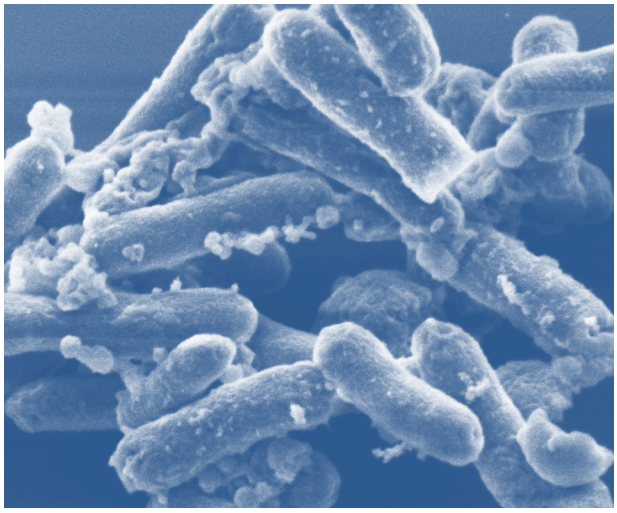
Bacterial resistance and susceptibility to antimicrobial peptides and peptidomimetics

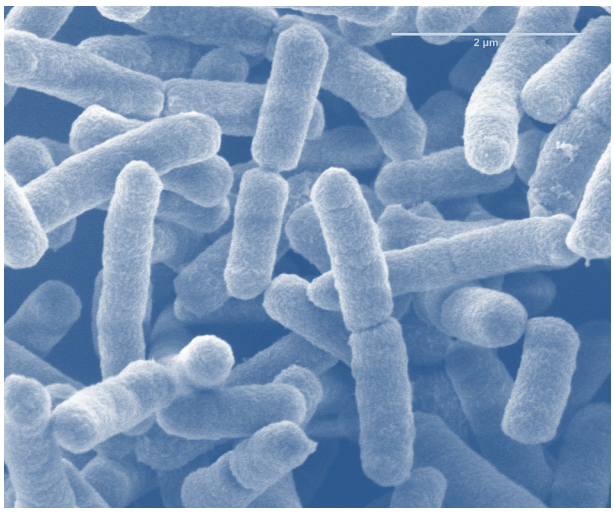
Citterio, Linda; Gram, Lone; Franzyk, Henrik

Publication date:
2017

Document Version
Publisher's PDF, also known as Version of record

Bacterial resistance and susceptibility to antimicrobial peptides and peptidomimetics





PhD thesis

Linda Citterio

Technical University of Denmark
Department of Biotechnology and Biomedicine

March 2017

Main supervisor: Lone Gram, Professor, Technical University of Denmark
Co-supervisor: Henrik Franzyk, Associate professor, University of Copenhagen

Front page:

Scanning Electron Microscopy (SEM) images of *Escherichia coli* treated with 8 μg/ml α-peptide/β-peptoid peptidomimetic and 25% human blood plasma (left) and un-treated (right). Ramona Valentina Mateiu; DTU CEN; 7-12-2014.

”One must care about a world one will not see.”

Bertrand Russell

Preface

This thesis is submitted as a partial fulfillment of the requirements to obtain a PhD degree.

The work presented in this thesis was carried out at the Department of Biotechnology and Biomedicine at the Technical University of Denmark, from the 1st of December 2013 to the 30th of November 2016.

Professor Lone Gram has been the main supervisor and associate professor Henrik Franzyk has been the co-supervisor of this project.

The Technical University of Denmark funded this project.

The results obtained during the three years are presented in three manuscripts, all included in the thesis.

Linda Citterio

Kongens Lyngby, March 2017

Abstract

Bacterial resistance to conventional antibiotics has become a global challenge and there is urgent need for new and alternative compounds. Antimicrobial peptides (AMPs) are under investigation as novel antibiotics. These are part of the immune defense of all living organisms; hence, they represent a valid candidate both for their antibacterial activity and for their immunomodulation features. However, these compounds have several disadvantages once administered *in vivo*. These shortcomings have led to extensive attempts of improving their features with rational synthetic design. Peptidomimetics are one class of such synthetic modified peptides. The purpose of this PhD project was to determine the antibacterial spectrum and potential use of synthetic antimicrobial peptides and peptidomimetics. Another key investigation has been the experimental development of resistance to these novel antibacterial agents.

We investigated (Article 1) the antibacterial effect of selected peptidomimetics in a simulated *in vivo* environment using human blood plasma and serum. We speculated that the activity of peptidomimetics was hampered by the presence of blood fluids. However, the antibacterial activity was enhanced in presence of human blood plasma but not in in presence of human blood serum. We hypothesized that complement system or clotting factors present in plasma but not in serum were causing the enhanced effect of peptidomimetics. Interestingly, in presence of heat-inactivated blood matrices, the activity of the compounds decreased dramatically or no enhancement was observed, indicating that inactivation of complement has occurred. We also determined whether the antibacterial activity of selected conventional antibiotics was affected by the presence of blood fluids and indeed the activity of a membrane active antibiotic was enhanced in presence of human plasma. We conclude that complement system and other factors present in human blood plasma interact synergistically with membrane active compounds such AMPs are. As a result, the concentrations of peptidomimetics and peptide antibiotics needed in vivo may be lower than predicted from standard antimicrobial susceptibility testing.

Unfortunately bacteria can easily adapt to AMPs in laboratory settings and we found (Manuscript 2) that in *Escherichia coli* through an adaptive evolution experiment. We hypothesized that evolution of resistance to the combination would be slower than to the single compounds. However, the lineages exposed to P9-4 (alone or in combination) were the slowest adapting as compared to the other treatments. We suggest that the AMP P9-4 could be considered a promising candidate for future application in clinical settings, because of its slow resistance development rate. Using whole-genome sequencing, we investigated the genetic basis of resistance in the adapted lineages and derived clones. Deletions in the gene encoding for the enzyme CDP-glycerophosphotransferase were the most common variants, indicating that a common sequence of mutation events has led to development of resistance. The zeta potential of adapted lineages was less negative than that of the wild type and we therefore hypothesized that a potential mechanism of resistance relies on surface charge modifications.

In Manuscript 3 we investigated the stability of the evolved resistance by re-cultivating selected resistant clones in absence of compound. Several clones retained resistance after re-cultivation in absence of compound. Genome analyses demonstrated that deletions in the gene encoding for the enzyme CDP-glycerophosphotransferase were still present after re-cultivation. Thus, this

enzyme may indeed play a key role in the mechanism of resistance. Cross-resistance is a common feature of resistant microorganisms and we therefore determined whether the adapted, resistant clones had altered susceptibility to other antibacterial compounds. The resistant clones were also resistant to compounds with intracellular activity. However, the same clones were as susceptible as the wild type when exposed to membrane-active compounds with specific features such as lipidation, incorporation of D-amino acids and presence of IR motifs. Thus, the concern that AMPs-resistant clones may be a threat to our immunity may be overestimated.

In conclusion, this PhD project supports the belief that bacteria hold the potential to develop resistance to each novel antibacterial agent. Nevertheless, strategies to circumvent resistance exist and must be pursued.

Resumé (Dansk)

Bakteriel resistens over for konventionelle antibiotika er en global udfordring, og der er et akut behov for nye og alternative forbindelser. Antimikrobielle peptider (AMP). Der er en del af alle levende organismers immunforsvar, er en stofgruppe, der undersøges som nye antibiotika. AMP har bred antibakteriel aktivitet og modulerer immunsystemet. AMPer indeholder positivt ladede aminosyrer, som interagerer med den negativt ladede bakterie-membran og ødelægger bakteriers cellemembran, så cellen lyseres og bakterien dør. På trods af disse lovende egenskaber, har , AMPer en række ulemper *in vivo,* bl.a. er nogle AMP cytotoksiske eller har lav stabilitet. Disse mangler har ført til forsøg på at forbedre deres funktioner med rationel syntetisk design. Peptidomimetika er én klasse af sådanne syntetiske modificerede peptidforbindelser.

I dette ph.d.-projektet testede vi bakteriel følsomhed og resistensrisiko over for både syntetiske og naturlige AMP. Den antibakterielle aktiviteten af stofferne forbindelserne blev øget, når interaktionen skete med tilsætning af humant blodplasma, men ikke med tilsætning af varme-inaktiverede blodmatricer. Det er vores hypotese, at der er synergi mellem blodproteiner, fx komplement, og peptidomimetika. Derfor kan lavere dosering af visse klasser af peptider anvendes i behandlingen af infektionssygdomme. Fra et klinisk perspektiv er dette et lovende fund og fremtidige undersøgelser skal fokusere på at få disse resultater implementeret i dyremodeller til proof-of-concept.

Bakterier synes ikke at udvikle resistens mode AMP, der er en del af vores naturlige medfødte immunsystem. Dog kan bakterierne nemt adaptere til AMP i laboratoriet i såkaldte adaptive evolution experiments. *Escherichia coli* udviklende nemt resistens over for både enkelte peptidomiketika og kombination af disse. Resistens var dog vanskelig at inducere over for en ni aminosyre lang AMP og en sådan forbindelse er derfor en lovende kandidat til fremtidige anvendelse i kliniske omgivelser.

Vi undersøgte det genetiske grundlag for bakteriernes resistens og identificerede et gen, i hvilket mutationer sandsynligvis er årsag til resistens. Mutationen resulterer formodentlig i ændringer af bakteriens overfladeladning. Mange af de resistente kloner bevarede modstand også når peptiderne blev fjernet, hvilket indikerer en stabil, genetisk kodet resistens. Nogle af de AMP-resistente mutanter var også resistente overfor andre udvalgte peptider, men bevarede følsomhed overfor de fleste. Dette resultat er lovende, og indikerer at brug af AMPs ikke vil gøre bakterier resistente overfor vores naturlige medfødte immunitet.

Peptidmimetika er lovende som fremtidens antibiotika, men det er nødvendigt med yderligere undersøgelser af resistensudvikling ligesom der kræves en bedre forståelse af krydsresistens og modtagelighed fænomen.

Research Articles

Included in the thesis

Article 1 - Citterio L, Franzyk H, Palarasah Y, Andersen TE, Mateiu RV, Gram L. Improved *in vitro* evaluation of novel antimicrobials: potential synergy between human plasma and antibacterial peptidomimetics, AMPs and antibiotics against human pathogenic bacteria. *Res Microbiol* 2016; 167:72-82.

Manuscript 2 - Citterio L, Franzyk H, Nielsen HM, Gram L. Adaptive laboratory evolution of *E. coli* reveals slow resistance development to the short antimicrobial peptide P9-4 and to a combination of three antimicrobial compounds. 2016; *Submitted*.

Manuscript 3 - Citterio L, Franzyk H, Gram L. Development of resistance to antimicrobial peptides and peptidomimetics: how critical is this challenge? 2016; *In preparation.*

Not included in the thesis

Article - Andreev K, Bianchi C, Laursen JS, **Citterio L**, Hein-Kristensen L, Gram L, Kuzmenko I, Olsen CA, Gidalevitz D. Guanidino groups greatly enhance the action of antimicrobial peptidomimetics against bacterial cytoplasmic membranes. *Biochim Biophys Acta (BBA)-Biomembranes* 2014; 1838(10):2492-2502.

Acknowledgments

I thank Lone for the supervision, especially for trusting me, for being direct and fair, for presenting me the ways to operate and pushing me to face my weaknesses and be more proud of my achievements.

I thank Henrik for the prompt help, great input to the project and impressive accuracy in reviewing manuscripts and proofs.

I thank Anna, Hanne, Ramona, Thomas and Yaseelan for contributing to the completion of my manuscripts, especially Ramona for her dedication in producing beautiful scanning electron microscopy images.

I thank the PhD students from "my" generation: Henrique, Sonia, Tom, Torben and the ones from the "new" generation: Bastian, Karen, Nicole, Sara, especially Bastian, Henrique and Sonia for sharing essential details.

I thank all the other past and present members of the Gram Lab for good advice and great company through the past three years. I thank Sandra from Lars´s group, Rachel from CfB and Thanassis from Copenhagen University for sharing their opinions on how to analyze genomes.

I thank the people who were aside me in these past three years, the ones that stayed and the ones that are gone too. A special thought goes to Dimitrios and R.K. for always presenting me life from the realistic and the idealistic point of view, respectively. I thank Mats for being a very quiet and respectful flatmate, especially during the last month of my PhD time.

And I thank Rozalia, of course, just for her amazing irreplaceable companionship.

I thank Matteo for his kind presence, for giving me the courage to face what I would call "problems" and making me believe that solutions can always be found.

I thank Clara for her passionate support, despite the distance and our divergent characters.

I thank Massimo and Monica for being outstanding examples of fortitude and patience in their everyday lives and for kicking me when I am drifted.

I thank anyone who will run into this thesis for the interest. I hope it will somehow enrich you.

Contents

Abbreviations

AMPs	Antimicrobial peptides
Ara4N	4-aminoarabinose
ATR-FTIR	Attenuated total reflectance – Fourier transform infrared spectroscopy
CD	Circular dichroism
CDC	Center for Disease Control and Prevention
ECDC	European Centre for Disease Prevention and Control
EMEA	European Medicines Agency (EMEA)
FDA	Food and Drug Administration
HIV	Human Immunodeficiency Virus
HTS	High-throughput sequencing
LPS	Lipopolysaccharide
MDR	Multi drug resistant
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin Resistant *Staphylococcus aureus*
NMR	Nuclear Magnetic Resonance
PD	Pharmacodynamics
PE	Phosphatidylethanolamine
PEtN	Phosphoethanolamine
PK	Pharmacokinetics
SEM	Scanning Electron Microscopy
SNP	Single Nucleotide Polymorphism
SNV	Single Nucleotide Variant
TA	Teichoic acids
WGS	Whole-genome sequencing
WHO	World Health Organization

Introduction

1. The challenge of antibiotic resistance development

Bacterial infectious diseases such as respiratory and gastro-intestinal infections are generally treated with conventional antibiotics. Most conventional antibiotics have still clinical relevance; however occurrence of resistance to them has become a global challenge (WHO 2012; Antimicrobial resistant threats, USA, 2013). The phenomenon of antimicrobial resistance has been known even before the introduction of penicillin as antibiotic (Abraham and Chain, 1940) and over the last century, it has been observed that resistance arises independently from the chemical group of drug (Figure 1). The issue has recently raised global awareness due to the increase of resistant organisms, the affected geographic locations and the breadth of resistance in single organisms (Levy and Marshall 2004).

Also, the number of people who died and related health costs contributed to the increased urgency of the problem. It is estimated that about 25,000 people die in Europe yearly due to infections related to antibiotic resistant bacteria, which represents 2/3 of all mortal hospital-acquired infections (Table 1). During a hospital stay 5% of the patients are infected, meaning 3.2 million/year. Moreover, resistant infections cause excess hospital days and related health care costs e.g. an estimation of $ 20 / € 16 billion in the US and EU, respectively, as reported by Fair and Tor, (2014). Countries with the lowest income may be even more affected by antibiotic resistance. In fact, spread of resistant bacteria is favored by poor hygiene, contaminated food and water and overcrowding. Moreover, susceptibility to infection increases with malnutrition (Laxminarayan et al., 2013).

Table 1: Estimated annual burden due to selected antibiotic-resistant bacteria in European Union, Iceland and Norway. Numbers in parentheses indicate percentage bloodstream infections.

Antibiotic-resistant bacteria	No. cases of infection*	No. extra deaths	No. extra hospital days
Antimicrobial resistant Gram-positive bacteria			
Methicillin-resistant *Staphylococcus aureus* (MRSA)	171 200 (12%)	5400 (37%)	1 050 000 (16%)
Vancomycin-resistant *Enterococcus faecium*	18 100 (9%)	1500 (28%)	111 000 (22%)
Antimicrobial resistant Gram-negative bacteria			
3rd generation cephalosporin-resistant *Escherichia coli*	32 500 (27%)	5100 (52%)	358 000 (27%)
3rd generation cephalosporin-resistant *Klebsiella pneumoniae*	18 900 (27%)	2900 (52%)	208 000 (27%)
Carbapenem-resistant *Pseudomonas aeruginosa*	141 900 (3%)	10 200 (7%)	809 000 (3%)

* Bloodstream infections, lower respiratory tract infections, skin and soft tissue infections, and urinary tract infections. Table 1.1, WHO 2012, adapted from ECDC and EMEA, (2009).

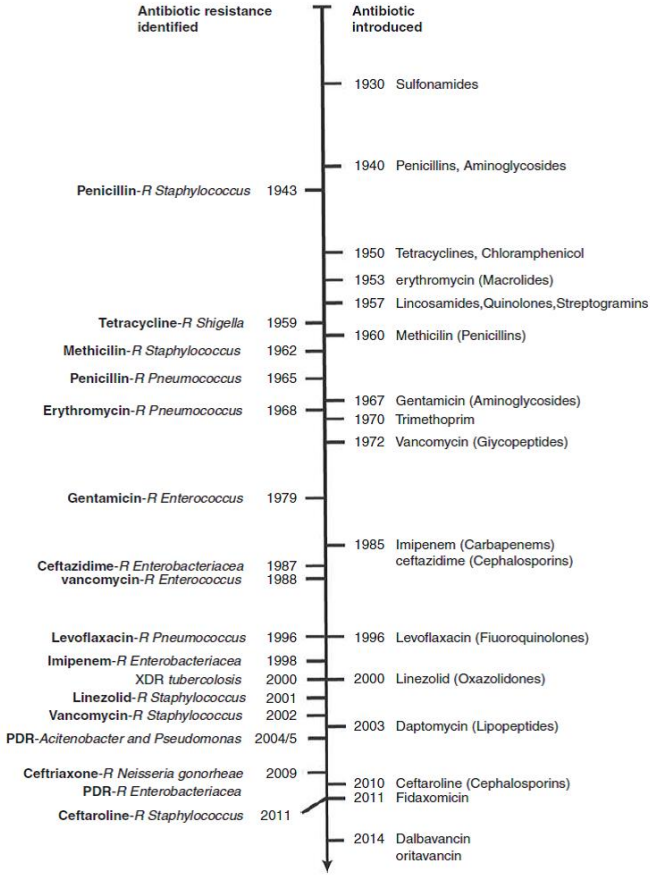


Figure 1: A timeline of introduction of various antibiotics and emergency of antibiotic resistance against them. Figure 5 in Penchovski and Traykovska, (2015).

Three different classes of drug-resistant bacterial pathogens were established by the Centers for Disease Control and Prevention (CDC 2013, *Threat Report*). Such classification into "urgent", "serious" and "concerning" pathogens can be used worldwide in order to recognize and prioritize the bacterial threats (Penchovski and Traykovska, 2015). In the category of "urgent" pathogens one finds the carbapenem-resistant Enterobacteriaceae (with *Klebsiella* spp. as the most recorded pathogen), *Clostridium difficile* and drug-resistant *Neisseria gonorrhoeae.* Among the "serious" threats, twelve pathogens have been included, some of whom are considered more deadly in the developing countries rather than in the US (Penchovski and Traykovska, 2015). The concern derives from accumulation of multiple genes, each resistant to a different drug, in the chromosome or in the plasmid of these bacteria, thus defined multi drug resistant (MDR) pathogens. In addition, the presence of multiple efflux pumps can extrude a great variety of drugs (Nikaido, 2009). Thus, it is evident that MDR pathogens are considered a growing challenge for healthcare environment and community too.

An example of "serious" MDR pathogen is the Gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA). In the USA it is estimated that approximately 100,000 deaths per year are

attributed to infections caused by selected MDR pathogens, 18,000 of these caused by MRSA (Klevens et al. 2007).
Examples of "serious" Gram-negative MDR pathogens are *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Antimicrobial resistant threats, USA, 2013; Sabtu et al., 2015). In accordance to this classification, it is estimated that the main pathogens causing hospital-acquired infections are the Gram-negative *Klebsiella* spp. (*K. pneumoniae*/*K. oxytoca*), *Escherichia coli*, *P. aeruginosa* and *Enterobacter* spp. (Magill et al., 2014; Amin and Deruelle, 2015).

The Gram-positive *Enterococcus faecium* and *Staphylococcus aureus*, along with the Gram-negative *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp., are also known as ESKAPE pathogens. According to the National Healthcare Safety Network (NHSN), around 40% of the hospital-acquired infections in US were related to the Gram-negative subset of the ESKAPE pathogens, in 2009-2010 (Amin and Deruelle, 2015). It is now believed that ESKAPE pathogens are the major cause of nosocomial infections throughout the world (Santajit and Indrawattana, 2016).

The spread of resistant clones coupled with the lack of effective antibiotics can cause serious consequences in health-care settings for vulnerable patients. Patients who are undergoing surgery, transplantation and chemotherapy have the highest risk of acquiring infections (Laxminarayan et al., 2007). In order to understand the relevance of this problem, it has been estimated that 30-40% of the patients having hip replacements would acquire a post-surgery infection, with fatality rate of 30% (Smith and Coast, 2013).

Despite the clinical challenge that these bacteria represent, the approval of new antibiotics has declined in the last 40 years (Amin and Deruelle, 2015). Oxazolidinones (e.g. linezolid in 2000) and lipopeptides (e.g. daptomycin in 2006) are the only two new classes of antibiotics that during the last 25 years that have reached clinical application against Gram-positive bacteria, while the others are older modified drugs (Sabtu et al., 2015). Also, antibiotic classes with activity against highly resistant Gram-negative bacteria are very scarce (Antimicrobial resistant threats, USA, 2013; Sabtu et al., 2015).

The scarcity of novel approved antibacterial compounds derives partially from the observation that pathogens rapidly will evolve resistance to novel compounds. This concern discourages investments in the development and approval of novel drugs (Carlet et al., 2012). Moreover, the cost of bringing new drugs into the market is considerable, with an estimation of US $ 1 billion per drug for developing and marketing a new antibiotic (Amin and Deruelle, 2015). In addition to these costs, and partially because of them, there is less interest in antibiotic development from the pharmaceutical companies. It is estimated that only 1.6% is the antibiotic allotment of all drugs in development by the major pharmaceutical companies (Shlaes, 2010). Moreover, multinational pharmaceutical companies with antibiotic divisions are only four (Boucher et al., 2013).

Given these premises, it becomes clear that the burden of antibiotic resistance requires prompt coordinated global interventions. Indeed, antibiotic resistance is considered one of the top-challenges for humanity, in accordance to the WHO (2014 Report).

2. How to tackle antibiotic resistance development

There is no universal consensus on which is the most appropriate way of addressing the problem of resistance to antibiotics (Sabtu et al, 2015) and there are several approaches to tackle resistance development (Table 2, Spellberg et al. 2003).

Table 2: New interventions to address the antibiotic-resistance crisis. Adapted from Spellberg et al., (2013).

Intervention	Status
Preventing infection and resistance	
Improvement of population health and health care systems to reduce admission to hospitals	Implementation research stage
Self-cleaning hospital rooms; automated disinfectant application e.g. through vapor	Some commercially available but require clinical validation; more needed
Novel drug-delivery systems to replace intravenous catheters; regenerative-tissue technology to replace prosthetics; noninvasive ventilation strategies	Basic science and conceptual stages
Niche vaccines to prevent resistant bacterial infections	Basic and clinical development stages
Refilling antibiotic pipeline by aligning economic and regulatory approaches	
Government or nonprofit grants and contracts to defray up-front R&D costs and establish nonprofits to develop antibiotics	Models in place, expansion needed, new nonprofit corporation needed
Institution of novel approval pathways (e.g. Limited Population Drug proposal)	Proposed, legislative and regulatory action needed
Preserving available antibiotics, slowing resistance	
Public reporting of antibiotic-use data for benchmarking and reimbursement	Policy action needed to develop and implement
Development of and reimbursement for rapid diagnostic and biomarker tests to enable appropriate use of antibiotics	Basic and applied research and policy action needed
Elimination of use of antibiotics to promote livestock growth	Legislation proposed
New waste treatments for degradation of antibiotics	One strategy approaching clinical trials
Developing microbe-attacking treatments with diminished potential to drive resistance	Preclinical, proof-of-principle stage
Immune-based therapies, such as infusion of monoclonal antibodies and white cells that kill microbes	
Antibiotics or biologic agents that do not kill bacteria but alter their ability to trigger inflammation or cause disease	

Developing treatments attacking host targets rather than microbial targets to avoid selective pressure driving resistance Direct moderation of host inflammation in response to infection (e.g. cytokine agonists or antagonists) Sequestration of host nutrients to prevent microbial access to nutrients Probiotics that compete with microbial growth	Preclinical, proof-of-principle stage

The main strategies would be to prevent infections from occurring, encourage investments in anti-infective treatments, delay resistance development and alter host-microbe interactions without directly killing the microbes. The latter approach may involve the usage of inhibitors of efflux pumps and/or quorum sensing inhibitors. The most popular suggested approach to delay resistance development is currently the reduction of usage of antibiotics. Indeed, a cost of $ 1.1 billion of unnecessarily prescribed antibiotics was estimated in the US. In addition to human usage, 24.6 million pounds of antibiotics per year were administered non-therapeutically on animals in the US, in the early 2000's. (Fair and Tor, 2014). In parallel to reduction of usage, developing of new compounds, especially against MDR bacterial pathogens, is strongly advocated (Penchovski and Traykovska, 2015).

Over the past twenty years several studies have investigated the potential of cationic antimicrobial peptides (AMPs) as novel anti-infective agents (Hancock and Lehrer, 1998; Marr et al., 2006). These naturally occurring compounds have been a model for extensive design of new classes of antibacterial compounds (McGrath et al., 2013). Hereafter we survey the features common to the natural compounds, their pros and cons and their potential applicability.

3. Hypotheses and objectives of the present investigation

The efficacy of synthetic natural and modified variants of antimicrobial peptides may be hampered when such compounds are used *in vivo*. We therefore hypothesized that the activity of α-peptide/β-peptoid peptidomimetics would be affected by the presence of body fluids such as human blood serum and plasma. Based on this hypothesis, the purpose of Article 1 was to investigate the antibacterial effect of α-peptide/β-peptoid peptidomimetics in laboratory media that would mimic *in vivo* conditions.

Bacterial resistance to antimicrobial peptides and peptidomimetics can develop as it does toward conventional antibiotics. AMPs used in combination may decrease the risk of bacterial resistance development as compared to single AMP-treatments. We therefore hypothesized that evolution of resistance to a combination of three selected antibacterial compounds would be slower than to the single compounds.

The objectives addressed in Manuscript 2 and 3 have been the following:

- to investigate in *E. coli* the development of adaptive resistance to two AMPs and one peptidomimetic, alone and in a combination of three;
- to identify the genetic basis of the evolved resistance in *E. coli* by whole-genome sequencing analysis;

- to investigate the stability of the evolved resistance;
- to search, in the adapted resistant bacteria, for the occurrence of cross-resistance and susceptibility to other antibacterial compounds.

4. Cationic antimicrobial peptides

AMPs are a group of compounds produced by most living organisms as part of their innate defense (Hancock and Lehrer, 1998); (Figure 2). AMPs can have different mechanisms of antibacterial activity. However, perturbation of the cell envelope is a key mechanism of activity of many AMPs. Use of AMPs as potential novel antibacterial agents is considered a promising approach to overcome bacterial resistance to conventional antibiotics. They constitute an innate defense component of all living organisms, have small size and have demonstrated activity against bacterial, viral and fungal infections (Tossi et al., 2000; Zasloff, 2002; Brogden et al., 2003).

AMPs exist in all multicellular organisms and have evolved in living organisms over 2.6 billion years (Kaufmann et al., 2004). It is known from the beginning of 20th century that body secretions as well as blood and polymorphonuclear leukocytes contain antimicrobial compounds (Skarnes and Watson, 1957). Example of secretions where human AMPs are present are saliva, tears, sweat and milk; they are also found in the skin and the tongue, bone marrow, plasma, kidneys, liver, heart, brain, eyes, intestine, sperm, urinary tract, amniotic fluid, and respiratory tract. Many different cell types such as epithelial/mucosal cells, macrophages, neutrophils, natural killer cells, monocytes, eosinophilic leukocytes, Paneth cells, T-cells and B-cells also contain AMPs (Wang and Wang, 2016).
The most well-known AMPs comprise lysozyme (isolated from the nasal mucous; Fleming, 1922), cecropins (from moths; Steiner et al., 1981), magainins (from frogs; Zasloff, 1987), β-defensins (Lehrer, 2004) and cathelicidins (Gennaro and Zanetti, 2000), of which the last two types are key components of the antimicrobial response in polymorphonuclear leukocytes in humans.
In fact, the human cathelicidin LL-37 chemoattracts neutrophils, monocytes, and mast cells (Yang et al., 2000) while β-defensins chemoattract leukocytes (Territo et al., 1989) as well as dendritic cells, which ultimately phagocytize and kill the pathogens (Liu, 2001). Hence, cathelicidins and β-defensins are key effectors both in the adaptive and innate immune system.

Mammalian AMPs can be expressed constitutively or be inducible (Martin et al., 1995), and they are produced by ribosomal translation of an mRNA template followed by proteolytic steps (Gudmundsson et al., 1996). Conversely, peptide-based antibiotics of bacterial origin (such as polymyxins, the glycopeptide vancomycin and the lipopeptide daptomycin) or fungal origin (β-lactams) are produced via non-ribosomal peptide synthesis.

AMPs typically possess a net positive charge and amphipathic properties. The positive charge of AMPs is believed to be key to their effect, interacting with and perturbing the negatively charged bacterial cell envelope. The net charge of human AMPs varies from −3 to +20, but most natural AMPs are cationic. In fact, it is often stated that a net positive charge of at least +2 is necessary for an AMP to exhibit a reasonable activity. AMPs normally contain two or more residues such as arginine or lysine that are positively charged at neutral pH (Hancock et al., 1995). Some AMPs contain histidine as positively charged residues in weakly acidic environments.

The length of human AMPs can vary from 10 to 150 amino acids (Wang, 2014). Some proteins are too big to be classified as AMPs or are produced following highly regulated immune processes (Kondos et al., 2010; Voskoboinik et al., 2010). AMPs longer than 10 residues may fold into a three-dimensional secondary structure, of which α-helices are most common. Notably, α-helices are barrel-shaped, and often two "sides" can be distinguished: a hydrophobic and a hydrophilic, consisting of non-polar amino acid side chains and positively charged residues, respectively (Hancock, 1997). The content of hydrophobic residues is approx. 40-60% enabling an amphiphilic structure (Tossi et al., 2000).

Up to 2600 AMPs are listed in an online antimicrobial peptide database, APD3, accessible at http://aps.unmc.edu/AP/main.html (Wang and Wang, 2016). A few examples of AMPs are shown in Figure 3. In the database, 112 are different human host-defense peptides, of which 100 were shown to have broad-spectrum antibacterial activity (Andersson et al., 2016).
In the APD3 database AMPs have been classified based on their 3D structure into four major families (Wang et al., 2016):

- α-family, composed of AMPs with α-helical structures, such as the human cathelicidin LL-37 (Agerberth et al.,1995)
- β-family, composed of AMPs with β-strands, such as the human α-defensins (Selsted et al., 1985)
- αβ-family, with AMPs with structures containing both α-helical and β-strands (Andersson et al., 2016)
- non-αβ-family contains AMPs with neither α-helical nor β-strands, such as indolicidin (from cattle; Selsted et al., 1992).

Other classification modes exist such as linear and cyclic peptides (Kohli et al., 2002). Peptides can also be grouped according to the most abundant amino acids e.g. Pro-rich (Gennaro et al., 2002; Otvos, 2002) and Arg-rich peptides (Chan et al., 2006).

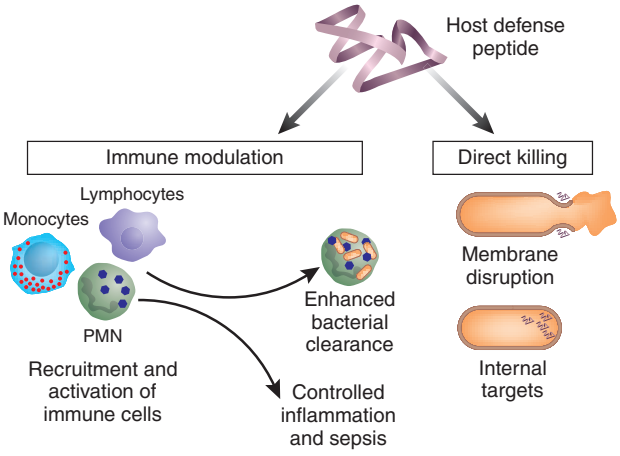


Figure 2: AMPs can act directly against the pathogens or indirectly by potentiating the immune host defense; certain peptides have one or the other activity preferentially (Figure 1 in Hancock and Sahl, 2006).

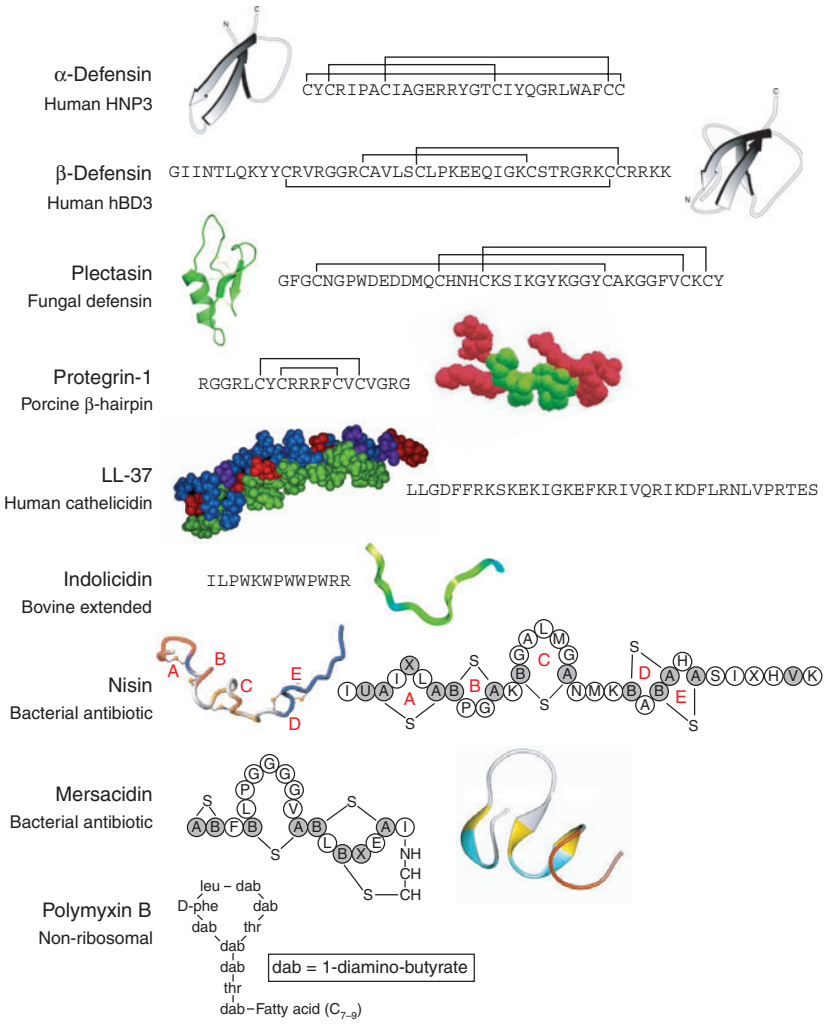


Figure 3: Selected structures and sequences of host-defense peptides (Figure 2 in Hancock and Sahl, 2006).

4.1 Advantages of AMPs over conventional antibiotics

Several features of AMPs (and derivatives thereof) make them potential promising novel antibiotics: AMPs have co-evolved with human pathogens. This long evolution time has allowed for emergence of high modularity and diversity in length, amino acid sequence and secondary structure (Hancock, 1997; Zasloff, 2002). AMPs exert bactericidal effect rather than being bacteriostatic; hence, cell membrane disruption and inhibition of cell functions occur during a short contact time and cause rapid killing of bacteria (Hirsch, 1956; Hultmark et al., 1980; Tu et al., 2015). They act in synergy with the immune system by inducing production of chemokines, accelerating angiogenesis, wound healing and modulating apoptosis in multicellular organisms (Gudmundsson and Agerberth, 1999; Lai and Gallo, 2009). All these are desirable features for a drug class alternative to conventional antibiotics.

4.2 Mechanism of action of AMPs

Net charge and amphipathic structure are the two interrelated features that confer antibacterial activity to AMPs. Often such peptides cause lysis of the bacterial cell and it is believed that cell lysis originates from the interaction between the bacterial, anionic membranes and the positively charged peptides. First, electrostatic interaction with the bacterial surface occurs (Skarnes and Watson, 1957; Raguse et al., 2002).

In Gram-negative bacteria, electrostatic interactions occur between the positively charged AMPs and the negatively charged lipopolysaccharide (LPS) or phospholipid head groups of the membrane (Figure 4). Teichoic acids (TAs) are the negatively charged components present in the bacterial envelope of Gram-positive bacteria. In Gram-positive bacteria AMPs interact with and diffuse into the cell membrane while in Gram-negative bacteria AMPs interact via high binding affinity for LPS and high permeabilization of the outer membrane leading to efficient and faster killing (Balakrishnan et al, 2013).

The factors influencing interactions of AMPs with membranes can be studied by modifying their charge and hydrophobic properties. The latter are assumed to be more important for activity against Gram-positive bacteria.

After the electrostatic interaction, the peptide is inserted into the bacterial cell membrane where the amphipathic nature of AMPs allows them to form pores. This process provokes disruption of the membrane integrity leading to osmotic lysis of the bacterial cell accompanied by leakage of cytoplasmic components, and consequently cell death (Van't Hof et al., 2001; Shai, 2002; Brogden, 2005; Kawasaki et al. 2008). Lysis generally increases as the charge increases (Ringstad et al., 2007; Malmsten, 2014). The nature of the charged group and charge distribution can also have an impact on the membrane interactions of AMPs (Pasupuleti et al., 2012).

Pore formation occurs through the following steps. First AMPs accumulate on the surface of the membrane (Epand and Vogel, 1999), and then after reaching a threshold concentration, AMPs self-assemble and are incorporated into the membrane. A series of models have attempted to predict the details of this process: (i) in the barrel-stave model, peptides associate and form a pore inserted perpendicularly in the bilayer; (ii) in the carpet mechanism, peptides lie parallel to the bilayer and ultimately disintegrate the membranes by a detergent-like effect; (iii) in the toroidal pore mechanism, peptides insert perpendicularly into the bilayer and cause a membrane curvature where the pore is flanked by both peptides and phospholipid head groups (Melo et al., 2009). These different models can all be valid for most AMPs under different conditions.

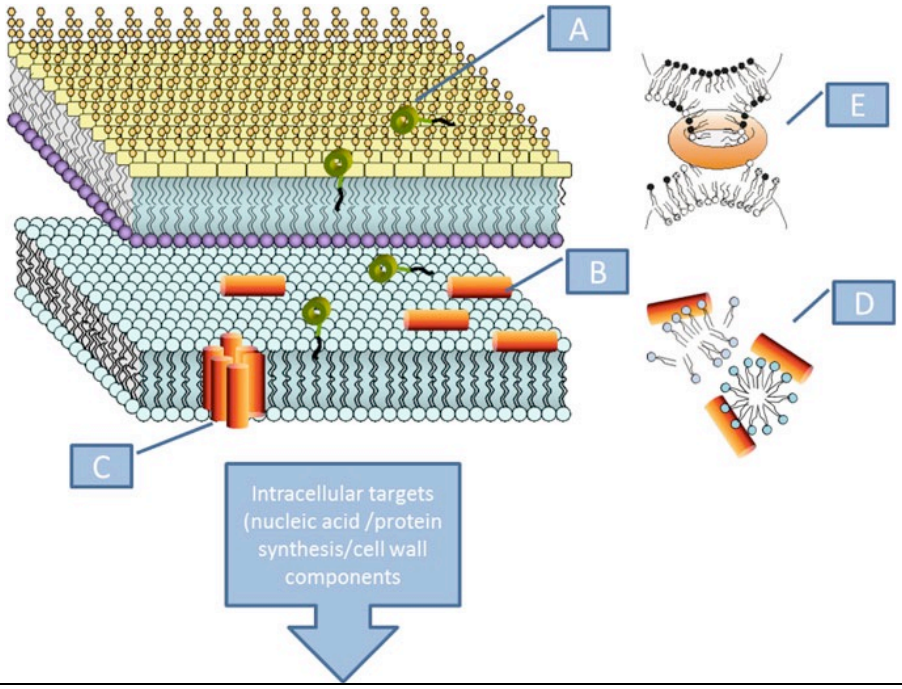


Figure 4: Model for the mechanism of action of AMPs. In Gram-negative bacteria, peptides must be able to penetrate the LPS-rich layer in the outer membrane in order to enter into the periplasmic space (A). Subsequently, peptides interact with the anionic cytoplasmic membrane while adopting an amphiphatic secondary structure (B). This structure is able to integrate into the outer leaflet, causing a thinning effect that is followed by channel formation, thus inducing depolarization and/or leakage of intracellular contents (C), or destruction of the bilayer structure by formation of micelles (D). In some cases, peptides induce contacts between outer and inner membranes, allowing mixing of lipids and therefore changing the membrane composition (E), thus resulting in osmotic imbalance. Finally, peptides may translocate across the cytoplasmic membrane and attack intracellular targets. Figure 1 in Rabanal and Cajal (Villa and Vinas, 2016).

Other mechanisms that involve much more specific interactions than described above have been suggested. Phoenix et al., (2015) and Stromsted et al., (2016) indicated phosphatidylethanolamine (PE) as a high-affinity lipid target for several AMPs. This lipid component is present in a high concentration on the surface of bacterial membranes, whereas it is only present on the cytoplasmic leaflet of mammalian membranes, and hence promotes specificity of AMPs for bacterial surfaces (Andersson et al., 2016). Bacterial membranes do not contain cholesterol (Tytler et al., 1995). Conversely, the plasma membranes of mammalian cells contain a significant amount (30-50 mol %) of cholesterol (Hao et al., 2001), along with neutral zwitterionic phospholipids, interspersed with low amounts of anionic lipids. Also these features ensure selectivity of AMPs toward bacteria over mammalian cell membranes (Lai and Gallo, 2009).

The mechanism of action of human AMPs is clearly targeted toward bacterial cell walls/membranes rather than mammalian plasma membranes. However, it is still not clear how the AMPs traverse the bacterial cell outer membrane. Membrane activity is the most frequently reported antibacterial mode of action for AMPs, but it may be an oversimplification of a much more complex process that does not always involve interactions with the bacterial surface (Huang et al, 2000; Guilhelmelli et al., 2013). Thus, it has been found that some AMPs may traverse bacterial envelope without damaging the membrane(s) and act intracellularly by blocking essential cellular processes (Patrzykat et al., 2002; Brogden, 2005; Sharma and Nagaraj, 2015). Suggested intracellular targets include the negatively charged DNA to which AMPs may bind through electrostatic interactions (Tu et al., 2015). Other intracellular mechanisms comprise inhibition of nucleic acid and/or protein synthesis as described by Brogden (2005) and Kawasaki et al. (2008). Moreover, other examples involve inhibition of chaperone-assisted protein folding, inhibition of enzymatic activity, and inhibition of cytoplasmic membrane septum formation and cell wall synthesis (Nicolas, 2009). Examples of AMPs with intracellular activity comprise α-defensin 5 (Chileveru et al., 2015) and indolicidin, a bovine cathelicidin with broad-spectrum bactericidal activity. The latter inhibits DNA synthesis (Subbalakshmi and Sitaram, 1998; Ghosh et al., 2014).

Nevertheless, also AMPs that target specific intracellular functions need to initially interact with the membrane (Otvos, 2002; Nicolas, 2009). Indeed some intracellularly-active AMPs may be translocated via certain membrane transporter proteins e.g. SbmA (Mattiuzzo et al., 2007; Corbalan et al., 2013).

Other studies have shown that membrane activity and intracellular targets are not clearly exclusive of each other (Podda et al., 2006; Gottschalk et al., 2015). It follows that interaction with microbial membranes and the resulting increased permeability cannot be the solely lethal steps for certain AMPs (Nicolas, 2009).

Therefore, AMPs are not merely pore formers as their mechanism of action is much more complex and diverse than previously believed. This diversity is not surprising considering the fact that host defense systems have evolved several strategies to fight pathogens (Nguyen et al., 2011).
The mechanism of action of AMPs also depends on the targeted bacteria. Furthermore, mechanism of action of AMPs differs not only between Gram-positive and Gram-negative bacteria, but also between different bacterial strains depending on the lipid composition of their membranes.

4.3 Methodologies for studying the mechanism of action of AMPs

It is essential to understand the mechanism of action of the antibacterial effect of AMPs. This is part of assessing potential toxicity and risks and can also facilitate the modifications of AMPs into potential drugs for human use.

Linear peptides (<40 residues) with α-helical domains can be produced by solid-phase synthesis and characterized by CD spectroscopy. The latter is used to infer the helical content of a given peptide, hence providing preliminary information on a peptide mechanism of action (Tossi et al., 1997). Structural definition of a given peptide can also be obtained by applying several other spectroscopic techniques in the presence of model systems such as cell wall components or liposomes. Examples of these techniques are NMR, ATR-FTIR, neutron scattering and atomic force

spectroscopy. In addition, surface plasmon resonance, fluorescent dye release from liposomes and electrical measurements of membrane conductance can be used to follow insertion, assembly and permeabilization events of AMPs in model systems (Zelezetski and Tossi, 2006). A typical approach is to use cytoplasmic phospholipid membranes mimicking bacterial membranes and study their morphological changes on a microscopic level before and after the insertion of the compound (Matsuzaki et al., 1994; Andreev et al., 2014).

Confocal and electron microscopy and flow cytometry are techniques used to study the mechanism of action of AMPs on whole cells (Chapple et al, 1998). The first ones can provide information on the morphological changes occurring on the bacterial cell, the second can be used to monitor changes in the bacterial cell wall potential.

In vitro studies with real bacteria such as antimicrobial susceptibility testing by determination of minimum inhibitory concentration (MIC) and time-kill assays are routinely applied.

Overall, the use of combined specific biophysical, biochemical and microbiological methods would be the best choice. Indeed, the analysis of the data inferred from both real and model system would provide a more complete understanding of the mechanism of action of peptides (Zelezetski and Tossi, 2006).

4.4 AMPs and potential application in treatments

On their way from pre-clinical phases to approval by FDA, AMPs are facing a series of challenges, partially due to the concern for their *in vivo* toxicity and resistance development.

The polymyxins B and E (the latter also known as colistin) are examples of AMPs in clinical use since the 1950s. They have been applied for both topical and systemic treatment of infections (Landman et al., 2008). Their therapeutic use has increased to combat multidrug-resistant pathogens (Falagas and Kasiakou, 2005). However, their intravenous use has been limited by the high nephrotoxicity and neurotoxicity (Falagas and Kasiakou, 2006). According to some authors, the concern for nephrotoxicity and neurotoxicity, already observed with the polymyxins, may be one of the reasons for preventing further development of AMPs for intravenous treatment. Nevertheless, these risks of toxicity seem overrated, considering that colistin is used continuously for years for treatment of patients affected by cystic fibrosis (Valerius et al., 1991; Schuster et al., 2013). The discrepancies between *in vitro* and *in vivo* trials may instead have discouraged clinical development of other AMPs (Falagas and Kasiakou, 2005; Zavascki et al., 2007; Landman et al., 2008).

Andersson et al., (2016) report cationic peptides that are currently in clinical development (Table 3). Among these, the human derived lactoferrin 1-11 and OP-145 (based on LL-37) are intended for intravenous administration and chronic ear infections, respectively. Pexiganan (analogue of magainin, frog-derived), iseganan (synthetic protegrin; from pig leucocytes) and omiganan (derivative of indolicidin; from bovine neutrophils) are also still in clinical development for treatment of diabetic foot ulcer, pneumonia and skin infections, respectively. Purely synthetic AMPs such as LTX-109 (Nilsson et al., 2015) and C16G2 (Kaplan et al., 2011) are in clinical trials for treatment of Gram-positive skin infections and dental diseases, respectively.

AMPs can also be used for other purposes than specifically bactericidal, e.g. for drug delivery into bacterial cells. Indeed intracellular-active AMPs can be used as vehicles for delivery of novel antibiotics such as the PNA (Antisense Peptide Nucleic Acid) oligomers (Hansen et al., 2016).

AMPs have also other applications than clinical. They are used as food preservatives, such is the case of the bacteriocin nisin (Papagianni, 2003). LL-37 and lysozyme are applied in cosmetics or in antifouling materials (Brogden and Brogden, 2011).

Table 3: Antimicrobial peptides in clinical development (Adapted from table 3 in Andersson et al., 2016).

Peptide	AMP source (host)	Status	Administration	Indication	Company
OP-145	LL-37 (human)	Phase I/II	Ear drops	Chronic bacterial ear infection	OctoPlus Inc.
hLF1-11 (Lactoferrin)	Lactoferrin	Not specified	Intravenous	Neutropenic stem cells transplantation patients	AM-Pharma B.V.
Pexiganan (MSI-78)	Magainin (frog)	Phase III	Topical cream	Diabetic foot infection	Dipexium Pharmaceuticals Inc.
		Phase III		Diabetic foot ulcers	MacroChem Corporation
Iseganan (IB-367)	Protegrin-1 (porcine leukocytes)	Phase III	Mouth wash	Prevention of chemotherapy-induced mucositis	National Cancer Institute
		Phase II/III		Prevention of ventilator-associated pneumonia	IntraBiotics Pharmaceuticals
Omiganan (MBI-226, CLS001)	Indolicidin (bovine neutrophils)	Phase III	Topical cream	Topical skin antisepsis, prevention of cetheter infections	Mallinckrodt
		Phase III		Rosacea	Cutanea Life Sciences Inc.
		Phase II		uVIN (usual type vulvaryl intraepithelial neoplasia)	Cutanea Life Sciences Inc.
		Phase II		Moderate to severe acne vulgaris	Cutanea Life Sciences Inc.
		Phase II		Mild to moderate atopic dermatitis	Cutanea Life Sciences Inc.

Lytixar (LTX-109)	Synthetic antimicrobial peptidomimetic	Phase II	Topical cream	Uncomplicated Gram-positive skin infections	Lytix Biopharma AS
		Phase I/IIa	Nasal	Nasal carriers of *Staphylococcus aureus*	
C16G2	Synthetic specifically targeted antimicrobial peptide	Phase II	Mouth wash	Prevention of tooth decay caused by *Streptococcus mutans*	C3 Jian Inc.

4.5 Disadvantages of AMPs

AMPs unfortunately come with several shortcomings (Table 4). One of these is the high cost of production as compared to conventional antibiotics, e.g. the penicillins. Yet this depends on the length of the compound.
On top of this, despite AMPs have been studied for more than three decades, a clear molecular understanding of their mechanism of action still represents a challenge (Wimley, 2010).

There may be bioavailability issues *in vivo*, as well as proteolytic instability (Chongsiriwatana et al., 2008). In fact, AMPs cannot be administered orally due to poor absorption after ingestion, because they are usually large and strongly cationic. They are rapidly degraded by proteases both in the bloodstream and in the gastrointestinal tract, and they do not diffuse easily into the Central Nervous System, due to the blood-brain barrier; they are rapidly degraded in the liver and excreted via the kidneys. They may lead to undesired effects due to interactions with several receptors; hence they may be too toxic to allow for systemic therapy (Bush et al., 2004). Moreover, there is an unknown risk of compromising the immune defense (Bell and Gouyon, 2003; Andersson et al., 2016).

Table 4: Advantages and disadvantages of AMPs as anti-infective drugs. Adapted from Gordon et al., (2005).

Advantages	**Disadvantages**
Broad-spectrum activity	Costs of synthesis, screening and manufacturing
Rapid killing	Patent exclusivity for economic viability
Bactericidal activity	Reduced activity in presence of salt, serum
Concomitant broad anti-inflammatory activity	Sensitivity to pH
Potential low levels of induced resistance	Susceptibility to proteolysis
High diversity	Local and systemic toxicity
Synergy with the immune system e.g. production of chemokines, accelerating angiogenesis, wound healing	Confounding biological functions (e.g. angiogenesis)
	Pharmacokinetic (PK) and pharmacodynamic (PD) issues
	Sensitization and allergy after repeated application
	Intrinsic resistance (e.g. in *Serratia marcescens*)

All these potential side effects make development of AMPs into future drugs challenging (Malmsten, 2014). Hence, they may be used for topical applications only, rather than systemic use (Cassone and Otvos, 2010). Indeed AMPs have proven to be successful when incorporated into topical therapeutic agents (Andersson et al., 2016).

The described shortcomings have led to an interest in designing synthetic AMPs and peptidomimetic analogues (Jahnsen et al., 2012; Liu et al., 2013; Jahnsen et al., 2015). Short-length synthetic AMPs (5-11 residues) have also received attention due to their structural simplicity (Lee et al., 2011; Lau et al., 2015) with an ensuing benefit in cost and ease of optimization as well as reduced side effects (Won et al., 2004; Oyston et al., 2009).

5. Synthetic variants of AMPs / peptidomimetics

Peptidomimetics exhibit antibacterial activity similar to AMPs. They are designed to improve the features of AMPs such as hydrophobicity, net charge and secondary structure (such as helicity) and provide reduced hemolytic activity and capability to assist in intracellular drug delivery (Marshall et al., 2003). Peptidomimetics possess non-peptidic backbones that confer proteolytic stability (Figure 5).

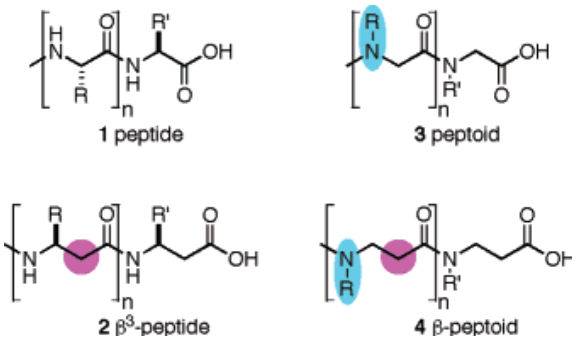


Figure 5: Backbone structures of natural α -peptides and peptidomimetic residues (Olsen et al., 2007).

Improved hydrophobicity is a desired feature in physiological environment, where high ionic strength can impair the membrane-disrupting effect of highly charged hydrophilic peptides (Ringstad et al., 2008). Yet, too hydrophobic peptides may interact both with bacteria and human cells (Malmsten, 2014). Hence, hydrophobicity shall be tuned carefully in order to maintain activity, but at the same time avoid toxicity. Higher hydrophobicity can be reached by incorporation of amino acids such as Leu, Ile, Phe or Trp (Qi et al., 2010) or by attachment of fatty acids to the peptide chain (Majerle et al., 2003).

Potency can be increased by introducing specific α-amino acids such as Leu or Lys (Beven et al., 2003). Such structure modifications stabilizes α-helical conformations (Chou and Fasman, 1974; Giangaspero et al., 2001), however, this is usually accompanied by decreased cell selectivity.

Similarly, tryptophan constitutes an amino acid that is abundant in natural AMPs (e.g. indolicidin and tritrpticin) (Epand and Vogel, 1999; Sitaram, 2006). Tryptophan may stabilize the α-helical structure of membrane-interacting peptides (Hu et al., 1993; Oh et al., 2000), and its indole side chain interacts favorably, via intercalation, with the outer membrane of the Gram-negative bacterial envelope (Jing et al., 2003). Hence, incorporation of Trp residues may confer antibacterial potency even in ultra-short synthetic AMPs (Won et al., 2002). Discouragingly, a high content of Trp also promotes hemolytic activity (Blondelle and Lohrer, 2000), raising the concern of lowered cell selectivity toward bacteria over mammalian cells. Nevertheless, this drawback may be circumvented by alternative molecular designs, *e.g.* Lee et al., (2011) found that Leu$\rightarrow$Trp substitutions in an LK-based amphipathic α-helical AMP afforded a short peptide (LLKWLKKWLKK-NH_2) with high antibacterial activity while retaining low hemolytic activity. In addition, C-terminal end-tagging with Trp or Phe residues was shown to improve potency and cell selectivity of the Pro/Arg-rich peptide RRPRPRPRP (Schmidtchen et al., 2011; Malmsten et al., 2011).

Also, a high content of Arg often confers high potency as it increases overall positive charge as well as hydrogen-bonding toward negatively charged head groups of negatively charged phospholipids abundant in the bacterial membrane (Chan et al., 2006). The nine-residue Pac-525 (Ac-KWRRWVRWI-NH_2) and its derivatives were reported to be essentially devoid of cytotoxicity (Qi et al., 2010; Li et al., 2015). Their high potency is believed to arise from the presence of Arg-Trp (RW) repeats, also present in other well-known natural AMPs, such as lactoferricin B, indolicidin and tritrpticin (Vogel et al., 2002; Liu et al., 2007). A screening of 30 ultra-short peptides (up to 9 residues) revealed that Pac-525 and an octapeptide (IRIRIRIR-NH_2), containing (RW) and isoleucine-arginine (IR) repeats, respectively, both displayed broad-spectrum activity (MIC 6.25 µM toward MRSA, *P. aeruginosa* and *Candida albicans*) as well as low cytotoxicity (Lau et al., 2015). The RW motif appears preferentially to induce membrane disruption rather than pore formation.

The presence of chiral hydrophobic β-peptoids, guanidinylated amino acid side chains and the relatively short length are some of the strategies adopted in order to keep a favorable balance between AMP potency and cytotoxicity (Liu et al., 2013).

Evaluation of peptide toxicity simply by hemolysis test is not enough to infer the toxicity of the compounds *in vivo*. Hence, considerable effort has been devoted to improved design towards enhanced selectivity against selected mammalian cell lines (Jahnsen et al., 2014).

An alternating cationic-hydrophobic design was shown to enhance selectivity against Gram-negative pathogens over mammalian benign cells (Jahnsen et al., 2014). Improved selectivity toward Gram-positive such as *Enterococcus faecium* and *Staphylococcus aureus* can be achieved by introduction of specific hydrophobic moieties at the N-terminus, unlike introduction of cationic moieties (Jahnsen et al., 2015).

Control of selectivity against mammalian cells can also be achieved by introduction of D-amino acids and fluorinated amino acids. These are believed to break the secondary structure and reduce the hydrophobic bonds involved in the interaction with mammalian cells.

5.1 Mechanism of action of peptidomimetics

Mechanism of action is inferred by studying the interaction of peptidomimetics with lipid bilayer membranes by microcalorimetric and spectroscopic analysis; by these means effect of length, charge and N-terminal end group can be studied. Membrane depolarization, dye leakage and Scanning Electron Microscopy (SEM) assays can be applied to study membrane interactions (Qi et al., 2010). The mechanism of action of peptidomimetics largely resembles that of AMPs. Hence, activity and membrane specificity are fine-tuned by keeping a balance between hydrophobicity and presence of cationic residues (Porter et al., 2002; Al Badri et al., 2008).

5.2 Disadvantages of peptidomimetics

Toxicity in the human body, due to high hemolytic activity, is one of the main current challenges for the application of peptidomimetics (Qi et al., 2010; Cruz-Monteagudo et al., 2011). High hemolytic activity can correlate with high hydrophobicity, high amphipathicity and high helicity. Hence, accurate rational design shall be pursued in light of developing peptides with lower or no toxicity over erythrocytes. Cruz-Monteagudo et al., (2011) proposed a chemoinformatic model where hemolytic activity of a given compound is predicted. This tool may aid in selection of compounds with great antimicrobial potency and at the same time low toxicity.
Potential undesirable side effects due to interaction of peptidomimetics with components of our immune system are also a concern and may prevent their further application.

5.3 Treatment applications of peptidomimetics

According to Gordon et al. (2005), no modified AMP has received FDA approval; in a more recent review, two compounds (synthetic mimics of defensin and protegrin, respectively) are in proccess of approval (Table 5); (Amin & Deruelle, 2015).

Table 5: Preclinical compounds currently in development against Gram-negative pathogens. Adapted from Amin and Deruelle, (2015).

Agent name	**Company**	**Antibiotic class**	**Target Gram-negative pathogens**	**Development status**
POL7080	Polyphor Ltd (Allschwil, Switzerland)	Protegrin mimetic	*Pseudomonas aeruginosa*	Phase I trial completed, recruiting participants for phase II trial
CTIX1278	Cellceutix (MA, USA)	Defensin mimetic	*Klebsiella pneumoniae*	*In vivo* animal model
RPX2014/RPX7009	The Medicines Company (NJ, USA)	Carbapenem/new β-lactamase inhibitor combination	*K. pneumoniae*	Phase I completed
BAL30072	Basilea	Monosulfactam	*Acinetobacter*	In phase I

	Pharmaceutica (Basel, Switzerland)		*baumannii, Enterobacter spp., Klebsiella oxytoca, and P. aeruginosa*	
DS-8587	Daiichi-Sankyo (Tokyo, Japan)	Quinolone	*Acinetobacter baumannii*	In phase I

6. The efficacy of AMPs and peptidomimetics *in vivo*

The efficacy of novel compounds is first evaluated *in vitro*, in standard laboratory medium (e.g. Mueller-Hinton broth). However, it is known that the Minimum Inhibitory Concentration (MIC) required for *in vitro* activity is generally higher than the concentration required *in vivo*, due to accumulation of the compound at the site of infection and synergy with other AMPs (Lai and Gallo, 2009). Also, interaction of AMPs with components of the human body may affect the actual efficacy *in vivo* (Deslouches et al., 2005). For instance, in presence of high salt concentrations (e.g. of NaCl and/or $MgCl_2$) the activity of AMPs may be lower than expected from MIC values determined *in vitro*. In fact, divalent cations such as Mg^{2+} and Ca^{2+} compete with the cationic AMPs for binding sites on the LPS (Tomita et al., 2000). Also, reduced activity of natural and synthetic peptides in presence of serum has been reported for several known AMPs (Knappe et al., 2010; Gottschalk et al., 2016). It follows that the efficacy of compounds *in vivo* may differ from that *in vitro*. Hence, it is necessary to find ways to predict the functionality of compounds *in vivo*.

An improved approach is to test the compounds in laboratory systems that mimic *in vivo* conditions i.e. in the presence of biologically relevant concentrations of blood matrices. Previous studies have shown that such conditions can be provided by human blood serum and plasma added to the standard laboratory medium (Yeaman et al., 2002; Hein-Kristensen et al., 2013(b). We applied a similar approach and found that the presence of human blood plasma increased the activity of two α-peptide/β-peptoid peptidomimetics against a broad range of Gram-negative and Gram- positive human pathogens (Table 1 in Citterio et al., 2016). This finding further supports the potential of two α-peptide/β-peptoid peptidomimetics *in vivo*. In fact, concentrations of peptidomimetics and peptide antibiotics may be lowered than usually predicted from standard antimicrobial susceptibility testing. Moreover, these compounds were selected for their low general cellular toxicity (Liu et al., 2013; Jahnsen et al., 2014). This means that cytotoxic side effects will decrease significantly when the concentration needed for bacterial killing is lowered 2- to 16-fold.

Hence, modification of AMPs in order to enhance their effect in presence of blood or at physiological ionic strength constitutes a viable strategy for assessing their utility *in vivo*. In these regards, Deslouches et al., (2005) designed an arginine-rich AMP that can retain its activity in presence of serum and plasma against *Pseudomonas aeruginosa.* Conversely, in the same study it was found that the human cathelicidin LL-37 loses its activity when in contact with serum and plasma. We also found a decreased activity of LL-37 in the presence of plasma (Table 5 in Citterio et al., 2016). Indeed, Wang et al. (1998) proposed that LL-37 binds to a 30-kDa plasma protein. It may be that LL-37 binds to blood components in order to balance its otherwise

cytotoxic effects *in vivo* (Panyutich and Ganz, 1991). In contrast, synthetic highly cationic non-helical compounds may not bind to blood components. Based on these observations, synthetic analogues gain a further advantage over natural AMPs (Brogden and Brogden, 2011; Godballe et al., 2011).

6.1 Causes of potentiation of antibacterial compounds by plasma

Endogenous blood components such as complement proteins as well as factors of the coagulation cascade may be involved in the potentiation effect. It is known that complement proteins can act in synergy with antibacterial compounds such as antibiotics (Dutcher et al., 1978) and AMPs (Yeaman et al., 2002). A previous study showed that terminal complement components could enhance the antibacterial effect of the membrane active polypeptide polymyxin B. Conversely the same effect was not seen for β-lactams or aminoglycoside antibiotics (Fierer and Finley, 1979). Similarly, in Article 1 we showed that plasma enhanced the activity of polymyxin B, as opposed to gentamicin or ampicillin (Table 5 in Citterio et al., 2016). Hence we hypothesized that potentiation of antibacterial compounds by plasma may preferentially occur for membrane-targeted compounds such as AMPs and peptidomimetics.

A connection between activation of complement proteins and the coagulation cascade has been discovered. Complement is known to enhance the coagulation process by inhibiting anticoagulation factors (Markiewski et al., 2007). Also, specific coagulation factors in plasma trigger the release of antimicrobial compounds in vivo (Frick et al., 2006). The human plasma that we have used in our study is devoid of platelets. Hence, we discarded the hypothesis that platelet-derived antimicrobial peptides may be involved in the potentiation of the peptidomimetics.

In these regards, AMPs derived from or based on human platelets are known for retaining activity in the presence of plasma (Darveau et al., 1992; Yeaman et al., 2002). In addition, peptides released by platelets such as platelet microbicidal proteins, defensins and kinocidins have gained increased attention for their involvement in clearance of pathogens (Yeaman et al., 2014). Thus, it is clear that platelets retain a hemostatic role as well as a key function in antimicrobial host defense. These observations lead us to speculate that all these processes may aid the activity of peptidomimetics *in vivo*. Nevertheless we acknowledge that these interconnected processes can only be confirmed in whole-blood models.

7. Causes of antibiotic resistance development

Antibiotic resistance is defined as an inherited feature of microorganisms that are able to grow at high concentrations of an antibiotic (Scholar and Pratt, 2000; Brauner et al., 2016).
Genetically encoded resistance can be located on chromosomal genes as well as on plasmids. Resistance already exists as an evolutionary trait; indeed resistance can be selected for within a window of drug concentrations high enough to inhibit wild type growth but low enough for some resistant mutants to grow (Drlica, 2003; Michel et al., 2008).
Since some antibiotics are themselves a bacterial product, bacterial adaptation to antibiotics produced by other bacteria occurred over the history of life (Spellberg et al, 2013). Indeed, several examples of resistant bacteria exist in nature. Moreover, resistance can develop in susceptible

bacteria following selection pressure from antibiotic use. Indeed, suboptimal concentrations of antibiotics are known to trigger a stepwise selection of mutations that confer competitive advantage (Laxminarayan et al., 2013). In fact, a major cause of resistance spread is represented by sub-optimal doses of antibiotics, according to US CDC (2013) and ECDC (2013).

Emergence of resistance is driven by the interplay between several factors such as mutation rate, strength of the selective pressure, fitness of the resistant mutant, compensatory evolution, epistasis (Hughes and Andersson, 2015). These concepts are briefly discussed below.

Mutation rate of antibiotics is the product of population size and rate of mutation, which is typically in the range of 10^{-5} to 10^{-10} per generation per cell. In an infected human, the bacterial population size may be unknown. However, some estimates exist and it is known that the bacterial population size can increase so that the presence of resistant mutants becomes more likely (i.e. population size x mutation rate >1). Hence, emergence of resistance becomes more likely in circumstances such as an infection.

Selection strength depends on the concentration of antibiotics to which bacterial pathogens are exposed. Both concentrations above and below the MIC of a given compound can induce selection of resistant mutants to such compound (Sabtu et al, 2015). In particular, sub-MIC (or sublethal) concentrations of antibiotics not only can induce selection of resistance, but also increase genetic and phenotypic variability in bacteria, as well as acting as signaling molecules. Hence, exposure to sublethal concentrations of antibiotics represents a growing concern for the control of resistance development and its environmental propagation (Andersson and Hughes, 2014).

Fitness is the evolutionary success of a drug-resistant pathogen in presence of a drug, as compared to that in absence of that drug. In other words fitness can be defined as the probability that a mutant emerges, fixes, transmits and remains within a host population.

Compensatory evolution refers to the emergence, in a drug-resistant mutant, of mutations that can reduce fitness cost of the mutations conferring resistance. Such compensatory mutations can increase the probability that resistance is maintained in absence of compound (Habets and Brockhurst, 2012; Hughes and Andersson, 2015).

Epistasis refers to a phenomenon where one component in a system is modulated by another component in the system. The relation of a phenotype, given by a certain mutation, to other mutations in the genome, is an example of epistatic interaction (Chou et al., 2011).
Examples of epistasis in antibiotic resistance may be synergy and antagonism between drugs (Yeh et al., 2009). Epistasis also involves phenomena such as cross-resistance and collateral sensitivity, meaning that resistance to a given drug can alter the susceptibility to other drugs (Macvanin and Hughes, 2005). Epistasis may drive the acquisition of resistance as well as contribute to the level of overall resistance within a population (Chou et al., 2011).

In addition to the factors described above, epidemiological factors such as structure, density and immunity of the host population can contribute to the spread of resistant bacteria. Moreover, interspecies gene transmission, poor hygiene both in communities and hospitals, as well as the increasing global travel and trade, all favor the dissemination of resistance (Laxminarayan et al., 2013).

8. Bacterial resistance to AMPs and peptidomimetics

It has been pointed out that bacteria would have encountered a great challenge in developing resistance towards AMPs (Hancock, 1997; Wimley and Hristova, 2011), or at least that the probability of resistance emergence would have been low, due rapid bactericidal activity of the compounds (Fox, 2013).
Despite these assumptions, development of resistance toward antimicrobial compounds is a naturally occurring phenomenon in bacteria and several mechanisms are known (Figure 6 and 7; Table 6). Indeed bacteria and AMPs have co-evolved and a parallel evolutionary race has taken place (Peschel and Sahl, 2006; Hale, 2012).
However, it is true that *in vivo*, injury and infections increase the expression of AMPs that are released by leukocytes, neutrophils and skin keratinocytes. In these conditions bacteria face the challenges to survive immune clearance and at the same time the metabolic cost of undergoing resistance-conferring mutations. It follows that the metabolic cost of the resistance mutations constrains adaptation to AMPs.
It is also generally believed that mutation frequencies are higher for antibiotics than for AMPs (Marr et al., 2006). This is because the latter lack a specific receptor as molecular target. Indeed AMPs act mainly by binding to different macromolecules in the bacterial cell membrane. As a result, when the bacterial cell is targeted in such multi-mode way, simultaneous alteration of several targets would appear less probable than it would be for single-target molecules (Sallum and Chen, 2008).

However, bacterial susceptibility to AMPs varies a lot in nature and the existing resistance mechanisms can be considered virulence phenotypes of pathogenic bacteria (Nizet, 2006). These AMP resistance mechanisms have been shown to contribute to pathogenesis; proof was given by comparison between wild type bacteria and AMP-sensitive mutants in *in vivo* infection models (Nizet, 2006). It thus appears that the ability to resist AMP killing is a typical feature of human pathogens (Joo et al., 2016).

A distinction between intrinsic resistance and inducible (or adaptive) resistance is usually made. Inducible (or adaptive) resistance is driven by environmental stimuli and can be reversible due to the fitness cost it imposes on bacteria (Andersson et al., 2016). Bacteria can indeed experience transient molecular modifications in the composition of bacterial membrane. The molecular mechanisms behind these modifications are known for both Gram-positive and Gram-negative bacteria (Yeaman and Yount, 2003; Kraus and Peschel, 2006; Nizet, 2006; Matamouros and Miller, 2015). Known strategies of resistance are membrane efflux pumps, that export AMPs, and bacterial-produced proteolytic enzymes, which cleave the peptides before they can act. Also, external trapping alterations can bind AMPs before they reach the cell membrane, with consequent neutralization. The most common strategies are cell surface charge alterations that induce overall decreased negative charges (Fernandez et al., 2010). This can occur by incorporation of positively charged molecules in the cell surface in order to reduce the interaction with AMPs (Andersson et al., 2016). Also, thickening of the peptidoglycan layer can contribute to resistance (Kramer et al., 2008) as well as modifications of membrane permeability, such as increased membrane rigidity or the opposite (Nizet, 2006).

Other strategies can be the downregulation of host AMP production (Nizet, 2006) and the activation of two components system in presence of compounds (Weatherspoon-Griffin et al., 2011).

Development of resistance to AMPs *in vivo* has been highlighted as a potential concern, if we consider that AMPs mimic natural compounds (Bell and Gouyon, 2003; Perron et al., 2006). Hence, the issue has been addressed experimentally and there is evidence that laboratory stress can cause intense selection and lead to bacterial adaptation to AMPs (Jochumsen et al, 2016). There is also evidence that resistance to peptide mimics of antimicrobial peptides can develop (Hein-Kristensen et al., 2013(a); Citterio et al., 2016; *Submitted*). Based on the current knowledge, we believe that every novel AMP or peptidomimetic may induce development of resistance in the bacterial target.

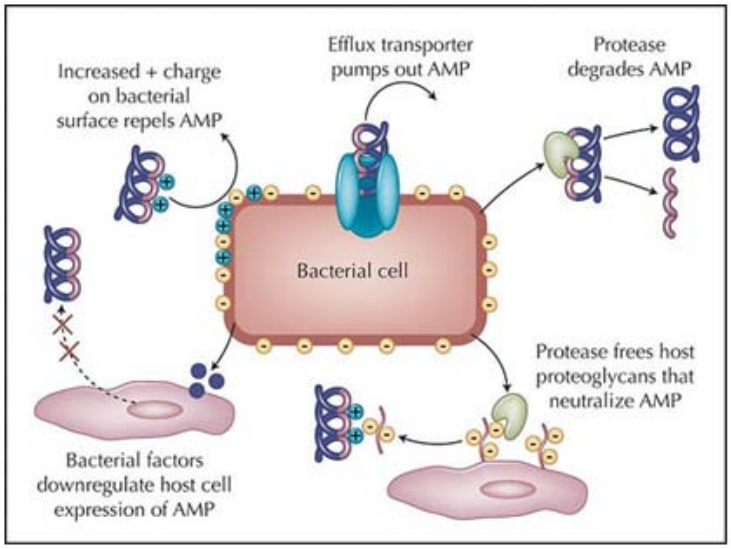


Figure 6: Potential mechanisms of resistance to antimicrobial peptides in pathogenic microorganism. Figure 2 in Gallo and Nizet, (2003).

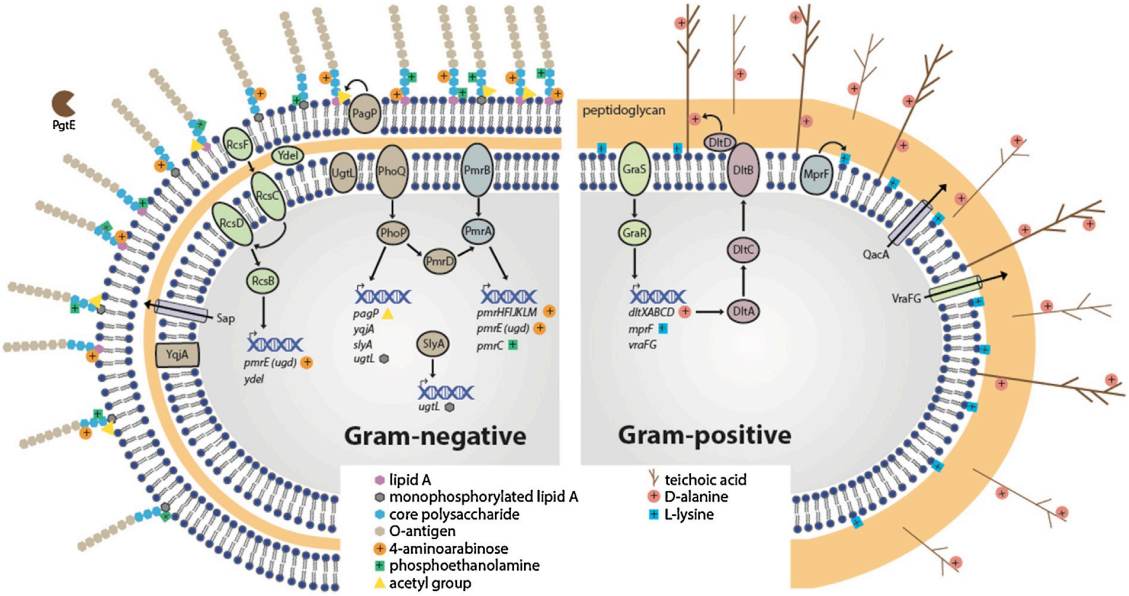


Figure 7: Summary of intrinsic antimicrobial peptide resistance mechanism in Gram-negative and Gram-positive bacteria. The main pathways resulting in transient high-level AMP resistance in bacteria are membrane modifications, increased efflux and proteolytic degradation. Figure 1 in Andersson et al., (2016).

8.1 Methodology of resistance development in laboratory settings

AMP and peptidomimetic resistance can be achieved experimentally by slowly adapting the bacteria through serial passages in liquid medium (Samuelsen et al., 2005; Perron et al., 2006) or direct plating (Roland et al., 1993; Sun et al., 2009). Mutants can be selected for with two methods: one is based on serial passaging in medium containing increasing concentrations of antibiotic or AMP; the other is based on selection on agar containing the compound in concentration higher than MIC (Figure 8). The serial passaging method is based on serial transfer of bacterial cultures in liquid medium supplemented with the compound, starting from sub-inhibitory concentrations (Perron et al., 2006; Jochumsen et al. 2016). Such exposed bacteria are defined “selection lines” (Perron et al., 2006) or “lineages”, (Hein-Kristensen et al., 2013(a). Lineages are replicates of populations that grow in parallel and provide a tool to study the sequential accumulation of adaptive mutations.

Perron et al., (2006) proposed a number of ten transfers at constant concentration of compound specifying that the number of transfers was reduced when the selection lines were growing vigorously. In Manuscript 2 (Citterio et al., 2016; *Submitted*) we kept the concentration constant for a number of five transfers, as previously done in Hein-Kristensen et al., 2013(a). Nevertheless, we transferred selected lineages for a number of times higher than five, once concentrations of compound were above MIC (Citterio et al., 2016; *Submitted*). Hence, it is clear that the choice of number of transfers is arbitrarily based on the experimental design.

This gradual selection in liquid medium can select for high fitness mutants, which is considered a disadvantage of this method. Also, genetic reconstitution is recommended, in order to confirm the individual mutations responsible for the acquired resistance.

Selection in solid medium has the disadvantage that it requires higher amount of compound and that some of them are less active in agar plate (Dhawan et al., 1997). Also, if evolution of resistance requires formation of several mutations, this would be detectable only with the serial passage method. As opposed to the first method, selection on agar has the advantages that low fitness mutants can be isolated and mutation rate can be determined (Andersson et al., 2016).

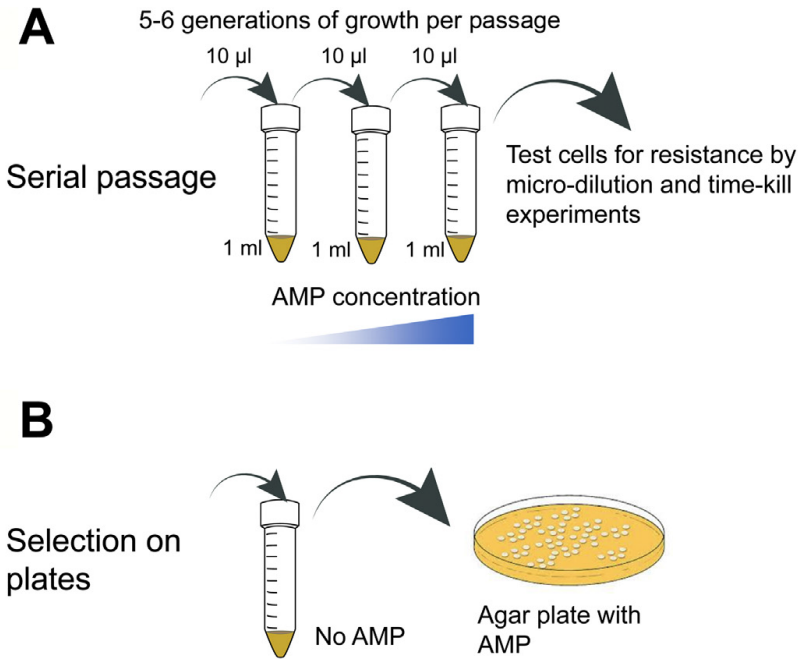


Figure 8: Methods used for isolation of AMP-resistant mutants. (A) Serial passage involves the growth of bacteria in liquid media containing progressively increasing concentrations of AMPs. (B) Selection on plates involves direct plating of bacteria on agar plates containing AMP concentrations above the MIC. Adapted from Figure 2 in Andersson et al., (2016).

8.2 Development of resistance to AMPs in laboratory settings

Several studies have used adaptive evolution to AMPs to address the issue of potential resistance development and overall show that AMP-resistant mutants can be easily obtained.

Evolution of resistance to increasing concentrations of the AMP pexiganan was studied in *E. coli* and *P. fluorescens* (Perron et al., 2006), where levels of resistance reached 2- to 64- fold and 32- to 512- fold the wild type MIC, respectively.
Samuelsen et al., (2005) exposed *S. aureus* to the AMP lactoferricin B and obtained a 30-fold increase in the wild type MIC after only four passages. MIC remained 10-fold higher than the wild type MIC in the same strains, re-cultivated for 30 passages in absence of compound, indicating stable resistance. Exposure of *S.* Typhimurium to LL-37, CNY100HL (a derivative of complement C3) and wheat germ histones also led to stable resistant mutants (Lofton et al., 2013).

Hong et al., (2016) studied bacterial evolution to the antimicrobial peptide tachyplesin I in three Gram-negative bacteria (*Pseudomonas, Escherichia* and *Aeromonas*) and also found stable resistance. This finding may pose a considerable risk in the clinical use of tachyplesin, despite its known promising antibacterial activity against bacteria, viruses and cancer cells (Nakamura et al., 1998).

Dobson et al., (2013) studied *in vitro* the adaptive evolution of *S. aureus* to AMPs already developed as drugs (selected from phylogenetically diverse taxa such as mammals, amphibians and insects) alone and in combination of two e.g. iseganan, pexiganan, melittin and a 1:1 combination of melittin and pexiganan, in parallel with vancomycin and streptomycin. The treatment of melittin and pexiganan in combination caused the earliest extinction among all the other treatments, indicating that bacteria encountered the highest challenge when exposed to two agents with different mechanism of action. Dobson et al., (2013) hypothesized that this is a general phenomenon that relies on the interaction between the compounds, as previously noted by Chait et al. (2007); Yeh et al., (2009); Fischbach (2011). These interactions may constrain evolution of resistance in natural settings, where multiple AMPs are present. This interpretation is partially in contrast with our results. Indeed in Citterio et al. (2016; *Submitted*), we showed that exposure to a single compound such as the AMP P9-4 caused the earliest extinction among all the other treatments, including a combination of three compounds. This finding indicated that bacteria were more challenged by exposure to P9-4 alone rather than exposure to the same compound in a combination of three. Hence, we suggested that bacterial resistance development is compound-dependent rather than combination-dependent.

Based on all these findings, we advocate early-stage investigation of resistance development for each novel promising compound.

8.2.1 Colistin example

Colistin represents a special case, despite being an AMP; it has the tendency of inducing resistance when administered in sublethal doses, which is common practice when the compound is used in synergistic combinations. However, this phenomenon seems to arise quite frequently with colistin rather than for other conventional antibiotics (Cassone and Otvos, 2010).
Roland et al. (1993) and Moffatt et al., (2010) studied the development of resistance to colistin in agar plates in *S.* Typhimurium and *A. baumannii,* respectively. In the *S.* Typhimurium colistin-resistant mutant there was constitutive activation of PmrAB, that caused increased Ara4N (4-aminoarabinose) with consequent phosphoethanolamine covalent modification of LPS. This modification also caused 1000-fold increased resistance to polymyxin B. The *A. baumannii* colistin-resistant mutants lost LPS consequent to the activation of one of the *lpx* genes, that are involved in lipid A biosynthesis.
Jochumsen et al., (2016) exposed *Pseudomonas aeruginosa* to colistin through a long-term adaptive evolution experiment based on serial transfers in liquid medium. They showed that evolution of resistance is a multistep process where mutations arise with a specific pattern, as previously formulated by Toprak et al., (2012), Palmer and Kishony, (2013) and De Visser and Krug, (2014). The resulting resistant phenotype is dependent on mutations in independent loci that act synergistically i.e. there is intergenic epistasis. Epistatic interactions can affect the number of

potential evolutionary pathways to resistance reducing them to a few trajectories, in which fitness increases monotonically with each single mutation (Weinreich et al., 2006).

Jochumsen et al., (2016) found mutations in the DNA mismatch repair gene *mutS*, in the regulators of LPS modification operon (*pho*, *pmr*) and in genes affecting biosynthesis of lipid A (*lpxC* and *lpxD*). Thus, evolution of resistance depends on mutations in transcriptional regulators that can fine-tune the effect of other mutations i.e. a series of modifications in the lipopolysaccharide. At least this is the case for resistance mechanisms towards AMPs in Gram-negative bacteria (Olaitan et al., 2014).

Table 6: Known mechanisms of resistance to AMPs, acquired with several methods. Adapted from Table 1 in Andersson et al. (2016). Ara4N = 4-aminoarabinose; PEtN = phosphoethanolamine.

Organism	Method for isolation	AMP resistance	Genes involved in AMP resistance	Proposed mechanism	Reference study
S. aureus	Clinical isolates	LL-37, human β-defensin 2 and 3, lactoferricin B	*hemB*	Inactivation results in small colony variants with reduced AMP binding/uptake	Glaser et al., 2014
E. coli, K. pneumoniae	Clinical isolates	Colistin, polymyxin B	*mcr-1*	Encodes a PEtN transferase modifies lipid A to reduce anionic charge	Hu et al., 2016
A. baumannii	Direct plating with colistin	Colistin, polymyxin B	*lpxA*, *lpxD* or *lpxC*	Inactivation results in loss of LPS production, reduced AMP binding	Moffatt et al., 2010
S. Typhimurium	Direct plating with colistin	Colistin, polymyxin B	*pmrA, pmrB*	Constitutive activation of *pmrAB*-regulated Ara4N and PEtN LPS modification reduce anionic charge	Roland et al., 1993; Sun et al., 2009
	Direct plating with protamine	Protamine, colistin, lactoferricin, human α-defensin 1	*hemA, hemB, hemC hemL*	Inactivation results in small colony variants with reduced AMP binding/uptake	Pränting and Andersson, 2010
	Serial passages with LL-37 or CNY100HL	LL-37, CNY100HL, wheat germ histones	*pmrB, phoP*	Constitutive activation of various LPS modifications reducing anionic charge	Lofton et al., 2013

8.2.2 Cross resistance

Mutants that are resistant to a specific drug can also be resistant to structurally similar drugs. This phenomenon is known as cross-resistance. Resistance to drugs belonging to different classes can be observed too, as pointed out by Sanders et al., (1984). The same study suggests that this

phenomenon was due to changes in the outer membrane proteins of the investigated microorganisms. Likewise, AMP-resistant bacterial mutants can display broad cross-resistance to a variety of AMPs with different structures and modes of action.

For AMP-resistant mutants, the derived concern is that resistance could develop toward other AMPs that are part of our immunity. Thus, cross-resistance clearly represents a potential risk for each novel antibacterial compound and requires more detailed investigations and understanding (Andersson et al., 2016).

For instance Hong et al., (2016) found that tachyplesin I-resistant mutants were also resistant to other AMPs such as pexiganan, tachyplesin III and polyphemusin I, as well as resistant to antibiotics such as cefoperazone and amikacin.

Hein-Kristensen et al., 2013(a) investigated, in peptidomimetic-resistant mutants, *in vitro* cross-resistance to other AMPs. We also addressed the development of cross-resistance in AMP- and peptidomimetic-resistant mutants in Manuscript 2 (Citterio et al., 2016; *Submitted*) and 3 (Citterio et al., 2016; *In preparation*). From these studies we infer that resistance and susceptibility patterns in resistant mutants are difficult to explain.

9. Investigation of resistance mechanisms by sequencing technologies

The development of high-throughput sequencing (HTS) technologies and their reduced cost now allow for deep and accessible knowledge on bacterial evolution (Metzker, 2010; Loman et al., 2012; McAdam et al., 2014). Indeed, Illumina currently provides the highest throughput per run coupled with the lowest per-base cost (Liu et al., 2012; Van Dijk et al., 2014).

The main challenge of WGS projects is the management of the amount and complexity of generated data. The chosen analysis workflow shall aim at obtaining meaningful biological results (Schadt et al., 2010). Such challenge of WGS data analysis has prompted the development of numerous tools, up to 205, as reported by Pabinger et al., (2013). Nevertheless, common to all these tools are the five main steps of the analysis pipeline: quality assessment, alignment, variant identification, variant annotation and visualization (Figure 9). In the alignment step reads can be mapped to a known sequence; alternatively, reads can be *de novo* assembled. This choice of analysis is made based on read length, availability of a known reference sequence and the biological application (Loman et al., 2012). Variant identification allows for detecting point mutations (also known as single nucleotide polymorphisms i.e. SNPs or single nucleotide variants i.e. SNVs). These positional data provide information down to the level of individual genomic variation. Such in-depth analysis may become the standard for genetic studies of natural populations (Ekblom and Wolf, 2014). Thus, the single-nucleotide resolution of HTS has enabled the identification of molecular mechanisms underlying bacterial resistance and pathogenesis (Köser et al., 2014; McAdam et al., 2014).

In Manuscript 2, we sequenced and analyzed the whole genomes of lineages and clones derived from the adaptive evolution experiment (Citterio et al., 2016; *Submitted*). We found single-nucleotide deletions in the gene encoding for the enzyme CDP-glycerophosphotransferase (CDP-glycerol: N-acetyl-β-D-mannosaminyl-1,4-N-acetyl-D-glucosaminyldiphosphoundecaprenyl

glycerophosphotransferase) as the most common variants in the adapted lineages and derived clones. We know that parallel populations can acquire similar mutations in a specific order, resulting in similar phenotypic trajectories (Toprak et al., 2012).
We then hypothesized that a common evolutionary trajectory has led to development of resistance, both to the individual compounds and to the combination of all three compounds. We speculated that the emergence of the same variant across lineages exposed to distinct compounds may also relate to the similar nature of such compounds (Band and Weiss, 2015).

The gene CDP-glycerophosphotransferase encoded for an enzyme consisting of 1266 amino acids. We described three deletion types in such gene (Citterio et al., 2016; *Submitted*). Two of these deletion types were located in the enzyme region responsible for the phosphotransferase activity. We measured the zeta potential of adapted lineages and found that it was less negative than that of the wild type. Thus, we speculated that the resistance mechanism was based on reducing the negative charge of the membrane, and limiting the interaction with positively charged AMPs. Such mechanism seems to constitute a novel variation as compared to the known strategies of limiting the attraction of cationic compounds (Band and Weiss, 2015).

However, it remains unproven whether this deletion is the actual resistance-conferring mutation. This can only be confirmed by introducing such variants back in wild type strains (Hachmann et al., 2011; Jochumsen et al., 2016).

In conclusion, the deep understanding of bacterial resistance mechanisms, supported by HTS, can markedly aid the discovery of potential targets in drug-resistant bacteria (Punina et al., 2015; Cole and Nizet, 2016).
Beyond the HTS analysis, increasing effort is now targeted toward metabolic network reconstructions, meaning that annotated genomes can serve as a basis for predicting a model of microbial physiology. This process aims at building a mechanistic genotype-phenotype relationship with the ultimate goal of designing antibiotics interfering with strain-specific capabilities (Feist et al., 2009).

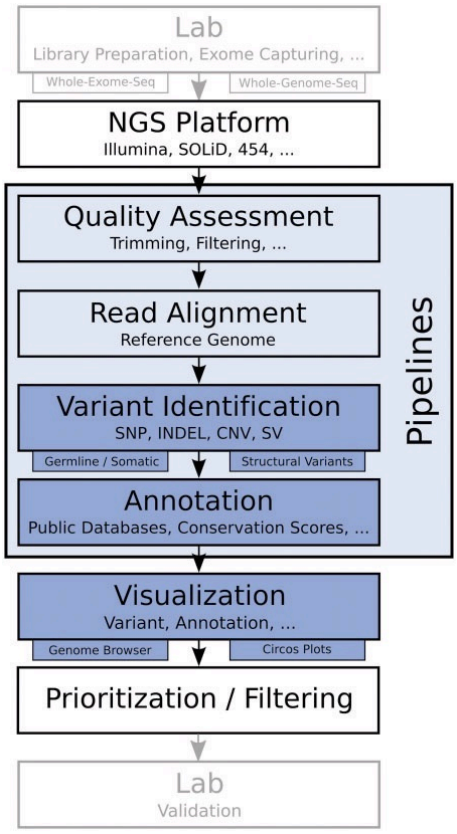


Figure 9: Basic workflow for whole-genome sequencing projects. After library preparation, samples are sequenced on a certain platform. The next steps are quality assessment and read alignment against a reference genome, followed by variant identification. Detected mutations are then annotated to infer the biological relevance. The found mutations can further be prioritized and filtered, followed by validation of the generated results in the lab. Figure 1 in Pabinger et al, (2014).

10. Strategies of circumventing resistance development

10.1 Drug combination strategy

Drug combinations can be applied in clinical settings due to a number of reasons. These can be the necessity for a long-term treatment, synergistic therapeutic effects between the drugs in the combination or a larger spectrum of activity (Tamma et al., 2012). Another relevant reason is the belief that combinations of drugs with different targets could disfavour the evolution of resistance (Dobson et al., 2013). This is because the pathogen is unlikely to acquire mutations and resistance against several targets at one time. Also the risk of resistance is reduced if the treatment clears the infection faster. A further advantage of combination treatment over monotherapy may be the diminished side effects of the drugs, due to reduction of dosage of the single drugs in the combination (Tu et al., 2015; Lin et al., 2015).

Drug combination therapy has been successful in the treatment of *Mycobacterium tuberculosis* since the late 1940s. Indeed, streptomycin combined with para-aminosalicylic acid was shown to markedly reduce evolution of resistance as compared to streptomycin alone (Dunner et al., 1949). Treatment of HIV infections has been another success in reducing drug resistance and increasing life expectancy of HIV patients (Palella et al., 1998). Examples of combination therapies in advanced development against Gram-negative bacterial infections are two β-lactam/β-lactamase inhibitor combinations and or one non β-lactam/β-lactamase inhibitor combination (Amin & Deruelle, 2015). Combination therapy is also advocated for cancer treatment (Glickman and Sawyers, 2012). Indeed Zhao et al., (2014) have explored the applicability of combination therapy based on α-helical peptides and chemical drugs (doxorubicin and epirubicin) against cancer cells. Their promising results in mouse model prompt for further clinical applicability. Promising results are also available for treatment of HIV with peptidomimetic inhibitors of HIV protease used in combination with antiretroviral agents (Randolph and DeGoey, 2004).

Some AMPs can increase the activity of conventional antibiotics with synergistic interactions *in vitro* (Cassone et al., 2008; Anantharaman et al., 2010; Feng et al., 2015; Lin et al., 2015). However their applicability in clinical is still unknown (Giacometti et al., 2000) or not thoroughly investigated (Ngu-Schwemlein et al., 2015). Niu et al., (2013) showed that the 4kD scorpion defensin potentiates the activity of conventional antibiotics *in vitro*. Hu et al., (2015) also showed synergy between the defensin plectasin and conventional antibiotics such as β-lactams and aminoglycosides, both *in vitro* and *in vivo.* Synergy between plectasin and cell wall-targeting antibiotics was also investigated in Breidenstein et al., (2015) who found synergism of plectasin with some of the tested agents. McGrath et al., (2013) showed synergy between a D-enantiomer peptidomimetic and piperacillin, an antibiotic commonly combined with tazobactam and applied in therapeutic applications. AMPs in combination with four selected conventional antibiotics have also proven their potential against bacterial biofilms (Reffuveille et al., 2014).
In contrast to all these studies in favor of combinations, He et al., (2014) did not find results that support combination of membrane-permeabilizing AMPs with antibiotics.

AMPs act naturally in combination of more than two compounds and Yu et al. (2016) found that three-AMPs combinations were more synergistic than two-AMPs combinations.
Moreover, AMPs have proven to act in synergy also with their modified counterparts *in vitro* (Chongsiriwatana et al., 2011; Zdybicka-Barabas et al., 2012). However, it is still unclear whether combinations solely made of AMPs and/or peptide mimics are more advantageous than (antibiotic + antibiotic) or (AMP + antibiotic) combinations.

It is believed that exposure to a combination of several AMPs can reduce the selective pressure needed for resistance development (Malmsten, 2014). Despite the extensive literature on the potential of drug combinations, much fewer studies have explored the actual risk of bacterial resistance development to such drugs combinations.

10.2 Disadvantages of combination strategy

Drug combination therapy based on synergistic couples of drugs can have adverse effect on resistance development. Indeed Vestergaard et al., (2016) showed that antibiotic combination therapy could select for high-fitness drug-resistant mutants of *Pseudomonas aeruginosa*.

In fact, synergistic drugs increase the selective advantage of single drug-resistant mutants (Torella et al., 2010). On the contrary, antagonistic drug combinations can limit evolution of resistance (Munck et al. 2014). This is supported by lower fitness in the bacteria that are induced to develop resistance to antagonistic as opposed to synergistic combinations (Chait et al., 2007; Hegreness et al., 2008; Michel et al., 2008).

Overall, drug interactions such as synergy, additive effect or antagonism as well as cross-resistance have an impact on selection (Michel et al., 2008); however it remains unclear which of these factors has higher impact on long-term evolution of drug resistance (Andersson et al., 2016).

10.3 Inversion of resistance

The main advocated strategies for tackling resistance development are the restriction of use along with the concept of combining antibiotics with compounds that inhibit their specific resistance mechanism. These strategies are certainly valid in neutralizing the advantage of resistant bacteria. However, they do not prevent the eventual selection of resistance over time.

Conversely, inversion of the selective advantage of resistance is believed to turn a resistant population susceptible again. Inversion of the selective advantage can be triggered by suppressive drug interactions e.g. in the situation where there is collateral sensitivity between two drugs. Indeed, resistance to a first applied drug can increase the sensitivity to a second applied drug (Imamovic and Sommer, 2013). The latter creates a concentration regime that inhibits the resistant bacteria, hence selecting for the sensitive ones (Baym et al., 2016).

So far a few studies have focused on the extent of collateral sensitivity for AMP-resistant mutants in presence of different AMPs and/or antibiotics (Berti et al., 2015; Garcia-Quintanilla et al., 2015). Hence, more efforts shall be devoted into understanding whether this strategy could be applicable in clinical settings.

11. Perspectives in the potential of AMPs and peptidomimetics as novel drugs

Several challenges still prevent novel compounds such as AMPs or peptidomimetics from proceeding more rapidly to approval. Among these challenges, a few are disclosed.

The mechanism of action and the role of immune response modulation remain unclear for many AMPs. Lower toxicity as a desirable feature remains challenging to achieve and test (Andersson et al., 2016). This testing can be impaired by lack of standardization and critical evaluation of testing methods (Cassone and Otvos, 2010). A matter of discussion could also be whether more effort shall be devoted to better characterize existing compound and the strategies to apply them most efficiently rather than investing in discovering novel compounds. In this regard, combining membrane permeabilizers may help potentiating the activity of existing approved compounds.
In addition, in light of translation of basic research into clinical application, a better characterization of the intercellular communication would be preferable to the investigation of microbiological-based pathways (Cassone and Otvos, 2010).

Nevertheless, AMPs and peptidomimetics do represent effective candidates as novel antibiotics for topical uses as well as helper drugs for conventional antibiotics or novel antibacterial compounds. Overcoming common misconceptions on peptide drugs, such as high-price, unfavorable pharmacodynamics and lack of delivery options, also represents a key goal for the advancement of peptide drugs (Otvos, 2014). Otvos and Wade (2014) highlight that strategies exist to overcome their alleged poor oral bioavailability and pharmacodynamics parameters. The acknowledgments of these strategies may also help expanding clinical application of AMPs beyond topical use.

Discussion and conclusion

The development of resistance to antibiotics has urged the search and discovery of new potential drug candidates. Many efforts are currently directed towards bioprospecting for novel compounds and assessing their spectrum of use. The compounds researched in the present thesis, the antimicrobial peptides (AMPs) and their synthetic analogues (peptidomimetics), constitute one avenue.

A key feature of a novel drug is the effect under *in vivo* conditions. It is well known that there is often a gap between the activity tested *in vitro* and actual *in vivo* applicability of novel compounds. For instance, AMPs are known to suffer from protease sensitivity and poor bioavailability *in vivo* while their synthetic derivatives are feared for the risk of toxicity. Thus, the choice of new compounds as antibiotics poses the challenge to test their actual applicability *in vivo.* On top of this challenge, the potential of bacterial resistance development may prevent novel compounds from entering the process of approval as future therapeutics. Based on these premises, the purpose of this thesis was to address the challenges of applicability of novel compounds, such as AMPs and peptidomimetics, and the development of bacterial resistance towards them.

Previous studies have found that the effectiveness of antimicrobial compounds was reduced in presence of body fluids. Nevertheless, a study in our group had found that the activity of selected peptidomimetics was instead potentiated in presence of body fluids (Hein-Kristensen et al., 2013). However, this was found in only one out of two tested bacterial species. Therefore, a broader spectrum of bacterial species was needed in order to infer whether the potentiation is a more general effect. Hence, the hypothesis behind Article 1 was that AMPs and their analogues could be affected by the presence of body fluids. From our results we inferred that the tested compounds were actually potentiated by human blood plasma in all tested (thirteen) bacterial species. Therefore, we believe that these compounds may be used *in vivo* in lower concentrations than those predicted by traditional *in vitro* testing. This is a significant advantage. Since high dosage of antibiotics is considered one of the causes of the spread of resistance in bacteria, the most advocated strategy is to reduce dosage. Hence, the possibility of decreased dosing of novel compounds while potentiating their effect is promising. Also, decreased concentrations *in vivo* may overcome a general concern about AMPs such is cytotoxicity.

The AMPs tested were potentiated to a different degree and this correlated with different mechanism of action, and from this we inferred that only compounds acting on the cell membrane

or envelope were potentiated by human blood plasma. We speculated that such effect is due to synergistic interaction between the compounds and factors present in blood, especially complement proteins that are in fact, as many AMPs, known to act primarily toward the bacterial membrane. Further studies in this direction shall confirm that there is a correlation between body fluid potentiation of the antimicrobial activity of the compounds and their mechanism of action. Nevertheless such investigation may be challenging for compounds whose mechanism of action is still unclear. Therefore, clarity on the mechanism of action remains a key issue for advancement of novel compounds. A consequent reflection is that a limit exists to the range of compounds for which decreased dosage could be applied.

The problem of identifying the real cause (mechanism) of potentiation remains unsolved. The selected compounds were indeed potentiated when exposed to human blood plasma, in a concentration that alone was not bactericidal. Based on previous knowledge on decreased activity of the compounds in presence of heat-treated blood, we inferred that proteins essential for the potentiation activity were denatured and we originally believed that complement proteins were the major cause of potentiation. However, complement proteins are present in both blood serum and plasma, and we observed a higher potentiation of the tested compounds in plasma as compared to serum. We therefore concluded that there must be another cause other than complement, which contributes to the effect of potentiation. Based on other studies on the functions of clotting factors and platelets, we speculated that clotting factors might be involved and synergistically interact with complement proteins. However, the plasma we have used is devoid of platelets and thus it becomes difficult to relate the observed potentiation to clotting factors, since other components of the coagulation cascade are missing. One consequent experiment would be to test whether platelet-rich plasma also potentiates the activity of the compounds.
Interactions between complement and the coagulation cascade may occur *in vivo* as a part of the immune response to pathogens. Hence, future work shall address the interaction between complement and clotting factors in order to clarify their impact on the activity of novel drugs *in vivo*.

The challenge of testing novel compounds in whole-blood models constitutes another unresolved issue. This testing is challenging due to the viscosity of whole blood. Also, the presence in blood of cell-derived immune factors may largely affect the interpretation of the results. On top of these challenges, the cost of such human sample and its limited shelf life may further limit the studies. Nevertheless, AMPs and peptidomimetics are acclaimed for their immune-modulating properties and they are supposed to act in the context of human immunity, where several factors would concur to potentiate their activity. Thus, another challenge is to evaluate whether traditional MIC is the appropriate test for such compounds. Serum stability has been considered for long a reliable screening tool in drug development. However, our results indicate that human blood plasma may represent a better predictor of the drug pharmacokinetics, as compared to serum. Based on these reflections, we suggest that future MIC testing uses traditional laboratory growth media supplemented with human blood plasma. In parallel, we believe that future studies shall also consider whether pre-clinical investigations using whole blood are actually feasible. Along these lines, it has already been suggested that future *in vitro* model systems shall at least contain phagocytic cells or even the patient's own blood (Nizet, 2015).

To summarize, Article 1 demonstrates that previous thinking, namely that synthetic compounds

may face a decreased activity in the presence of body fluids, is not always true. The goal of future research shall be to obtain pre-clinical results that are the most representative of *in vivo* conditions. We believe that compounds with promising increased activity in the presence of body fluids shall be preferred for proceeding studies for approval as future therapeutics.

For years it has been repeatedly stated that bacteria could not become resistant to AMPs, because AMPs are part of the natural innate human defense and it seems that they have retained effectiveness. However, studies have emerged and demonstrated that using an adaptive evolution approach, resistance can indeed evolve rapidly. Therefore, if AMPs and synthetic analogues are to be used, one must address and possibly overcome the resistance issue. In Manuscript 2 we investigated whether the use of a combination of three AMPs might prevent development of bacterial resistance as compared to single compounds.
This has been predicted based on the feature, typical of AMPs, of having multiple targets. Such feature is supposed to hinder resistance development in bacteria, which would be exposed to the great challenge of modifying several targets at once. This effect may be amplified once a combination of compounds with multiple targets is used. Indeed, we observed delayed resistance development in the lineages exposed to a combination of three compounds. However, we also found delayed resistance development toward a short AMP such as P9-4. This may occur due to the presence of RW groups, which are supposed to have an increased number of targets in the membrane, concomitantly with a strong membrane disruptive mode. It becomes evident that resistance development is compound-dependent and this suggests that other compounds with similar features to P9-4 are investigated for resistance development.

Our findings that resistance did develop against all individual AMPs tested lead to the recommendation that all novel compounds shall be tested by different experimental approaches (such as adaptive evolution) for resistance development before introduction and use. It follows that the choice of novel drugs shall be directed towards drug with confirmed delayed resistance development. The studies that provide a resistance assessment of new antibacterial agents tend to use only short time exposure to the compounds. By these means, there exists a risk of underestimating the actual potentiality of resistance development. Indeed, every bacterium could develop resistance after continuous exposure, provided that it is given enough time. Thus, it is evident that assessing the potential of resistance development over time is critical, as the temporal set-up allows several mutations to take place. A standardized procedure with a reasonably ample but still limited time frame for such testing is necessary. We propose a systematic protocol that other scientists can follow in order to investigate the pattern of resistance development to novel promising compounds. Automated ALE procedures are also available but are often intended for industrial or basic research purposes. Therefore we encourage further optimization of ALE in order to be specifically targeted to assess the emergence of resistance to novel drugs.
Overall, our results suggest that some compounds are more effective than others in circumventing resistance development in a limited time frame such could be the time of infection control. Hence, we consider our method highly informative both from a clinical but also from an evolutionary perspective.

In manuscript 2 we also investigated the adapted-evolved resistant lineages with a parallel genomic analysis. The purpose of such analysis was to identify variants that differentiate the adapted lineages from the wild type that was cultured in absence of compound. We found

deletions in the gene encoding for the CDP-glycerophosphotransferase and speculated that these independent mutations were involved in the adaptive resistance. Our results suggest that the mechanism of resistance may involve modification of the bacterial surface charge, resulting in a less negative membrane that could repel the positive compounds. We envision that further studies might confirm this prediction and clarify whether a single mutation or rather the interplay between different cooperative mutations is the cause of resistance. We first suggest the construction of wild type mutants with deletions in the CDP-glycerophosphotransferase, in order to confirm whether this indeed is the primary cause of the observed resistant phenotype.
The same deletions were found in bacteria exposed to structurally different compounds. Therefore we speculated that a common evolutionary trajectory has been followed. This may indicate that other compounds, not yet tested, could favor the emergence of the same mutation pathway. Therefore we encourage that a database system compiles upcoming knowledge on novel antimicrobial compounds, tested bacteria and relative predicted resistance genes. Such system may also largely benefit from distinguishing between resistance genes detected in experimentally evolved model bacteria and resistance genes already present in intrinsically resistant microorganisms. The derived shared accessible knowledge may improve the current understanding and overcome the existing misconceptions on the potential of resistance development to novel compounds.

Manuscript 3 highlights that some AMP-resistant mutants become susceptible as the wild type, simply after exposure to compounds with certain features. In particular, we found that all the six tested resistant mutants were susceptible as the wild type when exposed to an Arg-rich, IR-containing peptide. The relevance of this discovery has two main implications. One is that there may be a strong correlation between bacterial susceptibility and specific features of the compound. We therefore envision that future studies test a larger number of AMPs against resistant bacteria. The concern of resistance development is primarily toward AMPs that are part of our immunity. Thus, further testing of natural human AMPs (such as LL-37) towards AMP-resistant mutants shall be prioritized.
The second main implication is that the impact of the resistance trait on cross-resistance to other compounds may be overestimated. This overestimation may suggest that, in a clinical context, adaptive resistance to a widely used drug could be easily circumvented simply by exposure to a different drug. This strategy has been supported for conventional antibiotics (Baym et al., 2016, Stone et al., 2016). Hence, proven efficacy of such approach may reassure from considering that there is no way out from the issue of resistance development. On the other hand, if this strategy proves to be effective in clinical settings with conventional antibiotics, there will be higher discouragement in proceeding with the approval of AMPs as substitutes of conventional antibiotics. Nevertheless, AMPs and their analogues remain versatile molecules that can aid in innovative treatments, not simply aimed at killing the pathogens, but rather focused on restoring impaired immune functions or preventing damage to the local microbiota.

Overall, our study attempted to answer the question: “Can AMPs become the drugs of the future?” AMPs and their synthetic analogues retain a great potential for different applications. However, the substantial consideration they receive in basic research does not seem to attract adequate support in translational studies. In order to gain more chances to proceed in the process for approval, AMPs need to gain equal consideration in the clinical research.
We reaffirm that despite resistance to each novel compound is a reality, further investigation shall clarify under which conditions resistance and cross-resistance become actual threats.

References

Abraham EP, Chain E. An enzyme from bacteria able to destroy penicillin. *Nature* 1940; 146(3713):837.

Agerberth B, Gunne H, Odeberg J, Kogner P, Boman HG, Gudmundsson GH. FALL-39, a putative human peptide antibiotic, is cysteine-free and expressed in bone marrow and testis. *Proc Natl Acad Sci USA* 1995; 92:195–199.

Al-Badri ZM, Som A, Lyon S, Nelson CF, Nusslein K, Tew GN. Investigating the effect of increasing charge density on the hemolytic activity of synthetic antimicrobial polymers. *Biomacromolecules* 2008; 9:2805-2810.

Amin AN, Deruelle D. Healthcare-associated infections, infection control and the potential of new antibiotics in development in the USA. *Future Microbiol* 2015; 10(6):1049-1062.

Anantharaman A, Rizvi MS, Sahal D. Synergy with rifampin and kanamycin enhances potency, kill kinetics, and selectivity of de novo-designed antimicrobial peptides. *Antimicrob Agents Chemother* 2010; 54(5):1693-1699.

Andersson DI, Hughes D. Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat Rev Microbiol* 2010; 8(4):260-271.

Andersson DI, Hughes D. Microbiological effects of sublethal levels of antibiotics. *Nat Rev Microbiol* 2014; 12(7):465-478.

Andersson DI, Hughes D, Kubicek-Sutherland JZ. Mechanisms and consequences of bacterial resistance to antimicrobial peptides. *Drug Resist Updates* 2016; 26:43-57.

Andreev K, Bianchi C, Laursen JS, Citterio L, Hein-Kristensen L, Gram L, Kuzmenko I, Olsen CA, Gidalevitz D. Guanidino groups greatly enhance the action of antimicrobial peptidomimetics against bacterial cytoplasmic membranes. *Biochim Biophys Acta (BBA)-Biomembranes* 2014; 1838(10):2492-2502

Antibiotic resistance threats in the United States 2013, US Centers for Disease Control and Prevention; http://www.cdc.gov/drugresistance/pdf/ar-threats-2013-508.pdf

Antimicrobial resistance. Global Report on Surveillance. World Health Organization. 2014.

Balakrishnan VS, Vad BS, Otzen DE. Novicidin's membrane permeabilizing activity is driven by membrane partitioning but not by helicity: a biophysical study of the impact of lipid charge and cholesterol. *Biochim Biophys Acta (BBA)-Proteins and Proteomics* 2013; 1834(6):996-1002.

Band VI, Weiss DS. Mechanisms of antimicrobial peptide resistance in Gram-negative bacteria. *Antibiot* 2015; 4:18-41.

Baym M, Stone LK, Kishony R. Multidrug evolutionary strategies to reverse antibiotic resistance. *Science* 2016; 351(6268):aad3292.

Bell G, Gouyon PH. Arming the enemy: the evolution of resistance to self-proteins. *Microbiology* 2003; 149:1367-1375.

Berti AD, Baines SL, Howden BP, Sakoulas G, Nizet V, Proctor RA, Rose WE. Heterogeneity of genetic pathways toward daptomycin nonsusceptibility in *Staphylococcus aureus* determined by adjunctive antibiotics. *Antimicrob Agents Chemother* 2015; 59:2799-2806.

Beven L, Castano S, Dufourcq J, Wieslander Å, Wroblewski H. The antibiotic activity of cationic linear amphipathic peptides: lessons from the action of leucine/lysine copolymers on bacteria of the class Mollicutes. *Eur J Biochem* 2003; 270(10):2207-2217.

Blondelle SE, Lohner K. Combinatorial libraries: a tool to design antimicrobial and antifungal peptide analogues having lytic specificities for structure–activity relationship studies. *Peptide Science* 2000; 55(1):74-87.

Boucher HW, Talbot GH, Benjamin Jr DK, Bradley J, Guidos RJ, Jones RN, Murray BE, Bonomo RA, Gilbert D. 10 x '20 progress-development of new drugs active against Gram-negative bacilli: An update from the Infectious Diseases Society of America. *Clin Infect Dis* 2013; 56(12):1685-94.

Brauner A, Fridman O, Gefen O, Balaban NQ. Distinguishing between resistance, tolerance and persistence to antibiotic treatment. *Nat Rev Microbiol* 2016; 14(5):320-30.

Breidenstein EB, Courvalin P, Meziane-Cherif D. Antimicrobial activity of plectasin NZ2114 in combination with cell wall targeting antibiotics against VanA-Type *Enterococcus faecalis*. *Microbial Drug Resistance* 2015; 21(4):373-379.

Brogden KA, Ackermann M, McCray PB Jr, Tack BF. Antimicrobial peptides in animals and their role in host defences. *Int J Antimicrob Agents* 2003; 22:465-478.

Brogden KA. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat Rev Microbiol* 2005; 3(3):238-50.

Brogden NK, Brogden KA. Will new generations of modified antimicrobial peptides improve their potential as pharmaceuticals? *Int J Antimicrob Agents* 2011; 38(3):217-225.

Bush K, Macielag M, Weidner-Wells M. Taking inventory: antibacterial agents currently at or beyond Phase I. *Curr Opin Microbiol* 2004; 7(5):466-476.

Carlet J, Jarlier V, Harbarth S, Voss A, Goossens H, Pittet D. Ready for a world without antibiotics? The pensières antibiotic resistance call to action. *Antimicrob Resist Infect Control* 2012; 1:11.

Cassone M, Vogiatzi P, La Montagna R, Inacio VDO, Cudic P, Wade JD, Otvos L. Scope and limitations of the designer proline-rich antibacterial peptide dimer, A3-APO, alone or in synergy with conventional antibiotics. *Peptides* 2008; 29(11):1878-1886.

Cassone M, Otvos L. Synergy among antibacterial peptides and between peptides and small-molecule antibiotics. *Expert Rev Anti-Infect Ther* 2010; 8(6):703-716.

Center for Disease Control and Prevention. *Threat Report* 2013. https://www.cdc.gov/drugresistance/threat-report-2013/

Chait R, Craney A, Kishony R. Antibiotic interactions that select against resistance. *Nature* 2007; 446:668-671.

Chan DI, Prenner EJ, Vogel HJ. Tryptophan- and arginine-rich antimicrobial peptides: structures and mechanisms of action. *Biochim Biophys Acta* 2006; 1758:1184-1202.

Chapple DS, Mason DJ, Joannou CL, Odell EW, Gant V, Evans RW. Structure-function relationship of antibacterial synthetic peptides homologous to a helical surface region on human lactoferrin against *Escherichia coli* serotype O111. *Infect Immun* 1998; 66(6):2434-2440.

Chileveru HR, Lim SA, Chairatana P, Wommack AJ, Chiang IL, Nolan EM. Visualizing attack of *Escherichia coli* by the antimicrobial peptide human defensin 5. *Biochemistry* 2015; 54:1767-1777.

Chongsiriwatana NP, Patch JA, Czyzewski AM, Dohm MT, Ivankin A, Gidalevitz D, Zuckermann RN, Barron AE. Peptoids that mimic the structure, function, and mechanism of helical antimicrobial peptides. *Proc Natl Acad Sci USA* 2008; 105:2794-2799.

Chongsiriwatana NP, Wetzler M, Barron AE. Functional synergy between antimicrobial peptoids and peptides against gram-negative bacteria. *Antimicrob Agents Chemother* 2011; 55(11):5399-5402.

Chou HH, Chiu HC, Delaney NF, Segrè D, Marx CJ. Diminishing returns epistasis among beneficial mutations decelerates adaptation. *Science* 2011; 332(6034):1190-1192.

Chou PY, Fasman GD. Conformational parameters for amino acids in helical, α- sheet, and random coil regions calculated from proteins. *Biochemistry* 1974; 13:211-22.

Citterio L, Franzyk H, Palarasah Y, Andersen TE, Mateiu RV, Gram L. Improved *in vitro* evaluation of novel antimicrobials: potential synergy between human plasma and antibacterial peptidomimetics, AMPs and antibiotics against human pathogenic bacteria. *Res Microbiol* 2016; 167:72-82.

Citterio L, Franzyk H, Nielsen HM, Gram L. Adaptive laboratory evolution of *E. coli* reveals slow resistance development to the short antimicrobial peptide P9-4 and to a combination of three antimicrobial compounds. 2016; *Submitted*.

Cole JN, Nizet V. Bacterial evasion of host antimicrobial peptide defenses. *Microbiol Spectr* 2016; 4(1): VMBF-0006-2015.

Corbalan N, Runti G, Adler C, Covaceuszach S, Ford RC, Lamba D, Beis K, Scocchi M, Vincent PA. Functional and structural study of the dimeric inner membrane protein SbmA. *J Bacteriol* 2013; 195:5352-5361.

Cruz-Monteagudo M, Borges F, Cordeiro MND. Jointly handling potency and toxicity of antimicrobial peptidomimetics by simple rules from desirability theory and chemoinformatics. *J Chem Inf Mod* 2011; 51(12):3060-3077.

Darveau RP, Blake J, Seachord CL, Cosand WL, Cunningham MD, Cassiano-Clough L, Maloney G. Peptides related to the carboxyl terminus of human platelet factor IV with antibacterial activity. *J Clin Invest* 1992; 90**:**447-455.

De Visser JAGMM, Krug J. Empirical fitness landscapes and the predictability of evolution. *Nat Rev Genet* 2014; 15:480-490.

Deslouches B, Islam K, Craigo JK, Paranjape SM, Montelaro RC, Mietzner TA. Activity of the de novo engineered antimicrobial peptide WLBU2 against *Pseudomonas aeruginosa* in human serum and whole blood: implications for systemic applications. *Antimicrob Agents Chemother* 2005; 49(8):3208-16.

Dhawan VK, Yeaman MR, Cheung AL, Kim E, Sullam PM, Bayer AS. Phenotypic resistance to thrombin-induced platelet microbicidal protein *in vitro* is correlated with enhanced virulence in experimental endocarditis due to *Staphylococcus aureus*. *Infect Immun* 1997; 65:3293-3299.

Dobson AJ, Purves J, Kamysz W, Rolff J. Comparing selection on *S. aureus* between antimicrobial peptides and common antibiotics. *PLoS one* 2013; 8(10):e76521.

Drlica K. The mutant selection window and antimicrobial resistance. *J Antimicrob Chemother* 2003; 52(1):11-17.

Dunner E, Brown WB, Wallace J. The effect of streptomycin with para-amino salicylic acid on the emergence of resistant strains of tubercle bacilli. *Dis Chest* 1949; 16(6):661-666.

Dutcher BS, Reynard AM, Beck ME, Cunningham RK. Potentiation of antibiotic bactericidal activity by normal human serum. *Antimicrob Agents Chemother* 1978; 13:820-6.

Ekblom R, Wolf JB A field guide to whole-genome sequencing, assembly and annotation. *Evol Appl* 2014; 7(9):1026-1042.

Epand RM, Vogel HJ. Diversity of antimicrobial peptides and their mechanisms of action. *Biochim Biophys Acta (BBA)-Biomembranes* 1999; 1462(1):11-28.

European Centre for Disease Prevention and Control (ECDC) and European Medicines Agency (EMEA). ECDC/EMEA Joint Technical Report - The bacterial challenge: time to react. Stockholm, 2009.

European Centre for Disease Prevention and Control. *Annual Epidemiological Report* 2013. Reporting on 2011 surveillance data and 2012 epidemic intelligence data. Stockholm: ECDC; 2013. http://ecdc.europa.eu/en/publications/Publications/annual-epidemiological-report-2013.pdf

Fair RJ, Tor Y. Antibiotics and bacterial resistance in the 21st century. *Perspect Medicin Chem* 2014; 6:25-64.

Falagas ME, Kasiakou SK. Colistin: the revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. *Clin Infect Dis* 2005; 40:1333-1341.

Falagas ME, Kasiakou SK. Toxicity of polymyxins: a systematic review of the evidence from old and recent studies. *Crit Care* 2006; 10(1):1.

Feist AM, Herrgård MJ, Thiele I, Reed JL, Palsson BØ. Reconstruction of biochemical networks in microorganisms. *Nat Rev Microbiol* 2009; 7(2):129-143.

Feng Q, Huang Y, Chen M, Li G, Chen Y. Functional synergy of α-helical antimicrobial peptides and traditional antibiotics against Gram-negative and Gram-positive bacteria *in vitro* and *in vivo. Eur J Clin Microbiol Infect Dis* 2015; 34(1):197-204.

Fernandez L, Gooderham WJ, Bains M, McPhee JB, Wiegand I et al. Adaptive resistance to the ''last hope'' antibiotics polymyxin B and colistin in *Pseudomonas aeruginosa* is mediated by the novel two-component regulatory system ParR-ParS. *Antimicrob Agents Chemother* 2010; 54:3372-3382.

Fierer J, Finley F. Lethal effect of complement and lysozyme on polymyxin-treated, serum-resistant Gram-negative bacilli. *J Infect Dis* 1979; 140:581-9.

Fischbach MA. Combination therapies for combating antimicrobial resistance. *Curr Opin Microbiol* 2011; 14(5):519-523.

Fleming A. On a remarkable bacteriolytic element found in tissues and secretions. *Proc R Soc London B Biol Sci* 1922; 93(653):306-317.

Fox JL. Antimicrobial peptides stage a comeback. *Nat Biotechnol* 2013; 31(5):379-382.

Frick IM, Akesson P, Herwald H, Mörgelin M, Malmsten M, Nägler DK, et al. The contact system-a novel branch of innate immunity generating antibacterial peptides. *EMBO J* 2006; 25(23):5569-78.

Gallo RL, Nizet V. Endogenous production of antimicrobial peptides in innate immunity and human disease. *Curr Allergy Asthma Rep* 2003; 3:402-409.

Garcia-Quintanilla M, Carretero-Ledesma M, Moreno-Martinez P, Martin-Pena R, Pachon J, McConnell MJ. Lipopolysaccharide loss produces partial colistin dependence and collateral sensitivity to azithromycin, rifampicin and vancomycin in *Acinetobacter baumannii*. *Int J Antimicrob Agents* 2015; 46:696-702.

Gennaro R, Zanetti M. Structural features and biological activities of the cathelicidin-derived antimicrobial peptides. *Peptide Science* 2000; 55(1):31-49.

Gennaro R, Zanetti M, Benincasa M, Podda E, Miani M. Pro-rich antimicrobial peptides from animals: structure, biological functions and mechanism of action. *Curr Pharm Des* 2002; 8(9):763-778.

Ghosh A, Kar RK, Jana J, Saha A, Jana B, Krishnamoorthy J, Kumar D, Ghosh S, Chatterjee S, Bhunia A. Indolicidin targets duplex DNA: structural and mechanistic insight through a combination of spectroscopy and microscopy. *Chem Med Chem* 2014; 9:2052-2058.

Giacometti A, Cirioni O, Del Prete MS, Paggi AM, D'Errico MM, Scalise G. Combination studies between polycationic peptides and clinically used antibiotics against Gram-positive and Gram-negative bacteria. *Peptides* 2000; 21(8):1155-1160.

Giangaspero A, Sandri L, Tossi A. Amphipathic alpha helical antimicrobial peptides. *Eur J Biochem* 2001; 268:5589-5600.

Glaser R, Becker K, von Eiff C, Meyer-Hoffert U, Harder J. Decreased susceptibility of *Staphylococcus aureus* small-colony variants toward human antimicrobial peptides. *J Invest Dermatol* 2014; 134:2347-2350.

Glickman MS, Sawyers CL. Converting cancer therapies into cures: lessons from infectious diseases. *Cell* 2012; 148:1089-1098.

Godballe T, Nilsson LL, Petersen PD, Jenssen H. Antimicrobial β-peptides and a α-peptoids. *Chem Biol Drug Des* 2011; 77:107-16.

Gordon YJ, Romanowski EG, McDermott AM. A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs. *Curr Eye Res* 2005; 30(7):505-15.

Gottschalk S, Gottlieb CT, Vestergaard M, Hansen PR, Gram L, Ingmer H, Thomsen LE. Amphibian antimicrobial peptide fallaxin analogue FL9 affects virulence gene expression and DNA replication in *Staphylococcus aureus*. *J Medical Microbiol* 2015; 64(12):1504-1513.

Gottschalk S, Ingmer H, Thomsen LE. The lysine-peptoid hybrid LP5 maintain activity under physiological conditions and affects virulence gene expression in *Staphylococcus aureus*. *Peptides* 2016; 78:24-29.

Gudmundsson GH, Agerberth B, Odeberg J, Bergman T, Olsson B, Salcedo R. The human gene FALL39 and processing of the cathelin precursor to the antibacterial peptide LL-37 in granulocytes. *Eur J Biochem* 1996; 238:325-332.

Gudmundsson GH, Agerberth B. Neutrophil antibacterial peptides, multifunctional effector molecules in the mammalian immune system. *J Immunol Methods* 1999; 232(1-2):45-54.

Guilhelmelli F, Vilela N, Albuquerque P, Derengowski LDS, Silva-Pereira I, Kyaw CM. Antibiotic

development challenges: the various mechanisms of action of antimicrobial peptides and of bacterial resistance. *Front Microbiol* 2013; 4(353).

Habets MG, Brockhurst MA. Therapeutic antimicrobial peptides may compromise natural immunity. *Biol Lett* 2012; 8:416-418.

Hachmann AB, Sevim E, Gaballa A, Popham DL, Antelmann H, Helmann JD. Reduction in membrane phosphatidylglycerol content leads to daptomycin resistance in *Bacillus subtilis*. *Antimicrob Agents Chemother* 2011; 55(9):4326-4337.

Hale JD. Bacterial resistance to antimicrobial peptides. Bacterial pathogenesis: molecular and cellular mechanisms. Caister Academic Press UK 2012; 305-315.

Hancock REW, Falla T, Brown MM. Cationic bactericidal peptides. *Adv Microbial Physiol* 1995; 37**:**135-75.

Hancock REW. Peptide antibiotics. *The Lancet* 1997;349(9049):418-422.

Hancock REW, Lehrer R. Cationic peptides: a new source of antibiotics. *Trends Biotechnol* 1998; 16:82-88.

Hancock REW, Sahl HG. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat Biotechnol* 2006; 24(12):1551-57.

Hansen AM, Bonke G, Larsen CJ, Yavari N, Nielsen PE, Franzyk H. Antibacterial peptide nucleic acid-antimicrobial peptide (PNA–AMP) conjugates: antisense targeting of fatty acid biosynthesis. *Bioconjugate Chem* 2016; 27(4):863-867.

Hao M, Mukherjee S, Maxfield FR. Cholesterol depletion induces large scale domain segregation in living cell membranes. *Proc Natl Acad Sci USA* 2001; 98(23):13072-13077.

He J, Starr CG, Wimley WC. A lack of synergy between membrane-permeabilizing cationic antimicrobial peptides and conventional antibiotics. *Biochim Biophys Acta (BBA)-Biomembranes* 2014; 1848(1):8-15.

Hegreness M, Shoresh N, Damian D, Hartl D, Kishony R. Accelerated evolution of resistance in multidrug environments. *Proc Natl Acad Sci USA* 2008; 105:13977-13981.

a) Hein-Kristensen L, Franzyk H, Holch A, Gram L. Adaptive evolution of *Escherichia coli* to an α-peptide/β-peptoid peptidomimetic induces stable resistance. *PLoS one* 2013; 8(9):e73620.

b) Hein-Kristensen L, Knapp KM, Franzyk H, Gram L. Selectivity in the potentiation of antibacterial activity of a-peptide/b-peptoid peptidomimetics and antimicrobial peptides by human blood plasma. *Res Microbiol* 2013;164(9):933-40.

Hirsch JG. Phagocytin: a bactericidal substance from polymorphonuclear leucocytes. *J Exp Med* 1956; 103:589-611.

Hong J, Hu J, Ke F. Experimental induction of bacterial resistance to the antimicrobial peptide tachyplesin I and investigation of the resistance mechanisms. *Antimicrob Agents Chemother* 2016; 60(10):6067-6075.

Hu W, Lee KC, Cross TA. Tryptophans in membrane proteins: indole ring orientations and functional implications in the gramicidin channel. *Biochemistry* 1993; 32:7035-47.

Hu Y, Liu A, Vaudrey J, Vaiciunaite B, Moigboi C, McTavish SM, Kearns A, Coates A. Combinations of β-lactam or aminoglycoside antibiotics with plectasin are synergistic against methicillin-sensitive and methicillin-resistant *Staphylococcus aureus*. *PLoS one* 2015; 10(2):e0117664.

Hu Y, Liu F, Lin IY, Gao GF, Zhu B. Dissemination of the mcr-1 colistin resistance gene. *Lancet Infect Dis* 2016; 16(2):146-147.

Huang GT, Zhang HB, Kim D, Liu L, Ganz T. A model for antimicrobial gene therapy: demonstration of human beta-defensin 2 antimicrobial activities *in vivo*. *Hum Gene Ther* 2002; 13:2017-2025.

Hughes D, Andersson DI. Evolutionary consequences of drug resistance: shared principles across diverse targets and organisms. *Nat Rev Genet* 2015; 16:459-471.

Hultmark D, Steiner H, Rasmuson T, Boman HG. Insect immunity - purification and properties of 3 inducible bactericidal proteins from hemolymph of immunised pupae of *Hyalophora cecropia*. *Eur J Biochem* 1980; 106:7-16 .

Imamovic L, Sommer MOA. Use of collateral sensitivity networks to design drug cycling protocols that avoid resistance development. *Sci Transl Med* 2013; 5(204):204ra132.

Jahnsen RD, Frimodt-Møller N, Franzyk H. Antimicrobial activity of peptidomimetics against multidrug-resistant *Escherichia coli* - A comparative study of different backbones. *J Med Chem* 2012; 55:7253-7261.

Jahnsen RD, Sandberg-Schaal A, Vissing KJ, Nielsen HM, Frimodt- Møller N, Franzyk H. Tailoring cytotoxicity of antimicrobial peptidomimetics with high activity against multidrug-resistant *Escherichia coli. J Med Chem* 2014; 57:2864-73.

Jahnsen RO, Sandberg-Schaal A, Frimodt-Møller N, Nielsen HM, Franzyk H. End group modification: efficient tool for improving activity of antimicrobial peptide analogues towards Gram-positive bacteria. *Eur J Pharm Biopharm* 2015; 95:40-46.

Jing W, Hunter HN, Hagel J, Vogel HJ. The structure of the antimicrobial peptide Ac-RRWWRF-NH_2 bound to micelles and its interactions with phospholipid bilayers. *J Pept Res* 2003; 61:219-229

Jochumsen N, Marvig RL, Damkiær S, Jensen RL, Paulander W, Molin S, Jelsbak L, Folkesson, A. The evolution of antimicrobial peptide resistance in *Pseudomonas aeruginosa* is shaped by strong epistatic interactions. *Nat Commun* 2016; 7:13002.

Joo HS, Fu CI, Otto M. Bacterial strategies of resistance to antimicrobial peptides. *Phil Trans R Soc B* 2016; 371:20150292.

Kaplan CW, Sim JH, Shah KR, Kolesnikova-Kaplan A, Shi W, Eckert R. Selective membrane disruption: mode of action of C16G2, a specifically targeted antimicrobial peptide. *Antimicrob Agents Chemother* 2011; 55:3446-3452.

Kaufmann SHE, Medzhitov R, Gordon S (eds.) The innate immune response to infection. Washington DC. ASM Press; 2004: 465.

Kawasaki H, Koyama T, Conlon JM, Yamakura F, Iwamuro S. Antimicrobial action of histone H2B in *Escherichia coli*: evidence for membrane translocation and DNA-binding of a histone H2B fragment after proteolytic cleavage by outer membrane proteinase T. *Biochimie* 2008; 90:1693-1702.

Klevens RM, Morrison MA, Nadle J, Petit S, Gershman K, Ray S, Harrison LH, Lynfield R, Dumyati G, Townes JM, Craig AS, Zell ER, Fosheim GE, McDougal LK, Carey RB, Fridkin SK. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *JAMA* 2007; 298(15):1763-1771.

Knappe D, Henklein P, Hoffmann R, Hilpert K. Easy strategy to protect antimicrobial peptides from fast degradation in serum. *Antimicrob Agents Chemother* 2010; 54(9):4003-5.

Kohli RM, Walsh CT, Burkart MD. Biomimetic synthesis and optimization of cyclic peptide antibiotics. *Nature* 2002; 418(6898):658-661.

Kondos SC, Hatfaludi T, Voskoboinik I, Trapani JA, Law RHP, Whisstock JC, Dunstone MA. The structure and function of mammalian membrane-attack complex/perforin-like proteins. *Tissue Antigens* 2010; 76:341-351.

Köser CU, Ellington MJ, Peacock SJ. Whole-genome sequencing to control antimicrobial resistance. *Trends Genet.* 2014; 30:401-407.

Kramer NE, Hasper HE, van den Bogaard PT, Morath S, de Kruijff B, Hartung T, Smid EJ, Breukink E,

Kok J, Kuipers OP. Increased D-alanylation of lipoteichoic acid and a thickened septum are main determinants in the nisin resistance mechanism of *Lactococcus lactis*. *Microbiology* 2008; 154:1755-1762.

Kraus D, Peschel A. Molecular mechanisms of bacterial resistance to antimicrobial peptides. *Curr Top Microbiol Immunol.* 2006; 306:231-250.

Lai Y, Gallo RL. AMPed up immunity: how antimicrobial peptides
have multiple roles in immune defense. *Trends Immunol* 2009; 30:131-141.

Landman D, Georgescu C, Martin DA, Quale J. Polymyxins revisited. *Clin Microbiol Rev* 2008:21: 449-465.

Lau QY, Choo XY, Lim ZX, Kong XN, Ng FM, Ang MJ, Hill J, Chia CB. A head-to-head comparison of the antimicrobial activities of 30 ultra-short antimicrobial peptides against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*. *Int J Pept Res Ther* 2015; 21(1):21-8.

Laxminarayan R, Malani A, Howard D, Smith DL. Extending the cure: policy responses to the growing threat of antibiotic resistance. Washington DC: Resources for the Future, 2007.

Laxminarayan R, Duse A, Wattal C, Zaidi AK, Wertheim HF, Sumpradit N, ... Cars O. Antibiotic resistance - the need for global solutions. *Lancet Infect Dis* 2013; 13(12):1057-1098.

Lee SH, Kim SJ, Lee YS, Song MD, Kim IH, Won HS. De novo generation of short antimicrobial peptides with simple amino acid composition. *Regul Pept* 2011; 166(1):36-41.

Lehrer RI. Primate defensins. *Nat Rev Microbiol* 2004; 2:727-738.
Levy SB, Marshall B. Antibacterial resistance worldwide: causes, challenges and responses. *Nat Med* 2004; 10:S122-S129.

Li JY, Wang XJ, Wang LN, Ying XX, Ren X, Liu HY, ... and Ma GW. High *in vitro* antibacterial activity of Pac-525 against *Porphyromonas gingivalis* biofilms cultured on titanium. *BioMed Res Int* 2015; http://dx.doi.org/10.1155/2015/909870

Lin L, Nonejuie P, Munguia J, Hollands A, Olson J, Dam Q, ... Nizet V. Azithromycin synergizes with cationic antimicrobial peptides to exert bactericidal and therapeutic activity against highly multidrug-resistant Gram-negative bacterial pathogens. *EBioMedicine* 2015; 2(7):688-696.

Liu L, Li Y, Li S, Hu N, He Y, Pong R, Lin D, Lu L, Law M. Comparison of next-generation sequencing systems. *BioMed Res Int* 2012:251364.

Liu Y, Knapp KM, Yang L, Molin S, Franzyk H, Folkesson A. High *in vitro* antimicrobial activity of β-peptoid–peptide hybrid oligomers against planktonic and biofilm cultures of *Staphylococcus epidermidis*. *Int J Antimicrob Agents* 2013; 41(1):20-27.

Liu YJ. Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. *Cell* 2001; 106:259-262.

Liu Z, Brady A, Young A, Rasimick B, Chen K, Zhou C, Kallenbach NR. Length effects in antimicrobial peptides of the $(RW)_n$ series. *Antimicrobial Agents Chemother* 2007; 51(2):597-603.

Lofton H, Pränting M, Thulin E, Andersson DI. Mechanisms and fitness costs of resistance to antimicrobial peptides LL-37, CNY100HL and wheat germ histones. *PLoS ONE* 2013; 8(7):e68875.

Loman NJ, Constantinidou C, Chan JZ, Halachev M, Sergeant M, Penn CW, Robinson ER, Pallen MJ. High-throughput bacterial genome sequencing: an embarrassment of choice, a world of opportunity. *Nat Rev Microbiol* 2012; 10(9):599-606.

Macvanin M, Hughes D. Hyper-susceptibility of a fusidic acid-resistant mutant of *Salmonella* to different classes of antibiotics. *FEMS Microbiol Lett* 2005; 247:215-220.

Magill SS, Edwards JR, Bamberg W, Beldavs ZG, Dumyati G, Kainer MA, Fridkin SK. Multistate point-prevalence survey of health care-associated infections. *N Engl J Med* 2014; 370(13):1198-1208.

Majerle A, Kidric J, Jerala R. Enhancement of antibacterial and lipopolysaccharide binding activities of a human lactoferrin peptide fragment by the addition of acyl chain. *J Antimicrob Chemother* 2003; 51:1159-1165.

Malmsten M, Kasetty G, Pasupuleti M, Alenfall J, Schmidtchen A. Highly selective end-tagged antimicrobial peptides derived from PRELP. *PLoS One* 2011; 6(1):e16400.

Malmsten M. Antimicrobial peptides. *Ups J Med Sci* 2014; 119(2):199-204.

Markiewski MM, Nilsson B, Ekdahl KN, Mollnes TE, Lambris JD. Complement and coagulation: strangers or partners in crime? *TRENDS Immunol* 2007; 28(4):184-92.

Marr AK, Gooderham WJ, Hancock REW. Antibacterial peptides for therapeutic use: obstacles and realistic outlook. *Curr Opin Pharmacol* 2006; 6:468-472.

Marshall NJ, Andruszkiewicz R, Gupta S, Milewski S, Payne JW. Structure-activity relationships for a series of peptidomimetic antimicrobial prodrugs containing glutamine analogues. *J Antimicrob Chemother* 2003; 51:821-831.

Martin E, Ganz T, Lehrer RI. Defensins and other endogenous peptide antibiotics of vertebrates. *J Leukoc Biol* 1995; 58(2):128-136.

Matamouros S, Miller SI. *S.* Typhimurium strategies to resist killing by cationic antimicrobial peptides. *Biochim Biophys Acta (BBA)-Biomembranes* 2015; 1848(11):3021-3025.

Matsuzaki K, Murase O, Tokuda H, Funakoshi S, Fujii N, Miyajima K. Orientational and aggregational states of magainin 2 in phospholipid bilayers. *Biochemistry* 1994; 33:3342-3349.

Mattiuzzo M, Bandiera A, Gennaro R, Benincasa M, Pacor S, Antcheva N, Scocchi M. Role of the *Escherichia coli* SbmA in the antimicrobial activity of proline-rich peptides. *Mol Microbiol* 2007; 66:151-163.

McAdam PR, Richardson EJ, Fitzgerald JR. High-throughput sequencing for the study of bacterial pathogen biology. *Curr Opin Microbiol* 2014; 19:106-113.

McGrath DM, Barbu EM, Driessen WH, Lasco TM, Tarrand JJ, Okhuysen PC, ... Arap W. Mechanism of action and initial evaluation of a membrane active all-D-enantiomer antimicrobial peptidomimetic. *Proc Natl Acad Sci USA* 2013; 110(9):3477-3482.

Melo MN, Ferre R, Castanho MA. Antimicrobial peptides: linking partition, activity and high membrane-bound concentrations. *Nat Rev Microbiol* 2009; 7:245-250.

Metzker ML. Sequencing technologies - the next generation. *Nat Rev Genet* 2010; 11:31-46.
Michel JB, Yeh PJ, Chait R, Moellering Jr RC, Kishony R. Drug interactions modulate the potential for evolution of resistance. *Proc Natl Acad Sci USA* 2008; 105:14918-14923.

Moffatt JH, Harper M, Harrison P, Hale JD, Vinogradov E, Seemann T, Henry R, Crane B, St Michael F, Cox AD, Adler B, Nation RL, Li J, Boyce JD. Colistin resistance in *Acinetobacter baumannii* is mediated by complete loss of lipopolysaccharide production. *Antimicrob Agents Chemother* 2010; 54:4971-4977.

Munck C, Gumpert HK, Wallin AIN, Wang HH, Sommer MOA. Prediction of resistance development against drug combinations by collateral responses to component drugs. *Sci Transl Med* 2014; 6(262):262ra156.

Nakamura T, Furunaka H, Miyata T, Tokunaga, Muta T, Iwanaga S, Shimonishi, Y. Tachyplesin, a class of antimicrobial peptide from the hemocytes of the horseshoe crab (*Tachypleus tridentatus*) isolation and chemical structure. *J Biol Chem* 1988; 263:16709-16713.

Ngu-Schwemlein M, Dumond J, Rudd L, Rigaud JH. *In vitro* synergy between some cationic amphipathic cyclooctapeptides and antibiotics. *Aust J Chem* 2015; 68(2):218-223.

Nguyen LT, Haney EF, Vogel HJ. The expanding scope of antimicrobial peptide structures and their modes of action. *Trends Biotechnol* 2011; 29(9):464-472.

Nicolas P. Multifunctional host defense peptides: intracellular-targeting antimicrobial peptides. *FEBS J* 2009; 276(22):6483-6496.

Nikaido H. Multidrug resistance in bacteria. *Annu Rev Biochem* 2009; 78:119-146.

Nilsson AC, Janson H, Wold H, Fugelli A, Andersson K, Hakangard C, Olsson P, Olsen WM. LTX-109 is a novel agent for nasal decolonization of methicillin-resistant and -sensitive *Staphylococcus aureus. Antimicrob Agents Chemother* 2015; 59:145-151.

Niu M, Li X, Gong Q, Wang C, Qin C, Wang W, Chen P. Expression of 4kD scorpion defensin and its *in vitro* synergistic activity with conventional antibiotics. *World J Microbiol Biotechnol* 2013; 29(2):281-288.

Nizet V. Antimicrobial peptide resistance mechanisms of human bacterial pathogens. *Curr Issues Mol Biol* 2006; 8:11-26.

Nizet V. Stopping superbugs, maintaining the microbiota. *Sci Transl Med* 2015; 7:295ed8.

Oh D, Shin SY, Lee S, Kang JH, Kim SD, Ryu PD, et al. Role of the hinge region and the tryptophan residue in the synthetic antimicrobial peptides, cecropin A(1-8)- Magainin 2(1-12) and its analogues, on their antibiotic activities and structures. *Biochemistry* 2000; 39(39):11855-964.

Olaitan AO, Morand S, Rolain JM. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Front Microbiol* 2014; 5:643.

Olsen CA, Bonke G, Vedel L, Adsersen A, Witt M, Franzyk H, Jaroszewski JW. Alpha-peptide/beta-peptoid chimeras. *Org Lett* 2007; 9:1549-1552.

Ong ZY, Cheng J, Huang Y, Xu K, Ji Z, Fan W, Yang YY. Effect of stereochemistry, chain length and sequence pattern on antimicrobial properties of short synthetic β-sheet forming peptide amphiphiles. *Biomaterials* 2014; 35(4):1315-25.

Otvos L Jr. The short proline-rich antibacterial peptide family. *Cell Mol Life Sci* 2002; 59(7):1138-1150.

Otvos L Jr. Peptide-based drug discovery and development. Common misconceptions*. Pharm Outsourc* 2014; 15:40-43.

Otvos L Jr, Wade JD. Current challenges in peptide-based drug discovery. *Front Chem* 2014; 2:62.

Oyston PCF, Fox MA, Richards SJ, Clark GC. Novel peptides therapeutics for treatment of infections. *J Med Microbiol* 2009; 58:977-87.

Pabinger S, Dander A, Fischer M, Snajder R, Sperk M, Efremova M, ... Trajanoski Z. A survey of tools for variant analysis of next-generation genome sequencing data. *Brief Bioinform* 2014; 15(2):256-278.

Palella FJ Jr, Delaney KM, Moorman AC, Loveless MO, Fuhrer J, Satten GA, Aschman DJ, Holmberg SD. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. *N Engl J Med* 1998; 338(13):853-860.

Palmer AC, Kishony R. Understanding, predicting and manipulating the genotypic evolution of antibiotic resistance. *Nat Rev Genet* 2013; *14*(4):243-8.

Panyutich A, Ganz T. Activated alpha 2-macroglobulin is a principal defensin-binding protein. *Am J Respir Cell Mol Biol* 1991; 5:101-6.

Papagianni M. Ribosomally synthesized peptides with antimicrobial properties: biosynthesis, structure, function, and applications. *Biotechnol Adv* 2003; 21(6):465-499.

Pasupuleti M, Schmidtchen A, Malmsten M. Antimicrobial peptides: key components of the innate immune system. *Crit Rev Biotechnol* 2012; 32:143-71.

Patrzykat A, Friedrich CL, Zhang L, Mendoza V, Hancock REW. Sublethal concentrations of pleurocidin-derived antimicrobial peptides inhibit macromolecular synthesis in *Escherichia coli*. *Antimicrob Agents Chemother* 2002; 46:605-614.

Penchovsky R, Traykovska M. Designing drugs that overcome antibacterial resistance: where do we stand and what should we do? *Expert Opin Drug Discov* 2015; 10(6):631-650.

Perron GG, Zasloff M, Bell G. Experimental evolution of resistance to an antimicrobial peptide.

Proc Biol Sci 2006; 273:251-256.

Peschel A, Sahl HG. The co-evolution of host cationic antimicrobial peptides and microbial resistance. *Nat Rev Microbiol* 2006; 4:529-536.

Phoenix DA, Harris F, Mura M, Dennison SR. The increasing role of phosphatidylethanolamine as a lipid receptor in the action of host defence peptides. *Prog Lipid Res* 2015; 59:26-37.

Podda E, Benincasa M, Pacor S, Micali F, Mattiuzzo M, Gennaro R, Scocchi M. Dual mode of action of Bac7, a proline-rich antibacterial peptide. *Biochim Biophys Acta* 2006; 1760:1732-1740.

Porter EA, Weisblum B, Gellman SH. Mimicry of host-defense peptides by unnatural oligomers: antimicrobial β-peptides. *J Am Chem Soc* 2002; 124:7324-7330.

Pränting M, Andersson DI. Mechanisms and physiological effects of protamine resistance in *Salmonella enterica* serovar Typhimurium LT2. *J Antimicrob Chemother* 2010; 65:876-887.

Punina NV, Makridakis NM, Remnev MA, Topunov AF. Whole-genome sequencing targets drug-resistant bacterial infections. *Hum Genomics* 2015; 9:19.

Qi X, Zhou C, Li P, Xu W, Cao Y, Ling H, ... Chan-Park MB. Novel short antibacterial and antifungal peptides with low cytotoxicity: efficacy and action mechanisms. *Biochem Biophys Res Commun* 2010; 398(3):594-600.

Raguse TL, Porter EA, Weisblum B, Gellman SH. Structure-activity studies of 14-helical antimicrobial beta-peptides: probing the relationship between conformational stability and antimicrobial potency. *J Am Chem Soc* 2002; 124(43):12774-12785.

Randolph JT, DeGoey DA. Peptidomimetic inhibitors of HIV protease. *Curr Top Med Chem* 2004; 4(10):1079-1095.

Reffuveille F, de la Fuente-Núñez C, Mansour S, Hancock REW. A broad-spectrum antibiofilm peptide enhances antibiotic action against bacterial biofilms. *Antimicrobial Agents Chemother* 2014; 58(9):5363-5371.

Ringstad L, Andersson Nordahl E, Schmidtchen A, Malmsten M. Composition effect on peptide interaction with lipids and bacteria: variants of C3a peptide CNY21. *Biophys J*. 2007; 92:87-98.

Ringstad L, Protopapa E, Lindholm-Sethson B, Schmidtchen A, Nelson A, Malmsten M. An electrochemical study into the interaction between complement-derived peptides and DOPC mono- and bilayers. *Langmuir* 2008; 24:208-16.

Roland KL, Martin LE, Esther CR, Spitznagel JK. Spontaneous pmrA mutants of *Salmonella* Typhimurium LT2 define a new two-component regulatory system with a possible role in virulence. *J Bacteriol* 1993; 175:4154-4164.

Sabtu N, Enoch DA, Brown NM. Antibiotic resistance: what, why, where, when and how? *Brit Med*

Bull 2015; 116(1):105-113.

Sallum UW, Chen TT. Inducible resistance of fish bacterial pathogens to the antimicrobial peptide cecropin B. *Antimicrob Agents Chemother* 2008; 52:3006-3012.

Samuelsen O, Haukland HH, Jenssen H, Kramer M, Sandvik K, Ulvatne H, Vorland LH. Induced resistance to the antimicrobial peptide lactoferricin B in *Staphylococcus aureus*. *FEBS Lett* 2005; 579:3421-3426.

Sanders CC, Sanders WE, Goering RV, Werner V. Selection of multiple antibiotic resistance by quinolones, beta-lactams, and aminoglycosides with special reference to cross-resistance between unrelated drug classes. *Antimicrob Agents Chemothe*r 1984; 26:797-801.

Santajit S, Indrawattana N. Mechanisms of Antimicrobial Resistance in ESKAPE Pathogens. *BioMed Res Int* 2016; Article ID 2475067.

Schadt EE, Linderman MD, Sorenson J, Lee L, Nolan GP. Computational solutions to large-scale data management and analysis. *Nat Rev Genet* 2010; 11:647-57.

Schmidtchen A, Ringstad L, Kasetty G, Mizuno H, Rutland MW, Malmsten M. Membrane selectivity by W-tagging of antimicrobial peptides. *Biochim Biophys Acta*. 2011; 1808:1081-91.

Selsted ME, Harwig SS, Ganz T, Schilling JW, Lehrer RI. Primary structures of three human neutrophil defensins. *J Clin Invest* 1985; 76:1436-1439.

Selsted ME, Novotny MJ, Morris WL, Tang YQ, Smith W, Cullor, J.S. Indolicidin, a novel bactericidal tridecapeptide amide from neutrophils. *J Biol Chem* 1992; 267:4292-4295.

Scholar EM Pratt WB (eds.) The antimicrobial drugs. Oxford Univ Press USA 2000.

Schuster A, Haliburn C, Döring G, Goldman MH, for the Freedom Study Group. Safety, efficacy and convenience of colistimethate sodium dry powder for inhalation (Colobreathe DPI) in patients with cystic fibrosis: a randomised study. *Thorax* 2013; 68(4):344-350.

Shai Y. Mode of action of membrane active antimicrobial peptides. *Biopolymers* 2002; 66:236-248.

Sharma H, Nagaraj R. Human β-defensin 4 with non-native disulfide bridges exhibit antimicrobial activity. *PLoS ONE* 2015; 10(3):e0119525.

Shlaes DM. Antibiotics: The perfect storm. Springer Science and Business Media 2010.

Sievert DM, Ricks P, Edwards JR et al. National Healthcare Safety Network Team and Participating National Healthcare Safety Network Facilities. Antimicrobial-resistant pathogens associated with healthcare- associated infections: summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2009-2010. *Infect Control Hosp Epidemiol* 34(1):1-14 (2013).

Sitaram N. Antimicrobial peptides with unusual amino acid compositions and unusual structures. *Curr Med Chem* 2006; 13:679-96.

Skarnes RC, Watson DW. Antimicrobial factors of normal tissues and fluids. *Bacteriol Rev* 1957; 21:273-294.

Smith R, Coast J. The true cost of antimicrobial resistance. *BMJ (Clinical Research Ed.)* 2013; 346: f1493.

Spellberg B, Bartlett JG, Gilbert DN. The future of antibiotics and resistance. *N Engl J Med* 2013; 368:299-302.

Steiner H, Hultmark D, Engstrom A, Bennich H, Boman HG. Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature* 1981; 292:246-248.

Stone LK, Baym M, Lieberman TD, Chait R, Clardy J, Kishony R. Compounds that select against the tetracycline-resistance efflux pump. *Nat Chem Biol* 2016; 12(11):902-904.

Strömstedt AA, Kristiansen PE, Gunasekera S, Grob N, Skjeldal L, Göransson U. Selective membrane disruption by the cyclotide kalata B7: complex ions and essential functional groups in the phosphatidylethanolamine binding pocket. *Biochim Biophys Acta* 2016; 1858(6):1317-27.

Subbalakshmi C, Sitaram N. Mechanism of antimicrobial action of indolicidin. *FEMS Microbiol Lett* 1998; 160:91-96.

Sun S, Negrea A, Rhen M, Andersson DI. Genetic analysis of colistin resistance in *Salmonella enterica* serovar Typhimurium. *Antimicrob Agents Chemother* 2009; 53:2298-2305.

Tamma PD, Cosgrove SE, Maragakis LL. Combination therapy for treatment of infections with Gram-negative bacteria. *Clin Microbiol Rev* 2012; 25:450-470.

Territo MC, Ganz T, Selsted ME, Lehrer R. Monocyte-chemotactic activity of defensins from human neutrophils. *J Clin Invest* 1989; 84:2017-2020.

The evolving threat of antimicrobial resistance: options for action. Geneva: World Health Organization; 2012.

Tomita T, Hitomi S, Nagase T, Matsui H, Matsuse T, Kimura S, Ouchi Y. Effect of ions on antibacterial activity of human beta defensin 2. *Microbiology and immunology* 2000; 44(9):749-754.

Toprak E, Veres A, Michel JB, Chait R, Hartl DL, Kishony R. Evolutionary paths to antibiotic resistance under dynamically sustained drug selection. *Nat Genet* 2012; 44(1):101-105.

Torella JP, Chait R, Kishony R. Optimal drug synergy in antimicrobial treatments. *PLoS Comput Biol* 2010; 6(6):e1000796.

Tossi A, Tarantino C, Romeo D. Design of synthetic antimicrobial peptides based on sequence analogy and amphipathicity. *Eur J Biochem* 1997; 250(2):549-558.

Tossi A, Sandri L, Giangaspero A. Amphipathic, alpha-helical antimicrobial peptides. *Biopolymers* 2000; 55:4-30.

Tu J, Wu G, Zuo Y, Zhao L, Wang S. ZL-2, a cathelicidin-derived antimicrobial peptide, has a broad antimicrobial activity against gram-positive bacteria and gram-negative bacteria *in vitro* and *in vivo*. *Arch Pharm Res* 2015; 38(10):1802-1809.

Tytler EM, Anantharamaiah GM, Walker DE, Mishra VK, Palgunachari MN, Segrest JP. Molecular basis for prokaryotic specificity of magainin-induced lysis. *Biochemistry* 1995; 34:4393-4401.

Valerius NH, Koch C, Hoiby N. Prevention of chronic *Pseudomonas aeruginosa* colonisation in cystic fibrosis by early treatment. *The Lancet* 1991; 338(8769):725-726.

Van Dijk EL, Auger H, Jaszczyszyn Y, Thermes C. Ten years of next-generation sequencing technology. *Trends Genet* 2014; 30(9):418-426.

Van't Hof W, Veerman EC, Helmerhorst EJ, Amerongen AV. Antimicrobial peptides: properties and applicability. *Biol Chem* 2001; 382:597-619.

Vestergaard M, Paulander W, Marvig RL, Clasen J, Jochumsen N, Molin S, Jelsbak L, Ingmer H, Folkesson A. Antibiotic combination therapy can select for broad-spectrum multidrug resistance in *Pseudomonas aeruginosa*. *Int J Antimicrob Agents* 2016; 47(1):48-55.

Villa TG, Vinas M. (eds.) New weapons to control bacterial growth. Springer International Publishing 2016.

Vogel HJ, Schibli DJ, Jing W, Lohmeier-Vogel EM, Epand RF, Epand RM. Towards a structure-function analysis of bovine lactoferricin and related tryptophan-and arginine-containing peptides. *Biochem Cell Biol* 2002; 80(1):49-63.

Voskoboinik I, Dunstone MA, Baran K, Whisstock JC, Trapani JA. Perforin: structure, function, and role in human immunopathology. *Immunol Rev* 2010; 235:35-54.

Wang G. Human antimicrobial peptides and proteins. *Pharmaceuticals* 2014; 7(5):545-594.

Wang G, Li X, Wang Z. APD3: the antimicrobial peptide database as a tool for research and education. *Nucleic Acids Res* 2016; 44:D1087-D1093. http://aps.unmc.edu/AP/main.html

Wang Y, Agerberth B, Johansson J. Structure and activity of cathelicidin antibacterial proteins. *J Protein Chem* 1998; 17(6):522-523.

Weatherspoon-Griffin N, Zhao G, Kong W, Kong Y, Morigen et al. The CpxR/CpxA two-component system up-regulates two Tat-dependent peptidoglycan amidases to confer bacterial resistance to antimicrobial peptide. *J Biol Chem* 2011; 286(7):5529-5539.

Weinreich DM, Delaney NF, Depristo MA, Hartl DL. Darwinian evolution can follow only very few mutational paths to fitter proteins. *Science* 2006; 312:111-114.

Wimley WC. Describing the mechanism of antimicrobial peptide action with the interfacial activity model. *ACS Chem Biol* 2010; 5(10):905-917.

Wimley WC, Hristova K. Antimicrobial peptides: successes, challenges and unanswered questions. *J Membr Biol* 2011; 239(1-2):27-34.

Won HS, Park SH, Kim HE, Hyun B, Kim M, Lee B, et al. Effects of a tryptophanyl substitution on the structure and antimicrobial activity of C-terminally truncated gaegurin 4. *Eur J Biochem* 2002; 269:4367-74.

Won HS, Jung SJ, Kim HE, Seo MD, Lee BJ. Systematic peptide engineering and structural characterization to search for the shortest antimicrobial peptide analogue of gaegurin 5. *J Biol Chem* 2004; 279:14784-91.

Yang D, Chen Q, Schmidt AP, Anderson GM, Wang JM, Wooters J, Oppenheim JJ, Chertov O. LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. *J Exp Med* 2000; 192:1069-1074.

Yeaman MR, Gank KD, Bayer AS, Brass EP. Synthetic peptides that exert antimicrobial activities in whole blood and blood-derived matrices. *Antimicrob Agents Chemother* 2002; 46:3883-3891.

Yeaman MR, Yount NY. Mechanisms of antimicrobial peptide action and resistance. *Pharmacol Rev* 2003; 55:27-55.

Yeaman MR. Platelets: at the nexus of antimicrobial defence. *Nat Rev Microbiol* 2014; 12(6):426-437.

Yeh PJ, Hegreness MJ, Aiden AP, Kishony R. Drug interactions and the evolution of antibiotic resistance. *Nat Rev Microbiol* 2009; 7:460-466.

Yu G, Baeder DY, Regoes RR, Rolff J. The more the better? Combination effects of antimicrobial peptides. *Antimicrob. Agents Chemother* 2016; 60(3):1717-1724.

Zasloff M. Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proc Natl Acad Sci. USA* 1987; 84:5449-5453.

Zasloff M. Antimicrobial peptides of multicellular organisms. *Nature* 2002; 415:389-395.

Zavascki AP, Goldani LZ, Li J, Nation RL. Polymyxin B for the treatment of multidrug-resistant pathogens: a critical review. *J Antimicrob Chemother* 2007; 60:1206-1215.

Zdybicka-Barabas A, Mak P, Klys A, Skrzypiec K, Mendyk E, Fiołka MJ, Cytryńska M. Synergistic action of *Galleria mellonella* anionic peptide 2 and lysozyme against Gram-negative bacteria. *Biochim Biophys Acta (BBA)-Biomembranes* 2012; 1818(11):2623-2635.

Zelezetsky I, Tossi A. Alpha-helical antimicrobial peptides - using a sequence template to guide structure-activity relationship studies. *Biochim Biophys Acta (BBA)-Biomembranes* 2006; 1758(9):1436-1449.

Zhao J, Huang Y, Liu D, Chen Y. Two hits are better than one: synergistic anticancer activity of α-helical peptides and doxorubicin/epirubicin. *Oncotarget* 2014; 6(3):1769-1778.

Research Articles

Research in Microbiology 167 (2016) 72–82

www.elsevier.com/locate/resmic

Original article

Improved in vitro evaluation of novel antimicrobials: potential synergy between human plasma and antibacterial peptidomimetics, AMPs and antibiotics against human pathogenic bacteria

Linda Citterio [a], Henrik Franzyk [b], Yaseelan Palarasah [c], Thomas Emil Andersen [d], Ramona Valentina Mateiu [e], Lone Gram [a,*]

[a] *Department of Systems Biology, Matematiktorvet, Technical University of Denmark, 2800 Kgs Lyngby, Denmark*
[b] *Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark*
[c] *Thrombosis Research, University of Southern Denmark, Odense, Denmark*
[d] *Research Unit of Clinical Microbiology, University of Southern Denmark, Odense, Denmark*
[e] *DTU CEN, Fysikvej, Center for Electron Nanoscopy, Technical University of Denmark, 2800 Kgs Lyngby, Denmark*

Received 6 August 2015; accepted 10 October 2015
Available online 21 October 2015

Abstract

Stable peptidomimetics mimicking natural antimicrobial peptides (AMPs) have emerged as a promising class of potential novel antibiotics. In the present study, we aimed at determining whether the antibacterial activity of two α-peptide/β-peptoid peptidomimetics against a range of bacterial pathogens was affected by conditions mimicking in vivo settings. Their activity was enhanced to an unexpected degree in the presence of human blood plasma for thirteen pathogenic Gram-positive and Gram-negative bacteria. MIC values typically decreased 2- to 16-fold in the presence of a human plasma concentration that alone did not damage the cell membrane. Hence, MIC and MBC data collected in these settings appear to represent a more appropriate basis for in vivo experiments preceding clinical trials. In fact, concentrations of peptidomimetics and peptide antibiotics (e.g. polymyxin B) required for in vivo treatments might be lower than traditionally deduced from MICs determined in laboratory media. Thus, antibiotics previously considered too toxic could be developed into usable last-resort drugs, due to ensuing lowered risk of side effects. In contrast, the activity of the compounds was significantly decreased in heat-inactivated plasma. We hypothesize that synergistic interactions with complement proteins and/or clotting factors most likely are involved.

Keywords: Antimicrobial peptide; Peptidomimetics; Antibiotics; Plasma; Synergy

1. Introduction

Bacteria and other microorganisms are becoming resistant to conventional antibiotics at an alarming rate, and hence, novel antimicrobial compounds for treatment of infectious diseases are urgently needed [1]. Several strategies are being pursued in the search for novel anti-infectives, such as development of compounds with anti-virulence activity [2].

Almost all living organisms have an innate frontline defense against microbial infections. Antimicrobial peptides (AMPs) comprise a diverse group of natural defense compounds that have been isolated from most organisms ranging from bacteria to humans [3]. Most AMPs are highly cationic hydrophobic compounds that interact with the bacterial cell

* Corresponding author.
E-mail addresses: lincit@bio.dtu.dk (L. Citterio), henrik.franzyk@sund.ku.dk (H. Franzyk), ypalarasah@health.sdu.dk (Y. Palarasah), thandersen@health.sdu.dk (T.E. Andersen), ramona.mateiu@cen.dtu.dk (R.V. Mateiu), gram@bio.dtu.dk (L. Gram).

http://dx.doi.org/10.1016/j.resmic.2015.10.002
0923-2508/

membrane and cause cell lysis and cell death. AMPs have been suggested as new antibacterials [4]; however, many natural AMPs exhibit side effects, e.g. hemolysis or general cytotoxicity. Also, due to their peptide nature, many AMPs will rapidly be degraded in vivo by proteases, thereby diminishing their efficacy [5]. Stable synthetic variants of AMPs incorporating unnatural residues have been developed to overcome these shortcomings, and such peptidomimetics have also been suggested as potential future antibiotics [6,7]. In fact, structural modification may allow optimization of their bactericidal activity versus cytotoxicty toward human cells, and hence an improved therapeutic index [8].

Despite the vast plethora of antibacterial compounds being discovered and chemically synthesized, the transition from pre-clinical to clinical trial phases still represents a challenge [9]. In the first evaluation of potential efficacy of novel antibiotics, they are typically tested in laboratory media (e.g. Mueller-Hinton broth) for minimum inhibitory and minimum bactericial concentrations (i.e. MICs and MBCs). However, the actual efficacy in vivo may be different due to interactions with components of the human body [10]. Therefore, development of laboratory systems that mimic in vivo conditions will constitute important progress toward a more efficient selection of candidates for clinical trials. One obvious approach involves testing the compounds in the presence of biologically relevant concentrations of blood matrices and/or at physiological ionic strength. Reduced activity of natural and synthetic peptides in serum has often been raised as a concern [11]. It may, however, be possible to modify AMPs to overcome this issue or even enhance their effects in the presence of blood. Indeed, antibacterial peptides derived from or based on human platelets retain their activity in the presence of human plasma [12]. Also, Deslouches et al. [10] found that a de novo engineered AMP rich in arginine (WLBU2) maintained its activity against *Pseudomonas aeruginosa* in the presence of both serum and plasma. In contrast to these synthetic peptides, human cathelicidin LL-37 lost its activity in the presence of human serum as well as in plasma [10]. These findings highlight the advantages of synthetic analogs over natural AMPs (e.g. enhanced proteolytic stability in vivo and increased cell selectivity) [13–15], but also emphasize the need to include laboratory test methodologies mimicking physiological conditions.

We recently found that the presence of human blood plasma unexpectedly increased the activity of α-peptide/β-peptoid peptidomimetics against *Escherichia coli* [16]; however, we did not investigate whether this effect is limited to *E. coli* or may be extended to other pathogens. The purpose of the present study was to determine how the antibacterial effect of these peptidomimetics is influenced by plasma or serum for a broad range of pathogenic bacteria. Human plasma proved to potentiate peptidomimetics against all tested bacteria, and we hypothesized that synergy with components of the complement system might account for this observed potentiation. Thus, a major objective became the testing of this hypothesis.

2. Materials and methods

2.1. Bacterial strains

The effect of human plasma and serum on the antibacterial activity of peptidomimetics was tested against a panel of Gram-negative and Gram-positive pathogenic bacteria: *E. coli* ATCC 25922, *Salmonella typhimurium* L354, *Serratia marcescens*, *P. aeruginosa* PAO1, *Vibrio vulnificus* cmcP6, *V. vulnificus* DSM 10143, *Vibrio parahaemolyticus* RimD D2210633, *V. parahaemolyticus* ATCC 17802, *Staphylococcus aureus* 8325-4, *Staphylococcus epidermidis* RP62A, *S. epidermidis* 1457, *Listeria monocytogenes* EGDe BUG 1600 and *Bacillus cereus* ATCC 11778. References to these strains are reported in Supplemental Table S3.

2.2. Growth conditions and chemicals

The strains were stored at −80 °C and grown overnight at 37 °C (250 rpm) in cation-adjusted Mueller-Hinton II broth adjusted to pH 7.4 ± 0.2 (MHB, Becton Dickinson, 212322). The MICs of peptidomimetics were determined in MHB or MHB supplemented with 1.25% defibrinated horse blood (REF236999, Statens Serum Institute) to improve growth of *B. cereus* and *L. monocytogenes* [17]. The MBC was determined by plating from wells with no visible growth on brain heart infusion agar (BHI, Oxoid, CM1135) containing 1.5% agar (AppliChem, A7354). Human lyophilized plasma was reconstituted in MilliQ water to its original volume of 5 ml to a concentration of 100%. It was then stored at −20 °C. Human serum (Sigma Aldrich, H4522) was kept at −20 °C. Both plasma and serum were purchased as commercially available products. Plasma from human (Sigma Aldrich, P9523) was obtained from a pool of 1000–1500 donors, while human serum derived from human male AB plasma was obtained from a pool of approximately 200 donors. Ampicillin (Sigma Aldrich, A9518), gentamicin (Sigma Aldrich, G3632) and polymyxin B (Sigma Aldrich, P4932) were dissolved in MilliQ water to give stock solutions (10 mg/ml). Citrate solutions were prepared in MilliQ water with trisodium citrate dihydrate (Merck Millipore, 106448).

2.3. Peptidomimetics and antimicrobial peptide LL-37

The α-peptide/β-peptoid peptidomimetics 1 and 2 (Fig. 1) consist of alternating repeats of natural cationic α-amino acids and synthetic lipophilic β-peptoid residues, and they were prepared by solid-phase synthesis as previously reported [8,18]. These compounds were tested for cytotoxicity against HeLa cells in previous studies [19,20]. Their cytotoxicity (IC_{50} values) was 32 μM and 96 μM, respectively, while the cytotoxicity of closely related compounds towards HeLa cells was lower (i.e. IC_{50} values of 316 ± 20 and 228 ± 4 μM, respectively) [19]. The peptidomimetics exhibited very low toxicity toward Caco-2 and HUVEC cells (IC_{50} > 1000 μM) [19]. The sequence of the human cathelicidin LL-37 is LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES-

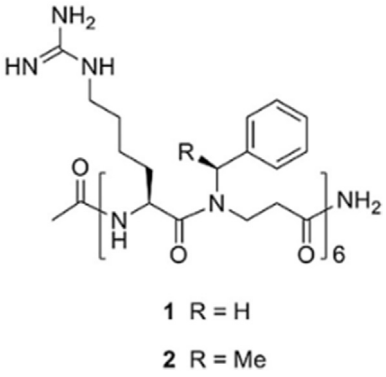


Fig. 1. Chemical structure of α-peptide/β-peptoid peptidomimetics 1 and 2.

$CONH_2$. The compounds were dissolved in MilliQ water to reach a stock concentration of 10 mg/ml. These stock solutions were stored at −20 °C.

2.4. Determination of MIC and MBC

MIC was determined by the microdilution broth method [21] in 96-well non-binding polypropylene microtiter plates (Thermo Scientific Nunc, 267245) with sterile polystyrene lids (Sigma Aldrich, CLS3930-100EA). Plates were autoclaved before use. To determine whether the materials used might influence the measured activity of the compounds, MIC and MBC were also tested in plates or vials of different materials. These included polystyrene plates (Thermo Scientific Nunc, 163320), UV-sterilized polypropylene plates (Thermo Scientific Nunc, 267334; as recommended by Hancock Laboratory Methods [22]), the above-mentioned non-binding polypropylene plates and glass vials (VWR Bie and Berntsen, 2775/378). Stock solutions of peptidomimetics 1 and 2, ranging from 4096 μg/ml to 32 μg/ml, were prepared in MilliQ water. Fifty μl of compound solution and 50 μl of the medium (MHB or MHB supplemented with plasma or serum) were dispensed into the first well of each row and then twofold diluted. The influence of native and heat-inactivated plasma and serum on the antibacterial activity of peptidomimetics and antibiotics was tested by adding plasma or serum to MHB in concentrations of 50% and 25%. After addition of peptide solution and bacterial culture, the resulting exposure concentrations were 25% and 12.5%. These tested concentrations of plasma and serum were based on previous work on *E. coli* [16]. However, several strains were unable to grow at these concentrations of serum and plasma, and therefore, MICs of serum and plasma for these strains were determined and then appropriate concentrations of serum and plasma were chosen based on these MIC values.

Overnight cultures of the strains were diluted in sterile 0.9% NaCl to OD_{546} = 0.2 (accepted range: 0.195–0.210) corresponding to approximately 1×10^8 CFU/ml. This suspension was further diluted 1:100 in MHB and 50 μl of this culture were inoculated into microtiter plates to reach a final concentration of 5×10^5 CFU/ml in a final volume of 100 μl. Plates were incubated for 20 h at 37 °C. Growth was determined visually either as turbidity or as formation of a pellet. MIC was determined to be the lowest concentration of peptidomimetics or antibiotics where no growth was observed. MBC was determined to be the lowest concentration where a 3-log reduction of the original CFU/ml was observed based on plating from the wells on BHI agar.

2.5. Potentiation of the antibacterial activity of peptidomimetics by human plasma and serum

The peptidomimetics had more pronounced antibacterial activity in the presence of plasma and serum (Tables 1–4; Supplemental Tables S1 and S2), and several experiments were conducted to determine the mechanism behind this potentiation. Plasma and serum were heated for 15 min at 56 °C as a non-specific procedure for inactivating the complement [23]. In addition, sodium polyanethole sulfonate

Table 1
MIC of plasma (expressed as % of plasma) for all strains used in the present study. MIC of peptidomimetic 1 in MHB, and in MHB supplemented with native plasma as well as with heat-treated plasma. Nd = not determined; ng = no growth; h.t. = heat-treated. The values are based on two individual experiments conducted in duplicate.

Target strain	MIC of plasma (%)	MIC of peptidomimetic 1 (μg × ml^{-1})					
		Added plasma (%)					
		0	3	6	12.5	25	12.5 h.t.
E. coli	≥75	2–8	nd	nd	0.5–1	0.25–0.5	>64
S. typhimurium	25–50	4–8	nd	1	1–4	ng	>512
S. marcescens	12.5	16–128	16	ng	ng-2	ng	64
P. aeruginosa	75	128–512	nd	nd	16–128	8	>512
V. vulnificus cmcP6	25–75	4–16	nd	nd	1–4	0.125–0.25	8–>64
V. vulnificus DSM 10143	25	4–8	nd	nd	0.5–2	ng	4–8
V. parahaemolyticus R.	25	4–8	2–4	2–4	ng	ng	>128
V. parahaemolyticus A.	6	4–8	ng-2	ng-2	ng	ng	>64
S. aureus 8325-4	75	8–128	nd	nd	8–128	2–4	16–>512
S. epidermidis RP62A	≥75	4–16	nd	0.5–8	nd	<0.25	8–64
S. epidermidis 1457	25	4–8	2	1	nd	ng	>128
L. monocytogenes	6–12.5	8–16	4–8	nd	ng	ng	8–16
B. cereus	≥75	2–8	nd	nd	0.5–1	0.5–1	4–8

Table 2
MBC of peptidomimetic 1 in MHB, MHB supplemented with native plasma and with heat-treated plasma, respectively. Nd = not determined; ng = no growth; h.t. = heat-treated.

Target strain	MBC for peptidomimetic 1 (μg × ml^{-1})					
	Added plasma (%)					
	0	3	6	12.5	25	12.5% h.t.
E. coli	4–16	nd	nd	1–8	1–2	>64
S. typhimurium	8–32	nd	1	0.5–4	ng	>64
S. marcescens	32–128	16–32	0.125	ng	ng	64
P. aeruginosa	512–>512	nd	nd	512	>16–>32	>512
V. vulnificus cmcP6	4–16	nd	nd	1–2	0.125–0.5	16–>64
V. vulnificus DSM 10143	4–8	nd	nd	0.5	ng	4–16
V. parahaemolyticus R.	4–8	2–4	2–4	nd	ng	>128
V. parahaemolyticus A.	4–8	2	nd	ng	ng	>64
S. aureus 8325-4	32–128	nd	nd	8	4–16	16–>64
S. epidermidis RP62A	4–16	nd	1–8	1	>0.25	8–16
S. epidermidis 1457	4–16	4	2	nd	ng	>128
L. monocytogenes	8–32	8–16	nd	≤0.125	ng	32
B. cereus	2–16	nd	nd	0.5–1	0.5–1	4–8

Table 3
MIC of serum (expressed as % of serum) for all the strains used in the present study. MIC of peptidomimetic 1 in MHB, and in MHB supplemented with native serum as well as with heat-treated serum. Nd = not determined; ng = no growth; r = resistant to 100% serum; h.t. = heat-treated. The values are based on two individual experiments conducted in duplicate.

Target strain	MIC of serum (%)	MIC of peptidomimetic 1 (μg × ml^{-1})					
		Added serum (%)					
		0	3	6	12.5	25	12.5 h.t.
E. coli	r	2–8	nd	nd	2–4	4	8–16
S. typhimurium	75	4–8	nd	nd	2	2	16–32
S. marcescens	25	16–128	2–32	2–8	ng-0.125	ng	>64
P. aeruginosa	75	128–512	nd	nd	128–256	64	>512
V. vulnificus cmcP6	50–75	4–16	nd	nd	4	2–4	8
V. vulnificus DSM 10143	75	4–8	nd	nd	2	2–4	8
V. parahaemolyticus R.	25	4–8	4–>64	4	8–16	<0.125	16–32
V. parahaemolyticus A.	12.5	4–8	2–4	ng-2	8–16	ng	8–16
S. aureus 8325-4	75	8–128	nd	nd	32–64	8	256
S. epidermidis RP62A	≥75	4–16	nd	nd	8	2	4–16
S. epidermidis 1457	r	4–8	nd	nd	8	2	8
L. monocytogenes	r	8–16	nd	nd	8–16	8	8–16
B. cereus	r	2–8	nd	nd	2–4	1–2	4

Table 4
MBC of peptidomimetic 1 in MHB, and in MHB supplemented with native serum and with heat-treated serum, respectively. Nd = not determined; ng = no growth; h.t. = heat-treated.

Target strain	MBC for peptidomimetic 1 (μg × ml^{-1})					
	Added serum (%)					
	0	3	6	12.5	25	12.5% h.t.
E. coli	4–16	nd	nd	2–8	4–8	16
S. typhimurium	8–32	nd	nd	4–8	2–4	32
S. marcescens	32–128	32	2–8	ng-0.125	ng	>64
P. aeruginosa	512–>512	nd	nd	>256	256	>512
V. vulnificus cmcP6	4–16	nd	nd	8	2–8	8–16
V. vulnificus DSM 10143	4–8	nd	nd	2–4	2–4	8–16
V. parahaemolyticus R.	4–8	>64	4	8–16	ng-4	16
V. parahaemolyticus A.	4–8	2–4	<0.25–2	8	ng	16
S. aureus 8325-4	32–128	nd	nd	64	16–64	512
S. epidermidis RP62A	4–16	nd	nd	8	4	8
S. epidermidis 1457	4–16	nd	nd	16	2–4	16
L. monocytogenes	8–32	nd	nd	16–32	16–32	32
B. cereus	2–16	nd	nd	2–8	2	4–8

(SPS; Sigma Aldrich P2008-5G), an inhibitor of activation of complement function [24], was dissolved in Tris-buffered saline (1 mg/ml) and added at 50% to plasma and serum for 5 min before MIC assays. Complement protein C3-depleted and complement-deficient sera were provided by the Department for Cancer and Inflammation, University of Southern Denmark. Complement protein C3-depleted and complement-deficient sera were stored at −80 °C.

2.6. Time-kill assay of E. coli by peptidomimetic 1 in presence of human plasma

One colony of *E. coli* grown overnight on BHI agar at 37 °C was inoculated into MHB and grown overnight at 37 °C with orbital shaking at 250 rpm. The culture was diluted 10^6-fold and grown for 18 h at 37 °C at 200 rpm. Optical density was adjusted to $OD_{546} = 0.2$ in MHB to give an initial inoculum of 10^8 CFU/ml. Peptidomimetic 1 was added at concentrations of 8, 16, and 32 μg/ml to a final volume of 1 ml. Killing kinetics was also tested in the presence of 25% plasma. For this experiment, 13 ml-centrifuge tubes made of polypropylene (Almeco 91016) were used. The peptidomimetic-exposed cultures were incubated at 37 °C at 200 rpm, and bacterial density was determined by plating of a dilution series on BHI agar plates after 1, 2, 5, 7 and 24 h of exposure. The experiment was performed as biological duplicates on two separate days (each with two technical replicates). Bactericidal activity of the peptidomimetic alone was compared to the peptidomimetic in the presence of 25% plasma by Student's *t*-test on log-transformed colony counts.

2.7. Visualization of the activity of peptidomimetics by scanning electron microscopy (SEM)

Many AMPs cause damage to the bacterial cell envelope, and SEM of peptidomimetic- and plasma-treated *E. coli* was used to visualize potential cell envelope damage. *E. coli* was grown and diluted ($OD_{546} = 0.2$) as described above. Peptidomimetic 1 was added at concentrations of 8, 16 or 32 μg/ml. The influence of 25% plasma (without addition of peptidomimetic) on cell morphology was also determined. MilliQ water was added to control samples. The peptidomimetic-exposed cultures were incubated at 37 °C at 200 rpm for 2 h; cell density was determined at the start of the exposure and after 2 h. The total volume after the 2 h treatment was transferred to Eppendorf tubes and centrifuged at 20,000 × *g* for 15 min in a MiniSpin-Plus Eppendorf 48276. The supernatant was removed and the cells were fixed for 16 h at 5 °C by treatment with 3% glutaraldehyde (Sigma Aldrich, G5882) in 0.5 ml MHB, pH adjusted to 7.3. Next, samples were washed three times in distilled water followed by staining with 1% osmium tetroxide (Sigma Aldrich, 75632) overnight at 5 °C. Dehydration was done by sequential treatment with ethanol solutions (30%, 50%, 70%, 80%, 90%, 100%) and acetone solutions (30%, 50%, 100%). Samples were stored overnight at 5 °C. All washing, staining and dehydration steps were carried out by applying 0.5 ml of substrate, incubating at room temperature for 10 min followed by centrifugation at 20,000 × *g* for 5 min. After dehydration, the samples were placed on a silicon disc and further dried in a Leica CPD300 critical point drier. The silicon disc was next attached to an aluminum stub by means of a double-sided C tape and coated with 2 nm Pt in a Cressington 208HR High Resolution Sputter Coater. The experiments were performed in two independent trials. Cell length was measured from a minimum number of 20 SEM micrographs using NIST Image J on cells lying flat on the surface. The length was defined as the median line along the cell length. Cell clustering was avoided by initial adjustment of the cell number. Also, a hydrophilic substrate such as a silicon wafer was used in order to ensure that the bacterial solution was well spread. This preparation favored the measurement of length of cells lying flat on the analyzed surface.

3. Results

3.1. MIC and MBC of peptidomimetics in the presence of plasma and serum

Plasma and serum were initially used at concentrations of 25% and 12.5% based on previous studies with *E. coli* [16]. Some of the strains in the present study did not grow at these concentrations, and therefore MICs and MBCs of plasma and serum were determined. In subsequent experiments, we used plasma and serum concentrations equivalent to ¼ or ½ of the MIC of plasma or serum for each strain (Tables 1–4).

Addition of plasma lowered the MIC of peptidomimetic 1 against all strains (Table 1). The presence of 25% plasma caused a higher potentiation (it lowered the MIC even more) than addition of 12.5% plasma. The MIC value of peptidomimetic 1 was reduced at least 4-fold in the presence of 12.5% plasma as compared to that in MHB alone for *E. coli*, *S. typhimurium*, *P. aeruginosa*, *V. vulnificus* and *B. cereus*, whereas no change was observed for *S. aureus*. For *S. marcescens*, *V. parahaemolyticus*, *S. epidermidis* and *L. monocytogenes*, plasma could only be tested at concentrations lower than 12.5%, as mentioned above, but in these cases, a reduction in the MIC value was observed as well.

A reduction in the MIC value for peptidomimetic 1 in the presence of 25% plasma, as compared to that found in MHB, was observed for all strains that grew well at this concentration (*E. coli*, *P. aeruginosa*, *V. vulnificus* cmcP6, *S. aureus* and *B. cereus*). For strains that did not grow at this concentration, a reduction in MIC values was observed when determined at lower plasma concentrations.

In the presence of serum at a concentration of 12.5%, a 2-fold reduction in the MIC values was typically observed for Gram-negative strains, except for *P. aeruginosa* and *V. parahaemolyticus*. For these strains as well as for Gram-positive bacteria, the MIC of peptidomimetic 1 was similar to that found in MHB alone. When the concentration of serum was doubled, a reduction in the MIC was observed for *P. aeruginosa*, *V. parahaemolyticus* and all Gram-positive bacteria tested. For *V. vulnificus*, *E. coli* and *S. typhimurium* there was

no further reduction in the MIC compared to that found in 12.5% serum.

MBC values of peptidomimetic 1 toward most bacteria in MHB alone were slightly higher than corresponding MIC values, and relative changes in MBC values observed in the presence of serum or plasma (Tables 2 and 4) followed trends analogous to those of MIC values (Tables 1 and 3), inferring that the apparent synergism between peptidomimetic 1 and serum/plasma indeed leads to significantly more efficient killing of a wide range of pathogenic bacteria.

Similar patterns for MIC and MBC were found for peptidomimetic 2 (Supplemental Tables S1 and S2).

The effect of plasma on the activity of conventional antibiotics and LL-37 was determined for four strains. Human LL-37 was moderately active against *E. coli* and *P. aeruginosa*, with a MIC of 32–64 μg/ml and 64 μg/ml, respectively; MIC values against *S. aureus* were less consistent, ranging from 32 to higher than 512 μg/ml. LL-37 was not potentiated by plasma, but rather, displayed a 4-fold increase in its MICs against both *E. coli* and *P. aeruginosa*. For *S. aureus*, MIC values remained higher than 512 μg/ml (Table 5).

No potentiation of ampicillin in the presence of plasma was observed (Table 5). The MICs of gentamicin against *E. coli* and *P. aeruginosa* were lowered from 1 to 0.25 μg/ml and from 1 to 2 to 0.5–1 μg/ml in 25% plasma, respectively, while no significant effects on the MICs were observed in *S. aureus* and *L. monocytogenes*.

In contrast, the presence of 25% plasma caused a ~10-fold reduction in the MIC of polymyxin B against *E. coli* and *P. aeruginosa*, while a 4-fold reduction was observed in *S. aureus* (Table 5). In *L. monocytogenes*, the MIC of polymyxin B was virtually unchanged in the presence of plasma.

3.2. Potentiation of activity of peptidomimetics by human plasma and serum

MICs and MBCs of peptidomimetic 1 and polymyxin B in the presence and absence of 25% plasma were assessed using plates of different materials (Supplemental Table S4). MICs of both peptidomimetic 1 and polymyxin B were reduced both in the absence and presence of 25% plasma in the two types of polypropylene plates. However, in polystyrene plates and glass vials, the MICs were much higher, probably as a consequence of substantial binding of these cationic compounds to the negatively charged surfaces of these materials. Plasma had no or only a limited effect on the MIC of peptidomimetic 1 in these systems (polystyrene, glass) most likely as a consequence of partial depletion of active compound in the medium due to surface adsorption, which inherently becomes relatively much more pronounced at the very low concentrations tested in the potentiation experiments.

The human blood used in the experiments was supplemented with 4% citrate as an anticoagulant in order to achieve citrate plasma. As citrate can chelate divalent cations that stabilize the bacterial cell envelope, it was speculated that it might be involved in plasma potentiation. Addition of citrate to serum and to MHB at concentrations of 2–8% and 0.5–4%, respectively, had no effect on the subsequent MIC values of peptidomimetic 1 (data not shown). Hence, the potentiation of peptidomimetics was not caused by the citrate added to plasma. An increase in pH from 7.2 to 8 was measured when plasma was added to the culture. This change in pH might affect the charge towards less overall protonation of the peptidomimetic, which would be expected to lower its affinity toward the cell membrane that would be equally or slightly more anionic when pH was raised to 8. However, the MIC of the peptidomimetics in MHB adjusted to pH 8 did not differ from the MIC in MHB at pH 7.2. Hence, the pH change caused by the addition of plasma did not contribute to the increased activity of the peptidomimetics, as the bacteria were able to grow equally well under these conditions.

Plasma and serum were heat-treated to abolish the inherent activity of the complement system (Tables 1–4). As opposed to untreated plasma, an increase in the MIC value was seen for all strains when grown in the presence of 12.5% heat-treated plasma, indicating lowered sensitivity to peptidomimetics, possibly due to inactivation of these by interaction with denatured plasma components.

Addition of 12.5% heat-treated serum, as compared to untreated serum, caused no major changes in the MIC values of peptidomimetic 1 for *V. parahaemolyticus* ATCC 17802 and

Table 5
MIC and MBC of conventional antibiotics and LL-37 for bacterial pathogens in MHB, and in MHB + 25% plasma. Nd = not determined; AMP = ampicillin, GEN = gentamicin, PMB = polymyxin B.

Medium Activity	Target strain	MIC and MBC (μg × ml^{-1})			
		MHB alone/MHB + 25% plasma			
		AMP	GEN	PMB	LL-37
MIC	*E. coli*	4–8/4–8	1/0.25	0.25–0.5/<0.03	32–64/128–256
	P. aeruginosa	>128/>128	1–2/0.5–1	1/0.06	64/>256
	S. aureus	0.125/0.06	0.125–0.25/0.125–0.25	8–16/2–4	32–>512/>512
	L. monocytogenes	0.06–0.125/0.25	0.06/0.06–0.125	8–64/32	nd
MBC	*E. coli*	4–8/4–8	1/0.25	0.25–0.5/<0.06	32–64/128–256
	P. aeruginosa	>128/>128	2–>2/1	2/0.125	64/256
	S. aureus	0.125/0.125	0.5/>0.125–1	16–>16/2–>8	>512/>512
	L. monocytogenes	0.125–0.25/0.25	0.125/0.06–0.125	16–64/32	nd

for all the Gram-positive strains except for *S. aureus*. An overall increase in the MIC values was found for *S. aureus* and all the other strains tested. A similar pattern was observed for peptidomimetic 2 (Supplemental Tables S1 and S2).

Given these preliminary results, subsequent experiments were aimed at clarifying the possible involvement of complement. Thus, the effect of sodium polyanethole sulfonate (SPS) was tested, as it is a specific inhibitor of the complement system [24]. A considerable increase in the MIC values was observed when SPS was added during the MIC test. Moreover, SPS alone abolished the activity of peptidomimetics (data not shown), and therefore it cannot be used in the present experimental setup to address the possible role of complement. MIC of peptidomimetics was also determined in the presence of complement C3-depleted serum and complement factor I- and H-deficient sera, derived from clinical samples. No change in the MIC was observed compared to non-depleted or non-deficient sera (data not shown).

3.3. Time-kill assay of E. coli by peptidomimetic 1 in the presence of human plasma

Peptidomimetic 1 was bactericidal to *E. coli*, as cell counts were significantly decreased after incubation for 2–3 h in a dose-dependent manner (Fig. 2; panel A). After treatment for 2 h, cell numbers (CFU/ml) were reduced with 1.5, 2.5 and 4 log units when treated with peptidomimetic 1 at 16, 32 and 64 μg/ml, i.e. 2 × MIC to 8 × MIC, respectively. The bacteria grew well in the presence of 25% plasma in MHB, and interestingly, a more rapid killing of *E. coli* was seen for peptidomimetic 1 when 25% plasma was added to MHB (Fig. 2; panel B). The combination of 8 μg/ml peptidomimetic and 25% plasma led to a cell reduction of 1.5 log units, which is similar to using 16 μg/ml peptidomimetic in MHB alone. A similar effect was observed at 16 μg/ml peptidomimetic + 25% plasma compared to 32 μg/ml, as well as 32 μg/ml + 25% plasma compared to 64 μg/ml (Fig. 2). After 24 h (data not shown), we observed a synergistic effect in the killing of *E. coli* when 25% plasma and peptidomimetic were used in combination at concentrations up to 16 μg/ml. Regrowth was observed in all samples treated with peptidomimetics only. For the samples treated with 8 and 16 μg/ml peptidomimetics, growth was comparable to the controls, while similar samples supplemented with plasma exerted a killing effect that was maintained after 24 h. Regrowth could be explained by partial depletion of the compound due to its binding to dead bacteria. Small-colony variants were observed in peptidomimetic-treated samples, both in the presence and absence of plasma; however, this phenotype was not maintained after colonies were re-grown on new agar plates.

3.4. Visualization of cell damage caused by human plasma

Since many AMPs and peptidomimetics act directly on the bacterial cell envelope, we speculated that human plasma might also disrupt the envelope and thereby cause potentiation via an additive effect. Scanning electron microscopy revealed no cell membrane damage on *E. coli* exposed to and grown in the presence of 25% plasma (Fig. 3, B). In contrast, different degrees of damage to the cell envelope were evident for samples treated with increasing concentrations of peptidomimetic 1 (Fig. 3, C–F). Damage could be seen as the formation of blebs (Fig. 3, D), which gave rise to protrusions of the outer cell membrane (Fig. 3, E) and formation of holes in the apical part of the cells (Fig. 3, C and F). Addition of 25% plasma to the samples treated with peptidomimetics at 8 μg/ml and 16 μg/ml, respectively, did not produce further damage (Fig. 3, D and F) compared to samples treated with 16 μg/ml and 32 μg/ml (Fig. 3, C and E). However, addition of plasma seemed to affect the average length of the cells. In fact, bacteria treated with plasma appeared longer (Supplemental Fig. S1). This cell elongation was observed after treatment with plasma, but not with peptidomimetics alone. A few selected samples were observed in an optical microscope as well (data not shown). Here, cell aggregation was detected in

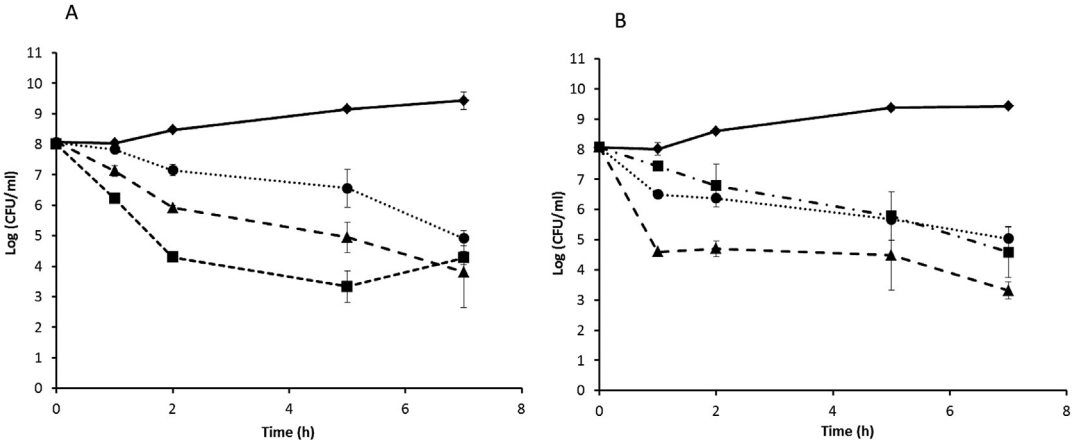


Fig. 2. Killing of *E. coli* by peptidomimetic 1 in MHB (A) and in MHB in the presence of 25% human plasma (B). Counts are averages of biological duplicates and error bars are standard deviations. 0 μg/ml: solid line; 8 μg/ml: dash dot line; 16 μg/ml: round dot line; 32 μg/ml: dash line; 64 μg/ml: square dot line. The experiment was performed in the absence and presence of plasma on the same day.

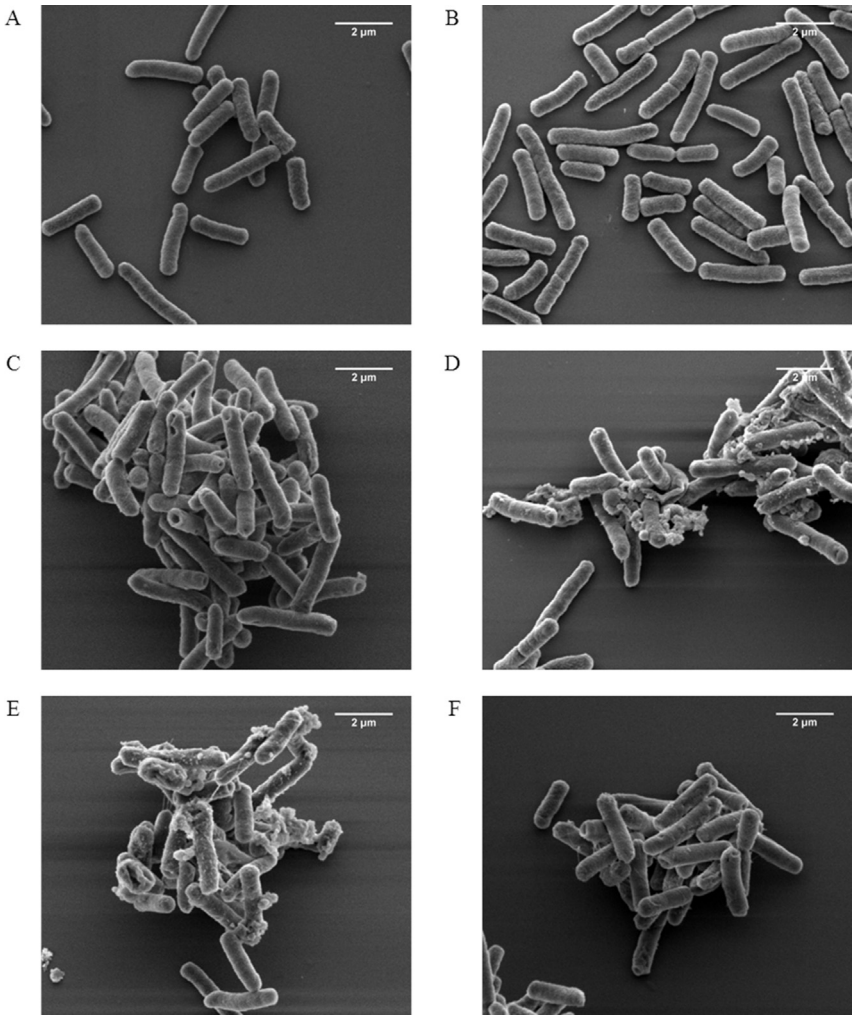


Fig. 3. Scanning electron microscopy (SEM) images of *E. coli*. (A) untreated; (B) untreated + 25% plasma; upon treatment with peptidomimetic 1: (C) 16 µg/ml, (D) 8 µg/ml + 25% plasma, (E) 32 µg/ml, (F) 16 µg/ml + 25% plasma.

specimens from samples treated with peptidomimetic plus plasma, whereas this was not observed for the controls, i.e. specimens not exposed to either compound or plasma and specimens exposed solely to plasma.

4. Discussion

Here we demonstrate that the antibacterial activity of two peptidomimetics against several Gram-negative and Gram-positive human pathogens is enhanced by the presence of human plasma and, to some extent, by the presence of human serum. From a clinical perspective, this is a promising finding, since it is likely that lower concentrations of peptidomimetics may be used in clinical settings than usually expected based on testing in laboratory growth media.

For the peptidomimetics investigated in the present study, the concentration that causes 10% hemolysis (HC_{10}) was previously found to be above 500 µg/ml [20], indicating low general cellular toxicity. In addition, peptidomimetic 1 was previously shown to exert acceptable cell selectivity between *S. epidermidis* and HeLa cells (i.e. the ratio between IC_{50} and MIC was above 200) [20] despite the fact that this cell line is more susceptible to these antibacterial peptidomimetics than other more relevant cell lines (e.g. HepG2, NIH 3T3 and HUVEC) [19]. Moreover, as shown by Jahnsen et al. [19], this type of peptidomimetics may be further optimized toward even lower cytotoxicity by partial hArg→Lys substitution. Thus, the finding that human plasma potentiates the antibacterial effect of compounds 1 and 2 further supports their potential use in vivo, as cytotoxic side effects are highly concentration-dependent and therefore will decrease significantly when the concentration needed for bacterial killing is lowered 2- to 16-fold.

The potentiation of antibacterial compounds by plasma could be caused by endogenous blood components such as complement proteins. Hein-Kristensen et al. [16] suggested that complement factors might be responsible for the plasma-enhanced activity of membrane-active AMPs and peptidomimetics against *E. coli* due to the fact that heat inactivation of complement [25] abolished this synergism, a finding corroborated by the present study in which we extended its validity to several bacterial species.

Activation of complement results in formation of the membrane attack complex (MAC) that generates pores in Gram-negative bacterial cell membranes. The role of AMPs as immune potentiators and signaling molecules is well-known, and it has recently been found that they share a number of features with complement [26]. Thus, given the membrane-perturbing effect of AMPs and antibacterial peptidomimetics, a synergistic effect between peptidomimetics and complement system appeared likely. Nevertheless, we observed no visible damage to the envelope when *E. coli* was exposed to plasma when examined by SEM. On the other hand, we found a dramatic increase in MIC in the presence of heat-inactivated plasma, supporting the assumption that one or more heat-sensitive protein(s) involved in the mechanism of plasma potentiation had suffered from denaturation.

Selected factors of the complement cascade such as factor H, factor I, and complement C3 did not seem, individually, to be the cause of potentiation based on testing of C3-depleted, factor H- and factor I-deficient sera. However, since MIC values were affected significantly more by plasma than by serum, and since both contain relevant complement proteins, future experiments should preferably involve purified complement factors and coagulation proteins.

Besides complement proteins, other factors, such as proteins of the coagulation cascade, could contribute to the effect of potentiation. This would explain why plasma gives rise to higher potentiation of peptidomimetics than serum. Unlike serum, the commercial plasma (P9523 www.sigmaaldrich.com) contains active clotting factors, which may respond to the presence of microorganisms. Activation of complement proteins and the coagulation cascade are connected [27], and complement is known to inhibit anticoagulation factors, thereby enhancing the coagulation process. Other studies [28] reported that specific coagulation factors in plasma promote the release of antimicrobial compounds directed toward the negatively charged surfaces of bacterial pathogens. From all these interconnected processes, it is inferred that higher potentiation of peptidomimetics by plasma could indeed arise from favorable interactions requiring the presence of both complement and clotting factors, given that the latter is absent in serum. However, it is also evident that only whole-blood models would mirror the cross-talk between the different systems acting in vivo [27].

Plasma enhanced the antibacterial effect of polymyxin B, but not that of gentamicin or ampicillin (Table 5). Given the membrane-perturbing effect of the peptidomimetics, as revealed by SEM and leakage of intracellular compounds [29], we conclude that the potentiation by plasma is related to the finding that the bacterial membrane appears to be the major target of these peptidomimetics. Other studies have reported that complement proteins can act in synergy with antibacterial compounds such as antibiotics [30] and AMPs [12], and for instance, the activity of polymyxin B is potentiated by serum [31]. Interestingly, it was found that terminal complement components are involved in the enhancement of the antibacterial effect of polymyxin B. In contrast, no synergistic effects between serum and β-lactams or aminoglycoside antibiotics were reported [32].

The activity of α-helical human cathelicidin LL-37 was decreased in the presence of plasma. Indeed, Wang et al. found that human plasma inhibits the antibacterial activity of LL-37, and it was proposed that LL-37 binds to a 30-kDa plasma protein [33]. LL-37 is a cationic natural human host defense peptide, and reducing its activity by binding to blood components might have evolved as a mechanism to balance its otherwise cytotoxic effects in vivo [34], whereas synthetic highly cationic non-helical and/or non-amphipathic compounds may not be bound by blood components. Also, LL-37 can be inactivated after binding to the Lpp receptor in *E. coli* [35]. This interpretation may explain the high MICs found for this AMP when determined in MHB.

The bacterial pathogens differed in their sensitivity to blood matrices. This could be attributed to differences in their propensity to interact with the bacteriolytic or bactericidal complement factors [36], since some pathogens are able to survive in serum or plasma at certain concentrations due to their natural virulence factors or resistance mechanisms. *Staphylococci* [37] and *Pseudomonas* spp. [38] are more resistant to blood than other pathogens, which is in accordance with our results, as *Pseudomonas* and *Staphylococcus* were among the strains that grew well in 25% plasma. In contrast, *Vibrio parahaemolyticus* is more sensitive to serum than *V. vulnificus* [39], as also seen in the present study for both serum and plasma.

However, since the killing efficiency of peptidomimetics was enhanced in the presence of plasma, it is suggested that: (i) the inherent resistance mechanisms may be impaired by the peptidomimetics, and/or (ii) potentiation of the peptidomimetics involves plasma components that alone do not exert a killing effect.

Electron microscopy (EM) is a valuable tool for visualizing the effects of antimicrobial peptides on the bacterial cell envelope [40], and here we demonstrate that the membrane-perturbing effects of α-peptide/β-peptoid peptidomimetics can also be documented by scanning EM (Fig. 3, C–F). Our results are consistent with previous studies where AMPs and peptoid mimics caused formation of blebs, pore formation and cell lysis [41,42]. In addition, we detected stronger occurrence of damage to the apical part of the cell, a feature that was noted earlier by Klainer and Perkins [43]. It is remarkable that cells treated with peptidomimetics in the presence of plasma often appeared longer than those treated in the absence of plasma. This elongation may well be a symptom of delayed or inhibited cell division, as noticed by Klainer and Perkins [43] when *E. coli* was treated with a sub-MIC concentration of penicillin. Chileveru et al. [44] also reported cell elongation after treatment of Gram-negative bacteria with human defensin-5, which was hypothesized to involve inhibition of cell division.

Overall, the present results show that the activity of membrane-active peptidomimetics against a range of Gram-positive and Gram-negative pathogenic bacteria is potentiated by the presence of human plasma. The potentiation effect

of plasma is dependent on the mode of action of the antibacterial compound used (as seen for the conventional antibiotics tested), since only compounds acting on the cell membrane or envelope appear to be potentiated by plasma. We hypothesize, but cannot conclude, that complement factors are involved in this potentiation. Also, we hypothesize that coagulation proteins may act in synergy with complement and increase the bactericidal effect of peptidomimetics, or vice-versa.

If future studies show that findings from such experiments may indeed be translated into animal proof-of-concept studies, a long-term perspective is that decreased dosing of certain classes of antibiotics might be applied in the treatment of infectious diseases.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgments

LC was supported by a PhD grant from the Technical University of Denmark.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.resmic.2015.10.002.

References

[1] European Centre for Disease Prevention and Control. Antimicrobial resistance surveillance in Europe 2011. Annual report of the European antimicrobial resistance surveillance network (EARSNet). Stockholm: ECDC; 2012.
[2] Rasko DA, Sperandio V. Anti-virulence strategies to combat bacteria-mediated disease. Nat Rev Drug Discov 2010;9(2):117–28.
[3] Zasloff M. Antimicrobial peptides of multicellular organisms. Nature 2002;415:389–95.
[4] Hancock REW, Sahl HG. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat Biotechnol 2006;24(12):1551–7.
[5] Ruiz J, Calderon J, Rondon-Villarreal P, Torres R. Analysis of structure and hemolytic activity relationships of antimicrobial peptides (AMPs). In: Castillo LF, editor. Advances in computational biology; advances in intelligent systems and computing, 232. Springer International Publishing Switzerland; 2014. p. 253–8.
[6] Chongsiriwatana NP, Patch JA, Czyzewski AM, Dohm MT, Ivankin A, Gidalevitz D, et al. Peptoids that mimic the structure, function, and mechanism of helical antimicrobial peptides. Proc Natl Acad Sci U S A 2008;105:2794–9.
[7] Jahnsen RD, Frimodt-Møller N, Franzyk H. Antimicrobial activity of peptidomimetics against multidrug-resistant *Escherichia coli*: a comparative study of different backbones. J Med Chem 2012;55:7253–61.
[8] Olsen CA, Ziegler HL, Nielsen HM, Frimodt-Møller N, Jaroszewski JW, Franzyk H. Antimicrobial, hemolytic and cytotoxic activities of β-peptoid-peptide hybrid oligomers: improved properties compared to natural AMPs. ChemBioChem 2010;11:1356–60.
[9] Otvos L, Wade JD. Current challenges in peptide-based drug discovery. Front Chem 2014;2:8–11.
[10] Deslouches B, Islam K, Craigo JK, Paranjape SM, Montelaro RC, Mietzner TA. Activity of the de novo engineered antimicrobial peptide WLBU2 against *Pseudomonas aeruginosa* in human serum and whole blood: implications for systemic applications. Antimicrob Agents Chemother 2005;49(8):3208–16.
[11] Knappe D, Henklein P, Hoffmann R, Hilpert K. Easy strategy to protect antimicrobial peptides from fast degradation in serum. Antimicrob Agents Chemother 2010;54(9):4003–5.
[12] Yeaman MR, Gank KD, Bayer AS, Brass EP. Synthetic peptides that exert antimicrobial activities in whole blood and blood-derived matrices. Antimicrob Agents Chemother 2002;46:3883–91.
[13] Godballe T, Nilsson LL, Petersen PD, Jenssen H. Antimicrobial β-peptides and α-peptoids. Chem Biol Drug Des 2011;77:107–16.
[14] Rotem S, Mor A. Antimicrobial peptide mimics for improved therapeutic properties. BBA Biomembranes 2009;1788:1582–92.
[15] Brogden NK, Brogden KA. Will new generations of modified antimicrobial peptides improve their potential as pharmaceuticals? Int J Antimicrob Agents 2011;38:217–25.
[16] Hein-Kristensen L, Knapp KM, Franzyk H, Gram L. Selectivity in the potentiation of antibacterial activity of α-peptide/β-peptoid peptidomimetics and antimicrobial peptides by human blood plasma. Res Microbiol 2013;164(9):933–40.
[17] EUCAST, E. C. for A. susceptibility T. of the E. S. of C. M. and I. D. (ESCMID). Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. 2003. p. 1–7. EUCAST Discussion Document E.Dis 5.1.
[18] Bonke G, Vedel L, Witt M, Jaroszewski J, Olsen C, Franzyk H. Dimeric building blocks for solid-phase synthesis of α-peptide-β-peptoid chimeras. Synthesis 2008;15:2381–90.
[19] Jahnsen RD, Sandberg-Schaal A, Vissing KJ, Nielsen HM, Frimodt-Møller N, Franzyk H. Tailoring cytotoxicity of antimicrobial peptidomimetics with high activity against multidrug-resistant *Escherichia coli*. J Med Chem 2014;57:2864–73.
[20] Liu Y, Knapp KM, Yang L, Molin S, Franzyk H, Folkesson A. High *in vitro* antimicrobial activity of β-peptoid-peptide hybrid oligomers against planktonic and biofilm cultures of *Staphylococcus epidermidis*. Int J Antimicrob Agents 2013;41(1):20–7.
[21] The Clinical and Laboratory Standards Institute (CLSI). Guideline M7–a9: methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, approved standard eight edition. 2009.
[22] Recently modified methods used by the Hancock Laboratory. 1998-2000. http://cmdr.ubc.ca/bobh/methods/MODIFIEDMIC.html.
[23] Hazen E. Effect of temperature of inactivation of human, rabbit and guinea-pig serum upon the haemolytic activity of complement. J Immunol 1943;46:341–6.
[24] Palarasah Y, Skjoedt MO, Vitved L, Andersen TE, Skjoedt K, Koch C. Sodium polyanethole sulfonate as an inhibitor of activation of complement function in blood culture systems. J Clin Microbiol 2010;48(3):908–14.
[25] Noguchi H. The thermostable anticomplementary constituents of the blood. J Exp Med 1906;8:726–53.
[26] Zimmer J, Hobkirk J, Mohamed F, Browning MJ, Stover CM. On the functional overlap between complement and anti-microbial peptides. Front Immunol 2015;5:1–10.
[27] Markiewski MM, Nilsson B, Ekdahl KN, Mollnes TE, Lambris JD. Complement and coagulation: strangers or partners in crime? TRENDS Immunol 2007;28(4):184–92.
[28] Frick IM, Akesson P, Herwald H, Mörgelin M, Malmsten M, Nägler DK, et al. The contact system-a novel branch of innate immunity generating antibacterial peptides. EMBO J 2006;25(23):5569–78.
[29] Hein-Kristensen L, Knapp KM, Franzyk H, Gram L. 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 Microbiol 2011;11(1):144.
[30] Dutcher BS, Reynard AM, Beck ME, Cunningham RK. Potentiation of antibiotic bactericidal activity by normal human serum. Antimicrob Agents Chemother 1978;13:820–6.
[31] Pruul H, Reynolds BL. Interaction of complement and polymyxin with Gram-negative bacteria. Infect Immun 1972;6:709–17.

[32] Fierer J, Finley F. Lethal effect of complement and lysozyme on polymyxin-treated, serum-resistant Gram-negative bacilli. J Infect Dis 1979;140:581−9.
[33] Wang Y, Agerberth B, Johansson J. Structure and activity of cathelicidin antibacterial proteins. J Protein Chem 1998;17(6):522−3.
[34] Panyutich A, Ganz T. Activated alpha 2-macroglobulin is a principal defensin-binding protein. Am J Respir Cell Mol Biol 1991;5:101−6.
[35] Chang TW, Lin YM, Wang CF, Liao YD. Outer membrane lipoprotein Lpp is Gram-negative bacterial cell surface receptor for cationic antimicrobial peptides. J Biol Chem 2011;287:418−28.
[36] Taylor PW. Bactericidal and bacteriolytic activity of serum against Gram-negative bacteria. Microbiol Rev 1983;47:46−83.
[37] Serruto D, Rappuoli R, Scarselli M, Gros P, Van Strijp JAG. Molecular mechanisms of complement evasion: learning from staphylococci and meningococci. Nat Rev Microbiol 2010;8:393−9.
[38] Lambris JD, Ricklin D, Geisbrecht BV. Complement evasion by human pathogens. Nat Rev Microbiol 2008;6(2):132−42.
[39] Carruthers MM, Kabat WJ. *Vibrio vulnificus* (lactose-positive vibrio) and *Vibrio parahaemolyticus* differ in their susceptibilities to human serum. Infect Immun 1981;32(2):964−6.
[40] Hancock REW, Rozek A. Role of membranes in the activities of antimicrobial cationic peptides. FEMS Microbiol Lett 2002;206:143−9.
[41] Hartmann M, Berditsch M, Hawecker J, Ardakani MF, Gerthsen D, Ulrich AS. Damage of the bacterial cell envelope by antimicrobial peptides gramicidin S and PGLa as revealed by transmission and scanning electron microscopy. Antimicrob Agents Chemother 2010;54(8):3132−42.
[42] Jahnsen RD, Haney EF, Franzyk H, Hancock REW. Characterization of a proteolytically stable multifunctional host defense peptidomimetic. Chem. Biol. 2013;20:1286−95.
[43] Klainer AS, Perkins RL. Surface manifestations of antibiotic-induced alterations in protein synthesis in bacterial cells. Antimicrob Agents Chemother 1972;1(2):164−70.
[44] Chileveru HR, Lim SA, Chairatana P, Wommack AJ, Chiang IL, Nolan EM. Visualizing attack of *Escherichia coli* by the antimicrobial peptide human defensin 5. Biochem 2015;54:1767−77.

Supplemental Table S1: MIC of peptidomimetic 2 in MHB, MHB supplemented with native serum or plasma as well as with heat-treated serum or plasma. Nd = not determined; ng= no growth.

Target strain	MIC for peptidomimetic 2 (µg × ml^{-1})										
	MHB	Added serum (%)				Added plasma (%)				12.5% heat-treated	
	Control	3	6	12.5	25	3	6	12.5	25	serum	plasma
E. coli	4-8	nd	nd	4-8	4-8	nd	nd	1-2	0.5-1	16-32	16->64
S. typhimurium	8-16	nd	nd	8-16	8-16	nd	4	2-8	ng	64-128	16->64
S. marcescens	16-512	4-64	1-4	ng-0.125	ng	16-32	ng	ng-4	ng	>64	>512
P. aeruginosa	128-512	nd	nd	128-256	128-256	nd	nd	16-128	16-32	>512	>512
V. vulnificus cmcP6	4-16	nd	nd	4-8	4	nd	nd	2-4	0.5-1	4-16	8->64
V vulnificus DSM 10143	4-16	nd	nd	2-4	4-8	nd	nd	1-4	ng	16	16
V. parahaemolyticus R.	4-8	4	8-16	8-16	ng-<0.125	4	2-4	ng	ng	32-64	>128
V. parahaemolyticus A.	4-8	1-4	ng	ng	ng	ng-2	ng	ng	ng	32	>64
S. aureus	8-64	nd	nd	8->32	4	nd	nd	4-8	2	64-128	>64->512
S. epidermidis RP62A	4-8	nd	nd	4	2	nd	0.25-2	0.5	ng-<0.25	8-16	16->64
S. epidermidis 1457	2-8	nd	nd	4	2	0.5-1	0.5-1	nd	ng	8	>128
L. monocytogenes	2-8	nd	nd	8	8	2-4	nd	ng	ng	8-16	8-16
B. cereus	2-8	nd	nd	4-8	2-4	nd	nd	1-2	1	8-16	8

Supplemental Table S2: MBC of peptidomimetic 2 in MHB, MHB supplemented with native serum or plasma as well as with heat-treated serum or plasma. Nd = not determined; ng= no growth.

Target strain	MBC for peptidomimetic 2 (µg × ml^{-1})										
	MHB	Added serum (%)				Added plasma (%)				12.5% heat-treated	
	Control	3	6	12.5	25	3	6	12.5	25	serum	plasma
E. coli	4-16	nd	nd	4-16	4-8	nd	nd	2-16	0.5-8	32	32->64
S. typhimurium	8-16	nd	nd	4-32	16	nd	4-8	0.5-8	0.25-ng	64	>64
S. marcescens	16-128	64	4	ng-0.125	ng	16-32	ng	ng	ng	>64	64
P. aeruginosa	128->512	nd	nd	>256	>512	nd	nd	512	>32-64	>512	>512
V. vulnificus cmcP6	8-32	nd	nd	8-16	4-8	nd	nd	4	0.5-2	8-16	16->64
V vulnificus DSM 10143	4-16	nd	nd	2-4	8	nd	nd	1	ng-0.125	16	16
V. parahaemolyticus R.	8-16	4	8-16	16	ng-4	4	2-4	nd	ng	32	>64
V. parahaemolyticus A.	4-8	4	ng	ng	ng	2	ng	ng	ng	32-64	>64
S. aureus	8-128	nd	nd	16->32	8-16	nd	nd	16	4-8	128	>64->512
S. epidermidis RP62A	4-8	nd	nd	4	2-4	nd	0.5-4	1	>0.25	16	>64
S. epidermidis 1457	4-8	nd	nd	4-8	2-4	1	1	nd	nd	8	>128
L. monocytogenes	2-8	nd	nd	16	8-16	4	nd	nd	nd	32	16
B. cereus	4-8	nd	nd	4-16	2-4	nd	nd	1-2	1	8-16	4

Supplemental Table S3: Bacterial strains used in the present study and relative reference.

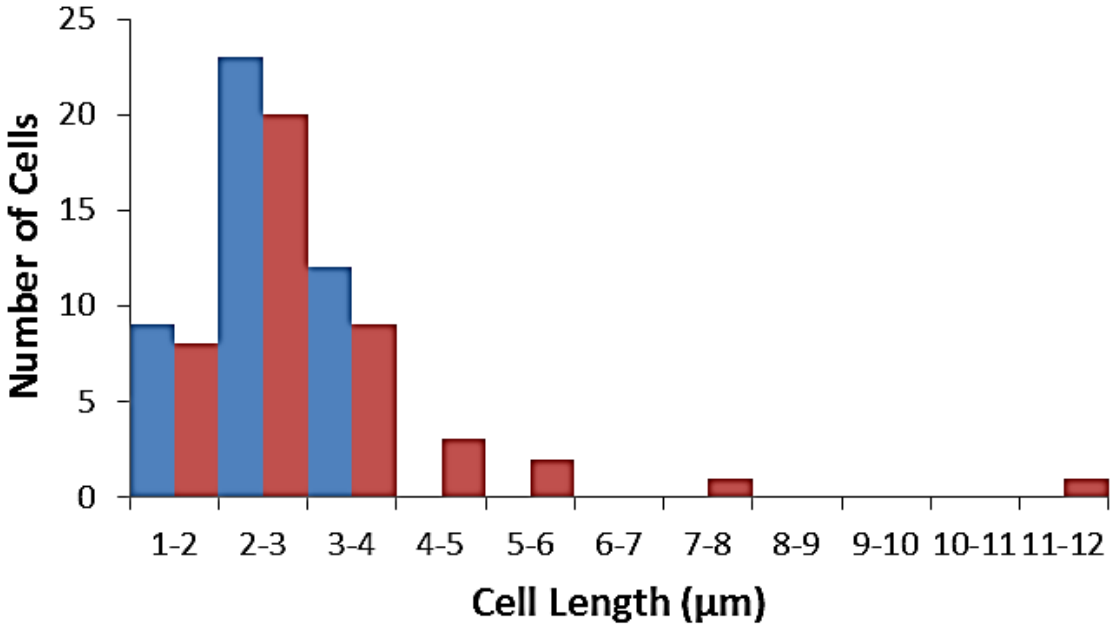
Strain name	Reference
Escherichia coli ATCC 25922	-
Salmonella typhimurium L354	Wray C. 1978. Experimental *Salmonella* Typhimurium infection in calves. Res Vet Sci 25(2):139-143.
Serratia marcescens	Hejazi A, Falkiner FR. 1997. *Serratia marcescens* J Med Microbiol 46(11):903-912.
Vibrio vulnificus DSM 10143	Reichelt JL, Baumann P, Baumann L. 1976. Study of genetic relationships among marine species of the genera *Beneckea* and *Photobacterium* by means of in vitro DNA/DNA hybridization. Arch Microbiol 110(1):101-120.
Vibrio vulnificus cmcP6	Kim YR, Lee SE, Kim CM, Kim SY, Shin EK, Shin DH, Chung SS, Choy HE, Progulske-Fox A, Hillman JD, Handfield M, Rhee JH. 2003. Characterization and pathogenic significance of *Vibrio vulnificus* antigens preferentially expressed in septicemic patients. Infect Immun 71(10):5461-5471.
Vibrio parahaemolyticus ATCC 17802	-
Vibrio parahaemolyticus RimD D2210633	Nasu H. 2000. A filamentous phage associated with recent pandemic *Vibrio parahaemolyticus* O3 : K6 strains. J Clin Microbiol 38(6):2156-2161.

Pseudomonas aeruginosa PAO1	Holloway B. 1955. Genetic recombination in *Pseudomonas aeruginosa*. J Gen Microbiol 13(3):572-581.
Listeria monocytogenes EGDe BUG 1600	Glaser P, Frangeul L, Buchrieser C, Rusniok C, Amend A, Baquero F, et al., Cossart P. 2001. Comparative genomics of *Listeria* species. Science 294(5543):849-52.
Bacillus cereus ATCC 11778	-
Staphylococcus aureus 8325	Novick R. 1967. Properties of a cryptic high-frequency transducing phage in *Staphylococcus aureus.* Virology 33(1):155-166.
Staphylococcus epidermidis RP62A	Christensen GD, Simpson WA, Bisno AL, Beachey EH. 1982. Adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth surfaces. Infect Immun 37(1):318-326.
Staphylococcus epidermidis 1457	Rupp ME, Ulphani JS, Fey PD, Mack D. 1999. Characterization of *Staphylococcus epidermidis* polysaccharide intercellular adhesin/hemagglutinin in the pathogenesis of intravascular catheter-associated infection in a rat model. Infect Immun 67(5):2656-2659.

Supplemental Table S4: MIC and MBC of peptidomimetic 1 (1) and polymyxin B (PMB) against *E. coli* in absence and presence of 25% plasma. MIC was assessed in the listed materials. Polypropylene[a]: plates sterilized by autoclave; polypropylene[b]: UV-sterilized plates. The experiments were run on the same day in two independent replicates.

Material	**MIC/MBC (µg/ml)**			
	1	**1 + 25% plasma**	**PMB**	**PMB + 25% plasma**
Polypropylene[a]	4-8 / 4-16	0.5-1 / 2-4	0.5 / 0.5	0.007-0.03 / 0.007-0.125
Polypropylene[b]	2-4 / 4-8	0.125-0.25 / 0.5-1	0.5-1 / 0.5-1	0.007-0.015 / 0.007-0.06
Polystyrene	16 / 16	4-8 / 16	2-4 / 2-4	>0.5-0.5 / >0.5-0.5
Glass	16 / 64	8-16 / >32	1 / 1	0.5 / 0.5-1

Supplemental Figure S1: Number of cells with relative length for MHB-treated sample (blue bars) and sample treated with MHB + 25% plasma (red bars). For each sample 44 cells were measured.



Adaptive Laboratory Evolution of E. coli Reveals Slow Resistance Development to the Short Antimicrobial Peptide P9-4 and to a Combination of three Antimicrobial Compounds

Linda Citterio[1], Henrik Franzyk[2], Hanne Mørck Nielsen[2], Lone Gram[1*]

[1]Technical University of Denmark, Denmark, [2]University of Copenhagen, Denmark

Submitted to Journal:
Frontiers in Microbiology

Specialty Section:
Evolutionary and Genomic Microbiology

Article type:
Original Research Article

Manuscript ID:
227051

Received on:
26 Aug 2016

Revised on:
10 Feb 2017

Frontiers website link:
www.frontiersin.org

Conflict of interest statement

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

Author contribution statement

LC, HF and LG designed the experiments. HMN provided guidance and facilities for the Zeta potential experiment. LC planned and carried out the experiments and drafted the manuscript. LG reviewed the manuscript. HF synthesized the compounds and took part in the revision of the manuscript. All authors have reviewed and approved the final manuscript.

Keywords

antimicrobial peptides, Peptidomimetics, Adaptive laboratory evolution, Resistance, E. coli

Abstract

Word count: 321

Antimicrobial peptides (AMPs) have for long been considered as potential new antimicrobials since resistance appears not to evolve readily in nature. However, adaptive laboratory evolution experiments (ALE) have demonstrated that bacteria may develop resistance also to AMPs. We, as others, hypothesize that the risk of resistance development decreases when two or more compounds are combined as compared to single-drug treatment. The purpose of the study was to investigate resistance development in E. coli ATCC 25922 when exposed to a combination of three compounds and to each of the compounds separately (peptidomimetic H-[Lys-βNSpe-hArg-βNSpe]3-NH2 and the AMPs novicidin and P9-4).

Surprisingly, none of the lineages exposed to P9-4 adapted to a 32-fold higher MIC, while all lineages exposed to H-[Lys-βNSpe-hArg-βNSpe]3-NH2 and three out of four lineages exposed to novicidin adapted to a 32-fold higher MIC than the wild type MIC, after passaging through approx. 350 generations. Only one out of four lineages exposed to the combination reached a 32-fold higher MIC of 256 µg/ml. The whole genomes of adapted lineages and individual clones were sequenced and analyzed, and an average of 6 single-nucleotide variants causing amino acid change were detected in the peptide-adapted lineages, while an average of 5 single-nucleotide variants were found in the control lineages. The most common variants in adapted lineages (and derived clones) were deletions in the gene encoding the enzyme CDP-glycerophosphotransferase, present in six out of eight adapted lineages and in eleven clones. The zeta potential of adapted lineages was less negative than that of the wild type, suggesting that a contributing factor to the gained resistance involve modification of the surface charge.

The results indicate that a common evolutionary trajectory has led to development of resistance both to the individual compounds and to a combination of all three compounds. The short antimicrobial peptide P9-4, giving rise to a slow resistance development rate, may be considered a promising candidate for further optimization and future application in clinical settings.

Funding statement

LC was supported by a PhD scholarship from the Technical University of Denmark.

Ethics statements

(Authors are required to state the ethical considerations of their study in the manuscript, including for cases where the study was exempt from ethical approval procedures)

Does the study presented in the manuscript involve human or animal subjects: No

Adaptive Laboratory Evolution of *E. coli* Reveals Slow Resistance Development to the Short Antimicrobial Peptide P9-4 and to a Combination of three Antimicrobial Compounds

Linda Citterio[1], Henrik Franzyk[2], Hanne Mørck Nielsen[3], Lone Gram[1*]

[1] Department of Bioengineering, Technical University of Denmark, Kgs. Lyngby, Denmark

[2] Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

[3] Department of Pharmacy, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

***Correspondence:**
Lone Gram
gram@bio.dtu.dk

Keywords: antimicrobial peptides, peptidomimetics, adaptive laboratory evolution, resistance, *E. coli*

Abstract

Antimicrobial peptides (AMPs) have for long been considered as potential new antimicrobials since resistance appears not to evolve readily in nature. However, adaptive laboratory evolution experiments (ALE) have demonstrated that bacteria may develop resistance also to AMPs. We, as others, hypothesize that the risk of resistance development decreases when two or more compounds are combined as compared to single-drug treatment. The purpose of the study was to investigate resistance development in *E. coli* ATCC 25922 when exposed to a combination of three compounds and to each of the compounds separately (peptidomimetic H-[Lys-βNSpe-hArg-βNSpe]$_3$-NH_2 and the AMPs novicidin and P9-4).

Surprisingly, none of the lineages exposed to P9-4 adapted to a 32-fold higher MIC, while all lineages exposed to H-[Lys-βNSpe-hArg-βNSpe]$_3$-NH_2 and three out of four lineages exposed to novicidin adapted to a 32-fold higher MIC than the wild type MIC, after passaging through approx. 350 generations. Only one out of four lineages exposed to the combination reached a 32-fold higher MIC of 256 µg/ml. The whole genomes of adapted lineages and individual clones were sequenced and analyzed, and an average of 6 single-nucleotide variants causing amino acid change were detected in the peptide-adapted lineages, while an average of 5 single-nucleotide variants were found in the control lineages. The most common variants in adapted lineages (and derived clones) were deletions in the gene encoding the enzyme CDP-glycerophosphotransferase, present in six out of eight adapted lineages and in eleven clones. The zeta potential of adapted lineages was less negative than that of the wild type, suggesting that a contributing factor to the gained resistance involve modification of the surface charge.

The results indicate that a common evolutionary trajectory has led to development of resistance both to the individual compounds and to a combination of all three compounds. The short antimicrobial peptide P9-4, giving rise to a slow resistance development rate, may be considered a promising candidate for further optimization and future application in clinical settings.

1 Introduction

Development of bacterial resistance to conventional antibiotics is a global concern (Fair and Tor, 2014), and it has recently been highlighted as one of the major challenges facing mankind (WHO Report, 2014). Consequently, there is an urgent need for exploration of new antibacterial compounds as well as more effective regimens for use of both existing and novel antibiotics. Antimicrobial peptides (AMPs) have been suggested as such novel antibacterial agents (Hancock and Sahl, 2006), despite common limitations such as cytotoxic side effects or sensitivity to proteolytic degradation (Chongsiriwatana et al., 2008). Peptidomimetics mimicking the biological activity of AMPs have been investigated, and some subclasses have proved to overcome these shortcomings of AMPs (Jahnsen et al., 2012).

AMPs have evolved to constitute an essential part of the antimicrobial defense system of most living organisms, and AMP resistance in natural innate immune system settings has not been reported (Malmsten, 2014). AMPs typically act as detergent-like compounds causing either pore formation or disruption of the cell envelope (Brogden, 2005), and it has been stated that resistance to AMPs will not develop as readily as it develops toward conventional antibiotics (Marr et al., 2006). Also, it is believed that development of resistance to AMPs is impeded by their rapid bactericidal activity (Fox, 2013).

However, since AMPs target conserved structures in the bacterial cell envelope, bacterial evolution have resulted in systems that circumvent their action, i.e. a parallel evolutionary race exists between pathogens and their hosts (Peschel and Sahl, 2006). Several authors (Devine and Hancock, 2002) (Nizet, 2006; Maria-Neto et al., 2015) have described a series of bacterial mechanisms of intrinsic resistance against AMPs. Also, for some compounds, resistant mutants are selected for when bacteria are exposed to sub-lethal concentrations (Cassone and Otvos, 2010) or when allowed to adapt over longer periods to AMPs (Perron et al., 2006) or peptidomimetics (Hein-Kristensen et al., 2013). Mutations leading to cell membrane modifications are likely to confer resistance to AMPs; however, the exact mechanisms of resistance to AMPs are not fully understood (Maria-Neto et al., 2015). Whole-genome sequencing of the end-point resistant lineages selected for may provide a more detailed insight into the molecular mechanisms underlying resistance (Köser et al., 2014). The pattern of mutation events may thus clarify whether resistance development involves a particular evolutionary trajectory (de Visser and Krug, 2014). Also, the understanding of resistance mechanisms is essential for discovery of potential targets useful for treatment of drug-resistant bacteria (Punina et al., 2015) (Cole and Nizet, 2016).

Most studies on AMPs and peptidomimetics have focused on their spectrum of activity, cost of production, the dose needed, and their efficacy in clinical trials (Brogden and Brogden, 2011). However, assessing the potential for resistance development is an important part of the evaluation of novel antibacterial compounds. All antimicrobials must be used in ways that optimize their effect at the lowest possible concentration and reduce the risk of resistance development. One strategy is to apply combination therapy that may provide an enhanced antibacterial effect *in vitro* via

synergistic effects (Zhuang et al., 2015; Galani et al., 2014). Thus, by combining drugs, the dose of each compound may be reduced whereby potential toxicity *in vivo* may be abolished (Pirrone et al., 2011). Further, a combination of several antibiotics may impose additional evolutionary restraints on the bacterial population (Lindsey et al., 2013) thereby suppressing the viability of mutants that otherwise might overcome the mode of action of the individual drugs.

Combination treatment has been successfully applied for HIV treatment (Lennox et al., 2009), malaria (Nyunt and Plowe, 2007), tuberculosis (Ortona et al., 1998), and it is used for treatment of severe infections with carbapenemase-producing Enterobacteriaceae (Qureshi et al., 2012). Several *in vitro* studies have shown the potential of combination treatment based on AMPs (Chongsiriwatana et al., 2011; Yu et al., 2016) and AMPs together with conventional antibiotics (Hindler et al., 2015). In fact, AMPs have the advantage of acting in synergy in the natural host environment (Cassone and Otovs, 2010).

AMPs act mainly by binding to different macromolecules in the bacterial cell membrane, and therefore typically have multiple low-affinity targets in contrast to conventional antibiotics. When the bacterial cell is targeted in a multi-mode way, resistance development is slowed down due to increased cost of fitness for the induced mutations. Hence, simultaneous alteration of several targets would appear less probable than it would be for single-target molecules (Sallum and Chen, 2008). In a combination approach, such multiple mutations would be of less probability due to an even higher number of potential target molecules.

The purpose of the present study was to assess the risk of resistance development to two AMPs and a peptidomimetic. We also addressed the hypothesis that resistance to a cocktail of compounds would develop slower than against each compound alone.

2 Material and methods

2.1 Bacterial strain and culture conditions.

Escherichia coli ATCC 25922 was grown in cation-adjusted Müller Hinton II broth (MHB) (Becton Dickinson 212322) adjusted to pH 7.4 and supplemented with 1.5% agar (Oxoid, CM0471) for culturing on solid medium. Stock cultures of this strain and resulting adapted lineages were stored at –80 ^{0}C in 25% glycerol.

2.2 Synthesis of peptidomimetics and AMPs.

The potential development of resistance was tested by using three compounds: the peptidomimetic H-[Lys-βNSpe-hArg-βNSpe]$_3$-NH_2 that displays an unnatural backbone with some degree of a non-helical secondary structure (Jahnsen et al., 2014; Figure 1), the AMP novicidin (KNLRRIIRKGIHIIKKYF-NH_2), a typical α-helical arginine/lysine-rich peptide (Nielsen and Otzen, 2010), and the AMP P9-4 (KWRRWIRWL) that most likely does not form a secondary structure due to its short length (Qi et al., 2010). H-[Lys-βNSpe-hArg-βNSpe]$_3$-NH_2, novicidin and P9-4 were used in the adaptation and were abbreviated as "1", "2", "3", respectively. Compounds used for assessment of cross resistance were the following: 5 (Ac-[hArg-βNSce-Lys-βNSpe]$_3$-NH_2), 6 (Ac-[Lys-βNphe]$_8$-NH_2), 7 (Lau-[Lys-βNphe]$_6$-NH_2) and 8 (Ac-[hArg-βNSpe]$_6$-NH_2) as shown in Figure 1.

All the compounds were prepared by solid-phase synthesis as previously described (Bonke et al., 2008; Olsen et al., 2010). The resulting lyophilized peptidomimetics and AMPs were dissolved in 10 mg/ml sterile MilliQ water and stored at –20 ^{0}C.

2.3. Determination of Minimum Inhibitory Concentration (MIC).

MIC of each compound alone, combinations of each of two compounds and of all three was determined. When testing mixtures of two compounds, each was included at 50% and when testing the combination of three, each was included at 33% of the total (Table 1). MIC was determined as described in (Citterio et al., 2016) in accordance with the CLSI guidelines (2012). Microdilution series of the compounds were prepared in UV-sterilized 96-well polypropylene plates (Thermo Scientific Nunc, 267334) with sterile polystyrene lids (Sigma Aldrich, CLS3930-100EA). Stock solutions of peptidomimetics and AMPs were prepared in MilliQ water. Fifty µl of compound solution and 50 µl of MHB were dispensed into the first well of each row, followed by two-fold dilutions giving a final concentration range of 128-0.25 µg/ml. Overnight culture of the strain was diluted in sterile 0.9% NaCl to OD_{546} 0.2 (accepted range: 0.195-0.210) corresponding to approximately 1×10^8 CFU/ml. This suspension was further diluted 1:100 in MHB and 50 µl of this culture were inoculated into microtiter plates to reach a final concentration of 5×10^5 CFU/ml in a final volume of 100 µl. Plates were incubated for 20 h at 37 °C. Growth was determined visually either as turbidity or as formation of a pellet. MIC was the lowest concentration of peptidomimetics or AMPs where no growth was observed. All MIC assays were performed as two independent replicates.

2.4 Adaptive Laboratory Evolution (ALE).

Adaptive laboratory evolution of *E. coli* was carried out as previously described (Perron et al., 2006) in presence of each compound alone and in a combination of all three compounds. An overnight culture of the strain was grown on MHB agar at 37 °C. A single colony was inoculated in liquid MHB and grown overnight at 37 °C at 250 rpm on orbital shaker. The initial culture was adjusted to OD_{546} = 0.2 and 10 µl were inoculated into 990 µl MHB giving 1 ml of final volume. Cultures were grown in Sterilin tubes (VWR, 212-7400) at 37 °C at 250 rpm. Throughout the experiment serial transfers of 10 µl of bacterial culture to fresh medium were performed when growth was visible as turbidity. After five transfers in un-supplemented medium, four different peptide treatments were tested: H-[Lys-βNSpe-hArg-βNSpe]$_3$-NH_2, novicidin, P9-4, and the combination of the three (mixed 1:1:1). Four replicates (lineages) were prepared for each treatment, with the addition of four un-supplemented cultures (i.e. non-exposed lineages), giving a total of 20 parallel lineages. As a simplification, the four lineages adapted to H-[Lys-βNSpe-hArg-βNSpe]$_3$-NH_2 (1) were referred to as "1a", 1b", "1c" and "1d" through the course of the manuscript. Lineages adapted to novicidin (2) were referred to as "2a", 2b", "2c" and "2d", lineages adapted to P9-4 (3) were indicated as "3a", 3b", "3c" and "3d", and lineages adapted to the combination of three were designated "4a", 4b", "4c" and "4d". Among non-exposed lineages, we selected lineages "0a" and "0b", that were transferred until the adaptation experiment was ended, and lineages "0c" and "0d" that were transferred until the first adaptation to 32-fold of the initial MIC was completed. Adaptation started from 1/16 of the wild type MIC (i.e. 0.25 µg/ml for 1a, 1b, 1c and 1d; 0.5 µg/ml for lineages 2a, 2b, 2c and 2d and 3a, 3b, 3c and 3d; and 0.5 µg/ml for 4a, 4b, 4c and 4d). Five transfers at constant concentration were performed. Then the concentration was doubled and the

experiment continued until a level of 32-fold higher MIC than wild type MIC was reached for most of the lineages. After each adaptation step, frozen stocks of the adapted culture were prepared.

Purity of the lineages was checked at each transfer by streaking on MHB agar plate. The total number of passages in presence of compound was 50, equivalent to approximately 350 generations. Re-inoculations were performed twice a day during the sub-MIC treatment and then reduced to once per day when growth became slower, as the concentration of compound was increased. In case no growth was observed the next day, the tube was left for another day. If growth still did not occur, the re-inoculation step was repeated in the same concentration as used in the previously grown culture. If still no growth was seen, concentration was halved. End of a lineage was considered to be reached when no growth was observed upon five consecutive re-inoculations.

To determine stability of the resistance, MIC was determined for each adapted lineage. Overnight cultures for this assay were prepared in presence of compound at the concentration each lineage was adapted to. For lineages that did not grow overnight under these conditions the compound concentration was decreased. Furthermore, the adapted lineages were re-inoculated five times in un-supplemented medium, and MIC was determined for all of them. To assess homogeneity of the lineage community, the lineages were streaked on agar plates, and then five individual clones (colonies) were isolated after overnight growth on solid medium. Clones were enumerated from 1 to 5. As an example, clone 1 isolated from the novicidin-adapted lineage "a" was indicated as 2a-1. MIC was also determined for all the clones.

2.5 Whole-genome sequencing and variant calling.

Lineages for whole-genome sequencing were chosen based on the results from the MIC assay performed on the adapted cultures and the respective clones. Two replicates of two control lineages and four adapted lineages were selected, giving a number of 12 genomes. Also, two out of five individual clones were considered for each replicate, giving a number of 24 genomes. Of the control lineages, half was derived from un-supplemented cultures that were transferred until the first adaptation to a 32-fold higher MIC had occurred; the other half was derived from un-supplemented cultures that were serially transferred until the experiment was completed. The wild type ancestral strain (*E. coli* ATCC 25922) was sequenced as well. Hence, a total number of 37 lineages and clones were whole-genome sequenced.

An overnight culture of *E. coli* (1.5 ml) was grown to stationary phase and genomic DNA was extracted with the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany). Quality of extracted DNA was assessed by 1% agarose gel electrophoresis and absorbance measurement at DeNovix DS-11 Spectrophotometer. Quantification was done by Qubit 2.0 Fluorometer (Invitrogen, United Kingdom). Preparation of genomic DNA libraries and sequencing was performed at the Novo Nordisk Foundation Center for Biosustainability (Hørsholm, Denmark). Libraries were prepared with TruSeq Nano HT (Illumina, San Diego, USA) and fragmented to an average size of 350-400 bp with CovarisE220. Average library size after adapter ligation was 500-550 bp, determined by Fragment Analyzer and Standard Sensitivity NGS kit. Concentrations of the final libraries were measured by Qubit, dsDNA broad range assay. Libraries were used for paired-end sequencing of genomes using the Illumina sequencing technology on a NextSeq v2 Mid Output 300 cycles (2x150). The obtained sequence reads of the wild type strain were *de novo* assembled in CLC Genomics Workbench, version 8 (CLC Bio, Aarhus, Denmark) resulting into 156 contigs,

231 comprising a total number of 5122028 bases, with a genome coverage of 89×. The assembled
232 genome has been deposited at GenBank under the BioProject PRJNA309047. All other 36 genome
233 sequences were submitted to the Sequence Read Archive (SRA) database under the accession
234 number SRP075796. All the sequenced genomes from adapted and control lineages were mapped
235 with the 156 contigs as a reference. Average genome coverage was 104×. The 156 contigs were
236 annotated by Rapid Annotation using Subsystem Technology (Aziz et al., 2008). Variants were
237 called by fixed ploidy variant detection in CLC Genomics Workbench, in order to identify point
238 mutations such as Single Nucleotide Polymorphisms (SNPs) and deletion-insertion-polymorphism
239 (DIPs). Variants with a frequency above 60% were considered for further analysis. Functional
240 consequences were predicted by searching for amino acid changes, and then filtering non-
241 synonymous mutations.
242 The analysis was repeated by assembling the wild type contigs to the previously annotated genome
243 of *E. coli* ATCC 25922, available at GenBank with the accession number CP009072 (Minogue et
244 al., 2014). Wild type variants, in comparison to this genome, were detected and shown in
245 Supplementary Table 2. All the adapted lineages were then mapped to the annotated genome.
246 Variants were detected and filtered against the known ones, already found in the wild type.
247
248 **2.6 Measurement of Zeta potential.**
249
250 The wild type strain, an adapted lineage (3c) and a clone (1a-1) were grown overnight in 2 ml liquid
251 MHB at 37 °C at 250 rpm on an orbital shaker. Cultures were grown both in presence and absence
252 of compound. Cells were harvested in Eppendorf 5810R centrifuge at 2250 × *g* for 20 min. Cell
253 pellets were washed five times in 0.5 mM potassium phosphate buffer solution (pH 7.4), in
254 Eppendorf 5417R centrifuge at 3000 × *g* for 5 min, as described in Halder et al., (2015). OD_{600} was
255 adjusted to 0.2 (corresponding to approximately 1×10^8 CFU/ml). Zeta potential was measured
256 with a Zetasizer Nano ZS90 device (Malvern, UK). The given values resulted from the average of
257 three technical replicates, each of them consisting of 20 measurements. The experiment was
258 repeated a second time on a separate day. Statistical significance was assessed by One-way analysis
259 of variance (ANOVA) with statistical F test.
260
261 **3 Results**
262
263 **3.1 Adaptive laboratory evolution.**
264 All four lineages exposed to H-[Lys-βNSpe-hArg-βNSpe]$_3$-NH_2 (1a to 1d) and three out of four
265 lineages exposed to novicidin (2b 2c, 2d) were adapted to a 32-fold higher MIC (128 and 256
266 µg/ml, respectively) after passaging through approximately 350 generations (Figure 2).
267 Unexpectedly, none of the lineages exposed to P9-4 (3a to 3d) adapted to a 32-fold higher MIC, but
268 only one of four lineages (3c) tolerated a 16-fold higher MIC of 128 µg/ml), whereas the other three
269 i.e. 3a, 3b, 3d remained at 8-, 4- and 4-fold higher MIC (64 and 32 µg/ml, respectively) as shown in
270 Figure 2. Only 4a out of four lineages exposed to the three-compound combination adapted to a 32-
271 fold higher MIC of 256 µg/ml), whereas the other three i.e. 4b, 4c and 4d remained at 4-, 8- and 8-
272 fold higher MIC (32 and 64 µg/ml, respectively) as shown in Figure 2.

273
274 **3.2 Re-growth of adapted lineages in presence of compound.**
275 All adapted lineages were revived from frozen stocks and re-grown at the compound concentration
276 that they were adapted to. All four 1-adapted lineages, 3c, and 4a (Table 2) were able to re-grow at

the concentration that they were adapted to, as opposed to the remaining lineages. The 2-adapted lineages were only able to re-grow at ¼ of the MIC that they were originally adapted to, and similar results were seen for 3a and 3b as well as for 4c. Lineages 3d, 4b and 4d were all able to re-grow at ½ of the MIC that they were originally adapted to (Table 2).

3.3 Assessment of resistance in adapted lineages.

Stability of resistance was tested by determining MIC after five transfers, corresponding to approximately 35 generations, in absence of compound (Table 2). Lineage 2a had a MIC of 4 μg/ml when re-tested, despite being adapted to 64 μg/ml. Only one of five clones i.e. 2d-4 had a high MIC of 64 μg/ml (Table 3) while for all the other clones MIC was comparable to *wt* MIC, i.e 4-16 μg/ml. 2a and 2d and selected derived clones were whole-genome sequenced, as they were found to be the most susceptible and resistant, respectively.

In the 1-adapted lineages the measured MIC was equivalent to a 32-fold higher MIC than the *wt* MIC (128 μg/ml) or even higher (256-512 μg/ml). Also, the high MIC was maintained in clones isolated from three out of four lineages, except for 1b-4 (Table 3). The lineages with the highest and lowest MIC values, respectively, 1a and 1c, were whole-genome sequenced as were two clones for each lineage.

MIC for 3c, originally evolved to a 16-fold higher MIC of 128 μg/ml, remained high (64-128 μg/ml) after re-growth in presence of compound and upon five subsequent re-inoculations in absence of compound. In the five isolated clones, the measured MIC varied from a lower limit equal to the *wt* MIC (8-16 μg/ml) to an upper limit of 64 μg/ml (Table 3).

MIC for 4a (adapted to 256 μg/ml) was 32-128 μg/ml after re-growth in presence of compound, and reached 16-32 μg/ml after five subsequent re-inoculations in absence of compound. In the isolated clones, a MIC range up to 128 μg/ml was found only in two out of 20 clones (Table 3). These results indicate that the resistance trait decreased in absence of compound, even though 3c and 4a previously adapted to a 16-fold higher MIC of 128 μg/ml and a 32-fold higher MIC of 256 μg/ml, respectively).

Lineages 3c and 4a were whole-genome sequenced as representatives of resistant lineages, along with two clones from each of them.

Lineage 4b (adapted to a 4-fold higher MIC of 32 μg/ml) and 4c and 4d (adapted to an 8-fold higher MIC of 64 μg/ml) as well as clones derived from these lineages displayed generally higher MIC than 3a (adapted to an 8-fold higher MIC of 64 μg/ml), 3b and 3d (both adapted to a 4-fold higher MIC of 32 μg/ml). In fact, 4c and 4d had MIC of 16-64 and 32 μg/ml, respectively, when grown in presence of compound (Table 2). In the clones isolated from such lineages, MIC values were between 8 and 128 μg/ml, while in all clones, isolated from 4a, 4b and 4d, MIC values did not exceed the *wt* MIC (8-16 μg/ml). Given these data, 4b and 3d were whole-genome sequenced as representatives of susceptible lineages.

Cross resistance was assessed in the lineages where resistance persisted to the highest degree (1a, 3c, 4a, 2d). MIC of peptidomimetics displaying structural variations as compared to 1 was also assessed (Table 4). Resistance was maintained toward compounds 5 and 6. It is noticeable that the lipidated compound 7 retained almost full activity against 1a (MIC was 16 μg/ml as compared to 4-8 μg/ml in the wild type) but not against the other resistant lineages. Compound 2 retained full activity against 1a and 3c, but not against 4a. This was also reflected in the MIC values of 2 in

combination with 1. In lineage 1a, resistance was retained toward 1 and 3. The two-compound combinations seemed to be less effective than the combination of three compounds. Compound 2 alone showed the best activity on resistant lineages adapted to the other two compounds.

3.4 Whole-genome sequencing.

The wild type *E. coli* ATCC 25922 genome was assembled into 156 contigs with 89× coverage. A fully closed genome is available at NCBI; however, we chose to genome-sequence the actual strain we worked with. The wild type genome showed 30 variants with frequencies above 60%, with fourteen of them occurring in coding regions (Supplementary Table 2), as compared to the fully closed genome. This number indicated that comparison of the adapted strains to the *de novo* sequenced wild type was justified.

Sequences from the adapted lineages and clones were mapped to the wild type contigs, and an average number of 26 variants per genome with a frequency above 60% were found. Variants in coding regions were on average 14. Among these, an average of 6 variants caused an amino acid change in proteins. In non-exposed lineages, we found an average number of 24 variants per genome with a frequency above 60%. Among these, an average of 14 were located in coding regions, and 5 variants caused amino acid changes in encoded proteins (Table 5; Supplementary Table 1).

Deletions in the CDP-glycerophosphotransferase (CDP-glycerol: N-acetyl-β-D-mannosaminyl-1,4-N-acetyl-D-glucosaminyldiphosphoundecaprenyl glycerophosphotransferase) were seen in 6 out of 8 exposed lineages and in 11 clones. Interestingly, deletions were seen in clone 3c-4, but not in its parent lineage (3c). The deletions occurred in different regions of the same protein (Figure 3). However, there was no pattern between variant type, peptide and lineage, except for 2d, where the same deletion type was found both in the community and in the clones.

Lineages that, based on MIC assessment, showed the highest level of resistance (the 1-adapted lineages as well as 3c and 4a) did not share the same variant pattern. In fact, 1a had a deletion in the genes encoding CDP-glycerophosphotransferase while its two isolated clones also showed a mutation in the outer-membrane protein assembly factor YaeT precursor. By contrast 1c displayed a mutation in three different proteins besides a deletion in the CDP-glycerophosphotransferase that was different from the one acquired by lineage a. In 4a, the highest number of variants was found (55 with a frequency above 60%). Among these, ten variants corresponded to non-synonymous mutations in coding regions, and three of these may be suggested to be involved in the resistance genotype: a deletion in the CDP-glycerophosphotransferase (present in 3d-4, 1a-1 and 1a-5), and mutations in the respiratory nitrate reductase δ chain and in the GTP pyrophosphokinase bifunctional (p)ppGpp synthase/hydrolase SpoT, respectively.

In 2a, which was less resistant than the other three lineages, a mutation in the diguanylate cyclase domain protein and one in the Zinc ABC transporter ZnuA were found in both the lineage community and in the derived clones. These mutations did not appear in any of the other adapted lineages. Also, this lineage, and its derived clones, did not show any deletion in the CDP-glycerophosphotransferase. By contrast, in 2d a deletion in the same region of the CDP-glycerophosphotransferase was present in both the lineage community and in the derived clones. Some of the mutations found in 3d-4, such as the ones in the CDP-glycerophosphotransferase,

respiratory nitrate reductase δ chain and GTP pyrophosphokinase, were also present in 4a and 4a-1. A mutation in the phage major capsid protein was found in both 3d and 4b-1.

The mutational events, common to both adapted and non-exposed lineages, are shown in Supplementary Table 1. A mutation in the YeeV toxin protein arose in one exposed lineage and two clones (3c, 3c-1 and 4a-1). A different mutation in the same protein was seen in non-exposed clone 0a-2. Interestingly, none of the 3-adapted lineages and clones had a mutation in the dihydrolipoamide acetyltransferase.

A few mutational events were typical for the non-exposed lineages, such as mutations in the LysR transcription regulator LrhA, the mannose-specific adesin FimH, the phosphatase CheZ, involved in chemotaxis response and the arginine pathway regulatory protein ArgR2C (Supplementary Table 1).

3.5 Measurement of Zeta potential.

The impact of the deletion in the CDP-glycerophosphotransferase gene on the resistance phenotype was assessed by measuring the Zeta potential of selected adapted lineages and clones. Average values for 3c and 1a-1 were –33 mV and –28 mV, respectively, both significantly higher than that found for the wild type (–48 mV).

4 Discussion

E. coli adapted readily to the peptidomimetic H-[Lys-βNSpe-hArg-βNSpe]$_3$-NH_2 (1) and the AMP novicidin (2). Indeed most of the lineages became resistant during the course of the adaptive evolution experiment. In contrast, resistance did not readily develop toward the P9-4 peptide (3), which differs from the other two compounds by displaying an arginine-tryptophan (RW) motif twice. This motif has been shown to increase the interaction of AMPs with membranes. In fact, for short peptides (6-12 residues) the presence of RW motifs has been highlighted as promoting membrane interactions, since arginine preferentially forms bidentate hydrogen bonds with the polar headgroups of phosphatidylglycerol, while tryptophan traverse the membrane by intercalation (Liu et al., 2007). Hence, the delayed resistance development may be related to the strongly membrane-disruptive mode of action that also confers some concomitant toxicity toward human cells. In addition, RW motifs preferentially induce inter-peptide rather than intra-peptide interactions (Liu et al., 2007). This latter feature may increase the number of molecular targets in the bacterial cell membrane, thereby delaying resistance development.

Common mutations were identified in the gene CDP-glycerophosphotransferase encoding an enzyme consisting of 1266 amino acids. Single-nucleotide deletions were found in three different regions of this gene (Figure 3). A deletion (found in the genomes of 1a, 3c-1, 3d and 3d-1) was located in a region encoding the tetratricopeptide (TPR) domain, which is a putative protein-binding surface. A second deletion type, found in the genomes of 1c, 1c-1, 2d, 2d-1 and 2d-4, had occurred in a domain putatively involved in outer membrane biosynthesis, as previously shown in *Klebsiella pneumoniae* (Frirdich et al., 2004). The third deletion type (present in 1a-1, 1a-5, 3d-4, 4a and the derived clones as well as in 4b and the derived clone 4b-1), was in a domain functionally related to cell wall biogenesis as well as lipid transport and metabolism. The second and third deletion types were located in the region responsible for the phosphotransferase activity thus inferring a putative

417 resistance mechanism based on deficient introduction of negative charge to the membrane. Such
418 mutations are likely to inhibit phosphorylation, thereby reducing the negative charge of the
419 membrane, and limiting the interaction with positively charged AMPs. We hypothesized that
420 lineages and clones which acquired a deletion that decreased the phosphotransferase activity would
421 possess less negative membranes. Indeed, higher zeta potential (–28 mV) was measured for clone
422 1a-1 as compared to both wild type (–48 mV) and 3c (–33 mV). The latter also possessed
423 significantly increased surface charge as compared to the wild type, indicating that it had acquired a
424 surface charge modification arising from deficiencies in at least one other membrane-related
425 maintenance system. This result suggests that for clone 1a-1 the mutation in CDP-
426 glycerophosphotransferase may affect the bacterial surface charge resulting in a less negative
427 membrane. This mechanism seems to constitute a novel variation as compared to the known
428 strategies of limiting the attraction of cationic compounds (Band and Weiss, 2015). Occurrence of
429 the same variant across lineages exposed to distinct compounds appears to be linked to the similar
430 nature of the antibacterial agents giving rise to the selection pressure (Band and Weiss, 2015). It is
431 unclear whether the distinct deletion types confer different levels of resistance. However, 1c
432 exhibited the same deletion in the CDP-glycerophosphotransferase as also found in 2d, and for both
433 the MIC values suggest that resistance was probably induced by activation of preexisting adaptive
434 systems, unlike the other 1-adapted lineages, where resistance was maintained. This shows that
435 diversification into different genotypes occurs both within lineages adapted to the same compound
436 and across lineages exposed to different compounds.

437 Despite the heterogeneity, the variants can be divided into four distinct groups, which represent
438 genotypes that have followed parallel evolutionary trajectories. The deletion in the CDP-
439 glycerophosphotransferase was not present in 2a, which instead had acquired mutations in the
440 diguanylate cyclase domain protein and in the periplasmic zinc transporter ZnuA. The former seems
441 to be involved in phosphorylation (like the CDP-glycerophosphotransferase), the latter in zinc
442 uptake (Hantke, 2005; Ammendola et al., 2007). A mutation in the zinc uptake system protein
443 ZnuA may be related to a known mechanism of resistance based on peptide degradation by zinc-
444 dependent metalloproteases (Kooi and Sokol, 2009).
445
446 Three genomes (3d-4, 4a and 4a-1) had acquired mutations in the respiratory nitrate reductase δ
447 chain, in the GTP pyrophosphokinase, alongside a deletion in the same region of the CDP-
448 glycerophosphotransferase. The respiratory nitrate reductase δ chain is essential for assembly of the
449 whole enzyme; hence, a mutation in this subunit may affect the overall function of the protein if the
450 assembly itself is compromised. Yet, the consequence of these mutations on resistance development
451 remains unclear.
452
453 Lineage 3c, the most resistant among the 3-adapted lineages, did not have a deletion in the CDP-
454 glycerophosphotransferase. Remarkably, despite the absence of a deletion in the CDP-
455 glycerophosphotransferase, this specific lineage developed stable resistance. In contrast, 2a, also
456 lacking this deletion, was less resistant than 3c. These findings may be related to the different
457 variant patterns that the lineages have acquired in presence of different compounds. However, the
458 deletion in the CDP-glycerophosphotransferase was present in 3c-4, inferring that there has been
459 more than one mutational trajectory within the same lineage community exposed to the same
460 compound. In fact, this selected clone may merely represent a rarely occurring genotype in the
461 lineage population. Nevertheless, 3c may represent a special case where the previously acquired
462 mutations were lost in the next generations, because they were deleterious (Török et al., 2012).

Potential loss of the mutations in the CDP-glycerophosphotransferase may be responsible for the prolonged period needed to reach adaptation to the highest concentration of exposure (128 μg/ml).

In conclusion, the four main mutational trajectories may be summarized as: a first one can be identified in the acquisition of a deletion in the CDP-glycerophosphotransferase, a second one in the diguanylate cyclase domain protein and in the periplasmic zinc transporter protein ZnuA, a third one had no deletion in the CDP-glycerophosphotransferase, while a fourth trajectory resulted in mutations in the respiratory nitrate reductase δ chain, in the GTP pyrophosphokinase, alongside a deletion in the CDP-glycerophosphotransferase.

The presence of the short peptide P9-4 in the combination appears to have slowed down the resistance development in three out of four lineages exposed to the combination. Nevertheless, the simultaneous exposure to three compounds may account for the decreased rate of resistance development, as inferred by the work of Pirrone et al. (2011). However, it has been highlighted that in clinical studies of Gram-negative infections there was no difference in emergence of resistance during combination therapy versus monotherapy (Tamma et al., 2012).

For lineage 4a resistance development was faster than for the other three 4-adapted lineages. However, despite adaptation to a 32-fold higher MIC of 256 μg/ml, this lineage proved incapable of retaining a high level of resistance as opposed to 1a, 1b and 1d. MIC results performed on adapted lineages and derived clones may overall indicate that for the three 1-adapted lineages as well as for 3c and 4a, resistance was relatively stable, and hence arises from genetic mutations, while for the others resistance may be a phenotypic switch trait, caused by inducible preexisting adaptive systems. In fact, exposure to sub-MIC concentrations may lead to upregulation of efflux pumps or simply decreased membrane permeability due to LPS modification with additional fatty acid chains (Nizet, 2006).

Overall, the resistant genotypes had acquired a few distinct but non-identical mutations that appear to be correlated to the mechanism of the evolved resistance.
We conclude that mutations in CDP-glycerophosphotransferase may be a prime contributing factor in the adaptation to the investigated compounds. On the other hand, the zeta potential measurement is not an exhaustive method for confirming this hypothesis. We envision that only the construction of wild type mutants with deletions in the CDP-glycerophosphotransferase would unequivocally confirm whether this indeed is the primary cause of the observed resistant phenotype.

It is not known whether independent mutations or rather the interplay between different cooperative mutations is responsible for resistance. As pointed out by Martinez and Baquero (2000), the emergence of independent or cooperative mutations depends on the specific bacterium-compound interaction. Also, it is known that a change of a single residue in an encoded protein may not interfere with its activity, unless the change occurs in the active site of an enzyme or in a residue that is involved in folding of the protein (Studer et al., 2013).

The three antibacterial compounds investigated for their potential risk of inducing resistance were selected to represent distinct structural differences, and thus comprised a non-helical 12-residue peptidomimetic H-(Lys-βNphe-hArg-βNphe)$_3$-NH_2 (denoted 1), with an alternating design of cationic Lys/hArg and aromatic peptoid residues, a typical 18-mer α-helical highly cationic AMP (i.e. novicidin also denoted 2) as well as a very short 9-residue Arg/Trp-rich AMP (i.e. P9-4 also

denoted 3). Interestingly, lineage 1a exhibited cross resistance to both peptidomimetics 6 and 8 (Ac-[Lys-βNphe]$_8$-NH_2 and Ac-[hArg-βNSpe]$_6$-NH_2) displaying only Lys or hArg residues, respectively, together with aromatic peptoid units, while susceptibility to the lipidated Lys-based analogue 7 was almost fully retained inferring that this structural modification most likely is associated with an altered mode of action. Nevertheless, this seems not just to be correlated with an increased overall lipophilicity of compound 7, since cross resistance to the hydrophobic analogue 5 had evolved also. Analogue 5 resembles compound 1 with respect to cationicity, but contains additional α-methyl groups in the peptoid side chains as well as a significant content of aliphatic cyclohexyl peptoid side chains, which collectively confer similar enhanced hydrophobicity as the single fatty acid in compound 7. Noticeably, the structurally most different compounds, 1 and 2, displayed less and no cross resistance, respectively. Somewhat surprisingly the combination of three and the 1+2 pair displayed similar activity against all resistant strains tested, whereas other two-compound combinations were less effective than the individual compounds. Compound 2 alone in fact showed unchanged activity on resistant lineages adapted to other compounds inferring that its killing mechanism appears to deviate significantly from those of 1 and 3.

We show that resistance development toward a combination of three AMPs was slower than to two of the three compounds when used alone. However, resistance to one of the peptides (P9-4) alone was surprisingly difficult to evolve. These findings indicate that resistance development is compound-dependent rather than combination-dependent. We propose that constrained evolutionary resistance-inducing trajectories are compound-dependent. Also, it is suggested that the influence of certain mutations can be explained, as they occurred in lineages upon exposure to different compounds. Our results infer that P9-4 may constitute a potential lead for a future drug candidate, and we encourage early-stage investigation of resistance development for all promising novel antibacterial compounds. In addition, the issue of the potential risk of cross-resistance seems to be quite complex as no clear-cut conclusions can be made from this limited study. However, more detailed studies comprising a larger number of AMPs are warranted in order to assess to which degree activity of other subclasses of AMPs will be compromised by resistance development to a single AMP or to a combination of antibacterials.

5 Conflict of Interest

The authors declare that they have no competing interests.

6 Author Contributions

LC, HF and LG designed the experiments. HMN provided guidance and facilities for the Zeta potential experiment. LC planned and carried out the experiments and drafted the manuscript. LG reviewed the manuscript. HF synthesized the compounds and took part in the revision of the manuscript. All authors have reviewed and approved the final manuscript.

7 Funding

LC was supported by a PhD scholarship from the Technical University of Denmark.

8 List of abbreviations

AMPs	antimicrobial peptides
ALE	adaptive laboratory evolution
MIC	minimum inhibitory concentration
HIV	human immunodeficiency virus
NCBI	national center for biotechnology information
CDP	conserved domain protein
ABC	ATP-binding cassette
TPR	tetratricopeptide repeat
LPS	lipopolysaccharide
MHB	Müller Hinton II broth
CLSI	clinical and laboratory standards institute
CFU	colony-forming units
wt	wild type
SRA	sequence read archive
SNPs	single-nucleotide polymorphisms
DIPs	deletion insertion polymorphisms

9 Acknowledgements

LC was supported by a PhD grant from the Technical University of Denmark. We wish to thank Jette Melchiorsen (Technical University of Denmark) for help in the ALE experiment and Anna Koza (Novo Nordisk Center for Biosustainability) for the library preparation for whole genome sequencing.

10 Availability of data and material

The data sets supporting the results of this article are available in the GenBank (NCBI) repository, under the BioProject PRJNA309047 and in the Sequence Read Archive (SRA) database under the accession number SRP075796. Data will be released to the public upon publication.

11 References

Ammendola, S., Pasquali, P., Pistoia, C., Petrucci, P., Petrarca, P., Rotilio, G., et al. (2007). High-affinity Zn^{2+} uptake system ZnuABC is required for bacterial zinc homeostasis in intracellular environments and contributes to the virulence of *Salmonella enterica*. *Infect. Immun.* 75, 5867–5876.

Antimicrobial resistance. Global Report on Surveillance. World Health Organization. 2014.

Aziz, R.K., Bartels, D., Best, A.A, DeJongh, M., Disz, T., Edwards, R.A, et al. (2008). The RAST server: rapid annotations using subsystems technology. *BMC Genomics* 9, 75. doi:10.1186/1471-2164-9-75.

Band, V.I., and Weiss, D.S. (2015). Mechanisms of antimicrobial peptide resistance in Gram-negative bacteria. *Antibiot.*4, 18–41.

Bonke, G., Vedel, L., Witt, M., Jaroszewski, J., Olsen, C., and Franzyk, H. (2008). Dimeric building blocks for solid-phase synthesis of α-peptide-β-peptoid chimeras. *Synthesis* 15, 2381–2390.

596 Brogden, K.A. (2005). Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?
597 *Nat. Rev. Microbiol.* 3, 238–50.
598 Brogden, N.K., and Brogden, K.A. (2011). Will new generations of modified antimicrobial peptides
599 improve their potential as pharmaceuticals? *Int. J. Antimicrob. Agents* 38, 217–225.
600 Cassone, M., and Otvos Jr. L. (2010). Synergy among antibacterial peptides and between peptides
601 and small-molecule antibiotics. *Expert.Rev.Anti.Infect.Ther.* 8, 703–716.
602 Chongsiriwatana, N. P., Patch, J. a, Czyzewski, A. M., Dohm, M. T., Ivankin, A., Gidalevitz, D., et
603 al. (2008). Peptoids that mimic the structure, function, and mechanism of helical antimicrobial
604 peptides. *Proc. Natl. Acad. Sci. U. S. A.* 105, 2794–2799. doi:10.1073/pnas.0708254105.
605 Chongsiriwatana, N.P., Wetzler, M., and Barron, A.E. (2011). Functional synergy between
606 antimicrobial peptoids and peptides against Gram-negative bacteria. *Antimicrob. Agents*
607 *Chemother.* 55, 5399–5402.
608 Citterio, L., Franzyk, H., Palarasah, Y., Andersen, T.E., Mateiu, R.V., and Gram, L. (2016).
609 Improved in vitro evaluation of novel antimicrobials: potential synergy between human plasma
610 and antibacterial peptidomimetics, AMPs and antibiotics against human pathogenic bacteria.
611 *Res. Microbiol.* 167, 72–82.
612 Cole, J.N., and Nizet, V. (2016). Bacterial evasion of host antimicrobial peptide defenses.
613 *Microbiol. Spectr.*, 1–22. doi:10.1128/microbiolspec.
614 Devine, D.A., and Hancock, R.E.W. (2002). Cationic peptides: distribution and mechanisms of
615 resistance. *Curr. Pharm. Des.* 8, 703–14.
616 Fair, R.J., and Tor, Y. (2014). Antibiotics and bacterial resistance in the 21st century. *Perspect.*
617 *Medicin. Chem.* 25–64. doi:10.4137/PMC.S14459.
618 Fox, J. L. (2013). Antimicrobial peptides stage a comeback. *Nat. Biotechnol.* 31, 379–382.
619 Frirdich, E., Vinogradov, E., and Whitfield, C. (2004). Biosynthesis of a novel 3-deoxy-D-manno-
620 oct-2-ulosonic acid-containing outer core oligosaccharide in the lipopolysaccharide of
621 *Klebsiella pneumoniae*. *J. Biol. Chem.* 279, 27928–27940.
622 Galani, I., Orlandou, K., Moraitou, H., Petrikkos, G., and Souli, M. (2014). Colistin/daptomycin: an
623 unconventional antimicrobial combination synergistic in vitro against multidrug-resistant
624 *Acinetobacter baumannii*. *Int. J. Antimicrob. Agents* 43, 370–374.
625 Halder, S., Yadav, K.K., Sarkar, R., Mukherjee, S., Saha, P., Haldar, S., et al. (2015). Alteration of
626 Zeta potential and membrane permeability in bacteria: a study with cationic agents.
627 *SpringerPlus* 4:672. doi:10.1186/s40064-015-1476-7.
628 Hancock, R.E.W., and Sahl, H.-G. (2006). Antimicrobial and host-defense peptides as new anti-
629 infective therapeutic strategies. *Nat. Biotechnol.* 24, 1551–1557.
630 Hantke, K. (2005). Bacterial zinc uptake and regulators. *Curr. Opin. Microbiol.* 8, 196–202.
631 Hein-Kristensen, L., Franzyk, H., Holch, A., Gram, L. (2013). Adaptive evolution of *Escherichia*
632 *coli* to an α-peptide/β-peptoid peptidomimetic induces stable resistance. *PLoS one*
633 8(9):e73620.
634 Hindler, J.A., Wong-Beringer, A., Charlton, C.L., Miller, S.A., Kelesidis, T., Carvalho, M., et al.
635 (2015). In vitro activity of daptomycin in combination with β-lactams, gentamicin, rifampin,
636 and tigecycline against daptomycin-nonsusceptible enterococci. *Antimicrob. Agents*
637 *Chemother.* 59, 4279–88.
638 Jahnsen, R.D., Frimodt-Møller, N., and Franzyk, H. (2012). Antimicrobial activity of
639 peptidomimetics against multidrug-resistant *Escherichia coli* - A comparative study of
640 different backbones. *J. Med. Chem.* 55, 7253–7261.
641 Jahnsen, R.D., Sandberg-Schaal, A., Vissing, K.J., Nielsen, H.M., Frimodt-Møller, N., and Franzyk,
642 H. (2014). Tailoring cytotoxicity of antimicrobial peptidomimetics with high activity against

multidrug-resistant *Escherichia coli*. *J. Med. Chem.* 57, 2864–2873.
Kooi, C., and Sokol, P.A. (2009). *Burkholderia cenocepacia* zinc metalloproteases influence resistance to antimicrobial peptides. *Microbiology* 155, 2818–2825.
Köser, C.U., Ellington, M.J., and Peacock, S.J. (2014). Whole-genome sequencing to control antimicrobial resistance. *Trends Genet.* 30, 401–407.
Lennox, J.L., DeJesus, E., Lazzarin, A., Pollard, R.B., Madruga, J.V.R., Berger, D.S., et al. (2009). Safety and efficacy of raltegravir-based versus efavirenz-based combination therapy in treatment-naive patients with HIV-1 infection: a multicentre, double-blind randomised controlled trial. *Lancet* 374, 796–806.
Lindsey, H.A., Gallie, J., Taylor, S., and Kerr, B. (2013). Evolutionary rescue from extinction is contingent on a lower rate of environmental change. *Nature* 494, 463–468.
Liu, Z., Brady, A., Young, A., Rasimick, B., Chen, K., Zhou, C., et al. (2007). Length effects in antimicrobial peptides of the (RW)n series. *Antimicrob. Agents Chemother.* 51, 597–603.
Malmsten, M. (2014) Antimicrobial peptides. *Ups. J. Med. Sci.*119,199-204.
Maria-Neto, S., de Almeida, K.C., Macedo, M.L.R., and Franco, O.L. (2015). Understanding bacterial resistance to antimicrobial peptides: from the surface to deep inside. *Biochim. Biophys. Acta - Biomembr.* 1848, 3078–3088.
Marr, A.K., Gooderham, W.J., and Hancock, R.E.W. (2006). Antibacterial peptides for therapeutic use: obstacles and realistic outlook. *Curr. Opin. Pharmacol.* 6, 468–472.
Martinez, J.L., and Baquero, F. (2000). MINIREVIEW-Mutation frequencies and antibiotic resistance. *Antimicrob. Agents Chemother.* 44, 1771–1777.
Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard - ninth edition. Clinical and Laboratory Standards Institute. 2012.
Minogue, T.D., Daligault, H.A., Davenport, K.W., Bishop-Lilly, K.A., Broomall, S.M., Bruce, D.C., et al. (2014). Complete genome assembly of *Escherichia coli* ATCC 25922, a serotype O6 reference strain. *Genome announcements*. 2, 4–5.
Nielsen, S.B., and Otzen, D.E. (2010). Impact of the antimicrobial peptide novicidin on membrane structure and integrity. *J. Colloid Interface Sci.* 345, 248–256.
Nizet, V. (2006). Antimicrobial peptide resistance mechanisms of human bacterial pathogens. *Curr. Issues Mol. Biol.* 8, 11–26.
Nyunt, M.M., and Plowe, C.V. (2007). Pharmacologic advances in the global control and treatment of malaria: combination therapy and resistance. *Clin. Pharmacol. Ther.* 82, 601–5.
Olsen, C.A., Ziegler, H.L., Nielsen, H.M., Frimodt-Møller, N., Jaroszewski, J.W., and Franzyk, H. (2010). Antimicrobial, hemolytic, and cytotoxic activities of β-peptoid- peptide hybrid oligomers: improved properties compared to natural AMPs. *ChemBioChem* 11, 1356-1360.
Ortona, L. and Antinori, A. (1998). Principles of therapy for tuberculosis. *Rays.* 23, 181-192.
Perron, G.G., Zasloff, M., and Bell, G. (2006). Experimental evolution of resistance to an antimicrobial peptide. *Proc. R. Soc. B Biol. Sci.* 273, 251–256.
Peschel, A., and Sahl, H.-G. (2006). The co-evolution of host cationic antimicrobial peptides and microbial resistance. *Nat Rev Microbiol* 4, 529–536.
Pirrone, V., Thakkar, N., Jacobson, J.M., Wigdahl, B., and Krebs, F.C. (2011). Combinatorial approaches to the prevention and treatment of HIV-1 infection. *Antimicrob. Agents Chemother.* 55, 1831–1842.
Punina, N.V, Makridakis, N.M., Remnev, M.A., and Topunov, A.F. (2015). Whole-genome sequencing targets drug-resistant bacterial infections. *Hum. Genomics* 9, 19. doi:10.1186/s40246-015-0037-z.
Qi, X., Zhou, C., Li, P., Xu, W., Cao, Y., Ling, H., et al. (2010). Novel short antibacterial and

antifungal peptides with low cytotoxicity: efficacy and action mechanisms. *Biochem. Biophys. Res. Commun.* 398, 594–600.

Qureshi, Z.A., Paterson, D.L., Potoski, B.A., Kilayko, M.C., Sandovsky, G., Sordillo, E., et al. (2012). Treatment outcome of bacteremia due to KPC-producing *Klebsiella pneumoniae*: Superiority of combination antimicrobial regimens. *Antimicrob. Agents Chemother.* 56, 2108–2113.

Sallum, U.W., and Chen, T.T. (2008). Inducible resistance of fish bacterial pathogens to the antimicrobial peptide cecropin B. *Antimicrob. Agents Chemother.* 52, 3006–3012.

Studer, R.A., Dessailly, B.H., and Orengo, C.A. (2013). Residue mutations and their impact on protein structure and function: detecting beneficial and pathogenic changes. *Biochem. J.* 449, 581–94.

Tamma, P.D., Cosgrove, S.E., and Maragakis, L.L. (2012). Combination therapy for treatment of infections with Gram-negative bacteria. *Clin. Microbiol. Rev.* 25, 450–470.

Török, M.E., Chantratita, N., and Peacock, S.J. (2012). Bacterial gene loss as a mechanism for gain of antimicrobial resistance. *Curr. Opin. Microbiol.* 15, 583–587.

de Visser, J.A.G.M.M., and Krug, J. (2014). Empirical fitness landscapes and the predictability of evolution. *Nat. Rev. Genet.* 15, 480–490.

Yu, G., Baeder, D.Y., Regoes, R.R., and Rolff, J. (2016). The more the better? Combination effects of antimicrobial peptides. *Antimicrob. Agents Chemother.*, AAC.02434–15. doi:10.1128/AAC.02434-15.

Zhuang, L., Sy, S.K.B., Xia, H., Singh, R.P., Mulder, M.B., Liu, C., et al. (2015). Evaluation of in vitro synergy between vertilmicin and ceftazidime against *Pseudomonas aeruginosa* using a semi-mechanistic pharmacokinetic/pharmacodynamic model. *Int. J. Antimicrob. Agents* 45, 151–160.

Table 1: MIC of the compounds used in the present study against *E. coli* ATCC 25922.

Compound	MIC (µg/ml)
1	4-8
2	4-16
3	8-16
4	8
5	8-16
6	8
7	4-8
8	4-8
1+2	4-8
1+3	8
2+3	8

1 (H-[Lys-βNSpe-hArg-βNSpe]$_3$-NH_2), 2 (novicidin), 3 (P9-4), 4 (1+2+3), 5 (Ac-[hArg-βNSce-Lys-βNSpe]$_3$-NH_2), 6 (Ac-[Lys-βNphe]$_8$-NH_2), 7 (Lau-[Lys-βNphe]$_6$-NH_2) and 8 (Ac-[hArg-βNSpe]$_6$-NH_2).

Table 2: Fold change in adapted lineages as compared to *wt* MIC of the compounds used in the adaptation.

	Fold increase as compared to *wt* MIC			
Lineage	**At the end of ALE**	**At re-growth upon freeze storage**	**After ALE and re-growth**	**After 5 re-inoculations in absence of compound**
1a	16-32	16-32	64	16-32
1b	16-32	16-32	64	16
1c	16-32	16-32	64	32
1d	16-32	16-32	32-64	32
2a *	4-16	1-4	1	1
2b *	16-64	4-16	1	2-4
2c*	16-64	4-16	2-4	1-2
2d*	16-64	4-16	4-16	2-8
3a *	4-8	1-2	1	1
3b *	2-4	1	1	1
3c	8-16	8-16	8	4-8
3d *	2-4	1-2	1	1
4a	32	32	4-16	2-4
4b *	4	2	1-2	1-2
4c *	8	2	2-8	2
4d *	8	4	4	2

Note: the fold increase presented in the first two columns refers to MIC values reported in Table 1. The actual concentration of adaptation is reported in Figure 2. *Lineages that did not grow at the concentration of adaptation and therefore were grown in lower concentration. Lineages in bold were whole-genome sequenced. Experiments were done in independent duplicates.

Table 3: Fold change in MIC of the compounds in five clones isolated from each adapted lineage.

	Fold change in MIC of the respective compound for each clone (1-5)				
Lineage	**1**	**2**	**3**	**4**	**5**
1a	**32-64**	32-64	32-64	32-64	**32**
1b	16	16	16	1	16
1c	**2**	4	2-8	4-8	**4-8**
1d	32	8-32	16-32	32	64
2a	**1**	1	**1**	1	1
2b	1	1	1	1	1
2c	1	1	1	1	1
2d	**1**	1	1	**4-16**	1
3a	1	1	1	1	1
3b	1	1	1	1	1
3c	**4**	2-4	4	**2-4**	4
3d	**1**	1	1	**1**	1
4a	**2-4**	**4**	4	4	2-4
4b	**1-2**	2	1-4	**2-4**	1-2
4c	2	1-2	1-8	1	1
4d	1	2-16	2-4	8-16	2

All clones were tested against the respective compound used for adaptation. Fold changes in bold relate to the clones that were whole-genome sequenced. Experiments were done in independent duplicates.

801 **Table 4:** Cross resistance in selected resistant lineages.
802

Compound	Fold change in MIC (µg/ml) in adapted lineages			
	1a	**3c**	**2d**	**4a**
1	32-64	16	4-8	64-128
2	1	1	4-16	2-8
3	2-4	8-16	2-4	8-16
4	1-2	2-4	4	8
5	8-16	16	8	32
6	64 - > 64	64	32	>64
7	2-4	8	4-8	16-32
8	8-16	4-8	2	16-32
1 + 2	1	4	2-4	8
1 + 3	8	16	4	32
2 + 3	2	4-8	4-8	8

803
804 1 (H-[Lys-βNSpe-hArg-βNSpe]$_3$-NH_2), 2 (novicidin), 3 (P9-4), 4 (1+2+3), 5 (Ac-[hArg-βNSce-Lys-
805 βNSpe]$_3$-NH_2), 6 (Ac-[Lys-βNphe]$_8$-NH_2), 7 (Lau-[Lys-βNphe]$_6$-NH_2) and 8 (Ac-[hArg-βNSpe]$_6$-NH_2).

806

Table 5: Single-nucleotide variants (frequency >60%) causing amino acid change in adapted lineages and respective clones. In italic: variant causing synonymous mutations.

	Mutations in adapted lineages and clones																							
	1						**2**						**3**						**4**					
Gene product	**a**	**a-1**	**a-5**	**c**	**c-1**	**c-5**	**a**	**a-1**	**a-3**	**d**	**d-1**	**d-4**	**c**	**c-1**	**c-4**	**d**	**d-1**	**d-4**	**a**	**a-1**	**a-2**	**b**	**b-1**	**b-4**
CDP-glycerophosphotransferase	x	x	x	x	x					x	x	x			x	x	x	x	x	x	x	x	x	
Respiratory nitrate reductase δ chain																		x	x	x				
GTP pyrophosphokinase																		x	x	x				
Unsaturated fatty acid biosynthesis repressor FabR2C TetR																						x		
Diguanylate cyclase							x	x	x															
Outer membrane protein assembly factor YaeT precursor		x	x																					
Uncharacterized Yrbk											x													
S-formylglutathione hydrolase												x												
Transcriptional activator of maltose regulon 2C MalT												x												
Zinc ABC transporter ZnuA							x	x	x															
Phage major capsid protein																x							x	
T1SS secreted agglutinin RTX								x															x	x
Mobile element protein								x		x														
Antirestriction protein klcA					x			x			x	x									x			
Exopolygalacturonate lyase										x	x	x												
FecD (Iron transport protein)					x																		x	
Glutamate decarboxylase															x		x			x				
Ferredoxin reductase																		x						
Periplasmic binding protein																						x		

We show lineages 1a and 1c and clones 1a-1, 1a-5, 1c-1, 1c-5 for H-[Lys-βNSpe-hArg-βNSpe]$_3$-NH_2, lineages 2a and 2d and clones 2a-1, 2a-3, 2d-1, 2d-4 for novicidin, lineages 3c and 3d and clones 3c-1, 3c-4, 3d-1, 3d-4 for P9-4, lineages 4a and 4b and clones 4a-1, 4a-2, 4b-1, 4b-4 for the combination. We report only variants that were not also present in non-exposed lineages. We do not show variants occurring in hypothetical proteins.

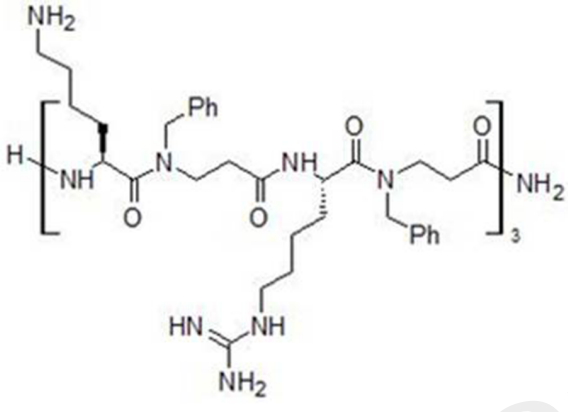
12 Figure legends

Figure 1: Structure of the peptidomimetics used in the present study. From the top left, clockwise: compound 1 (H-[Lys-βNSpe-hArg-βNSpe]$_3$-NH_2) 5 (Ac-[hArg-βNSce-Lys-βNSpe]$_3$-NH_2) 6 (Ac-[Lys-βNphe]$_8$-NH_2) 7 (Lau-[Lys-βNphe]$_6$-NH_2) and 8 (Ac-[hArg-βNSpe]$_6$-NH_2).

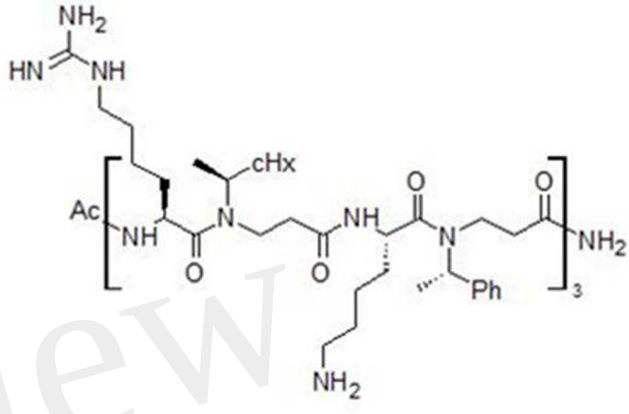
Figure 2: Adaptive laboratory evolution of *E. coli* to the single compounds and the combination. Four independent lineages (black line: lineage a, red line: lineage b, blue line: lineage c, green line: lineage d) were adapted for each treatment: H-[Lys-βNSpe-hArg-βNSpe]$_3$-NH_2 (compound 1), novicidin (2), P9-4 (3), and the combination of the three compounds (4).

Figure 3: Location of the three deletion types in the gene encoding for the CDP-glycerophosphotransferase. The precise location of the deletions is marked with a purple bar. The deletions were found in a region encoding for the TPR domain (yellow) and in two distinct regions related to the phosphotransferase activity (light orange and orange).

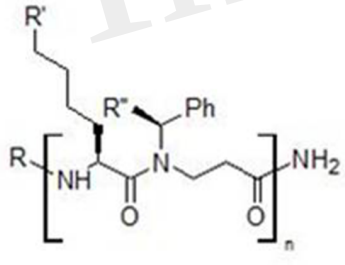
Figure 1.TIF



1 = H-(Lys-βNphe-hArg-βNphe)$_3$-NH$_2$



5 = Ac-(hArg-βNSce)$_6$-Lys-NSpe)$_3$-NH$_2$



6 = Ac-(Lys-βNphe)$_8$-NH$_2$: R = Ac; R' = NH2; R" = H; n = 8

7 = Lau-(Lys-βNphe)$_6$-NH$_2$: R = Lau; R' = NH$_2$; R" = H; n = 6

8 = Ac-(hArg-βNSpe)$_6$-NH$_2$: R = Ac; R' = NH(C=NH)-NH$_2$; R" = Me; n = 6

Abbreviations used:

βNphe = N-phenylmethyl-β-alanine

βNSpe = N-(*S*)-1-phenylethyl-β-alanine

βNSce = N-(*S*)-1-cyclohexylethyl-β-alanine

Lau = lauroyl = H_3C-$(CH_2)_{10}$(C=O)-

Ph = phenyl

cHx = cyclohexyl

hArg = homoarginine

Figure 2.JPEG

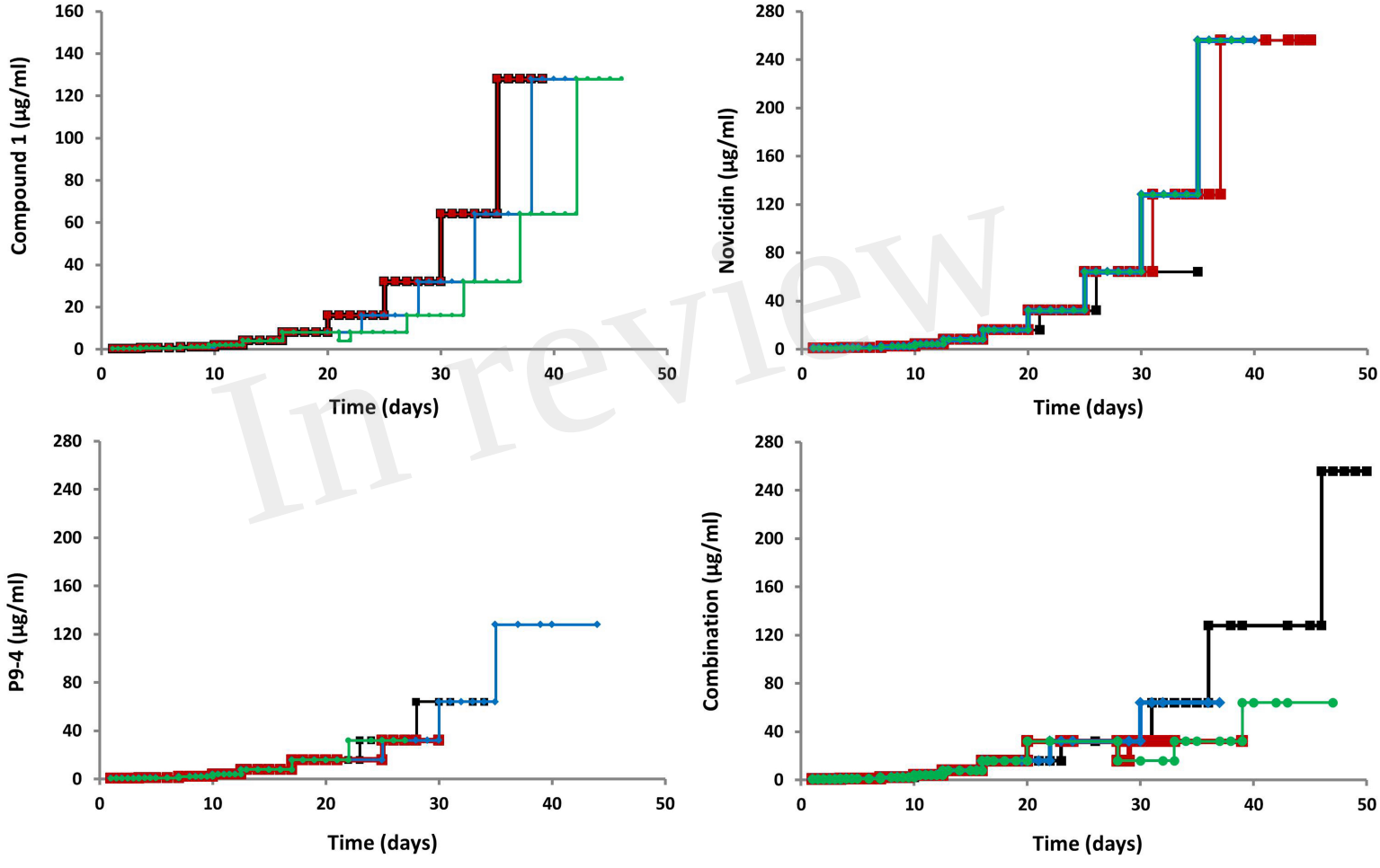
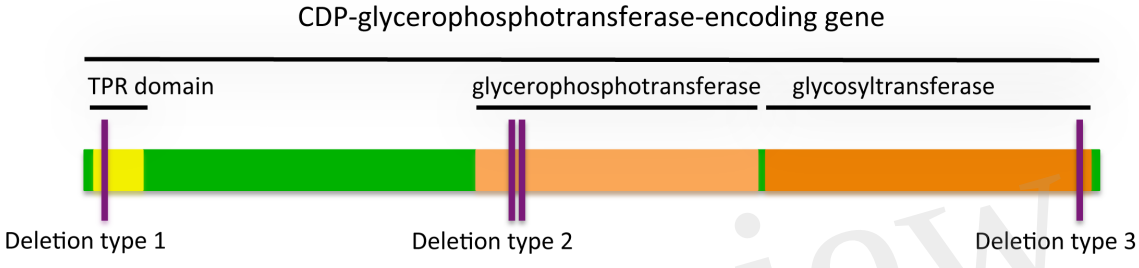


Figure 3.TIF



Supplementary Material

Adaptive Laboratory Evolution of *E. coli* Reveals Slow Resistance Development to the Short Antimicrobial Peptide P9-4 and to a Combination of three Antimicrobial Compounds

Linda Citterio[1], Henrik Franzyk[2], Hanne Mørck Nielsen[3], Lone Gram[1*]

***Correspondence:** Lone Gram gram@bio.dtu.dk

Supplementary Table 1: Single nucleotide variants (frequency >60%) causing amino acid change in both adapted and non-exposed lineages.

	Adapted lineages and clones																																		
	1						**2**										**3**									**4**						**0**					
Gene product	**a**	**a-1**	**a-5**	**c**	**c-1**	**c-5**	**a**	**a-1**	**a-3**	**d**	**d-1**	**d-4**	**c**	**c-1**	**c-4**	**d**	**d-1**	**d-4**	**a**	**a-1**	**a-2**	**b**	**b-1**	**b-4**	**a**	**a-1**	**a-2**	**b**	**b-1**	**b-2**	**c**	**c-1**	**c-2**	**d**	**d-1**	**d-2**
Mannose-specific adesin FimH																									x					x	x	x			
phosphatase CheZ																											x		x				x		x
Arginine pathway regulatory protein ArgR2C																																		x	
LysR family transcription regulator lrhA																									x					x	x	x			
YeeV toxin protein													x	x						x						x									
Mobile element protein	x			x	x	x	x		x	x		x	x	x		x	x		x	x			x	x	x	x	x	x	x	x	x	x		x	x
DNA repair protein RadC	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x
Dihydrolipoamide acetyltransferase	x	x				x	x	x	x	x		x			x		x		x	x	x	x	x	x					x	x		x	x			x
Membrane protein b2001					x							x							x									x								
BigB		x																								x										
Uncharacterized YkfH	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x

Variants typical of non-exposed lineages (0) are shown in the upper part of the table. In non-exposed lineages, 0a, 0b and respective clones were transferred until the adaptation experiment was ended while lineages 0c, 0d and respective clones were transferred until the first adaptation to 32× was completed. Single nucleotide variants detected both in adapted and in non-exposed lineages and clones are shown in the lower part of the table.

Supplementary Table 2: Single nucleotide variants (frequency >60%) occurring in coding regions of the wild type genome used in the present study, as compared to the reference strain *Escherichia coli* ATCC 25922.

Gene product	**Number of variants in coding regions**
LPS-assembly protein lptD	1
Rhs element Vgr family protein	3
Phage major capsid E family protein	1
deoR C term sensor domain protein	2
Glycosyl transferase 21 family protein	1
Integrase core domain protein	1
Putative membrane protein	1
Putative entS/YbdA MF S transporter	1
Propionate CoA ligase	1
Hypothetical proteins	2

Development of resistance to antimicrobial peptides and peptidomimetics: how critical is this challenge?

Linda Citterio[1], Henrik Franzyk[2] and Lone Gram[1*]

[1] Department of Biotechnology and Biomedicine, Technical University of Denmark, Matematiktorvet 301, DK-2800 Kgs. Lyngby, Denmark

[2] Department of Drug Design and Pharmacology, Section for Natural Products and Peptides, University of Copenhagen, DK-2100, Copenhagen, Denmark

* corresponding author

e-mail: gram@bio.dtu.dk

phone: +45 2368 8295

Keywords: antimicrobial peptides, peptidomimetics, cross-resistance, *E. coli*

Introduction

The rapid emergence and spreading of bacterial antibiotic resistance highlight the urgent demand for discovery and development of novel antimicrobial compounds and treatment strategies. In the last two decades, antimicrobial peptides (AMPs) have been considered as potential novel antibiotics due to the lack of widespread co-evolution of resistance in nature (Ulvatne, 2003; Zaiou, 2007). Initially, emergence of resistance toward AMPs was assumed to be low due to their rapid bactericidal activity (Wimley and Hristova, 2011; Fox, 2013). Nevertheless, development of resistance to AMPs has been raised as a potential risk as general resistance mechanisms might compromise the innate immune defense in humans (Bell and Gouyon, 2003; Perron et al., 2006). Resistance is a natural phenomenon that depends on the environmental stimuli, and e.g. stress induced by AMPs under laboratory conditions may exert a selection pressure that confers bacterial adaptive resistance (Hong et al., 2016).

By using an adaptive laboratory evolution approach, resistant *E. coli* lineages, exhibiting substantially increased minimal inhibitory concentrations (MICs) toward AMPs and peptidomimetics, were readily developed (Citterio et al., 2016, submitted). Hence, any AMP or synthetic analogue may select for resistant mutants present (or evolving) in a bacterial population. In addition, it has been found that AMP-resistant bacterial mutants can display cross-resistance to other AMPs with different structures and modes of action (Andersson et al., 2016). Thus, it is crucial to investigate the potential of such cross-resistance as it represents a possible risk for any novel antibacterial compound. Interestingly, Baym et al., (2016) recently reported that inversion of the selective advantage of resistance could turn a resistant population susceptible again. Such inversion of the selective advantage is triggered by suppressive drug interactions. In fact, resistance to the first applied drug can increase the sensitivity to a second applied drug (Imamovic and Sommer, 2013). The latter creates a concentration regime that inhibits the growth of resistant bacteria, hence selecting for the sensitive part of the population (Baym et al., 2016). Based on these observations, we hypothesized that suppressive drug interactions can select against AMP-resistant mutants, hence inducing susceptibility in a resistant population.

Cross-resistance and susceptibility patterns were difficult to explain when only a few compounds were included in the studies (Hein-Kristensen et al., 2013; Citterio et al., 2016, submitted). Hence, the present purpose was to assess whether previously evolved AMP-resistant *E. coli* clones were resistant or susceptible to a structurally more diverse range of compounds. Thus

we selected an array of synthetic AMPs and peptidomimetics for an extended study of cross-resistance. These compounds represent a variety of structural features such as length, hydrophobicity, lipidation, incorporation of D-amino acids, and presence of characteristic motifs (e.g. RW or IR) or high abundance of certain amino acids.

Our hypothesis was that combinations of compounds displaying different typical features of synthetic AMPs might give rise to drug-suppressive interactions in AMP-resistant clones. Clones derived from lineages adapted in the laboratory upon exposure to sub-lethal concentrations of the AMPs P9-4 and novicidin as well as peptidomimetic H-[Lys-βNSpe-hArg-βNSpe]$_3$-NH2 (HF-1002-2) individually and in a combination of all three compounds were examined (Citterio et al., 2016, submitted). From whole-genome sequence analysis of these AMP-adapted clones, we identified a novel putative distinct mode of resistance leading to an overall loss of negative charge of the bacterial membranes. We hypothesized that this mechanism of resistance was genetically encoded; hence it would be maintained in absence of the selective pressure exerted by the compound. Therefore we re-cultivated highly resistant clones in absence of compound and investigated whether the single nucleotide variants, identified immediately after the adaptive evolution experiments, were still present in the populations of these clones after fifteen re-inoculation steps.

The primary purpose of the present study was to investigate the stability of resistance in AMP-resistant mutants as well as estimating the degree of and trends for cross-resistance in such highly resistant clones.

Methods

Bacterial strain and culture conditions.

Escherichia coli ATCC 25922 was grown in cation-adjusted Müller Hinton II broth (MHB) (Becton Dickinson 212322) adjusted to pH 7.4 and supplemented with 1.5% agar (Oxoid, CM0471) for culturing on solid medium. Stock cultures of this strain and re-cultivated clones were stored at -80 ^{o}C in 25% glycerol.

Synthesis of peptidomimetics and AMPs.

The compounds (peptidomimetics and AMPs) were prepared by solid-phase synthesis as previously described (Bonke et al., 2008; Olsen et al., 2010). The resulting lyophilized compounds and the

antibiotic polymyxin B (Sigma Aldrich, P4932) were dissolved in 10 mg/mL sterile MilliQ water and stored at -20 ^{o}C.

Stability of resistance.

A number of lineages of *E. coli* were previously adapted to three compounds, alone and in a combination of three (Citterio et al., 2016, submitted). Seventeen clones were isolated from these lineages and re-cultured in absence of compound for five, ten and fifteen re-inoculation steps. An overnight culture of the strain was inoculated in liquid MHB and grown overnight at 37 ^{o}C at 250 rpm on orbital shaker. Cultures were grown in Sterilin tubes (VWR, 212-7400) at 37 ^{o}C at 250 rpm. An amount of 10 µl was inoculated into 990 µl MHB giving 1 ml of final volume. Throughout the experiment serial transfers of 10 µl of bacterial culture to fresh medium were performed when growth was visible as turbidity. Purity of the lineages was checked by streak on MHB agar plates. The total number of passages in absence of compound was fifteen, equivalent to approximately 105 generations. Frozen stock cultures were prepared after five, ten and fifteen re-inoculation steps, respectively.

Determination of Minimum Inhibitory Concentration (MIC).

MIC of the compounds or MIC of the combination toward which they were adapted to was tested after the five, ten and fifteen re-inoculation steps, respectively (Table 2). When testing the combination of three, each was included at 33% of the total. MIC was determined as described in (Citterio et al., 2016) in accordance with the CLSI guidelines (2012). Microdilution series of the compounds was prepared in UV-sterilized 96-well polypropylene plates (Thermo Scientific Nunc, 267334) with sterile polystyrene lids (Sigma Aldrich, CLS3930-100EA). Working solutions of peptidomimetics and AMPs were prepared in MilliQ water. Fifty µl of compound solution and 50 µl of MHB were dispensed into the first well of each row and then two-fold diluted giving a final concentration range of 128-0.25 µg/ml. Starting concentration was increased to 1024 µg/ml for the most resistant clones. Overnight culture of the strain was diluted in sterile 0.9% NaCl to OD_{546} 0.2 (accepted range: 0.195-0.210) corresponding to approximately 1 x 10^8 CFU/ml. This suspension was further diluted 1:100 in MHB and 50 µl of this culture were inoculated into microtiter plates to reach a final concentration of 5 x 10^5 CFU/µl in a final volume of 100 µl. Plates were incubated for 20 h at 37 ^{o}C. Growth was determined visually either as turbidity or as formation of a pellet. MIC

was the lowest concentration of peptidomimetics or AMPs where no growth was observed. All MIC assays were performed as two independent replicates.

Whole-genome sequencing and variant calling.

Twelve clones were whole genome sequenced immediately after adaptation (Table 4); five of these have been published in Citterio et al., (2016, submitted) whereas seven genomes were sequenced *de novo* as part of the present study. Of the twelve clones, six that retained high MIC after fifteen re-inoculation steps were selected for whole-genome sequencing along with two clones, for which MIC have decreased to MIC in wild type *E. coli* (Table 5). An overnight culture of *E. coli* (1.5 ml) was grown to stationary phase and genomic DNA was extracted with the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany). Quality of extracted DNA was assessed by 1% agarose gel electrophoresis and absorbance measurement at DeNovix DS-11 Spectrophotometer. Quantification was done by Qubit 2.0 Fluorometer (Invitrogen, United Kingdom). Preparation of genomic DNA libraries and sequencing was performed at the Novo Nordisk Foundation Center for Biosustainability (Hørsholm, Denmark). Libraries were prepared with TruSeq Nano HT (Illumina, San Diego, USA) and fragmented to an average size of 350-400 bp with CovarisE220. Average library size after adapter ligation was 500-550 bp, determined by Fragment Analyzer and Standard Sensitivity NGS kit. Concentrations of the final libraries were measured by Qubit, dsDNA broad range assay. Libraries were used for paired-end sequencing of genomes using the Illumina sequencing technology on a NextSeq v2 Mid Output 300 cycles (2x150). Average genome coverage was 104×. All the sequenced genomes from adapted clones were mapped with the wild type strain (*Escherichia coli* ATCC 25922) as reference. The obtained sequence reads of the wild type strain were *de novo* assembled in CLC Genomics Workbench, version 8 (CLC Bio, Aarhus, Denmark) resulting into 156 contigs, comprising a total number of 5122028 bases, with a genome coverage of 89×. The assembled genome has been deposited at GenBank under the BioProject PRJNA309047. The 156 contigs were annotated by Rapid Annotation using Subsystem Technology (Aziz et al., 2008). Variants were called by fixed ploidy variant detection in CLC Genomics Workbench, in order to identify point mutations such as Single Nucleotide Polymorphisms (SNPs) and deletion-insertion-polymorphism (DIPs). Variants with a frequency above 60% were considered for further analysis. We inferred functional consequences by searching for amino acid changes, and then filtering non-synonymous mutations.

Results

We selected a number of clones from an adaptive evolution experiment, which were highly resistant to peptoids/AMPs (Citterio et al., 2016, submitted). In the present work these clones were sub-cultured for several generations in order to determine whether resistance was maintained in absence of a selection pressure exerted by the respective compounds. A reduction in MIC following this prolonged culturing in absence of peptide was observed for six out of the seventeen original clones upon the 5^{th} re-inoculation (n-iv4, P94-iii1, P94-iii4, c-i2, c-ii4, c-iv4). Only slight additional reduction of MICs was seen after the 10^{th} re-inoculation. Interestingly, for three clones (n-iv4, P94-iii1, c-i2) MIC values were even higher than they were after the 5^{th} re-inoculation. Upon the 15^{th} re-inoculation, eight of 17 clones still retained resistance, *i.e.* there was a 8- to 64-fold increase in their MICs as compared to the original MIC in wild-type *E. coli* (Table 2).

After fifteen re-inoculation steps, we selected six clones with retained elevated MICs along with two clones (P94-iii4 and c-iv4), for which MIC had reverted to the original MIC in the wild-type *E. coli* (Table 2; depicted in bold). MIC of eleven compounds along with the well-known peptide antibiotic polymyxin B was assessed in the same clones in order to determine susceptibility and potential cross-resistance (Table 3). Almost all clones, retaining resistance to P9-4, HF-1002-2, novicidin, and to the combination of all three, were also resistant to compounds **1**, **3** (both with intracellular targets) and to some extent to compound **6** (Lys-rich, 20 amino acid long, membrane-active). By contrast, the resistant clones were sensitive to compounds **9** (Arg-rich; with IR motifs; 8 amino acid long), **11** (D-peptide, Lys/Phe-rich, 15 amino acids long) and **8** (Lys-rich; 4 amino acid long), all assumed to be membrane-active compounds. The resistant clones were also moderately sensitive to compound **2** (Arg-rich; with RW motifs; 12 amino acid long). There was a similar MIC pattern of the membrane-active compounds **5** and **6** (both Lys-rich) for all the resistant clones (n-iv4; c-i2; P94-iii5; HF-i5; HF-iii5; HF-iv5).

Clone P94-iii4 was susceptible to all the compounds, as opposed to clone c-iv4, that showed resistance to compounds **1** and **3**, despite the fact that resistance to the combination was lost after the fifteen re-inoculation steps.

MIC of polymyxin B (PmB) was higher than the wild-type *E. coli* for all the tested clones. In particular the MIC of PmB against c-i2, HF-i5 and HF-iv5-PMB was 16-fold higher than MIC of PmB against wild-type *E. coli*.

The clones tested for cross-resistance were also whole-genome sequenced in order to determine whether the mutations acquired after adaptation to P9-4, novicidin, HF-1002-2 and the combination of all three compounds, respectively, were preserved after continued culturing with fifteen re-inoculation steps in absence of compound.

Discussion

All clones that were evolved to gain resistance against novicidin, P9-4, HF-1002-2 and the combination of all three compounds, were also resistant to compounds **1**, **3** and **6** indicating that they share a common mode of action, and thus may be susceptible to similar resistance mechanisms. This evolved resistance in these clones comprises compounds **1** and **3** that both have intracellular activity (Zhu et al., 2007; Lele et al., 2013), and this may indicate that compound **6** also act via an intracellular target. AMPs with intracellular mechanisms are known to act slower than membrane-active AMPs (Giacometti et al., 1998). The killing mechanism of membrane-active compounds is known to involve multiple possible target molecules, while AMPs with intracellular activity are more likely to have a specific target (Jenssen et al., 2006). Hence, AMP-resistant mutants may be able to evolve resistance to such compounds more readily than to membrane-active AMPs. For some novel synthetic AMPs it has been found that their mechanism of action is not clarified as yet, and it may involve a combination of both membrane activity and intracellular targets (Cassone and Otvos, 2010). However, so far there have been no previous reports with clear evidence supporting that cross-resistance to intracellularly acting compounds occurs more likely than to membrane active ones, as our study infers. Also, proteolysis and extrusion by efflux pumps are very common means by which bacteria adapt to AMPs (Band and Weiss, 2015; Andersson et al., 2016). Hence, the specific structure (i.e. sequence) of an AMP may be responsible for its susceptibility toward proteolytic degradation. In order to investigate this, the array of AMPs might be tested against a few selected known mutant strains with characteristic resistance patterns.

The specific subclasses of AMPs included in the test array were initially chosen based on previous studies on rational design of AMPs. Interestingly, the subtypes comprised by this array appear to correlate well with susceptibility of resistant clones. There was susceptibility in all tested clones toward compounds **2** and **9** (which both are Arg-rich), and the ultra-short lipidated compound **8** as well as the Lys/Phe-rich D-peptide **11**. These four compounds may have a number of targets within the bacterial membrane that allows them to overcome the mechanisms of resistance acquired by all tested evolved clones. In this respect, it was already pointed out that

small differences in the mechanism of action might conserve the activity of a compound in AMP-resistant clones (Macwana and Muriana, 2012). Compound **2** is a 12-residue peptide; compound **8** is Lys-rich (consisting of 4 amino acids). The 8-residue compound **9** contains IR motifs and represents an analogue of an octapeptide previously highlighted for its broad-spectrum and lack of toxicity (Ong et al., 2013; Lau et al., 2015). Hence, these structural features might be further explored in order to confirm their advantages in synthetic drugs for which resistance development is a prominent concern.

Other experimentally adapted clones could be tested or, even better, naturally resistant bacteria or clinical resistant isolates.

This study highlights that AMP-resistant mutants become susceptible as the wild-type strain upon prolonged exposure to sub-lethal concentrations of compounds with certain features. The relevance of this discovery will be further acknowledged once natural human AMPs such as LL-37 are tested towards AMP-resistant mutants.

References

Andersson, D.I., Hughes, D., Kubicek-Sutherland, J.Z. (2016). Mechanisms and consequences of bacterial resistance to antimicrobial peptides. *Drug Resist Updates* 26, 43-57. http://doi.org/10.1016/j.drup.2016.04.002

Aziz, R.K., Bartels, D., Best, A.A, DeJongh, M., Disz, T., Edwards, R.A, et al. (2008). The RAST server: rapid annotations using subsystems technology. *BMC Genomics* 9, 75. doi:10.1186/1471-2164-9-75.

Band, V.I., Weiss, D.S. (2015). Mechanisms of antimicrobial peptide resistance in Gram-negative bacteria. *Antibiot* 4, 18-41.

Baym, M., Stone, L. K., Kishony, R. (2016). Multidrug evolutionary strategies to reverse antibiotic resistance. *Science* 351(6268), aad3292.

Bell, G., Gouyon, P.H. (2003). Arming the enemy: the evolution of resistance to self-proteins. *Microbiology* 149, 1367-1375.

Bonke, G., Vedel, L., Witt, M., Jaroszewski, J., Olsen, C., and Franzyk, H. (2008). Dimeric building blocks for solid-phase synthesis of α-peptide-β-peptoid chimeras. *Synthesis* 15, 2381-2390.

Cassone, M., Otvos, L. (2010). Synergy among antibacterial peptides and between peptides and small-molecule antibiotics. *Expert Rev Anti-Infect Ther* 8(6), 703–716. doi:10.1586/eri.10.38

Citterio, L., Franzyk, H., Palarasah, Y., Andersen, T.E., Mateiu, R.V., and Gram, L. (2016). Improved in vitro evaluation of novel antimicrobials: potential synergy between human plasma and antibacterial peptidomimetics, AMPs and antibiotics against human pathogenic bacteria. *Res Microbiol* 167, 72-82.

Citterio, L., Franzyk, H., Nielsen, H.M. and Gram, L. (2016). Adaptive laboratory evolution of *E. coli* reveals slow resistance development to the short antimicrobial peptide P9-4 and to a combination of three antimicrobial compounds. *Submitted.*

Fox, J.L. (2013). Antimicrobial peptides stage a comeback. *Nat Biotechnol* 31(5), 379-382.

Giacometti, A., Cirioni, O., Greganti, G., Quarta, M., Scalise, G. (1998). *In Vitro* activities of membrane-active peptides against Gram-positive and Gram-negative aerobic bacteria. *Antimicrob Agents Chemother* 42, 3320-3324.

Hein-Kristensen, L., Franzyk, H., Holch, A., Gram, L. (2013). Adaptive evolution of *Escherichia coli* to an α-peptide/β-peptoid peptidomimetic induces stable resistance. *PloS ONE 8*(9), e73620.

Hong, J., Hu, J., Ke, F. (2016). Experimental induction of bacterial resistance to the antimicrobial peptide tachyplesin I and investigation of the resistance mechanisms. *Antimicrob Agents Chemother* 60(10), 6067-6075.

Imamovic, L., Sommer, M. O. A. (2013). Use of collateral sensitivity networks to design drug cycling protocols that avoid resistance development. *Sci Transl Med* 5(204), 204ra132.

Jenssen, H.; Hamill, P.; Hancock, R. E. W. (2006). Peptide Antimicrobial Agents. *Clin Microbiol Rev* 19, 491-511.

Jochumsen, N., Marvig, R. L., Damkiær, S., Jensen, R. L., Paulander, W., Molin, S., Jelsbak, L., Folkesson, A. (2016). The evolution of antimicrobial peptide resistance in Pseudomonas aeruginosa is shaped by strong epistatic interactions. *Nat Commun* 7, 13002.

Lau, Q. Y., Choo, X. Y., Lim, Z. X., Kong, X. N., Ng, F. M., Ang, M. J., Hill, J., Chia, C.B. (2015). A head-to-head comparison of the antimicrobial activities of 30 ultra-short antimicrobial peptides against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*. *Int J Pept Res Ther* 21(1), 21-8.

Lele, D. S., Talat, S., Kaur, K. J. (2013). The presence of arginine in the Pro-Arg-Pro motif augments the lethality of proline rich antimicrobial peptides of insect source. *Int J Pept Res Ther* 19(4), 323-330.

Macwana, S., Muriana, P.M. (2012). Spontaneous bacteriocin resistance in *Listeria monocytogenes* as a susceptibility screen for identifying different mechanisms of resistance and modes of action by bacteriocins of lactic acid bacteria. *J Microbiol Methods* 88, 7-13.

Olsen, C.A., Ziegler, H.L., Nielsen, H.M., Frimodt-Møller, N., Jaroszewski, J.W., Franzyk, H. (2010). Antimicrobial, hemolytic, and cytotoxic activities of β-peptoid- peptide hybrid oligomers: improved properties compared to natural AMPs. *ChemBioChem* 11, 1356-1360.

Ong, Z. Y., Gao, S. J., Yang, Y. Y. (2013). Short synthetic b-sheet forming peptide amphiphiles as broad spectrum antimicrobials with antibiofilm and endotoxin neutralizing capabilities. *Adv Funct Mater* 23, 3682-3692.

Perron, G.G., Zasloff, M., Bell, G. (2006). Experimental evolution of resistance to an antimicrobial peptide. *Proc Biol Sci* 273, 251-256.

Ulvatne, H. (2003). Antimicrobial peptides: potential use in skin infections. *Am J Clin Dermatol* 4,

591-5.

Wimley, W.C., Hristova, K. (2011). Antimicrobial peptides: successes, challenges and unanswered questions. *J Membr Biol* 239 (1-2), 27-34.

Zaiou M. (2007). Multifunctional antimicrobial peptides: therapeutic targets in several human diseases. *J Mol Med* 85, 317-29.

Zhu, W.L., Hahm, K.-S., Shin, S.Y. (2007). Cathelicidin-derived Trp/Pro-rich antimicrobial peptides with lysine peptoid residue (Nlys): therapeutic index and plausible mode of action. *J Pept Sci* 13(8), 529–35.

Table 1: Characteristics of the compounds used in the present study and their MIC in *E. coli* ATCC 25922.

c* = combination of novicidin + P9-4 + HF-1002-2. PMB = polymyxin B.

Predicted mechanism of action	Sub-class	No/name	Amino acid/chemical sequence	Length (aa)	Charge	MW (g/mol)	MIC in *E. coli*	
							µg/ml	µM
Intracellular target	Arg/Pro-rich	3	RRIRPRPPRLPRPRPRP-NH_2	17	+9	3198.89	4	1.25
	Trp/Arg-rich	1	VRRFkWWWkFLRR-NH_2	13	+7	2761.55	8	2.89
Membrane active	Lys/Trp-rich	10	$(KW)_4$-NH_2	8	+5	1844.68	16	8.67
	Trp/Arg-rich	P9-4	KWRRWIRWL-NH_2	9	+5	1968.85	8-16	6.09
	Arg-rich	2	RRWRIVVIRVRR-NH_2	12	+7	2462.26	2-4	1.21
	Arg-rich; β–sheet	9	$(IRIR)_2$-NH_2	8	+5	1664.53	8	4.8
	Lys/Arg-rich	novicidin	KNLRRIIRKGIHIIKKYF-NH_2	18	+9	3322.09	4-16	3.01
	Lys-rich	4	KWKLFKKVLKVLTTG-NH_2	15	+6	2472.41	4	1.6
	Lys-rich	5	KFLKKAKKFGKAFVKIL-NH_2	17	*+8*	2905.80	4	1.3
	Lys-rich	6	KWKSFIKKLTKKFLHSAKKF-NH_2	20	+9	3519.35	2-4	0.85
	Lys-rich; lipidated	8	Pam-KK*K*K-NH_2 Lipidated Lys-rich	4	+4	1224.22	4-8	4.9
	Lys/Phe-rich; D-peptide	11	Ac-**FKKLKKLFSKLFSFK**-NH_2	15	+6	2614.58	8	3.05
	Ala-rich	7	ALWKTLLKKVLKAAAK-NH_2	15	+6	2465.45	2-4	1.21
	Polypeptide with fatty acid chains	PMB	$C_{55}H_{96}N_{16}0_{13}2H_2SO_4$	-	-	1385.61	0.25-0.5	0.27
Unknown	Achiral peptoid / Lys-hArg	HF-1002-2	H-(Lys-βNphe-hArg-βNphe)$_3$-NH_2	12	+7		4-8	
	Lys/Arg-rich+ Trp/Arg-rich+ Achiral peptoid / Lys-hArg	c*	-	-	-		8	

Table 2: MIC values for *E.coli* clones selected after adaptive laboratory evolution, at the end of the adaptation, and after five, ten and fifteen re-inoculation steps in absence of compound, respectively. The assay was repeated in two independent experiments. Clones are named after the compound they were adapted to (n = novicidin, HF = HF-1002-2, c = combination), the lineage (i to iv) and the clone number (1 to 5). In bold, clones that were selected for whole-genome sequencing analysis (Table 4) and assessment of cross-resistance (Table 3).

Clone	**MIC (µg/ml) after ALE**	**MIC (µg/ml) after culturing steps without compound**			**Fold increase from *wt* MIC**
		5th	**10th**	**15th**	
n-iv4	64	16	32-64	32-64	4-8
HF-i1	256	256	128	64	8-16
HF-i2	256	256	128-256	64-128	16
HF-i3	256	512	128	64-128	16
HF-i4	256	256	128	64-128	16
HF-i5	128-256	256	128	64-128	16
HF-iii5	16-64	32	32	16-32	4
HF-iv5	256-512	512	256	128-256	32
P94-iii1	32-64	16	16-32	16	1-2
P94-iii3	32-64	32	16-32	32	2-4
P94-iii4	32	16	16	8-16	1
P94-iii5	32-64	32-64	16	64	4-8
c-i1	16-32	16	8	8-16	1-2
c-i2	32	16	16-32	16-32	2-4
c-ii4	16-32	8-16	8-32	8-16	1-2
c-iv2	16-128	8-32	8-16	8-16	1-2
c-iv4	64-128	8	4-8	4-16	1.25

Table 3: Fold increase of *E. coli* MIC to peptides as compared to *wt* MIC values of compounds **1**-**11** and polymyxin B (PMB) for selected clones that were re-cultivated for fifteen steps in absence of compound used in the adaptation. The assay was repeated in two independent experiments. n = novicidin, HF = HF-1002-2, c = combination, lineage number from i to iv, clone number from 1 to 5. * MIC of the compound (or the combination) to which resistant and susceptible clones were previously adapted to.

Clones considered susceptible

Compound	**MIC (µg/ml)**	**Fold increase from *wt* MIC in selected clones**							
	wt	**n-iv4**	**c-i2**	**c-iv4**	**P94-iii4**	**P94-iii5**	**HF-i5**	**HF-iii5**	**HF-iv5**
*****	-	4-8	2-4	1.25	-	4-8	16	4	32
1	8	16	>16	16	0.5-1	32	64	32	64
3	4	8-16	32	16	0.5-4	16-32	32	8-32	32
6	2-4	16-32	32-64	8	1-2	8-16	2-4	4-8	2
5	4	8-16	32	2-4	2-4	8	2	2-4	2
PMB	0.25-0.5	2-4	16	4	2	8	16	2-4	16
7	2-4	8	8-16	2	2	4-8	2-4	2-4	1-2
4	4	8	8	2	1-2	8	4-8	4	4
10	16	1-2	8	2	1	2	4	2-4	8
2	2-4	0.5-1	2-4	2-8	1	2-4	2-4	4	2-4
8	4-8	2	2-4	1-2	1-2	2	1-2	1	1-2
11	8	2-4	2	2-4	1-2	2-4	2	1-2	0.5
9	8	1	2	1	1-2	1-2	1	1	1

Table 4: Single nucleotide variants (SNVs) in clones that were whole-genome sequenced after adaptation and after fifteen re-inoculation steps. The total number of amino acid changing SNVs and a selection of the related mutational changes are shown. CDP = CDP-glycerol: N-acetyl-β-D-mannosaminyl-1,4-N-acetyl-D-glucosaminyldiphosphoundecaprenyl glycerophosphotransferase; n = novicidin, HF = HF-1002-2, c = combination, lineage number from i to iv, clone number from 1 to 5.

Clones	**Number of SNVs after ALE**	**Mutational changes**	**Number of SNVs after 15 re-inoculation steps**	**Mutational changes**
n-iv4	3	- CDP - Transcriptional activator of maltose regulon 2C MalT - S-formylglutathione hydrolase	10	- CDP - Transcriptional activator of maltose regulon 2C MalT - S-formylglutathione hydrolase
c-i2	1	- CDP	9	- CDP - GTP pyrophosphokinase - Respiratory nitrate reductase - Propionate CoA ligase
c-iv4	7	- CDP	10	- CDP - poT spermidine ABC transporter - Glutamate synthase
P94-iii4	1	- CDP	8	- T1SS secreted agglutinin RTX
P94-iii5	7		3	- Glucose-1-phosphate thymidylyltransferase
HF-i5	2	- CDP - Outer membrane protein assembly factor YaeT precursor	12	- CDP - 1 2 C4-α-glucan glycogen branching enzyme 2C - Outer membrane protein assembly factor YaeT precursor
HF-iii5	1		4	- CDP
HF-iv5	64	- CDP	57	- CDP