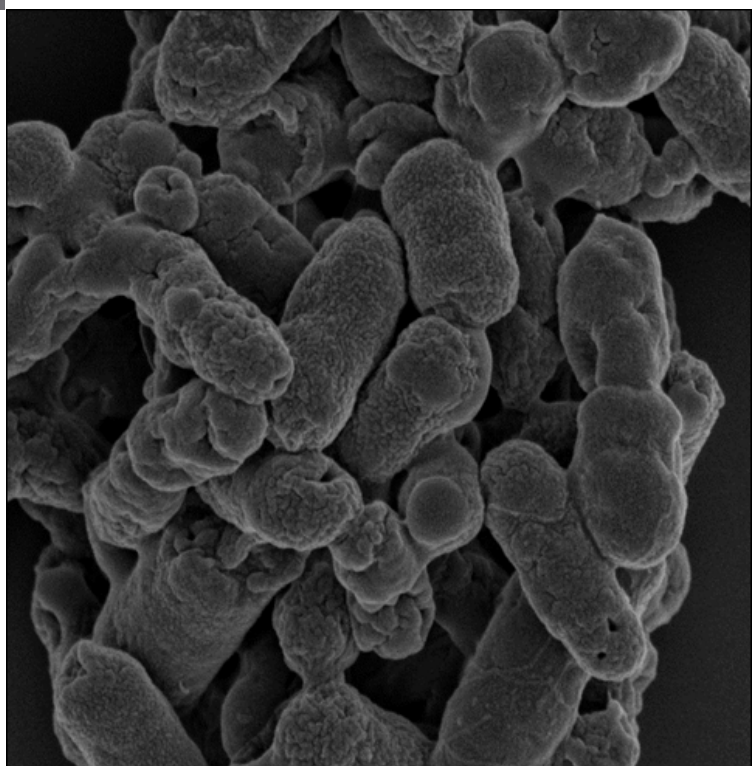

DTU Food
National Food Institute

Spectrum and activity of novel antimicrobial peptidomimetics

PhD thesis

by

Line Hein-Kristensen

2012

National Food Institute

Technical University of Denmark

Division of Industrial Food Research

Front page photos:
Upper left: Chimera-resistant isolate of *Escherichia coli*
Upper right: Molecular structure of chimera 30 and 34
Bottom right: Scanning Electron Microscopy (SEM) of *Serratia marcescens* treated with chimera 30

Preface

The work presented in this thesis is the result of a PhD study following the PhD programme at the Technical University of Denmark. The PhD study has been carried out at the National Food Institute, Division of Industrial Food Research from March 2009 to February 2012. The project was financed by the Research Council for Technology and Production (project 09-065902) and the Technical University of Denmark.

I would like to thank my supervisor Lone Gram (DTU Food) for her many ideas, great enthusiasm and for always finding the time to discuss my work. I would also like to thank Henrik Franzyk (KU Pharma) for his dedication in explaining his ideas and thoughts behind peptidomimetic design to a non-chemist.

Also, I would like to acknowledge the Center for Electron Microscopy (DTU) for introducing me to Scanning Electron Microscopy and for producing the amazing images of chimera-treated bacteria. Thanks to the DTU Multi-assay core for instructions on how to use the Qubit fluorometer and to Jørgen Kurtzhals (Rigshospitalet) for taking the time to draw my blood.

A special thanks to my colleagues in the micro-group for providing a nice atmosphere and an inspiring scientific environment.

Finally I would like to thank my closest family and friends for their continuous support. Especially I would like to thank my boyfriend Anders for his encouragement and for always being able to put me in a good mood.

Line Hein-Kristensen
Kgs. Lyngby, February 2012

This thesis is based on the following papers:

Paper 1

Line Hein-Kristensen, Kolja M. Knapp, Henrik Franzyk & Lone Gram (2011). Bacterial membrane activity of α-peptide/β-peptoid chimeras: Influence of amino acid composition and chain length on the activity against different bacterial strains. *BMC Microbiology* 11:144.

Paper 2

Line Hein-Kristensen, Kolja M. Knapp, Henrik Franzyk & Lone Gram (2012). Continuous selection of *Escherichia coli* to an α-peptide/β-peptoid peptidomimetic leads to inherent resistance against similar compounds. Manuscript in preparation.

Paper 3

Line Hein-Kristensen, Kolja M. Knapp, Henrik Franzyk & Lone Gram (2012). Effect of human blood plasma and immune effectors on the antibacterial activity of an α-peptide/β-peptoid chimera against wild-type *Escherichia coli* and chimera-resistant mutants. Manuscript in preparation.

Summary

Antibiotics have been an effective weapon against bacterial infections for over 50 years. However, bacterial resistance towards conventional antibiotics has increased considerably within the last decades and the number of antibacterial agents available for treating complicated bacterial infections is becoming increasingly limited. In the search for alternatives therapies, antimicrobial peptides (AMPs) have received considerable attention since they target the bacterial Achilles' heel i.e. their distinct membrane structure. These host defence molecules are ubiquitous in nature by forming part of the immune system among all classes of life. Several of these compounds have therefore been characterised and developed into future antibacterials. Furthermore, in an attempt to improve the antibacterial activity, synthetic analogues i.e. peptidomimetics have been designed based on the structural properties of natural AMPs.

The purpose of this PhD study was to establish the potential correlation between structure and antibacterial activity for a series of α-peptides/β-peptoid peptidomimetics and additionally to determine if mechanistic differences could explain observed variations in activity. We determined the activity of the peptidomimetics against a range food borne and nosocomial pathogenic bacteria. These structure-activity studies demonstrated that peptide length was important for high antibacterial activity since analogues with a length shorter than 12 residues were virtually inactive. In the present design, with a 1:1 ratio between cationic α-amino acids and hydrophobic β-peptoids, amino acid composition and chirality in the β-peptoid unit only had a minor influence on antibacterial activity.

By using an ATP leakage assay we determined that the mechanism of action of the chimeras was permeabilization or disruption of the bacterial cell membrane. The resulting changes to the cell surface were visualised with Scanning Electron Microscopy (SEM). Importantly, our leakage studies were performed with viable bacterial cells and using a concentration that was close to the Minimum Inhibitory Concentration (MIC). The findings show that all of the chimeras included in the study have a similar mechanism of action that was independent on bacterial species. However, the study showed that the detailed interaction with the cell membrane may be different, since there were large variations in the amount of leaked ATP and subsequent loss of viability. A series of three peptides differing only in length all caused ATP leakage but only the longest of the three caused complete depletion of intracellular ATP, which correlated with a substantial loss in the number of viable cells.

In a continuous selection protocol encompassing 500 generations, 10 out of 10 lineages of *Escherichia coli* developed resistance towards the chimera they had been exposed to. This was the first time resistance was successfully developed towards peptidomimetics, though several studies have reported resistance towards AMPs. Resistance was specific to compounds within the peptidomimetics library, since we were unable to demonstrate cross-resistance to other AMPs. We sequenced the entire genome of six highly resistant isolates from two separate lineages, and identified a single-nucleotide-polymorphism (SNP) in the gene encoding the MltD protein. This protein functions in the reorganization of the peptidoglycan layer, and we consider it likely that a change in this protein is the cause of resistance, since the SNP was found exclusively in isolates with high levels of resistance.

Conversely, these resistant isolates displayed increased sensitivity towards human blood plasma possibly due to immune effector compounds present in this. The addition of 50 % blood plasma also increased the activity of the chimeras against wild type bacteria by up to 32 times. This effect was abolished by heat-treatment, which is a method known to inactivate the complement system.

The findings in this thesis have elucidated how central structural determinants influence antibacterial activity. Peptidomimetics can be regarded as promising future antibiotics since the possibility to optimize their properties through structural modification allows for continuous variation. This thesis concludes that antibacterial activity can be improved further and that in the future resistance may be circumvented by optimizing the existing scaffold.

Resumé (in Danish)

Antibiotika har været brugt til behandling af bakterieinfektioner gennem mere end 50 år. Bakterie-resistens overfor konventionelle antibiotika er dog steget drastisk de seneste årtier, hvilket begrænser antallet af behandlingsmuligheder mod komplicerede bakterieinfektioner. I jagten på alternative antibakterielle stoffer har de antimikrobielle peptider (AMPer) fået meget opmærksomhed eftersom deres aktivitet er rettet mod bakteriernes akilleshæl dvs. deres særlige membranstruktur. Disse forsvarsstoffer findes overalt i det naturlige miljø, da de udgør en del af immunforsvaret hos alle livsformer. Adskillige af disse stoffer er derfor blevet karakteriseret og udviklet som fremtidens antibiotika. Derudover er syntetiske analoger, peptidomimetics, blevet designet ud fra strukturen af naturlige AMPer, hvilket giver mulighed for at forbedre den antibakterielle aktivitet.

Formålet med dette ph.d. projekt var at etablere en sammenhæng mellem struktur og antibakteriel aktivitet i en række α-peptid/β-peptoid peptidomimetics, og derudover at klarlægge hvilke mekanismer, der kunne forklare eventuelle forskelle i aktivitet. Vi bestemte aktiviteten af disse peptidomimetics mod en række fødevarebårne og hospitals-erhvervede patogene bakterier. Dette studie af forholdet mellem struktur og aktivitet viste, at en vis peptid-længde var nødvendig for at opnå høj antibakteriel aktivitet, da analoger med en længde på mindre end 12 enheder var næsten ineffektive. I det indeværende design, hvor der er en 1:1 ratio mellem de kationiske α-aminosyrer og hydrofobiske β-peptoider, havde aminosyre-komposition og chiralitet i β-peptoid enheden kun ringe indflydelse på den antibakterielle aktivitet.

Vi brugte et ATP lækage assay til at vise, at den antibakterielle aktivitet skyldtes permeabilisering eller ødelæggelse af bakteriens cellemembran. Dette forårsagede ændringer af celleoverfladen, som blev visualiseret gennem Skanning Elektron Mikroskopi (SEM). Det er vigtigt at understrege, at lækage studierne blev lavet med levende bakterier og at vi brugte en koncentration, som var tæt på den Minimum Inhibitoriske Koncentration (MIC). Vores undersøgelser viste, at alle analogerne har den samme virkningsmekanisme og at denne er uafhængig af bakterien. Resultaterne viste dog også, at den præcise interaktion med membranen kan variere eftersom der var store variationer i mængden af lækket ATP og det efterfølgende fald i antallet af levende celler. En serie på tre peptider, som kun varierede i længden, forårsagede alle ATP lækage, men kun den længste bevirkede, at cellen blev tømt for ATP. Dette korrelerede med et markant fald i antallet af levende celler.

Kontinuerlig selektion gennem 500 generationer resulterede i, at 10 ud af 10 linjer af *Escherichia coli* udviklede resistens mod den benyttede analog. Dette var det første bevis på udvikling af resistens mod peptidomimetics, selvom adskillige studier har vist, at AMP resistens kan udvikles. Vi anser resistensen for at være specifik for analogerne i indeværende peptid-bibliotek, da vi ikke fandt tilfælde af kryds-resistens til en række andre AMPer. Vi sekventerede hele genomet fra seks høj-resistente isolater fra to forskellige linjer, og identificerede en enkeltmutation (SNP) i genet, der koder for MltD proteinet. Dette protein er involveret i reorganiseringen af peptidoglycan laget og eftersom denne mutation kun forekom i isolater, som var meget resistente, anser vi det for sandsynligt, at en ændring i dette protein er årsagen til resistens.
Disse resistente isolater havde forøget sensitivitet overfor humant blodplasma, hvilket kunne skyldes tilstedeværelsen af innate effektormolekyler. Tilsætning af 50 % blodplasma forøgede samtidig aktiviteten af analogerne op til 32 gange. Denne effekt forsvandt efter varme-behandling, som er en metode, der benyttes til at inaktivere komplementsystemet.

Arbejdet i denne afhandling har medvirket til en øget forståelse for hvordan centrale strukturelle egenskaber påvirker den antibakterielle aktivitet. Peptidomimetics kan betragtes som et godt bud på fremtidens antibiotika, fordi det er muligt at optimere deres egenskaber gennem ændringer i deres struktur, hvilket betyder uanede muligheder for variation. Denne afhandling konkluderer, at den antibakterielle aktivitet kan forbedres yderligere, og at fremtidig resistens måske kan afværges ved optimering af den nuværende design.

Contents

1. Introduction and scope of the PhD project

Since the discovery of penicillin in 1928 and the market introduction a decade later, antibiotics has been part of the standard arsenal for treating infectious diseases. In the antibiotic era of the 40's and 50's several novel classes of antibiotics were discovered. This gave hope for a future where all infections could be controlled. Indeed, today it is difficult to imagine infectious medicine where treatment with antibiotics is not an option. However, bacterial resistance to conventional antibiotics has increased considerably within the last decades (Agersø et al., 2010; Jensen et al., 2009). Thus, the number of antibacterial agents available for treating complicated bacterial infections is becoming increasingly limited. Particular problematic bacteria include *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus* and extended-spectrum Lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae* (Boucher et al., 2009). Importantly, the increased proportion of resistant isolates seems to be correlated with an increased consumption of "broad-spectrum" antibiotics (Jensen et al., 2009). Novel antibiotics are urgently needed but due to the large R&D expenses and modest gain (Projan, 2003), the pipeline of many pharmaceutical companies is unacceptably lean and very few novel antibiotic classes are marketed (Jabes, 2011) (Figure 1.1).

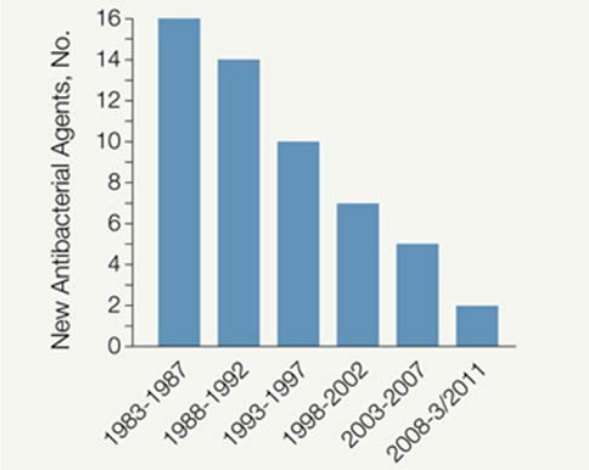

Figure 1.1. Number of new systematic antibiotics approved by the FDA (United States) from 1983 through March 2011. From (Kuehn, 2011).

Consequently, antibacterial drug development relies on modifying already existing drugs (Fischbach and Walsh, 2009), which may accelerate the development of resistance as the mechanism of action resembles that of the parent drug (Livermore, 2011).

This has lead to an intensified search for alternative antibacterial therapies. Antimicrobial peptides (AMPs) constitute one of the most promising novel classes of novel antibacterial drugs (Yeung et al., 2011). These compounds form part of the antimicrobial defence among all classes of life including

humans (Maroti et al., 2011), and has received considerable attention because they target the Achilles' heel of bacteria i.e. their distinct membrane structure (Zasloff, 2002). Hence, immense effort has been put into characterising and developing these compounds into future therapeutics. Currently, the biggest obstacles for the AMPs to fulfil their therapeutic promise are poor pharmacokinetics due to their sensitivity to proteases, unknown *in vivo* toxicity profiles and high manufacturing cost associated with chemical synthesis (Hancock and Sahl, 2006). Additionally, the approval of AMPs for systemic use relies on their ability to show superior efficacy compared to conventional already marketed antibiotics, which trials up until now have failed to demonstrate.

Synthetic modification of natural AMPs or mimicking their structural properties for developing novel antimicrobials constitute promising approaches for optimizing the activity while limiting the level of toxicity (Palermo and Kuroda, 2010). Indeed, new tools for rapid chemical synthesis has made it possible to assess the effect of small structural modifications in libraries of synthetic analogues i.e. peptidomimetics. Consequently, structure-activity studies of a variety of peptidomimetics have been reported by several research groups.

A library of such peptidomimetics were designed and synthesized by the Natural Products Research group at the Faculty of Pharmaceutical Sciences (now Health Sciences), University of Copenhagen. These compounds consist of alternating units of cationic α-amino acids and hydrophobic β-peptoids and possess structural properties similar to that of AMPs and other peptidomimetics. The α-peptide/β-peptoid chimeras display limited hemolytic activity and are resistant towards proteases (Olsen et al., 2007). They are hence being characterised and optimised for future therapeutic use. This involves testing their activity on microbial biofilms (DTU Systems biology) and determining their activity *in vivo* using animal models (Statens Serum Institut). Additionally, these peptidomimetics have been investigated for their capabilities to assist in intracellular drug delivery.

Novel peptidomimetics exhibit considerable antibacterial activity similar to natural AMPs, and it is hypothesized that it is possible to further improve this by structural modifications of the existing scaffold. The central purpose of this project has been to establish a possible correlation between peptidomimetic structure and antibacterial activity. Consequently, we hypothesized that substitutions with different types of α-amino acids or β-peptoids or modifications of peptide length would alter the activity against a range of food borne and nosocomial bacterial pathogens, since such modifications have been reported to alter the activity of other peptidomimetics. Most AMPs and peptidomimetics target the bacterial cell membrane leading to formation of pores, cell lysis and cell death. We

hypothesised that the same would be the case for the present peptidomimetics, and as an essential part of the characterisation, we therefore wanted to determine the mechanism of action of the most active compounds. Furthermore, since resistance to AMPs is considered unlikely or very difficult to select for, we were interested in investigating if this was also the case for the peptidomimetics. Here, it would be particular interesting to study if potential resistance would also confer resistance to other groups of AMPs and thereby potentially hamper the human innate immune defence.

This thesis consists of an overview section and three papers. The overview section describes the nature of AMPs and the approaches for using their properties to develop novel antimicrobials i.e. peptidomimetics (Chapter 2). The overview also includes a review of the activity and mechanism of action of AMPs/peptidomimetics (Chapter 3) and describes the mechanisms bacteria have evolved circumvent these activities (Chapter 4). Finally, the potential effects the immune system may have on activity of these compounds *in vivo* will be described (Chapter 5). The experimental work and the results obtained during the PhD will be described in the three papers although selected results will also be covered in relevant chapters of the overview section.

2. Natural antimicrobial peptides and novel peptidomimetics

Antimicrobial peptides (AMPs) have the potential for developing into future therapeutic agents for treatment of infections caused by human pathogenic bacteria. Hence understanding how, when and where they function has become of considerable interest. In the next chapters I will review how these compounds affect bacteria both *in vivo* and *in vitro*, and the mechanisms bacteria have developed to counteract the activities of AMPs. This chapter will give an introduction to antimicrobial peptides in nature, and the synthetic approaches for developing new compounds that mimic natural AMPSs i.e. peptidomimetics. Several different classes of peptidomimetics have been developed and the α-peptide/β-peptoids chimeras that are the focus of this thesis constitute one of these groups. Accordingly, the library of peptidomimetics and how this was designed will be reviewed in detail.

2.1. Cationic antimicrobial peptides

Antimicrobial peptides (AMP) constitute a structurally diverse group of compounds. They are characterised by having a net negative (cationic) charge and an ability to assume an amphipathic structure enabling insertion into bacterial membranes (Chapter 3). As this does not demand a stringent organization of their sequence or structure, several diverse architectures have evolved (Giangaspero et al., 2001). The diversity in sequence and structure of natural AMPs will be reviewed in this section. Human AMPs will be covered in Chapter 5, where they will be discussed as part of their role in the innate immune defence in humans.

2.1.1. The nature of antimicrobial peptides

Cationic AMP are defined as peptides of less than 50 residues with a net positive charge, conferred by cationic amino acid residues, and a substantial portion (above 30 %) of hydrophobic residues (Hancock and Sahl, 2006). AMPs form part of the immune defence among all classes of life and are hence ubiquitous in nature (Maroti et al., 2011). Consequently, more than 1000 natural AMPs have been identified to date (Godballe et al., 2011; Wang et al., 2009). These fall into two classes: the non-ribosomally synthesized produced only by bacteria and the ribosomally synthesized produced by all species of life including bacteria (Hancock and Chapple, 1999). The former include among others the polymyxins and are made on multi-enzyme complexes formed by a series of peptide synthetases performing the peptide synthesis in an ordered fashion (Hancock and Sahl, 2006). Most AMPs are however transcribed from specific genes and synthesized on ribosomes (Zasloff, 2002). In invertebrates, AMPs serve as the primary defence against bacterial infections (McPhee et al., 2005),

whereas they in higher animals and plants constitute part of the generic defence response known as the innate immune system (Maroti et al., 2011). The expression profiles of genes encoding AMPs differ depending on the organism: local or systemic, constitutive or induced by infectious or inflammatory stimuli (Andres, 2011). In the majority of cases the response is local as that produced by phagocytic cells and epithelium in animals (Chapter 5). However, intruding bacteria induce a systemic response in insects (Andres, 2011). The diversity of sequences is such that the same peptide sequence is rarely recovered from two different species (Zasloff, 2002). This suggests that each AMP have evolved to act optimally in the environment in which it is produced and against local microorganisms (Hancock and Chapple, 1999). Diversification of the AMP repertoire will enable the host to better cope with microbial challenges (Patil et al., 2004). Intriguingly, it has been proposed that bacterial resistance mechanisms (Chapter 4) direct and shape the diverse repertoire of AMPs (Peschel and Sahl, 2006).

2.1.2. Peptide secondary structures

Due to the large variations in amino acid sequence, AMPs are classified based on their secondary structure of which there are four main classes: β-sheet, α-helical, extended and loop peptides, with the first two classes being the most common in nature (Powers and Hancock, 2003) (Figure 2.1).

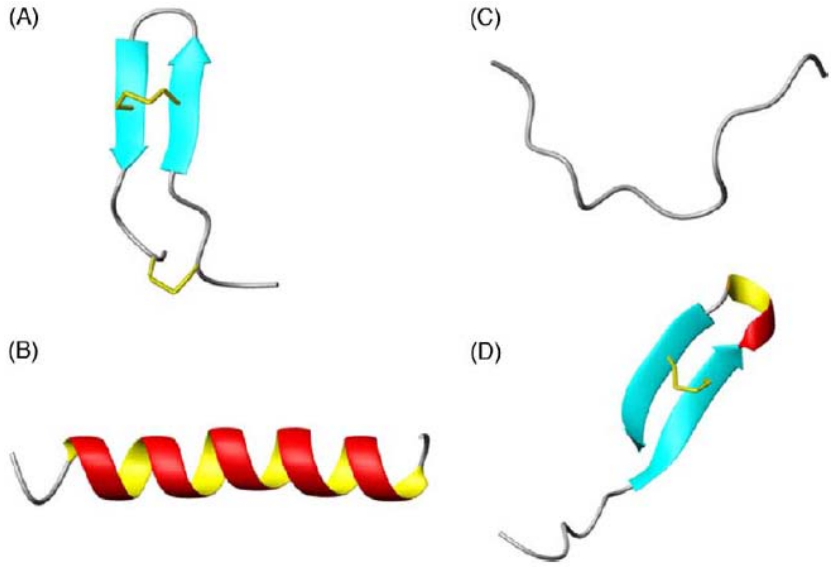

Figure 2.1. Structural classes of antimicrobial peptides displayed by one representative AMP of each group: A) β-sheet, tachyplesin I; B) α-helical, magainin 2; C) extended, indolicidin; D) loop, thanatin. Disulfide bonds are indated in yellow. From (Powers and Hancock, 2003).

Peptide structures are frequently determined when AMPs are interacting with model membranes or are in a membrane-mimetic environment (McPhee et al., 2005). The AMPs typically display

unordered structures in aqueous solutions (Friedrich et al., 1999; Javadpour et al., 1996). Methods for determining the structural characteristic of a given compound will be described in the next section. In addition to natural AMPs, a range of synthetic variants (peptidomimetics) have been produced which also fall into one of these structural classes (Powers and Hancock, 2003), with the large majority of these designed to form an α-helical structure.

The α-helical peptides are the most abundant class of natural peptides. These peptides will upon interaction with target membranes fold into an amphipathic α-helix with one face of the helix predominantly containing the hydrophobic amino acids and the opposite face the charged amino acids (McPhee et al., 2005). Of note, though there within this group also is little conservatism in the amino acid sequence, there is a pronounced trend when it comes to the distribution of different types of residue i.e. charged, polar etc. (Tossi et al., 2000) (Figure 2.2).

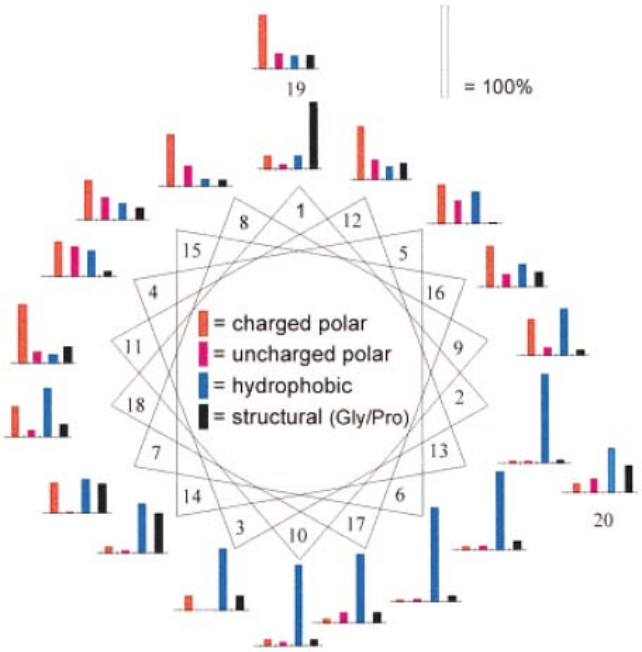

Figure 2.2. Helical wheel projection of the residue distribution in the 20 residue N-terminal stretch of 150 α-helical AMPs from natural sources. Residue numbers are indicated. The analysis indicates considerable positional conservatism in terms of residue type within the secondary structure of the helix. From (Tossi et al., 2000).

β-sheet peptides are classified by the presence of an antiparallel β-sheet stabilized by two or more disulfide bonds (Powers and Hancock, 2003) (Figure 2.2a), and include among others the defensins of vertebrates, plant and insects (Andres, 2011). Other AMPs contain high proportions of certain amino acids such as tryptophan, histidine and proline (Hancock and Patrzykat, 2002). For instance the bovine indolicidin contain high amounts of tryptophan (Ando et al., 2010). Most of these peptides adopt extended structures upon interaction with the membrane and this is stabilized by hydrogen bonds and van der Waals forces with lipids rather than interresidue hydrogen bonds

(McPhee et al., 2005). Lastly, the structure of loop peptides is imparted by the presence of a single bond i.e. disulfide, amide or isopeptide (Powers and Hancock, 2003) (Figure 2.1d).

2.2. Approaches for developing novel antimicrobial structures

The potential of AMPs as therapeutic agents shows great promise with a variety of natural and synthetic compounds currently in development (Yeung et al., 2011). Novel AMPs can be developed in one of three ways; i) discovery and characterization of previously unknown natural AMPs, ii) synthetic optimization of natural AMP or iii) using the properties of natural AMPs as templates for designing synthetic AMPs i.e. peptidomimetics. In this section I will describe each of these approaches for gaining new compounds. The antibacterial activity of specific structures and the effect optimization may have on this will be covered in Chapter 3.

2.2.1. Discovery and optimization of natural antimicrobial peptides

Traditionally, novel antimicrobial compounds were isolated from natural sources (Livermore, 2011). This process frequently starts with confirmed biological activity e.g. direct inhibition of bacterial growth either using crude natural extracts or biological peptides libraries (Silva et al., 2011). Alternatively, organisms may be screened for secretory proteins using transposon assisted signal trapping (Mygind et al., 2005) or unknown cDNA sequences identified through amplification using specific primers as in the case of novel cathelicidins (Tossi et al., 1997a). Genome mining constitutes another promising tool for discovering novel antimicrobial compounds (Velasquez and van der Donk, 2011). The production of antimicrobial compounds is often an unstable trait e.g. due to regulatory systems making it difficult to define optimal growth conditions in screening systems (Nes and Johnsborg, 2004). The increasing availability of genome sequences and rapid developments in bioinformatics means that it is now possible to use *in silico* screening to identify novel members of antimicrobial compound groups (de Jong et al., 2010). This approach has been used for identification of antimicrobials from both prokaryotic (Begley et al., 2009; Wang et al., 2011b) and eukaryotic (Tian et al., 2010) sources.

Primary antibacterial evaluation forms the starting point for precipitation, purification and structural characterisation of the potential compound (Silva et al., 2011). After the identification of promising compounds, lead candidates may be optimized to further increase their antibacterial activity or spectrum of activity. Peptide libraries containing millions of AMP variants are subjected to high-throughput-screening (HTS) computational or *in vitro*-based methods for determining activity

(Blondelle and Lohner, 2000; Raventos et al., 2005; Silva et al., 2011). Alternatively, high-throughput-synthesis on cellulose support membranes followed by screening against microbes expressing the luciferase gene cassette can elucidate effective amino acid substitutions of a parent peptide (Hilpert and Hancock, 2007).

Several modified variants of natural AMPs are now in clinical development (Andres, 2011; Brandenburg et al., 2011). AMP congeners are chemical compounds that are closely related to each other in composition and prepared by relaxing the structure, replacing specific amino acids within the parent AMP sequence or truncating the N-terminus and/or the C-terminus ends of the parent peptide (Brogden and Brogden, 2011). Primary sequence modifications of natural AMPs are employed to increase the net charge, overall amphipathicity or improve their predicted folding patterns (Giangaspero et al., 2001; Kondejewski et al., 1996). For instance, variants based on the membranolytic protegrin I contained loop sequence related to the parent compound but was linked to a proline template which stabilized the β-hairpin conformation (Srinivas et al., 2010).

Hybrid AMPs are constructed from the active regions of two or three natural AMPs to combine the benefits of each individual fragment (Brogden and Brogden, 2011). They are constructed through chemical synthesis or expressed as a fusion protein in a recombinant system (Jung et al., 2011). The cecropin-bee melittin hybrid peptide CEME contains the N-terminal domain of cecropin and the C-terminal domain from melittin and has now additionally been further developed into amino acid-replaced analogues (Friedrich et al., 1999). Similarly, chimeric peptides containing the N-terminal region of the human β-defensin 3 and the middle region of human β-defensin 2 were more potent than their parent peptides (Jung et al., 2011). Very recently, the design of hybrid peptides based on cecropin A, LL-37 and magainin II was described (Fox et al., 2012) (Figure 2.3).

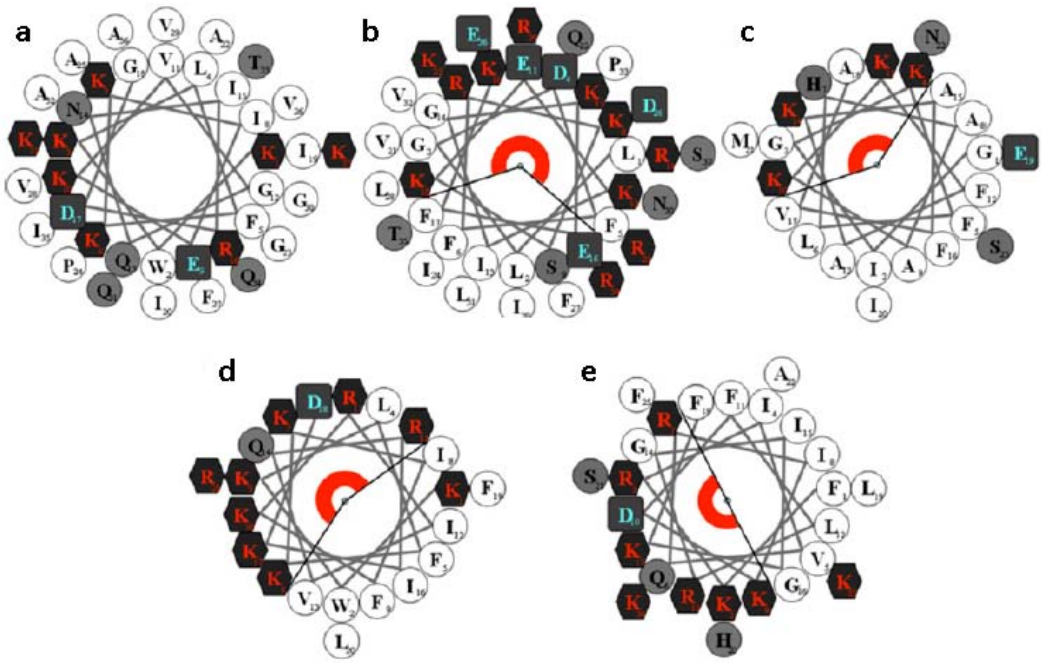

Figure 2.3. Helical wheel models of a) cecropin A, b) LL-37, c) magainin II and two hybrid peptides d) CaLL (cecropin A residues 1-8 followed by LL-37 residues 17-29 and e) LLaMa (LL-37 residues 17-29 followed by magainin II residues 1-12). Residues coloured by type (hydrophobic, charged etc.). The predicted angle subtended by the positively charged residues on the formation of the α-helix is shown. From (Fox et al., 2012).

For this, fragments of the parent peptides were selected if they made up the core helix structure and/or were responsible for antibacterial activity. Fragments were then combined *in silico* to determine which hybrid peptides would form the best amphipathic α-helixes. This yielded four hybrid peptides that were more potent than their parent peptides (Fox et al., 2012).

Comparison and extraction of sequence patterns of natural AMPs is an alternative method for optimizing the antimicrobial activity. The most frequent amino acids in the first 20 position of natural α-helical AMPs have been determined (Figure 2.2). This sequence analogy reveals the distribution of residue types in the archetypal α-helical AMP and can thus be used to create a sequence template useful in guiding the design of novel peptides (Tossi et al., 1997b).

2.2.2. De novo synthesis of peptidomimetics

Several natural AMPs have been associated with high levels of toxicity and additionally these compounds may suffer from a variety of pharmacokinetic shortcomings including poor bio-availability, low metabolic stability and formulation difficulties (Findlay et al., 2010). A variety of novel and structurally diverse cationic compounds that mimic the amphiphilic topology have been

developed to overcome these problems, the classes of which will be reviewed in the next section. Chemical synthesis is the main method for production of peptidomimetics though it is a both time-consuming and costly process particularly for longer peptides (Silva et al., 2011).
Peptidomimetics are created by using solid-phase peptide synthesis (SPPS), where the molecule is bound to a resin bead and synthesized in a step-by-step process by adding reagents in a consecutive fashion. As a standard Fmoc (fluorenylmethoxy-carbonyl) active ester chemistry is applied (Fields and Noble, 1990). Due to the complexity of these reactions it will not be reviewed in detail here; for details of the chemical reactions involved in synthesizing the α-peptide/β-peptoid chimera I refer to Olsen et.al (2007). In short, the C-terminal Fmoc amino acid (or in the case of the chimeras, a peptide/peptoid dimer) is attached to the resin bead (Chou et al., 2008; Olsen et al., 2007). Thereafter Fmoc building blocks are attached in a consecutive manner to the growing chain for the number of cycles needed to obtain the desired length. After synthesis is complete the compound is cleaved from the resin bead and Fmoc removed from the N-terminal amino acid (Radzishevsky et al., 2008).
A variant of solid-phase synthesis enables mixtures of compounds to be synthesized in separate groups each being defined by individual residues or building blocks at one or more given positions (Blondelle et al., 1996). This form of semi-random synthesis creates synthetic combinatorial libraries, which subsequently can be screened for inhibitory activity.

2.2.3. Purification and structural characterisation

After synthesis samples are purified using reversed-phase high-performance liquid chromatography (RP-HPLC) and subjected to matrix-assisted laser desorption ionization (MALDI) mass spectroscopy to verify correct mass (da Silva et al., 2008; Junkes et al., 2011; Park et al., 2011; Yeaman et al., 2002).

HPLC is used to purify the finished product from by-products giving final products that are > 95 % pure (Dathe et al., 2001; Olsen et al., 2007; Wu et al., 1999). The compounds are separated based on their affinity for a stationary hydrophobic phase and a mobile polar phase, respectively (Schmitt et al., 2007). Retention times indicate how strongly the compound will interact with the hydrophobic phase, and will be unique for each compound based on its structural and chemical properties (Blondelle and Houghten, 1992). HPLC may also be used analytically to determine if the specific compound can be degraded by proteolytic enzymes (Porter et al., 2002; Schmitt et al., 2007). where compound cleavage will be evident as extra peaks in the chromatogram (Schmitt et al., 2007).
Peptoids are protease resistant compared to their homologous peptide counterparts (Rotem and Mor,

2009). The chimeras used in this thesis are resistant to enzyme degradation, which is believed to be due to the inclusion of this unit (Olsen et al., 2007).

The secondary structure of peptidomimetics, which is induced when the compound binds to the hydrophobic stationary phase, may influence the retention time (Blondelle and Houghten, 1992). Hence, the level of amphipathicity (segregation of hydrophilic and hydrophobic residues) may be determined using HPLC (Chen et al., 2005b; Schmitt et al., 2007) (Figure 2.4).

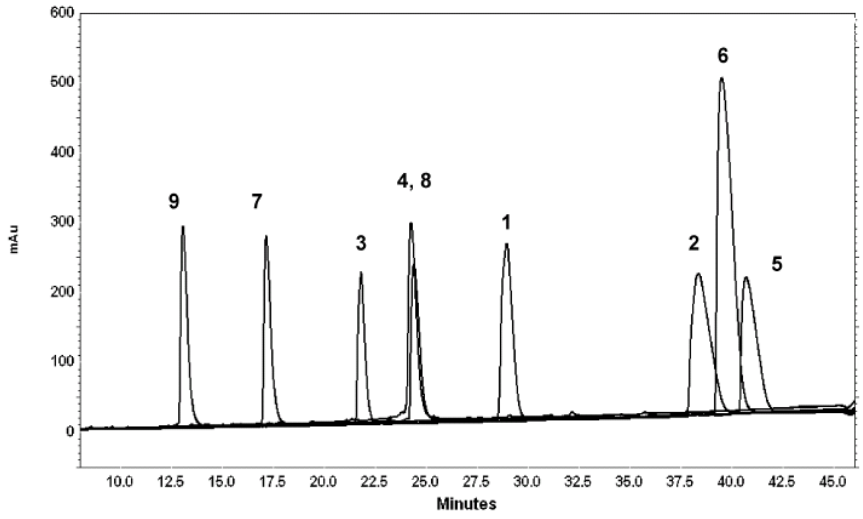

Figure 2.4. Overlay of HPLC traces for a series of nine peptides with different conformations. The longest retention times were seen for compounds that adopt a globally amphipathic helical structure and are extremely hemolytic. From (Schmitt et al., 2007).

A preliminary indication of peptide secondary structure is obtained from analysis of a circular dichroism spectrum (CD). In order to predict the structure both outside and within the cytoplasmic membrane the analysis is commonly performed in both a hydrophilic and a hydrophobic environment (Chen et al., 2005b). The pattern for α-helical peptides is very distinctive since these produce large negative bands at ~222 nm and ~208 nm and a large positive band at ~193 nm (Azad et al., 2011) (Figure 2.5a). CD spectra of some of the early α-peptide/β-peptoid chimeras revealed that lack of chirality (see next section) leads to a lower degree of secondary structure in these compounds (Olsen et al., 2007) (Figure 2.5b).

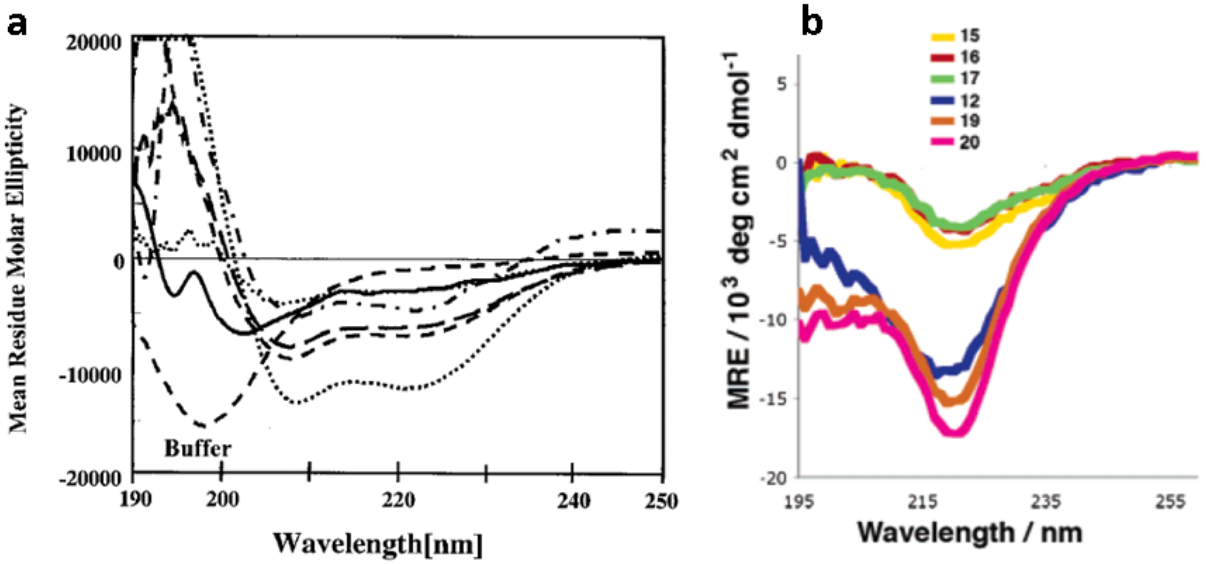

Figure 2.5. Peptide CD spectra: a) CD spectra of a series of CEME analogues in the presence of liposomes or buffer (dashes, random coil); all peptides except one (solid line, random coil) had the characteristic spectrum of α-helixes (Friedrich et al., 1999), b) CD spectra of a series of α-peptide/β-peptoid chimeras; a substantial drop was seen in the ellipicity amplitudes at 219nm for peptides 15-17 which had a non-chiral β-peptoid unit. This indicates a lower degree of secondary structure for these compounds (Olsen et al., 2007).

An amphipathic helical conformation is not expected for the chimeras since these have a design of alternating hydrophobic and cationic residues, though it is likely, based on the CD spectrum and antibacterial activity, that these compounds have another type of amphipathic conformation. Though CD spectroscopy is often used to elucidate the peptide secondary structure, it may not always be accurate since two peptides expected to differ in secondary can actually have a similar CD spectrum (Glattli et al., 2002). Alternative and more accurate methods for determining structure are crystallography or nuclear magnetic resonance (NMR) (Hicks et al., 2007; Mandal et al., 2009; Yu et al., 2002), which also makes it possible to define the peptid tertiary structure.

2.3. Classes of antibacterial peptidomimetics

Mimics of natural AMPs are collectively called peptidomimetics (Godballe et al., 2011). These mimics are often based on a different backbone i.e. they are not solely based on α-amino acids (Rotem and Mor, 2009). These modifications often render the compounds protease-resistant and additionally they may have improved bioavailability compared to peptide analogues (Patch and Barron, 2002). All peptidomimetics are modelled after the structural requirements known to be important for antibacterial activity (section 3.3.3), and hence all contain elements of positively charged and hydrophobic residues resulting in a compound with amphipathic properties (Godballe et

al., 2011). In this section I will outline selected representative classes of peptidomimetics, which have received increased focus over the last decade (Figure 2.6).

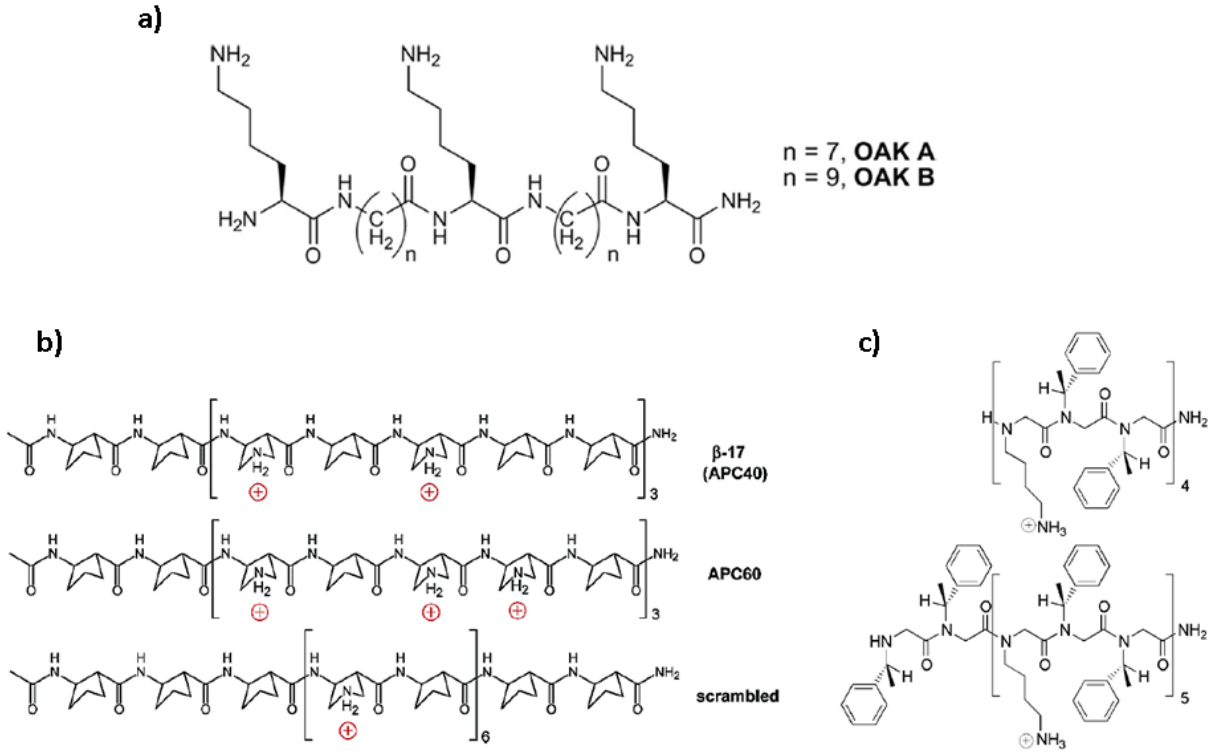

Figure 2.6. Selected types of antimicrobial peptidomimetics. a) oligo acyl lysines (OAKs) (Findlay et al., 2010; Radzishevsky et al., 2007); b) β-peptides (Porter et al., 2002); c) peptoids (Patch and Barron, 2003).

Oligo acyl lysines (Figure 2.6a) were designed by Mor's group and are composed of repeats of an acyl (fatty acid) chain of variable length and a charged amino acid i.e. lysine (Radzishevsky et al., 2007). Hence, it is possible to dissect the relative roles of charge and hydrophobicity by modifying the number of lysine-acyl subunits and the acyl length, respectively (Radzishevsky et al., 2008). Interestingly, this design prevents the formation of stable secondary structures because of the rotational freedom of the carbon atoms in the acyl chain (Rotem and Mor, 2009). Conversely, these compounds tend to aggregate thereby organizing themselves into a stable amphipathic supra-molecular structure (Radzishevsky et al., 2008).

In nature, most peptides are composed of α-amino acids and hence β-peptides containing an extra methylene group (Figure 2.7) are rare (Godballe et al., 2011). Because of this extra methylene group they are not recognised by traditional proteases and are therefore intrinsically resistant to enzymatic degradation (Porter et al., 2002). In fact, a design of alternating α- and β-amino acids also increase proteolytic stability (Schmitt et al., 2007), thereby making peptidomimetics containing β-amino acids

promising antibacterial candidates. These compounds adopt helical structures with greater conformational freedom than α-peptides because of the additional methylene unit present in the backbone (Porter et al., 2002). Consequently, whereas sequences of natural α-peptides most commonly adopt an α-helix conformation, β-peptides sequences adopt distinct helical conformations (Patch and Barron, 2002). Several groups have designed β-peptides that adopt different helical conformations. The example given in Figure 2.6b is by Gellman's group, which includes an intra-residue five-membered ring which restrict the conformational freedom compared to other β-peptides (Porter et al., 2002). Analogues differ in their number of cationic and hydrophobic residues and in the predicted distribution of positive charges around the helix i.e. scrambled (Porter et al., 2002).

Incorporating unnatural D-amino acids into the peptide structure is an alternative approach for increasing resistance towards proteolysis (Hancock and Sahl, 2006). In nature, most peptides are composed of L-amino acids, and peptides containing D-amino acids (i.e. the mirror image of L-amino acids) are rare (Papo et al., 2002). Hence, incorporation of D-amino acids could lead to lack of protease sequence recognition. Replacing natural amino acids with D-amino acid disrupts the structure of α-helical peptides (Chen et al., 2005b), and this may also abolish the cytotoxic effects on mammalian cells (Vooturi and Firestine, 2010). However, if peptide activity does not depend on the stereospecific recognition of a cellular target (Fehlbaum et al., 1996), activity is retained (Merrifield et al., 1995). Since most peptide target the bacterial membrane, D-peptides analogues will generally be as active as L-peptides (Tossi et al., 2000).

Peptoids share the same structure as amino acids, with the exception that the side chain has been removed from the α-carbon to the amide nitrogen (Findlay et al., 2010) (Figure 2.7). Oligomers of these building blocks are called α-peptoids. The backbone structure renders the compound protease resistant (Vooturi and Firestine, 2010), and decreases the level of hemolysis (Patch and Barron, 2003). Peptoids are unable to form intra-molecular hydrogen bonds that help to stabilizes α-helical and β-sheet structures, but incorporation of bulky aromatic side chains can lead to the formation of helices (Wu et al., 2001). This knowledge leads to the formation of a library of ampeptoids (antimicrobial peptide oligomers) with peptoid analogous composed of both charged and hydrophobic peptoid monomers (Chongsiriwatana et al., 2008) (Figure 2.6c). As for other peptidomimetics the design is modified by changing the number of cationic and hydrophobic residues or by increasing the number of subunits (Figure 2.6c).

Inspired by the heterogeneous backbone structures of α/β-peptides (Schmitt et al., 2004) and the structural features of peptoids, the present α-peptide/β-peptoid chimeras were developed (Olsen et al., 2007). β-peptoids combine the structural features of β-peptides and peptoids, and hence add to the existing repertoire of peptidomimetics (Bonke et al., 2008) (Figure 2.7).

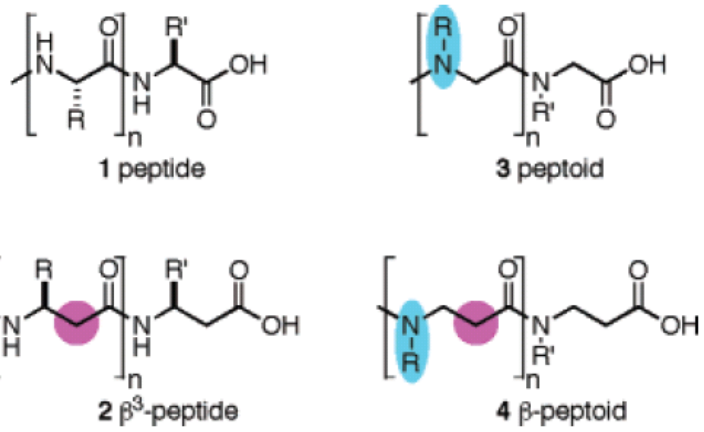

Figure 2.7. Backbone structures of natural α-peptides and novel peptidomimetic residues. From (Olsen et al., 2007).

Dimeric building blocks composed of β-peptoid units and amino acid moieties were designed for use in SPPS (Olsen et al., 2007). The advantage of novel heteromers over homomers composed only of β-peptoids is the possibility of diversification of side-chains functionalities via inclusion of various commercially available α-amino acids (Bonke et al., 2008). Lysine and homoarginine have been used as the α-amino acid residues in most of the chimeras; the use of homoarginine over arginine makes it possible to construct both homoarginine and lysine containing building blocks using the same intermediate. The structure of guanidino-group containing amino acids such as arginine facilitate stronger interaction with the bacterial membrane (section 3.3.3) compared to amino-group containing amino acids (e.g. lysine). Additional diversity can be created by using a combination of the two types of amino acids (Chimeras 45-49, Figure 2.8). For the β-peptoid residues a chiral and a non-chiral residue have been designed (Figure 2.8). These form the hydrophobic part of the chimeras. The six chimeras that have been used for the experimental work in this thesis are given in Figure 2.8.

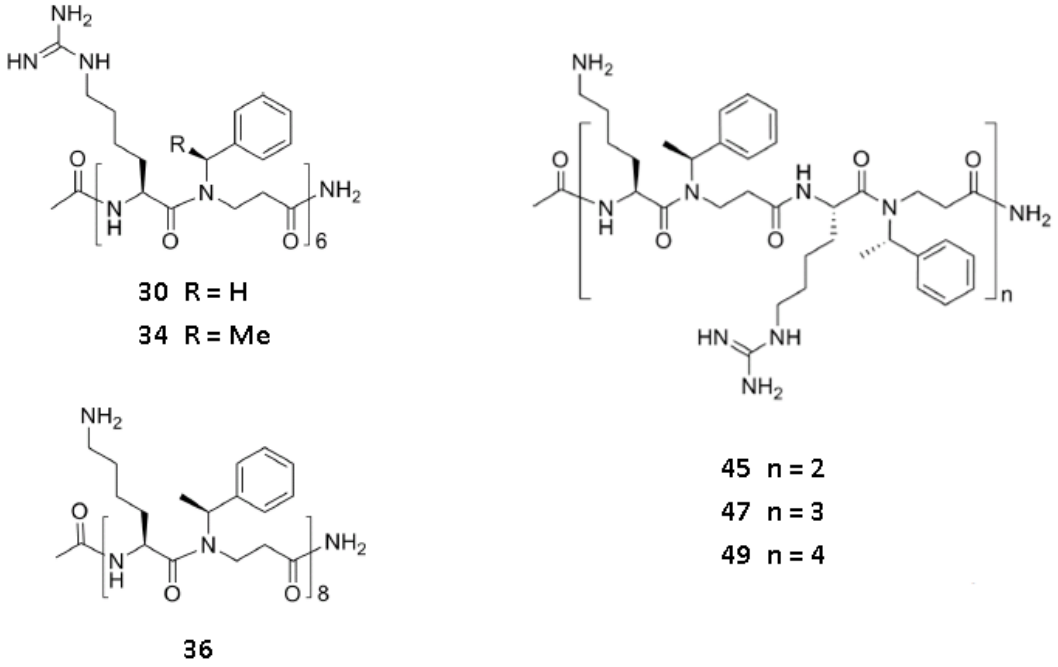

Figure 2.8. Chemical structures of the six α-peptide/β-peptoid chimeras that have been the primary focus of this thesis. The structures of compounds 30 and 34 include a homoarginine residue, whereas compound 36 have lysine as the α-amino acid. Chimeras 34 and 36, as well as 45-49, contain a chiral β-peptoid, whereas the β-peptoid in 30 is non-chiral. Additionally, length varies in chimeras 45-49. Modified from Paper I.

Also, it is possible modify length by increasing the number of subunits (chimeras 45-49) though the maximally feasible limit to this is around 16 residues. Lastly, chimeras with a palmitoyl end group and fluorescent-coupled oligomers has been synthesized. The peptidomimetic library now comprises close to 100 α-peptide/β-peptoid chimeras, and hence allows for ample investigation of the effect structural modifications may have on activity.

2.4. Conclusions from chapter 2

Antimicrobial peptides (AMPs) are defined as peptides less than 50 residues in length with a net positive charge and a substantial portion of hydrophobic residues. Natural AMPs are a diverse group of compounds and due to large variations in amino acid sequence they are often classified based on their secondary structures i.e. α-helical, β-sheet, extended or loop. Several novel antibacterial structures have been developed by optimizing natural AMPs or by using the properties of natural AMPs as templates i.e. peptidomimetics. The latter is prepared using solid-phase synthesis and the level of secondary structure elucidated using circular dichroism. The present chimeras contain cationic α-amino acids and hydrophobic β-peptoids, and small modifications in the type and/or number of residues has resulted in the development of a peptidomimetic library.

3. Activity and mechanism of action of antimicrobial peptides

The immense diversity of natural antimicrobial peptides (AMPs) and peptidomimetics have led to a vast number of structure-activity studies in an effort to elucidate their mechanisms of action (MoA). Though a few comprehensive studies have revealed the exact activity of selected AMPs, the complexity of the AMP-bacteria interaction means that it can be difficult to elucidate the detailed mechanisms behind the activity of natural AMPs or mimetics thereof (Hale and Hancock, 2007). In this chapter I will first define some commonly used terms and concepts for determining AMP activity before I review the MoA of natural AMPs and peptidomimetics. Particular focus will be on the structural determinants of peptidomimetic activity and how this relates to their interaction with the bacterial membrane. Finally, I will describe the parameters essential for high selectivity towards prokaryotic membranes whereby damage of eukaryotic cells is prevented.

3.1. Spectrum of activity of antimicrobial peptides and peptidomimetics

Most AMPs have a wide spectrum of antimicrobial activity. Hence, though studies often focus on their antibacterial properties, some are also effective against pathogenic fungi (Fehlbaum et al., 1996; Ryge et al., 2008) or possess anti-viral activities (Hancock and Rozek, 2002). Additionally, natural AMPs also have immune-modulatory actions and can thus enhance the host defence against pathogens as reviewed by others (Brown and Hancock, 2006). Therefore, synthetic AMPs though designed for optimal antimicrobial activity may also possess such activities (Easton et al., 2009). The immune-modulatory properties of AMPs will not be covered in this thesis, however, it is interesting that the immune-modulatory and antibacterial activity of natural AMPs is associated with different structural moieties of the parent molecule (Braff et al., 2005; Jung et al., 2011).

Determination of the Minimum Inhibitory Concentration (MIC) is considered the “golden standard” for assessing the activity of an antimicrobial drug (Andrews, 2001). The MIC is defined as the lowest concentration of a given compound that inhibits visible growth of bacteria after overnight incubation. In addition, determination of the Minimum Bactericidal Concentration (MBC), the concentration that kills an arbitrary 99.9 % of bacteria, is a useful measure (Pearson et al., 1980). It follows from this that if the MIC=MBC then a compound has a bactericidal effect, whereas if MBC>MIC then a compound is bacteriostatic i.e. at MIC it only inhibits. Since AMPs predominantly target the bacterial plasma membrane, the majority of them are bactericidal. The MIC value of natural AMPs is typically in the range of 1-10 μg/mL, which is somewhat lower than the very high *in vitro* activities

seen for the smaller conventional antibiotics (Hancock, 1997b; Maloy and Kari, 1995; Piers et al., 1994). Due to large differences in the size of the antimicrobial molecule (Chapter 2) it is better to express activity in µmol/L units instead µg/mL (Paper I), but unfortunately this is neglected in most structure-activity studies. Hence, when the activity of AMPs is expressed in molar terms it becomes closer to that of conventional antibiotics (Hancock and Patrzykat, 2002).

AMPs have been described as "dirty drugs" that disturb many bacterial functions with low potency rather than having a single high-affinity target (Peschel and Sahl, 2006), which is especially apparent at very low concentrations (section 3.5). However, in general the mechanisms behind the activity of AMPs can be divided into two functional classes; membrane-active and non-membrane-active (Hale and Hancock, 2007). Several models have been proposed for explaining the AMP MoA (section 3.3.1), and summarized in the Shai-Matsuzaki-Huang model (Zasloff, 2002) named after the authors of three different models (Figure 3.1).

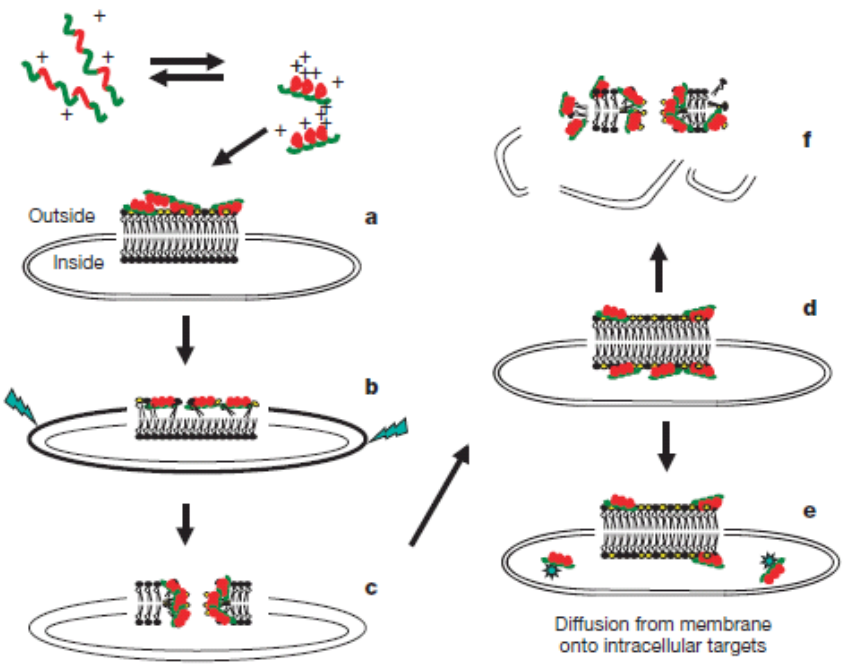

Figure 3.1. The Shai-Matsuzaki-Huang model of AMP activity. The model proposes the interaction of AMP with the membrane, followed by displacement of lipids, alterations of membrane structure, and in some cases internalisation. This model is based on individual models that propose non-receptor mediated AMP-lipid interactions (for details of these models see section 3.1.1). From (Zasloff, 2002).

Some AMPs are active against both Gram-positive and –negative bacteria, as we saw with our peptidomimetics (Paper I), or only active against one group (Fehlbaum et al., 1996; Giangaspero et al., 2001). There are some controversies as to which bacterial group the AMPs are most effective

against (Epand et al., 2010; Hancock and Rozek, 2002), however it appears that the more hydrophil (polar) an AMP is the more ineffective it will be against Gram-negative bacteria (Epand et al., 2010). The AMP MoA is generally similar in Gram-positive and –negative bacteria, but as explained in the subsequent sections the precise interaction will vary due to differences in the outer cell layers (teichoic acids vs. LPS). Indeed, gram-selectivity appears to be primarily influenced by the initial interaction between the AMP and the outer cell surface, which has been extensively studied for peptidomimetics (Dathe et al., 1997; Giangaspero et al., 2001).

The design of peptidomimetics is based on the optimization of the structural properties (i.e. hydrophobicity, charge, secondary structure etc.) of natural AMPs that favour interaction with bacterial cell membranes. Hence, since they are designed for optimal membrane interaction, the cell membrane is their primary target (Godballe et al., 2011; Palermo and Kuroda, 2010), though they may also display other activities at lower concentrations (Hong et al., 2003). Semi-synthetic AMPs i.e. optimized variants of natural AMPs (Chapter 2), will predominantly exert the same mechanism as its parent peptide with most of the studied cases being membrane-active (Dathe et al., 2001; Kasetty et al., 2011; Maloy and Kari, 1995). One interesting exception from this is a series of protegrin peptidomimetics, which has a different target than the membrane-active parent peptide (Srinivas et al., 2010). I will use the phrases “natural AMPs and peptidomimetics” when discerning the differences in their mechanism of action, but otherwise continue to use the term “AMP” to cover both natural and synthetic AMPs.

3.2. Initial peptide interaction with the bacterial cell surface

Irrespective of which of the two functional classes a given compound belongs to, the initial interaction of a AMPs with a bacterial cell happens through similar mechanisms (Hale and Hancock, 2007). The cationic AMPs are first attracted to the negative charge of the bacterial cell surface, which in Gram-negative bacteria is due to the lipopolysaccharide (LPS) layer and in Gram-positive the presence of teichoic acids and carboxyl groups in the multilayered peptidoglycan that they have to traverse (Tossi et al., 2000). Some AMPs effectively bind and neutralize LPS (endotoxin) (Chapple et al., 1998; Junkes et al., 2011; Piers et al., 1994; Wu and Hancock, 1999) or teichoic acids (Scott et al., 1999a), potentially making these compound useful to prevent bacterial sepsis and inflammation caused by both Gram-positive and –negative bacteria (Brandenburg et al., 2011).

The process by which AMPs gain entry to the plasma membrane has been more extensively studied for Gram-negative bacteria due to the focus on the barrier properties of the outer membrane (Powers and Hancock, 2003). The outer membrane of Gram-negative bacteria is asymmetric with the inner leaflet composed of phospholipids and the outer leaflet composed of the polyanionic glycolipid LPS (Hancock, 1997c). The negative charges of LPS are due to phosphates and acidic sugars, which are bridged by divalent cations that partially neutralize the negative charge and stabilize the outer membrane (McPhee et al., 2005). After being attracted to Gram-negative bacteria, AMPs are subsequently taken up by the self-promoted uptake pathway (Hancock, 1997b). The initial association with the bacterial membrane occurs through electrostatic interaction between the AMP and the anionic LPS in the outer membrane (Powers and Hancock, 2003). Because cationic AMPs have affinities for LPS that are at least three orders of magnitude higher than those for the native cations Ca^{2+} and Mg^{2+}, they competitively replace these ions and, being bulky, disrupt the normal barrier property of the outer membrane (Hancock and Chapple, 1999). Hence, the lower activity of AMP often seen in high salt containing media or in the presence of divalent cations is a direct consequence of enhanced competition for LPS binding sites (Friedrich et al., 1999; Sanchez-Gomez et al., 2008). AMP binding to LPS means that the affected membrane area becomes destabilized and develops transient cracks, which permits passage of a variety of small hydrophobic compounds and small proteins including the AMPs itself hence the term self-promoted uptake (Hancock, 1997b) (Figure 3.2).

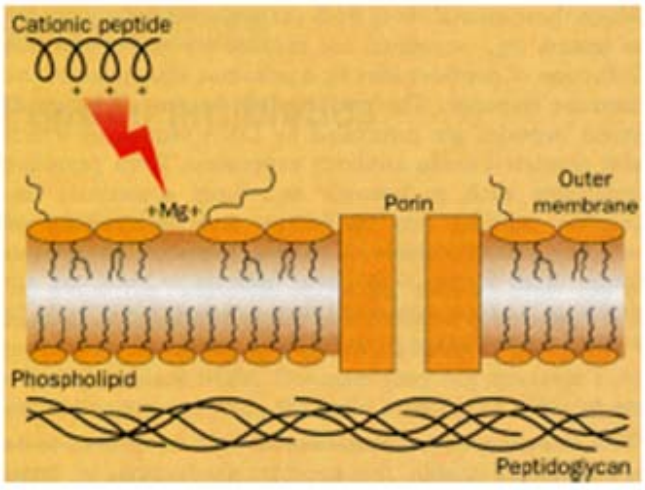

Figure 3.2. Self-promoted uptake of AMPs across the outer membrane of Gram-negative bacteria. Antibiotic entry is mediated through the water-filled channels formed by porins. From (Hancock, 1997b).

The ability of AMPs to disrupt the barrier function of the outer membrane, also explain their ability to increase the activity (i.e. entry) of conventional antibiotics (Cassone and Otvos, Jr., 2010). It has been proposed that some AMPs are Gram-positive selective because they are unable to assess or

utilize the self-promoted uptake pathway needed for Gram-negative activity (Hancock, 1997b). However, some AMPs with lower affinities for LPS binding are still effective permeabilizers and hence these may permeabilize by a distinguishable method involving neutralization of a patch of the outer membrane, thereby creating the cracks needed for entry (Hancock and Chapple, 1999). In line with this, it was intriguingly proposed that bacterial aggregation of Gram-negative bacteria following treatment with the non-membrane active insect AMP thanatin, was due to reduced LPS surface charge following AMP binding which thus reduced the electrostatic repulsion between bacteria allowing aggregation (Fehlbaum et al., 1996).

Binding to LPS is required for Gram-negative activity, but disruption of this is not believed to be the direct cause of lethality since the cells can remain viable even after the outer membrane is removed (i.e. as in the case of spheroblasts) (McPhee et al., 2005; Scott et al., 1999b). Interestingly, transmission electron microscopy revealed visible detachment of the outer membrane from the inner cytoplasmic membrane following treatment of *Escherichia coli* with lactoferrin (Chapple et al., 1998). Very recently, an outer membrane protein Lpp of the Enterobactericeae family was identified as an alternative binding site for AMPs leading to internalization of the Lpp-AMP complex and hence suggesting that LPS binding may not be the universal first step in AMP MoA (Chang et al., 2012).

The importance of the outer membrane in mediating AMP activity is made obvious in studies using bacterial strains that are outer-membrane deficient or LPS mutant strains that lack different LPS moieties (Junkes et al., 2011). In fact, removal of even single moieties of LPS resulted in a drastic reduction in the activity of cyclic peptidomimetics, suggesting that LPS interaction is a prerequisite for the self-promoted uptake pathway (Junkes et al., 2011). The outer membrane permeability can be assessed using a hydrophobic probe that is excluded by an intact outer membrane, but fluoresces strongly when it enters the membrane due to AMP disintegration of the outer membrane (Wu and Hancock, 1999). In contrast, the activity of AMPs can be increased by the addition of EDTA (a strong permeabilizer of the outer membrane due to cation chelation) (Epand et al., 2010; Kondejewski et al., 1996), which was also the case for our peptidomimetics (Figure 3.3.).

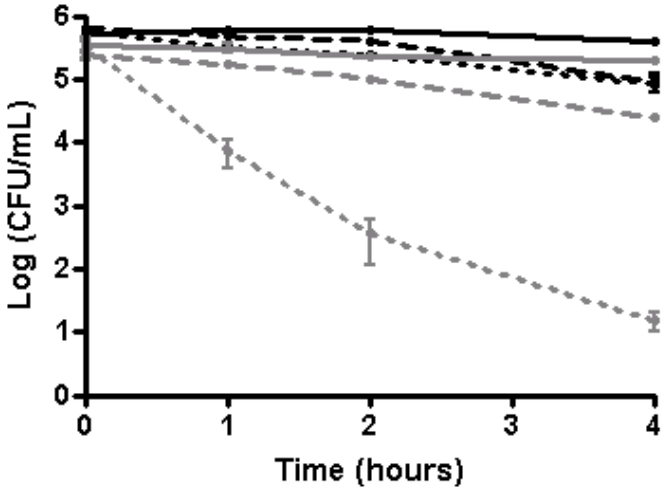

Figure 3.3. Effect of EDTA pre-treatment on the killing activity of one of the α-peptide/β-peptoid chimeras. *Staphylococcus aureus* (black) and *E.coli* (grey) treated with EDTA (solid), chimera (punctuated) or EDTA+ chimera (dotted). Unpublished results.

Disruption of the outer membrane will increase the amount of AMP/peptidomimetic that reaches the plasma membrane. The cell wall of Gram-positive bacteria will not be disrupted by EDTA treatment, and the activity against this group will therefore unaffected by this (Figure 3.3).

The thick cell wall of Gram-positive bacteria is composed of peptidoglycan embedded with polymers of alternating phosphate and alditol groups called teichoic acids (Peschel and Collins, 2001), that AMPs have to traverse before they can interact with the plasma membrane (Brogden, 2005). No model has been proposed for how AMPs traverse this layer, only that after attraction to the Gram-positive bacterium, the resulting interionic forces simply facilitate AMP accumulation in the cell wall (Peschel and Collins, 2001). In contrast to the requirement for LPS binding in utilizing the self-promoted uptake pathway need for Gram-negative activity, binding to teichoic acid does not correlate with the ability of AMPs to kill bacteria, indicating that AMPs use this mechanism to contact other targets (Brogden, 2005; Scott et al., 1999a). However, lantibiotics such as nisin use lipid II, the fatty acid anchor for the growing peptidoglycan chain, as a docking molecule from which it can diffuse into the cell membrane (Brotz et al., 1998; Sahl et al., 2005). The self-promoted uptake pathway of Gram-negatives allow quicker access to the cell membrane and hence activity is often higher and killing kinetics more rapid than for Gram-positive bacteria which rely on diffusion after initial interaction (Giangaspero et al., 2001; Hancock and Rozek, 2002).

3.3. Mechanism of action of membrane-active antimicrobial peptides

When reaching the plasma membrane the mechanisms of membrane-active and non-membrane AMPs diverge (Powers and Hancock, 2003). Though both functional groups will have to interact with the plasma membrane to exert their activity only the membrane interaction of membrane-active

AMPs leads to loss of bacterial cell viability. From the sequence alone, it can be difficult to predict the activity and MoA of a natural AMP (Hancock and Chapple, 1999). The variation in charge and hydrophobicity of peptidomimetics makes it possible to predict the interaction with the membrane by slightly modifying these parameters (Palermo and Kuroda, 2010). Because of the complexity of AMP-membrane interaction, several theoretic models have been proposed, some more controversial and others easily accepted, and since these are the foundation of most studies of membrane-active AMPs, these will be reviewed first.

3.3.1. Models for membrane interaction

At the cytoplasmic membrane, the cationic AMPs interact with the negatively charged groups of the external leaflet of phospholipids. It is generally accepted that an amphipathic conformation of the AMP is required for insertion into the membrane, where the hydrophilic face interacts with the phospholipid head groups whereas the hydrophobic face is inserted into the bilayer core (Jenssen et al., 2006; Rotem and Mor, 2009). Various models have been proposed for explaining the mechanisms behind the resulting damage to the membrane architecture (Figure 3.4).

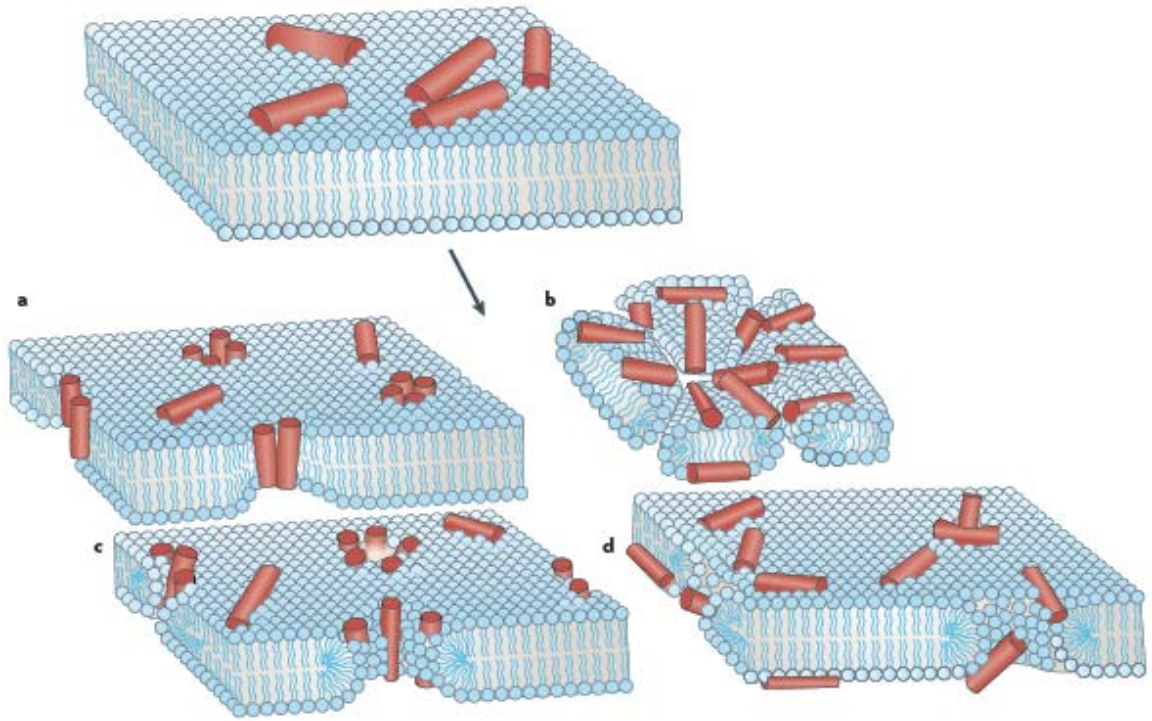

Figure 3.4. Models for AMP-membrane interaction: a) barrel-stave, b) carpet, c) toroidal pore and d) aggregate. Modified from (Melo et al., 2009).

The first model that was proposed was the barrel-stave model (Figure 3.4a). It has been used to interpret the voltage dependent ion-permeable channels in planar lipid bilayer membranes that are extremely heterogeneous in size and lifetime (Kordel et al., 1988). In this model the peptides forms the staves of the barrel-like pore in which the number of peptides in the pore will determine the size (and conductance) of the channel (Wu et al., 1999). This is followed by the progressive recruitment of additional monomers to increase pore size and stability (Hancock and Chapple, 1999). Following the attraction to the plasma membrane, the AMPs will, at a critical peptide:lipid ratio, reorient themselves perpendicular to the lipid bilayer, which they are believed to span (Hale and Hancock, 2007). The hydrophobic side chains will then face the hydrophobic membrane core, whereas the hydrophilic side chains face towards the hydrophilic core that span the width of the membrane. The critique of this model points to fact that pores would be too irregular in size and duration and that AMPs too short to be able to cover the width of the membrane are still able to form pores (Wu et al., 1999).

The detergent-like properties of some AMPs have been explained using the carpet model (Figure 3.4b) (Shai, 1995). In this model peptides saturate the surface of the cytoplasmic membrane before causing a complete detergent-like disruption of the membrane (Hancock and Chapple, 1999). A distinct feature of this model is that no pores are formed but rather that the membrane integrity is completely disrupted. It has been disputed due to the fact that though certain peptides completely depolarize the membrane at their MIC, they also cause partial collapse of their membrane potential at concentrations below the MIC (Wu et al., 1999). This contradicts the carpet model which suggests that a certain threshold concentration is needed before the membrane is destroyed (Brogden, 2005). Additionally, though membrane dissolution will happen for most AMPs at very high concentration it there is limited evidence that this will happen at the minimal effective concentration (Jenssen et al., 2006).

In the toroidal pore model (Figure 3.4c) insertion of AMPs into the membrane is envisioned to induce lipids to bend around peptide aggregates until a continuous channel between the outer and inner leaflet is formed lined by both peptide and lipid head groups (Ludtke et al., 1996). This model differ from the barrel-stave model in that the peptide remains associated with the lipid head groups (Hale and Hancock, 2007). The aggregate model (Figure 3.4d) (Matsuzaki et al., 1996) have some reassemblies with toroidal pore model (Jenssen et al., 2006). This model is used for explaining how cationic AMPs can kill through both membrane permeabilization and internal target attack (Hale and

Hancock, 2007). In this model local aggregation of varied number of peptide molecules within the membrane provides a transient passage for irons and internalisation of AMPs (Wu et al., 1999).

It is important to recognize that all of these models may be valid under different conditions and for different structural groups of AMPs (Jenssen et al., 2006). Indeed, the existence of all of these proposed models have been demonstrated (Table 3.1)

Table 3.1. Proposed models for the membrane activity of selected natural AMPs and peptidomimetics

Mechanism of membrane action	Compound	Reference
Barrel-stave	Almethicin	(Bechinger, 1999)
Carpet	Melittin diastereomers	(Oren and Shai, 1997)
	α-helical peptides	(Giangaspero et al., 2001)
	Peptidomimetic	(Yu et al., 2009)
Toroidal	Peptidomimetic	(Azad et al., 2011)
	Magainin	(Ludtke et al., 1996)
	LL-37	(Lee et al., 2011)
	Protegrin	(Yamaguchi et al., 2002)
	Melittin	(Yang et al., 2001)
Aggregate	Indolcidin	(Wu et al., 1999)
	Mastoparan X	(Matsuzaki et al., 1996)

Naturally, all of the above models require some kind of threshold concentration before membrane activity can be measured (Melo et al., 2009) for example in leakage or membrane depolarisation studies (section 3.3.4). Hence, it is proposed that AMPs have two distinct physical states of binding to lipid bilayers (Huang, 2000; Yang et al., 2001); at low peptide:lipid ratios the AMP adsorb in the lipid head group region in a functionally inactive state. When a threshold value is reached the AMPs forms a pore that is lethal to the cell. The threshold value is determined by both the lipid composition of the membrane, which varies between bacterial species, and the secondary structure of the AMP (Huang, 2000). Such variations in the interaction between membranes of different lipid compositions and different structural groups of AMP may explain the validity of the various models.

3.3.2. Mechanisms of membrane-active natural antimicrobial peptides

The interaction between natural peptides and the bacterial membrane has been investigated in several studies. The least common model is the barrel-stave, which is considered unique for alamethicin (a fungal peptide with a large proportion of hydrophobic residues) (Bechinger, 1999). When in contact

with lipid membranes it adopts an α-helical configuration, where after the hydrophobic regions align with the lipid core region of the bilayer and the hydrophilic regions form the interior of the pore (Brogden, 2005). Analysis of multi-channel kinetics using patch-clamp techniques have revealed rapid and pronounced conductance fluctuations (Bechinger, 1999) as also proposed in the model.

The majority of membrane-active AMPs that are cationic and water-soluble form toroidal pores (Lee et al., 2011). These pores have a pore radius in the range of 15-25 Å (3-5 nm), and are thus substantially larger than barrel-stave pore size of 9 Å (1.8 nm) (Yang et al., 2001). Representative AMPs of this group include magainin (Ludtke et al., 1996), protegrin (Yamaguchi et al., 2002; Yang et al., 2001), melittin (Yang et al., 2001) and LL-37 (Lee et al., 2011), and hence encompass several different structural groups (see Chapter 2).

The carpet model has previously been proposed to explain the MoA of α-helical peptides (which include LL-37) (Giangaspero et al., 2001; Shai, 1999). The ability of an AMP to transform into a helical conformation is one of the most controversial topics in peptidomimetic design (Section 3.3.3). It is speculated that helical peptides due to their secondary structure have the optimal separation of cationic and hydrophobic residues that would then mediate interaction with lipid head groups and the hydrophobic core, respectively (Agawa et al., 1991). Consequently, this would speak in favour of the toroidal model as opposed to the carpet model.

The aggregate model is a variation of the toroidal pore model and explains how AMPs may interact with the membrane without leading to loss of viability. Thus it is possible that the ability of AMPs to act on lipid membranes reflect their mechanism of passage across the membrane and that their actual targets are in the cytoplasm (Wu et al., 1999). For instance, pseudin dissipate the membrane potential and cause leakage from the cell, but also bind to RNA and inhibit protein synthesis (Park et al., 2011). This is reflected by a slower killing kinetics compared to other membrane-active peptides, and hence membrane activity could by a secondary MoA or only work in conjunction with other cellular activities (Park et al., 2011). The mechanism of defensins is similarly not well defined (Brogden, 2005). Early on they were believed to be membrane active by an unknown mechanism (Lehrer et al., 1989; Shimoda et al., 1995; Yeaman et al., 1998), but this has been disputed since they are also able to interact with intracellular targets (Ganz, 2003) (section 3.4). Hence, membrane interaction seen in early studies could only be a consequence of the molecule having to traverse the membrane to reach an intracellular target.

3.3.3. Structural determinants of peptidomimetic activity

The recent advances in design strategies and synthesis protocols have facilitated the structural optimization of natural peptides and the generation of novel scaffolds of peptidomimetics.
The synthesis and diversity of peptidomimetics was reviewed in Chapter 2. Here this will be put in context by reviewing the importance of structure-activity relationship studies in fine-tuning their physiochemical attributes for improved antibacterial properties (Rotem and Mor, 2009).

Studies of the activity of peptidomimetics have demonstrated that membrane activity of a given compound depends on its overall physiochemical properties rather than the precise details of its structure (Tossi et al., 2000). However, there a two common functional requirements; a net charge cationicity that facilitates attraction with the negatively charged surface, and the ability to assume amphipathic structures that permit integration into the bacterial cell membrane (Giangaspero et al., 2001). Additionally, since these compounds primarily are developed for therapeutic use, it is important to assure membrane selectivity. The selectivity of peptidomimetics for lysis of bacterial cells rather than mammalian cells depends on the appropriate balance between hydrophobicity and the distribution of charged side chains in the peptide; if the compounds are too polar hey have little affinity for the bacterial membranes, and if they are too hydrophobic they fail to discriminate between bacterial and mammalian membranes (Liu et al., 2004) (section 3.6).

Charge of natural AMPs vary widely, from 0 to +16 though most active peptides fall into the intermediate range (Tossi et al., 2000). For peptidomimetics charge may be modified by the inclusion of basic amino acid such as lysine or arginine, which are protonated at physiological pH generating a net cationic charge (Palermo and Kuroda, 2010). Several studies have shown a clear correlation between charge and potency (Al-Badri et al., 2008; Dathe et al., 2001; Jiang et al., 2008; Taira et al., 2010). However, it is only within a certain range that increased cationicity is associated with increased antimicrobial activity; in a study of magainin analogues a net charge above +5 reduced antibacterial activities (Dathe et al., 2001). This decrease may result in part from excessive strong peptide interaction with phospholipid head groups, thereby preventing translocation (Yeaman and Yount, 2003). Similarly, other studies did not find an increase in antimicrobial activity if charge was increased above moderate levels (Al-Badri et al., 2008; Chongsiriwatana et al., 2008; da Silva et al., 2008; Friedrich et al., 1999; Scott et al., 1999b).

The percentage of hydrophobic residues in natural AMPs varies but is generally around 40-60 % consistent with the requirement for an amphiphilic structure (Tossi et al., 2000). Hydrophobicity

governs the extent to which a peptidomimetic can partition into the lipid bilayer. Since increased hydrophobicity is associated with loss of membrane specificity, it is kept relatively constant (Yeaman and Yount, 2003), and has primarily been studied as part of the ability of compounds to adopt an amphipathic secondary structure (Dathe et al., 1997; Friedrich et al., 1999; Taira et al., 2010). The levels of charge and hydrophobicity is not necessarily inversely proportional as some peptidomimetics also contain high numbers of noncharged polar residues (Tossi et al., 2000). Studies of both charge and hydrophobicity highlight the importance of maintaining a balance between the hydrophobic and cationic components (Al-Badri et al., 2008; Porter et al., 2002). The range of MIC in a series of oligoacyllysines (OAKs) showed that the level of hydrophobicity and charge should be in a relatively narrow window to retain full activity (Radzishevsky et al., 2008) (Figure 3.5).

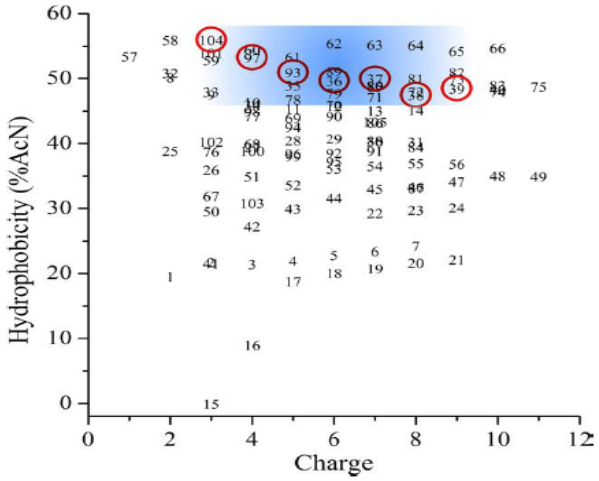

Figure 3.5. Relationship between HQ (hydrophobicity/charge) properties and antibacterial activity against *E. coli* of 103 short oligoacyllysines (OAK) leading to the visualisation of an optimal HQ window (blue). Potent OAKs (MIC≤ 3μM) are highlighted (red circles). From (Rotem and Mor, 2009) modified from (Radzishevsky et al., 2008).

It has been suggested that the chemical structure of cationic amino acids, and not simply their positive charge, is an important activity determinant. Arginine contains a guanidinium group, which has a strong affinity for phospholipids thereby facilitating membrane interaction (Palermo and Kuroda, 2010), and hence guanidinylation may enhance antimicrobial activity (Choi et al., 2009). No changes in antimicrobial activity were seen by globally substituting several amino acids as long as these were of similar polarity and did not change secondary structure (Chen et al., 2005b; Epand et al., 2010; Javadpour et al., 1996; Taira et al., 2010). Single amino acid substitutions at central positions in the peptide chain may however have an impact on antibacterial activity likely because it hinders proper folding into the secondary structure (Friedrich et al., 1999; Kasetty et al., 2011; Lim et al., 2005; Perez-Paya et al., 1995). Additionally the attachment of fatty acids to the peptide chain has been shown to increase antibacterial activity (Majerle et al., 2003). We saw no increase in antimicrobial activity when chimera 34 or 47 was modified with palmitoyl (unpublished results).

According to the models of pore formation described above, the peptide has to span the membrane to exert its membrane permeabilizing activities. For α-helical peptides it has been predicted that a length of 20 amino acid residues is required to span the lipid bilayer (Agawa et al., 1991). Length correlates extensively with antimicrobial activity (Agawa et al., 1991; Blondelle and Houghten, 1992; Javadpour et al., 1996) until activity stagnates or even decreases at a length of around 20 amino acid residues (Blondelle and Houghten, 1992; Deslouches et al., 2005b). Some suggestions have been given to the mechanisms of membrane interaction for shorter peptides (Agawa et al., 1991). Hence, one intriguing study indicated different membrane interactions for an OAK hexamer (charge of + 6) and an octamer (charge of + 8); due to differences in charge only the latter caused membrane disruption whereas the other translocated into the cytoplasm (Rotem et al., 2008).

In our peptidomimetics library hydrophobicity is constant at 50 % due to the alternating design of natural cationic amino acids (lysine/homoarginine) and the hydrophobic β-peptoids (see Chapter 2). We found no influence of type of amino acid (chimera 34 vs. 36), but increased chain length (chimera 45-49) had a pronounced effect on a wide spectrum of bacterial species (Table 3.2).

Table 3.2. Minimum Inhibitory Concentration (μM) of six selected chimeras from the present library (Paper I).

	Chimera 30	Chimera 34	Chimera 36	Chimera 45	Chimera 47	Chimera 49
S. aureus 8325	5.9	2.8	18.7	141.2	23.8	4.5
K. pneumoniae ATCC 13883	1.5	2.8	37.5	282.4	23.8	9.0
S. marcescens ATCC 8100	46.8	45.5	150.0	> 282.4	190.3	71.8
E. coli ATCC 25922	1.5	2.8	9.4	141.2	3.0	2.2
E. coli MG1655	1.5	2.8	4.7	141.2	5.9	2.2
E. coli AAS-EC-009	1.5	2.8	9.4	141.2	11.9	4.5
E.coli AAS-EC-010	1.5	1.4	9.4	141.2	3.0	2.2
L. monocytogenes 4446	2.9	1.4	1.1	70.6	3.0	1.1
L. monocytogenes N53-1	2.9	2.8	1.1	70.6	5.9	1.1
L. monocytogenes EGD	1.5	2.8	1.1	70.6	3.0	1.1
V. vulnificus ATCC^T	1.5	1.4	2.3	35.3	3.0	2.2
V. parahaemolyticus ATCC^T	1.5	1.4	2.3	70.6	3.0	1.1

A higher degree of secondary structure had been found previously for analogues with chiral β-peptoid side chains (as in 34 and 36) as compared chimera with achiral β-peptoid residues (as in 30) (Olsen et al., 2007), but as seen in table 3.2 this had no influence on antimicrobial activity.

The ability of a peptidomimetic to adopt a secondary structure has been extensively debated. Traditionally, secondary structure was considered necessary in order to obtain an amphipathic residue arrangement of the molecule (Mondal et al., 2010). Especially, the view that a helical structure is required for antimicrobial activity has been favoured (Chapple et al., 1998; Chen et al., 2005b; Porter et al., 2002) and the structure of the helix was determined to be particularly important for activity against Gram-positive bacteria (Dathe et al., 1997; Giangaspero et al., 2001). Additionally, a significant positional conservation in terms of type of residues types has been found for a range of natural AMPs, where hydrophobic residues dominate one face of the helix and polar residues the other for optimal membrane interaction (Tossi et al., 2000). The hypothesis that peptides need a globally amphiphilic helical conformation has now been abandoned by several groups (Ilker et al., 2004; Oren and Shai, 1997; Schmitt et al., 2004) In fact, recent studies have shown that neither global amphiphilicity nor regular secondary structure is required for efficient interaction with the membrane (Mondal et al., 2010; Radzishevsky et al., 2007), and now also suggested by us (Paper I).

3.3.4. Methods for determining activity against model membranes and viable bacteria

Virtually all cationic AMPs cause severe membrane disruption if high enough concentrations are administered (Hancock and Rozek, 2002; Zhang et al., 2001), which could give false indication of the MoA and masking any potential intracellular activity. Consequently if the primary MoA should be determined, experiments should not be performed above the MIC (Figure 3.6).

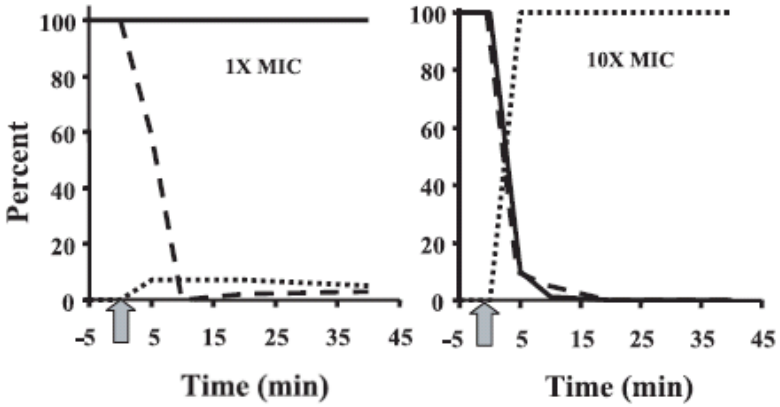

Figure 3.6. Influence of concentration on the MoA of a peptidomimetic against *E. coli*. Peptide was added at time zero (arrow) and cell viability (bold line), rate of RNA synthesis (dashed line) and membrane potential (dotted line) measured. RNA synthesis was inhibited at 1xMIC and the membrane disrupted at 10xMIC. From (Hancock and Rozek, 2002).

Model membrane has been widely used to determine cell membrane damage induced by peptides (Brogden, 2005). For this, single or mixed phospholipid suspensions are prepared as lipid monolayer, lipid bilayers or liposomes (Koo et al., 1997; Zhang et al., 2001) or as dye-containing vesicles (Mazzuca et al., 2010; Park et al., 2011) and these are then incubated with AMPs. Lipid mixtures try to mimic the lipid composition of specific bacteria since this vary pronouncedly; in Gram-negative

bacteria phosphatidylglycerol (PG) and phosphatidylethanolalamine (PE) dominate, whereas in Gram-positive bacteria PG and cardiolipin (CL) are the most important (Epand and Epand, 2010). Formation of pores or general membrane disruption can be experimentally demonstrated by measuring the extent of membrane depolarisation or the leakage of intracellular contents to the extracellular environment that is a consequence of membrane disruption. Monitoring voltage-dependent channels in membrane bilayers is useful for assessing the formation and not least the stability of the peptide-induced pore (Brogden, 2005). Several studies have measured the conductivity of an electrical current generated by the formed pore or used assays with membrane-potential sensitive dyes as an indicator for membrane-activity (Agawa et al., 1991; Koo et al., 1997; Kordel et al., 1988). Membrane depolarisation can however occur due to the activity of both membrane-active and non-membrane active AMPs and hence changes in the membrane potential is not the lethal event per se (Friedrich et al., 2000; Park et al., 2011; Wu and Hancock, 1999; Yeaman et al., 1998). Leakage studies make use of fluorescent dyes (Friedrich et al., 1999; Junkes et al., 2011; Liu et al., 2004) or measure the amount of leaked intracellular compounds (Johansen et al., 1997; Zaknoon et al., 2009), although these assays varies widely in sensitivity (O'Neill et al., 2004). Model membranes do not capture the heterogeneity of a real bacterial membrane, and hence it is better to use viable cells or protoplasts when possible (Hancock and Rozek, 2002). We measured ATP leakage from *E. coli* treated with chimera 49 at $1.68x10^7$ peptide molecules/bacterium, where the MIC value equals $5.4x10^9$ peptide molecules/bacterium (Paper I) (Figure 3.8).

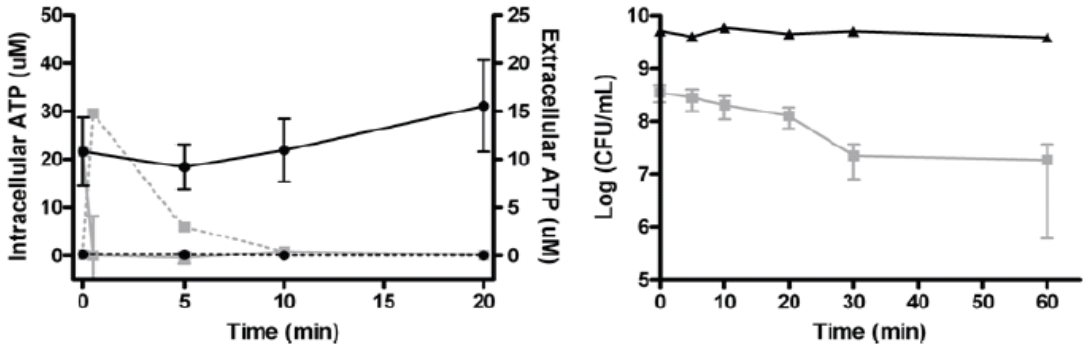

Figure 3.7. ATP leakage from *S. aureus* after the addition of 1000 µg/mL of chimera 49 (left) and the corresponding change in the number of viable bacterial cells (right). Left: Mean (SEM) intracellular (IC, solid line) and extracellular (EC, punctuated line) ATP concentration for cells treated with chimera 49 (grey) compared to MilliQ-treated control (black). Right: Mean (SEM) number of viable cells after addition of chimera 49 (grey line) compared to MilliQ-treated control (black line). From Paper I.

Additionally, Scanning Electron Microscopy (SEM) was performed on several species of chimera-treated bacteria to visualise the damage to the cell envelope (Figure 3.8) (unpublished).

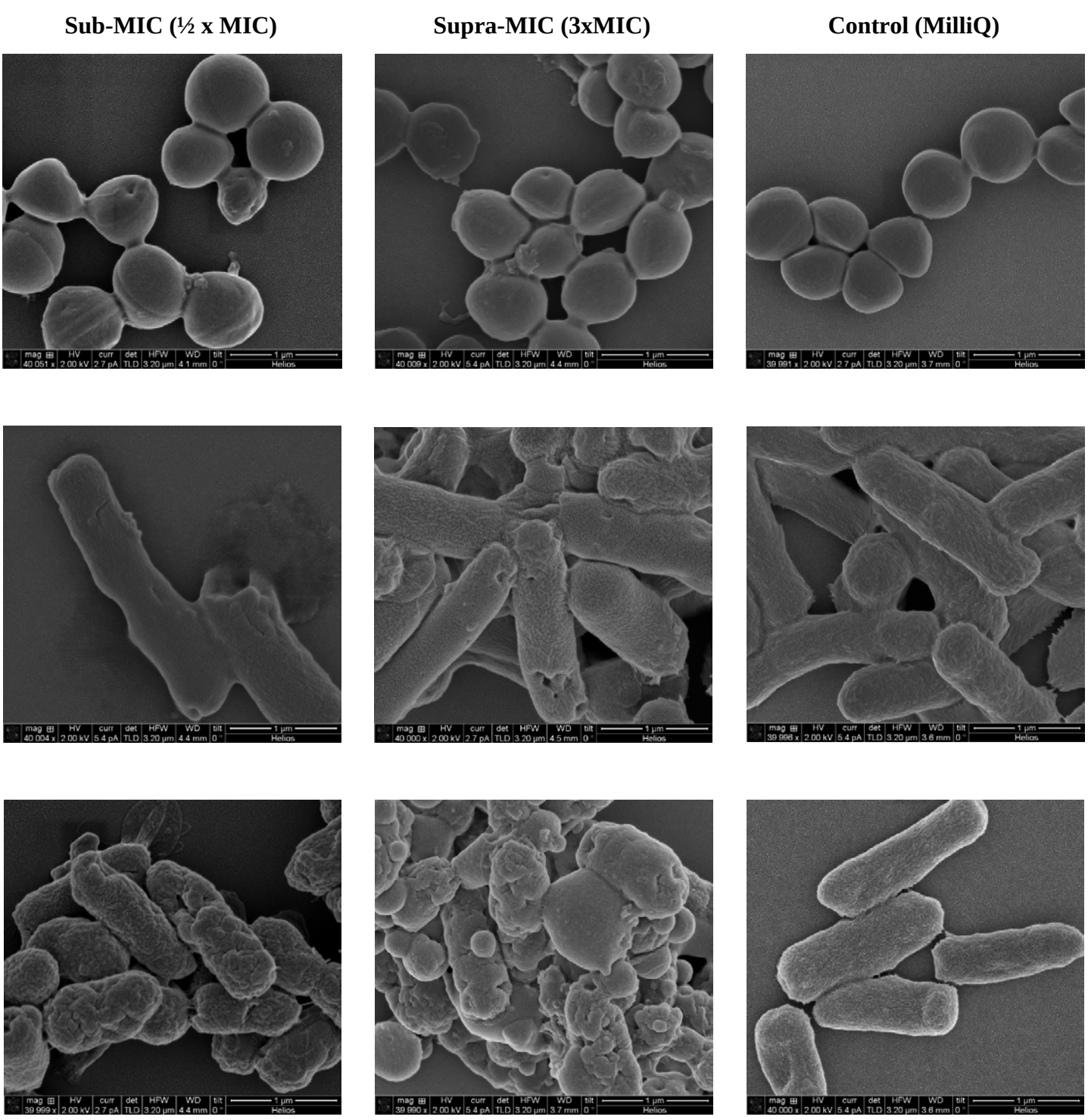

Figure 3.8. Scanning Electron Microscopy (SEM) of *Staphylococcus aureus* (top row), *Escherichia coli* (middle row) and *Serratia marcescens* (bottom row) treated with sub-inhibitory or supra-inhibitory concentrations of chimera 30. MIC was determined for an inoculum at 10^8 CFU/ml to correct for higher bacterial number needed for SEM imaging i.e. MIC levels at 128 µg/mL, 32 µg/mL and 256 µg/mL for the three species, respectively. Unpublished results.

Damage to the outer cell surface was especially evident for the two Gram-negative bacteria and in fact damage to both of them was evident also at sub-MIC concentrations. Expansion of the outer membrane leading to the formation of blebs due to peptide activity has been demonstrated earlier for *E. coli* (Hancock and Rozek, 2002). The present chimeras are bactericidal so MIC=MBC, and hence it is expected that at MIC levels the bacteria will die (i.e. ATP will leak from the cell and visible damage can be seen). However, the results from the ATP leakage assay (for several chimeras) and from SEM for chimera 30 suggest that lower levels also exert damage to at least a proportion of the bacterial population. None of our results have suggested that the MoA differ between the chimeras in the library. Additionally, cell aggregation was evident in SEM images of chimera 30-treated cells but was also seen using light microscopy of bacteria treated with the other chimeras, which would be expected for a membrane-disrupting peptidomimetic as demonstrated by others (Loit et al., 2010).

Electron microscopy is an excellent method for visualising the actions of AMPs, but it is important to use relevant concentration around MIC i.e. we re-determined MIC to correct for the higher inoculums needed for SEM (Figure 3.8). SEM or TEM (transmission electron microscopy) has been used in several studies for determining membrane activity (Azad et al., 2011; Hartmann et al., 2010; Sallum and Chen, 2008). For instance melittin diastereomers caused complete lysis of *E. coli* at MIC, whereas patches were observed below MIC (Oren and Shai, 1997). Additionally, confocal laser-scanning microscopy has been used to visualize the localization of peptide (Park et al., 2011; Sochacki et al., 2011) and solid-state NMR spectroscopy used to measure the orientation and penetration of AMPs into lipid bilayers or bacterial cells (Bechinger, 1999; Pius et al., 2012).

3.4. Non-membrane and intracellular targets of antimicrobial peptides

The majority of mechanistic studies of AMP activity have focused on the membrane disruption abilities of these compounds, but it is clear that some AMPs also possess non-membrane activities, which may either be their primary activity or work in conjunction with their activities against the membrane (Otvos, Jr., 2005). Hence, rather than disrupting the membranes, these peptides are able to traverse the membrane and subsequently bind to targets in the cytoplasma disrupting vital functions (Palermo and Kuroda, 2010).Translocation has been proposed to occur by a process related to the aggregate model (section 3.3.1) by transient disruption of the membrane without leading to permeabilization (Powers and Hancock, 2003).

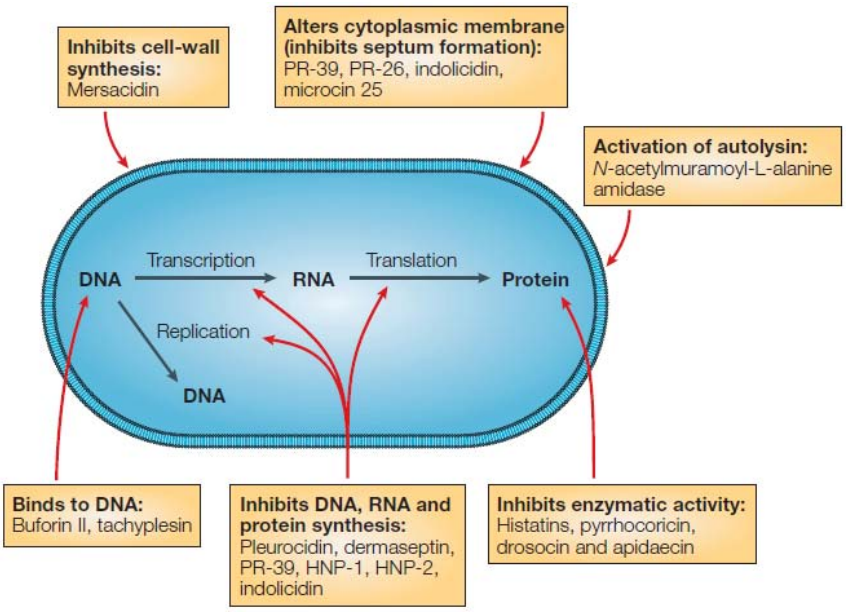

Figure 3.9. Mechanisms of action for intracellular antimicrobial peptide activity displayed here with *E. coli* as the target organism. From (Brogden, 2005).

The internalization of AMPs may be determined experimentally. It can be visualized by confocal laser microscopy as elegantly demonstrated for distinguishing the localisation of polymyxin B and the non-lytic proline rich $Bac7_{1-35}$ (Benincasa et al., 2009). In the same study a flow-cytometry method based on fluorescence quenching was described (Benincasa et al., 2009). Additionally, recently transposon libraries was used to unveil the intracellular target and route of cellular entry of antibiotics which possibly could be modified for elucidating Amp targets as well (Wang et al., 2011a).

Once in the cytoplasm, AMPs can interact with DNA, RNA and/or cellular proteins or inhibit the synthesis of these compounds. Consequently, loss of viability is often much slower than for membrane-active AMPs and may take several hours (Kragol et al., 2001). However, the toad AMP buforin II penetrates *E. coli* and binds to nucleic acids in the cytoplasma while still causing rapid cell death (Park et al., 1998). Interestingly, this AMP has a homology with DNA-binding histones, which are also being developed as therapeutic AMPs (Pavia et al., 2011), and which may thereby also explain its affinity for DNA. Another α-helical AMP, pseudin-2, isolated from the South American frog *Pseudis paradoxa*, inhibits protein synthesis by binding to RNA (Park et al., 2011). Additionally, a short OAK peptidomimetic displayed the ability to translocate across the membrane and inhibit DNA functions (Rotem et al., 2008). Though it was originally concluded that the MoA of bovine neutrophil AMP indolcidin was through membrane permeabilization (Hale and Hancock, 2007), this was since disputed (Wu et al., 1999) and an alternative MoA proposed, when it was

discovered that indolicidin induce filamentation of *E.coli* which is thought to result from DNA synthesis inhibition (Subbalakshmi and Sitaram, 1998). An AMP that targets DNA repair has also been characterised (Su et al., 2010). Another class of intracellular targets are the ones that inhibit enzymatic activity either by sequestrating ATP (Hilpert et al., 2010) or by inhibiting the ATPase action such as pyrrhocoricin, which inhibits the activity of the heat shock protein DnaK thereby preventing chaperone-assisted folding (Kragol et al., 2001).

In the case of lantibiotics, several antibacterial activities are combined for increased potencies against Gram-positive bacteria; in addition to the pore-forming abilities of these compounds they also interact with pyrophosphate-binding sites on the membrane-bound cell wall precursor lipid II leading to inhibition of peptidoglycan biosynthesis (Breukink and de, 2006) (Figure 3.10).

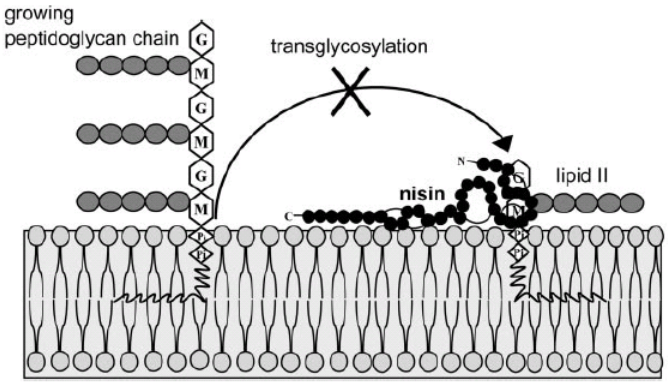

Figure 3.10. Lipid II-mediated activity of lantibiotics. Lipid II carries the completed subunit of the cell wall, which needs to be polymerised to the murein network. Binding of nisin to lipid II block the polymerisation reaction. From (Sahl et al., 2005).

Such a non-membrane external target leading to the inhibition of cell wall biosynthesis, is similar to that of the glycopeptide antibiotic vancomycin (Sahl et al., 2005) and has recently been demonstrated for other AMPs. The MoA of the fungal defensin, plectasin, was recently reported to also be related to lipid II binding, although in a different step in the biosynthesis pathway than lantibiotics or vancomycin (Schneider et al., 2010). Similarly, studies on a family of protegrin peptidomimetics showed that the MoA against *Pseudomonas* involved inhibition of LptD, an outer-membrane protein that functions in the assembly of LPS, and hence a bacteriostatic activity different from the lytic activity of most peptidomimetics(Srinivas et al., 2010). Recently, a human defensin was shown to inhibit cell wall biosynthesis in *Staphylococcus aureus* (Sass et al., 2010), though the MoA of this peptide has always been thought to be a result of membrane permeabilization. In line with this, seminalplasmin, an AMP from bovine seminal plasma, lyses *E. coli* by stimulating autolytic enzymes(Chitnis and Prasad, 1990). Cell wall lytic enzymes of *S. simulans* were also activated by the lantibiotic Pep 5 leading to weakened cell wall and cell lysis (Bierbaum and Sahl, 1987).

Lack of membrane activity has indicated that other natural AMPs and peptidomimetics may have an intracellular target (Fehlbaum et al., 1996; Friedrich et al., 2000; Junkes et al., 2011; Wu et al., 1999). The models for membrane activity predicts that AMP MoA is not receptor based which is why D-peptides are generally as activity as L-analogues (Maloy and Kari, 1995). Some studies where membrane activity was not found or was only minor additionally indicated the existence of a stereospecific target such as a receptor. Notably, it was demonstrated that both D- and L-enantiomers of thanatin are active against Gram-positive bacteria, only L-thanatin is activity against Gram-positive bacteria (Fehlbaum et al., 1996). Similarly, a non-pore forming mechanism involving stereospecificity was also proposed for apidaecin, a proline-rich insect AMP, since the D-enantiomer was completely devoid of antibacterial activity (Casteels and Tempst, 1994).

3.5. Effects of sub-lethal concentrations of antimicrobial peptides

The realization that several AMPs have intracellular targets and that some cationic compounds may even contain a delivery module that allows entry of foreign cargo into the bacterial cell (Foged et al., 2008) has lead to the realization that AMPs may also have more subtle intracellular effects (Otvos, Jr., 2005). At concentration well below the MIC value the membrane may be depolarised (Friedrich et al., 2000; Wu et al., 1999) and peptide-mediated flip-flop of phospholipid may also occur at concentrations much lower than those that for instance cause calcein released across the membrane (Hancock and Rozek, 2002; Zhang et al., 2001). Also, electron microscopy of bacterial cells treated with mellitin diastereomers indicated membrane structural changes well below the MIC (Oren and Shai, 1997). These results indicate that low amounts of AMP may translocate into the bacterial cell when these are exposed to sub-lethal concentrations without causing damage to the cytoplasmic membrane (Patrzykat et al., 2002).

Several bacteria respond to the presence of AMPs by activating two-component systems, which induces structural changes that confer increased tolerance to subsequent exposure of these compounds (section 4.2). Obviously this is induced at concentrations that do not kill the bacterial cell. Stress responses are often detected using reporter fusions (e.g. lacZ) and may help to characterize the mode of antibacterial action of new compounds (Bianchi and Baneyx, 1999; Davies et al., 2006). Stress-responses can also be examined using transcription analyses, which has shown that considerable change in the up- or down-regulation of genes may occur at low concentrations (Chiu et al., 2010; Hong et al., 2003; Kruse et al., 2009). The critical parameter in providing

meaningful expression profiles is the concentration of the inhibitor, since low concentrations are needed to provide a specific response (O'Neill and Chopra, 2004).

We saw considerable differences in the reduction of growth, when different bacteria where exposed to sub-lethal concentrations of chimera revealing that stress-responses (and thereby possible the MoA) may vary between different bacteria (Paper I). Transcription profiles from exposed bacteria may provide a better understanding of the MoA of the chimeras. A study of pleurocidin-derived AMPs showed that though these compounds damage the membrane at concentration at or above the MIC, at concentration below the MIC only macromolecular synthesis (DNA, RNA, proteins) was inhibited (Patrzykat et al., 2002).

3.6. Membrane selectivity and determination of toxicity

The application of AMPs as therapeutic antibacterial agents is often limited by unfavourable toxicity profiles towards human cells and can restrict their use to topical applications (Hancock and Chapple, 1999). Indeed, the MIC value of a potential new compound reflects its therapeutic potential, since moderate MIC values will necessitate the use of higher doses which could lead to unwanted activity towards host cells (Zasloff, 2002). Several antibiotics are toxic in high doses but are still usable due to their very low MIC values. Host toxicity is predominantly evaluated in terms of hemolytic activity, although the susceptibility of erythrocytes is not necessarily extendable to other host cells (Tossi et al., 2000). Toxicity can occur as a consequence of the structural determinants that are modified for higher antibacterial activity. Excessively hydrophobic peptides are indiscriminately toxic to both human and bacteria cells because their hydrophobic nature mediates insertion into human cell membrane without the aid for electrostatic interaction needed for bacterial activity (Palermo and Kuroda, 2010), (Figure 3.10).

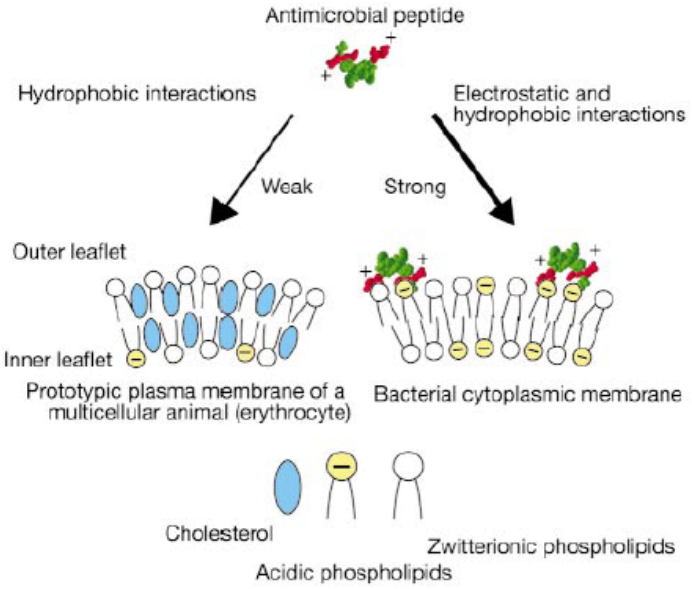

Figure 3.11. Membrane targets of AMPs and the basis of membrane selectivity. From (Zasloff, 2002).

Bacterial cytoplasmic membranes contain a large proportion of negatively charged (anionic) phospholipids such as phosphatidylglycerol and cardiolipin as wells as neutral (zwitterionic) phospholipids e.g. phosphatidylcholine (McPhee et al., 2005). This latter phospholipid is also found in eukaryotic membranes, which is composed principally of lipids with no net charge (Zasloff, 2002). Hence, due to their cationic charge, AMPs have selectivity for bacterial membranes. The negatively charged bacterial cell surface and a high transmembrane potential provide increased attraction towards bacteria (Yeaman and Yount, 2003).

The comparison between toxicity profiles has been hampered by the fact that a standardised protocol for determining hemolysis has been lacking (Palermo and Kuroda, 2010). Selectivity is often reported as the therapeutic index defined as the ratio of bacterial cell killing to eukaryotic cell killing i.e. MHC/MIC, where the former equals to maximum concentration that produces no hemolysis (Chen et al., 2005b; Jiang et al., 2008). Additionally, the concentration that causes 50 % hemolysis (EC_{50}) has been widely used (Dathe et al., 1997; Ilker et al., 2004). Significant hemolysis occurring only at concentration much higher than the antibacterial MIC value indicates selectivity for bacterial cells over mammalian cells (Porter et al., 2002).

Studies of peptidomimetic have shown that strong hemolysis generally correlated with high hydrophobicity (Dathe et al., 2001; Ilker et al., 2004), high amphipathicity (Al-Badri et al., 2008; Chen et al., 2005b) and high helicity (Dathe et al., 1997; Giangaspero et al., 2001; Javadpour et al., 1996; Oren and Shai, 1997). For our series of chimeras (where hydrophobicity is kept constant) length is the most important determinant for increased cytotoxicity (H. Franzyk, personal

communication). It is not always that increased hemolysis correlates with increased antibacterial activity (Giangaspero et al., 2001; Kondejewski et al., 1996; Schmitt et al., 2004). The effect of secondary structure on hemolysis indicates that structuring is more important for the interaction with eukaryotic membranes, which may suggest that different molecular mechanisms are underlying the antimicrobial and haemolytic activities of these compounds (Carotenuto et al., 2008).

3.7. Conclusions from chapter 3

Antimicrobial peptides (AMPs) have a wide spectrum of activity. The cationic nature of antimicrobial peptides facilitates their attraction to the negatively charged bacterial surface. Though they have been described as “dirty drugs” simultaneously disturbing many cellular functions, most antimicrobial peptides target the bacterial cell membrane, although distinct intracellular actions also have been reported. Several models for membrane interaction have been proposed. The most important structural determinants of peptidomimetic activity include charge, length, hydrophobicity, amphipathicity and secondary structure, since these variables may optimize the interaction with the plasma membrane. Small modifications in structural parameters can improve antibacterial activity, though this may also lead to increased hemolytic activity. Membrane activity can be determined using different methods including assays for membrane depolarisation and permeabilization, microscopy and solid state NMR spectroscopy. We used an ATP leakage assay and scanning electron miscopy to demonstrate that the present chimeras are likely to be membrane-active.

4. Bacterial resistance to antimicrobial peptides

The discovery of antibiotics for use against bacterial pathogenic bacteria gave hope for a future where all infectious diseases could be controlled (Fernandez et al., 2010). With the emergence and rapid spread of bacterial resistance to antibiotics it soon became clear that this would not be the case. A variety of compounds are being tested as future alternatives to the conventional antibiotics, but now more effort is put into predicting and preventing potential resistance development (Baquero, 2001). Part of the promise of antimicrobial peptides (AMPs) is that since they target the Achilles' heel of bacterial membrane structure (Zasloff, 2002) and have co-evolved with bacteria without development of widespread resistance (Peschel and Sahl, 2006), resistance to these compounds is considered unlikely. However, it is vital that this holds true, since resistance to AMPs could ultimately compromise the activity of our own immune system (Bell and Gouyon, 2003) (Chapter 5). In this chapter I will review the most common mechanisms that confer bacterial resistance to AMPs and relate this to resistance development against the α-peptide/β-peptoid peptidomimetics. Additionally, the concept of cross-resistance within groups of AMPs will be reviewed.

As for antibiotics, three major types of resistance to AMPs are known to exist; intrinsic, acquired and adaptive (Fernandez et al., 2011) (Table 4.1.).

Table 4.1. Outline of the characteristics of three major types of resistance to antimicrobials. Modified from (Fernandez et al., 2011).

Type of resistance	Intrinsic	Acquired	Adaptive
Acquisition	Not acquired, part of the genetic make-up of the strain or species	Mutation	Changes in the gene expression triggered by environmental factors or presence of antimicrobials
		Horizontal transfer	
Characteristics	Inheritable	Inheritable	Not inheritable
	Stable	Stable	Transient
	Irreversible	Irreversible	Generally reverts upon removal of inducing signal
	Independent of environment	Independent of environment	Dependent of environment

Whereas intrinsic resistance is an inherent genetic feature of the bacterial strain, exposure to AMPs can result in bacteria acquiring resistance either through mutations or by exploiting the phenomenon of adaptive resistance (Fernandez et al., 2011). Hence, the therapeutic use of AMPs can potentially result in the development of acquired and adaptive resistance, and since therapeutic use is also the

goal for the peptidomimetics, these two types of resistance will therefore be the primary focus of this chapter. The following discussion will be focused on resistance towards AMPs; as discussed in section 4.3.2 very few studies have dealt with resistance towards peptidomimetics. However, due to their similarity in the mechanism of action, it is likely that acquired resistance mechanisms against peptidomimetics will be similar to the ones described for AMPs.

4.1. Mechanisms of intrinsic and acquired resistance

Both Gram-negative and –positive bacteria use multiple strategies to prevent killing by AMPs (Figure 4.1). These include changing the charge and permeability of the different layers of the cell envelope, removing AMPs by efflux transporters and digesting by bacterial proteases.

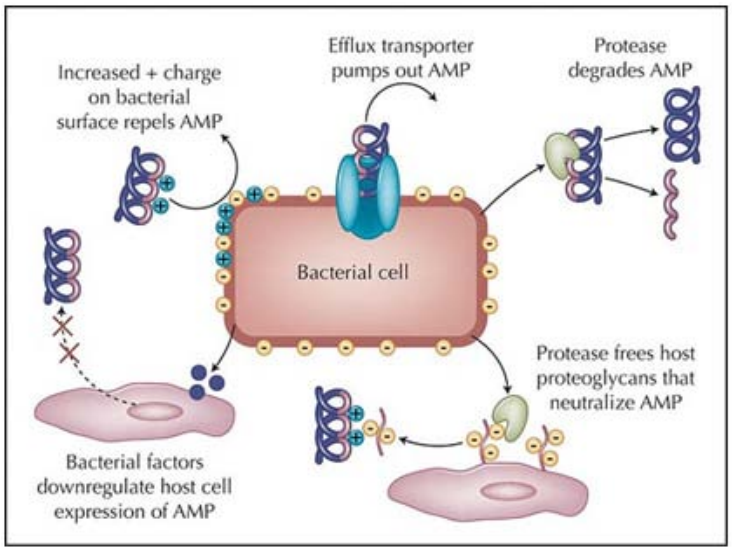

Figure 4.1. Mechanism of bacterial resistance to antimicrobial peptides. From (Gallo and Nizet, 2003).

The mechanisms involved in the different types of resistance are generally similar whether these constitute an intrinsic bacterial feature or develop through environmental stimuli or mutations, and the mechanisms will therefore be reviewed simultaneously.

4.1.1. Modification of outer cell layers

The anionic charge of the outer cell layer of bacteria mediates the initial attraction between the cationic AMP and the bacterial cell (Zasloff, 2002) (Chapter 3). Hence, modulating cell surface charges is a common strategy for bacteria to prevent AMP activity.

Gram-positive bacteria such as staphylococci are surrounded by a thick cell wall composed of peptidoglycan and polymers of alternating phosphate and alditol groups termed teichoic acids.

However, teichoic acids are negatively charged which facilitate electrostatic attraction of cationic AMPs. Esterification of teichoic with the positively charged D-alanine decreases the negative net charge of teichoic acids promoting AMP resistance (Otto, 2009). The mechanism is catalysed by enzymes encoded within the *dlt* operon and contributes to bacterial resistance in many Gram-positive bacteria including *Staphylococcus aureus*, *Clostridium difficile*, *Streptococcus pneumoniae* and *Lactococcus lactis* (Kovacs et al., 2006; Kramer et al., 2008; McBride and Sonenshein, 2011; Peschel et al., 1999). In addition, thickening of the peptidoglycan layer has also been shown to occur in *L. lactis* upon acquisition of resistance (Kramer et al., 2008).

The outer membrane of Gram-negative bacteria constitutes a semi-permeable barrier that in many species mediates intrinsic resistance to AMPs as well as antibiotics (Goldstein et al., 1983; Hancock, 1997c). The enterobacterial outer membrane consists of an inner monolayer of phospholipids and an outer surface monolayer of lipopolysaccharide (LPS) (see Figure 4.3). LPS molecules have a high negative charge, providing an anionic external surface similar to that seen for teichoic acid in Gram-positive bacteria. This facilitates attraction of cationic AMPs, however Gram-negative bacteria have developed multiple ways of modulating LPS charge by changing its chemical composition. These changes are often associated with the activity of two-component systems, the mechanisms of which will be reviewed in section 4.2. The most common method of reducing LPS charge is by the addition of 4-amino-deoxy-arabinose to lipid A, a component of LPS (Trent et al., 2001) (Figure 4.2).

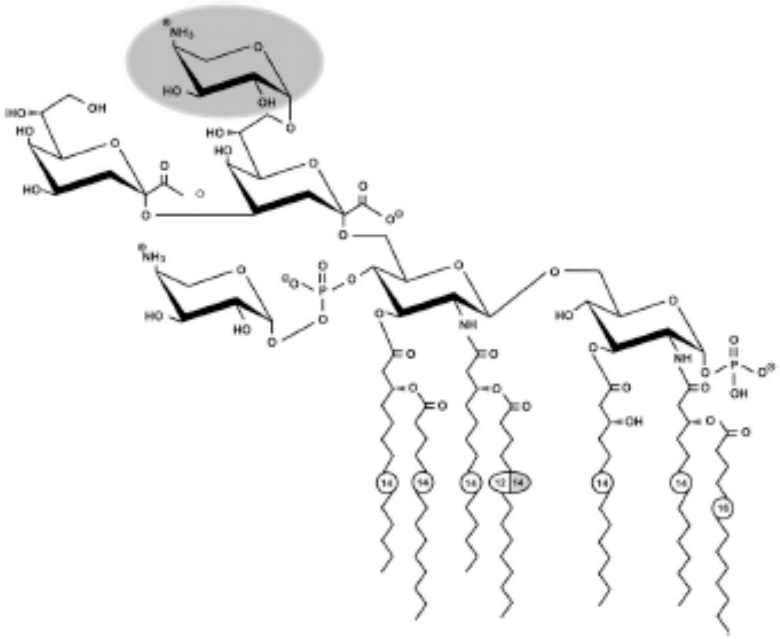

Figure 4.2. Chemical structure of LPS from AMP-sensitive (*Salmonella minnesota*) and resistant (*Proteus mirabilis*) strains. The resistant strain has ~50 % substitution with amino-arabinose in the LPS layer (dark area) which reduces the negtaive net charge compared to the suscpetibile strain. This is similar to that seen for enterobactericaeae.
From (Howe et al., 2007).

This partly neutralizes the negative charge of lipid A, thereby reducing the electrostatic charges with AMPs. Lipid A modification with amino-arabinose has been investigated in enterobactericeae and found in polymyxin B-resistant strains of *Escherichia coli*, *Salmonella typhimurium* and *Klebsiella pneumoniae* (Helander et al., 1996; Nummila et al., 1995; Zhou et al., 2001). This may be a result of constitutive expression of the PmrAB regulon (Froelich et al., 2006) (Section 4.2).

Decreased susceptibility to AMPs may also occur due to decreased permeability of the outer membrane as seen in a polymyxin B-resistant *E. coli* (Rahaman et al., 1998), where the extent of permeability was determined by using a fluorescent dye. The mutant displayed changes in the composition of LPS and the amounts of outer membrane proteins including porins making this the likely mechanism of resistance (Rahaman et al., 1998). The outer membrane has been described as a molecular sieve and the porins as the holes of the sieve (Hancock, 1997c). Hence, tight regulation of porins may provide resistance to AMPs (Mathur and Waldor, 2004). Scanning electron microscopy (SEM) of the cell surface of AMP-resistant strains has revealed visible ultra-structural changes to the outer membrane following acquisition of resistance (Sallum and Chen, 2008). It is likely that this is mediated by structural changes to both LPS and outer membrane proteins.

Some inherently AMP-resistant species such as *Serratia* and *Morganella* express an outer membrane that lacks the appropriate density of acidic lipids to provide AMP-binding sites (Zasloff, 2002). *S. marcescens* is well-known for its resistance to AMPs, and the outer membrane is likely to be the cause of this (Goldstein et al., 1983; Viljanen and Vaara, 1984). Perhaps as a consequence of this, it was also the only of the tested bacterial species which was resistant to the α-peptide/β-peptide peptidomimetics (Paper I).

Though peptidoglycan is primarily a feature of the Gram-positive cell wall, Gram-negative bacteria posses a thin layer of peptidoglycan in the periplasmic space between the inner and outer membranes, which ensures structural integrity to the cell by preventing osmolysis (Laubacher and Ades, 2008) (Figure 4.3).

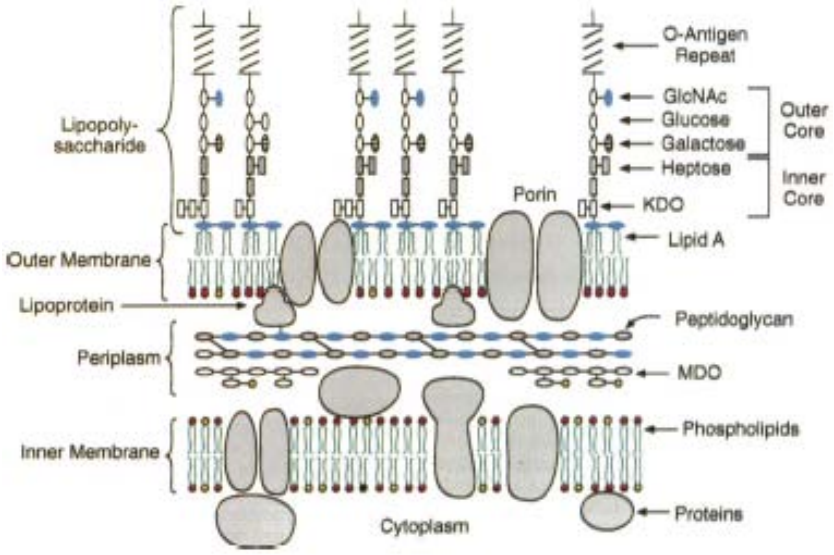

Figure 4.3. Structure and composition of the cell envelope of Gram-negative bacteria. From (Raetz, 1993).

When the cell grows and divides, murein hydrolases cut the peptidoglycan, while penicillin-binding-proteins (PBPs) ligate new strands to the existing peptidoglycan layer; a process which evidently needs to be tightly regulated (Typas et al., 2011; Uehara and Bernhardt, 2011). Peptidoglycan stress due to antibiotic-mediated inhibition of PBPs activates the Rcs phosphorelay in *E. coli* leading to enhanced survival in the presence of antibiotics (Laubacher and Ades, 2008). It is not clear what genes in the Rcs regulon mediate survival, but the authors speculate that the Rcs pathway may strengthen the outer membrane permeability barrier (Laubacher and Ades, 2008). In *E. coli* a variety of peptidoglycan hydrolases are responsible for remodelling of the peptidoglycan layer (van Heijenoort, 2011). As part of characterizing the resistant isolates developed through continuous selection of *E. coli* to compound 34 (Paper II, Section 4.3), we decided to whole-genome sequence some of these isolates and compare them to the ancestral wild type (*E. coli* ATCC 25922). An amino-acid changing SNP in the gene encoding the membrane-bound lytic murein transglycosylase (MltD) was one out of four SNPs found only in high-resistant isolates (Paper II). Overproduction of MltD in *E. coli* has been shown to lead to spheroplast formation and cell lysis (van Heijenoort, 2011). Conversely, inactivation of the mltD gene in *Vibrio anguillarum* by homologous recombination facilitated resistance to antibiotics, which the authors speculate could be due to an inability of MltD to weaken the outer membrane structure (Xu et al., 2011). We will elucidate the role of this SNP in resistance acquisition by introducing this into the wild type strain.

4.1.2. Decreased charge or permeability of the bacterial plasma membrane

Many AMPs exert their antibacterial effect through pore formation or disintegration of the bacterial plasma membrane leading to leakage of cell contents and ultimately cell death (Brogden, 2005).

Therefore, the bacterial cell membrane can therefore be considered the primary target of AMPs. This target is very different from the distinct targets of most antibiotics, where resistance in some cases can be achieved through single point mutations of target enzymes (Hancock, 1997a). The overall design of the bacterial membrane structure is not easily redesigned (i.e. mutated) and would, in theory, require a myriad of mutations to retain integrity and function, which has been proposed to make AMP resistance unlikely (Zasloff, 2002). In spite of this hypothesis, multiple studies have shown that structural changes of the plasma membrane can mediate AMP resistance without leading to fitness cost. The cationic charge of AMPs allows them to interact and be inserted into the anionic plasma membrane (Zhang et al., 2001) (Chapter 3). Hence, bacteria may prevent AMP activity by reducing the negative charge of the plasma membrane, which reduces bacterial affinity for AMPs and renders the bacteria more tolerant to these (Ernst and Peschel, 2011). The reduction is negative net charge is created by modifying the anionic membrane phospholipids with cationic amino acids; in *S. aureus* the modification of phosphatidylglycerol with lysine confers resistance to human AMPs (Peschel et al., 2001). Interestingly, modification of phosphatidylglycerol with alanine (a neutral amino acid) also conferred resistance to the tested AMP protamine (Klein et al., 2009) (Figure 4.4).

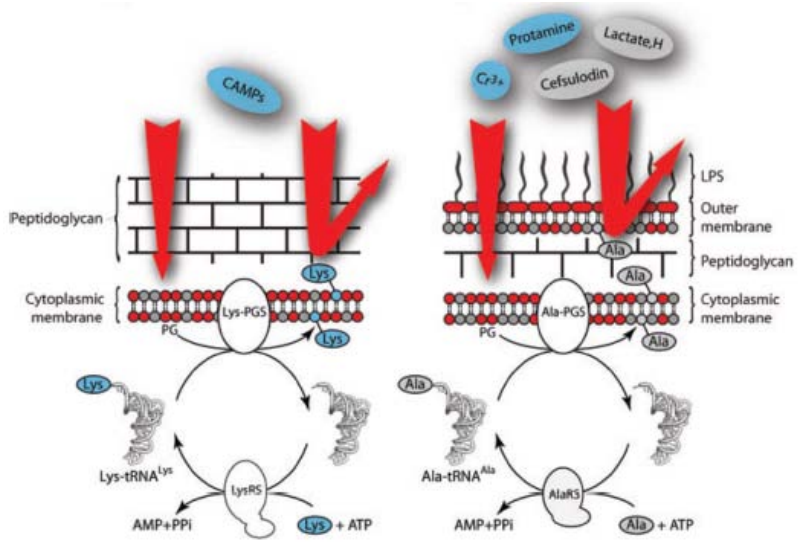

Figure 4.4. Aminoacylation with lysine or alanine in the membranes of *S. aureus* (left) or *Pseudomonas aeruginosa* (right). Though only lysine is charged, modification with both types of amino acids leads to AMP resistance. Positively charged molecules are indicated in blue and neutral ones in grey. From (Roy et al., 2009).

Lysine-modified phosphatidylglycerol are naturally present in many Gram-positive bacteria (Ernst and Peschel, 2011), and consequently the responsible MprF enzyme was not discovered until its inactivation increased the AMP susceptibility of a *S. aureus* mutant (Peschel et al., 2001).

Translocation or "flipping" of lysyl-phosphatidylglycerol to the outer membrane leaflet (leading to asymmetry in the membrane phospholipid composition) will hence also increase the net positive surface charge leading to increased resistance (Jones et al., 2008). Similarly, reduced levels of phosphatidylglycerol as a result of mutations in its synthase can also lead to reduced net negative charge creating resistance to daptomycin (a membrane-active antibiotic) (Hachmann et al., 2011). It has however also been proposed that the observed resistant phenotype of bacteria with increased lysyl-phosphotidylglycerol content could be a consequence of increased membrane stability rather than repulsion of AMPs (Kilelee et al., 2010). Increased membrane rigidity has been seen for resistant mutants which is likely to reduce membrane penetration by AMPs (Mehla and Sood, 2011; Mishra et al., 2011a). It is interesting that the other extreme (very fluid membranes) has also been associated with increased resistance (Bayer et al., 2000; Jones et al., 2008) indicating the existence of a membrane order optimum for AMP activity.

4.1.3. Efflux transporters

If the AMP is successful in traversing the outer layers of the cell envelope, many bacteria make use of efflux systems that expel these again before they can reach the plasma membrane or an intracellular target (Hancock, 1997c). Efflux systems fall into four categories: ATP-binding proteins, major facilitator proteins, resistance-nodulation-division proteins and small multidrug resistance proteins with many bacteria expressing more than one protein (Bell and Gouyon, 2003). Efflux pumps are a very common strategy in bacterial resistance to antibiotics (Li and Nikaido, 2004). However, though there are examples of efflux-mediated AMP resistance, the requirement for direct recognition of certain AMP sequences or structural motifs may have compromised the ability of efflux transporters to serve as a general mechanism of resistance against AMPs (Peschel and Sahl, 2006). Accordingly, producer self-protection by ATP-dependent EpiFEG in *S. epidermidis* is specific to the epidermin produced (Otto et al., 1998). This is in opposition to efflux proteins that expels a broad range of compounds such as organic cations or several classes of antibiotics; a major facilitator protein is encoded by the *qacA* locus on the psK1 plasmid which confers staphylococci resistance to several classes of antibiotics and to platelet microbicidal protein 1 (Kupferwasser et al., 1999). Similarly, the MltrCDE multi-drug efflux pump of *Neisseria gonorrhoeae* provides resistance against a diverse range of AMPs, though not to human defensins (Shafer et al., 1998). Temperature-dependent efflux systems (Bengoechea and Skurnik, 2000) and proteins with a dual role as K^+ transporters (Parra-Lopez et al., 1994) has also been shown to be implicated in AMP resistance.

4.1.4. Proteolysis and inactivation of AMPs

It is apparent that bacteria may obstruct AMP activity by proteolysis. However, the success of this is widely determined by the structure of the AMP; a simple linear structure or α-helical structure such as that of the human cathelicidin LL-37 is relatively susceptible to proteolysis by the outer membrane protease PgtE of *Salmonella enterica* (Guina et al., 2000). Therefore making AMP structures more rigid through the introduction of disulfide bridges renders AMP considerably more resistant to proteolysis (Peschel and Sahl, 2006) and the occurrence of disulfide-bridged defensins in a variety of organism underscore the virtue of this structure (Ganz, 2003). In *E. coli* the OmpT protease is responsible for the recovery of growing cells after protamine treatment (Stumpe et al., 1998).

Since proteases only have limited effect on more complex AMP structures, some bacteria have evolved mechanisms for extracellular trapping and neutralization of AMPs, which can however be quite specific for certain AMPs. *Staphylococcus aureus* produce staphylokinase, which binds α-defensins and completely eliminate the bactericidal effect of these compounds (Jin et al., 2004). Another example is given by the streptococcal inhibitors of complement (SIC) in *Streptococcocs pyogenes* (Fernie-King et al., 2004), however, the specificity of this mechanism is in the extremes since SIC binds strongly to human β-defensin 2 and 3 but not at all to human β-defensin 1 (Fernie-King et al., 2004).

4.2. Adaptive resistance via Gram-negative two-component systems

Though resistance mechanisms confer an immense advantage for bacteria in the presence of AMPs, the constitutive expression of genes that mediate structural changes or confer inactivation of AMPs may entail a fitness cost when the compound is are not present (Andersson, 2006). Many bacteria have therefore developed systems that restrict the resistance-conferring response to conditions where AMPs are actually present in the environment. Two-component systems consist of a sensory kinase and a response regulator, which allow the bacteria to sense and respond to environmental changes or stimuli (Stock et al., 2000). Such systems are an intrinsic feature of these bacteria, however, they are only activated when induced thereby mediating adaptive (or inducible) resistance. The systems are present in both Gram-negative and –positive bacteria (Fernandez et al., 2011) of which only the two-component systems present in Gram-negative bacteria will be reviewed in this section.

Adaptive resistance is described as an auto-regulated phenomenon characterised by induction of resistance in the presence of the AMP and reversal to the sensitive phenotype in its absence (Skiada et al., 2011). Consequently, exposure to a compound will increase tolerance to subsequent exposures (Daikos et al., 1990) (Figure 4.5).

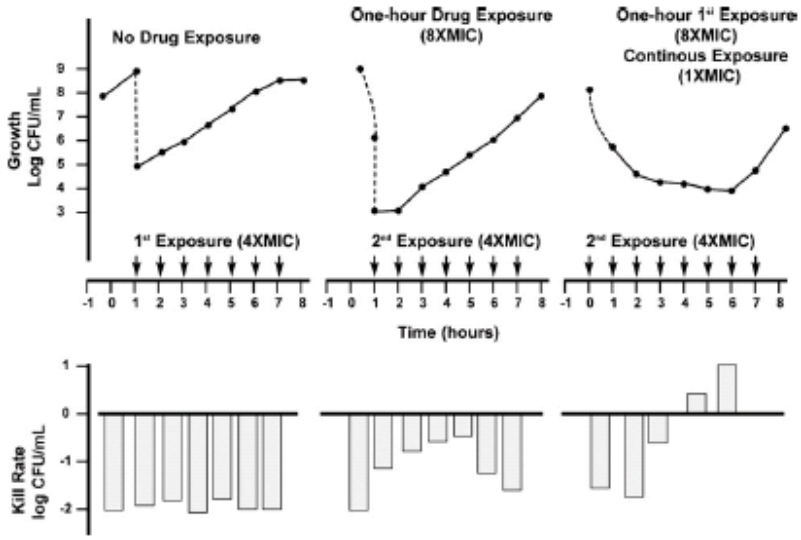

Figure 4.5. How previous exposure to an antibacterial (here aminoglycoside) leads to adaptive resistance in *P. aeruginosa*. Upper graphs show the number of variable bacteria at different times without prior drug explosive (control, left panel) with 1 hour prior drug exposure to 8xMIC and with continuous drug exposure to 1xMIC after the initial drug exposure of 8xMIC. Lower graphs show the bactericidal rate measured after addition of 4xMIC at successive hourly intervals. Adaptive resistance is evident in the middle and right panel; adaptive resistance is enhanced and prolonged with grown in continuous presence of the drug (right panel). From (Skiada et al., 2011).

Extensive research carried out in *Pseudomonas* and *Salmonella* has within the last decade provided a much clearer picture of the mechanism behind adaptive resistance and the regulatory pathways involved (Fernandez et al., 2011).

In *P. aeruginosa* three two-component systems involved in the process of adaptive resistance have been described to date. These systems all result in the incorporation of 4-aminoarabinose in lipid A leading to a decreased negative charge of the LPS (section 4.1.1 and Figure 4.2). The model AMPs used has primarily been of the polymyxin group (polymyxin B and colistin) though adaptive resistance to other AMPs has also been described (Fernandez et al., 2011). All three two-component systems result in the up-regulation of genes in the *arn* operon formerly known as the polymyxin resistance operon or *pmr* (Falagas et al., 2010) (Figure 4.6).

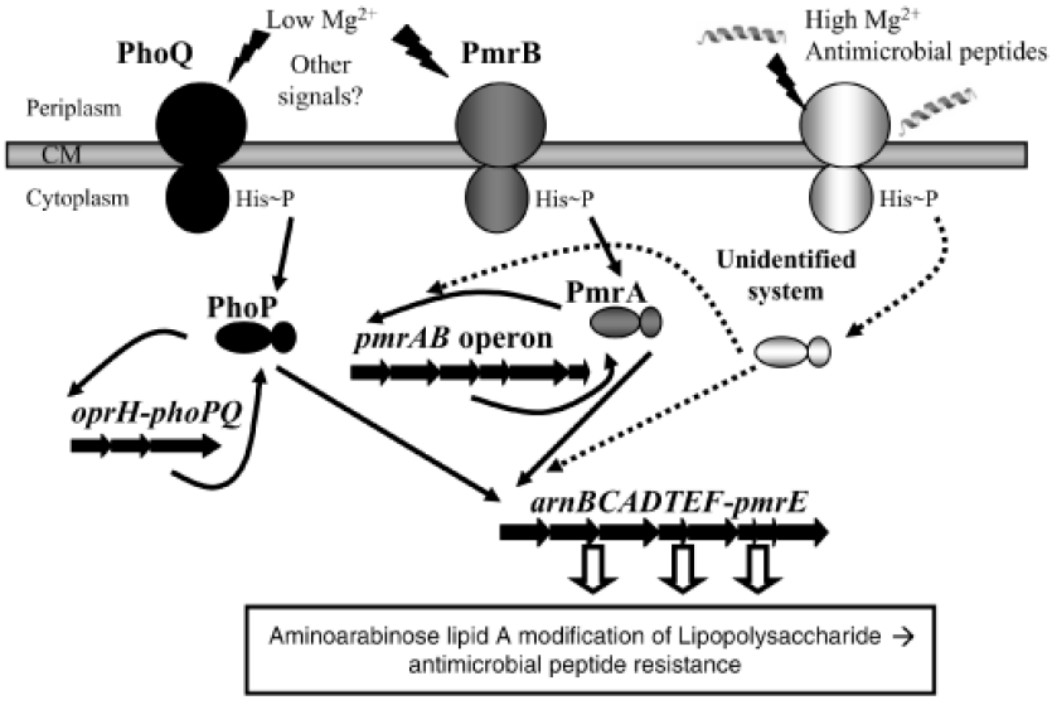

Figure 4.6. Model of *P. aeruginosa* PhoPQ and PmrAB regulatory networks in resistance to AMPs. The "unidentified system" to the lower right has now been identified and named the ParRS system (see text for details). From (Gooderham et al., 2008).

The level of divalent cations in the environment has a large role in the activation of two-component systems; in *P. aeruginosa* both PhoPQ and PmrAB are activated independently by low concentrations of Mg^{2+}, likely to reflect starvation conditions (Gooderham and Hancock, 2009). The PhoPQ was the first two-component system described in *P. aeruginosa*, identified due to its high similarity to the PhoPQ system of *Salmonella typhimurium* (Macfarlane et al., 1999). This was followed by the identification of the PmrAB system (McPhee et al., 2003). Under low concentrations of $Mg2^{+}$ these two systems induce the modification of lipid A by up-regulating the *arn* operon, and it has been shown that mutants in these systems may lead to constitutive expression of the system and stable resistance to polymyxins (Macfarlane et al., 1999; McPhee et al., 2003). However, AMPs induce the *arn* operon independently of the PhoPQ and PmrAB systems and are able to do this under higher (physiologically relevant) concentrations of divalent cations (Gooderham et al., 2008). The puzzle was finally solved in 2010 with the identification of the ParRS two-component system, which is activated by AMPs regardless of the concentration of Mg^{2+} (Fernandez et al., 2010) making it relevant for mediating adaptive resistance *in vivo*. Mutations in the ParRS system only affect sensing of polymyxins and indolicidin indicating the presence of specific recognition pathways for different structural groups of AMPs (Fernandez et al., 2010). Interestingly, the ParRS system has recently been shown to confer resistance to several different classes of antibacterials (AMPs as well as

conventional antibiotics) by inducing an arsenal of different resistance mechanisms i.e. LPS modifications, increased drug efflux and reduced permeability of porins (Muller et al., 2011).

The regulatory network of two-component systems and divalent cation sensing in *Salmonella* is different from that of *P. aeruginosa* though they possess similar systems encoded by homologues genes. Notably, in *Salmonella* AMPs are able to activate the PhoPQ system (Bader et al., 2005) (Figure 4.7).

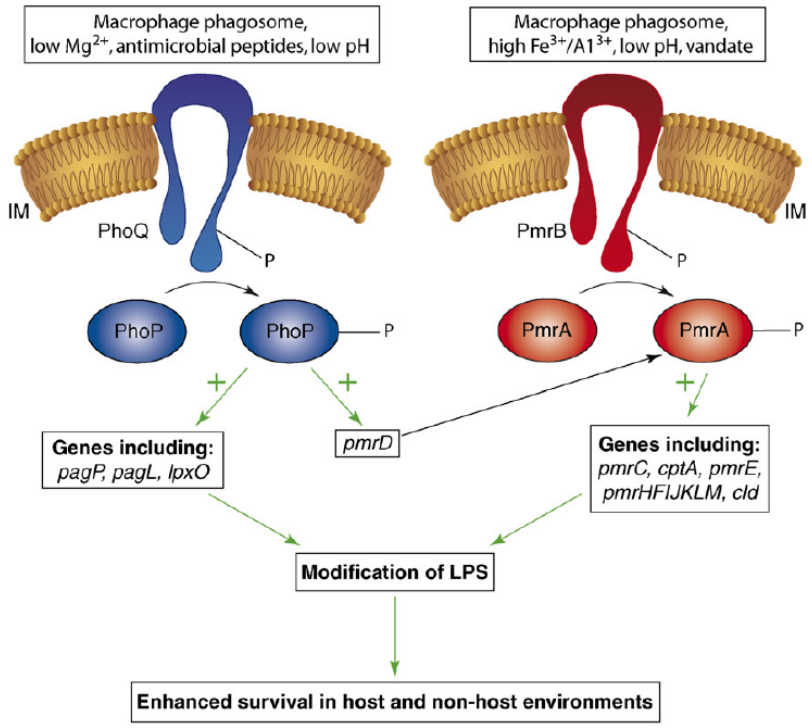

Figure 4.7. Model of *Salmonella* spp. PhoPQ and PmrAB regulatory networks in resistance to AMPs. From (Gunn, 2008).

AMPs are detected via their interaction with the sensor domain of PhoQ (Bader et al., 2005). Accordingly, it has elegantly been proposed to use the PhoQ-activating potential, a predicament of the development of resistance, in the optimization of novel AMP-like structures (Kindrachuk et al., 2007). PhoPQ regulates genes involved in cell envelope remodelling and proteolytic cleavage of AMPs (Fernandez et al., 2011; Guina et al., 2000). In addition, the activated PhoP transcriptional regulator induces the transcription of the *pmrD* gene, the product of which leads to stabilization of the phosphorylated form of PmrA (Fernandez et al., 2011). PmrA is then able to induce the expression of genes in the *pmr* operon (homologue to the *Pseudomonas arn* operon) involved in the modification of LPS (Gunn, 2008). Hence, both the PhoPQ and PmrAB systems are involved in the modification of LPS (Ernst et al., 2001; Zhou et al., 2001) (Figure 4.8).

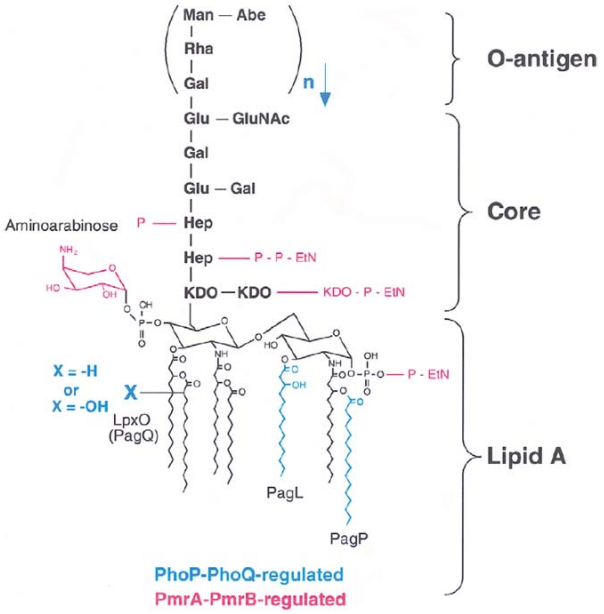

Figure 4.8. Chemical structure of *S. typhimurium* LPS with PhoPQ- and PmrAB-mediated structural modifications. From (Ernst et al., 2001).

In *Salmonella* the PhoPQ and PmrAB systems also respond to the different environments encountered during the lifestyle of this species. PhoPQ responds to decreased Mg^{2+} concentrations and both systems are activated in environments with low pH i.e. two parameters that are both known to present within the macrophage phagosome (Ernst et al., 2001; Gunn, 2008). Another interesting two-component system in *S. typhimurium* named the CprxRA was shown to mediate protamine resistance by up-regulating the expression of peptidoglycan amidases (section 4.1.1) (Weatherspoon-Griffin et al., 2011). The authors believe it is plausible that this system confer resistance to AMPs by combating the damage of the cell envelope caused by these or alternatively by modifying the outer membrane to prevent AMP binding.

This proposed mechanism is very similar to how we propose a mutation in the mltD gene could confer resistance to one of our chimeras (Paper II). Two-component system have not been extensively studied in *E. coli* as it has for *Pseudomonas* and *Salmonella*; peptidoglycan stress responses also mediate resistance to antimicrobials in *E. coli* but this is mediated by a phosphorelay system (Laubacher and Ades, 2008). Resistance mediated by either perturbations or structural changes of the peptidoglycan layer is interesting as it is not normally considered the primary target for novel resistance mechanisms in Gram-negative bacteria, since this layer is very thin compared to what is seen in Gram-positive bacteria. In *E. coli* PmrA also activate genes involved in the modification of lipid A with 4-aminoarabinose and subsequent resistance to AMPs (Froelich et al., 2006; Gunn, 2008; Guo et al., 1998). The activation of PmrA is, however, different from that of *Salmonella*, since PmrD is present but fails to inactivate PmrA (Gibbons et al., 2005). Regardless of

this, we consider it unlikely that two-component system or other sensing systems are involved in the resistance to the chimeras, and neither to the resistance to polymyxin B evolved in the same study. This conclusion is made from the fact that resistance to both of these compounds was heritable (i.e. resistance was not eliminated in the absence of the drug) (Paper II). The expression of the *pmr* operon in yet another enterobactericeae, *Klebsiella pneumoniae*, is regulated by PhoP, PmrD and PmrA in a similar way to *Salmonella* (Cheng et al., 2010).

4.3. Acquisition of resistance through cumulative changes

The potential for development of resistance to new antimicrobial agents is often described in terms of resistance frequencies (O'Neill and Chopra, 2004). Such frequencies are determined by plating bacterial cultures on selective plates with compound concentrations above MIC and counting the number of resistant mutants arising after a defined time period. Low resistance potentials are often associated with antibacterials with more than one cellular target (O'Neill and Chopra, 2004), as described for AMPs. Though resistance frequencies have been mostly examined for antibiotics, spontaneous resistance towards AMPs have been reported (Macwana and Muriana, 2012). Transposon mutant libraries constitute another approach for developing AMP resistant mutants. Since the transposon element randomly inserts into the genome, this may disrupt genes involved in bacterial susceptibility, which allows for subsequent identification of colonies with increased resistance when plated on selective plates (Thomsen et al., 2010). AMPs have often been described as “dirty drugs” that disturb many biological functions with low potency rather than blocking a specific high affinity target (Peschel and Sahl, 2006). Hence, mutations in multiple specific pathways or structures are often needed to obtain high levels of AMP resistance, which is only rarely achieved with the above methods. Instead resistance may be evolved by the sequential accumulation of mutations of small individual effect.

4.3.1. The application of continuous selection for resistance development

Bacterial populations will inevitably evolve both *in vivo* (Lieberman et al., 2011) and *in vitro* (Barrick et al., 2009). As they do so resistant bacteria arise at low frequency by mutation (Bell and Gouyon, 2003; Conrad et al., 2011). Continuous exposure to an antibacterial will select for those mutations that confers increased fitness or tolerance in the presence of the selective agent since resistant bacteria will have an advantage and still be able to grow. For example *E. coli* has a large mutational target size for increased tolerance to antibacterial drugs (Girgis et al., 2009). Continuous selection is the process by which bacteria adapt to the presence of antimicrobial drugs (Bell and

Gouyon, 2003). Interaction between antibacterial agents and microbial populations can occur even at very low concentration (Girgis et al., 2009) hence concentrations several times lower than the MIC are often the starting point for adaptation. The progressive enrichment in low-level resistant populations can then favour secondary selection for more specific and effective mechanisms of resistance i.e. directional selection (Baquero, 2001).

4.3.2. Evolution of AMP resistance in the laboratory

Several studies have attempted to develop resistance to AMPs by continuous selecting for growth in the presence of the compound, a process which has been termed adaptive laboratory evolution (Conrad et al., 2011). The success rate (i.e. the successful development of resistance) of these studies varies extensively and seems to depend on the number of passages, initial concentrations, number of individual lineages and whether or not the concentration is kept constant or gradually increased (Farrell et al., 2010; Kramer et al., 2006; Perron et al., 2006; Steinberg et al., 1997; Zaknoon et al., 2009). In general, resistance development is tracked over time (Figure 4.9).

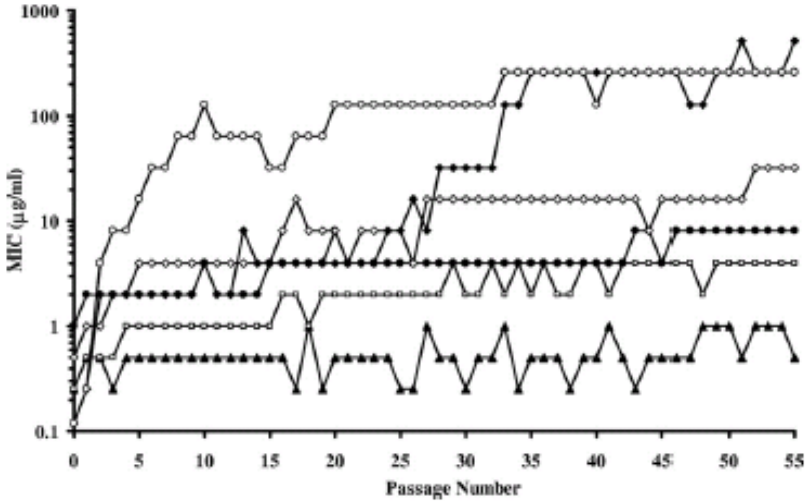

Figure 4.9. Resistant development profiles of XF-73, a dicationic porphyrin (black triangles) compared with selected conventional antibioitcs against *S. aureus*. From (Farrell et al., 2010).

As done in the study with XF-73, a membrane-active antimicrobial (Farrell et al., 2010) (Figure 4.9) resistance development of novel antimicrobials is often compared to that of conventional antibiotics (Steinberg et al., 1997; Zaknoon et al., 2009). However, this is complicated by the fundamental differences between antibiotics and AMPs; the mutation rates for are generally higher for antibiotic resistance, and most AMP resistance mechanism often only result in reduced susceptibility to AMPs i.e. low levels of resistance (Peschel and Sahl, 2006). Development of resistance to a specific AMP should therefore ideally only be compared to that of other AMPs.

In paper II we describe the development of resistance to a synthetic AMP peptidomimetic (compound 34) and the natural AMP polymyxin B. Heritable resistance to compound 34 was demonstrated in several lineages (Table 4.2).

Table 4.2. Changes in the Minimum Inhibitory Concentration (μg/mL) of compound 34 after continuous selection and the stability of this after five passages in unsupplemented media. From Paper II.

Lineage number	After selection	Stability
1	n.a.	128-256
2	256	256
3	n.a.	128
4	256	128
5	64-128	128
6	n.a.	64-128
7	128	128
8	n.a.	32-64
9	n.a.	128
10	256	32

MIC of *E. coli* ATCC 25922: 8 μg/mL; n.a.: Could not be revived in supplemented media

The pronounced variation in the level of resistance and the stability of this (i.e. lineage 10) indicate the presence of multiple resistance mechanisms and/or bacterial population structures in the different lineages. This was the first time stable, heritable resistance was demonstrated to a synthetic AMP, though another successful study has been performed with the AMP analogue pexiganan (Perron et al., 2006). The best parameter for successful development of resistance consequently seems to be graduate increases in concentration (Paper II).

Continuous selection experiments have also been shown to be useful for developing resistance to antibiotics (Camargo et al., 2008; Girgis et al., 2009; Lee et al., 2010; Palmer et al., 2011). Since antibiotic resistance is fairly feasible to develop in the lab, the focus is often on the spread of resistance-conferring mutations and bacterial heterogeneity in the evolved populations (Lee et al., 2010). The development of next generation sequencing technologies has provided the means to study the molecular basis of evolution of a genome scale (Conrad et al., 2011). Indeed, whole-genome sequencing of highly resistant isolates makes it possible to elucidate the mechanisms behind resistance (Hachmann et al., 2011; Lee et al., 2010; Palmer et al., 2011). Point mutations found in evolved isolates can then be introduced into the wild type to determine that these are indeed

responsible for resistance (Hachmann et al., 2011). The use of hyper-mutator strains has greatly facilitated the process by which bacteria are continuous selected for growth in the presence of antibacterials (Miller et al., 2002; O'Neill and Chopra, 2001). Hyper-mutator strains can be used to predict the emergence of very rare mutants (Baquero, 2001); additionally mutator phenotypes may also evolve in bacterial populations (Barrick et al., 2009), which greatly accelerates the rate of evolution.

Resistance to AMPs is seldom seen in nature (Zasloff, 2002), where bacteria and natural AMPs seem to have co-evolved (Peschel and Sahl, 2006). In nature bacteria are exposed to a range of AMPs with different mechanisms of action, whereas in the laboratory lineages can be exposed to a particular stress that limits growth creating an intense selection likely to cause specific adaptation (Perron et al., 2006). Hence, it is possible that the development of AMP for therapeutic use will not be retarded by the observation that AMP resistance can be developed *in vitro*.

4.3.3. Resistance formed by population-based resistance mechanisms

Isogenic clones will evolve differently if given enough time (Blount et al., 2008), and this will happen with or without the selection pressure of an antimicrobial agent. However, though some heterogeneity is expected, it is puzzling how less resistant isolates are able to survive (and evolve?) in the presence of high concentrations of antibacterials (Figure 4.10).

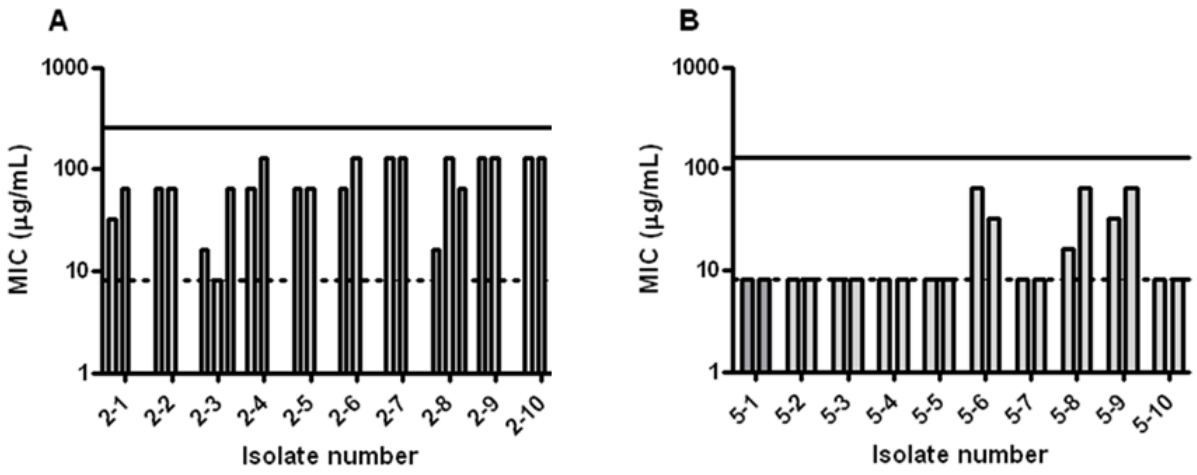

Figure 4.10. MIC (µg/mL) for compound 34 against individual isolates from lineage 2 (A) and lineage 5 (B). The MIC of all isolates is below the population MIC. Solid line: population MIC, punctuated line: wild type MIC. From Paper II.

We (Paper II) and others (Lee et al., 2010) have demonstrated that the majority of individual isolates are less resistant than the population as a whole (Figure 4.10). One obvious explanation is that the population is not stable enough to maintain high levels of resistance, but other population-based resistance mechanisms could also be responsible for this discrepancy. Microenvironments has been shown to accelerate the emergence of bacterial antibiotic resistance (Zhang et al., 2011), and although a structured environment is not formed during liquid growth it is possible that there slight differences in the local concentrations of the compound. This will lead to the development of subpopulations e.g. bacterial aggregation has been seen in the presence of antimicrobials.

In a study of antibiotic resistance following continuous culturing of *E. coli* it was proposed that a few highly resistant isolates improve the survival of the less resistant members of the population (Lee et al., 2010). They do this through the production of indole, a signal molecule that induces protective mechanisms in the less resistant cells. Since the production of indole entails a fitness cost, this mechanism is equivalent to a form of bacterial kin selection that enhances the survival capacity of the overall population (Lee et al., 2010). The presence of a few highly resistant isolates in a bacterial population has also been demonstrated with colistin as the selective agent (Adams et al., 2009).

The term "epigenetics" describes the heritable variations in gene expression pattern that exists within an isogenic population. It has been demonstrated that epigenetic inheritance can mediate resistance to antibiotics (Adam et al., 2008) (Figure 4.11).

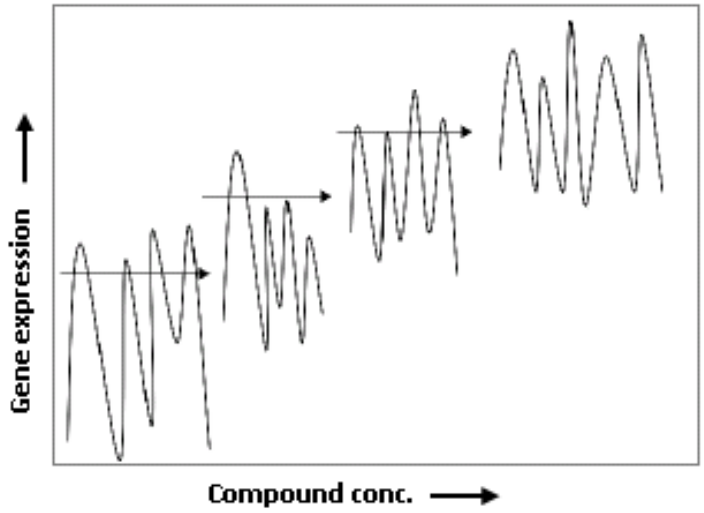

Figure 4.11. Model for the evolution of antibacterial resistance based on epigenetic inheritance. Within isogenic populations there is random variation in the expression levels of genes. Antibacterial exposure (horizontal arrows) selects cells with gene expression that favours survival. Modified from (Adam et al., 2008).

Characteristics of antibiotic resistance based on epigenetic-based inheritance include extremely high reversion rates to antibiotic sensitivity (over 50 %) and altered gene expression patterns in resistant

cells (Adam et al., 2008). For instance over-expression of the gene encoding glutamate decarboxylase resulted in increased ampicillin resistance. Spontaneous epigenetic modifications has also been studied in the expression of cell surface antigens and antibiotic persister cells (Avery, 2006).

Finally, epistasis interactions i.e. how the phenotypic effects of a mutation depends on the other mutations in the genome (Chou et al., 2011) may drive the acquisition of resistance and contribute to the overall level of resistance within the population (MacLean et al., 2010; Trindade et al., 2009).

4.4. Cross-resistance between AMPs and peptidomimetics

Development of resistance is most often a result of selection pressure with a single selective agent, which explain the effectiveness of combination therapies in preventing antibiotic resistance (Fischbach, 2011). However, the evolved resistance mechanism may also confer resistance to other compounds besides the primary agent i.e. cross-resistance. Resistance to AMPs varies greatly in specificity: in some cases it is highly specific and only protects bacteria against a narrow range of peptides, whereas other cases involve mechanisms that confer broad resistance against many structural types of AMPs (Bell and Gouyon, 2003). Indeed, several resistance mechanisms are based on the recognition of specific sequences or motifs, making cross-resistance less likely (Fernie-King et al., 2004; Kupferwasser et al., 1999; Peschel and Sahl, 2006).

In studies where no cross-resistance is seen, the inability of a specific resistance mechanism to protect against AMP activity is likely to be a result of slight differences in the AMP mechanism of action (Macwana and Muriana, 2012). Nevertheless, the difficulties in predicting AMP cross-resistance is clearly evident, since other studies have demonstrated that the resistance developed against structurally unrelated AMPs share a common mechanism (Mathur and Waldor, 2004; Mehla and Sood, 2011). Two-component systems has also show to be induced by unrelated AMPs, thereby forming the potential for cross-resistance between many structural groups (Farris et al., 2010; Jochumsen et al., 2011; McPhee et al., 2003).

We demonstrated pronounced levels of cross-resistance within the peptidomimetics library, when *E. coli* had been cultured with a single chimera (Paper II) (Figure 4.12).

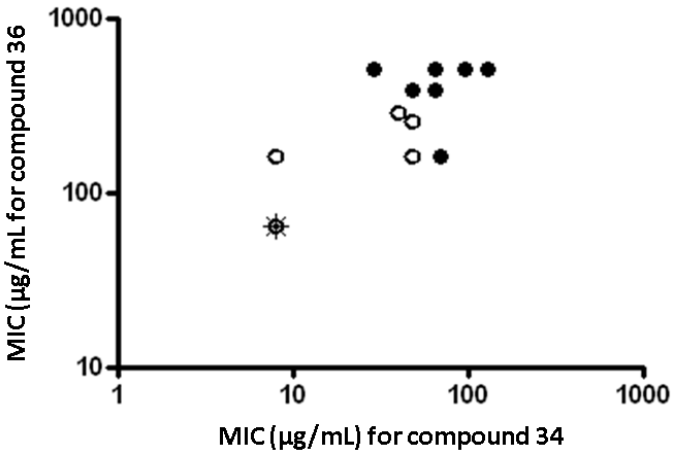

Figure 4.12. *E. coli* cross-resistance between compound 34 and 36. Asterisk marks wild type MIC for both compounds. From Paper II.

Cross-resistance was evident between compound 30, 34 and 36 which displays different amino acid side groups (lysine/homoarginine), length (12/16 amino acids) and chirality (chiral/non-chiral) (see Chapter 2 for the structural design of the different compounds). These results indicate that resistance developed against the overall design of the peptidomimetics rather than the specific structural moieties. Of note, the level of chirality is believed to have a large impact on the secondary structure of the compound, which could affect the interaction with the bacterial membrane (Deslouches et al., 2005b; Zhang et al., 2001). We did not find cross-resistance to other membrane-active peptides, making it even more interesting that AMP resistance mechanisms based on structural changes of the cell envelope, and of the outer membrane in particular, have been shown to also confer resistance to conventional antibiotics which nonetheless interact with the membrane in a substantially different way than AMPs (Fernandez et al., 2010; Muller et al., 2011; Rahaman et al., 1998).

4.5. Conclusions from chapter 4

Bacteria use several different resistance mechanisms for protecting themselves against the activity of AMPs. The most common of these include modification of the cell envelope or membrane (leading to decreased charge or permeability), efflux pumps and proteases. Additionally, resistance may be induced by AMP sensing of two-component systems. Continuous selection provides a way of acquiring resistance through cumulative changes, which was successfully employed for the peptidomimetics. Modification of the peptidoglycan layer in the cell envelope of *E. coli* is the likely cause of resistance to the α-peptide/β-peptoid chimeras.

5. Antibacterial activity under physiologically relevant conditions

A critical step in the further advancement of novel antimicrobial compounds is the transition from test tube activity to characterisation in biological environments. Due to the inherent properties of the peptidomimetic (or antimicrobial peptide, AMP), the compound may interact with blood or tissue components leading to changes in activity. Changes in activity due to the presence of various immune factors constitute a major obstacle in predicting peptide activity *in vivo* and this topic is therefore the primary focus of this chapter. Understanding the effect physiological environments may have on the antibacterial activity of these compounds will enables us to provide a preliminary evaluation of their therapeutic potential.

5.1. Factors influencing AMP activity in a physiological environment

Three years ago a study was published in Nature on the activity of Platensimycin, an inhibitor of the type II fatty acid synthesis pathway, in a model that simulate environments relevant to infection i.e. human serum (Brinster et al., 2009). Platensimycin is one of many natural antibiotics characterised within the last years, which has showed great potential against Gram-positive bacteria in standard laboratory media. However, serum (and thereby also blood) contains large amounts of fatty acids, and once activity was determined under these biologically relevant conditions, inhibition of fatty acid biosynthesis was completely abolished since the bacteria naturally will use the fatty acids that are available to them (Brinster et al., 2009). This is an excellent example of the need to determine and understand activity of new antimicrobial compounds under relevant physiological conditions.

Physiological relevant conditions can lead to a decrease in or a complete loss of activity of otherwise promising compounds making these unusable for systemic use (O'Neill and Chopra, 2004; Powell et al., 1993; Sabath, 1978). The structure of AMPs makes them particularly sensitive to biological environments and to date many clinical trials (i.e. Phase III) have therefore been restricted to topical applications (Andres, 2011; Yeung et al., 2011). A well known example of a therapeutic AMP currently on the market is colistin (or polymyxin E), a bacteriocin structurally similar to polymyxin B and isolated from *Bacillus polymyxa*. Colistin is widely used for aerosol treatment of *Pseudomonas aeruginosa* in cystic fibrosis patients (Michalopoulos and Papadakis, 2010), although high levels of resistance and toxicity issues has strongly affected its use (Falagas et al., 2010; Fernandez et al., 2010). The increased salinity of the bronchopulmonary fluids in cystic fibrosis patients constitute a large obstacle in the development of new AMPs against lung infections in these

patients since the activity of AMPs is extremely sensitive to salt concentrations, thereby creating a market for the development of salt-resistant AMPs (Deslouches et al., 2005a; Friedrich et al., 1999; Goldman et al., 1997). It is not clear if the mechanism behind salt sensitivity is obstruction of binding sites on the bacterial membrane or reduced stability of the compound (Deslouches et al., 2005a).

Degradation of AMPs by body proteases can lead to premature blood clearance creating unfavourable pharmacokinetics (Yeung et al., 2011). Testing serum stability has therefore become an essential part of AMP development (Jenssen and Aspmo, 2008) and strategies to stabilize peptides against serum proteases is an important part of the structural design (Knappe et al., 2010). The α-peptides/β-peptoids, which are the focus of this thesis, have previously been found to be resistant to proteases (Olsen et al., 2007; Olsen et al., 2010), which can be attributed to structural features of the peptoid moiety (Chongsiriwatana et al., 2008; Godballe et al., 2011) (Chapter 2). In addition to protease degradation, protein binding may also decrease serum levels of the unbound antibacterial thereby indirectly reducing antibacterial activity (O'Neill and Chopra, 2004). Binding to albumin or lipoproteins can therefore also explain lower activity in serum or whole blood compared to what is seen in standard media (Deslouches et al., 2005a; McKay et al., 2009; Sorensen et al., 1999).

One of the major worries regarding the therapeutic use of AMPs or peptidomimetics is that their mechanism of action is very similar to the action of natural AMPs, from which they have been designed, as well as other compounds of the innate immune system. Interaction with components of our own immune system makes it difficult to predict peptide activity *in vivo* and could potentially have unknown consequences on our ability to fight infections (Bell and Gouyon, 2003).

5.2. Antibacterial compounds of the innate human immune system

Before discussing the effects the simultaneous activity of immune factors and antimicrobial peptides, I will first give a brief introduction to the immune factors which may affect peptide activity. I will only mention other parts of the immune system (e.g. immune cells) in their role as producers or mediators of activity.

5.2.1. The ubiquitous innate defence molecules

The neutrophils are central cellular effectors of the innate system with many of the blood active antimicrobial proteins and peptides in their granule-associated arsenal (Levy, 2000). These cells

contain two types of cytoplasmic granules; the primary (azurophil) and secondary (specific) after the order in which they are synthesized (Levy, 2000). The content of the primary granule is largely released into the phagolysosome thereby exposing ingested bacteria to its contents (Figure 5.1).

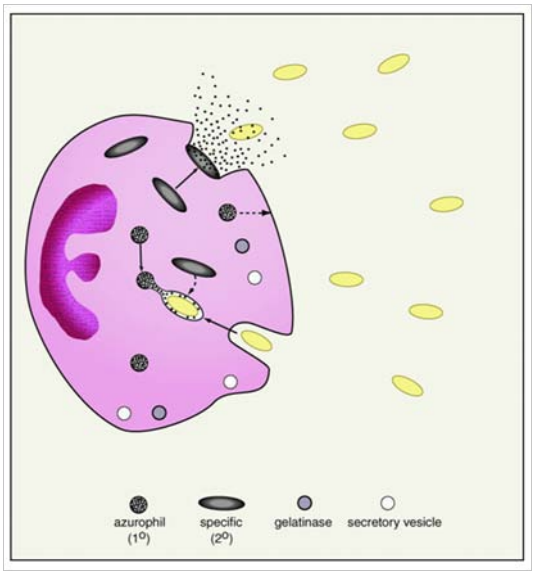

Figure 5.1. Neutrophil degranulation of antimicrobial proteins and peptides. Secondary granules are more prone to degranulate their contents into the extracellular fluid, whereas primary granules are predominately degranulated into the phagolysosome. From (Levy, 2000).

The phagolysosome is the mature phagosome, which is also an essential feature of the activity of macrophages and dendritic cells, and is designed for degradation of engulfed microorganism (Flannagan et al., 2009). The phagosome has a central role in the generation of oxygen-dependent immune factors, which will be described later. The contents of the primary granule include defensins, AMP-like compounds and lysozyme. Since these compounds are primarily released intracellularly their concentration in the blood is low; additionally the defensins are strongly inhibited by cations and blood proteins and their activity would therefore be inhibited in the extracellular environment (Levy, 2000). In contrast to the primary granule of neutrophils, the contents of the secondary granule is primarily released into the extracellular space (Figure 5.1), and larger concentrations of these compounds which include cathelicidins, lactoferrin and lysozyme will therefore be present in the blood (Levy, 2000; Sorensen et al., 1999).

The peptidomimetics designed by KU Pharma mimic the structure and mechanism of action of natural AMPs. The diversity of natural AMPs was reviewed in Chapter 2, but some additional explanations seem justified for human AMPs. As for other mammals a cocktail of AMPs belonging to different structural classes are expressed in humans (Zasloff, 2002), which are likely to have overlapping activities (Hancock and Sahl, 2006; Rieg et al., 2011) or ability to form synergy with each other (Chen et al., 2005a; Yan and Hancock, 2001). Multiple studies have shown that AMPs

protect against invasive bacterial infections particularly at epithelial surfaces, which is a common place of expression of these compounds (Goldman et al., 1997; Islam et al., 2001; Nizet et al., 2001).

In humans two families of AMPs exist, the cathelicidins and the defensins (Brown and Hancock, 2006; Maroti et al., 2011). The sole representative of the cathelicidins in humans is LL-37 (Bowdish et al., 2005), which is produced by proteolytic cleavage of the hCAP-18 precursor protein (Nijnik and Hancock, 2009). As indicated by the name it is a 37 amino acid long AMP, and has an α-helical secondary structure (Figure 5.2., for more information on the different structural families of AMP see Chapter 2). LL-37 is produced by both neutrophils and epithelial cells (Nijnik and Hancock, 2009), and is an important part of the innate immune defence at epithelial surfaces for protection against skin infections (Nizet et al., 2001). Due to its release from neutrophils into the extracellular fluid large concentrations in the µg/mL range have been found in plasma (Zahner et al., 2010) though often bound to lipoproteins (Sorensen et al., 1999). Like many other AMPs, LL-37 target the bacterial plasma membrane leading to permeabilization of this followed by rapid cell death though the exact mechanisms behind membrane interaction are still unclear (Lee et al., 2011).

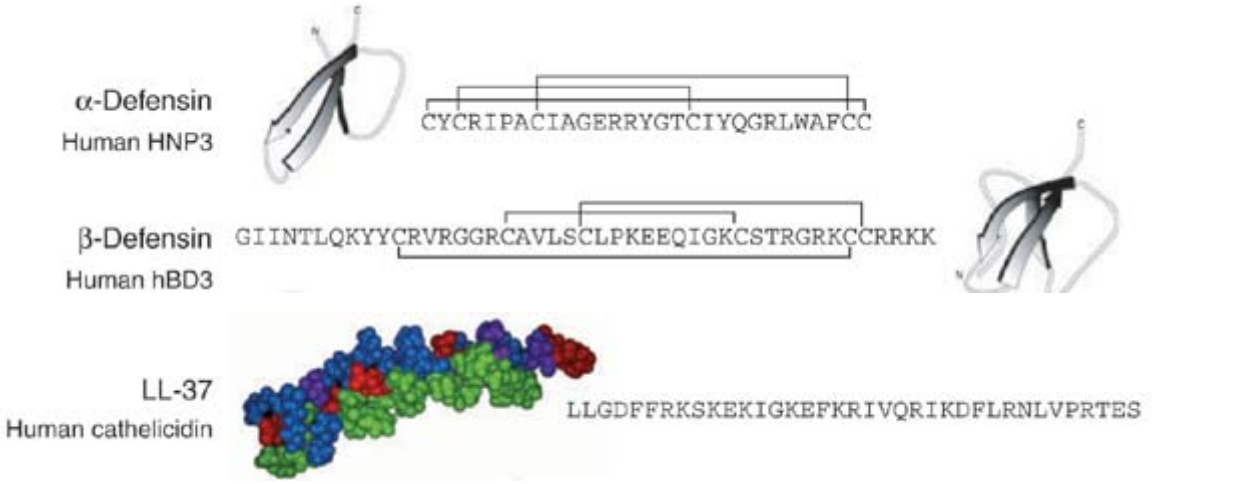

Figure 5.2. Structure of the two families of human AMPs; the defensins and the cathelicidin (LL-37). The defensins can be further sub-divided based on the linking pattern between cysteine. Modified from (Hancock and Sahl, 2006).

Defensins constitute the other group of AMPs in humans (Hazlett and Wu, 2011). They have a β-sheet structure linked by three disulfide bonds formed by six cysteine residues and can be classified into two main classes, α- and β-defensins based on the linking pattern between these cysteines (Ganz, 2003) (Figure 5.2). The α-defensins are concentrated in the neutrophils granules but also in the Paneth cells of the small intestine, while β-defensins are primarily secreted by mucosal surface

epithelia (Hazlett and Wu, 2011). In humans six α-defensins (human neutrophil peptide, hNP 1-4 and human defensin, hD 1-2) and four β-defensins (human beta defensins, hBD 1-4) have been found to date (Hazlett and Wu, 2011). The mechanism of action of defensins is, similarly to that of LL-37, permeabilization of the bacterial plasma membrane leading to cell lysis (Lehrer et al., 1989). Some strains of *Staphylococcus aureus* are resistant to defensins (Peschel et al., 2001) though resistance is not reflected in the clinical manifestations of infection with this bacterium (Rieg et al., 2011).

Other antimicrobial compounds of the innate immune defence with an AMP-like mechanism of action include the bactericidal/permeability-increasing protein (BPI), lactoferrin and the serprocidins (Levy, 2000). The BPI protein has a prominent role in the activity against Gram-negative bacteria in that it displays high activity in blood, serum and plasma, exerts multiple activities against bacteria (i.e. lysis, opsonisation, neutralization of LPS) and can act in synergy with defensins, LL-37 and the complement system (Elsbach and Weiss, 1998; Schultz and Weiss, 2007).

The enzymatic activity of lysozyme involves hydrolysis of the peptidoglycan layer of the bacterial cell envelope, and since this is primarily found in Gram-positives its activity is predominantly limited to these bacteria. It is produced by both primary and secondary granules and is present in μg/mL concentrations in the blood (Taylor, 1983) but is also abundant in body secretions. Lysozyme activity is mainly mediated through synergy with other antimicrobial compounds of the innate immune defence and synergy has been found to both AMPs (Bals et al., 1998; Yan and Hancock, 2001) and the antibody-complement system (Taylor, 1983). This suggests that lysozyme gains access to the peptidoglycan substrate only after the integrity of the outer membrane has been compromised by another compound. For this reason, lysozyme alone is not considered effective against Gram-negative bacteria. Modification of the peptidoglycan backbone of Gram-positive bacteria decreases the bacterial sensitivity to lysozyme thereby preventing cell lysis (Davis and Weiser, 2011).

Activated neutrophils and macrophages increase oxygen consumption in what has been termed the respiratory burst (Levy, 2000) leading to the production of reactive oxygen (ROS) and nitrogen species (RNS), which form the oxygen-dependent part of the innate immune defence (Fang, 2004). The NADPH-dependent phagocyte oxidase found in neutrophils release superoxidase into the phagosomal lumen upon phagocytosis which is then converted into hydrogen peroxide (Flannagan et al., 2009; Radtke and O'Riordan, 2006). Experimental wound models in zebrafish has demonstrated that concentrations of hydrogen peroxide can reach 50 μM near a wound margin (Niethammer et al., 2009). Nitric oxide is particularly associated with macrophages where it is produced by the inducible

nitric oxide synthase (iNOS) before it is converted to reactive nitrogen species (Radtke and O'Riordan, 2006). Both ROS and RNS interact with numerous bacterial targets leading to protein inactivation, lipid conversion and irreversible DNA damage (Flannagan et al., 2009).

5.2.2. Formation and role of the complement-antibody complex

The bactericidal and bacteriolytic properties of serum or plasma against susceptible Gram-negative bacteria stems primarily from the complement cascade (Taylor, 1983). The complement system is present in the blood as inactive pre-cursor proteins but will when triggered be activated through one of three distinct cascades, the classical, alternative or lectin pathway of complement activation (Trouw and Daha, 2011). Activation of either of these pathways by Gram-negative bacteria leads to the formation and insertion into the bacterial envelope of a multi-component complex, the membrane attack complex (MAC), leading to pore formation and bacteriolysis (Kondos et al., 2010) (Figure 5.3).

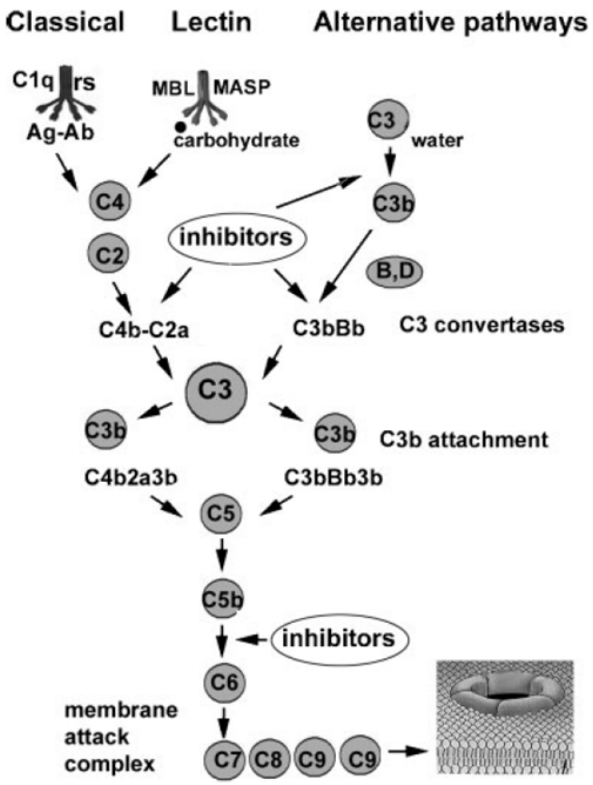

Figure 5.3. Complement cascade, In vertebrates there are three cascade pathways: classical, lectin and alternative. This can lead to the formation of the membrane attack complex (MAC) causing pore formation in the membrane. From (Danilova, 2006).

Several studies (Frank et al., 1987; Tanaka et al., 2010; Taylor, 1983) have shown that pore formation due to complement activity is dependent on the presence of antibodies since antibody-depleted serum is unable to form pores on the bacterial surface (Figure 5.4).

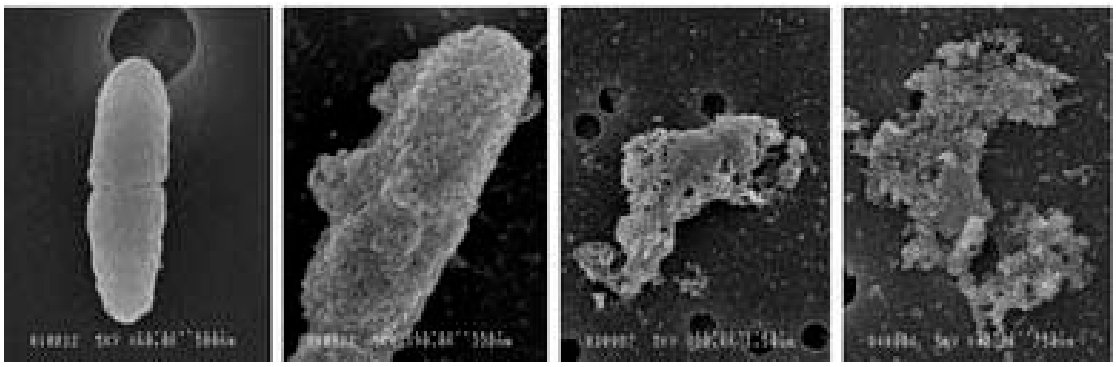

Figure 5.4. Bacteriolysis of serum-treated *Pseudomonas aeruginosa* caused by visible pore formation. Pictures from left to right show pre-treated cells (far left) and cells in different stages of bacteriolysis. Lysis was associated with disposition of complement component C9 but only in the presence of antibodies. Modified from (Tanaka et al., 2010).

It has been suggested that antibodies directs the complement attack by enhancing the specificity of the reaction (Frank et al., 1987; Joiner et al., 1987) but it also has an involvement in the formation of the pore complex itself (Tanaka et al., 2010).

Heat-treatment of plasma (Hazen, 1943; Zinsser and Johnson, 1911) proved early on to be a simple way to inactivate the complement system, and this method can be used to get a preliminary indication of the role of the complement in studies where the bactericidal effect of plasma or synergy with antimicrobial compounds needs elucidation (as explained in the next section). Another more direct method for complement inactivation is the use of antibodies to inhibit specific components of this system (Darveau et al., 1991; Dutcher et al., 1978).

The evasion of complement activity by bacteria can result in persistence of infection (Granoff, 2009; Zipfel, 2009). In such serum-resistant strains the MAC is formed but not properly inserted into the membrane (Frank et al., 1987).

5.3. Activity of antibacterials in the presence of innate immune factors

Since the activity of the innate immune system is formed by a complex network of antimicrobial compounds, the introduction of foreign antimicrobial drugs into this system can have consequences. Antibiotics for treatment of bacterial infections were a landmark in modern medicine and are now the common route when the immune system fails. The structure and mechanism of AMPs are different and could potentially have more dire consequences on the function of the immune system.

Hence, it is important to investigate the activity of these compounds in the presence of an active immune system.

5.3.1. Activity in the presence of single immune factors

It has been stated that the therapeutic use of AMPs could "arm the enemy" (Bell and Gouyon, 2003) i.e. allow the bacteria to develop AMP resistance, which could affect bacterial susceptibility to human AMPs. In fact a few studies have indicate that this concern is justified (Habets and Brockhurst, 2012; Jones et al., 2008; Mishra et al., 2011b), but similar studies with antibiotics have suggested that they too could be a cause of concern (Kristian et al., 2007; Zahner et al., 2010). Cross-resistance of daptomycin-resistant *Staphylococcus aureus* to human AMPs (i.e. hNP-1 and thrombin-induced platelet bactericidal protein (tPMP)) has been found in two separate studies (Jones et al., 2008; Mishra et al., 2011b). Daptomycin is a lipopeptide antibiotic, but have a similar membrane-disrupting mechanism of action as many AMPs. Bacteriostatic antibiotics may similarly impair the activity of LL-37 against *Escherichia coli* and *S. aureus in vitro* as well as the complement-mediated killing of *E. coli* (Kristian et al., 2007).

The combination of antimicrobial drugs and immune factors can also result in synergy. Consequently, sub-inhibitory concentrations of antibiotics increase the bacterial susceptibility to hNP-1 and tPMP (Xiong et al., 1999), the stability of membrane attack complex of the complement cascade (Schweinle and Nishiyasu, 1992) and neutrophil phagocytosis (Mandell and Afnan, 1991). I*n vitro* synergy has also been demonstrated between lysozyme and different groups of AMPs (Bals et al., 1998; Yan and Hancock, 2001). However, we were not able to demonstrate synergy between a chimera and various immune factors (a short analogue of LL-37, lysozyme or hydrogen peroxide) in their activity against *E. coli* nor did we find a change in the susceptibility to these compounds after acquisition of chimera resistance (Paper III).

The mechanisms behind the interaction of immune factors and antimicrobials are often unknown, and it seems like that the exact mechanisms vary between different combinations. One study showed that the antagonistic effect of bacteriostatic antibiotics on LL-37 activity was due to preferential targeting at the plane of cell division of the latter, a condition of heightened susceptibility that the bacteriostatic antibiotics prevented (Kristian et al., 2007). Generally, for drugs having intracellular targets, the activity of immune factors (AMPs, lysozyme or complement) is likely to disrupt the cell envelope thereby potentially increasing the intracellular concentrations of the compound (Dutcher et al., 1978). Increasing the permeability of the bacterial cell membrane is also an effective method in

combinatory antimicrobial drug treatment or for sensitization of resistant strains (Pomares et al., 2010). Many immune factors are active against different components of the cell envelope, of which the cell membrane is an often described target for natural AMPs and the principle target of synthetic AMPs (Vooturi and Firestine, 2010). However, since large variations occur in the exact interaction of drugs or immune factors with components of cell envelope, seen by the lack of cross-resistance often found between membrane-active AMPs, it is possible that the activity of one compound primes the bacterial cell for activity of the other (Dutcher et al., 1978; Schweinle and Nishiyasu, 1992; Vaara and Vaara, 1983).

In line with this, we saw that disruption of the outer membrane by the chelating agent EDTA subsequently increases the susceptibility of *E. coli* to the chimeras (Figure 3.3) indicating that combining the chimeras with another compound that has the cell envelope as a target can at least in some cases result in synergy.

5.3.2. Activity in a simulated immune system environment

Human or mammalian whole blood and cell-free derivatives thereof are widely used simulated *in vivo* systems for predicting the potential systemic activity of antimicrobial compounds (Taylor, 1983). Whole blood demands easy access to donors, and the majority of studies have therefore been done with blood serum; blood which has been allowed to clot after which blood cells are removed. However, adding anticoagulant to freshly drawn blood before removal of blood cells will result in blood plasma, which essentially is the cell-free fraction of whole blood while still containing the same soluble components and is hence likely to be a more correct model system than serum.

Determining the effect single immune factors may have on the activity of antimicrobial drugs (as described above) is relatively straightforward and ideas to the mechanism behind interactions can even be proposed if the bacterial target of both individual compounds is known. Determining cause of effects in a less easily defined immune system environment such as blood plasma or blood serum is more difficult because it is often not known what plasma or serum components actually mediates an effect. In cases where the blood plasma in itself seem to exert an effect on the bacteria it becomes even more difficult to distinguish plasma-mediated potentiation of drug activity from the bactericidal activity of blood plasma (Paper III).

As part of the pre-clinical development of new antimicrobials, the activity of these compounds is often determined in whole blood, plasma or serum to determine the potential *in vivo* activity in a

simple (and safe) model. Hence, activity has been determined in these fluids for both antibiotics (Dutcher et al., 1978; Pruul and McDonald, 1992) and AMPs (Deslouches et al., 2005a; Viljanen et al., 1986; Yeaman et al., 2002; Zhanel et al., 1998). Since it is our aim to use the present peptidomimetics for therapeutic use, we similarly wanted to determine their activity in a simulated *in vivo* system such as human blood plasma and chose a single α-peptide/β-peptoid chimera (compound 30) from the library for this. We found a pronounced increase in activity in the presence of blood plasma (Table 5.1, Paper III).

Table 5.1. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of compound 30 against *Escherichia coli* grown in MHB with/without human blood plasma, From Paper III.

Growth conditions	MIC (μg/mL)	MBC (μg/mL)
MHB media	4	4
MHB + 50 % plasma	0.125-1	1
MHB + 50 % inactivated plasma	8	8

The potentiation by plasma was abolished following heat-treatment similar to observations in previous studies of AMP activity (Fierer and Finley, 1979; Vaara et al., 1984; Yeaman et al., 2002; Zhanel et al., 1998). It is well known that heat-treatment of plasma inactivates the complement system (Hazen, 1943). It has therefore become a widely used method to establish the role of complement in plasma potentiation of antimicrobial drugs (Kristian et al., 2007; Odio et al., 1984; Pruul and McDonald, 1992). The relevance of heat-inactivation in these studies is emphasized by the proposal that the bacteriolytic activity of plasma and serum against Gram-negatives stems primarily from complement-mediated killing (Taylor, 1983).

Other studies have used complement-directed antibodies (Dutcher et al., 1978) or complement deficient sera (Darveau et al., 1991; Darveau and Cunningham, 1990; Fierer and Finley, 1979; Vaara et al., 1984) to investigate the role of complement in plasma potentiation. Consequently, in the majority of studies with heat-treatment or complement-deficient serum it is suggested (or even concluded) that complement is responsible for observed plasma potentiation of antimicrobial drugs ((Darveau et al., 1991; Davidson et al., 1991; Dutcher et al., 1978; Fierer and Finley, 1979; Tateda et al., 1993); only in a single study did heat-inactivation of serum not inhibit the enhancement of antibiotic activity (Pruul and McDonald, 1992), but no suggestions was made to what serum components then mediates the observed effect.

The knowledge that blood components may potentiate the activity of antimicrobial drugs is important in the fight against AMP- and antibiotic-resistant bacteria, since it can be speculated if *de facto* resistance actually will be diminished once bacteria enter the body. We demonstrated that plasma susceptibility of three chimera-resistant isolates increased following resistance acquisition and that chimera MIC against these isolates returned to wild type level in the presence of plasma (Paper II). A few other studies have investigated plasma susceptibility of antibiotic-resistant strains (Alexander et al., 1980; Dutcher et al., 1978; Kristian et al., 2007; Odio et al., 1984), but several of these studies were done with unstable (i.e. inducible resistant) strains or found no changes in the effect of serum addition compared to what is seen for antibiotic-susceptible strains. One study (Odio et al., 1984) did, however, show that the MIC of a mezlocillin-resistant *E. coli* approached the MIC of mezlocillin-susceptible *E. coli* in the presence of serum. Though the results from our study are still preliminary, they speak strongly against the notion that the use of AMP therapeutics would "arm the enemy" as have been previously suggested (Bell and Gouyon, 2003).

5.4. Conclusions from chapter 5

Evaluation of the therapeutic potential of AMPs and peptidomimetics includes determining the activity of these compounds under physiologically relevant conditions. However, once in body fluids AMPs are prone to bind to proteins, be degraded by proteases or interact with the immune system making it difficult to predict the potential *in vivo* activity. The activity of the immune system is mediated by a complex network of antimicrobial compounds that can diminish or increase the activity of therapeutic AMPs. Blood plasma or blood serum can be used as simulated *in vivo* systems. Several studies have shown that potentiation of AMP activity in this environment is due to interaction with the complement system, and our results indicated that the same was the cause for the observed plasma potentiation of α-peptide/β-peptide activity. Using chimera-resistant isolates we additionally showed that acquisition of resistance lead to increased plasma susceptibility. Our results significantly increase the therapeutic potential of these compounds.

6. Concluding remarks and future perspectives

Antimicrobial peptides (AMPs) are considered promising future drugs for treatment of bacterial infections. Several AMPs are now in clinical development, and an additional few have already been marketed. However, due to unfavourable toxicity towards human cells the application of these compounds is often restricted to topical use. The balance between retaining high antibacterial activity and limiting toxic effects can be fine-tuned by the use of chemical synthesis to create novel peptidomimetics. This involves minor structural modifications of synthetic AMP analogues allowing repeated optimization of antibacterial activity, while making it possible to limit undesirable side-effects. One of the main purposes of this thesis was to establish how peptide structure and antibacterial activity are correlated, and additionally to determine if mechanistic differences could explain resulting changes in activity.

All work in this thesis was done using a series of α-peptide/β-peptoid chimeras designed and synthesized at the Faculty of Pharmaceutical Sciences, Copenhagen University. These compounds are distinctive because their design does not allow the compound to form the amphipathic helical secondary structure observed for several other synthetic analogues. A heterogeneous design allows for a higher number of structural modifications and has previously been most studied for α/β-peptides. For the present work we focused on establishing the antibacterial effect of different α-amino acid compositions, the presence of chirality in the β-peptoid unit and modifications of peptide chain length. Only chain length had a pronounced effect on MIC values since analogues shorter than 12 residues were virtually inactive. Chimeras that were non-chiral in the β-peptoid unit have been reported to form a lower degree of secondary structure than chiral analogues. Our findings indicate that the ability to form secondary structures only has a minor effect on activity. This warrants for additional studies of how compounds with different levels of chirality interact with the bacterial cell membrane.

We studied the antibacterial mechanism of action for selected chimeras. In line with others we found that the chimeras permeabilize the membrane leading to leakage of intracellular compounds, in our case ATP. Whereas most studies have been performed on model membranes, we chose to use viable bacterial cells. Importantly, the chimera concentration used for achieving leakage was lower than the observed MIC values when bacterial cell numbers are taking into account. This discrepancy suggests that i) the cell membrane is damaged but the cells remain viable or ii) that only the cell membranes

of a proportion of cells is disrupted. Our findings from using series of chimeras of different length (i.e. 8, 12 and 16) residues clearly demonstrated that though some ATP is leaked from cells this does not necessarily lead to a loss in number of viable cells. Hence, bacteria may survive despite of (minor) damage to the membrane; a conclusion that is important considering the number of studies done on peptide activity against model membranes. The length series included both lysine and homoarginine in the repeat, and only the analogue with a length of 16 residues caused complete depletion of intracellular ATP. This is in line with what has been found previously for α-helical peptides, but since we don't yet know the precise secondary structure of these compounds, a model for their conformation during pore formation/disruption is presently lacking. Conversely, two other chimeras (30 and 34) with a length of 12 residues and only containing homoarginine as the α-amino acid caused a critical degree of membrane disruption for the number of viable cells to decrease. Collectively, our results suggest that despite of minor structural differences between the chimeras, their interaction with the membrane varies profoundly.

The considerable attention AMPs has received over the last decades stems primarily from the notion that resistance development is unlikely. If resistance to these compounds also confers resistance to AMPs of the human immune defence, it is important that this claim holds true since it would otherwise compromise our ability to resist infections. Nevertheless, several research groups have successfully developed resistance towards AMPs and it was therefore important to clarify the resistance potential of the α-peptide/β-peptoid chimeras. Accordingly, we demonstrated that continuous exposure to one of the synthetic analogues did select for resistance, which was the first time resistance towards a peptidomimetic was reported. It can be speculated that it may be less desirable to conduct comprehensive studies of potential resistance development for in house designed analogues. Importantly, we demonstrated that though resistance to peptidomimetics may develop, no cross-resistance was found to a range of membrane-active AMPs. The details of the interaction between the bacterial membrane and AMPs/peptidomimetics have been extensively debated and several models have been proposed for the interaction which additionally may differ for different compounds. Our results illustrate that the resistance mechanism towards peptidomimetics does not confer universal protection against other membrane-active AMPs.

Comparing genome sequences of wild type and peptidomimetic resistant mutant, led us to consider a SNP in the gene encoding the membrane-bound lytic murein transglycosylase (mltD) as the most likely cause for resistance to the chimeras. Consequently, we plan to introduce this SNP into the ancestral wild type bacteria to see if this confers a decrease in chimera susceptibility. Since no other

studies have successfully developed resistance to peptidomimetics, it is unknown if the mechanism is specific for the present peptidomimetics. Resistance mechanisms involving modifications to the cell wall thereby causing reduced AMP entry has been reported numerous times. Consequently, it is peculiar that we did not observe any cross-resistance to AMPs outside the peptidomimetics library. Our findings could suggest that the α-peptide/β-peptoid chimeras in addition to its cell-permeabilizing activities also have an effect on the peptidoglycan layer. If these compounds have a distinct way of interacting or traversing the peptidoglycan layer that differs from that of natural AMPs this would explain why resistance does not confer universal protection against AMPs. We only investigated chimera resistance development in *Escherichia coli* and it would be interesting to investigate if the mechanisms differ for other bacteria. A similar study has been performed recently for *Staphylococcus epidermidis*, but a single apparent resistance mechanism was not found (Y. Liu, personal communication). Interestingly, this study indicated that two distinct SNP profiles develop when the bacteria are exposed to one of two different chimeras. Hence, the development of resistance may be different for compounds with minor structural differences, which is in direct line with what we have found to occur for membrane activity. These observations have led us to hypothesize that resistance development may be circumvented through structural modifications of the present scaffold, which we plan to pursuit in future years. Hence, different structures will be probed for their ability to induce resistance leading to repeated optimization of promising structures. If resistance development can be prevented completely, this will increase the therapeutic potential of peptidomimetics considerably.

The findings in this thesis have demonstrated how structure affects the antibacterial activity and mechanism of action of peptidomimetics. In addition, we showed that resistance may develop, which cautions against the indiscriminate use of these compounds. We strongly recommend that comprehensive studies of potential resistance development should be an integrated part of the characterisation of novel peptidomimetics. This can be concluded even though we observed that resistant isolates were in fact more sensitive to (immune) factors present in human plasma. We need to conduct further studies to provide an explanation for these results, before we can determine the effect resistance may have on the human immune system. Collectively, our results do not discourage the development of peptidomimetics as potentially useful antibacterial agents. The great potential of these compounds is based on the possibility to optimize their properties through structural modification. Hence, AMPs and synthetic analogues thereof in particular, can still be considered promising future antibacterials.

7. References

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Paper 1

Line Hein-Kristensen, Kolja M. Knapp, Henrik Franzyk & Lone Gram (2011)

Bacterial membrane activity of α-peptide/β-peptoid chimeras: Influence of amino acid composition and chain length on the activity against different bacterial strains.

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Bacterial membrane activity of α-peptide/β-peptoid chimeras: Influence of amino acid composition and chain length on the activity against different bacterial strains

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Abstract

Background: Characterization and use of antimicrobial peptides (AMPs) requires that their mode of action is determined. The interaction of membrane-active peptides with their target is often established using model membranes, however, the actual permeabilization of live bacterial cells and subsequent killing is usually not tested. In this report, six α-peptide/β-peptoid chimeras were examined for the effect of amino acid/peptoid substitutions and chain length on the membrane perturbation and subsequent killing of food-borne and clinical bacterial isolates.

Results: All six AMP analogues inhibited growth of twelve food-borne and clinical bacterial strains including Extended Spectrum Beta-Lactamase-producing *Escherichia coli*. In general, the Minimum Inhibitory Concentrations (MIC) against Gram-positive and -negative bacteria were similar, ranging from 1 to 5 μM. The type of cationic amino acid only had a minor effect on MIC values, whereas chain length had a profound influence on activity. All chimeras were less active against *Serratia marcescens* (MICs above 46 μM). The chimeras were bactericidal and induced leakage of ATP from *Staphylococcus aureus* and *S. marcescens* with similar time of onset and reduction in the number of viable cells. EDTA pre-treatment of *S. marcescens* and *E. coli* followed by treatment with chimeras resulted in pronounced killing indicating that disintegration of the Gram-negative outer membrane eliminated innate differences in susceptibility. Chimera chain length did not influence the degree of ATP leakage, but the amount of intracellular ATP remaining in the cell after treatment was influenced by chimera length with the longest analogue causing complete depletion of intracellular ATP. Hence some chimeras caused a complete disruption of the membrane, and this was parallel by the largest reduction in number of viable bacteria.

Conclusion: We found that chain length but not type of cationic amino acid influenced the antibacterial activity of a series of synthetic α-peptide/β-peptoid chimeras. The synthetic chimeras exert their killing effect by permeabilization of the bacterial cell envelope, and the outer membrane may act as a barrier in Gram-negative bacteria. The tolerance of *S. marcescens* to chimeras may be due to differences in the composition of the lipopolysaccharide layer also responsible for its resistance to polymyxin B.

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Background

Antimicrobial peptides (AMPs) are host defence molecules that constitute an essential part of the innate immune system among all classes of life [1]. Most AMPs permit the host to resist bacterial infections by direct killing of invading bacteria or other microorganisms, however, many AMPs are also immuno-modulatory and thus enhance the host defence against pathogens [2-5].

In addition to their natural role in combating infections, AMPs are recognized as promising alternatives to conventional antibiotics for which development of resistance has become an ever-increasing concern [6-8]. Peptide based drugs are often hampered by a rapid *in vivo* degradation, however, this may be circumvented by stabilizing natural AMPs by single-site substitutions or by designing novel synthetic analogues with an altered backbone that confers complete stability to the compounds. Careful investigation of structure-activity relationships may eventually allow design of optimised antimicrobial compounds with high activity and minimal side effects [9-15].

Many AMPs fold into an amphipathic structure, and it is believed that this topology enables pore formation or disintegration of bacterial cell membranes leading to bacterial cell death. The amphipathic properties usually include cationic patches that promote interaction with the anionic bacterial membrane as well as hydrophobic patches that favor integration into the membrane. Since this is the most common mode of action for AMPs there has been an intense focus on their ability to adapt an amphipathic conformation [16,17]. In particular, design of peptides with a high propensity to fold into a helical amphipathic conformation has attracted considerable interest [13,18-20].

We have previously described a synthetic approach for design of α-peptide/β-peptoid chimeras possessing a design with alternating N-alkylated β-alanine (β-peptoid) and α-amino acid units (Figure 1). In addition, preliminary investigations showed that such peptidomimetics constitute a novel subclass of proteolytically stable antimicrobial compounds [21-23]. This design displays chiral unnatural β-peptoid residues that appear to contribute with structure-promoting effects and lipophilicity, while strongly cationic properties and intramolecular hydrogen bonding capacity are introduced via the α-amino acids lysine and/or homoarginine [24]. The precise secondary structure of these chimeras still remains to be elucidated, nevertheless, circular dichroism (CD) spectroscopy clearly indicates the presence of some degree of secondary structure [22,23]. Interestingly, a higher degree of secondary structure was found for analogues containing chiral side chains in the β-peptoid units (i.e. compounds 2 and 3 in Figure 1) as compared to chimeras with achiral β-peptoid residues (i.e. compound 1 in Figure 1) [22], but the effect of this on antibacterial activity remains largely unresolved [23].

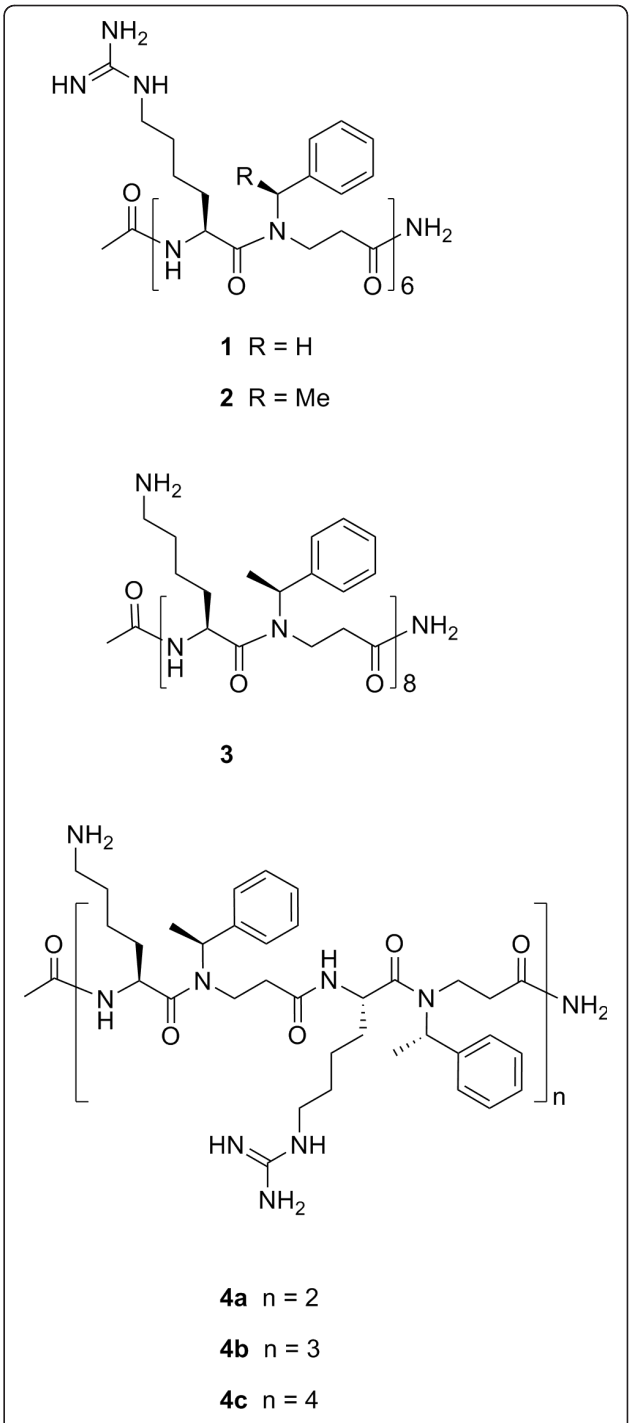

Figure 1 Chemical structure of the six α-peptide/β-peptoid chimeras

The membrane-destabilizing effects of the chimeras have only been investigated in model liposomes prepared from phosphatidylcholine, a phospholipid found

predominantly in eukaryotic cells, and several of the chimeras permeabilized such liposomal membranes [24]. Most studies on membrane activity of antimicrobial peptides have in fact been performed on model membranes [25-28] while the effects on cell membranes of viable bacteria have often not been tested. Also, the effect of membrane permeabilization on killing of bacteria has not been tested [27].

Here, we test the antibacterial effect of six chimeras against a spectrum of bacterial strains that include several important clinical and food-borne pathogens. The main purpose was to examine how the type of cationic amino acid and sequence length affected the antibacterial activity and to correlate this to a potential membrane-related mode of action in viable bacteria.

Part of this work was presented at the 50th InterScience Conference on Antimicrobial Agents and Chemotherapy in Boston 12-15th of September 2010.

Methods

Bacterial strains and culture conditions

Initial activity experiments were carried out with twelve strains from seven bacterial species representing common laboratory strains and clinical strains derived from both food-borne and nosocomial infections (Table 1). Stock cultures were stored at -80°C in 4% (w/v) glycerol, 0.5% (w/v) glucose, 2% (w/v) skimmed milk powder and 3% (w/v) tryptone soy powder. All experiments were carried out with bacteria incubated for one night (i.e. approximately 18 hours) at 37°C. Experiments were performed in cation-adjusted Mueller Hinton II broth (MHB) (Becton Dickinson 212322) adjusted to pH 7.4 or Tryptone Soy Broth (TSB) (Oxoid CM0129) for the ATP leakage assays. Brain Heart Infusion (BHI) (CM1135) with agar (VWR 20768.292) 1.5% as gelling agent was used throughout for colony plating.

Table 1 Origin and reference of bacterial strains used in the present study

	Origin	Ref
S. aureus 8325-4	Wildtype	[59]
K. pneumoniae ATCC 13883	Human, clinical	-
S. marcescens ATCC 8100	Human, clinical	-
E. coli ATCC 25922	Wildtype	-
E. coli MG1655	K-12 F^- lambda$^-$	[60]
E. coli AAS-EC-009	Human, clinical	[a]
E.coli AAS-EC-010	Human, clinical	[a]
L. monocytogenes 4446	Human, clinical	[61]
L. monocytogenes N53-1	Food processing	[62]
L. monocytogenes EGD	Wildtype	[b]
V. vulnificus ATCC^T	Human, clinical	-
V. parahaemolyticus ATCC^T	Human, clinical	-

Susceptibility testing were carried out with a selection of twelve different bacterial strains comprising common laboratory strains and clinical strains derived from food-borne pathogens as well as pathogens responsible for nosocomial infections. [a] ESBL-producing clinical samples from Danish patients in 2007; [b] This strain was kindly provided by Werner Goebel, University of Würzburg.

Peptide synthesis and selection

α-Peptide/β-peptoid chimeras consisting of alternating repeats of natural cationic α-amino acids and synthetic lipophilic β-peptoid residues were prepared by solid-phase synthesis as previously described [21,22]. Six chimeras were investigated in this study. The possible differences in sensitivity of different bacterial species were evaluated by testing the analogues 1, 2 and 3, distinguished by different degrees of chirality and type of cationic amino acid. Additionally, the mixed series 4a, 4b and 4c, differing only in the chain length, was used for evaluating the effect of this on antimicrobial activity (Figure 1). Compounds 1, 2 and 3 have been described previously [23,24,29], while the series 4a, 4b and 4c were synthesized using the already established synthesis protocols involving known dimeric building blocks [21,22]. The identity of the primary peptidomimetic sequences 4a, 4b and 4c were confirmed by high-resolution MS (Bruker MicroTOF-Q LC mass spectrometer equipped with an electrospray ionization source): compound 4a, (m/z) $[M+4H]^{4+}$ obsd. = 339.9727 (calcd. = 339.9719, ΔM 2.3 ppm); compound 4b, (m/z) $[M+5H]^{5+}$ obsd. = 402.0614 (calcd. = 402.0608, ΔM 1.4 ppm); compound 4c, (m/z) $[M+6H]^{6+}$ obsd. = 443.2880 (calcd. = 443.2879, ΔM 0.2 ppm). Peptides were solubilized to a stock of 10 mg/mL in sterile MilliQ water and stored at -20°C.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The Minimum Inhibitory Concentration (MIC) of the chimeras was determined against the spectrum of bacteria using the microdilution method according to guidelines of the Clinical and Laboratory Standards Institute (CLSI) [30]. Chimera 1:2 serial dilutions were prepared from 1,024 μg/mL stock solutions to give a final range of 512-0.5 μg/mL in the wells. This corresponds to a final range of 144 to 0.14 μM for the heaviest chimera (i.e. chimera 4c) and of 282 to 0.27 μM for the lightest chimera (i.e. chimera 4a). Colonies grown overnight (i.e. approximately 18 hours) on BHI agar were suspended in 0.9% saline to give a turbidity of 0.13 at OD_{546} (approximately 1×10^8 CFU/mL), and then diluted in MHB pH 7.4 to a final concentration of 5×10^5 CFU/mL in each well. Following CLSI guidelines the media for testing of *Listeria monocytogenes* strains were supplemented with 2.5% lysed horse blood. Polypropylene plates (Nunc 442587) were used to minimize peptide binding and incubation time was 18-20 hours at 37°C. MIC was determined in a minimum of two technical replicates as the lowest concentration of the

peptide analogue where no visible growth was found. The Minimum Bactericidal Concentration (MBC) was determined by plating 10 μL of the suspension from the first three wells without growth on BHI agar and incubating these for 24 hours at 37°C. MBC was the lowest concentration at which a 99.9% reduction in CFU/mL was observed. Activity is expressed in μmol/L to enable a direct comparison of analogues with different length (= size).

Killing kinetics of *Staphylococcus aureus* and *Serratia marcescens*

In vitro time-kill curves for chimera 1, 2 and 3 were determined against *S. aureus* 8325 (MIC μM: chimera 1 5.9; chimera 2 2.8; chimera 3 18.7) and *Serratia marcescens* ATCC 8100 (MIC μM: chimera 1 46.8; chimera 2 45.5; chimera 3 150.0). These two bacterial strains represent organisms susceptible and tolerant to the chimeras, respectively. The bactericidal effect of the three chimeras was tested at MIC in two independent experiments; additionally the effect of chimera 2 was tested at ¼ and 1/2 times MIC. In brief, a suspension prepared from fresh overnight colonies as described above was transferred to 2 mL PBS or cation-adjusted MHB with chimera added (from a 10 × MIC solution) to give a similar bacterial cell density as employed in the MIC determination; the resulting suspension was then incubated at 37°C, 300 rpm. Samples for colony determination were taken at 0, 1, 2, 4, 6 and 8 hours after addition and transferred to a ten-fold dilution row. Colony counts were determined after incubation for 24 hours at 37°C.

ATP leakage assay

Pore formation as caused by peptide addition was determined by measuring ATP leakage from the bacterial cell using a bioluminescence assay [31]. The assay was used to estimate differences between sub-typical chimeras 1, 2 and 3 on *S. aureus* and *S. marcescens* and to evaluate the effect of chain length of mixed type chimeras 4a, 4b and 4c on *S. aureus*. In brief, bacteria were grown in TSB at 37°C for 24 hours and then re-inoculated in TSB at 37°C for 6-8 hours until an absorbance at 546 nm of 2.5 for *S. aureus* and 2.0 for *S. marcescens* and then harvested (10 min at 2,000 × g). The bacteria were grown to a high absorbance since a high concentration of bacteria was necessary in order to get a measurable response in the ATP leakage assay. Cells were washed once in 50 mM potassium phosphate buffer (pH 7.0) and once in 50 mM HEPES buffer (pH 7.0), before the pellet was resuspended in HEPES buffer to an OD_{546} ~ 10, and then stored on ice. Before chimera addition bacteria were pre-incubated with 0.2% (w/v) glucose to energize the cells. In general a chimera dose of 1000 μg/mL (corresponding to 280-552 μM for all chimeras) was used for all assays; however, for determining dose response curves additional doses of 100 (28-55 μM), 250 (71-137 μM) and 500 (140-276 μM) μg/mL were tested, and only the immediate release was noted. Total ATP and extracellular ATP were determined with a luminometer (Pharmacia Biotech Novaspec II Visible Spectrophotometer). Intracellular volumes [32] of *S. aureus* and *S. marcescens* (0.85 μm^3 and 1.7 μm^3, respectively) were subtracted from the total volume before calculating the extracellular ATP concentration; the intracellular ATP concentration could then be calculated from this and the total ATP. ATP leakage kinetics was determined on a bacterial suspension prepared as above. Samples were taken at time 0, 5, 10, 20, 30 and 60 minutes and viable counts determined. Both the ATP leakage assay and killing kinetics performed under the same assay conditions were performed in two independent experiments.

Results

Based on our previously published work on α-peptide/β-peptoid chimeras [23,24,29] we selected six compounds for the present study. Our main purpose was to examine the influence of the type of cationic amino acid and chain length on antibacterial activity and specificity. Also we aimed at elucidating the mechanism of action against live bacterial cells and determine if this (membrane perturbation) was influenced by the chimera structural characteristics. We measured ATP leakage from chimera-treated cells as an indication of membrane pertubation. Comparing the ATP leakage with time-kill studies allowed us to establish if there was a direct correlation between permeabilization of the membrane and killing of bacterial cells.

MIC and MBC against clinical and food-borne pathogens

Twelve strains representing seven bacterial species were tested for their susceptibility to the peptide analogues. The analogues exhibited a broad-spectrum activity with no distinct differences between Gram-positive and -negative bacteria (Table 2). Five of the six chimeras had a strong antibacterial effect with MIC values below 5 μM. Important food-borne pathogens were included in the susceptibility assay panel. Thus, three *L. monocytogenes* strains representing both a clinical lineage 1 strain (strain 4446) and a persistent lineage 2 strain from a food-processing plant (strain N53-1) as well as clinical isolates of *V. vulnificus* and *V. parahaemolyticus* were examined.

The MIC values of chimeras 1, 2 and 3 were similar, indicating that the β-peptoid side chain chirality (i.e. 1 vs. 2) had no effect on antibacterial activity and that the 12-meric homoarginine (hArg) based sequence 2 was likely equalled by the longer 16-meric lysine-containing analogue 3. Generally, low MIC values were found for these

Table 2 Minimum Inhibitory Concentration (μM) of the six α-peptide/β-peptoid chimeras in the present study

	Chimera 1	Chimera 2	Chimera 3	Chimera 4a	Chimera 4b	Chimera 4c
S. aureus 8325	5.9	2.8	18.7	141.2	23.8	4.5
K. pneumoniae ATCC 13883	1.5	2.8	37.5	282.4	23.8	9.0
S. marcescens ATCC 8100	46.8	45.5	150.0	> 282.4	190.3	71.8
E. coli ATCC 25922	1.5	2.8	9.4	141.2	3.0	2.2
E. coli MG1655	1.5	2.8	4.7	141.2	5.9	2.2
E. coli AAS-EC-009	1.5	2.8	9.4	141.2	11.9	4.5
E.coli AAS-EC-010	1.5	1.4	9.4	141.2	3.0	2.2
L. monocytogenes 4446	2.9	1.4	1.1	70.6	3.0	1.1
L. monocytogenes N53-1	2.9	2.8	1.1	70.6	5.9	1.1
L. monocytogenes EGD	1.5	2.8	1.1	70.6	3.0	1.1
V. vulnificus ATCC^T	1.5	1.4	2.3	35.3	3.0	2.2
V. parahaemolyticus ATCC^T	1.5	1.4	2.3	70.6	3.0	1.1

Minimum Inhibitory Concentration of the six peptidomimetics in this study against the spectrum of bacteria expressed in μM. Values were obtained from a minimum of two independent trials. The Minimum Bactericidal Concentration (MBC) was in all assays equal to or a maximum of one two-fold higher than the MIC value indicating a bactericidal mode of action.

three compounds, however, the activity of chimera 3 was slightly lower than for chimera 1 and 2 against some of the bacteria i.e. *S. aureus*, *K. pneumoniae* and *S. marcescens*.

Chimeras 4a, 4b and 4c all have a 1:1 mixture of Lys and hArg residues, but differ in length (8-16 residues), and this had a marked effect on their antibacterial activity. The pattern was the same against all bacterial strains tested. The longest of the three, chimera 4c, was the most active compound with MIC values of 1.1-2.2 μM against the foodborne pathogens *L. monocytogenes* and *Vibro spp*. Chimera 4c was also active against the clinical strains of *E. coli*, *S. aureus* and *K. pneumoniae* with MIC values in the range of 2.2-9.0 μM (Table 2). Chimera 4b, with a length of 12 residues, was less antibacterial with MIC values approximately 2-3 times higher than those of the 16-mer 4c (Table 2). Chimera 4a being only half the length of chimera 4c was the least antibacterial as the MIC values were 15-70 times higher than those of chimera 4c (Table 2). Thus, the relative increase in activity was much larger for elongation with a third repeating unit (i.e. from 8-mer 4a to 12-mer 4b), than the further elongation of 4b with a fourth repeating unit to afford 4c, revealing the minimally required length of an active AMP analogue to be approximately 12 residues.

Two Extended Spectrum Beta-Lactamase (ESBL)-producing *E. coli* clinical isolates (AAS-EC-009 and AAS-EC-010) were included to determine if this antibiotic resistance affected chimera sensitivity. However, the chimeras were as effective against these strains as against non-ESBL strains indicating that resistance mechanisms conferring resistance to conventional antibiotics do not diminish the activity of the present peptidomimetics. Interestingly, *S. marcescens*, which is known to be intrinsically resistant to other antimicrobial peptides, was tolerant to all six chimeras (MICs above 46 μM; Table 2), and it most likely possesses resistance mechanisms that are different from those present in the two multi-resistant *E. coli* strains.

All six chimeras had a Minimum Bactericidal Concentration (MBC) equal to or double the MIC. The high similarity between the MIC and MBC values indicates that the chimeras exhibit a bactericidal mode of action.

Killing kinetics in two bacteria with different susceptibility

S. marcescens was the only bacterial strain tested that was tolerant to the α-peptide/β-peptoid chimeras. The strain is the only one considered intrinsically resistant to the polymyxin group of AMPs, and this could explain its resistance to our peptidomimetics. If so, this would indicate that a very similar resistance mechanism was responsible for the observed decrease in susceptibility. Therefore we performed a comparative mechanistic study that also included *S. aureus* and *E. coli* as susceptible reference strains.

We exposed *S. aureus* and *S. marcescens* to peptidomimetics 1, 2 and 3 at three different concentrations in MHB as well as at their MIC concentration in PBS buffer in order to determine whether these chimeras were only active against growing bacterial cells. *S. marcescens* was killed rapidly by chimera 2 (Figure 2A), and the lethal effect was clearly concentration-dependent (Figure 2C). In contrast, *S. aureus* was killed more slowly and with a less pronounced effect of dose (Figure 2B and 2D). Treatment of *S. marcescens* with chimera 2 at its MIC caused a 2 log decrease in the number of viable bacteria within the first hour after which cell numbers declined over the next 5 hours. When the bacteria were treated with the chimera in PBS, the killing occurred very rapidly and no viable cells remained after the first hour. When *S. aureus* was treated with chimera 2 at the MIC in MHB, the number of viable cells did not decrease until after 6

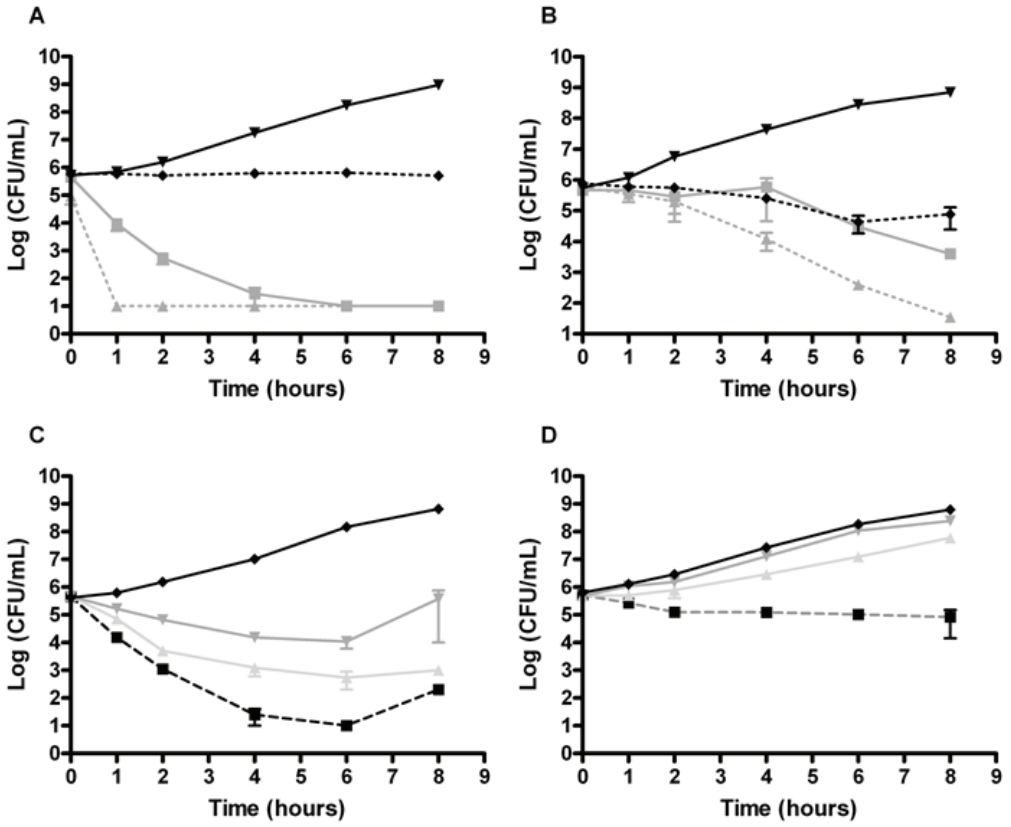

Figure 2 Killing kinetics of chimera 2 against *S. marcescens* (A+C) and *S. aureus* (B+D) displayed as mean number of viable cells with standard error of the mean (SEM). The assays were performed in two independent experiments. Time-kill of the chimera was determined at MIC in MHB (grey solid) and PBS (grey punctuated) and compared to MilliQ-treated control in MHB (black solid) and PBS (black punctuated) for *S. marcescens* (A) and *S. aureus* (B). The effect of chimera concentration on time-kill was determined in MHB at ¼ MIC (dark grey), 1/2 MIC (light grey) and MIC (black punctuated) and compared with MilliQ-treated control (black solid) for *S. marcescens* and (C) and *S. aureus* (D).

hours, however, when treated in PBS, viable cell numbers decreased with log 2 after 4 hours (Figure 2B). Even though a slightly decreased growth rate was observed for *S. aureus* upon treatment with concentrations below MIC as compared to the control, a concentration close to the MIC value was needed to completely inhibit growth of the culture (Figure 2D). In comparison, as low as ¼ MIC resulted in a reduction in cell number of *S. marcescens* (Figure 2C) revealing a more pronounced concentration-dependent killing for this bacterium.

Since the MIC value found for *S. marcescens* was considerably higher than that seen for *S. aureus*, we performed time-kill on *E. coli*, which exhibited a similar susceptibility in terms of MIC to that of *S. aureus*, to test if the rapid lethal effect against *S. marcescens* was due to the higher concentrations of peptidomimetics (*E. coli* ATCC 25922 MIC μM: chimera 1 1.5; chimera 2 2.8; chimera 3 9.4). However, a rapid killing effect was also found for this bacterial species (data not shown) ruling out that the elevated concentrations solely could be responsible for the high killing rate seen for *S. marcescens*.

Membrane perturbation effects in two bacteria with different sensitivity

Killing kinetics often reflect the mode of action, and we hypothesized that differences between *S. aureus* and *S. marcescens* regarding their sensitivity and time-kill might be due to different modes of interaction with the peptidomimetics. Therefore, an ATP bioluminescence assay was employed to determine (i) whether cell envelope perturbation was involved in the antibacterial effect, and (ii) if so, whether the organisms differed in the degree of ATP leakage.

Chimera 1, 2 and 3 caused leakage of ATP from both *S. aureus* and *S. marcescens*, but all three peptidomimetics gave rise to an ATP leakage from *S. aureus* that was substantially larger than that from *S. marcescens* (see Figure 3 for results with chimera 1). The intracellular ATP concentration rapidly approached zero for both bacteria within the first few minutes, whereas the extracellular ATP concentration increased more rapidly during the first minutes for *S. aureus* (~20 μM) than for *S. marcescens* (~5 μM). To examine if this could be due to the fact

that the two bacteria were treated with the same dose despite their very different MIC values, we determined their dose response curves. For both bacteria a minimum chimera dose of 500 μg/mL (i.e. 145-180 μM) was needed to obtain the maximum immediate response (data not shown) ruling out that the rapid release of ATP from *S. aureus* seen in Figure 3A is due to a higher concentration/MIC ratio than employed for *S. marcescens*.

To investigate if the degree of ATP leakage from the bacterial cell corresponded to the simultaneous decrease in the number of viable cells (i.e. if *S. marcescens* cells on the basis of their elevated MIC were in fact able to survive even after a moderate ATP leakage) we determined time-kill under *exactly* the same conditions as the ATP bioluminescence assay had been performed. Irrespective of which of the three chimeras that were used, both bacteria were reduced 2-3 log from an initial value of log ~9.5 per mL within the first 20 minutes before the ATP leakage tailored off and no further decrease in viable count was seen for up to 60 minutes (not shown). This indicates that the degree of ATP leakage from the two bacteria (i.e. the concentration of the extracellular ATP) does *not* reflect differences in viability. No reduction in the number of viable bacteria was seen for the control (not shown), and the intracellular concentration of ATP did not change (Figure 3A and 3B).

Although there was no systematic difference in the MIC values between Gram-positive and -negative bacteria, we speculated that the Gram-negative outer membrane could act as a barrier to the penetration of AMPs, since polymyxin B resistance in *S. marcescens* has been linked to induced changes in the amount and composition of lipopolysaccharide (LPS) in the outer membrane [33]. Moreover, similar resistance-conferring membrane alterations have also been seen for other bacteria in response to polymyxin B treatment [34-36]. Accordingly, we studied how a membrane-destabilizing pre-treatment of *S. marcescens*, *E. coli* and *S. aureus* with the divalent metal cation-chelating agent EDTA would affect the killing caused by chimera 1. In these experiments we used a non-lethal 0.5 mM concentration of EDTA together with the non-lethal 1.5 μM concentration of the tested AMP analogue. A slight reduction in the number of viable cells corresponding to 0.5 log was seen for *S. aureus* when treated with chimera 1 alone while *E. coli* and *S. marcescens* were reduced with 1.5 log (data not shown). No discernable difference in the number of viable cells remaining was observed between *S. aureus* treated successively with EDTA and peptidomimetic and *S. aureus* treated only with the peptidomimetic. In contrast, cell numbers of both *S. marcescens* and *E. coli* were reduced with 4-5 log from an initial value of log ~5.5 within the first 4 hours (not shown) upon treatment with a sub-lethal EDTA concentration together with the chimera. This indicates that the intact outer membrane indeed appears to act as a protective barrier against the antibacterial chimeras.

The effect of chimera chain length on membrane perturbation activity

Peptidomimetics 4a, 4b and 4c consist of the same repeating unit of four residues (Figure 1; n = 2, 3 and 4, respectively), and thus differ only in length. The MIC values increased dramatically when going from 8-mer (4a) to 12-mer (4b) while further elongation to 16-mer (4c) only led to a slight enhancement in potency (Table 2). Hence, we were intrigued to establish whether mechanistic differences could explain this strong correlation.

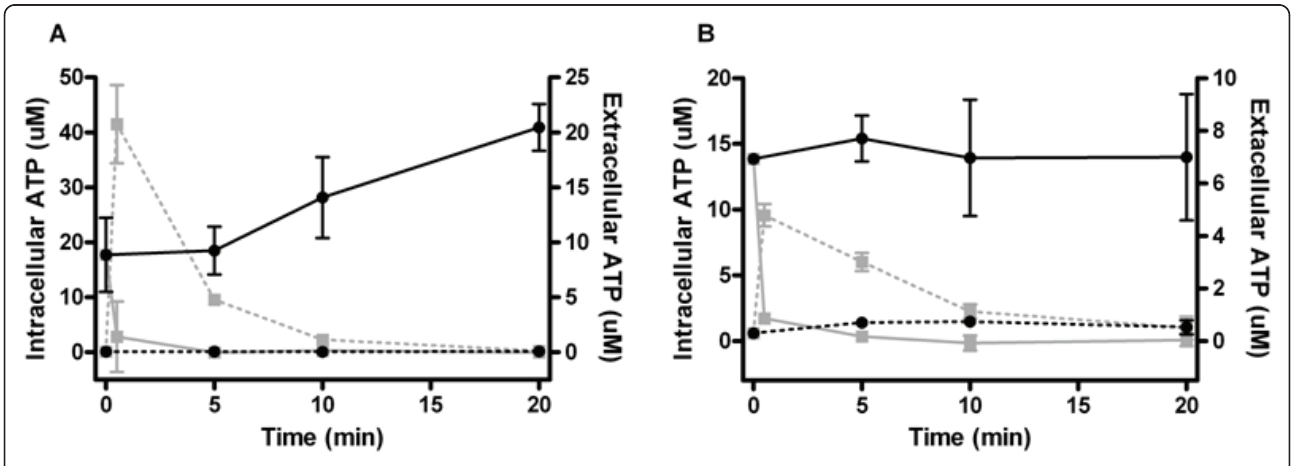

Figure 3 Chimera-induced ATP leakage in *S. aureus* (A) and *S. marcescens* (B) after treatment with 1000 μg/mL chimera. The assays were performed in two independent experiments. Mean (SEM) intracellular (IC, solid line) and extracellular (EC, punctuated line) ATP concentration for *S. aureus* cells (figure A, grey lines) and *S. marcescens* cells (figure B, grey lines) treated with chimera 1 compared to MilliQ-treated control (black lines).

We determined ATP leakage from *S. aureus* when treated with chimeras 4a, 4b and 4c to evaluate the effect of chain length on the extent of pore formation or membrane disintegration caused by the chimeras. Peptidomimetic-induced ATP leakage was markedly different for *S. aureus* treated with chimera 4a (Figure 4A) as compared to *S. aureus* treated with chimera 4c (Figure 4C). The immediate ATP release was approximately 15 μM for both peptidomimetics; however, the intracellular ATP concentration remained at approx. 5 μM, when the bacterial cells were treated with the shorter analogue 4a, whereas cells treated with chimera 4c were immediately depleted of intracellular ATP. Since the leakage was continuous it seemed that the cells were able to maintain the ATP production. *S. aureus* cells treated with the intermediate length 12-meric chimera 4b had the same leakage pattern as induced by chimera 4a. Dose-response profiles were also determined (as already described in the previous section), and despite differences in MIC values between chimeras 4a and 4c, both reached the immediate maximum ATP release at 500 μg/mL (i.e. 276 μM and 140 μM, respectively). Likewise, the observed ATP release was similar immediately upon treatment with either chimera 4a or 4c, and again cells treated with chimera 4a were able to maintain a low intracellular level of ATP.

The fact that some ATP remained in the cell after treatment with chimera 4a could point to an incomplete disruption of the bacterial cell membrane as compared to bacterial cells treated with chimera 4c. To determine if an intracellular ATP concentration of 5 μM had a physiological effect and would allow the bacterial cells to survive, time-kill was again performed under *exactly* the same conditions as used in the ATP assay to allow comparison of ATP leakage with killing kinetics. After treatment with chimera 4c, cell numbers were reduced with 2 log within the first 20 minutes (Figure 4D), however,

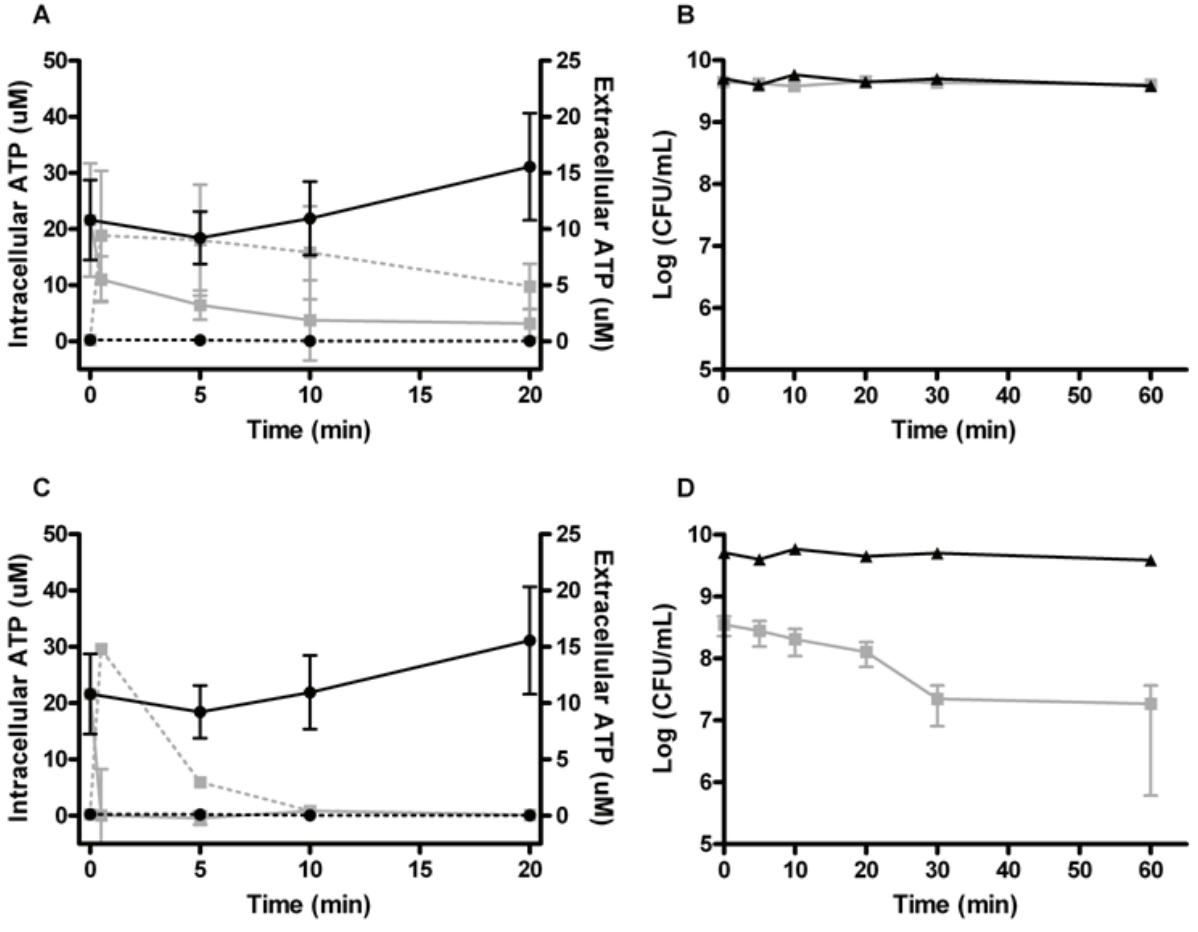

Figure 4 The effect of chimera chain length on ATP release from *S. aureus* after treatment with 1000 μg/mL chimera and the corresponding change in the number of viable cells after treatment with chimera 4a (A+B) or chimera 4c (C+D). The assays were performed in two independent experiments. Mean (SEM) intracellular (IC, solid line) and extracellular (EC, punctuated line) ATP concentration for cells treated with chimera 4a (figure A, grey lines) or 4c (figure C, grey lines) compared to MilliQ-treated control (black lines). Mean (SEM) number of viable cells after addition of chimera 4a (figure B, grey line) or 4c (figure D, grey line) compared to MilliQ-treated control (black line).

after treatment with chimera 4a (Figure 4B) or chimera 4b (not shown) no killing was observed. The pool of intracellular ATP in the peptidomimetic-treated bacterial cells can therefore, as opposed to the amount of leaked ATP, be considered as indicative for the number of viable cells remaining.

Discussion

The aim of this study was to determine the mechanism of action for a series of peptidomimetics, and specifically we set out to probe the importance of amino acid composition and chain length for antibacterial activity. We included a strain intrinsically resistant to AMPs, and addressed whether killing kinetics and AMP mechanism of action in viable bacteria could provide a mechanistic explanation for the much lower susceptibility of *S. marcescens* as compared to the more sensitive bacteria.

We examined the effect of having exclusively lysine or homoarginine cationic residues as well as of substituting the chiral β-peptoids with achiral counterparts as represented by the α-peptide/β-peptoid chimeras 1, 2 and 3 (Table 2). All three peptidomimetics had MIC values of 1-3 μM against most bacterial strains, which compared to many natural AMPs is a high activity [14,19,37-39]. Noticeably, a considerably lower activity against *S. aureus* and *K. pneumoniae* was observed for the lysine-containing chimera 3 (6-13 fold) as compared to the homoarginine-based chimera 2, while only a slightly lower activity of chimera 3 (2-7 fold) was seen compared to chimera 2 when tested against *E.coli*. The reduced chirality in chimera 1 did not give rise to any significant loss of activity as compared to chimera 2. In a preliminary antimicrobial characterization these peptidomimetics were tested against four common bacteria and a fungus [23], whereas the present study also included important food-borne pathogens *L. monocytogenes*, *V. vulnificus* and *V. parahaemolyticus* against which the chimeras also were active (Table 2).

Additionally we investigated the effect of chain length on activity by studying a series of three peptidomimetics (i.e. chimera 4a, 4b and 4c based on the same repeating unit of four residues), which indicated that the minimally required length for an active peptidomimetic is around 12 residues (Table 2). It has previously been reported that 14 amino acids is the minimal sequence required for an active antimicrobial peptide [25], however, this and other studies focused on the effect of length on helicity which implies structural restrictions in the design to enable it to span the lipid bilayer [26,40]. Also, it is clearly established that the low activity earlier reported for the shorter homologues of chimera 3 (e.g. the 12-mer exhibited almost no activity [23]) may be compensated for by a longer sequence. Chimera 4c corresponds to the analogue where half of the lysines in chimera 3 are replaced by homoarginines, and similarly chimera 4b may be considered an analogue derived from chimera 2 by exchanging half of the homoarginines with lysines. Comparison of the activities found for these two pairs indicates that a high content of homoarginines generally induces a somewhat higher potency; especially, the activity against *S. aureus* and *K. pneumoniae* is clearly promoted by a prevalence of guaninido-functionalized residues.

A high activity was also found against two isolates of ESBL-producing *E. coli* (AAS-EC-09 and AAS-EC-010) indicating that resistance towards conventional antibiotics do not affect the sensitivity towards these peptidomimetics, further supporting a different mode of action. Many AMPs exhibit a cell envelope-perturbing effect [41-43], and hence their target is different from traditional antibiotics of which many act by inhibiting cell wall synthesis or on intracellular targets [44-46]. Notably, *S. marcescens* was the only bacterial strain that proved tolerant to the peptidomimetics, and thus must harbour specific resistance mechanisms involving induction of changes in the cell envelope.

Time-kill experiments showed that *S. marcescens* was killed more rapidly than the susceptible strain of *S. aureus* when treated with chimera 1, 2 or 3 at concentrations close to their MIC values (Figure 2). Polymyxin B and other cationic AMPs may at high doses in themselves act like chelating agents allowing them to penetrate the outer membrane [47,48], however, a noticeable effect was also seen against *S. marcescens* at concentrations lower that the MIC value (Figure 2C). Rapid killing was also demonstrated for *E. coli* exposed to the peptidomimetics, indicating that this could be a phenomenon associated with Gram-negative bacteria. Shorter exposure times caused a significant killing of Gram-negative bacteria when treated with some α-helical AMPs that act by permeabilization of the membrane [37]. Another explanation for the observed differences in the rate of killing could be that either the degree or mode of membrane disruption differs among bacteria i.e. the chimeras may exert their effect by a combination of several mechanisms. The fact that cell membranes of different bacteria differs in lipid composition [49] could influence the interaction between phospholipids and AMPs. However, there is no unequivocal evidence demonstrating that an AMP may exhibit different pore-forming properties in different bacteria, as the proposed co-existence of several disruption modes in fact still is a topic of debate [50,51].

Many AMPs exert their antibacterial effect by interactions with the bacterial cell membrane [38,41,52] involving pore formation or membrane disintegration that in turn causes leakage of the cell contents, which ultimately leads to cell death. Nevertheless, there is a growing amount of indirect evidence that the mechanisms of some very potent AMPs in fact involves an initial period

of intracellular accumulation prior to the actual bacterial killing indicating that they act on intracellular targets [38,53,54]. To further investigate the effect of the present peptidomimetics on the cell membrane in *S. marcescens* and *S. aureus* and to determine how structural features of these peptidomimetics might affect the potential membrane-related mode of action we examined their ability to cause leakage of intracellular compounds e.g. ATP. A considerable body of data on the leakage of intracellular compounds has already been obtained by using model membranes thus confirming that many membrane-active peptides indeed exert a permeabilizing effect [24-26,28]. These studies have, however, not demonstrated whether there is a direct kinetic relationship between cell membrane damage and loss of viability, and for this reason we combined leakage assays with a time-kill experiment under exactly the same conditions.

Treatment of both *S. marcescens* and *S. aureus* with peptidomimetics 1, 2 and 3 caused leakage of ATP from the bacterial cells with a similar simultaneous reduction in the number of viable cells, and therefore we conclude that even though *S. marcescens* is tolerant to the peptidomimetics their mode of action against this bacterium is similar to that of *S. aureus*. Earlier, chimera 3 was investigated for its ability to induce calcein leakage in unilamellar liposomes mimicking human cell membranes with a positive response [24], but based on the consistent results in the present work all three peptidomimetics are likely to permeabilize both model and bacterial membranes. Leakage of intracellular compounds has been determined to be the mode of action for many AMPs [55-57], but here we have established this mode of action for a series of peptidomimetics. We conclude that variation of the type of cationic amino acid (i.e. lysine versus homoarginine) did not have an effect on the mode of action in viable bacteria.

Since *S. marcescens* was tolerant to all peptidomimetics tested, their mode of action must therefore involve a target that is ultimately changed by resistance mechanisms in this species. It is well-known that *S. marcescens* is tolerant to the polymyxin group of antimicrobials, and the main hypothesis is that this is due to inherent changes in the composition of the LPS of the Gram-negative outer membrane that acts as a barrier [33]. We demonstrated that the outer membrane also seems to play an important role in the tolerance of *S. marcescens* towards our chimeras as a combined treatment including the chelating agent EDTA resulted in a reduction in the number of viable cells comparable to that seen for a more susceptible Gram-negative strain of *E. coli* treated similarly (not shown). This indicated that the innate differences in susceptibility between the two Gram-negative species could be completely eliminated after destabilization of the outer membrane.

When designing new antimicrobial peptides it is generally accepted that a minimum length is required in order for the peptide to span or transverse the cell membrane. However, the majority of studies have focused on optimizing the length of AMPs assuming it to adopt a helical conformation [25,26,40]. By contrast, due to their design with alternating hydrophobic and cationic residues our peptidomimetics are not expected to adopt an amphipathic helical active confirmation, but rather an extended conformation with some degree of secondary structure as indicated by analysis of their CD spectra [22,23]. Recently, it has been shown that neither global amphipathicity nor regular secondary structure may be required for short peptides to effectively interact with bacterial membranes [19,58], but the optimal length of such peptides has not been rationalized by mechanistic experiments. Only oligomers with a chain length above 12 residues, i.e. the 16-meric peptidomimetic 4c were able to cause such a substantial leakage of ATP that the number of viable cells were reduced (Figure 4C and 4D). We attribute this to the inability of chimeras 4a and 4b to produce a critical degree of membrane disruption thus leaving a sufficient level of intracellular ATP for the cells to survive (Figure 4A and 4B for chimera 4a).

This is to our knowledge the first time that the effect of chain length has been investigated on the membrane-perturbing activity of peptidomimetics without a dominant secondary structure. Also, we believe that our study is the first that directly, in a kinetic fashion, correlate membrane permeabilization with actual killing kinetics.

Previously, the interaction of α-peptide/β-peptides chimeras with liposomal model membranes and murine fibroblast was described [24]. Most recently, we investigated their cytotoxicity and haemolytic activity towards human HeLa cells and erythrocytes, respectively [23]. Besides confirming that members of this subclass of peptidomimetics exhibit a broad antimicrobial activity that includes resistant strains and food-borne pathogens, the purpose of the present study was to undertake a more detailed investigation of their mode of action. The present contribution describes their interaction with viable bacterial cells, and we found that these antimicrobial peptidomimetics have a mode of action involving the cell membrane. The observed membrane disruption depends strongly on chain length, and it may be impeded if the outer membrane in a Gram-negative bacterium possesses an innate altered composition.

Conclusion

Several α-peptide/β-peptoid chimeras were bactericidal against important food-borne and clinical pathogens with MIC values in the range of 1-5 μM. We examined

the effect of changing the ratio between amino- and guanidino-functionalized cationic residues as well as the influence of chain length on both antibacterial activity and ATP leakage. Although, minor differences in the antimicrobial profile of the chimeras may be ascribed to the degree of chirality and/or type of cationic amino acids, by far the most pronounced impact stems from the chain length. Only one bacterial species, *S. marcescens*, was tolerant to the peptidomimetics most likely due to the composition of its outer membrane; however, the ATP leakage was as pronounced as seen for more sensitive bacteria. We conclude that these synthetic antimicrobial peptidomimetics exert their effect through permeabilization of the cell membrane, and that this corresponds to a simultaneous reduction in the number of viable bacteria with the pool of intracellular ATP being indicative of viability. This is the first time that a relationship is established between permeabilization and killing within a peptidomimetics library.

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Authors' contributions
LHK planned and carried out all experiments and drafted the manuscript. HF designed the peptidomimetics and participated in the revision of the manuscript. KMK synthesized the peptidomimetics. LG helped in the design of the experiments and the drafting of the manuscript. All authors have seen and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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Paper 2

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Continuous selection of *Escherichia coli* to an α-peptide/β-peptoid peptidomimetic leads to inherent resistance against similar compounds.

Manuscript in preparation

Continuous selection of *Escherichia coli* to an α-peptide/β-peptoid peptidomimetic leads to inherent resistance against similar compounds

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ABSTRACT

Antimicrobial peptides (AMPs) and synthetic analogues thereof target conserved structures of bacterial cell membranes and consensus has been that resistance is unlikely to evolve. Hence, such compounds are considered promising alternatives to conventional antibiotics. The purpose of the present study was to assess the potential of a novel α-peptide/β-peptoid peptidomimetic to induce resistance in a clinical isolate of *Escherichia coli.*

In a continuous re-inoculation culturing, *E. coli* ATCC 25922 was exposed to a synthetic α-peptide/β-peptoid chimera (10 lineages), polymyxin B (10 lineages), or MilliQ water (4 lineages). The concentration was increased in ten steps from 1/16 MIC (Minimum Inhibitory Concentration for chimera: 8 µg/mL; polymyxin B: 1 µg/mL) to 32×MIC encompassing ~500 generations. All 10 lineages exposed to the chimera adapted to 32×MIC while 8 out of 10 of the polymyxin B-exposed lineages adapted to the highest concentration. All adapted lineages were tested for MICs. All lineages exposed to 32×MIC of either the chimera or polymyxin B had a significantly increased MIC to the respective compound (MIC for chimera-exposed lineages: 128-256 µg/mL; polymyxin B-exposed lineages: 32 µg/mL). Five transfers in unsupplemented media did not abolish resistance indicating that this is heritable. No cross-resistance was found between chimeras and conventional antibiotics, and cross-resistance between the chimera- and polymyxin B-adapted lineages was low indicating separate mechanisms of resistance. Isolates obtained by plating lineage populations differed in their MIC against the chimera revealing heterogeneous populations (MIC: 16-256 µg/mL). These isolates showed no cross-resistance against a panel of membrane-active AMPs, but cross-resistance was found to two similar peptidomimetics.

Whole-genome sequencing identified four identical single-nucleotide-polymorphisms (SNPs) in all six highly chimera-resistant isolates (from two lineages). One SNP was in the gene encoding the

membrane-bound lytic murein transglycosylase D (mltD), and we hypothesise that alterations in the peptidoglycan layer was the cause of chimera resistance, however, further studies are required to elucidate the mechanism by which this SNP confers chimera resistance.

INTRODUCTION

Resistance of human bacterial pathogens to conventional antibiotics has increased drastically worldwide within the last decades (Boucher et al., 2009). This has led to an intense search for safer alternatives for which resistance is less likely to evolve (Fischbach and Walsh, 2009;Peschel and Sahl, 2006). These include novel natural compounds with antimicrobial activity (Porsby et al., 2011), inhibitors of quorum sensing (Bjarnsholt *et al.*, 2010;Quave *et al.*, 2011), and antimicrobial peptides (AMPs) (Hancock and Sahl, 2006;Yeung *et al.*, 2011). The latter group comprises host defence molecules constituting part of the innate immune defence in all higher forms of life, where they display both direct antimicrobial activity and a broad range of immuno-modulating effects (Bowdish *et al.*, 2005;Gallo and Nizet, 2003;Brown and Hancock, 2006). Development of resistance to these peptides is considered unlikely due to their co-existence with bacteria during the long period of evolution, and moreover their preferred target is the ”Achilles´ heel” of bacterial cells i.e. their distinct membrane structure (Zasloff, 2002). Consequently, there has been increased focus on the characterization of natural AMPs (Mygind *et al.*, 2005;Park *et al.*, 2011) and on semi-synthetic (Zhu *et al.*, 2006;Lim *et al.*, 2005;Srinivas *et al.*, 2010) and synthetic analogues (Chen *et al.*, 2005;Chongsiriwatana *et al.*, 2008;Radzishevsky *et al.*, 2007), as well as on the development of these leads into future antibacterials against human bacterial infections.

Exposure to antimicrobials can result in resistance, which generally can be divided into two types, i.e. mutational or adaptive resistance. While the former is considered stable and arises after mutations or acquisition of a genetic element the latter term describes an auto-regulated phenomenon characterised by rapid induction of resistance in the presence of the drug followed by reversal to the sensitive phenotype when drug is absent (Skiada et al., 2011). The outer membrane of Gram-negative bacteria acts as a semi-permeable barrier mediating decreased sensitivity to antimicrobial compounds (Hancock, 1997). In addition to this, AMP-induced stress has in some species been

shown to activate innate two-component systems leading to further modifications of the cell envelope thereby decreasing the negative charge or permeability of this barrier (Fernandez *et al.*, 2010;Cheng *et al.*, 2010;Weatherspoon-Griffin *et al.*, 2011;Gunn, 2008;Sallum and Chen, 2008). However, the observed decrease in susceptibility is most often eliminated once the AMP is removed, and it is therefore considered a tolerance induction rather than resistance caused by genetic alterations.

Since AMPs and their analogues mimic structures that are part of the innate immune defence in humans, development of resistance towards such compounds could potentially compromise our innate immune defence (Bell and Gouyon, 2003). Therefore it is important to consider the likelihood and potential consequences of emergence of AMP-resistant strains before these compounds are to be used for systemic infections (Bell and Gouyon, 2003;Perron *et al.*, 2006). Only very few studies have dealt with this problem, and in most published work, the possible AMP-induced mutation frequencies have been compared to the much higher mutation frequencies usually observed for conventional antibiotics. In general, earlier experiments have been short-time studies (Zaknoon *et al.*, 2009;Steinberg *et al.*, 1997). A single comprehensive study, focusing on continuous selection to the magainin-analogue pexiganan, showed that prolonged exposure to an AMP indeed may result in heritable resistance (Perron et al., 2006) despite the common opinion that resistance towards AMPs is unlikely to evolve. The study unequivocally shows that this issue calls for an increased focus in AMP development programs.

We have previously described a synthetic approach towards peptidomimetics exhibiting antimicrobial properties, and in the present study we investigate α-peptide/β-peptoid chimeras possessing a design with alternating N-alkylated β-alanine (β-peptoid) and α-amino acid units (Bonke *et al.*, 2008;Olsen *et al.*, 2007). Previously, we have demonstrated that these compounds are

active against a range of nosocomial and food-borne pathogenic bacteria cell lysis, and we have also shown that the length of the peptide has a marked influence on activity (Olsen *et al.*, 2010;Hein-Kristensen *et al.*, 2011). Here, we investigate the risk of resistance development in a human pathogenic bacterium as this is a key parameter required when assessing the therapeutic potential of AMPs and their analogues.

MATERIALS AND METHODS

Bacterial strain and culture conditions. All experiments were performed with *Escherichia coli* ATCC 25922. Stock cultures of the wild type strain and lineage isolates were stored at –80^{o}C in 4 % (w/v) glycerol, 0.5 % (w/v) glucose, 2 % (w/v) skimmed milk powder and 3 % (w/v) tryptone soy powder. Lineage populations were frozen at –80^{o}C in 50 % (w/v) glycerol. All experiments were performed at 37^{o}C. Experiments were carried out in cation-adjusted Mueller Hinton II broth (MHB) (Becton Dickinson 212322) adjusted to pH 7.4 or 1 % (w/v) peptone (Becton Dickinson 211677) for MIC determination of natural AMPs. Brain Heart Infusion (BHI) (CM1135) with agar (VWR 20768.292) 1.5 % as gelling agent was used throughout for colony plating.

Chimera synthesis, antibiotics and natural AMPs. α-peptide/β-peptoid chimera 1, 2 and 3 consisting of alternating repeats of cationic natural α-L-amino acids and synthetic lipophilic β-peptoid residues were synthesized by solid-phase synthesis as previously described (Bonke *et al.*, 2008;Olsen *et al.*, 2007). Polymyxin B (P4932), protamine (P4020), gentamicin (G3632), ampicillin (A9518) and erythromycin (E6376) were purchased from Sigma Aldrich.
KR-12 (Wang, 2008), IsCTp (Lim et al., 2005), PEP-1-K (Zhu et al., 2006) and Melittin were prepared by automated microwave (MW)-assisted solid-phase Fmoc-based synthesis on a CEM Liberty microwave peptide synthesizer using a Rink amide resin (loading: 1.0 mmol/g). Fmoc deprotection was performed with 20 % piperidine-DMF at 75°C (30 sec followed by 180 sec), while

coupling was performed by using the appropriate Fmoc-protected building block (5.0 eq) with DIC (5 eq.) and HOBt (5 eq.) in DMF at 75°C for 15 min. Capping was applied after every fourth coupling with Ac_2O-DIPEA-NMP (1:2:3) at 65°C (30 sec, repeated once). Final deprotection of the N-terminus was followed by cleavage and simultaneously side chain deprotection with TFA-TIS-H_2O (95:2.5:2.5; 3 mL) for 60 min. The filtrate was collected and the resin was eluted with DCM (2 mL) and TFA (2× 2 mL). The combined filtrates were conc. *in vacuo*, and then co-evaporated with toluene (3×). The crude product was triturated with Et_2O, dissolved in MeCN-H_2O (50:50) containing 0.1 % TFA, and then purified by preparative HPLC. Finally the product was dissolved in water (1 mL) and lyophilized. Analytical HPLC was carried out on a Phenomenex Luna C18 (2) (3 μm) column (150×4.60 mm) using binary mixtures of eluent A (H_2O-MeCN-TFA 95:5:0.1) and eluent B (H_2O- MeCN-TFA 5:95:0.1) for elution with a flow rate of 0.8 mL/min by using a linear gradient of 10-60 % B during 30 min. Peptides were detected with UV at λ = 220 nm. Preparative HPLC was performed on a Luna C18 (2) (5 μm) column (250 × 21.20 mm) with an Agilent 1100 LC system with a multiple-wavelength UV detector. Elution was performed with a linear gradient of 10-40 % during 20 min at a flow rate of 20 mL/min. Peptides were detected with UV at λ =220 nm. LC-HRMS was performed with a Phenomenex Luna C18 (2) (3 μm) column (150×4.6 mm) using binary mixtures of eluent C (H_2O-MeCN-HCOOH 95:5:0.1) and D (H_2O-MeCN-HCOOH 5:95:0.1). Elution was performed with a linear gradient of 10-60 % D during 30 min at a flow rate of 0.5 mL/min. HRMS spectra were obtained using a Bruker MicrOTOF-Q II Quadropol MS detector. Analyt. HPLC (>97 % purity at 220 nm) retention times (RT): RT = 16.85 min for KR-12; RT = 28.75 min for mellitin; RT = 17.74 min for IsCTp; RT = 17.79 min for Pep-1-K.

Chimeras and natural AMPs were solubilised to a stock of 10 mg/mL in sterile MilliQ water and stored at -20^{o}C. Polymyxin B was solubilised to a stock of 10 mg/mL in sterile MilliQ water, filter-sterilized and stored at 5^{o}C. Protamine was solubilised to a concentration of 1024 μg/mL in sterile MilliQ water and used immediately. Gentamicin and ampicillin were solubilised to a stock of 25

mg/mL in sterile MilliQ water, filter-sterilized and stored at 5°C and -80°C, respectively. Erythromycin was solubilised to a stock of 10 mg/mL in 96 % ethanol and prepared fresh for each experiment.

Continuous selection experiment. Continuous selection of resistance was performed for chimera 1 (Figure 1) and for the clinically used polymyxin B. A bacterial suspension of a single colony of *E. coli* ATCC 25922 was re-inoculated (10 µl) five times in unsupplemented MHB (990 µl). It was then re-inoculated in MHB supplemented with chimera 1 (ten lineages) or polymyxin B (ten lineages), and after ten re-inoculations at constant chimera or polymyxin B concentration, the concentrations were doubled. The starting concentration was 1/16 of the Minimum Inhibitory Concentration, MIC (i.e. 0.5 µg/mL and 0.0625 µg/mL, respectively), and this was increased to 32×MIC during the course of the experiment. Four lineages grown in MHB with MilliQ water were included as control. Re-inoculations were performed twice a day, but were reduced to once a day when growth was slower as assessed by turbidity of the cultures. The total number of passages (supplemented with AMP) was 77 times encompassing ~500 generations. For each increase in concentration the lineages were preserved as freezing stocks. Ten colony isolates were randomly selected from each lineage population at 32×MIC and preserved as freezing stocks.

Determination of MIC. MIC was determined by using the micro-dilution method according to guidelines of the Clinical and Laboratory Standards Institute (The Clinical and Laboratory Standards Institute (CLSI), 2006). Chimera and erythromycin 1:2 serial dilutions were prepared from 1024 µg/mL stock solutions to give a final range of 512-0.5 µg/mL in the wells. Ampicillin 1:2 serial dilutions were prepared from a 256 µg/mL stock solution to give a final range of 128-0.13 µg/mL in the wells. Polymyxin B and gentamicin 1:2 serial dilutions were prepared from a 64 µg/mL stock solution to give a final range of 32-0.03 µg/mL in the wells. Also, 1:2 serial dilutions of the AMPs KR-12, IsCTp, Melittin and PEP-1-K were prepared from a 64 µg/mL stock solution to give a final

range of 32-0.03 µg/mL in the wells; however this was performed in 1 % peptone instead of in MHB since activity was drastically diminished in the latter (not shown).

The populations were tested for their susceptibility towards chimera 1, polymyxin B, gentamicin and ampicillin. Isolates selected from these populations were additionally tested against chimera 1, polymyxin B, erythromycin and the reference AMPs. To estimate the level of cross-resistance to related peptidomimetics, MIC determinations were performed for chimeras 2 and 3 (Figure 1) towards selected isolates. The bacterial suspensions were grown overnight in MHB at 37^{o}C for determination of the population MIC, or individual isolates were grown overnight on non-selective BHI agar at 37^{o}C. MIC determination performed on the wild type strain using re-suspended colonies, as well as on a culture grown overnight in broth, showed that pre-growth in broth had minimal effect on the MIC results. In order to determine the population MIC, the respective compound (chimera 1 or polymyxin B) was added to a concentration corresponding to 32×MIC to maintain the selection pressure of the freezer-revived lineages, and MIC determination performed on the outgrown population (i.e. ~10^9 CFU/mL). In all experiments, bacterial cells were suspended in 0.9 % saline to give a turbidity of 0.13 at OD_{546} (approximately 1×10^8 CFU/mL) and diluted in MHB pH 7.4 to a final concentration of 5×10^5 CFU/mL in each well. Polypropylene plates (Nunc 442587) were used to minimize peptide binding, and the incubation time was 18-20 hours at 37^{o}C. Incubation time was additionally extended up to 44 hours for the individual isolates due to a potential effect of growth rate on the outcome, but no effect was seen of this (not shown). MIC was found in a minimum of two technical (population MIC) or biological (isolate MIC) replicates as the lowest concentration of the AMP or antibiotic where no visible growth was found.

Stability of resistance. Frozen stocks were reconditioned in unsupplemented MHB media for five transfers (~35 generations) at 37^{o}C. The heritability of resistance was established through MIC determination of bacterial populations as described above.

Fitness cost. To evaluate whether resistance altered the growth properties of the mutant isolates, we determined the growth rate of three isolates from lineage 2 (i.e. 2-7, 2-9 and 2-10) in unsupplemented MHB and MHB supplemented with chimera 1 at sub-MIC concentrations 1 (i.e. 4 and 8 µg/mL). Optical density readings were obtained at 570 nm in 10-12 minute increments on cultures grown at 37^{o}C over 24 hours (unsupplemented MHB) or 72 hours (supplemented MHB) using the automated Bioscreen C system (Labsystems, Helsinki, Finland). Growth rates were found using linear regression on ln(initial CFU/mL) vs. detection time (i.e. the time until a 0.5 unit increase in absorbance was reached) for inoculum sizes ranging from 10^2-10^7 CFU/mL (Dalgaard and Koutsoumanis, 2001). Data were analysed in GraphPad Prism version 4.03 using One-Way ANOVA followed by Dunnett's Test to correct for multiple testing. Control groups were *E. coli* ATCC 25922 (absence of chimera) or isolates grown in unsupplemented media (presence of chimera). Significance levels were in all tests set at $p < 0.05$. All experiments were done in duplicate on at least two independent days.

Whole-genome sequencing. A total of fourteen isolates from chimera-exposed lineages were chosen for whole-genome sequencing. We chose six high-level resistant isolates from lineage 2 (numbers 4, 6, 7, 9 and 10) and lineage 7 (number 7), three low-level resistant isolates from lineage 5 (number 6, 8 and 9) and five isolates with wild type MIC from lineage 4 (number 4 and 6), lineage 5 (numbers 2, and 4) and lineage 7 (number 9). For comparison we chose one isolate from control 2 (number 3) and two isolates from control 1 (numbers 3 and 5). The genome sequence of the ancestral wild type *E. coli* ATCC 25922 has not previously been published and was therefore included as reference.

Genomic DNA was extracted from each sample by using phenol:chloroform:isoamyl alcohol and then precipitated with isopropanol. Samples were RNAse treated before quantification and quality analysis using 1 % agarose gel electrophoresis, NanoDrop Spectrophotometer (Saveen Werner, Sweden) and Qubit 2.0 Analyser (Invitrogen, United Kingdom). Libraries of 500 bp were used for 100 bp paired-end sequencing of genomes using the Illumina sequencing technology on a HiSeq2000

with a minimum coverage of 100 (Beijing Genomics Institute, Hong Kong, China). *E. coli* ATCC 25922 was *de novo* assembled into contigs using the CLCbio Genomics Workbench (Aarhus, Denmark) resulting in 135 contigs comprising 5,116,439 bp. Using this procedure the 17 isolates were mapped with the 135 contigs as a reference with a minimum coverage of 100 and single-nucleotide-polymorphism (SNPs) were then detected.

RESULTS

The aim of this study was to investigate the development and spectrum of resistance in a clinical isolate of *Escherichia coli* following exposure to an α-peptide/β-peptoid chimera. Additionally, we were interested in elucidating the mechanism underlying the observed resistance.

Continuous selection towards AMP resistance. *Escherichia coli* ATCC 25922 was continuously re-cultured from a concentration of 1/16 of the wild type MIC until a concentration of 32 times the wild type MIC of either chimera 1 (i.e. 256 µg/mL) or polymyxin B (i.e. 32 µg/mL) was reached encompassing ~500 generations. All ten lineages supplemented with chimera was eventually outgrown at 32×MIC; for lineages supplemented with polymyxin B this was the case for eight out of ten (Figure 2). It is evident that growth was affected at an earlier stage for the polymyxin B-supplemented lineages, i.e. growth was inhibited for all strains already at ¼ MIC where two lineages failed to grow further; for the chimera-supplemented growth was not visibly inhibited until a concentration at or above the MIC (Figure 2). Only once was the concentration decreased for a lineage supplemented with chimera (i.e. lineage 8) due to lack of growth at 32×MIC. After two additional passages at 16×MIC the lineage successfully grew at 32×MIC.

The four controls were transferred into fresh unsupplemented media simultaneously with the transfer of the two other groups of lineages. At the time point corresponding to 4×MIC of the supplemented lineages (i.e. 32 µg/mL for the chimera and 4 µg/mL for polymyxin B) we investigated the ability of

these controls to grow in the presence of the same concentration as the supplemented lineages. Two controls supplemented with chimera at 4×MIC failed to grow, but one of the two controls supplemented with 4×MIC of polymyxin B became outgrown. Growth was retained for three additional passages at the same concentration of polymyxin B indicating that the spontaneous mutation rate for polymyxin B may be higher than that of chimera 1.

Population MIC after selection. MIC was determined for the selective AMP against revived outgrown cultures at 32×MIC of all lineages in the two groups (Table 1). Only five out of ten of the freezing stocks of the chimera-supplemented lineages could be re-cultured in chimera-supplemented media, though all of the eight polymyxin B-supplemented freezing stocks fully outgrew. We ascribe this to stress induced by freezing in some lineages as the cultures subsequently grew in unsupplemented substrate and retained resistance. A dramatic increase in MIC was seen for both the chimera- and the polymyxin B-supplemented lineages showing that resistance had developed against the selective agent (Table 1). For the five chimera-supplemented lineages, MIC against chimera 1 was increased to 128-512 μg/mL from an initial level of 8 μg/mL for the wild-type corresponding to a 16- to 64-fold increase. The eight polymyxin B-supplemented lineages all had a MIC value at 32 μg/mL against polymyxin B, i.e. a 32-fold increase in MIC as compared to the wild-type strain. The four controls only showed minor increases in MIC compared to the wild type.

We determined potential cross-resistance to the other selective agent (i.e. chimera 1 vs. polymyxin B) and to an aminoglycoside (gentamicin) and a cell wall-active antibiotic (ampicillin). No cross-resistance was found against the two conventional antibiotics for any of the lineages (not shown). Some cross-resistance was found against polymyxin B for some of the chimera-supplemented lineages (2- to 16-fold increase in MIC compared to wild type, not shown), and against chimera 1 for the polymyxin B-supplemented lineages (2- to 8-fold increase in MIC as compared to the wild type, not shown).

Stability of resistance. To establish if the acquired resistance was heritable we revived all lineages in unsupplemented media and cultured them for five passages (corresponding to ~35 generations) in the absence of the compound applied for selection. All ten lineages tolerant to the chimera could now successfully be revived. Again we performed MIC determinations against both selective agents for all lineages. The lineages exposed to the chimera displayed high levels of resistance against this compound even after growth in unsupplemented media confirming the heritability of resistance (Table 1). The ten lineages displayed MIC values against chimera 1 between 32 µg/mL (4×MIC) and 256 µg/mL (32×MIC); the lowest being lineage no. 10 which showed a strong decrease in the MIC value compared to the initial level of 256-512 µg/mL indicating that this lineage may be less stable than the others. Similarly, the MIC for polymyxin B against the polymyxin B-exposed lineages remained high upon culturing for five passages in unsupplemented media (Table 1). The low level of cross-resistance seen in the two groups of lineages against chimera 1 and polymyxin B, respectively, was lost after removal of the selection pressure, and became similar to that of the controls (not shown).

Isolate MIC after selection. From each of the lineages cultured at 32×MIC, ten individual isolates were randomly selected from plating the population on non-selective plates. We chose to focus specifically on the isolates from two of these lineages; lineage no. 2 which had a population MIC of 256 µg/mL, was quickly outgrown in supplemented media and displayed various colony morphologies (normal as well as larger more light colonies) and lineage no. 5, which had a population MIC of 128 µg/mL, appeared to have a reduced growth rate and looked homogenous when plated. To this end, it seemed likely that different genetic events had occurred in these two populations, and that this may be reflected in the resistance profiles of the isolates from the two populations. In order to establish whether heterogeneity was a general trend in all lineages, two randomly selected isolates from lineage 4 (4-4 and 4-6) and lineage 7 (7-7 and 7-9) were included in the further investigation.

We determined the MIC against chimera 1 for the twenty isolates from lineage 2 and 5 (Figure 3). Both populations were heterogeneous displaying large differences in MIC values of individual isolates. The ten isolates from lineage 2 displayed MIC values within the range 8-128 µ/mL (Figure 3). Two of the ten isolates appeared to be particularly unstable; in three biological replicates isolates 2-3 exhibited MIC values of 8, 16 and 64 µg/mL, while the MIC values for isolate 2-8 were 16, 64 and 128 µg/mL for the three biological replicates. Remarkably, from lineage 5, seven out of ten isolates had the same MIC value as the wild type (i.e. 8 µg/mL) while none of the other three isolates displayed the same MIC as the whole population i.e. 128 µ/mL (Figure 3). Overall, this population was evidently less resistant (or stable) than lineage 2. Differences in colony morphology were not reflected in a significantly altered MIC value. Additionally, individual colonies were verified as *E. coli* by 16S rRNA sequencing as well as by a range of standard phenotypic tests. Interestingly, the two isolates from lineage 4 had the same MIC value as the wild type *E. coli*. Moreover, the MIC value of one of the isolates from lineage 7 (i.e. 7-9) was the same as that found for the wild type, whereas the other isolate (7-7) was highly resistant (MIC = 64 µg/mL). Therefore it appears that it is a general trend that the vast majority of cells have a lower MIC than the population as a whole and that a large degree of heterogeneity exists within the lineage populations.

Cross-resistance. Since AMPs are found widely in nature, the potential of cross-resistance to natural AMPs constitutes an important issue to clarify. Therefore we determined MIC values against polymyxin B (bacterial), protamine (salmon), KR-12 (a short analogue of the human cathelicidin LL-37), IsCTp (scorpion), Pep-1-K (viral) and mellitin (honey bee venom) for all twenty isolates from lineages 2 and 5. We did not find increased MIC values for any of these as compared to the wild type (not shown). Next, the level of cross-resistance towards the related chimera 2 (Figure 1) was addressed. We found high levels of resistance against this chimeras for all resistant isolates, whereas isolates with wild type MIC towards chimera 1 also had considerably lower MIC values towards this chimera (Figure 4). Furthermore, MIC determination for a third chimera (i.e. chimera 3, Figure 1)

against the three most resistant isolates from lineage 2 gave MIC levels that were 8 times higher than seen for the wild type (i.e. 4 µg/mL vs. 32 µg/mL). The evaluation of cross-resistance against different groups of antimicrobials including conventional antibiotics, natural AMPs and peptidomimetics confirmed that the developed resistance was specific for these peptidomimetics.

Fitness cost. Another interesting issue was to determine whether acquisition of resistance had altered the growth properties of the resistant isolates. Thus, the growth rates of the twenty isolates from lineages 2 and 5 were compared with that of the wild type strain in the absence of the selective chimera. None of the twenty isolates displayed a significant change in growth rate compared to *E. coli* ATCC 25922 ($p > 0.05$ for all). Likewise, no correlation was seen between MIC of the isolates (Figure 3) and their growth rates. We also determined the growth rate of the three most resistant isolates found, namely isolates 2-7, 2-9 and 2-10 in the presence of sub-lethal concentrations of chimera 1. Despite the high MIC value (128 µg/mL for all three isolates) a pronounced effect on growth was seen even at very low concentrations of chimera 1. The growth rate of all three isolates were significantly reduced at a chimera concentration of 8 µg/mL corresponding to the wild type MIC or 1/16 of the isolate MIC (2-7: $p<0.01$; 2-9: $p<0.05$; 2-10: $p<0.01$). Remarkably a significantly reduced growth rate was also seen for two of the isolates (2-7: $p < 0.01$ and 2-10: $p < 0.05$) at a concentration of 4 µg/mL i.e. 1/32 of the MIC of the isolate.

Whole-genome sequencing. Fourteen isolates exposed to chimera 1 and three isolates cultured with MilliQ water as well as the ancestral wild type were whole-genome sequenced. The wild type (*E. coli* ATCC 25922) was *de novo* assembled creating 135 contigs encompassing 5.12 MB. The 135 contigs of the ancestral strain then served as a reference for indentifying SNPs in the evolved isolates. The sequenced isolates were divided into three groups based on their susceptibility towards chimera 1: (i) isolates with wild type MIC (8 µg/mL), (ii) isolates with low levels of resistance (4-8 ×wild type MIC), and (iii) isolates that were highly resistant (8-16 ×wild type MIC) (Table 2). We identified four SNPs, which were present in the six highly resistant isolates (i.e. with MIC 8-16 times

above wild type MIC) from lineages 2 and 7, but not in isolates with low or intermediate levels of resistance or in the controls not exposed to chimera 1. Using BLAST it was determined that the four SNPs were located in genes encoding a hypothetical protein, a putative outer membrane transporter, a macrolide transporter and the membrane-bound lytic murein transglycosylase D (mltD), respectively. No change was seen in the susceptibility of the five isolates to the macrolide erythromycin as compared to the wild type (not shown). The SNP in the mltD gene caused a change of amino acid 55 from proline (nonpolar) to glutamine (polar), but the effect of this on the secondary structure of the protein is unknown (Bateman and Bycroft, 2000). No consistent pattern was seen in the presence of SNPs in the three isolates displaying low levels of resistance to chimera 1 (5-6, 5-8, 5-9, Figure 3).

DISCUSSION

In this study it was demonstrated that *Escherichia coli* may develop resistance during continuous exposure to increasing concentrations of an α-peptide/β-peptoid peptidomimetic (Table 1) and that this was observed in all of ten lineages. To our knowledge this is the first time heritable resistance has been developed to a synthetic AMP analogue (i.e. a peptidomimetic). We have previously shown that these peptidomimetics are membrane-active (Hein-Kristensen et al., 2011). For long, it was believed that AMP resistance development was unlikely since these compounds target the bacterial Achilles' heel i.e. the plasma membrane structure (Zasloff, 2002). Hence, though these peptidomimetics indeed target the structural features of the bacterial membrane, the investigated wild-type strain of *E. coli* developed a mechanism of resistance that apparently circumvents the membrane-activity of the peptidomimetics. A few studies have shown that resistance may develop towards other AMPs. The most comprehensive of these studies was performed with pexiganan, an analogue of the natural AMP magainin against *Pseudomonas fluorescens* and *E.coli* (Perron et al.,

2006). The authors demonstrated high levels of heritable resistance in both bacteria showing that resistance mechanisms may evolve as a result of a continuous selection pressure exerted by a single compound. Other studies have not found AMP resistance as a result of continuous selection with natural or synthetic AMPs (Farrell *et al.*, 2011;Zaknoon *et al.*, 2009;Steinberg *et al.*, 1997), however, these studies were performed with sub-inhibitory concentrations of the AMP without any attempts at increasing the concentration. We speculate that the development of resistance shown by Perron et.al. (2006) and demonstrated in the present study might be due to the gradual 2-fold increments in the concentration starting from a very low level (1/16 of MIC) of the AMP used for selection.

E. coli bacteria possess a large mutational reservoir for increased resistance to antibiotics (Girgis et al., 2009). SNP detection in the sequenced genomes revealed an average number of 500 SNPs in each isolate as compared to the ancestral wild type (not shown), i.e. statistically one SNP occurs per generation indicating a very high mutation rate. The differences observed in the level of resistance and stability (Table 1) indicate that distinct mutational events may have taken place between and within the lineages. We have shown that the MIC values for the chimera varied profoundly between individual isolates from lineages 2 and 5 (Figure 3) as well as from lineage 7. It is interesting that we did not find any isolates displaying the population MIC of either lineage 2 or 5; we hypothesize that this could be due to: (i) lack of stability of the population, or (ii) presence of low proportions of highly resistant isolates. In the development of resistance to antibiotics, bacterial charity (i.e. production of indole) has been proposed to confer protection to less resistant clones in a heterogeneous population (Lee et al., 2010). Also, it is possible that epigenetic events such as changes in gene expression may provide temporary protection of the entire population (Adam et al., 2008).

Since earlier studies had indicated moderate mutation rates for polymyxins (Rahaman *et al.*, 1998;Falagas *et al.*, 2010), we selected for polymyxin B resistance in parallel experiments. Thus, we found indications of a higher mutation rate for polymyxin B than that of chimera 1 as spontaneous growth of one of the controls at 4×MIC was only seen for the former (not shown). No cross-resistance was seen between chimera 1 and polymyxin B, independently of which compound had been used as the selective agent, demonstrating that different mechanisms must confer resistance against these compounds. Resistance to polymyxins has been demonstrated several times to be related to modification of the bacterial outer membrane, in particular of the lipopolysaccharide (LPS) layer (Rahaman *et al.*, 1998;Nummila *et al.*, 1995;Zhou *et al.*, 2001). Such modifications are most often mediated by two-component systems, which have been widely studied for their role in resistance to AMPs (Cheng *et al.*, 2010;Fernandez *et al.*, 2010;Weatherspoon-Griffin *et al.*, 2011). However, two-component systems are associated with adaptive (inducible) resistance, but as resistance to polymyxin B in our case proved heritable in unsupplemented media, two-component systems are unlikely to be responsible for the development of resistance to polymyxin B in the present study.

Similarly, resistance to chimera 1 was heritable indicating that tolerance involving two-component systems are not responsible for conferring resistance against chimera 1 in the resistant isolates. To investigate the resistance mechanism, we tested for cross-resistance to conventional antibiotics and natural AMPs. Resistance to aminoglycosides may be mediated by LPS modifications (Rahaman et al., 1998), but we could not demonstrate any cross-resistance to this type of antibiotics. Similarly, no change was seen in the susceptibility of the isolates to β-lactams. Importantly, we found no evidence of cross-resistance to a range of natural and semi-synthetic AMPs indicating that the resistance mechanism for membrane-active AMPs is not universal. However, pronounced cross-resistance was seen to other peptidomimetics with the same scaffold (e.g. 2 and 3), i.e. cross resistance was

demonstrated to a chimera where: (i) homoarginine was replaced by lysine and extra repeating units were added (i.e. 16-meric 2 vs. 12-meric 1), and (ii) the structure was non-chiral (i.e. 3) as opposed to the chiral structure of chimera 1 (Figure 1). Especially, cross-resistance to the latter chimera is interesting since the extent of chirality influences the secondary structure of the compound, which generally is believed to have a marked impact on the mechanism of action of membrane-active AMPs and peptidomimetics (Deslouches et al., 2005).

Whole-genome sequencing of resistant isolates revealed that resistance possibly was mediated by a SNP in the gene encoding the MltD protein, a membrane-bound lytic murein transglycosylase responsible for peptidoglycan reorganization. This SNP causes a change in amino acid 55 of the protein from proline (a nonpolar amino acid) to glutamine (a polar amino acid), and it is possible that this changes the secondary structure and thereby the activity of MltD. A study in *Vibrio anguillarum* has shown that inactivation of the mltD gene results in resistance to conventional antibiotics (Xu et al., 2011). Since we found this SNP in six isolates from two different lineages, it appears not to be deleterious to the bacteria. Additionally, the potential modification of the cell envelope does not seem to entail a fitness cost in resistant isolates since we found no change in the growth rate of the isolates as compared to the ancestral wild-type strain. However, in a separate study we have found evidence that resistant isolates have an increased susceptibility to the innate antibacterial action of human blood plasma (Hein-Kristensen *et al.*, 2012). Further studies are needed to confirm that this SNP in the gene encoding mltD is responsible for the observed resistance to the peptidomimetics.

In conclusion, we found that *E. coli* may develop heritable resistance during continuous selection to an α-peptide/β-peptoid peptidomimetic, which we believe is the first time this has been demonstrated for a backbone-modified AMP analogue. Cross-resistance was demonstrated to other compounds belonging to the same peptidomimetic subclass indicating that cross-resistance most likely is

correlated with the scaffold. Importantly, the mechanism of resistance appear to be closely linked to changes in the organization of the peptidoglycan layer due to a common mutation in the gene encoding the MltD protein in all highly resistant isolates.

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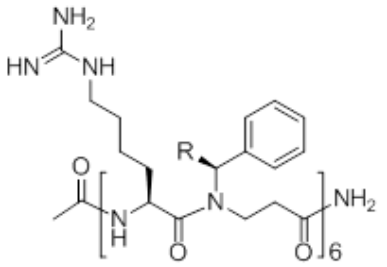
1

2



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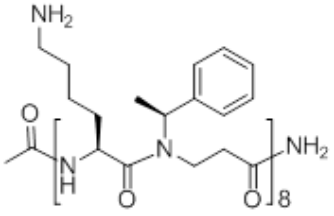

2

Figure 1. The structure of chimera 1 used for continuous culturing of *Escherichia coli* and the structure of chimeras 2 and 3 to which cross-resistance was demonstrated.

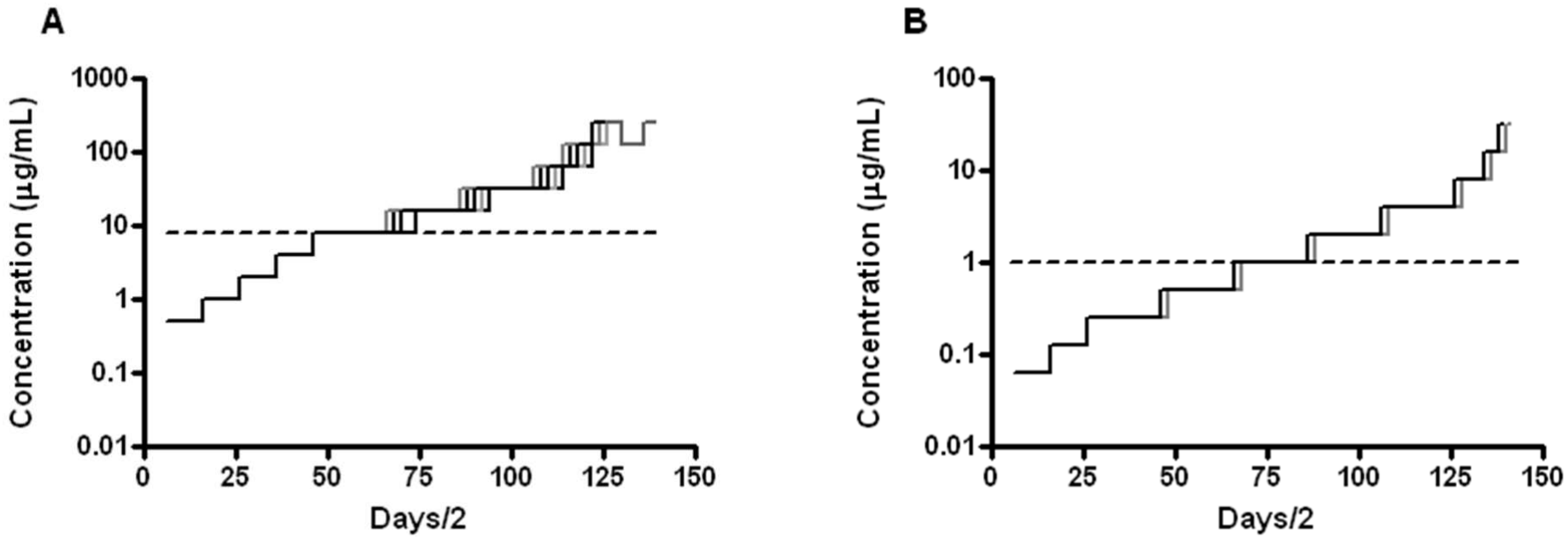

Figure 2. Continuous selection exerted by chimera 1 (A), or by polymyxin B (B) leads to tolerance in *E. coli*. All lineages exposed to the peptidomimetic were cultured up to 32×MIC (A); for lineages selected against polymyxin B this was eight out of ten (the last two were not able to grow above ¼ MIC i.e. 0.25 µg/mL). Punctuated lines indicate wild type MIC of the selective agents (8 µg/mL and 1 µg/mL, respectively).

Table 1. Minimum Inhibitory Concentration (µg/mL)

	Chimera 1 (chimera-exposed lineages)		Polymyxin B (polymyxin B-exposed lineages)	
Lineage number	After selection	Stability	After selection	Stability
1	n.a.	128-256	n.a.	n.a.
2	256	256	32	32
3	n.a.	128	32	32
4	256	128	32	32
5	64-128	128	32	32
6	n.a.	64-128	32	32
7	128	128	n.a.	n.a.
8	n.a.	32-64	32	32
9	n.a.	128	32	32
10	256	32	32	32
Control 1	32	16-32	8	0.5
Control 2	8	8	1	0.5-1
E. coli ATCC 25922	8		1	

Table 1. Minimum Inhibitory Concentration (µg/mL) of the cultured lineages against the two selective agents, chimera 1 and polymyxin B. MIC values were determined for exposed lineages while keeping the selection pressure as well as after the selection pressure had been removed for ~ 35 generations (i.e. stability). MIC values are based on two technical duplicates. All exposed lineages displayed increased MIC values compared to the ancestral *E. coli* strain, and resistance was heritable for all but one lineage.

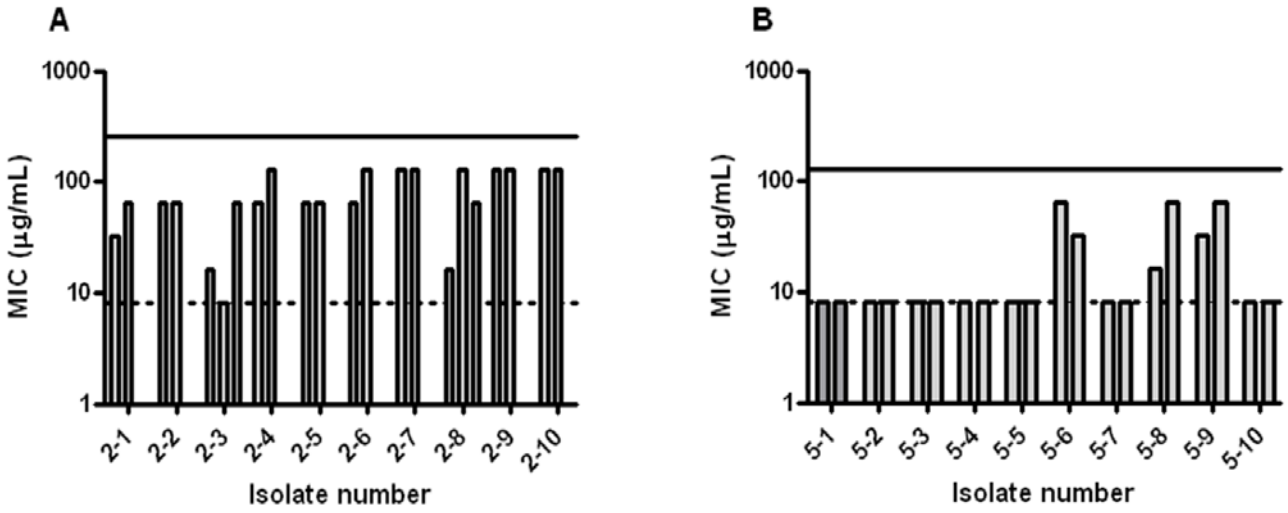

Figure 3. Minimum Inhibitory Concentration (MIC) for chimera 1 against population isolates of lineage 2 (A) and lineage 5 (B). Bars indicate biological replicates; MIC for all isolates was determined twice, a third replicate was performed for isolates 2-3 and 2-8 due to large variations in results. Susceptibility to chimera 1 varies widely within the populations. Solid line: population MIC; punctuated line: wild type MIC (8 µg/mL).

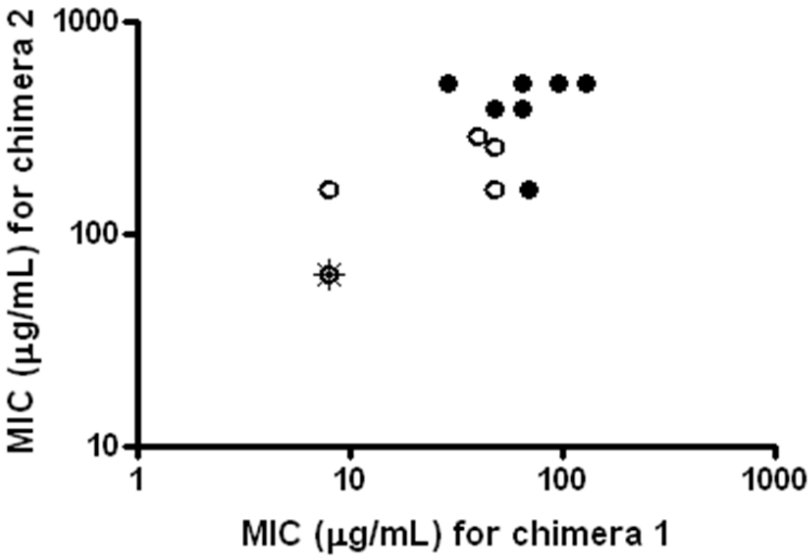

Figure 4. Association between Minimum Inhibitory Concentration (MIC) for chimera 1 and chimera 2 against lineage 2 (black circles) and lineage 5 (white circles). *E. coli* ATCC 25922 MIC is highlighted with an asterisk (i.e. MIC values of 8 µg/mL and 64 µg/mL for chimera 1 and 2, respectively). This data point is shared with six of the ten isolates from lineage 5. All values based on two biological replicates; the mean value is displayed when results varied.

Table 2. Levels of resistance in sequenced isolates grouped by MIC values

Wild type MIC (8 µg/mL)	**Low levels of resistance** (4-8 × wild type MIC)	**High levels of resistance** (8-16 × wild type MIC)
4-4	5-6	2-4
4-6	5-8	2-6
5-4	5-9	2-7
5-6		2-9
7-9		2-10
		7-7

Table 2. Levels of resistance in the three groups of sequenced isolates. MIC was determined in a minimum of two biological replicates according to CLSI guidelines. Isolates were grouped based on the average of these determinations. Only the six highly resistant isolates contained a SNP in the gene encoding MltD.

Paper 3

Line Hein-Kristensen, Kolja M. Knapp, Henrik Franzyk & Lone Gram (2012)

Effect of human blood plasma and immune effectors on the antibacterial activity of an α-peptide/β-peptoid chimera against wild-type *Escherichia coli* and chimera-resistant mutants.

Manuscript in preparation

Effect of human blood plasma and immune effectors on the antibacterial activity of an α-peptide/β-peptoid chimera against wild-type *Escherichia coli* and chimera-resistant mutants

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ABSTRACT

Cationic antimicrobial peptides (AMPs) are considered promising leads for novel antibiotics, however, their activity may be compromised under physiologically relevant conditions due to inherent instability towards proteolytic degradation. The purpose of the present study was to determine the activity of a series of stable α-peptide/β-peptoid peptidomimetics against *Escherichia coli* in the presence of human blood plasma and relevant immune effector molecules. We also addressed if acquired bacterial resistance towards the chimeras might compromise plasma antibacterial activity.

The Minimum Inhibitory Concentration (MIC) of an α-peptide/β-peptoid chimera against a clinical strain of *E.coli* decreased from 4 μg/mL in MHB media to 0.125-1 μg/mL with the addition of 50 % blood plasma. Similarly, the Minimum Bactericidal Concentration (MBC) decreased 4-fold in the presence of 50 % blood plasma. To elucidate the cause of this enhanced antibacterial activity, the interaction between the chimera and effector molecules of the immune system was examined. No effect (synergy or antagonism) was found between the chimera and immune effector molecules (a shortened LL-37 analogue, lysozyme, or hydrogen peroxide), however, the enhancement of antibacterial activity observed in blood plasma was abolished following heat-induced inactivation of the complement system. Chimera-resistant mutants of *E.coli*, exhibiting a MIC 8 times above the MIC of a wild-type strain, were highly susceptible to plasma antibacterial activity and were killed when blood plasma concentrations exceeded ~30 %. Additionally, chimera MIC of the resistant mutants returned to wild-type level upon addition of 25 % plasma.

The enhancement of chimera antibacterial activity in a physiological environment and the increased susceptibility to blood plasma following acquisition of resistance significantly increases the therapeutic potential of these compounds.

INTRODUCTION

The number of new classes of antimicrobials released for clinical use has been extremely limited since the golden-age of antibiotics in the 1930-1960s (Fischbach and Walsh, 2009). Only four new classes of antibiotics have been introduced within the last decade, instead most novel antibacterial drugs are designed through modification of existing scaffolds (Jabes, 2011;Livermore, 2011). Furthermore, treatment of infectious diseases has been hampered by the rapid emergence of bacterial resistance to such varieties of antibiotics soon after their introduction, and it seems that in spite of low mutation rates resistance to all classes of conventional antibiotics will inevitability evolve over time (Theuretzbacher, 2011). One strategy to win this evolutionary battle involves the use of the very widespread class of naturally occurring antimicrobial peptides (AMPs). However, AMPs must in general be developed into compounds suitable for therapeutic use (Hancock and Sahl, 2006). These short, cationic compounds form part of the innate immune defence among all classes of life, where they display direct antimicrobial activities as well as immuno-modulatory properties (Zasloff, 2002;Nijnik and Hancock, 2009;Easton *et al.*, 2009). The co-evolution of AMPs and bacteria has obviously avoided development of resistance, and have thus made the structural properties and diversity of these compounds very interesting research objectives (Peschel and Sahl, 2006;Woolhouse *et al.*, 2002). Yet, concerns have been raised that systemic therapeutic use of AMPs might disrupt this delicate balance allowing bacteria to develop resistance, and thereby compromise our own immune defence (Bell and Gouyon, 2003).

One of the great obstacles in the further advancement of lead AMPs is the transition from *in vitro* activity to efficacy in animal models because of the sensitivity of many AMPs to biological environments (Deslouches et al., 2005). Biological relevant conditions may lead to a decrease or complete loss of activity of otherwise promising compounds making these unsuitable for systemic use (Sabath, 1978;Otvos and Cudic, 2007;O'Neill and Chopra, 2004;Powell *et al.*, 1993). The

activity of AMPs is especially sensitive to the saline conditions present in plasma. In particular divalent cations compete with the peptide for bindings sites on the negatively charged bacterial surface (Deslouches et al., 2005). Moreover, AMPs are susceptible to degradation by serum proteases (Knappe et al., 2010), and for these reasons many AMPs have only been developed as topical agents. A simple model for determining the potential *in vivo* activity involves biological evaluation in the presence of human blood plasma, which may be obtained from freshly drawn blood by removal of red and white blood cells. Therefore it contains the same components as whole blood as opposed to blood serum. Plasma contains both factors known to facilitate the growth of bacteria (i.e. vitamins, amino acids etc.) as well as factors of the immune defence promoting killing or inhibition of bacteria (e.g. complement, lysozyme, antibodies and AMPs), and hence blood plasma exhibits an innate antibacterial effect (Taylor, 1983). However, some bacterial strains are resistant to plasma and are therefore not efficiently cleared from the blood by natural immune processes.

The soluble components of the immune system (i.e. complement, antibodies and AMPs) may act synergistically with antimicrobial therapeutics, and such interaction has been demonstrated for both AMPs and antibiotics (Dutcher *et al.*, 1978;Deslouches *et al.*, 2005;Vaara and Vaara, 1983). Although the exact mechanisms are not known it appears that the combined effects lead to disruption of the permeability barrier of the outer membrane of Gram-negative bacteria thereby enabling immune factors and antibacterial drugs to reach their targets more readily (Dutcher *et al.*, 1978;Yeaman *et al.*, 2002). Synergy as well as negative interactions (i.e. antagonism) between antimicrobial drugs and human AMPs have been reported by numerous investigators (Mishra *et al.*, 2011;Jones *et al.*, 2008;Zahner *et al.*, 2010;Kristian *et al.*, 2007;Xiong *et al.*, 1999).

Previously, we have described the synthesis of an array of α-peptide/β-peptoid chimeras designed with alternating N-alkylated β-alanine (β-peptoid) and α-amino acid residues (Bonke *et al.*,

2008;Olsen *et al.*, 2007). The structure-activity relationship against food borne and nosocomial bacterial pathogens was investigated, and the cell envelope was shown to be the primary target of the chimeras (Hein-Kristensen et al., 2011). The ultimate aim is to test and use these chimeras *in vivo*, and the present study concerns determination of activity of a single α-peptide/β-peptoid chimera in a simulated *in vivo* system as an initial step in this development process. We used a clinical strain of *Escherichia coli* that is one of the principal test strains for determining antimicrobial activity (The Clinical and Laboratory Standards Institute (CLSI), 2006). Also, this strain produces hemolysin making it relevant to test in a model for potential activity in blood. Additionally, we used three chimera-resistant mutants of *E.coli* to determine whether the acquisition of heritable resistance affected the tolerance of the bacteria to innate immune factors present in blood.

MATERIALS AND METHODS

Bacterial strains and culture conditions and human blood plasma. All experiments were performed with *Escherichia coli* ATCC 25922 or isolates derived from this strain. Stock cultures were stored at –80^{o}C in 4 % (w/v) glycerol, 0.5 % (w/v) glucose, 2 % (w/v) skimmed milk powder and 3 % (w/v) tryptone soy powder. Unless otherwise stated experiments were carried out with bacteria incubated for one night (i.e. approximately 18 hours) at 37^{o}C. Experiments were performed in cation-adjusted Mueller Hinton II broth (MHB) (Becton Dickinson 212322) adjusted to pH 7.4. Brain Heart Infusion (BHI) (CM1135) with agar (VWR 20768.292) 1.5 % as gelling agent was used for colony plating. Human blood plasma was obtained from two healthy individuals by centrifugation of freshly drawn blood for 10 minutes at 4000×g. Sodium citrate was used as anticoagulant. Blood plasma was stored at –20^{o}C in 2 mL aliquots, whereby multiple freeze-thaw cycles was avoided.

Chimera synthesis. A library of α-peptide/β-peptoid peptidomimetics consisting of alternating repeats of natural cationic α-amino acids and synthetic lipophilic β-peptoid residues was synthesized by solid-phase synthesis as previously described (Bonke *et al.*, 2008;Olsen *et al.*, 2007). From this library a single compound i.e. chimera 1 was chosen for all experiments, Figure 1. The chimera was solubilised to a stock concentration of 10 mg/mL in MilliQ water and stored at -20^{o}C.

Growth of wild type bacteria in human blood plasma. The ability of *E. coli* ATCC 25922 to grow in human plasma was investigated in a spectrum of 25-50 % plasma (based on preliminary experiments). A volume of 50 µl 100 % plasma, plasma diluted with MHB pH 7.4 to 2 × assay concentration or pure MHB as control was added to each well of a polypropylene microtiter plate (Nunc 442587). Colonies grown on BHI agar overnight (i.e. approximately 18 hours) were suspended in 0.9 % saline to give a turbidity of 0.13 at OD_{546} (approximately 1×10^8 CFU/mL), and then diluted in MHB pH 7.4 to a final concentration of 5×10^5 CFU/mL in each well. Growth was assessed after 18-20 hours at 37^{o}C. Experiments were performed on at least two independent days for each blood donor. *E.coli* formed a dense slime precipitate when grown in plasma. To test whether extracellular DNA could be a component of this, the slime pellets were transferred to a new microtiter-plate and DNase treated. 50 µl 20× or 100× DNase (Sigma Aldrich D5319) diluted in 0.9 % saline were added, and the plates were incubated at 37^{o}C for up to 20 hours.

Determination of chimera Minimum Inhibitory Concentration (MIC) with/without blood plasma. MIC was determined using the micro-dilution method according to guidelines of the Clinical and Laboratory Standards Institute (CLSI) (The Clinical and Laboratory Standards Institute (CLSI), 2006). Chimera 1:2 serial dilutions in MHB pH 7.4 were prepared from a 32 µg/mL stock solution to give a final range of 0.016-16 µg/mL in the wells. Bacterial suspensions were prepared as described above to give a final bacterial density of 5×10^5 CFU/mL in each well. Polypropylene plates (Nunc 442587) were used to minimize peptide binding, and the incubation time was 18-20 hours at 37^{o}C. MIC was determined in a minimum of two biological replicates as the lowest

concentration of the peptide analogue where no visible growth was found. The Minimum Bactericidal Concentration (MBC) was determined by plating 10 µL of the suspension from the first three wells without growth on BHI agar and incubating these for 24 hours at 37°C. MBC was the lowest concentration at which a 99.9 % reduction in CFU/mL was observed.

To determine whether chimera activity was affected by human blood plasma, we compared the activity (i.e. MIC) in MHB with the activity in MHB supplemented with 50 % plasma. We chose this amount of plasma because 50 % is the natural proportion of plasma in human blood, and because growth of *E. coli* was not diminished by the addition of 50 % plasma. For MIC determination in 50 % plasma, we used the standard protocol, but made the chimera 1:2 serial dilutions in 100 % plasma instead of MHB. In each experiment a row of MHB 1:2 serial dilutions were included as control. MBC determination was performed as described above. Experiments were performed in at least two independent experiments for each blood donor. Additionally, we performed the MIC determination in 50 % plasma on a shaken culture (300 rpm) since preliminary experiments indicated that stagnant growth in plasma could protect against peptide activity. For this, chimera was added in a concentration of 8, 1, 0.125 or 0 µg/mL to a bacterial suspension at 5×10^5 CFU/mL with/without 50 % plasma for a total volume of 0.5 mL. Cultures were incubated at 37°C, 300 rpm for 20 hours and MBC was determined as described above.

The presence of plasma strongly reduced chimera MIC, and to determine if plasma had to be present simultaneously to exert an effect or if pre-incubation with blood plasma also had an influence on peptide activity, we pre-incubated bacteria in plasma for seven passages (~50 generations). Preliminary experiments showed no effect on growth at 6.25 %, and we therefore chose this concentration for the experiments. *E. coli* ATCC 25922 grown on BHI agar overnight at 37°C was re-inoculated (10 µl) seven times in MHB (990 µl) supplemented with plasma or MilliQ water as control with transfer into fresh media once a day. After the last passage, cells were either (i) harvested and washed three times (0.9 % saline, 2000×g, 10 min) before being re-suspended in 0.9 %

saline to give a turbidity of 0.13 at OD_{546} (approximately 1×10^8 CFU/mL), and then diluted in MHB pH 7.4 to a final concentration of 10^6 CFU/mL, or (ii) transferred directly into fresh MHB pH 7.4 to a concentration at 10^6-10^7 CFU/mL. Time-kill was performed by adding chimera to a final concentration of ½×MIC (i.e. 2 µg/mL) and incubating the suspension at 37°C, 300 rpm. Samples for colony determination were taken at 0, 2, 4, 6, 8, 10 and 24 hours after addition and transferred to a ten-fold dilution row. Colony counts were determined after incubation for 24 hours at 37°C. The whole experiment was performed twice.

Interaction with innate immune factors. To investigate which compounds in plasma may be responsible for enhanced antibacterial effect of the chimera, we used the checkerboard method (Yan and Hancock, 2001) to investigate possible synergy with relevant immune effector molecules. Since the present chimera has a structure and mode of action that mimic natural AMPs belonging to the innate immune defence (Hein-Kristensen et al., 2011), where synergy is known a to be prominent part of the overall activity (Peschel and Sahl, 2006), we focused on a LL-37 analogue i.e. KR-12 (Wang, 2008), hydrogen peroxide, and lysozyme from human neutrophils (Sigma Aldrich L8402) (Yan and Hancock, 2001). The chimera was serially diluted along the rows of the microtiter plate and the immune effector molecules diluted along the columns. Chimera 1:2 serial dilutions were prepared from a 64 µg/mL stock solution to give a final range of 0.25-16 µg/mL in the wells. Lysozyme 1:2 serial dilutions were prepared from a 256 µg/mL stock solution to give a final range of 1-64 µg/mL in the wells. Hydrogen peroxide 1:2 serial dilutions were prepared from a 32 mM stock solution to give a final range of 0.13-8 mM in the wells. Also, 1:2 serial dilutions of the natural peptide KR-12 were prepared from a 64 µg/mL stock solution to give a final range of 0.25-16 µg/mL in the wells; however this was performed in 1 % (w/v) peptone (Becton Dickinson 211677) instead of in MHB since activity was drastically diminished in the latter (not shown). For testing synergy with KR-12 we therefore also diluted the chimera in 1 % peptone, which had no effect on chimera activity (not shown). The bacterial suspension was prepared as described for standard MIC

determination. Polypropylene plates (Nunc 442587) were used to minimize peptide binding, and the incubation time was 18-20 hours at 37^{o}C. Synergy, defined as a reduction in the MIC value of the chimera in the presence of one of the effector molecules, was investigated in two biological replicates.

Heat-inactivation of plasma. Human blood plasma contains parts of the complement system, which can be inactivated by high temperatures (Zinsser and Johnson, 1911;Hazen, 1943). To determine whether complement enhanced the effect of the chimera, we determined chimera activity in heat-inactivated plasma, and compared this with untreated plasma. Plasma was heat-inactivated by incubation at 56^{o}C for 15 minutes followed by rapid cooling on ice. MIC of the chimera in the presence of 50 % heat-inactivated plasma, 50 % untreated plasma or MHB as control was determined as described above. The assay was performed in two independent experiments.

Effect of bacterial chimera-resistance on the activity of blood plasma. *E.coli* is capable of developing heritable resistance to chimeras following continuous selection (Hein-Kristensen *et al.*, 2012). Resistant variants of *E.coli* were not resistant to other natural or synthetic AMPs, but increased MICs were seen for compounds with a similar design, indicating that the activity of the chimeras within the array were influenced by the same resistance mechanism (Hein-Kristensen *et al.*, 2012). The MIC value of the ancestral wild type to chimera 1 is 4 µg/mL whereas it is 32 µg/mL for resistant isolates (Hein-Kristensen *et al.*, 2012). Since it has been claimed that AMP resistance is a potential health risk due to impairment of our own immune system, we determined plasma activity against three resistant isolates and compared this to the effect on the (ancestral) wild type bacteria to see whether the activity of plasma had been changed following acquisition of resistance.
Three resistant isolates (strains 2-7, 2-9 and 2-10, numbered according to isolation from lineage 2 (Hein-Kristensen *et al.*, 2012)) were revived from freeze storage. The three isolates were grown in 25-50 % blood plasma and 25-50 % heat-inactivated plasma as described above for the wild type. Experiments were performed on at least two independent days for each blood donor. MIC

determination of a short analogue (KR-12) of LL-37, hydrogen peroxide, and lysozyme was performed in biological duplicates as described above to see whether resistance also conferred elevated MIC values to immune factors. Lastly, MIC against chimera 1 was determined as described above in the presence of 25 % plasma (chosen due to lack of growth of the three isolates in 50 % plasma) to determine if this could decrease or abolish the high MIC values seen for the resistant isolates.

RESULTS

The purpose of this study was to determine whether physiologically relevant compounds (human blood plasma and human immune factors assumed to be present in this) influenced the activity of an α-peptide/β-peptoid chimera as compared to the activity seen in conventional laboratory assay media. Additionally, we wanted to test growth and susceptibility of chimera-resistant *E. coli* mutants towards these compounds in order to establish how development of chimera resistance influenced the sensitivity of bacteria to factors of the innate human immune defence.

Growth of wild type bacteria in human blood plasma. *E. coli* ATCC 25922 grew well in MHB supplemented with 25-50 % human blood plasma, but formed visible, large (0.5 mm) pellets when grown in 25-50 % plasma (Figure 2b). Microscopy revealed a dense 3D matrix of cells clustering within a slime matrix (Figure 2e). We suspected that eDNA was responsible for the slime/biofilm matrix, however, the slime pellet showed no signs of disintegration after treatment with DNAse for 20 hours.

Chimera MIC with/without human blood plasma. MIC for chimera 1 in MHB was 4 µg/mL against *E. coli* ATCC 25922, which corresponds to the MBC value reported previously (Hein-Kristensen et al., 2011). With 50 % plasma added, the MIC decreased to 0.125-1 µg/mL, i.e., a 4- to 32-fold decrease (Table 1). This decrease in MIC was observed with plasma from two different

donors. The change in MIC was less pronounced when the assay was performed in 25 % plasma (i.e., MIC was only diminished to 1-2 µg/mL) indicating a dose-response effect of plasma on chimera activity. Though MIC values in plasma differed substantially (most likely reflecting variations in the plasma composition itself), MBC values never exceeded 1 µg/mL. Hence, compared to chimera activity in MHB media, where MIC equals MBC, the activity of chimera 1 in 50 % plasma was bacteriostatic. The same pellet morphology consisting of a dense slime matrix of cells observed when *E. coli* was grown in 50 % plasma only was also seen upon addition of sub-inhibitory concentrations of chimera (Figure 2a).

The slime pellet was only observed when *E. coli* was grown with plasma under stagnant conditions. To determine whether the slime pellet influenced the bactericidal activity of chimera 1, we determined MIC on a shaken culture. Due to limited amounts of chimera only three concentrations were used in MHB media with or without 50 % plasma. At 8 µg/mL (2×MIC) no growth was seen in MHB with or without plasma added. At 1 and 0.125 µg/mL, growth was only seen in MHB without plasma added. Colony counts of tubes with 1 or 0.125 µg/mL of chimera in 50 % plasma revealed bacterial densities that were very close to inoculation levels indicating that bacterial growth had only been inhibited. Microscopy revealed normal single motile cells. Since the tolerance to the chimera was decreased under both stagnant and shaking growth conditions, and no slime pellet was observed in the latter, this phenomenon does not appear to be the cause of the observed increase in tolerance towards chimera 1.

To determine whether the decrease in MIC in the presence of plasma was due to a synergistic activity between chimera and plasma, or a direct effect of plasma that caused enhanced susceptibility to the following treatment, we pre-incubated *E. coli* for 7 days (i.e. 50 generations) in MHB supplemented with 6.25 % plasma, and then performed time-kill using a concentration of ½×MIC. No change was seen in chimera inhibition of cells pre-incubated in plasma compared to cells pre-

incubated in MilliQ water (not shown), neither at inoculum levels of 10^6 nor 10^7 CFU/mL (not shown).

Effect of plasma components on chimera activity. The checkerboard method was used to test for synergy between chimera 1 and immune effector molecules: the LL-37 analogue KR-12, hydrogen peroxide, and lysozyme. However, neither of these compounds changed the MIC of the chimera (not shown).

We hypothesised that complement activity in plasma could be the cause of increased sensitivity to chimera 1, and therefore we inactivated complement by heating (Hazen, 1943). Heat treatment completely abolished the effect of plasma on chimera activity (Table 1). The MIC of chimera 1 against *E. coli* is 4 µg/mL when determined in MHB, but in 50 % plasma the MIC value decreased to 0.125-1 µg/mL. In the presence of 50 % heat-inactivated plasma, the MIC value was 8 µg/mL (Table 1). The development of the slimy pellet seen in 50 % normal plasma was not observed for *E. coli* grown in 50 % heat-inactivated plasma

Effect of chimera resistance on the activity of blood plasma. Similarly to the ancestral wild type *E. coli*, from which the three resistant isolates had been developed, the resistant isolates were grown in a range of 25-50 % plasma. While the wild type grew well and formed a visible pellet at 50 % plasma concentration, the resistant isolates were not able to grow at plasma concentrations above 30 %. At concentrations where the isolates were able to grow, a dense slime matrix of cells was formed, though less pronounced than for the wild type (Figure 2b versus 2d). Colony counts of cells recovered from wells with high plasma concentrations gave none or a very low number of surviving cells showing that high concentrations of plasma was bactericidal to the chimera-resistant isolates. When plasma was heat-treated as described above the mutants grew well in 50 % plasma similar to the wild type. Additionally, no changes were seen in the MIC of KR-12, hydrogen peroxide or lysozyme following acquisition of chimera resistance. Lastly, MIC determination was performed

against the three isolates in 25 % plasma to see whether the presence of plasma counteracted the mechanism of resistance. In 25 % plasma the MIC value of chimera 1 against the wild type *E. coli* was only marginally affected i.e. MIC was decreased from 4 µg/mL in MHB media to 1-2 µg/mL. However, MIC determined against the three resistant isolates decreased from 32 µg/mL in MHB media to 1 µg/mL in 25 % plasma. Hence, the presence of plasma was found to abolish the acquired resistance to chimera 1.

DISCUSSION

One of the major obstacles in using cationic AMPs as antimicrobial agents is their inactivation under physiologically relevant conditions. This inactivation may be caused by binding of the cationic AMPs to blood components (e.g. serum albumin) (Deslouches et al., 2005), degradation by proteases (Jenssen and Aspmo, 2008), or obstruction of bacterial membrane binding sites by divalent cations of dissolved salts (Friedrich et al., 1999). Since all of these processes may take place during circulation in the bloodstream, determination of the peptide activity in blood plasma provides a suitable simple model for *in vivo* systemic use of AMPs and peptidomimetics.

We found that blood plasma potentiated chimera activity (Table 1) to a surprisingly high extent. The bacteriolytic activities of plasma are well-known (Taylor, 1983), however, microscopy revealed that *E. coli* cells were alive within the slime matrix formed during growth without shaking. We speculate that the matrix may be formed by the contents of lysed cells thereby protecting the other cells from plasma. Possibly, this might lead to a decreased initial cell number, which in turn then affects the MIC value. However, the notion that the slime matrix provides protection against the chimera is not likely since a similar result was obtained under shaken incubation conditions, where the slime matrix was not formed.

Synergy between chimera 1 and plasma components offers an alternative explanation for the increased activity of chimera 1 in the presence of plasma. Previously, we have shown that α-peptide/β-peptoid chimeras target the cell membrane and leads to leakage of intracellular contents (Hein-Kristensen et al., 2011). Many immune factors present in plasma (i.e. AMPs, lysozyme, complement) have a similar effect on the cell envelope though the exact mechanisms vary between different factors (Levy, 2000). In the present investigation we did not find evidence of synergy between the chimera and KR-12, lysozyme or hydrogen peroxide (not shown) although synergy between antibacterial drugs and human AMPs has previously been shown (Mishra *et al.*, 2011;Jones *et al.*, 2008;Xiong *et al.*, 1999). This is interesting since the cathelicidin LL-37 and lysozyme are both present in the blood at a concentration in the µg/mL range (Taylor, 1983;Zahner *et al.*, 2010), and synergy could therefore have explained the observed decrease in MIC. However, heat-inactivation of plasma, a method commonly used for eliminating complement activity (Hazen, 1943), rendered the plasma inefficient in reducing the MIC value of the chimera (Table 1). The bactericidal activity of the complement system stems from the formation of the membrane-attack-complex (MAC) that efficiently lyses intruding bacteria (Taylor, 1983;Kondos *et al.*, 2010). Synergy between the complement system and AMPs (Yeaman *et al.*, 2002;Fierer and Finley, 1979) or antibiotics (Darveau and Cunningham, 1990;Dutcher *et al.*, 1978;Schweinle and Nishiyasu, 1992) has been reported, but it is not known whether this is due to the compounds acting in concert or sequentially. We did not find any effect of pre-exposure to plasma on the killing activity of chimera with low concentrations (6.25 %) of plasma, although sequentially enhanced activity of plasma and antibiotics has been shown by others (Dutcher et al., 1978). This indicates that the chimera and plasma components potentially act in concert, however, we have not tested the effect of opposite order (i.e. the effect of pre-exposure to chimera on subsequent plasma activity) so it is possible that chimera could prime the bacteria for complement activity, which has been demonstrated for antibiotics (Alexander *et al.*, 1980;Taylor *et al.*, 1981;Schweinle and Nishiyasu, 1992). In line with this, serum

has been shown to potentiate the post-antibiotic effect (Davidson et al., 1991). Furthermore, cationic AMPs similar to these chimeras, possess an ability to traverse the outer membrane of Gram-negative bacteria, and some studies have shown that this may lead to disintegration of the outer membrane structure even at low doses (Epand *et al.*, 2008;Vaara and Vaara, 1983). If the integrity of the outer membrane is compromised due to chimera activity, this would facilitate insertion of MAC into the plasma membrane, providing a potential mechanistic explanation for the observed decrease in chimera MIC. Further studies are needed to validate the role of the complement system in the observed increase in chimera activity in the presence of blood plasma.

One potential problem in using AMP peptidomimetics against systemic infections is that resistance to these compounds could potentially compromise our own immune defence since these compounds mimic the structure of our own defence peptides (Bell and Gouyon, 2003). We investigated this by using a resistant mutant of the same *E. coli* strain to determine whether acquisition of resistance influences the activity of innate immune factors present in the blood. Surprisingly, the growth of the three chimera-resistant isolates 2-7, 2-9 and 2-10 was significantly hampered in the presence of plasma. Hence, the acquisition of resistance to an AMP peptidomimetic in fact renders the bacteria more susceptible to at least the soluble components of the innate immune defence in blood plasma. The mechanism of resistance of these isolates is likely a single-nucleotide-polymorphism (SNP) in the gene for the membrane-bound lytic murein transglycosylase D (mltD) (Hein-Kristensen *et al.*, 2012), which is involved in reorganization of the peptidoglycan layer (Xu et al., 2011). We hypothesize that changes in the structure of the outer membrane confer an increased susceptibility to yet unknown membrane-acting plasma components. Inactivation of complement by heat treatment eliminated the bactericidal activity of plasma against the three chimera-resistant isolates. It is possible that changes in the organization of the peptidoglycan layer due to changes in the mltD protein leads to easier access of complement components and subsequent MAC insertion into the

plasma membrane. We were also interested in investigating chimera activity towards the resistant isolates in the presence of plasma. Interestingly, chimera resistance was abolished in the presence of 25 % plasma, suggesting that the activity of plasma components renders the resistance mechanism against chimera activity ineffective. Plasma potentiation against resistant strains has been demonstrated for antibiotics (Odio et al., 1984). We suspect that the mechanisms behind this are similar to that for the chimera-susceptible strain.

In conclusion, chimera activity was significantly increased when determined in a physiological environment, and under these conditions the resistance mechanism of chimera-resistant isolates was rendered ineffective. These results add further support to the therapeutic potential of the peptidomimetics and to AMPs in general.

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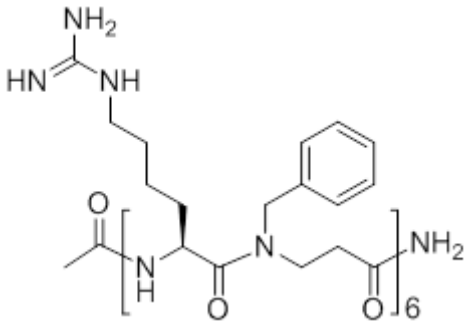

Figure 1. Structure of chimera 1

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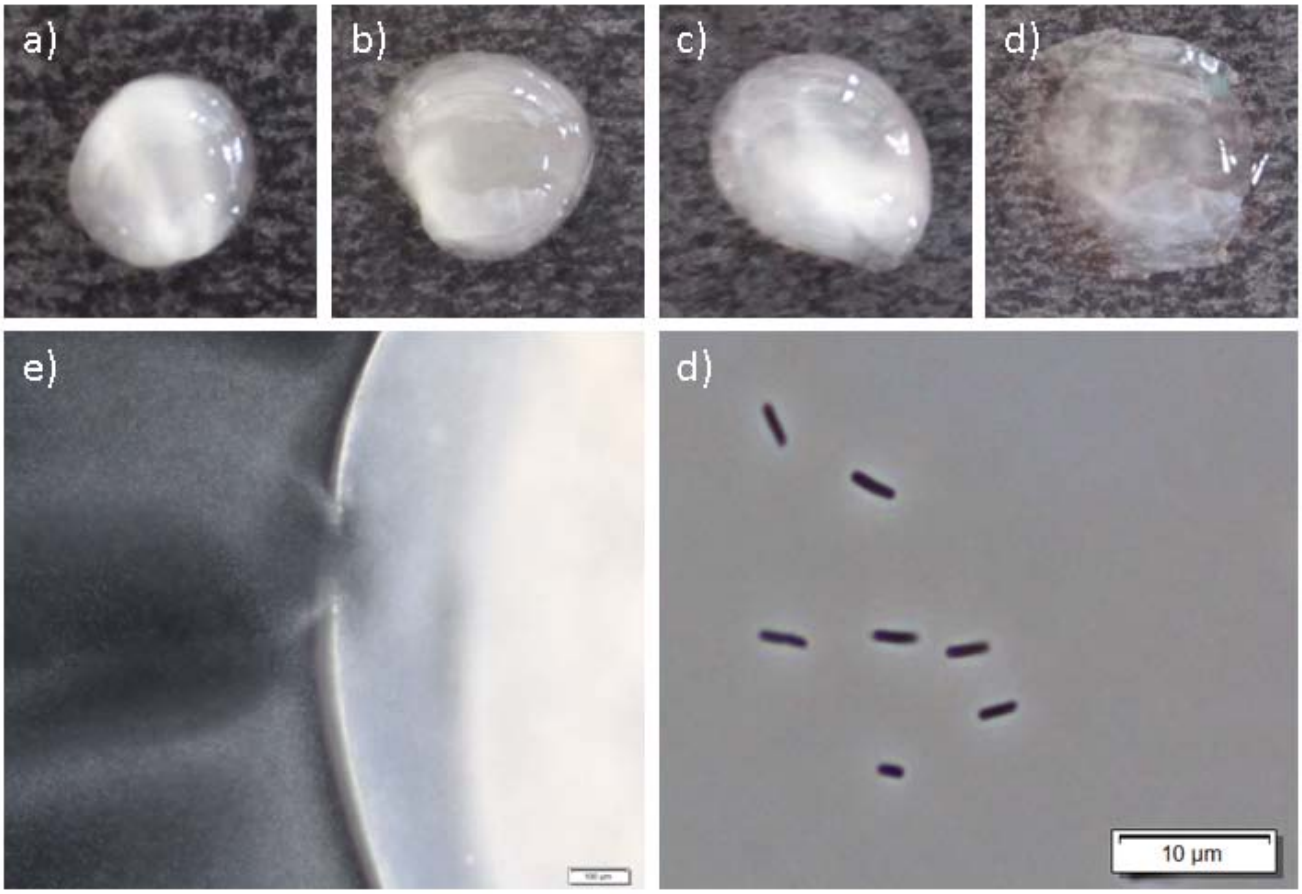

Figure 2. Formation of slime matrix after stagnant growth of *Escherichia coli* ATCC 25922 (a and b), or one of the resistant isolates (c and d) with chimera 1 (a and c) or without chimera 1 (b and d). A more dense slime matrix was formed after addition of chimera 1, in particular for the resistant isolate (d). Light microscopy at 10× magnification showed release of contents after puncture of the slime matrix (e); 100× magnification clearly identified the contents as *E. coli* cells

1

Table 1. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of chimera 1 against *E. coli* grown in the presence/absence of human blood plasma

Growth conditions	MIC (μg/mL)	MBC (μg/mL)
MHB (stagnant)	4	4
50 % plasma (stagnant)	0.125-1	1
50 % inactivated plasma (stagnant)	8	8
MHB (shaken)	8	8
50 % plasma (shaken)	0.125	> 1

Table 1. Minimum Inhibitory Concentration and Minimum Bactericidal Concentration against *E. coli* ATCC 25922 in MHB media with/without 50 % human blood plasma under stagnant and shaken incubation conditions. Values were obtained from a minimum of two independent tests with two blood donors. Plasma was inactivated by heat treatment for 15 min at 56^{o}C**.**

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