CURBING MULTIDRUG-RESISTANT BACTERIA WITH NOVEL HOST DEFENSE PEPTIDE FRAGMENTS

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Abbreviations

AMP	Antimicrobial Peptide
AMR	Antimicrobial resistance
ATCC	American Type Culture Collection
CCCP	Carbonyl cyanide m-chlorophenyl hydrazine
CFU	Colony forming units
DC	Dendritic cell
$DiBAC_4(3)$	Bis-1,3-dibutylbarbituric acid-trimethine-oxonol
$DiOC_2(3)$	3,3'- diethyloxacarbocyanine iodide
DMEM	Dulbecco Modified Eagle Medium
DSMO	Dimethyl sulfoxide
DSMZ	German Collection of Microorganisms and Cell Cultures GmbH
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
GALT	Gut associated lymphatic tissue
GIT	Gastrointestinal tract
GOT	Glutamate-oxalacetate-transaminase
HAc	Acetic acid
HBD	Human beta defensin
HD	Human defensin
HDP	Host defense peptide
HNP	Human neutrophil peptide
HPLC	High performance liquid chromatography
IL	Interleukin
kDA	kiloDalton
LB	Luria-Bertani broth
LD	Lethal dose
LDH	Lactate dehydrogenase
MIC	Minimal inhibitory concentration
MRGN	Multidrug-resistant Gram-negative
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass spectrometry
Na_2HPO_4	Sodium hydrogen phosphate

NaH_2PO_4	Sodium dihydrogen phosphate
NH_4HCO_3	Ammonium bicarbonate
OD	Optical density
PBS	Phosphate buffered saline
PI	Propidium iodide
RBC	Red blood cell
RDA	Radial Diffusion Assay
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
TLR	Toll like receptor
TSB	Tryptic soy broth
Triton X-100	Polyethylene glycol *tert*-octylphenyl ether
VRE	Vancomycin-resistant *Enterococcus*
w/v	Weight per volume
YPD	Yeast peptone dextrose

Summary

The increasing occurrence of multidrug-resistant microorganisms represents a substantial threat for mankind. To-date, antibiotic-resistant bacteria are responsible for more than 670,000 infections and nearly 33,000 deaths in the European Union every year - with upward tendency. None of the common antibiotics are effective against these bacteria. Because of this alarming development, WHO has been forced to publish a dossier to draw attention to the prioritization of research and development of novel antibiotics.

Host defense peptides (HDPs) – formerly known as antimicrobial peptides – are an important component of the innate immune system and possess a broad range of antimicrobial and immune modulatory properties to control infections. Defensins, the most prominent class of HDPs in humans, has been subject to extensive research focus as potential therapeutics. These small cationic molecules can be classified into α- and β-defensins by their characteristic arrangement of disulphide bridges. Unfortunately, the production of these precisely folded peptides on an industrial scale is a technically complex and expensive process. Thus, new cost-effective strategies more resilient to multidrug resistance are urgently needed. An innovative concept to overcome some of the production-associated hurdles is to generate antimicrobial active peptide fragments by proteolytic cleavage.

Herein, we used the strategy of proteolytic digestion of HDPs to generate new biologically active fragments. The linearized forms of the human neutrophil peptide-4 and human β-defensin-1 are susceptible to proteolytic degradation and served as precursors. This way, we discovered HNP-$4_{1\text{-}11}$ and an eight-amino acid carboxyl-terminal fragment of hBD1, called octapeptide. Both fragments displayed a broad antimicrobial spectrum without showing toxicity to human cells.

In addition, we modified the octapeptide N-terminal with palmitic acid together with different spacers such as sugars or amino acids to create a set of unique lipopeptides (Pam's) with increased stability and bactericidal activity. The most promising peptide, Pam-3 exhibited prominent antimicrobial activity against multidrug-resistant ESKAPE pathogens and additionally eradicated already established biofilms *in vitro*, without inducing drug-resistance. In mouse models, Pam-3 selectively reduced acute intestinal *Salmonella* and established *Citrobacter* infections, without compromising the core microbiota, hence displaying an added benefit to traditional broad-spectrum antibiotics.

Taken together, we demonstrated that small peptide fragments on the basis of human defensins have great potential to combat multidrug-resistant infections. Further studies are warranted to unleash their full potential and hopefully opening a new chapter of effective treatment strategies.

Zusammenfassung

Das zunehmende Auftreten von multiresistenten Mikroorganismen stellt eine ersthafte und nicht zu unterschätzende Gefahr für die Menschheit dar. Schon heute sind antibiotika-resistente Bakterien jährlich für mehr als 670,000 Infektionen und annährend 33,000 Tote in der Europäischen Union verantwortlich – Tendenz steigend. Denn gegen multiresistente Bakterien hilft oft keines der gängigen Antibiotika, was besonders in Krankhäusern zu einem immer häufigeren Problem werden wird. Aufgrund dieser besorgniserregenden Entwicklung sah sich WHO genötigt ein Dossier zu veröffentlichen, um auf die Priorisierung von Forschung und Entwicklung neuer Antibiotika gegen multiresistente Bakterien aufmerksam zu machen.

Ein vielversprechender Ansatz sind antimikrobielle Peptide (AMPs), die ein wichtiger Bestandteil des angeborenen Immunsystems sind und ein breites Wirkspektrum zur Kontrolle von Mikroorganismen besitzen. Defensine, eine der bedeutendsten Gruppen humaner AMPs, stehen seit einiger Zeit vermehrt als potentielle Therapeutika im Fokus. Diese werden durch ihre charakteristische Anordnung von Disulphidbrücken in α- und β-Defensine eingeteilt. Die Herstellung dieser präzise gefalteten Peptide in industriellem Maßstab ist jedoch technisch hoch komplex und teuer. Ein neues innovatives Konzept um diese Hürden zu umgehen, stellt die Generierung antimikrobiell aktiver Peptidfragmente durch proteolytische Spaltung dar.

In dieser Arbeit wurden deshalb Fragmente von α- und β-Defensine auf ihr Potential zur Bekämpfung antibiotika-resistenter Bakterien untersucht. Dafür wurden zunächst das humane α-Defensin 4 (HNP-4) und das humane β-Defensin 1 (hBD1) proteolytisch gespalten, um verschiedene Fragmente zu generieren. Von den so entstandenen Fragmenten wurden HNP-$4_{1\text{-}11}$ und ein 8-Aminosäure-langes hBD1 Fragment (Octapeptid) auf ihre antimikrobielle Aktivität gegen Bakterien und Pilze sowie die Toxizität in humanen Zelllinien untersucht. Beide Fragmente zeigten bakterizide Wirkung und eine gute *in vitro* Verträglichkeit.

Auf Basis des Octapeptids wurden verschiedene Lipopetide (Pam-1-5) entwickelt. Pam-3 zeigt herausragende bakterizide Wirkung gegen multiresistente Bakterien des ESKAPE-Panels und eliminiert zusätzlich etablierte Biofilme *in vitro*. Dabei kommt es bei einer verlängerten Exposition von Bakterien nicht zur Entwicklung von Resistenzen. Zusätzlich führte die orale Gabe von Pam-3 in intestinalen Infektionsmodellen in *vivo* zu einer selektiven Reduzierung der pathogenen Bakterien ohne die Mikrobiota zu beeinträchtigen.

Abschließend betrachtet bestärkt diese Arbeit die Erforschung und Entwicklung neuer antimikrobieller Substanzen auf Basis von humanen Defensin-Fragmenten. Weitere Studien sind daher nötig, um das volle Potential dieser vielversprechenden Kandidaten im Kampf gegen multiresistente Bakterien zu erhalten.

1 Introduction

1.1. The human gastrointestinal system

The human gastrointestinal (GI) tract is an amazing organ system with area size of up to 40 square meters in an adult person. It spans multiple organs and crosstalk with all parts of the human body. The GI tract consists of mouth, esophagus, followed by the stomach, small and large intestines and ends with the rectum and anus. Main function is digestion, absorption of nutrients and energy, but it also plays an important role in immunity, fluid and electrolyte balance, detoxification, neuroendocrine axis and expulsion of waste as feces (Kim & Pritts, 2017).

The GI tract is constantly colonized by an immense number of microorganisms, collectively called the gut microbiota. This symbiotic relationship affects many aspects of host physiology, starting from GI tract development, nutrient metabolism (Bäckhed *et al*, 2004), immune system maturation and function (Hooper *et al*, 2012; Belkaid & Hand, 2014; Honda & Littman, 2016; Rooks & Garrett, 2016), up to shaping the epigenetic landscape (Kelly *et al*, 2018). In addition, the gut microbiota can even influence brain development, function and behavior (Collins *et al*, 2012; Stilling *et al*, 2014; Sampson & Mazmanian, 2015). But this unique symbiosis also bears risks and requires close monitoring. Our knowledge is still limited, studies are ongoing, but by understanding this complex interaction many questions will hopefully be answered.

1.1.1. Host barrier

The human body interacts with the environment at multiple sites, including oral cavity, mucosae of the airways, genitourinary tract, skin and GI tract. Latter one covers a surface of approximately 32 m^2 and is the most exposed system to the outside world (Helander & Fändriks, 2014). Besides digestion and resorption of nutrients and water, the immense surface is in constant contact with commensal microorganisms, pathogens and harmful substances. Protection of the host against microbial invasion as well as shaping the composition of the gut microbiome is only possible due to a complex defense system including an effective intestinal host barrier (König *et al*, 2016). The intestinal barrier, which is a demarcation between the intestinal lumen and the inner body tissue (Fig. 1), is organized as a multilayer system of two main components – first, the physical barrier composed of an epithelial layer and the overlaying mucus. Second, the biochemical barrier generated by various components of the immune system (Vancamelbeke & Vermeire, 2017).

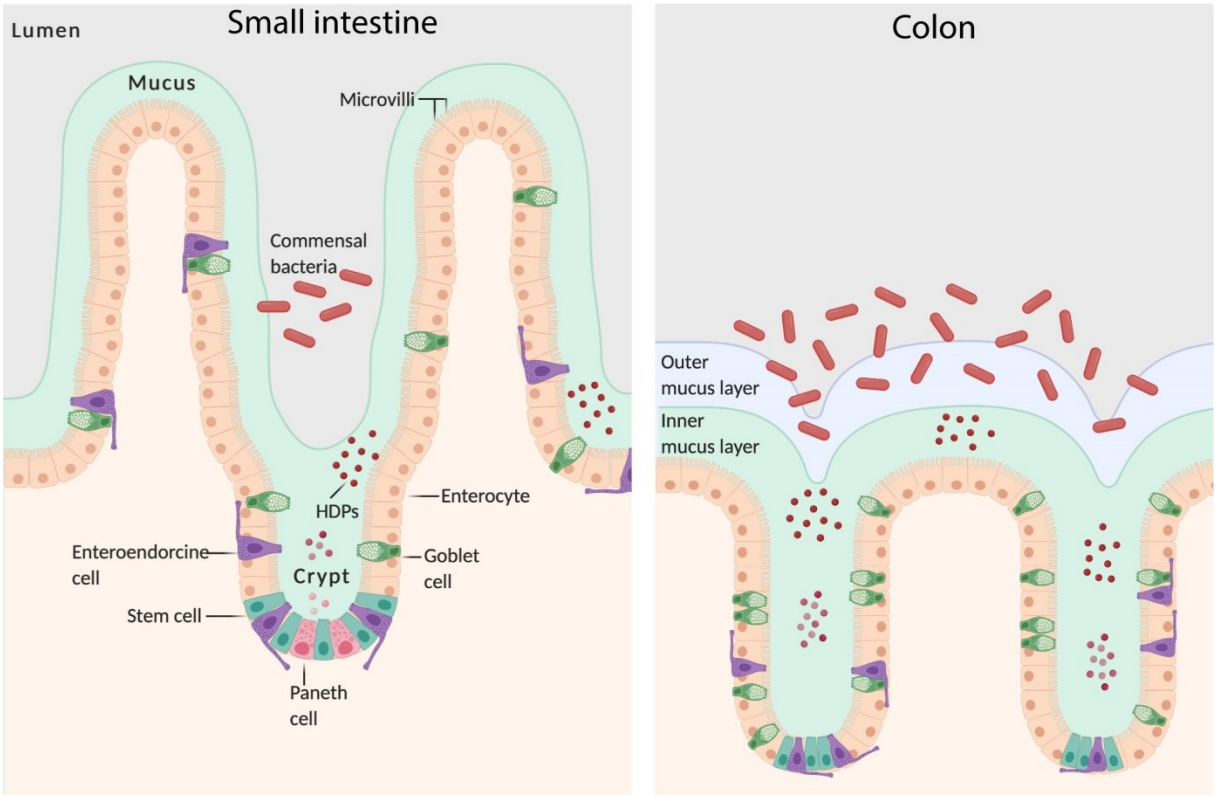

Figure 1: Composition of the intestinal barrier. The intestinal barrier consists of a physical and biochemical component to separate effectively the luminal microbiota and the mucosal immune system. Intestinal epithelium cells (enterocytes) and various specialized cells form together with the overlaying mucus the physical barrier. Stem cells regulate the continuous renewal of the epithelial cell layer, whereas goblet cells produce mucins that form the mucus layer. Host defense peptides (HDPs) are secreted by Paneth cells and enterocytes and are important component for the biochemical barrier. Adapted from (Mowat & Agace, 2014) and created with BioRender.com.

1.1.1.1. Physical barrier - Epithelium and mucus

Key to the coexistence of the gut microbiota and mucosal immune system is the capacity to maintain the segregation between luminal microbial communities and the host. The intestinal epithelium cells are by far the strongest element of the physical intestinal barrier against bacterial invasion. Pluripotent intestinal epithelial stem cells that reside at the crypts spawn various distinct cell types such as absorptive enterocytes, goblet cells, Paneth cells, enteroendocrine cells and microfild cells (Peterson & Artis, 2014). Connected via tight junctions, adherent junctions and desmosomes, these cells together form a continuous, sealed cellular barrier separating the lumen from the lamina propria (Groschwitz & Hogan, 2009).

The intestinal mucus layer represents the first line of physical defense against microbial encroachment, created by goblet cells secreting highly glycosylated mucins into the lumen (Vancamelbeke & Vermeire, 2017). The most prominent of these mucins, mucin 2 is essential for the organization of the two mucus layer in the colon: an outer, loose layer and an inner dense layer. While these layers are structurally different and physically separated, they contain similar biochemical components as the outer layer is built by the inner. Surprisingly, they differ in their

characteristics. As such, the outer layer is loose and allows long-term colonization of commensal bacteria, thus contrasting the – in a healthy state - sterile inner mucus layer (Johansson *et al*, 2008; Hansson & Johansson, 2010). Unlike the colon, the small intestine has only a single, loose and unattached mucus layer, allowing limited diffusion of bacteria. Peristaltic and secretion keeps the epithelium free by moving detached mucus with trapped microorganisms forward for expulsion in the feces (Ermund *et al*, 2013).

1.1.1.2. Biochemical barrier - Innate and adaptive immune factors

The intestinal barrier is reinforced by the innate immune system, which provides an effective biochemical barrier. Most notably host defense peptides (HDPs), secreted by various intestinal epithelia cells throughout the small intestine and colon (Hooper & Macpherson, 2010). HDPs are produced by virtually all intestinal epithelial cells, including enterocytes, goblet cells and Paneth cells. Paneth cells of the small intestine are capable of producing many additional HDPs, such as α-defensins, cathelicidins and lysozyme in the crypts (Su *et al*, 2009; Turner, 2009; Bevins & Salzman, 2011). Most of these peptides disrupt highly conversed and essential features of bacterial biology. Thus commensal and pathogenic bacteria are controlled and resistance development limited (Gallo & Hooper, 2012). The expression of HDPs is regulated by different mechanisms. Several HDPs like the human α-defensin-6 or the human β-defensin 1 are expressed constitutively, whereas others requires bacterial signals through pattern recognition receptors. These special receptors include the outer membrane Toll-like receptors and intracellular NOD-like receptors, which recognize unique molecular patterns of bacteria and other microorganism (Peterson & Artis, 2014).

In addition, cells of the adaptive immune system present in the lamina propria of intestine and the gut-associated lymphoid tissue (GALT) are involved in the mucosal immune defense (Ahluwalia *et al*, 2017). The cellular components of GALT, such as isolated lymphoid follicles or mesenteric lymph nodes are located along the entire small and large intestine. In the small intestine, another important component of GALT, so called Peyers' patches, can be found (Mowat, 2003). The immune cells of GALT consist of T and B cells, dendritic cells (DCs) and unique cell types, including microfold cells and intraepithelial lymphocytes. Microfold cells are sampling luminal antigens and transcytose them to the epithelium where they are endocytosed by DCs. Apart from macrophages, DCs are the only professional antigen presenting cells. The antigen-loaded DCs initiate priming and maturation of naïve T and B lymphocytes, which results in the activation of adaptive immune responses (Wershil & Furuta, 2008; Koboziev *et al*, 2010).

1.2. Human microbiota

Humans are colonized by countless microorganisms mainly bacteria, but also protozoa, archaea, fungi and viruses (including bacteriophages). They reside in and on multiple body parts such as oral cavity, urogenital tract, GI tract, respiratory tract and skin (Aas *et al*, 2005; Grice *et al*, 2009; Ling *et al*, 2010; Arumugam *et al*, 2011; Dickson *et al*, 2013) . Each compartment appears to have its own unique microbiome with environment specific microorganism.

The oral microbiome is one of the most diverse in the human body with around 1000 species (Dewhirst *et al*, 2010), similar in complexity to the gut microbiome, and tend to be dominated by *Streptococcus*. Species of the commensal microbiota have been linked to the two commonest oral diseases – dental caries and periodontitis (Wade, 2013).

Similarly, the vaginal microbiota has also been associated with multiple urogenital diseases, including bacterial vaginosis, urinary tract infections and sexually transmitted diseases. In reproductive-aged women, the healthy vagina falls into at least one of five reproducible community types, four are dominated by a single Lactobacillus species, whereas the remaining one consists of a mixture with a greater abundance of anaerobic species (Ravel *et al*, 2011). But the vaginal microbiota is not static construction, although some are more stable it is not unusual that the communities frequently fluctuate between the several community types (Gajer *et al*, 2012). Remarkably, the vaginal microbiota changes specifically as a function of gestational age by increasing Lactobacillus species and decreasing anaerobic species during pregnancy (Romero *et al*, 2014).

The skin is the human body's largest organ. Major role of the skin is to serve as a physical and biochemical barrier, protecting the host from invading pathogens and infections. These are the results of a broken barrier or an imbalance between commensals and pathogens (Byrd *et al*, 2018). Skin sites differ in their physiological characteristics: sebaceous (oily), moist or dry and are colonized by *Corynebacterium*, *Propionibacterium*, and *Staphylococcus* (Grice *et al*, 2009). Similar to those in the gut, commensal skin microorganisms fulfil essential roles, including protection against invading pathogens, support of the immune system by educating T cells found in the skin and the breakdown of natural products. In special circumstances such host immune suppression even commensals like *Staphylococcus epidermidis* are capable of causing infections (Otto, 2009). Thus, the balance between health and disease depends on the interaction between the microbiota and various host components (Belkaid & Tamoutounour, 2016; Egert *et al*, 2017; Chen *et al*, 2018).

None of the listed compartments harbors more microorganisms than the GI tract, the probably most powerful system in our body.

1.2.1. Gut microbiota

The past decades have marked an explosion of research focusing on the gut microbiota and its interactions with the host. It was originally proposed that bacteria outnumber human cells by a ratio of at least 10:1. Yet, more recent research corroborate that the ratio is closer to 1:1 (Sender *et al*, 2016). The small and large intestines of the GI tract are colonized by trillions of microorganisms, including archaea, bacteria, fungi, microbial eukaryotes, protozoa and viruses (Eckburg *et al*, 2005; Ley *et al*, 2008). The composition of the gut microbiota various between individuals, genetically background, lifestyle, dietary, usage of antibiotic and geographical location (Lloyd-Price *et al*, 2016), but also among one individual depending on the period of life (Yatsunenko *et al*, 2012; Human Microbiome Project Consortium, 2012; Voreades *et al*, 2014).

The development of the infant gut microbiota is affected by many environmental and host factors (Rodríguez *et al*, 2015). Several perinatal conditions, such as mode of delivery, antibiotic usage and type of feeding have been reported to influence the composition (Mueller *et al*, 2015; Tanaka & Nakayama, 2017). Additionally, the mother's age, lifestyle and metabolic status affect the establishment of the infant microbiota, too (Milani *et al*, 2017; Ventura *et al*, 2018). The initial microbiota is mainly characterized by low diversity and undergoes multiple alterations in very early life, before becoming more diverse and reaching a more adult-like microbiota by the end of the first 3–5 years of life (Rodríguez *et al*, 2015; Derrien *et al*, 2019).

Due to the various conditions at different body sites of the intestine, the microbial density and diversity gradually increase from proximal to distal gut (Sekirov *et al*, 2010; Hillman *et al*, 2017). Only very few ingested microorganism are able to pass through the acidic environment of the stomach. Therefore, with the exception of few specialist adapted to these conditions like *Helicobacter pylori* (Montecucco & Rappuoli, 2001), the stomach and the following duodenum are barely colonized. In the small intestine, the number increases continuously from duodenum to ilium concluding with the highest density and diversity found in the colon (Sommer & Bäckhed, 2013). The vast majority present in the intestine belongs to one of only five phyla - Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria and Verrucomicrobia, however abundance and diversity on the species level can vary considerably (Schroeder & Bäckhed, 2016). Remarkably, Firmicutes and Bacteroidetes representing 90% of the gut microbiota (Arumugam *et al*, 2011). More than 200 different genera are forming the Firmicutes phylum with key members such as *Clostridium*, *Lactobacillus*, *Ruminococcous*, and important short-chain fatty acid (SCFA) producers like *Eubacterium* and *Fecalibacterium*. Bacteroidetes consists of predominant genera such Bacteroides and Prevotella, which are efficient degraders of dietary fibers (Schroeder & Bäckhed, 2016). Actinobacteria are primarily represented by the *Bifidobacterium* genus, whose taxa is mainly known as probiotics, whereas the proportionally less abundant phylum Verrumcomicrobia includes only *Akkermansia*. The last

remaining the Proteobacteria includes *Escherichia*, *Desulfovibrio* and *Helicobacter* (Schroeder & Bäckhed, 2016; Rinninella *et al*, 2019), and many common human pathogens are found in this phylum (Rizzatti *et al*, 2017).

1.2.2. Human gut microbiota in health and disease

The human gut microbiota plays a key role in host physiology. Symbiotic bacteria such as *Bacteroides*, *Lactobacillus* and *Bifidobacerium* are digesting for the host non-digestible dietary fibers through fermentation and supply this way essential nutrients (Larsbrink *et al*, 2014; Goh & Klaenhammer, 2015). Main products of fermentation are short-chain fatty acids (SCFAs), including butyrate, propionate and acetate, and serve as an important energy source (Bergman, 1990). In addition, these SCFAs are also important signals to regulate gut motility, inflammation and glucose homeostasis (Flint *et al*, 2012; Cani *et al*, 2013; Valdes *et al*, 2018). Furthermore, the gut microbiota is capable of producing and deliver vitamins to the host (Rowland *et al*, 2018). Besides nutrition, the intestinal microbiota is also a component of the gut barrier, and contribute significantly in maturation of immune system (Round & Mazmanian, 2009), lymphocyte homeostasis (Belkaid & Harrison, 2017), tissues and organ development and protecting against pathogens (Pickard *et al*, 2017; Das & Nair, 2019). Moreover, the microbiome seems to influence the development of the brain and the nervous system (Diaz Heijtz *et al*, 2011; Sharon *et al*, 2016; Ma *et al*, 2019). However, not all bacteria have beneficial effects and many diseases have been associated with alterations in the microbiota.

Disrupting the gut homeostasis results in a so called dysbiosis, a compositional and functional imbalance in the gut microbiota associated with a wide range of diseases affecting various organs (Schroeder & Bäckhed, 2016; Gorkiewicz & Moschen, 2018). A distinction can be drawn between intestinal and extra-intestinal diseases, such as atherosclerosis (Jonsson & Bäckhed, 2017; Ma & Li, 2018), asthma (Arrieta *et al*, 2015), autism spectrum disorder (MacFabe *et al*, 2007; Wang *et al*, 2012), cardiovascular disease (Tang *et al*, 2017), Alzheimer's and Parkinsons disease (Sampson *et al*, 2016; Jiang *et al*, 2017), non-alcolholic fatty liver disease (Henao-Mejia *et al*, 2012; Strowig *et al*, 2012), obesity and diabetes (Ley *et al*, 2006; Kovatcheva-Datchary *et al*, 2015; Pedersen *et al*, 2016; John & Mullin, 2016; Anhê *et al*, 2020). Prominent representatives of microbiota associated intestinal diseases include colorectal cancer (Arthur *et al*, 2012; Brennan & Garrett, 2016; Wong & Yu, 2019), irritable bowel syndrome (Chong *et al*, 2019) and inflammatory bowel disease (Wehkamp *et al*, 2005a; Halfvarson *et al*, 2017). Apart from this, the composition of the commensal microbiota protects the intestines from potential pathogens – a phenomenon called colonization resistance (Buffie & Pamer, 2013). Microbiota-mediated colonization resistance can be indirect and direct – the prior examplified by nutrient depletion and the latter by bacteriocins attacks or type 6 secretion systems (Pamer, 2016). A

secondary indirect strategy is immune-mediation, e.g. by producing and modifying host-derived metabolites such as SCFAs and bile salts, or by activating innate immune defenses in the mucosa to secret antimicrobial peptides that kill pathogens (Libertucci & Young, 2019). SCFAs can inhibit the growth of *Salmonella* Typhimurium in high concentrations and it has been shown that they can induce the production of antimicrobial peptides (Zhao *et al*, 2018). These peptides are essential components of innate immune defense and crucial for health.

1.3. Host defense peptides

Host defense peptides (HDPs), also known as *antimicrobial peptides*, are endogenous, naturally produced peptides which are found in all multicellular organisms from plants to humans (Zasloff, 2002). The antimicrobial peptide database has catalogued more than 3000 natural antimicrobial peptides to date (Wang *et al*, 2016b). In humans, they have been identified at almost all body sites and in many different cell types including epithelial cells, macrophages andneutrophils. (Ostaff *et al*, 2013).

HDPs constitute an important first line of defense, protecting the host against infections and modelling the host microbiota. HDPs exhibit a broad range of activities from killing both Gram-positive and Gram-negative bacteria as well as fungi, preventing biofilm formation or anti-viral properties (Lehrer & Ganz, 1999; Hancock & Diamond, 2000). Besides having direct antimicrobial activity, many HDPs are capable to modulate the innate immune response (Hancock *et al*, 2016).

HDPs are of amphipathic, cationic character containing amino acids with hydrophobic and cationic residues (Zasloff, 2002). Based on their 3D structure, amino acid composition and number of disulphide bonds, HDPs are classified into four major families (Fig. 2) (Wang *et al*, 2016b):

(i). The α-family consists of HDPs with α-helical structures, for example the human cathelicidin LL-37.

(ii). The β-family is composed of HDPs with beta-strands, for example the human α-defensins.

(iii). The αβ-family is composed of both helical and beta-strands in the same 3D structure, for example the human β-defensins.

(iv). The non-αβ-family contains neither helical nor beta-strands, for example the bovine indolicidin.

Although more than 3000 HPDs are identified only for less than 400 of them 3D structures are known, which is why many other systems are used besides this classification (Andersson *et al*, 2016). The three major groups of HDPs expressed in humans include the cathelicidin LL-37, histatins and defensins.

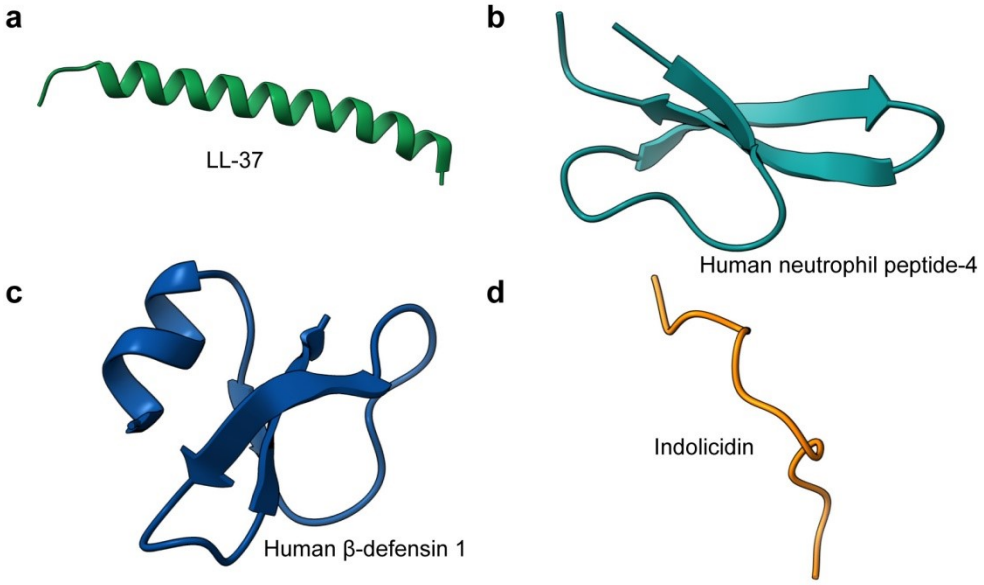

Figure 2 Structural classes of host defense peptides. **(a)** α-helical human cathelicidin LL-37 (PDB code 5nmn) (Sancho-Vaello *et al*, 2017), **(b)** β-looped human neutrophil peptide-4 (PDB code 1zmm) (Szyk *et al*, 2006), **(c)** mixed structure of human β-defensin 1 (PDB code 1iju) (Hoover *et al*, 2001) and **(d)** bovine indolicidin (PDB code 1g89) (Rozek *et al*, 2000). High-resolution images of peptide backbones were obtained from PDBsum and generated using UCSF ChimeraX (Goddard *et al*, 2018). Adapted from (Brogden, 2005).

1.3.1. Defensins

Defensins are the most widely studied family of HDPs (Wehkamp *et al*, 2005a; Harder *et al*, 2007). These small molecules consist of 28-47 amino acids, a molecular weight of 3 – 5 kDa and a cationic charge. They have a characteristic β-sheet core stabilized with six disulphide-linked cysteines, and are divided into α- and β-defensins. The θ-defensins, a third group, is inactivated in humans and only occurs in old-world monkeys (Ganz, 2003; Selsted & Ouellette, 2005).

1.3.1.1. α-Defensins

α-defensins have been identified only in mammals and are an evolutionary young group of host defense peptides (Lynn & Bradley, 2007). To date, six different α-defensins have been characterized in humans. Four of these are expressed by immune cells, namely human neutrophil peptides 1 to 4 (HNPs), and were, as the name indicate, initially found in neutrophils (Ganz *et al*, 1985; Selsted *et al*, 1985), but are also expressed in monocytes and natural killer cells (Selsted & Ouellette, 2005). The remaining two, human α-defensin 5 and 6 (HD-5 & HD-6) are expressed by Paneth cells in the small intestine (Lehrer & Lu, 2012).

All HNPs exist as propeptides within polymorphonuclear neutrophils azurophilic granules and are directly activated during their trafficking by tryptic cleavage into the active form (Liu & Ganz, 1995). These granules fuse with the lysosome after phagocytosis of pathogens allowing context specific antimicrobial activity (Ganz *et al*, 1985; Selsted *et al*, 1985; Ganz, 2003). While HNP-1; HNP-2 and HNP-3 have almost identical amino acid sequences and thus only differ in the first amino acid residues at the N-terminus, HNP-4 is quite dissimilar with respect to sequence and composition (Lehrer & Lu, 2012). Both, HNP-1 and HNP-3 consist of 30 amino acid residues, with one additional amino acid residue compared to HNP-2. In contrast, HNP-4 is composed of 33 amino acid residues with a distinct sequence from that of other HNPs (Wilde *et al*, 1989).

All HNPs exhibit antimicrobial activity against bacteria, fungi and enveloped viruses (Ganz *et al*, 1985; Daher *et al*, 1986; Lehrer *et al*, 1989; Holly *et al*, 2017). In addition to their antimicrobial activity, HNPs possess immune-modulatory properties that depend on the concentration range (Yang *et al*, 2002; Rehaume & Hancock, 2008). HNP-1 and HNP-2 show chemotactic activity for naïve T cells, immature dendritic cells and monocytes at low concentrations (Territo *et al*, 1989; Yang *et al*, 2000), whereas HNPs demonstrate cytotoxic at high concentrations (Hashimoto *et al*, 2012). The concentration of HPN-1-3 within neutrophils is very high (representing 30-50% of the total protein (Rice *et al*, 1987)), while HNP-4 accounts only for approximately 2% of the total defensin content in azurophilic granules. One possible explanation is the significantly increased antimicrobial efficacy of HNP-4 against Gram-negative bacteria, although it is less active against Gram-positive bacteria (Harwig *et al*, 1992; Ericksen *et al*, 2005).

Similar to HNPs, the two human enteric α-defensins (HD-5 and HD-6) are stored as propeptides in Paneth cells and secreted mainly upon bacterial stimulation. They are then activated in the lumen by tryptic cleavage into their mature forms (Ghosh *et al*, 2002). While HD-5 is a very potent antimicrobial peptide with strong activity against various pathogens (Ericksen *et al*, 2005), HD-6 demonstrates its antimicrobial potential only under reducing conditions - as present in the gut (Schroeder *et al*, 2015). Otherwise HD-6 forms nanonets to entrap bacteria (Chu *et al*, 2012).

1.3.1.2. β-Defensins

β-defensins are ubiquitous and the evolutionary older family of defensins present in all vertebrates (Zhu & Gao, 2013). To date, more than 30 potential genes coding for β-defensins have been described based on human genome analysis, of which only human β-defensins 1-4 (hBD1-4) have been studied in detail (Pazgier *et al*, 2006; Donnarumma *et al*, 2016). These peptides are primarily secreted by epithelial cells in skin, the urogenital, gastrointestinal and respiratory tracts (McCray & Bentley, 1997; Bals *et al*, 1998; Dunsche *et al*, 2002; García *et al*,

2001), but also immune cells such as monocytes, macrophages and dendritic cells can express these peptides (Duits *et al*, 2002; Yin *et al*, 2010; Ryan *et al*, 2011). β-defensins are either constitutively expressed as observed for hBD1 in various tissues or upon demand by either commensal, probiotic or pathogenic bacteria as well as pro-inflammatory stimuli (O'Neil *et al*, 1999; Harder *et al*, 2001; Sørensen *et al*, 2005; Schlee *et al*, 2007; Semple & Dorin, 2012).

All of the β-defensins have the capacity to kill or inhibit a wide variety of bacteria and fungi. But the spectrum of antimicrobial activity varies for each defensin. In addition, the bactericidal activity of β-defensins depends on environmental conditions such as high concentrations of salt and plasma proteins (Goldman *et al*, 1997; Bals *et al*, 1998); only exception is hBD3 which is salt-insensitive (Taylor *et al*, 2008). One of the first identified defensins was hBD1 which was isolated from blood plasma (Bensch *et al*, 1995). For a while it was thought hBD1 exhibit only minor antimicrobial activity (Tollin *et al*, 2003), but by mimicking the *in vivo* situation in the gut, leading to the reduction the disulfide bridges unmasked the broad activity against several bacteria and fungi (Schroeder *et al*, 2011b). Similar to HD-6, both forms of hBD1 have distinct antimicrobial profiles and functions, only the reduced form of hBD1 is capable of forming net-like structures entrapping bacteria (Raschig *et al*, 2017). Both, hBD2 and hBD3 have been shown to exhibit broad activity against bacteria, fungi and viruses (Harder *et al*, 2001; Feng *et al*, 2005; Weinberg *et al*, 2012). Several studies demonstrated that the human immunodeficiency virus-type 1 (HIV-1) induces their expression and that both β-defensins inhibit HIV-1 infections of oral epithelial cells (Quiñones-Mateu *et al*, 2003; Pace *et al*, 2017). The last member of the human β-defensin family hBD4 also revealed antimicrobial activity against bacteria and fungi (García *et al*, 2001; Schneider *et al*, 2005).

In addition to their antimicrobial potential, β-defensins also have immune modulation properties such as chemoattracting innate and adaptive immune cells, inducing cytokines or chemokines, and modulate cellular functions (Yang *et al*, 1999; Lai & Gallo, 2009; Hancock *et al*, 2016; Koeninger *et al*, 2020). They play a crucial role for health and impairments in induction are associated with colonic Crohn's disease (Wehkamp *et al*, 2005b).

1.4. Antimicrobial resistance crisis

The global occurrence and spread of new multidrug-resistant bacteria along with a decline in the discovery of new antimicrobials represents a prominent and emerging health care threat. Loss of antibiotic efficacy causes increased number of hospitalizations, treatment failures and spread of drug-resistant pathogens (Martens & Demain, 2017). The Centers for Disease Control and Prevention (CDC) estimates that the annual infection rate exceeds 2.8 million cases only in USA, resulting in more than 35,000 deaths caused by multidrug-resistant bacteria and fungi (CDCP, 2019) hence mirroring the 33,000 annual death caused my AMR in Europe (Cassini *et al*, 2019).

For this reason, the World Health Organization (WHO) felt obliged to call out for new strategies to fight these devastating pathogens. The WHO assigned the highest priority to antimicrobial drug research and development for Gram-negative bacteria of the so-called ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species) panel (Tacconelli *et al*, 2018). These pathogens account for the majority of nosocomial infections worldwide being attributed to ≥ 700,000 deaths annually (Kelly & Davies, 2017).

At large, the pharmaceutical industry and governments alike have failed to develop new antibiotics. Many pharmaceutical companies are shying away from this opportunity because of a lack of financial return, inefficiency and the rapid occurrence of resistance (The Lancet, 2020). The major challenge is the divergent motivation from society versus companies, where novel strategies are welcomed yet shelved by regulatory authorities. The rationale behind such decisions, mitigating the risk of multidrug resistance while ensuring these novel therapies in case of an outbreak, is justified, yet jeopardizes the costly development of novel antibiotics. To meet this request, alternatives to conventional antibiotics are urgently needed (Ghosh *et al*, 2019; Theuretzbacher *et al*, 2019).

1.5. Aim of this work and rational of individual publications

The occurrence and spread of multidrug-resistant microorganisms is considered to be one of the biggest public health threats on a global scale. Already today, antibiotic-resistant bacteria occur for more than 670,000 infections and approximately 33,000 deaths every year in the European Union (Cassini *et al*, 2019). The lack of new, effective antibiotics has urged WHO to call out for new strategies to fight these frightful pathogens. Therefore, WHO has published a list of the 12 most devastating bacteria for whose cure new antibiotic substances are urgently needed (Tacconelli *et al*, 2018). Thus, new strategies, including those of antimicrobial peptide-derivate must be developed in the battle against multidrug-resistant bacteria (Mookherjee *et al*, 2020). Host defense peptides could be a promising treatment option. HDPs show a broad range of antimicrobial and immunological properties (Zasloff, 2002). Herein, I focused on investigating the antimicrobial activity of different defensin fragments, namely HNP-$4_{1\text{-}11}$ and octapeptide, on various multidrug-resistant bacteria. Additionally, I aimed to improve the antimicrobial activity of the octapeptide by modification with palmitic acid, to increase *in vivo* stability as well.

1.5.1. Human neutrophil α-defensin 4 fragmentation

Human neutrophil peptides 1-4 (HNPs) are expressed by immune cells and exhibit potent antimicrobial activities (Lehrer & Lu, 2012). The most potent peptide of this family against Gram-negative bacteria is HNP-4, which is approximately 100 times more potent against

Escherichia coli than the other three family members (Wilde *et al*, 1989). Inspired by the work of Ehmann *et al*, we used HNP-4 as a precursor to generate various peptide fragments via proteolytic digestion (Ehmann *et al*, 2019). We aimed to identify new antimicrobial active fragments to overcome the antibiotic-resistance crisis. Therefore, we analyzed the potency and efficacy of a single fragment against multidrug-resistant bacteria compared to HNP-4. Furthermore we wanted to investigate the mode of action of this fragment as well as if it was resistant to further degradation by bacterial proteases.

1.5.2. The human β-defensin 1 fragment called octapeptide

The human β-defensin 1 (hBD1) was the first defensin found in humans and is constitutively produced by epithelia and immune cells (Bensch *et al*, 1995; Ryan *et al*, 2011). For a long time was unclear why the human organism continuously produces hBD1, a peptide with limited antimicrobial activity (Tollin *et al*, 2003). Mimicking the environmental conditions in the gut or using thioredoxin, which catalyze the reduction, unmasked the full potential of hBD1 against several microorganisms (Schroeder *et al*, 2011b; Jaeger *et al*, 2013). Under these conditions, the reduced form of hBD1 is sensitive to the intestinal protease trypsin and can be degraded in in various fragments, while the oxidized form is resistant (Schroeder *et al*, 2011a). Here, we analyzed the antimicrobial activity of an eight amino acid carboxyl-terminal fragment of hBD1 and influence on human cells.

1.5.3. Modifications of octapeptides results in novel lipopeptides

In an era of multidrug resistance the search for new substances is becoming increasingly important. One promising approach are host defense peptides, which possess various mechanisms to control infections (Mookherjee *et al*, 2020). Defensins, the most prominent class of HDPs in humans have been in focus as potential therapeutics for some time. But to-date, large scale production of accurately folded defensins is challenging and cost-intensive. We have recently shown that newly generated peptide fragments based on human defensins could be considered as a potential cost-effective option (Wendler *et al*, 2019). Here, we used these findings to develop novel synthetic lipopeptides. We determined theirs *in vitro* bactericidal activity against various bacteria. Based on these results, the most promising peptide was further analyzed in terms of potency against multidrug-resistant bacteria and biofilms. Additionally, we determined its efficacy *in vivo* in murine gastrointestinal infection models.

2 Fragmentation of human neutrophil α-defensin 4 to combat multidrug-resistant bacteria

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Abstract

The occurrence and spread of multidrug-resistant bacteria is a prominent health concern. To curb this urgent threat, new innovative strategies pursuing novel antimicrobial agents are of the utmost importance. Here, we unleashed the antimicrobial activity of human neutrophil peptide-4 (HNP-4) by tryptic digestion. We identified a single 11 amino acid long fragment (HNP-4_{1-11}) with remarkable antimicrobial potential, exceeding that of the full length peptide on both mass and molar levels. Importantly, HNP-4_{1-11} was equally bactericidal against multidrug-resistant and non-resistant strains; a potency that was further enhanced by N- and C-terminus modifications (acetylation and amidation, respectively). These observations, combined with negligible cytotoxicity not exceeding that of the full length peptide, presents proteolytic digestion of innate host-defense-peptides as a novel strategy to overcome the current health crisis related to antibiotic-resistant bacteria.

2.1. Introduction

The spread and occurrence of new multidrug-resistant bacteria represents a prominent and emerging health care threat on a global scale. At large, the pharmaceutical industry and governments alike have failed to develop new antibiotics which has urged World Health Organization (WHO) to call out for new cost-effective strategies to fight these devastating pathogens (Tacconelli *et al*, 2018). A major challenge is the divergent motivation from society versus companies, where novel strategies are welcomed yet shelved by regulatory authorities. The rationale behind such decisions, mitigating the risk of multidrug resistance while ensuring these novel therapies in case of an outbreak, is justified, yet jeopardizes the costly development of novel antibiotics. Thus, new cost-effective strategies more resilient to multidrug resistance are urgently needed (Sukkar, 2013; Falagas et al., 2016). Host-defense-peptides (HDP) – previously known as antimicrobial peptides (AMPs) – possess a broad range of antimicrobial properties, which could be useful to develop new antimicrobials in the fight against resistant pathogens (Zasloff, 2002). Defensins are the most prominent class of HDPs in humans. These small cationic molecules share as a common motive six conserved cysteines, which form three disulphide bonds classifies them into α- and β-defensins (White et al., 1995; Ganz, 2003; Selsted and Ouellette, 2005). Four of the six human α-defensins are expressed by immune cells, namely human neutrophil peptides 1 to 4 (HNPs), whereas the remaining two, human α-defensin 5 and 6 (HD-5 & HD-6) are expressed by Paneth cells in the small intestine (Lehrer and Lu, 2012). All HNPs are processed from propeptide to mature form during their trafficking activated by proteolytic digestion in polymorphonuclear neutrophils azurophilic granules (Valore and Ganz, 1992). These granules fuse with the lysosome after phagocytosis of pathogens allowing for

context specific bactericidal activity (Ganz et al., 1985; Selsted et al., 1985). Based on the biological control of these processes it is hypothesized that synthetic production of said peptides could be used as an antibiotic tool against extracellular pathogens. Yet, large-scale expression of accurately folded defensins is a major cost-challenge. Inspired by our recent observation that duodenal fluid degrades full length HD-5 to multiple biological active fragments with different antimicrobial properties including potency, efficacy and bacterial spectrum (Ehmann et al., 2019), we hypothesized that enzymatic digestion of mature HDPs could unleash their antimicrobial capacity and concomitantly solve the production-cost challenge of full length peptides. To this end, the least expressed HNP, HNP-4 (Harwig et al., 1992; Hu et al., 2019), is more bactericidal against Gram-negative bacteria than any of HNP-1-3 (Ericksen et al., 2005). While HNP-1-3 only differs internally in the first amino acid sequence, HNP-4 is more divergent combined with an increased negative charge ultimately enhancing antimicrobial activity (Lehrer and Lu, 2012). We used HNP-4 as precursor to identify new therapeutic agents. To this end, tryptic digestion of the linearized full length peptide liberated its antimicrobial potential. We identified a single fragment with a remarkable bactericidal potency, exceeding the MIC of the full length peptide on molar level. Surprisingly, we observed the antimicrobial efficacy of said peptide to be equally efficient against multidrug-resistant and non-resistant strains, hence presenting HDP fragmentation (Latendorf et al., 2019) as an innovative and cost-effective strategy to aid curbing the emerging threat of antibiotic resistance.

2.2. Material and Methods

Bacterial strains

B. adolescentis Ni3,29c and *B. breve* were provided by Ardeypharm GmbH (Herdecke, Germany). *L. rhamnosus* GG was obtained from InfectoPharm Arzneimittel and Consilium GmbH (Heppenheim, Germany). *A. baumannii* DSM30007, *B. vulgatus* DSM1447, *E. coli* MC1000 DSM6214, *E. coli* DSM8695 (EPEC), *E. coli* DSM10729 (UPEC), *E. faecalis* DSM20478, *E. faecium* DSM20477, *K. pneumoniae* DSM30104 and *S. epidermidis* DSM20044 were obtained from German Collection of Microorganisms and Cell Culture GmbH (Braunschweig, Germany). *A. baumannii* 4-MRGN, *B. longum*, *E. coli* ATCC25922, *E. faecium*, *E. faecalis* ATCC29212, *K. pneumoniae* 3-MRGN, *L. fermentum*, *L. salivarius*, *P. aeruginosa* ATCC27853, *P. aeruginosa* 4-MRGN, *S. enterica* serovar Enteritidis, *S. aureus* ATCC25923 and *S. salivarius* were obtained as clinical isolates from the Robert-Bosch-Hospital Stuttgart, Germany. *B. subtilis* (trpC2), *E. coli* JM83, *P. aeruginosa* PAO1, *P. aeruginosa* XPAT1, *P. aeruginosa* XPAT2, *S. aureus* USA300 and *Y. enterocolitica* were provided by the Interfaculty Institute for Microbiology and Infection Medicine, Tübingen, Germany.

Peptides

HNP-4 (Purity ≥ 99%) was obtained from PeptaNova GmbH (Sandhausen, Germany). All peptide fragments, HNP-$4_{1\text{-}11}$ and HNP-$4_{1\text{-}11\text{mod}}$ were chemically synthesized by EMC Microcollections GmbH (Tübingen, Germany) and purified by precipitation. EMC Microcollections guarantees a purity >> 90% by HPLC analysis (Figure S3). All peptides were dissolved in 0.01% acetic acid.

Screening for fragments of HNP-4 using LC/MS

As previously described (Ehmann et al., 2019), 2.5 µg of HNP-4 were incubated in 50 mM NH_4HCO_3 buffer (pH 8.0; Fluka) with 2 mM tris (2 carboxyethyl) phosphine for 15 minutes at 37°C. Afterwards 0.05 µg trypsin (1:50 (w/w)) was added and incubated for additional 30 minutes at 37°C. Lastly, formic acid and acetonitrile in a final concentration of 0.5% and 10% were added, respectively, and the samples analyzed by mass spectrometry. Mass spectrometry was performed as a LC/MS system using an Agilent 1200 series HPLC with an Agilent Advanced Bio Peptide Map (2.1x150 mm, 2.7 µm) column with a flow of 0.4 ml/min at 55°C column temperature and a 6540 UHD Q TOF LC/MS system (Agilent) for mass analysis. The samples were separated by a gradient of acetonitrile in 0.1% formic acid. The gradient started at 2% acetonitrile for 4 minutes and then increases during 35 minutes to 45%. Mass spectrometric

analyses were performed in single MS mode from 100 to 3400 m/z with positive ion polarity and were analyzed by Agilent MassHunter Quantitative Analysis B 06.00 software.

Screening for potential dimers of HNP-$4_{1\text{-}11}$ and HNP-$4_{1\text{-}11mod}$ using HPLC-MS

To analyze possible inter-/intramolecular dimer formation HPLC-MS were performed by EMC Microcollections GmbH Tübingen. HPLC-MS was performed using a Chromolith Fast Gradient RP18e, 50 x 2 mm column (Merck) with detection at a wavelength of 214 nm, followed by an ESI-MS analysis. The samples were separated by a gradient of MeCN (acetonitrile) containing 0.1% FA (monofluoroacetic acid) from 0 to 100% in 30 min.

Radial Diffusion Assay

Antimicrobial activity of all peptides was assessed with a modified version of the radial diffusion assay as described earlier (Schroeder et al., 2011b). Briefly, bacteria were cultivated (anaerobic bacteria in anaerobic jars with AnaeroGen, Oxoid, UK) for up to 18 hours in liquid TSB medium. Log-phase bacteria were washed with 10 mM sodium phosphate buffer; pH 7.4 and diluted to 4 x 10^6 CFU/ml in 10 ml agar (10 mM sodium phosphate buffer, pH 7.4 with 0.3 mg / ml TSB powder and 1% (w/v) low EEO-agarose (AppliChem). Bacteria were incubated under aerobic or anaerobic conditions, respectively, with 2 µg HNP-4 or 4 µg of each fragment for three hours at 37°C. Afterwards, plates were covered with 10 ml of an overlay-gel containing 6 % (w/v) TSB powder, 1 % (w/v) agar and 10 mM sodium phosphate buffer and incubated for 24 hours. The diameter of the inhibition zones corresponds to the antimicrobial activity, when subtracting the diameter of 2.5 mm corresponding to the diameter of the punched well. Experiments were repeated at least three times.

Turbidity Broth Assay

Log-phase bacteria were washed twice with 10 mM sodium phosphate buffer containing 1 % (w/v) TSB. Approximately 4 x 10^5 CFU/ml bacteria were incubated with serial peptide concentrations (1.56 – 100 µM) in a final volume of 100 µl in 10 mM sodium phosphate buffer containing 1% (w/v) TSB for 2 hours at 37°C. Afterwards, 100 µl of 6% TSB (w/v) were added and absorbance was measured at 600 nm (Tecan, Switzerland) and monitored for 12 hours. Experiments were carried out at least three independent times.

Time-kill Assay

Log-phase bacteria (5 x 10^5 CFU/ml) were incubated with 6.25 µM of HPN-4_{fl}, HNP-$4_{1\text{-}11}$, HNP-$4_{1\text{-}11mod}$ or 0.01% acetic acid as a control in 10 mM sodium phosphate buffer containing 1% (w/v)

TSB. After incubation at 37°C and 150 rpm for 0 to 120 min, a sample was taken from the suspension and added to a 0.05% (v/v) sodium polyanethole sulfonate (Sigma-Aldrich) solution, which neutralizes remaining peptide activity, and plated on LB agar to determine the number of viable bacteria. Experiments were carried out at least three independent times.

Reduction Assay

The amino acid sequences of HNP-$4_{1\text{-}11}$ and HNP-$4_{1\text{-}11mod}$ contain cysteines which might form disulfide bonds with another fragment. As reducing agent Dithiothreitol (DTT) was used. Both peptides, HNP-$4_{1\text{-}11}$ and HNP-$4_{1\text{-}11mod}$ were pre-incubated with either 0.1 mM or 1 mM DTT for 1 h at room temperature followed by a turbidity broth assay with approximately 5 x 10^5 CFU/ml bacteria as described above. The MIC of HNP-$4_{1\text{-}11}$ and HNP-$4_{1\text{-}11mod}$ was determined against different bacteria strains. Experiments were carried out at least three independent times.

Protease Inhibitor Assay

Log-phase bacteria were cultivated for up to 18 hours in TSB containing different concentrations (0.01 or 0.1) of Bacterial ProteaseArrest™ (G-Biosciences) and 0.5 M EDTA. Bacteria were washed with twice with 10 mM sodium phosphate buffer containing 1% (w/v) TSB and the optical density at 600 nm was adjusted to 0.1. Approximately 5 x 10^5 CFU/ml bacteria were incubated with serial peptide concentrations (1.56 – 12.5 µM) in a final volume of 100 µl in 10 mM sodium phosphate buffer containing 1% (w/v) TSB and (0.01 or 0.1) of Bacterial ProteaseArrest™ and 0.5 M EDTA for 2 hours at 37°C. After incubation, 100 µl of 6% TSB (w/v) were added and absorbance was measured at 600 nm (Tecan, Switzerland) and monitored for 12 hours. Experiments were carried out at least three independent times.

Cell Toxicity Assay

Experiments were conducted with the human colonic epithelial adenocarcinoma cell line CaCo2 subclone TC7 which was obtained from the Robert-Bosch-Hospital Stuttgart, Germany. HT29 MTX cells subclone E12 (Merck, Germany) were used as an additional colorectal carcinoma cell line. Cells were used at an internal early passage of about 25 – 40. For experiments, 1500 cells/well were seeded in a 96-well plate in 90 µl media. Cells were treated with serial peptide concentrations (1.56 – 100 µM) in a final volume of 100 µl and incubated for 96 hours. Afterwards, the CellTiter-Glo® 2.0 Cell Viability Assay (Promega, USA) was performed based on the company's protocol. Experiments were carried out at least three independent times.

Hemolytic activity of HNP-4 fragments

Hemolytic activity assay was performed as described earlier (Oddo and Hansen, 2017). Briefly, 1 ml O neg whole blood was washed twice with PBS, centrifuged and 1 % (v/v) erythrocytes suspension prepared. Erythrocytes were incubated with serial peptide concentrations (1.56 – 100 µM) for 1 hour at 37°C. Then, samples were centrifuged, supernatant collected and optical density measured at 414 nm. Toxicity against erythrocytes was relative determined to the hemolytic activity of 0.1 % Triton X-100. Experiments were carried out in duplicates and performed twice.

Ethics statement

The study protocol was previously approved by the Ethical Committee of the University Hospital Tübingen, Germany. Patients and controls who were included in this study all gave their written and informed consent after the study purpose, samples procedure, and potential adjunctive risks were explained. All experiments were conducted in accordance with the relevant guidelines and regulations.

2.3. Results

2.3.1. Identification of a novel HNP-4 fragment after tryptic digestion

To generate possible fragments out of HNP-4 we used trypsin as a serine protease. It is known from previous work that folded defensins seemed to be stable against proteolytic digestion (Schroeder et al., 2011a). We incubated HNP-4 with 2 mM TCEP (tris(2-carboxyethyl)phosphine; Sigma-Aldrich) to open the disulphide bonds leading to a more linear structure susceptible to proteolytic digest. We analyzed the trypsin-incubated reduced HNP-4 via LC/MS methods and were able to detect several fragments according to the observed ions and their mass to charge ratio (Fig. 1A). Identified fragments were mostly located in the N-terminal region based on the cleaving sites of trypsin (Fig. 1B). As it is commonly accepted that the net charge of AMPs could play an important role to their antimicrobial activity, we focused on HNP-$4_{1\text{-}11}$ with a positive net charge of +3 Fig. 1B, marked in red.

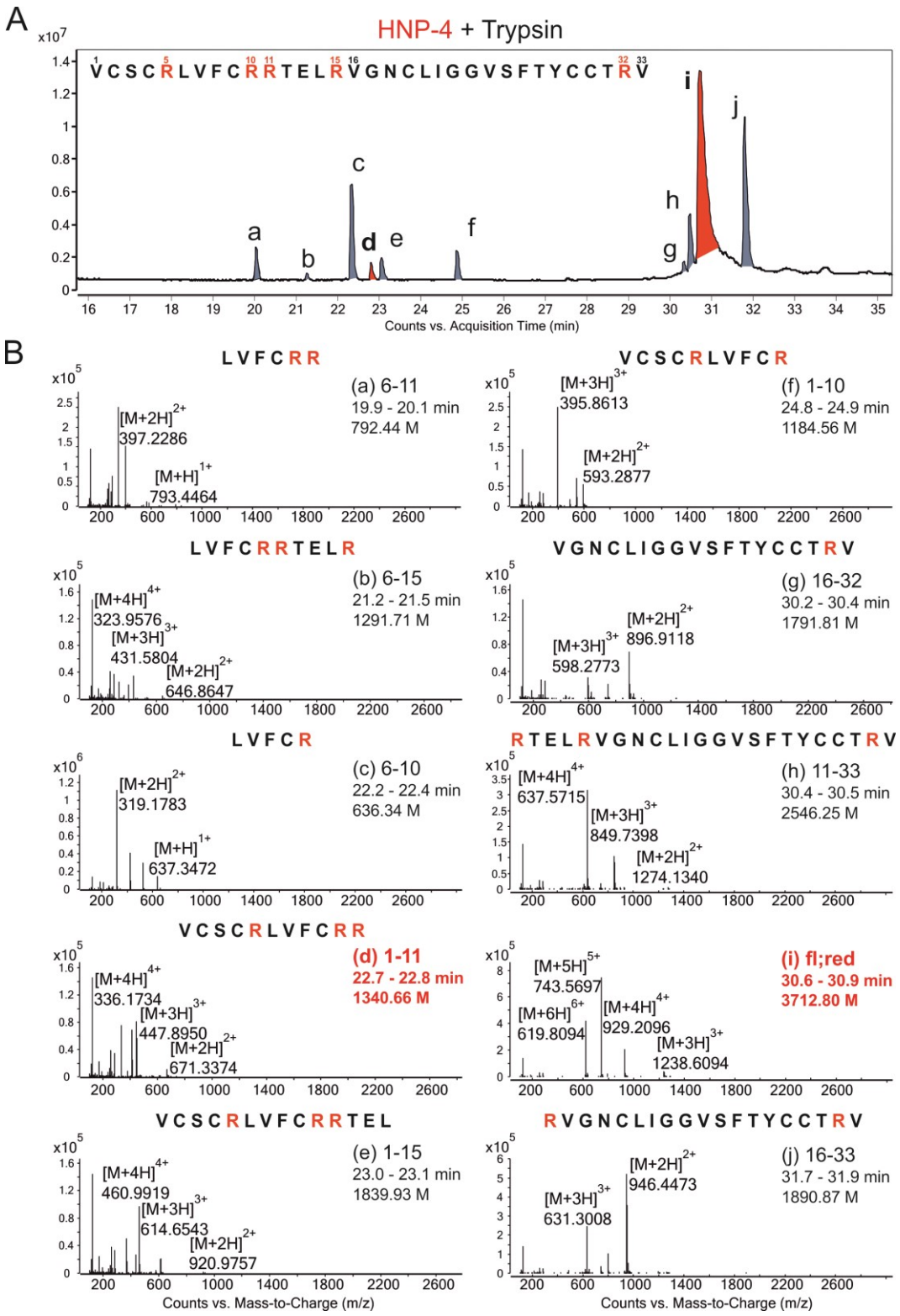

Figure 1. Proteolytic digestion of reduced HNP-4 by trypsin produced different fragments. **(A)** Displays an overview of the chromatogram from an incubation of reduced HNP-4 with trypsin after reduction with 2 mM TCEP. All detectable fragments were marked in red or grey (a-j) and listed due to their retention time. **(B)** Show the mass-to-charge (m/z) graphs of all detected fragments. In all mass-to-charge graphs we pointed out the neutral mass based on the detected ions. All peptides marked in red were chose for synthesis and further investigations.

2.3.2. Antimicrobial efficacy of HNP-$4_{1\text{-}11}$ and HNP-$4_{1\text{-}11mod}$

The natural in vivo stability of short linear peptides is generally weak; we therefore used an additional modified form of HNP-$4_{1\text{-}11}$ (HNP-$4_{1\text{-}11mod}$). Here we exchanged the L-amino acids with D-amino acids and modified the N-terminus (acetylation) and C-terminus (amidation). Both modifications should result in a gain of stability (Brinckerhoff et al., 1999; Hong et al., 1999), hence potentially leading to a stronger antimicrobial activity. To analyze the antimicrobial activity of HNP-4_{fl}, HNP-$4_{1\text{-}11}$ and HNP-$4_{1\text{-}11mod}$ we used RDAs against a subset of different commensal and pathogenic bacteria (Figures S1 & S2). All of our tested peptides showed an antimicrobial activity against tested bacteria (Fig. 2).

Commensal bacteria	HNP-4		
	fl	1-11	1-11mod
B. subtilis 168trpC			
B. vulgatus			
B. adolescentis Ni3,29c			
B. breve			
B. longum			
E. coli MC1000			
L. fermentum			
L. rhamnosus			
L. salivarius			
S. salivarius ssp.*salivarius*			

Pathogenic bacteria	HNP-4		
	fl	1-11	1-11mod
A. baumannii 4-MRGN			
A. baumannii DSM30007			
E. faecalis DSM20478			
E. faecium DSM20477			
E. coli (EPEC) DSM8695			
E. coli (UPEC) DSM10729			
K. pneumoniae 3-MRGN			
K. pneumoniae DSM30104			
P. aeruginosa 4-MRGN			
P. aeruginosa ATCC27853			
S. Enteritidis			
S. aureus ATCC25923			
S. aureus USA300			
S. epidermidis DSM20044			

High activity | Low activity | No activity

Figure 2. HNP4-derivates display a high antimicrobial activity against commensal and pathogenic bacteria. We analyzed the antimicrobial potential of the identified fragment and its modified version against commensal and pathogenic bacteria. In this heat map, we listed all bacteria and the activity of the fragments in RDA against them. We used 2 µg of the full-length peptide and 4 µg of each fragment. An inhibition zone greater than 8 mm was determined as highly active, between 2.5 and 8 mm as low active, while a diameter of 2.5 mm (diameter of the punched well) was marked as no activity. The heat map is based on three independent experiments.

While the RDA is the suitable assay to determine a general antimicrobial activity of different peptides, a comparison between different peptides is not possible according to their different abilities (like diffusion) in an agarose gel. We therefore next used a turbidity broth assay to determine the minimal inhibitory concentration (MIC) of HNP-4_{fl}, HNP-$4_{1\text{-}11}$ and HNP-$4_{1\text{-}11mod}$ against pathogenic (some multidrug-resistant) Gram negative and positive bacteria (Fig. 3A). While all peptides displayed antimicrobial activity against tested bacteria (sole exception: HNP-4_{fl} against *K. pneumoniae* DSM30104), HNP-$4_{1\text{-}11}$ was surprisingly equimolar to HNP-4_{fl}, indicating that the antimicrobial potency of the natural complex-to-produce HNP-4_{fl} is chiefly

driven by the first 11 amino acids (HNP-$4_{1\text{-}11}$), at least in its linear form. To this end, Hu and colleagues recently observed some dependency of specific residues post position 11 in the fully folded native peptide (Hu et al., 2019). Pointing further towards enhanced bactericidal efficacy of this linear fragment, HNP-$4_{1\text{-}11mod}$, which is expected to exhibit increased stability over the non-modified version, was superior to both HNP-4_{fl} and HNP-$4_{1\text{-}11}$ with a MIC several fold lower than the one observed for the natural occurring full length peptide. Additionally, we performed a time-kill assay to investigate the efficacy of HNP-$4_{1\text{-}11}$ and HNP-$4_{1\text{-}11mod}$ compared to the HNP-4_{fl}. Although we observed a higher potency of HNP-$4_{1\text{-}11}$, the efficacy was similar to HNP-4. In contrast, HNP-$4_{1\text{-}11mod}$ was superior in both aspects (Fig. 3B).

A

	MIC after 12 hours	HNP-4_{fl}		HNP-$4_{1\text{-}11}$		HNP-$4_{1\text{-}11mod}$	
		µM	µg/ml	µM	µg/ml	µM	µg/ml
Gram negative	*A. baumanii* 4-MRGN	12.5	46.4	25	33.5	6.25	8.6
	A. baumanii DSM30007	3.125	11.6	6.25	8.4	6.25	8.6
	E. coli JM83	12.5	46.4	6.25	8.4	6.25	8.6
	K. pneumoniae 3-MRGN	>>>	>>>	25	33.5	6.25	8.6
	K. pneumoniae DSM30104	100	371.5	25	33.5	6.25	8.6
	P. aeruginosa 4-MRGN	50	185.8	50	67.1	6.25	8.6
	P. aeruginosa ATCC27853	6.25	23.2	12.5	16.8	6.25	8.6
	P. aeruginosa PAO1	12.5	46.4	12.5	16.8	12.5	17.3
	P. aeruginosa XPAT1	12.5	46.4	12.5	16.8	12.5	17.3
	P. aeruginosa XPAT2	12.5	46.4	12.5	16.8	12.5	17.3
	S. Enteritidis	100	371.5	25	33.5	12.5	17.3
	Y. enterocolitica	12.5	46.4	12.5	16.8	6.25	8.6
Gram positive	*E. faecium* 475747	3.125	11.6	3.125	4.2	3.125	4.3
	E. faecium DSM20477	6.25	23.2	12.5	16.8	6.25	8.6
	S. aureus ATCC25923	6.25	23.2	12.5	16.8	3.125	4.3
	S. aureus USA300	6.25	23.2	50	67.1	12.5	17.3

B

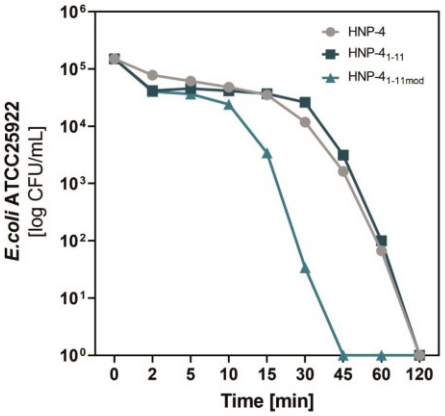

Figure 3. Comparison of the potency (MIC) and efficacy (killing rate) of HNP-4_{fl}, HNP-$4_{1\text{-}11}$ and HNP-$4_{1\text{-}11mod}$. (A) The minimal inhibitory concentration (MIC) in µM and µg/ml as a concentration without any bacterial growth. Peptides were incubated with tested bacteria and changes in optical density (OD600) were measured after 12 hours at 37°C. If we were able to observe an antimicrobial effect but did not detect a total inhibition of bacterial growth we marked it with ">>>". Each experiment was carried out three independent times. (B) Killing of *E. coli* ATCC25922 after 0 to 120 min exposure to 6.25 µM (1x MIC) HNP-4_{fl}, HNP-$4_{1\text{-}11}$ and HNP-$4_{1\text{-}11mod}$. Results are expressed as the number of viable bacteria (in log10 CFU) per milliliter. Values are means of three independent experiments.

2.3.3. *In vitro* stability of HNP-$4_{1\text{-}11}$ and HNP-$4_{1\text{-}11mod}$

We modified the turbidity broth assay to determine the stability and potential resistance against proteolysis and/or natural degradation. To this end, we determined the antimicrobial activity of HNP-$4_{1\text{-}11}$ and HNP-$4_{1\text{-}11mod}$ against *E. coli* ATCC25922 in presence a protease inhibitor cocktail (Fig. 4A). Increasing amounts of protease inhibitors did not improve the bactericidal potential of any of the tested fragments, indicating bacterial proteases do not further degrade mentioned fragments, hence corroborating their stability. Instead, the data points towards a potential fragment: protease interaction, as high concentrations of protease inhibitors reduced the bactericidal efficacy of both fragments. Enhanced prevalence of cysteine residues on most HDPs

led to the current models of multimer formation, combined with a high net charge, as a mechanism to interact with the surface of microorganisms (Brogden, 2005; Mukherjee and Hooper, 2015). To address if multimers were essential for bactericidal efficacy, we determined the MIC of HNP-$4_{1\text{-}11}$ and HNP-$4_{1\text{-}11mod}$ against *E. coli* ATCC25922 in the presence of increasing levels of the reducing agent, DTT (Fig. 4B). Elevated DTT concentrations did not affect antimicrobial activity of neither HNP-$4_{1\text{-}11}$ nor HNP-$4_{1\text{-}11mod}$, suggesting that monomeric peptides were sufficient to kill *E. coli* ATCC25922. To further substantiate these observations, we next performed a HPLC-MS analysis to determine possible inter-/intramolecular dimer formation (Fig. 4C & D). In line with the results from our reduction assay, we did not detect any formation of oligomeric or polymeric peptide fragments.

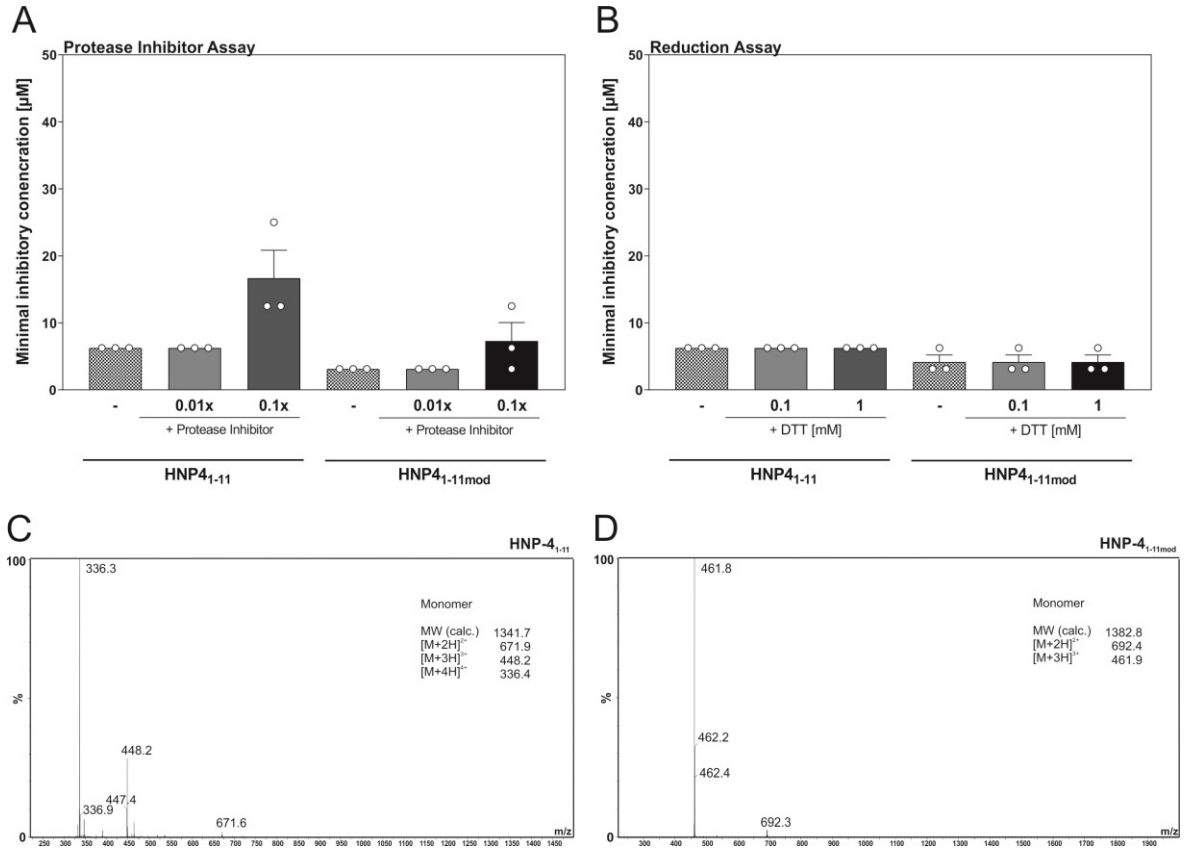

Figure 4. Reduction as well as proteolysis of HNP-$4_{1\text{-}11}$ and HNP-$4_{1\text{-}11mod}$ have no influence on the antimicrobial activity. **(A)** Changes in the antimicrobial activity against *E. coli* ATCC25923 were analyzed in the presence of a protease inhibitor cocktail. **(B)** The minimal inhibitory concentration of HNP-$4_{1\text{-}11}$ and HNP-$4_{1\text{-}11mod}$ was determined against *E. coli* ATCC25922 under reducing conditions due to the optical density after 12 h. Results from three independent experiments with ± SEM are represented. **(C)** ESI-MS analysis of HNP-$4_{1\text{-}11}$ to detect potential dimer's after peptide dilution. **(D)** Analysis of HNP-$4_{1\text{-}11mod}$ using ESI-MS to detect potential dimer's after peptide dilution.

2.3.4. Cytotoxic and hemolytic effects of HNP-$4_{1\text{-}11}$ and HNP-$4_{1\text{-}11mod}$

To determine the potential of HNP-$4_{1\text{-}11}$ and HNP-$4_{1\text{-}11mod}$ for in vivo applications as therapeutic agents, we used two different cell lines to investigate their cytotoxic abilities. While we only

observed minor cytotoxic effects on CaCo2/TC7 cells at higher peptide concentration (Fig. 5A), HT29 MTX E29 cells were more susceptible to both peptide-derivates (Fig. 5B). Importantly, at lower concentrations (e.g. 12.5 µM, where HNP-$4_{1\text{-}11mod}$ has a strong antibacterial effect), the fragments exhibited only modest cytotoxicity. We additionally examined the hemolytic activity of said peptides (Fig. 5C). While HNP-$4_{1\text{-}11mod}$ has a 20 % hemolytic effect at 150 µM (by far exceeding the highest concentration needed for bactericidal efficacy) there was negligible toxicity at ≤ 18.75 µM, i.e. the highest biological relevant concentration. Thus, compared to the honey bee toxin, Melittin, which showed an 80 % hemolytic effect at 1.25 µM both HNP-$4_{1\text{-}11}$ and HNP-$4_{1\text{-}11mod}$ appeared with low hemolytic activity. In conclusion, the cytotoxic concentrations identified were magnitudes higher than the corresponding bactericidal concentration.

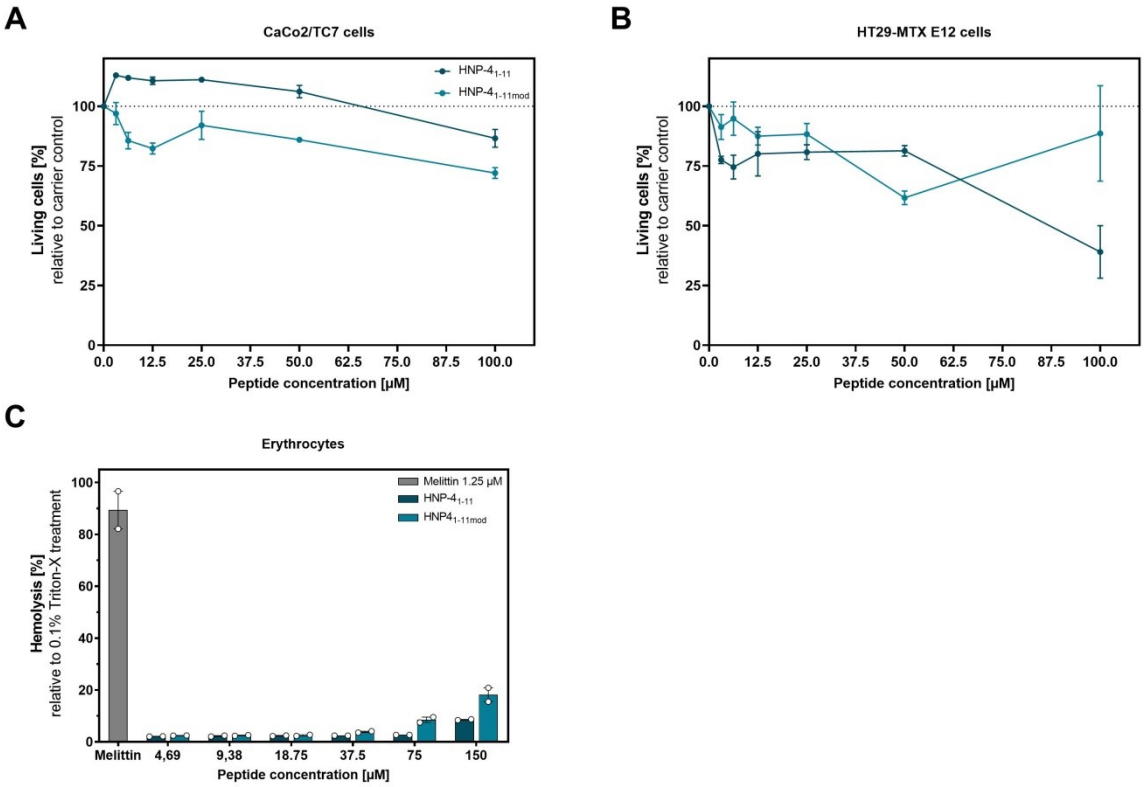

Figure 5. HNP-$4_{1\text{-}11}$ and HNP-$4_{1\text{-}11mod}$ show only minor cytotoxic and hemolytic activity at high concentrations. We investigated the cytotoxic activity of HNP-$4_{1\text{-}11}$ and HNP-$4_{1\text{-}11mod}$ against **(A)** CaCo2/TC7 or **(B)** HT29 MTX E 29 cells. We seeded 1500 cells per well and treated them after 24 hours with different peptide concentrations. Living cells were determined after 96 hours treatment using a CellTiter Glo2.0 assay. Results from three independent experiments with ± SEM are shown. **(C)** Hemolytic activity on human erythrocytes of the peptides compared to 0.1% Triton-X treatment. Results from two independent experiments with ± SEM are represented.

2.4. Discussion

Loss of antibiotic efficacy causes increased number of hospitalizations, treatment failures and spread of drug-resistant pathogens (Martens and Demain, 2017). WHO called out to develop new strategies against Gram-negative bacteria in general, and in particularly those from the WHO priority list (Tacconelli et al., 2018). To meet this request, alternatives to conventional antibiotics are urgently needed (Ghosh et al., 2019; Theuretzbacher et al., 2019, 2020). Thus, new strategies, including those of antimicrobial peptide-derivates must, be developed in the battle against multi-drug resistant bacteria (Fosgerau and Hoffmann, 2015; Breij et al., 2018). To this end proteolysis of HD-5 generated various antimicrobial active peptides with selectivity to certain bacteria (Ehmann et al., 2019). These fragments possess abilities to shift microbiota composition without decreasing diversity. Moreover, mice treated with HD-$5_{1\text{-}9}$, the most potent fragment identified, harbored an increased amount of *Akkermansia* sp. (Ehmann et al., 2019). The same could be shown for the human β-defensin 1, where digestion also led to a diverse set of biological active antimicrobial fragments (Wendler et al., 2019). This study complements our earlier reports with the discovery that proteolytic digestion of HNP-4 led to a highly active easy-to-produce 11 amino acids short fragment (HNP-$4_{1\text{-}11}$) with a broad antimicrobial spectrum against Gram negative and Gram positive bacteria. We hypothesize that this interesting phenomenon represents a general feature of HDPs rather than being specific to HNP-4, in part based on the observation that also the N-terminal part of HNP-1 is antimicrobial active (Varkey and Nagaraj, 2005). It is thus possible that this method of tryptic digestion of HNP-4 may be used as a general technique to unleash the antimicrobial potential of endogenous expressed HDPs to aid curbing the antibiotic resistance crises.

Interestingly, HNP-$4_{1\text{-}11}$ possesses equal or better antimicrobial activity against bacteria than the full-length peptide on molar level. A modified version of this fragment further improved both potency and efficacy. Remarkably, HNP-$4_{1\text{-}11\text{mod}}$ was highly effective in vitro against various multidrug-resistant bacteria including *A. baumannii* 4-MRGN, *K. pneumoniae* 3-MRGN and *P. aeruginosa* 4-MRGN; all top 'members' of the WHO priority and Centers for Disease Control and Prevention lists (Tacconelli et al., 2018; CDCP, 2019). Lending credence to the hypothesis of modified HDPs representing an underexplored plethora of drug candidates against multidrug-resistant bacteria, a recent study elegantly corroborated that this exact class of bacteria are more susceptible to HDPs (Lázár et al., 2018), hence stressing their potential as new therapeutic agents. While we were able to show that HNP-$4_{1\text{-}11}$ and HNP-$4_{1\text{-}11\text{mod}}$ displayed a broad spectrum antimicrobial activity pattern, we did not focus on their antimicrobial mechanisms, but the capacity to induce rapid killing of Gram-negative bacteria indicates membrane interactions as part of the mode(s) of action. From a general point of view cysteines and charged amino acids are often relevant for antimicrobial activity (Jiang et al., 2008). Importance of those amino acids

led to the current models of HDP mechanism forming multimers as well as the need of charged amino acids to interact with the surface of microorganisms (Brogden, 2005; Mukherjee and Hooper, 2015). Due to these observations, we initially assumed that also the antimicrobial activity of the here presented fragments depended on dimerization. Yet, our reducing assays followed by HPLC-MC analysis illustrated that monomeric formation was sufficient for the observed bactericidal activity, pointing towards a different mode of action of these hallmark peptide fragments, disputing the current dogma in the field. Although covalent dimers are absent, non-covalent oligomeric forms of both peptides cannot be entirely excluded. Additional analyses are necessary to determine the importance of supramolecular peptide forms for antimicrobial activity, as non-covalent oligomerisation can be relevant for antimicrobial activity of several and in particular amyloid-forming peptides (Latendorf et al., 2019).

A challenge with HDPs in therapeutic contexts is their susceptibility to proteolysis by bacterial proteolytic enzymes (Reijmar et al., 2007), in particular in reduced environments (Schroeder et al., 2011a), as exemplified by the outer membrane protease of *Salmonella enterica* which degrades and thereby inactivates HDPs, thus supporting an essential role of bacterial proteases in bacterial resistance to HDPs (Guina et al., 2000). The conceptual advancement of utilizing protease-degraded biologically active fragments, as showcased here by trypsin digest is therefore intriguing. Such fragments should, by nature, be resistant to further degradation and may prove valuable to aid fight multi-drug resistant pathogens. In keeping with this notion, our analysis revealed that HNP-$4_{1\text{-}11}$ and HNP-$4_{1\text{-}11\text{mod}}$ activity was not further boosted by protease inhibitors, suggesting that proteases per se do not hamper their function. Instead, high levels of protease inhibitors appeared to limit the bactericidal efficacy of both HNP-$4_{1\text{-}11}$ and HNP-$_{41\text{-}11\text{mod}}$ suggesting that these fragments conversely interact with proteases, rather than being annulled by them, to induce bacterial killing. Future studies are warranted to elucidate the extent of such potential fragment:protease interaction. For potential therapeutic application, we assessed toxicity of HNP-$4_{1\text{-}11}$ and HNP-$4_{1\text{-}11\text{mod}}$. Both peptides showed cell-type dependent cytotoxicity and hemolytic activity at higher concentrations. To this end, HNP-$4_{1\text{-}11\text{mod}}$ exerted a greater impact on CaCo-2 cells, whereas HNP-$4_{1\text{-}11}$ possessed higher cytotoxicity against HT29-MTX E12 cells, but for both tested cell types the cytotoxic concentration range were magnitudes higher than the concentrations needed for antimicrobial activity.

In summary, although future *in vivo* experiments are warranted to determine the full potential of HNP-$4_{1\text{-}11}$ and HNP-$4_{1\text{-}11\text{mod}}$, our results demonstrate promising efficacy of HNP-$4_{1\text{-}11}$ and HNP-$4_{1\text{-}11\text{mod}}$ against multidrug-resistant bacteria. From this point of view, proteolytic digestion of HDPs could be used to generate new biologically active fragments to overcome the antibiotic-resistance crisis.

Acknowledgments

We thank Marion Strauss and Jutta Bader for excellent technical assistance.

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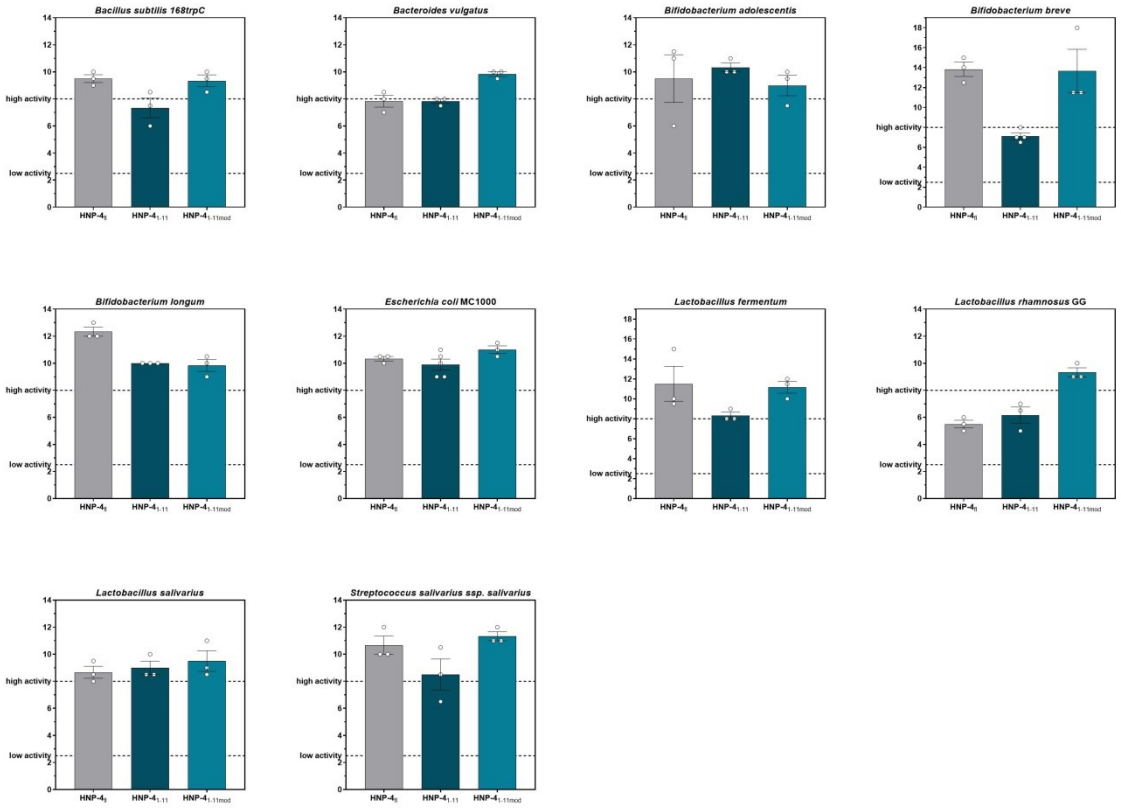
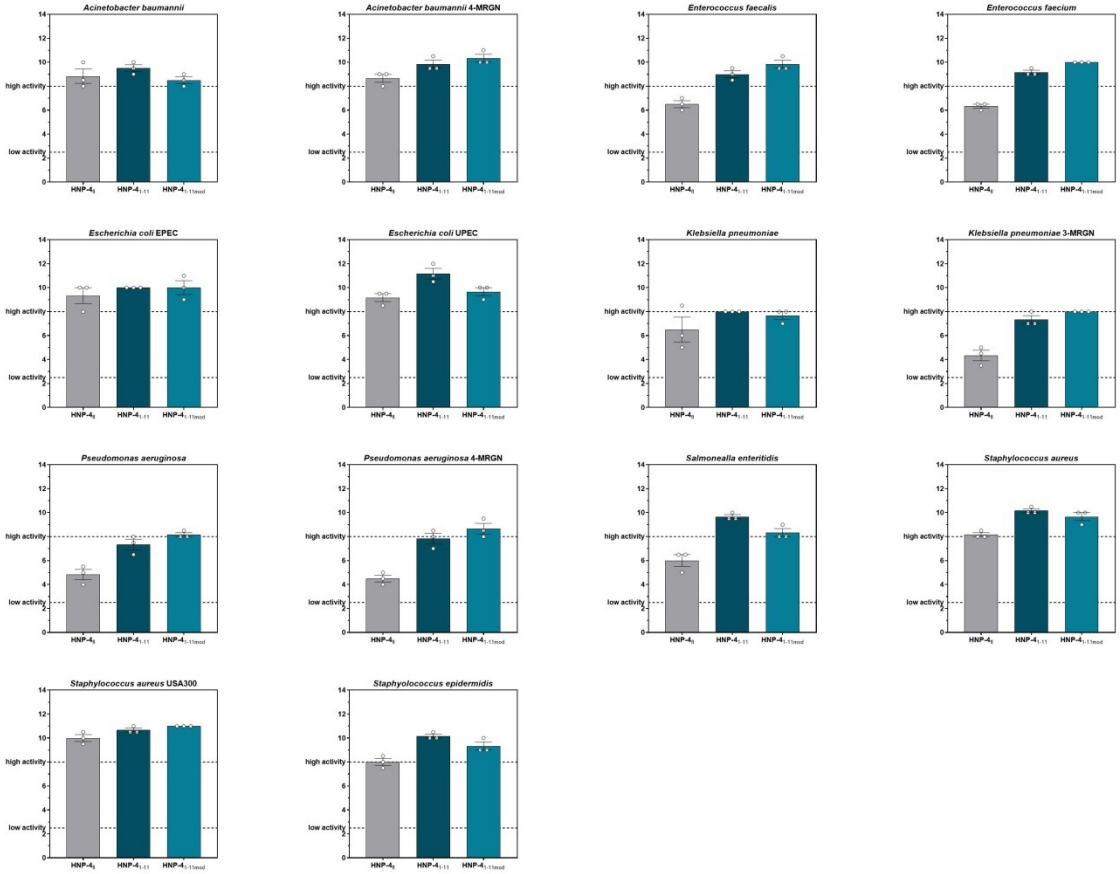
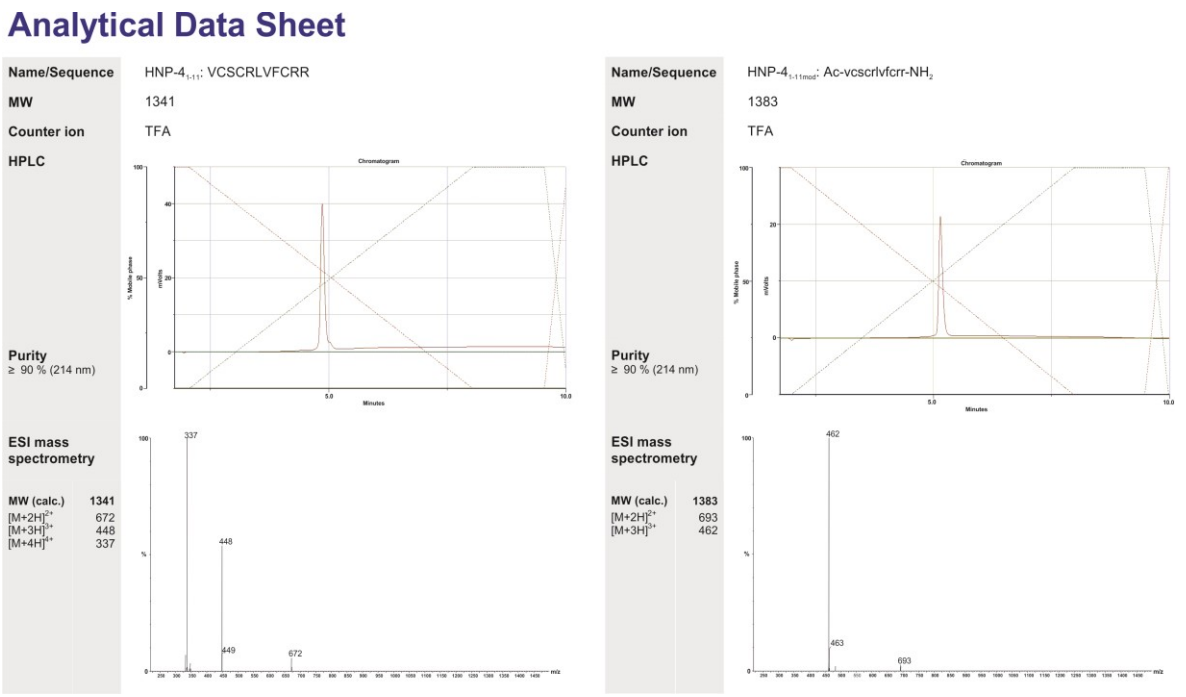
2.5. Supplementary material



Supp. Fig. 1. RDA with the HNP-4 fragments against commensal bacteria. Here we show the detailed results of the RDA experiments. Data are presented as mean ± SEM. Experiments were carried out three independent times.



Supp. Fig. 2. RDA with the HNP-4 fragments against pathogenic bacteria. Here we show the detailed results of the RDA experiments. Data are presented as mean ± SEM. Experiments were carried out three independent times.



Supp. Fig. 3. Analytical data sheet of HNP-4$_{1-11}$ and HNP-4$_{1-11mod}$. Here we show the detailed analysis of purity of HNP-4$_{1-11}$ and HNP-4$_{1-11mod}$.

3 Proteolytic degradation of reduced human beta defensin 1 generates a novel antibiotic octapeptide

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Wendler J, Schroeder BO, Ehmann D, Koeninger L, Mailänder-Sánchez D, Lemberg C, Wanner S, Schaller M, Stange EF, Malek NP, Weidenmaier C, LeibundGut-Landmann S & Wehkamp J (2019) Proteolytic Degradation of reduced Human Beta Defensin 1 generates a Novel Antibiotic Octapeptide. *Sci. Rep.* 9: 3640.

Abstract

Microbial resistance against clinical used antibiotics is on the rise. Accordingly, there is a high demand for new innovative antimicrobial strategies. The host-defense peptide human beta-defensin 1 (hBD-1) is produced continuously by epithelial cells and exhibits compelling antimicrobial activity after reduction of its disulphide bridges. Here we report that proteolysis of reduced hBD-1 by gastrointestinal proteases as well as human duodenal secretions produces an eight-amino acid carboxy-terminal fragment. The generated octapeptide retains antibiotic activity, yet with distinct characteristics differing from the full-length peptide. We modified the octapeptide by stabilizing its termini and by using non-natural D-amino acids. The native and modified peptide variants showed antibiotic activity against pathogenic as well as antibiotic-resistant microorganisms, including *E. coli*, *P. aeruginosa* and *C. albicans*. Moreover, in an *in vitro C. albicans* infection model the tested peptides demonstrated effective amelioration of *C. albicans* infection without showing cytotoxity on human cells. In summary, protease degradation of hBD-1 provides a yet unknown mechanism to broaden antimicrobial host defense, which could be used to develop defensin-derived therapeutic applications.

3.1. Introduction

Antimicrobial peptides (AMPs) are evolutionary ancient peptide antibiotics produced by all multicellular organisms. They are part of the primary defense against microbial infections and exhibit antimicrobial activity against bacteria, fungi and some enveloped viruses (Zasloff, 2002; Bevins, 2003). Humans produce different classes of AMPs, one of them are the defensins. These secreted peptides are characterized by their small size (3 to 5 kDa), cationic net charge and six conserved cysteine residues, which are connected via three disulphide bridges (Martin *et al,* 1995; Zasloff, 2002; Harder *et al,* 2007). Human beta-defensin 1 (hBD-1) was the first beta-defensin identified in humans and is produced by epithelia, monocytes, plasmacyoid dendritic cells and platelets (Bensch *et al,* 1995; Zhao *et al,* 1996; Ryan *et al,* 2011; Kraemer *et al,* 2011). In contrast to inducible beta-defensins 2 and 3, hBD-1 is produced constitutively and its expression can be regulated by peroxisome proliferator-activated receptor gamma (PPARγ) and hypoxia-inducible factor alpha (HIF1-α) (Zhao *et al,* 1996; Peyrin-Biroulet *et al,* 2010; Kelly *et al,* 2013). We could recently show that antimicrobial activity of hBD-1 is strongly increased after reduction of its three disulphide bridges independent of bacterial Gram-status (Schroeder *et al,* 2011b; Raschig *et al,* 2017). Activation of the peptide could be executed by a reducing environment or enzymatically by the oxido-reductase thioredoxin (Schroeder *et al,* 2011b; Jaeger *et al,* 2013). Due to their ancient evolutionary origin and the strong demand for novel antimicrobial strategies, AMPs have been considered as potential antibiotic drug candidates. Mainly because

they target "Achilles heels" of microorganisms, only few resistance mechanisms have been evolved over long time (Andersson *et al,* 2016; Fleitas *et al,* 2016). Still, large-scale chemical synthesis of defensins containing three native disulphide-bridges has been a challenge and rendered the production expensive. Accordingly, the production of smaller, but yet antibiotic, fragments of defensins without disulphide bridges is a promising option. We have shown previously that reduced hBD-1 can be degraded by the intestinal protease trypsin (Schroeder *et al,* 2011a). Here, we evaluate a carboxy-terminal fragment of hBD-1 that is generated after proteolytic digestion by intestinal proteases. We investigate its antimicrobial activity and its potential to be exploited as a possible candidate for future antibiotic drug development.

3.2. Material and Methods

Bacterial and fungal strains

Bacterial strains (*Bifidobacterium adolescentis* Ni, 29c (clinical isolate), *Bifidobacterium breve* (from probiotic VSL#3) and *Streptococcous salivarius ssp. thermophiles* DSM 20617 were obtained from Ardeypharm (Germany). *Escherichia coli* ATCC 25922, *Escherichia coli* K12, *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans* ATCC 10231, *Enterococcus faecalis* ATCC 29212 as well as antibiotic-resistant clinical isolates of *Acinetobacter baumannii*, *Enterococcus faecalis*, *Enterococcus faecium*, *Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* were provided by the Department for Laboratory Medicine at Robert-Bosch-Hospital Stuttgart, Germany. *Candida albicans* SC5314 was obtained from Salomé LeibundGut-Landmann (Institute of Immunology, Vetsuisse Faculty, University of Zürich, Switzerland).

Peptides

Carboxy-terminal octapeptides were chemically synthesized by EMC Microcollections (Tuebingen, Germany) and purified by precipitation. The oxidized peptides were obtained from Peptide Institute (Japan). All peptides were dissolved in 0.01 % acetic acid.

Protease digestion and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS)

2 µg of oxidized or reduced hBD-1 were digested with pepsin or chymotrypsin at a protease: peptide ratio of 1:20 in HCl-acidified water, pH 3 (pepsin) or 10 mM sodium phosphate, pH 7.4 (chymotrypsin) for 90 min. Human duodenal secretion (pH 6.5 – 7) was taken during a routine gastroscopy by rinsing the duodenum with saline. Oxidized and reduced hBD-1 were incubated

with human duodenal secretion for 30 min at 37°C. As a control both peptides were incubated with 0.9% NaCl. Peptides were enriched with ZipTip (Millipore), co-crystallized with a-cyano-4-hydroxy cinnamic acid and analyzed with an ultraflex TOF/TOF machine (Bruker, Germany).

Radial Diffusion Assay

Antimicrobial radial diffusion assay was modified from reference (Lehrer *et al,* 1991) and performed as described earlier (Schroeder *et al,* 2011b). Briefly, microorganisms were cultivated (anaerobic bacteria with AnaeroGen, Oxoid, UK) for up to 18 hours in liquid TSB medium. Log-phase cultures were washed and diluted to 4 x 10^6 colony forming units in 10 ml agar. Incubation was carried out in 10 ml of 10 mM sodium phosphate, either pH 7.4 or 5.7, containing 0.3 mg/ml of TSB powder and 1% (w/v) low EEO-agarose (AppliChem) with 0 or 1 mM dithiothreitol (DTT, Sigma-Aldrich) under anaerobic or aerobic conditions for three hours. 1 or 4 µg of synthetic, oxidized hBD-1 (Peptide Institute, Japan) and 1 or 4 µg of synthetic peptides (EMC Microcollections, Tuebingen) were filled into small punched wells in a final volume of 4 µl. This concentrated peptide solution dilutes while diffusing into the gel, thereby generating concentration-dependent, round-shaped inhibition zones when killing immobilized microorganisms. An overlay-gel containing 6% (w/v) TSB powder, 1% agarose and 10 mM sodium phosphate buffer without DTT was poured onto the plates and after incubation for up to 48 h at 37°C the diameter of inhibition zones was measured. Experiments were repeated at least three times; mean ± SEM is shown.

Microdilution Broth Assay

To differentiate between microbistatic and microbicidal activity we performed a broth microdilution assay. For that, *E. coli* ATCC25922 bacteria were incubated overnight at 37°C, 150 rpm. *C. albicans* ATCC 10231 was grown at 30°C overnight, 150 rpm in liquid TSB. Cells were collected by centrifugation (2500 rpm, 10 min, 4°C), washed twice and resuspended in 10 mM sodium phosphate buffer containing 1% (w/v) TSB broth. Required *C. albicans* cell density was adjusted using a hemocytometer. For bacteria the optical density of OD600nm = 0.1 was determined. Approximately 5 x 10^5 CFU/ml bacteria or fungi were mixed with indicated peptide concentrations (1.25 – 200 µg/ml) in a final volume of 100 µl in 10 mM sodium phosphate buffer containing 1% (w/v) TSB broth and incubated for 2 hours at 37°C. After incubation 10 µl per well were plated on LB-/ or YPD-agar plates to determine the CFU/ml. After that 100 µl of 6% TSB (w/v) were added and absorbance was measured at 600 nm (Tecan, Switzerland) and monitored for 18 hours. Growth relative to the positive control in % was plotted against peptide concentration. Experiments were carried out at least three times; mean ± SEM is shown.

Flow Cytometry Assay

Approximately 1.5 x 10^6 CFU log-phase bacteria or overnight cultured *C. albicans* ATCC 10231 were used in a final volume of 100 µl TSB (1:6 diluted H2O). We added peptides in concentrations 50 and 100 µg/ml in a final volume of 10 µl and incubated theses suspensions for 1h at 37°C. Subsequently 2 µl of membrane potential sensitive dye $DiBAC_4(3)$ [bis-[1,3-dibutylbarituric acid)trimethine oxonol] (Thermo Scientific, USA) (50 µg/ml) or Propidium Iodide (Thermo Scientific, USA) (50 µg/ml) were added and incubated for 10 min at room temperature. Then samples were centrifuged (5 min, 7000 rpm, RT) and re-suspended in 300 µl PBS. The percentage of fluorescent positive cells was determined using Canto II flow cytometer (BD Bioscience) with DIVA software (BD Bioscience) as described earlier (Nuding *et al,* 2006). Experiments were repeated at least three times and mean ± SEM is shown.

Transmission Electron Microscopy

Approximately 1 x 10^8 CFU of *E. coli* ATCC 25922 and *C. albicans* ATCC 10231 were incubated with 400 µg/ml peptides or control solution for 2 hours at 37°C. Treated microorganism were fixed with Karnovsky's fixative (3% Paraformaldehyd, 3.6% Glutaraldehyd, pH 7,2) and embedded in 3.5% agarose at 37°C, coagulated at room temperature, and fixed again in Karnovsky's fixative. Post-fixed samples (1% OsO_4, 1 h) were rinsed with distilled water, block-stained with uranyl acetate (2% in distilled water), dehydrated in alcohol (stepwise 30-96%), immersed in propylene oxide and embedded in glycine ether (polymerized 48 h at 60 C, Serva, Heidelberg). Ultra-thin sections were examined with a LIBRA 120 (Carl Zeiss AG, Oberkochen) at 120 kV.

Metabolic Activity Assay

To assess the metobalic activity of Caco-2 cells we used the WST-1 Cell Proliferation Reagent (Roche, Germany). Briefly, 1 x 10^5 Cells /ml were seeded and incubated with 100 µg/ml or 200 µg/ml octapeptides for 24h at 37°C, 5% CO_2. After incubation the supernatant was removed and cells were washed with PBS and incubated with 20 µl Cell Proliferation Reagent WST-1 for 1h 37°C, 5% CO_2. Finally the absorbance was measured at 450 nm and 620 nm. Experiments were repeated three times, mean ± SEM.

Hemolytic Activity of Antimicrobial Peptides

A hemolytic activity assay for testing antimicrobial peptides was performed as described earlier (Oddo & Hansen, 2017). Briefly, 150 µl of melittin (5 µM) was added to the positive control wells and incubated overnight. On the next day 1 ml blood was added to 3 ml PBS, mixed gently and

centrifuged for 8 min, 700 x g. The supernatant was discarded and cells were re-suspended in 4 ml PBS and centrifuged again. After removing the supernatant, cells were centrifuged for 8 min at 1000 x g. Supernatant was discarded. For each well we used 75 µl of 1% Red blood cell (RBC) suspension in PBS. RBC suspension was mixed with indicated peptide concentration (2.5 – 200 µg/ml) in a final volume of 150 µl and incubated for 1h at 37°C. Finally the plate was centrifuged at 1000 x g for 10 min and 60 µl of supernatant was quickly transferred into a new plate. The absorbance was measured by 405nm and 540 nm. Hemolytic activity was plotted relative to the 0.2 % Triton X-100. Experiments were repeated three times; mean ± SEM is shown.

Culture of a Model Human Oral Epithelium

1 x 10^6 TR146 cells (derived from a squamous cell carcinoma of the buccal mucosa; SkinEthic, France) were seeded into polycarbonate plastic inserts (Millipore) in DMEM (Lonza) and cultured for 8 days to form a multilayered epithelium. Medium in the wells (basal) and in the inserts (apical) was changed daily. On day 5 (airlift) medium was aspired from the apical side and cells were fed from the basal side for the rest of culture time in 6-well-plates. No antibiotics were used for the entire time of culture and the experiments (Mailänder-Sánchez *et al,* 2017). The octapeptides were diluted to 100 µg/ml in 0.01% acetic acid and 50 µl of these dilutions were applied to the apical side of the model epithelia and incubated for 24h. The supernatant was used for cytotoxicity assays and enzyme-linked immunosorbent assay.

LDH-Cytotoxicity Assay

To analyze the damage of the epithelial cells caused by octapeptides, release of lactate dehydrogenase (LDH) into the supernatant of RHOEs was quantified, using the cytotoxicity detection kit with L-LDH solution as standard, according to the manufacturer's instructions (Roche, Germany). Supernatants of RHOEs were analyzed 24h post infection.

Enzyme-linked Immunosorbent Assay

Interleukin 8 (IL-8) and IL-1α were quantified in the supernatants of RHOEs using DuoSet ELISA-Kits (RnD Systems, US) according to the manufacturer's instructions. Further details are explained above (Culture of a model human oral epithelium).

Infection of Model Human Oral Epithelium with *C. albicans*

Octapeptides were diluted to 50 µg/ml in PBS, or an equivalent volume of 0.01% acetic acid were applied to the apical side of the RHOEs and incubated for 1h. *C. albicans* SC5314 was synchronized as described previously (Schaller *et al,* 2006). Yeast cells were washed three times

in PBS and 1 x 10^5 CFU were used for infection of pre-treated RHEs. After 24h RHEs were fixed with Karnovsky´s fixative. Post-fixed samples (1% OsO_4, 1 h) were rinsed with distilled water, block-stained with uranyl acetate (2% in distilled water), dehydrated in alcohol (stepwise 30-96%), immersed in propylene oxide and embedded in glycide ether (polymerized 48h at 60°C, Serva, Heidelberg). Semi-thin sections were stained with toluidine blue and examined with a Nikon Eclipse 80i light microscope (magnification 1:400). Afterwards, fungal cells were simply coloured in red. Epithelial damage was evaluated by two independent experts in a blinded manner on a scale between 0 and 5 with 0= intact epithelia, 1= damage only in topmost cell layer; 2 = damage in top third of epithelium; 3 = damage in top half of epithelium; 4 = damage in all cell layers except lowermost; 5 = damage in all epithelial layers.

Ethics statement

The study protocol was previously approved by the Ethical Committee of the University Hospital, Tuebingen, Germany. Patients and controls who were included in this study all gave their written and informed consent after the study purpose, samples procedure, and potential adjunctive risks were explained. All experiments were conducted in accordance with the relevant guidelines and regulations.

Statistical Analysis

Results are presented as mean ± SEM from at least three independent experiments. Statistical analysis was performed using GraphPad Prism 7.03. The Data were not normally distributed and a Kruskal-Wallis test was performed. * indicate statistically significant differences compared to infected control. P-values, showing the statistical significance, were displayed by asterisks: $p>0.05$ = ns; $p\leq0.05$ = *; $p\leq0.01$ = **; $p\leq0.001$ = ***; $p\leq0.0001$ = ****.

3.3. Results

3.3.1. Degradation of reduced hBD-1 generates an antimicrobial octapeptide

Reduction of the three disulphide-bridges of hBD-1 yields a linear peptide which not only differs structurally from the oxidized form (Schroeder *et al,* 2011b), but is also more prone to proteolytic degradation by the protease trypsin. (Schroeder *et al,* 2011a). To further analyze proteolytic susceptibility of hBD-1 (Figure 1a) towards physiological gastro-intestinal proteases, we treated oxidized and reduced hBD-1 with pepsin and chymotrypsin (Figure 1b). Similar to trypsin digestion, oxidized hBD-1 was protease resistant while the reduced form was readily digested. Focusing on the degradation products we detected a fragment having an m/z of 893.5, corresponding to the eight carboxy-terminal amino acids of hBD-1, NH_2-RGKAKCCK-COOH (RGKAKCCK). To assess the in vivo relevance of our findings in more detail, we incubated oxidized and reduced hBD-1 with human duodenal secretion, which is rich in proteolytic enzymes. In agreement with our in vitro data, ex vivo digestion generated a fragment having an m/z of 893.5 (Figure 1c) for reduced hBD-1 but not for oxidized hBD-1. While the in vivo presence of the octapeptide in the human gut remains to be proven, this finding supports the hypothesis that proteolytic cleavage of reduced hBD-1 could generate a novel antimicrobial peptide in the human intestine. To test whether antibiotic activity is retained in this degradation product we next analyzed antimicrobial activity of the terminal octapeptide. In a radial diffusion assay (RDA) (Lehrer *et al,* 1991) we thus compared activity of oxidized and reduced hBD-1 with the octapeptide RGKAKCCK against selected commensal and pathogenic microorganisms (Figure 1d). The octapeptide exhibited convincing activity against *Bifidobacterium adolescentis*, *Streptococcus salivarius ssp. thermophilus*, *Escherichia coli*, *Candida albicans* but neglectable antibiotic activity against *Pseudomonas aeruginosa*. While RGKAKCCK and oxidized hBD-1 generated no inhibition zones against *Bifidobacterium breve*, only reduced hBD-1 inhibited its growth. As expected, however, semi-quantitative evaluation on a molar base (4 µg reduced hBD-1 is equivalent to 254 µM in the RDA while 1 µg of octapeptide is equivalent to 280 µM) revealed that activity of the isolated terminus is less potent compared with the full length-peptide (Figure 1d). We found previously that cysteine residues are crucial for antimicrobial activity of hBD-1 against *E. coli* and *Bif. adolescentis* (Schroeder *et al,* 2011b; Raschig *et al,* 2017). We confirmed these results for the terminal octapeptide, as replacing both cysteines (RGKAKAAK) completely abolished activity against *E.coli* and *C. albicans* (Figure 1e, Supplementary Figure 1). However, replacing either Cys6 (RGKAKACK) or Cys7 (RGKAKCAK) by alanine resulted in strongly decreased activity against *C. albicans*, while antibiotic activity of those peptides increased

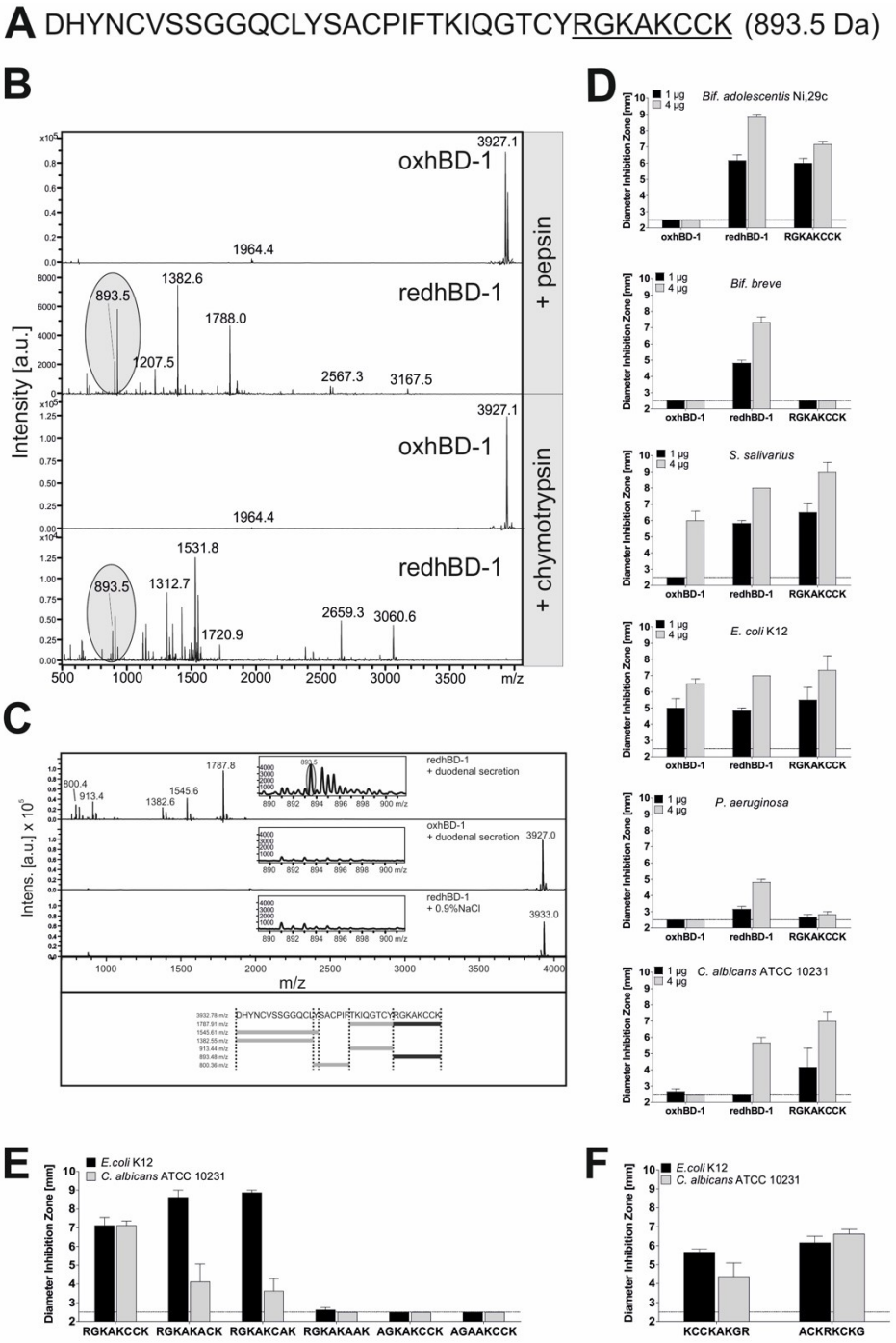

Figure 1. Proteolytic digestion of reduced hBD-1 generates an antimicrobial octapeptide. **(A)** Aminoacid sequence of hBD-1, depicted as one-letter code. **(B)** The oxidized and the reduced form of hBD-1 were digested with pepsin or chemotrypsin and fragments were analyzed by MALDI-MS. The carboxy-terminal fragment RGKAKCCK (893.5 Da, highlighted in a) was further investigated. **(C)** The oxidized and the reduced hBD-1 were digested with human duodenal secretion and fragments were analyzed by MALDI-MS. Fragments were identified by comparison with an in silico digest using ExPASy software. **(D)** Different peptide concentrations of oxidized (ox) and reduced (red) as well as the carboxy-terminal octapeptide RGKAKCCK were tested in an antimicrobial diffusion assay against several microbial strains. Diameter of inhibition zones indicates antimicrobial activity; a diameter of 2.5 mm (dotted line) is the diameter of an empty well. **(E,F)** Modifications of RGKAKCCK (4 µg) were tested in an antimicrobial diffusion assay against *E. coli* and a fungal strain *C. albicans*. Letters indicate amino acid one-letter code. All diffusion assays were carried out at least three times, mean +/- SEM is shown.

against *E.coli*. Consequently, cysteine residues seem to have an important, yet different, role for the antibiotic mechanism against the tested fungi and bacteria. As antimicrobial peptide activity also relies on a positive net charge (Taylor *et al,* 2008; Lehrer, 2011) we further investigated the role of positively charged amino acids (Figure 1e). Despite having two cysteine residues, the variants lacking Arg1 (AGKAKCCK, net charge +3) or Arg1 and Lys3 (AGAAKCCK, net charge +2) were completely inactive against both tested microorganism. Thus, antimicrobial activity of the octapeptide RGKAKCCK (net charge +4) depends on cysteine residues as well as a particular positive charge. Yet, as a reversed-order peptide had lower activity than the wild-type peptide or a scrambled version, especially against *C. albicans* (Figure 1f), not only the amino acid composition but also its sequential order or its position seem to be involved in the peptides' activity.

3.3.2. Characterization of RGKAKCCK and its modified variants

Since the discovery of antimicrobial peptides there is anticipation to exploit them as antibiotic drugs (Kruse & Kristensen, 2008). To test the potential of our octapeptide to be used as a therapeutic agent, we first generated peptide variants to improve its stability. To prevent non-specific cleavage by amino-carboxypeptidases, we chemically stabilized its termini by amino-terminal acetylation and carboxy-terminal amidation (Ac-RGKAKCCK-NH_2) and generated both peptides also in D-amino acid configuration (rGkakcck and Ac-rGkakcck-NH_2, respectively).

Next, to evaluate the antibiotic activity, we tested wild-type and modified peptide variants in their ability to inhibit growth of (opportunistic) pathogenic microorganisms (Figure 2a). Direct comparison revealed that those variants with stabilized termini had promising activity against *E. coli* and *C. albicans*. Moreover, while all peptides displayed antimicrobial activity at pH 7.4, a reducing environment (DTT), acidification (pH 5.7), or a combination of acidification and reducing conditions (pH 5.7 + DTT) strongly decreased antimicrobial activity against *E. coli* (Supplementary Figure 2a) and *C. albicans* (Supplementary Figure 2b). Thus, antimicrobial activity of the generated octapeptides can be influenced by environmental conditions, in particular by reducing conditions with an acidic pH.

The widespread use of antibiotics in agriculture and to treat bacterial infections has led to a rapid emergence of microbial resistance (Taubes, 2008; Chait *et al,* 2012). As a consequence, in hospitals several multi-drug resistant strains exist that threaten effective therapy of microbial infections (Ventola, 2015). We therefore tested if the hBD1-derived peptide and its modified forms are also active against drug-resistant clinical isolates. As shown in figure 2b, we identified antimicrobial activity against clinical isolates of antibiotic-resistant *E. coli*, *Pseudomonas aeruginosa* and *Enterococcus faecium*. In contrast, *Acinetobacter baumanii* was only susceptible towards Ac-rGkakcck-NH_2 whereas *Enterococcus faecalis* and *K. pneumoniae* were not sensitive.

Direct comparison of the tested peptide revealed that the peptides RGKAKCCK and Ac-rGkakcck-NH_2 inhibited growth of the most tested antibiotic-resistant bacteria, making them the most promising candidates for further drug development among the four tested peptides.

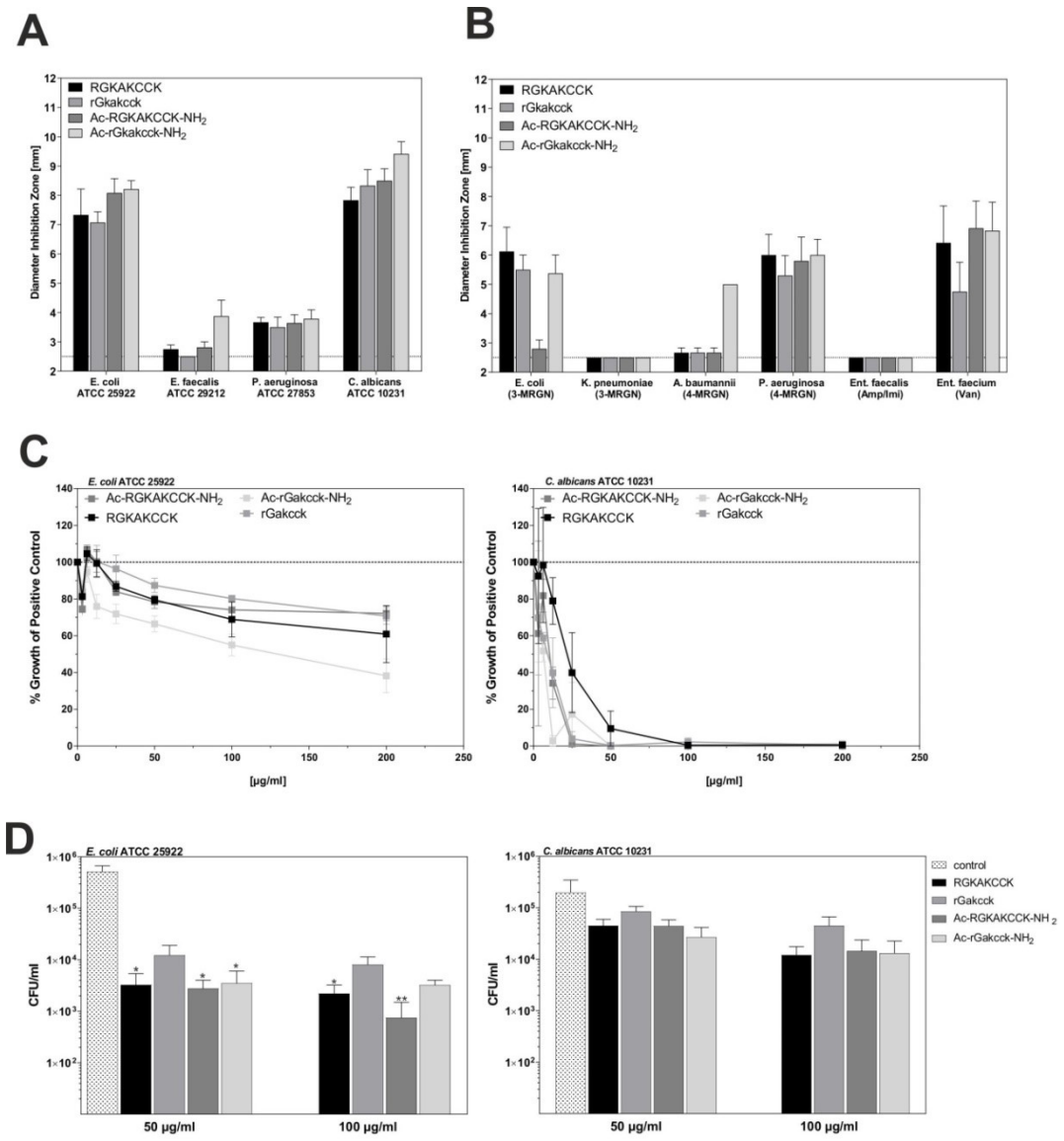

Figure 2. Antimicrobial activity of RGKAKCCK and its modified variants. The carboxyterminal octapeptide RGKAKCCK was stabilized at its termini by acetylation of the amino-terminus and amidation of the carboxy-terminus (Ac-RGKAKCCK-NH_2). Both variants were also synthesized by using D-stereoisomeric amino acids (indicated by small letters). Antimicrobial activity of octapeptides (4 µg) was tested against pathogenic microorganisms of clinical relevance (A) and with antibiotic resistance (B) in radial diffusion assay. 3-MRGN: multi-resistant Gram negative pathogen (3 out of 4 antibiotic classes), 4-MRGN: multi-resistant Gram negative pathogen (4 out of 4 antibiotic classes) MRSA: Methicillin-resistant *Staphylococcus aureus*, Amp/Imi: Ampicillin/Imipinem, Van: Vancomycin. (C) Different concentrations of octapeptides were tested in a turbidity liquid assay against *E. coli* ATCC 25922 and *C. albicans* ATCC 10231. Peptides were incubated with tested microorganisms and change in optical density (OD600nm) was measured and % growth of untreated control was plotted after 12 hours. (D) Aliquots were plated on agar plates and colony forming units (CFUs) were calculated the next day. Data are presented as mean +/- SEM of at least three independent experiments. The statistical significance was evaluated by using Kruskal-Wallis test compared to control and marked with *= $p < 0.05$ and **$p < 0.01$.

As the radial diffusion assay does not differentiate between microbistatic and microbicidal activity and contains immobilized bacteria or fungal cells, we complemented our antimicrobial tests with a broth microdilution assay to investigate susceptibility of *E. coli* and *C. albicans*. The tested octapeptides completely inhibited *C. albicans* growth at concentrations of 100 µg/ml, while the growth of E. coli was only inhibited to 40 % of the untreated control by Ac-rGkakcck-NH_2 (Figure 2c). In addition, microbial cultures were incubated with the different peptides and colony forming units (CFU) were determined. For *E. coli* incubated with 100 µg/ml of RGKAKCCK or Ac-RGKAKCCK-NH_2, a more than 100-fold decrease in CFU was observed when compared to untreated controls (Figure 2d). In contrast, CFU reduction of *C. albicans* was less pronounced, thus, indicating bactericidal activity against *E.coli* and a combination of fungicidal and fungistatic activity against *C. albicans* of the tested octapeptides.

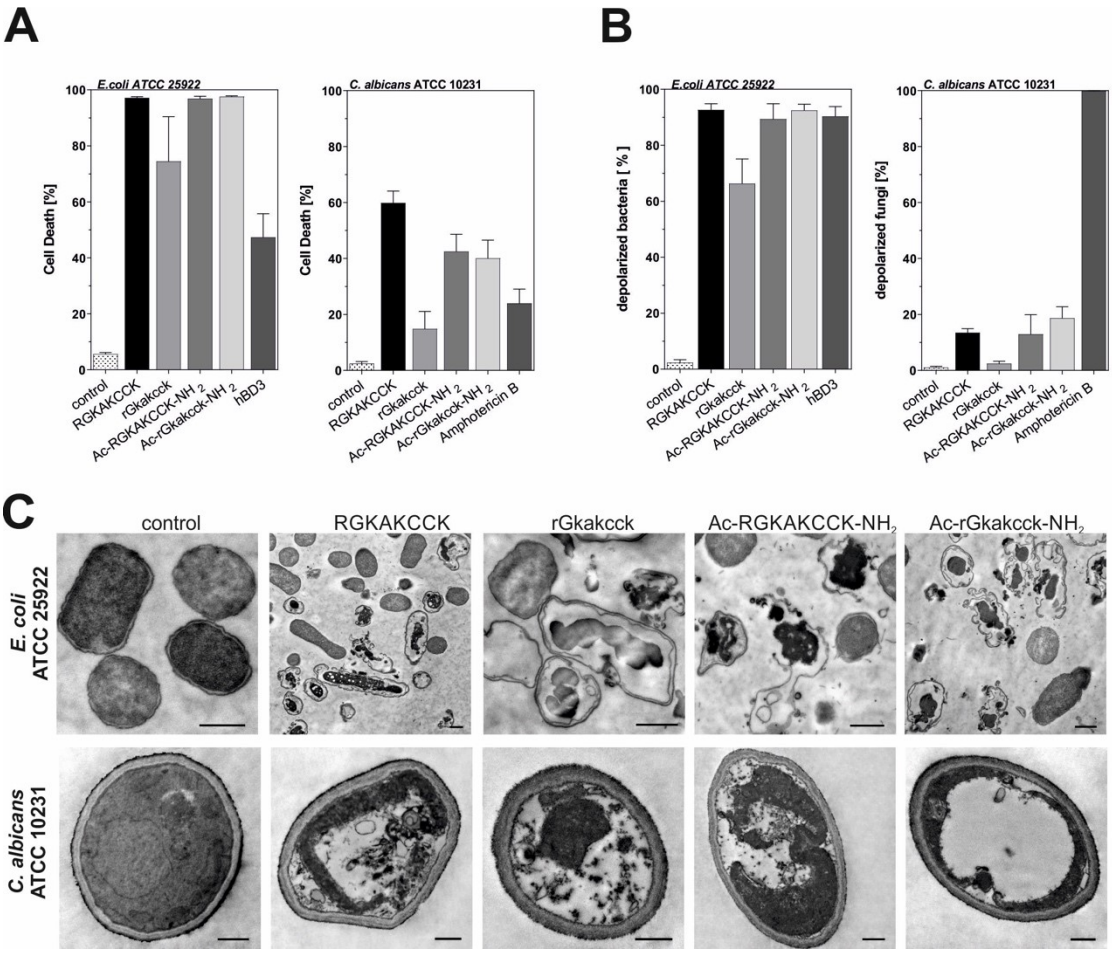

Figure 3. Characterization of mode of action in *E. coli* and *C. albicans*. (A) Membrane pores or (B) Membrane depolarization of 1x10^6 CFU *E. coli* ATCC 25922 or *C. albicans* ATCC 10231 in response to 100 µg/ml octapeptides were tested. Microorganisms were treated 1h with 100 µg/ml peptide and living organisms were analyzed by flow cytometry. As control we used hBD3 (50 µg/ml) and Amphotericin B (20 µg/ml) and untreated strains. Data are presented as mean +/- SEM of at least three independent experiments. (C) Transmission electron micrograph of *E. coli* (upper panel) and *C. albicans* (lower panel) treated with 400 µg/ml peptide. Magnification bar: 0.5 µm.

Many antimicrobial peptides target the microbial membrane (Mathew & Nagaraj, 2017; Mukherjee *et al,* 2014). To test whether this is also true for the octapeptides, we used a flow cytometric assay measuring membrane permeability by the dye propidium iodide (PI), which cannot permeate intact membranes. *E. coli* and *C. albicans* were incubated with the peptides and PI uptake, which indicated cell death, was analyzed. For *E. coli*, all tested peptides exhibited at least 70% bacterial killing, while treatment with RGKAKCCK, Ac-RGKAKCCK-NH_2 or Ac-rGkakcck-NH_2 led to almost 100% cell death (Figure 3a). In contrast, only the wild-type peptide RGKAKCCK induced up to 60% PI uptake when incubated with *C. albicans*.

In addition to PI uptake, we analyzed cellular membrane potential by using the membrane potential sensitive dye $DiBAC_4(3)$ (Figure 3b). When incubating the peptides with *E.coli*, we observed strong membrane depolarization for the same peptides that caused PI uptake. In contrast, *C. albicans* displayed less than 20 % of membrane depolarization. Thus, our results support a bactericidal effect of RGKAKCCK and Ac-rGkakcck-NH_2 against *E.coli* by targeting the bacterial membrane, while the antibiotic effect against *C. albicans* seems rather membrane-independent. To further investigate whether octapeptide treatment leads to structural damage of the microorganisms we used transmission electron microscopy (TEM) to visualize peptide-treated bacteria and fungi. Incubation of *E. coli* and *C. albicans* with all tested variants of the octapeptide led to different degrees of structural disintegration (Figure 3c). This was especially pronounced in *E. coli*, where all peptides caused detachment of the cell membrane from the cytosol, cell wall and membrane disruption as well as disintegration of cytosolic structures. In *C. albicans*, however, no destruction of the fungal cell wall could be observed, confirming results from the flow cytometric experiments (Figure 3a/b). Still, disintegration of cytosolic structures could also be observed in fungal cells, similar to the damage observed in *E. coli*. Taken together, our results demonstrate a bactericidal and fungicidal/fungistatic effect of the tested octapeptides.

3.3.3. Potential of the octapeptides for therapeutic drug development

For potential therapeutic application, toxicity of the peptides needs to be excluded. We therefore analyzed cell-toxicity of all peptide variants against the intestinal epithelial cell line CaCo-2 and against erythrocytes. By utilization of a WST viability test we could not observe any cell toxicity in the relevant antimicrobial concentration ranges of 100 µg/ml (Figure 4a) and 200 µg/ml (Supplementary Figure 4a) or any hemolytic effect against erythrocytes (Figure 4b and Supplementary Figure 4b).

We furthermore tested the toxicity of the octapeptides by using in vitro reconstituted human oral epithelium (RHOE), which was analyzed by histology and lactate dehydrogenase (LDH) activity (Figure 4c). No LDH release was detected with peptide variants, whereas the

cytotoxic compound Triton-X-100, used as a positive control, induced a strong release of LDH. Similarly, when quantifying the release of the pro-inflammatory cytokines Interleukin-8 (IL-8) and IL-1α after incubation of the RHOE with the octapeptides we did not detect any inflammatory response in the RHOE (Figure 4d).

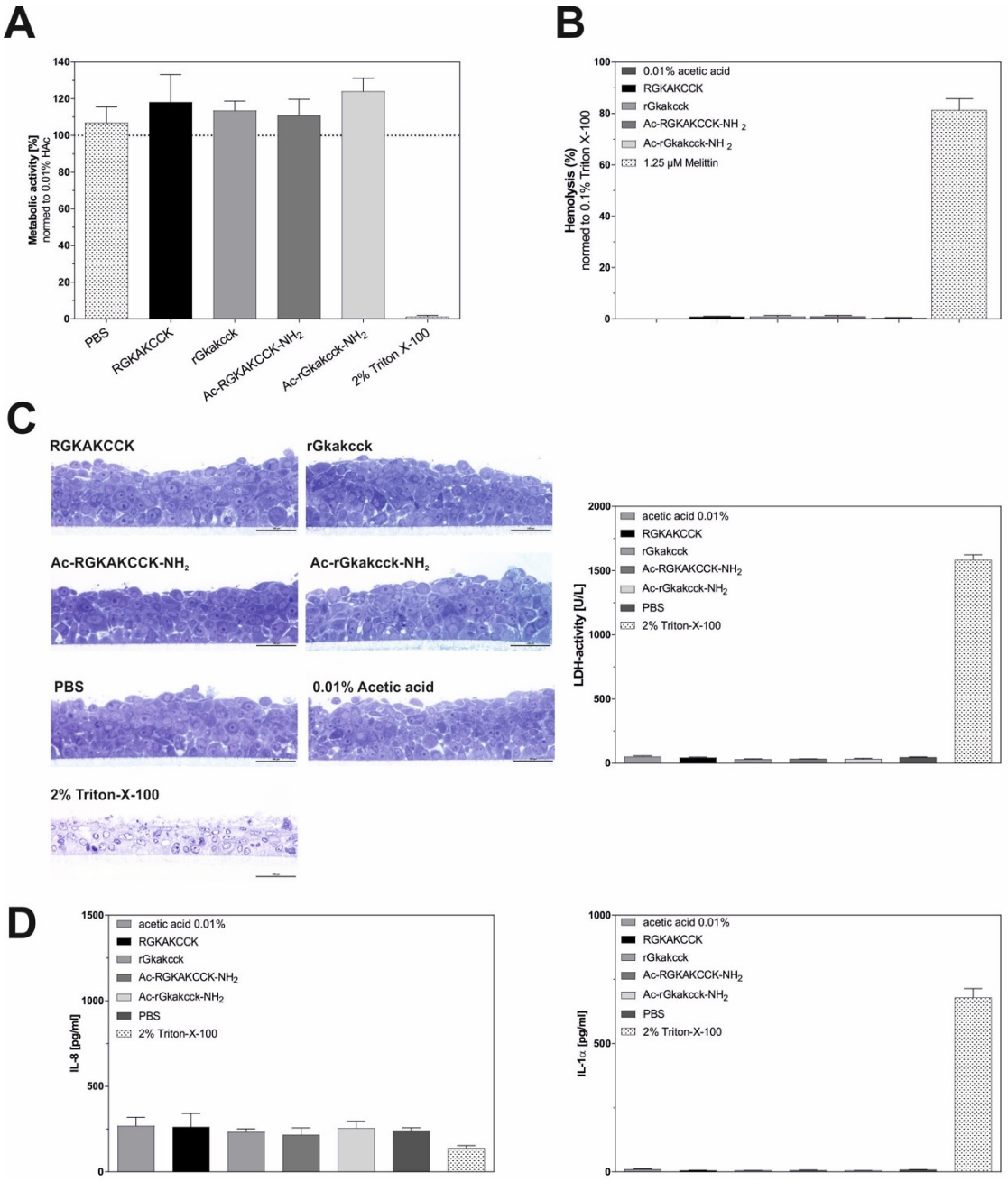

Figure 4. Modified octapeptides are not cytotoxic. Cytotoxicity of octapeptides (100 µg/ml) was investigated by **(A)** WST-1 based test against the human intestinal epithelial cell lines CaCo-2 and **(B)** Hemolytic Activity assay against erythrocytes, using 2% Trition-X-100 and 1.25 µM Melittin as positive control. **(C)** Histological analysis of model oral epithelia treated with 100 µg/ml peptide and cytotoxicity was additionally tested by lactate dehydrogenase release against this model human oral epithelium. **(D)** Cytokine release of model oral epithelia was analyzed by ELISA. Mean +/- SEM of three independent experiments is shown.

Suitability of the octapeptides as novel candidates for antimicrobial drug development was further substantiated in a model or oral candidiasis (Schaller *et al,* 2006). In this model a multilayer of RHOE was preincubated with 50 µg/ml (Figure 5a) or 100 µg/ml octapeptides (Supplementary Figure 5) for 1h before infecting the cells with *C. albicans* for 24h (Figure 5a). Epithelial damage of RHOEs was quantified by independent experts in a blinded manner on a scale between 0 and 5 (Figure 5b). Untreated cells infected with *C. albicans* displayed evident

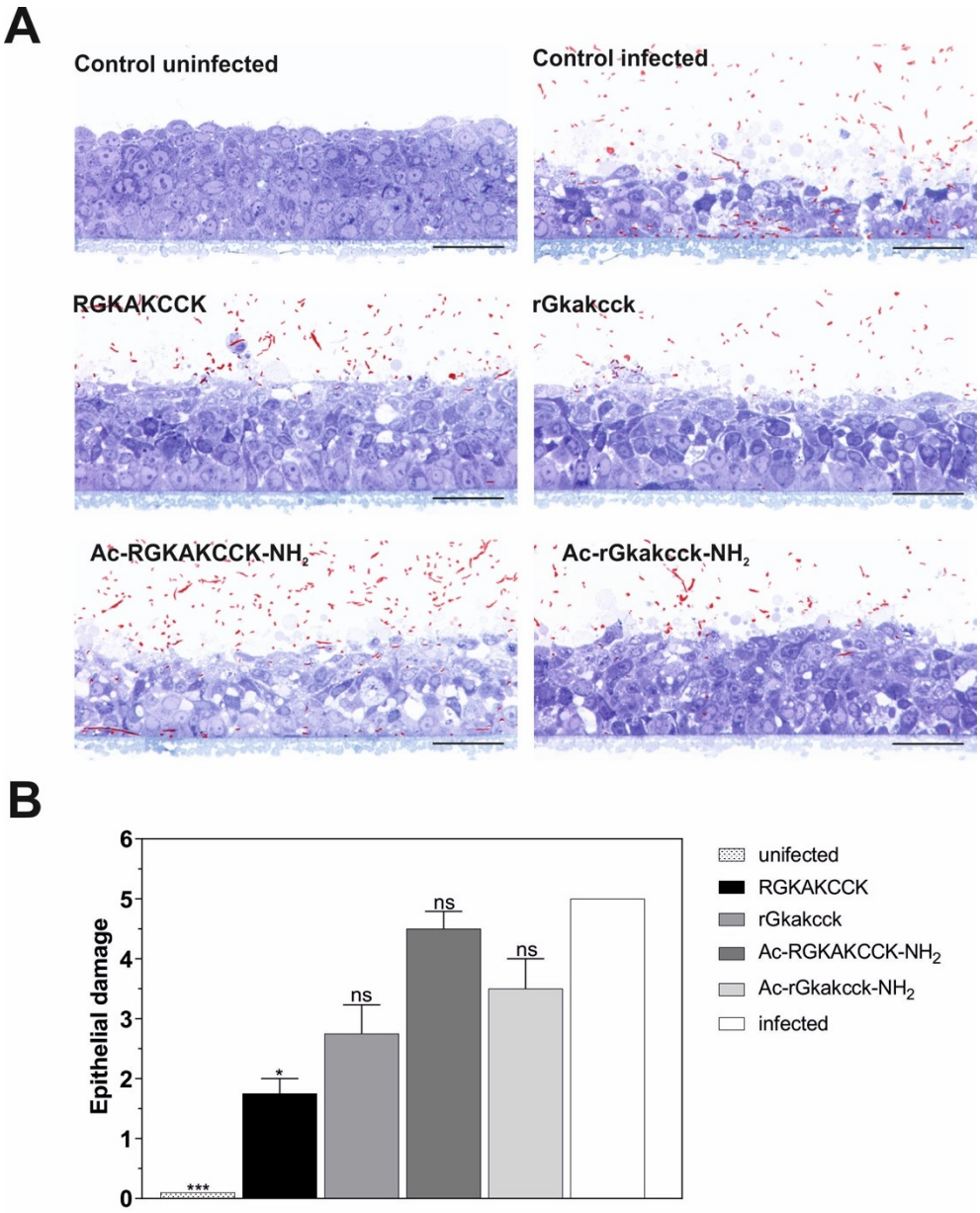

Figure 5. Octapeptides are potential candidates for antimicrobial drug development. **(A)** Reconstituted human oral epithelia were pre-incubated with PBS (control uninfected) or 50 µg/ml of octapeptides as indicated. Subsequently cells were infected with *C. albicans* SC5314 (highlighted in red). Representative images are shown (magnification 400x, bar = 100 µm). **(B)** Epithelial damage was evaluated by four independent experts and the combined evaluation (mean +/- SEM, criteria described in methods) is shown. The statistical significance was evaluated by using Kruskal-Wallis test with ns = not significant and *= $p < 0.05$.

epithelial damage and cell lysis and detectable fungi (colored in red) in all layers of the epithelium. However, pre-treatment with RGKAKCCK convincingly reduced the fungal load in the epithelium and ameliorated epithelial damage. Additionally, a histological analysis confirmed the protective effect of RGKAKCCK (Figure 5b) whereas a pre-treatment with 50 µg/ml rGkakcck moderately improved epithelial damage. In contrast, epithelial damage and a high amount of detectable fungal cells in lower epithelium were observed with a pre-treatment with the modified octapeptides Ac-RGKAKCCK-NH_2 and Ac-rGkakcck-NH_2: While *C. albicans* cells invaded the whole epithelium with a pre-treatment with Ac-RGKAKCCK-NH_2, *C. albicans* invaded and damaged only the upper epithelium with a pre-treatment with Ac-rGkakcck-NH_2. Consequently, our eight amino-acid peptide RGKAKCCK of the carboxy-terminus of hBD1 has the best potential to be optimized for topical application against infectious microbes on epithelial surfaces.

3.4. Discussion

So far, no multicellular organism has been identified which does not produce antimicrobial peptides. Even over a long time of evolution those host defense molecules have retained their antimicrobial capacity with only minor resistance mechanisms at the microbial target (Peschel & Sahl, 2006; Fleitas *et al,* 2016). Thus, an improved knowledge of these antibacterial molecules may help to identify novel targets for antimicrobial therapy (Nizet, 2006). In fact, in the recent years short antimicrobial peptides have gained increased interest as new opportunities for therapeutics (Fosgerau & Hoffmann, 2015). In here, we characterized a carboxy-terminal octapeptide of hBD-1 that was generated after proteolytic digestion by gastro-intestinal proteases and might thus occur in vivo in the human gastrointestinal tract.

While reduction of disulphide bridges increases activity of several antimicrobial peptides (Masuda et al, 2011; Schroeder et al, 2011b, 2015), it also increases their susceptibility towards proteolytic digestion. This is especially relevant in the intestinal tract, where duodenal secretions contain high amounts of proteases to facilitate digestion of dietary proteins. However, our observation that a degradation product of hBD-1 retains antibiotic activity indicates effective utilization of a scare resource: we speculate that once the reduced peptides diffuse from the intestinal mucus layer towards the lumen, they can be degraded as soon as they come into contact with intestinal proteases. But instead of being inactivated, degradation products can retain their antimicrobial activity, thereby making the most effective use of these antimicrobial host defense molecules.

While a major shift in antimicrobial activity and activity spectrum can be observed after reduction of the oxidized hBD-1 (Figure 1d, consistent with (Schroeder *et al,* 2011b; Raschig *et al,* 2017; Wendler *et al,* 2018), only minor alterations can be observed after degradation of the

reduced peptide. However, on a molar basis the octapeptide does not exhibit the full potency of the full-length peptide, suggesting that the remaining 28 amino acids of hBD-1 hold additional features that enhance and modulate antibiotic activity as, for instance, observed for *Bifidobacterium breve* (Figure 1d). Remarkably, and in contrast to hBD-1, a reducing environment diminished antimicrobial activity of the tested octapeptides against *E. coli* and *C. albicans* (Supplementary Figure 2). It is possible that due to their small size the peptides need to form dimers or higher-order oligomers to effectively penetrate the microbial cell wall. It is likely that such oligomerization occurs by forming intermolecular disulphide-bridges, thus explaining the strong dependence on cysteine residues for the activity of RGKAKCCK against *E. coli* and *C. albicans* (Figure 1e). We could observe that human blood serum can influence bacterial growth and the antimicrobial activity (Supplementary Figure 3). Similarly, acidic pH led to lower antimicrobial activity as compared to pH 7.4 (Supplementary Figure 2). This is in accordance with previous studies, which could show that antimicrobial peptides can bind to human plasma proteins (de Breij *et al,* 2018; Wang *et al,* 2004). Thus, further optimization of a potential peptide formulation would be required to employ the octapeptide as a topical skin therapeutic, as human skin has a pH of about 5.5.

The cell envelope is a commonly discussed target for antimicrobial peptides (Wilmes *et al,* 2011). While the full length hBD-1 targets the bacterial cell wall and entrapped bacteria in net-like structures, the octapeptides seem to have distinct antimicrobial mechanisms (Figure 2d). Our methods revealed a breakdown of membrane potential and loss of membrane integrity in bacteria. In contrast, treated fungal cells displayed a functional membrane but cytosolic defects (Figure 3). These data highlight that our octapeptides have diverse antibiotic strategies for different microorganisms. Remarkably, even for the same microbial species, we observed strain specific differences in susceptibility. While the *P. aeruginosa* ATCC type strain was not susceptible towards our octapeptides (Figure 1d and Figure 2a), the multi-resistant *P. aeruginosa* 4-MRGN strain was susceptible (Figure 2b). This is in accordance with previous studies, which could show that antibiotic-resistant bacteria show an increased sensitivity against antimicrobial peptides (Lázár *et al,* 2018).

Different peptides derived from β-defensins have already been investigated on their antimicrobial activity against bacteria and fungi (Krishnakumari *et al,* 2006, 2009). For instance, 19-mer peptides derived from the carboxy-terminus of hBD-1 or -2 and a 22-mer derived from the terminus of hBD-3 were analyzed on their antibiotic activity. These peptides retained one disulphide bridge and had lethal concentrations in the low micromolar range. With our carboxy-terminal octapeptide we can facilitate synthesis by significantly shortening the amino acid sequence and by omitting the disulphide bridge. Most studies investigating defensin-based peptides have been focused on hBD-3, which is one of the most potent AMPs. By generating different amino-carboxy-terminal peptides, Hoover *et al*. identified several carboxy-terminal

peptides with 9 to 14 amino acids having activity against *E. coli* or *P. aeruginosa*, but not against *S. aureus* (Hoover *et al,* 2003). In these peptides, cysteine residues were replaced by serine residues, which we found to be also crucial for activity in our peptides. Also, Reynold *et al*. described that antimicrobial activity of hBD-3 was mainly localized in the amino-terminal half (Reynolds *et al,* 2010). Similar to our results, they reported that distinct amino acids are important for activity against different strains, suggesting that the strain-selectivity of such peptides can be modulated by varying the sequence.

To be utilized as potential antimicrobial molecules, it has to be excluded that the generated peptides provoke resistance of the treated microorganism. By using a host-derived antimicrobial peptide as therapeutic, this is of major importance, as resistance or cross-resistance towards other AMPs might be fatal for the host. While further testing's are required to determine whether our identified peptides provoke such resistance, we believe that the octapeptide can serve as a backbone structure that could be optimized to enhance and/or specialize its activity and to diverge from its natural structure, which would decrease the risk of resistance induction. For example, due to their difference in activity against *E.coli* and *C. albic*ans, optimization of the peptides RGKAKACK and RGKAKCAK (Figure 1e) could generate a peptide that is effective against *E.coli*, but not against *C. albicans*.

In conclusion, we identified that the host can broaden its antimicrobial arsenal by generating several antibiotic molecules from the AMP hBD-1, depending on its redox state and proteolytic degradation. We believe that this strategy can be therapeutically exploited and that our identified hBD-1 derived carboxy-terminal peptides can be optimized for topical application against bacterial or fungal infections.

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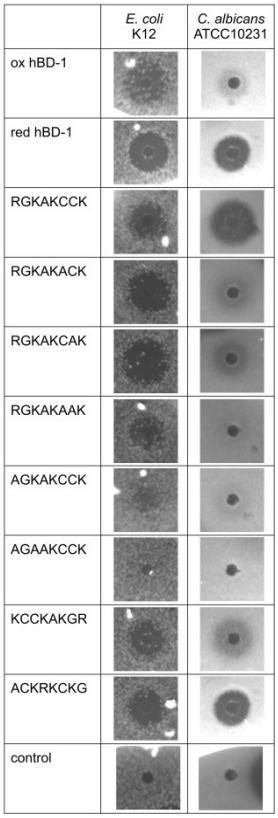
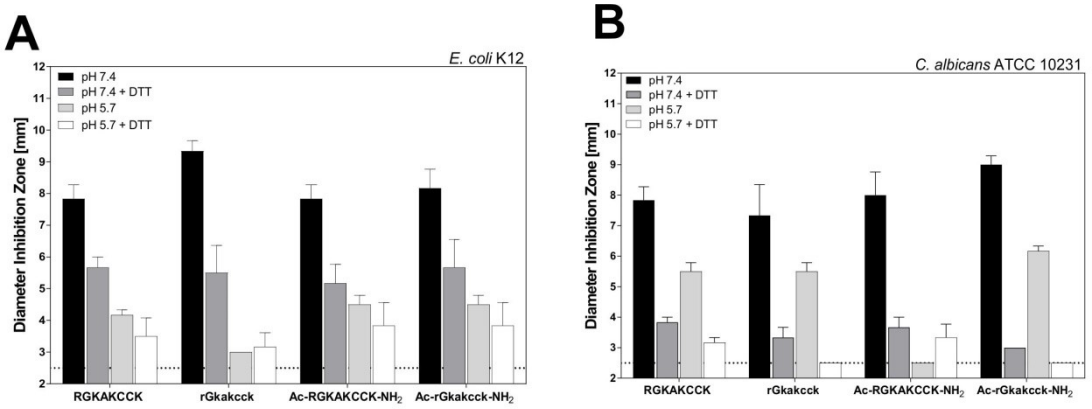
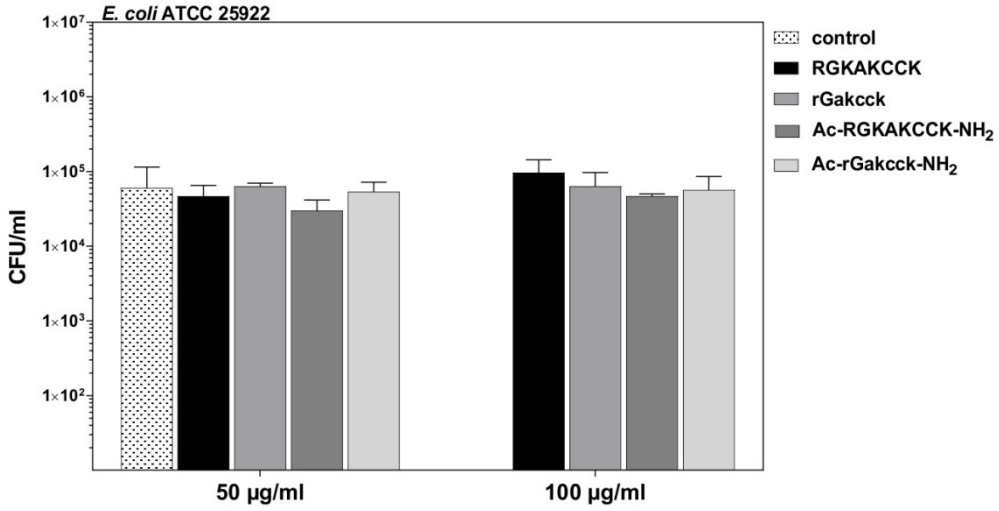
3.5. Supplementary material



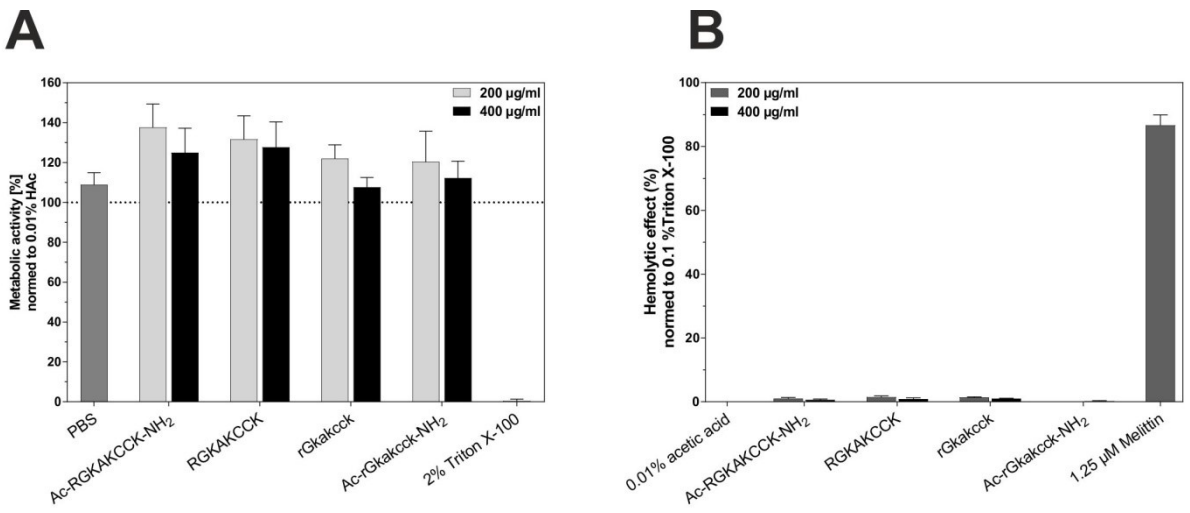
Supp. Fig. 1. Representative Images of radial diffusion assays. Different peptides (4 µg) were tested in an antimicrobial diffusion assay against several microbial strains. Diameter of inhibition zones indicates antimicrobial activity; a diameter of 2.5 mm is the diameter of an empty well. Representative images are shown.



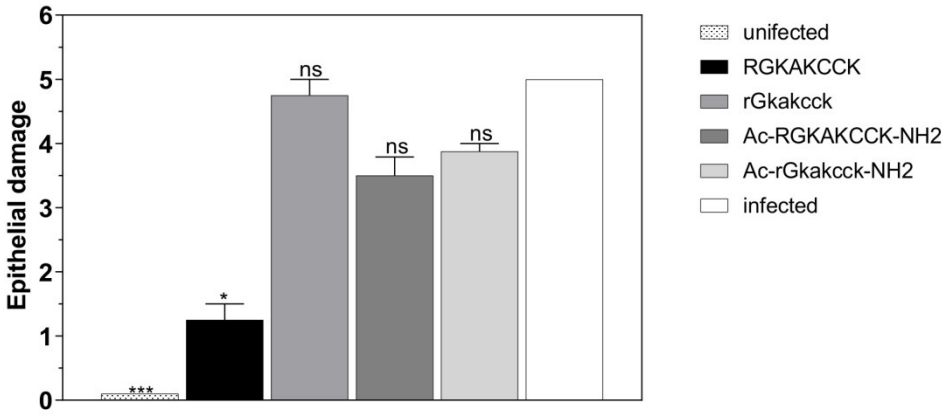
Supp. Fig. 2. Chemical environment influences antimicrobial activity of RGKAKCCK and its modified variants. The carboxy-terminal octapeptide RGKAKCCK was stabilized at its termini by acetylation of the amino-terminus and amidation of the carboxy-terminus (Ac-RGKAKCCK-NH_2). Peptides (4 µg) were investigated on their antimicrobial activity against **(A)** *E. coli* K12 and **(B)** *C. albicans* ATCC 10231. Radial diffusion assay was carried out under standard conditions (black), reducing conditions (dark grey) or by using an acidic pH without DTT (light grey) and with DTT (transparent). Letters indicate amino acid one-letter code. Diameter of inhibition zones indicates antimicrobial activity; a diameter of 2.5 mm (dotted line) is the diameter of an empty well. Experiments were carried out at least three times, mean + SEM is shown.



Supp. Fig. 3. Presence of human blood serum influences antimicrobial activity of octapeptide variants. Two different concentrations of octapeptides were tested in a turbidity liquid assay against *E. coli* ATCC 25922. Peptides were incubated in human blood serum with tested microorganism. After 2h aliquots were plated on agar plates and colony forming units (CFUs) were calculated the next day. Data are presented as mean +/- SEM of at least three independent experiments.



Supp. Fig. 4. Modified octapeptides are not cytotoxic in higher concentrations. 200 µg/ml and 400 µg/ml of different octapeptides were tested in two different cytotoxicity assays. **(A)** A WST-1 based test with human intestinal epithelial cell lines CaCo-2 and **(B)** Hemolytic Activity assay (Oddo & Hansen, 2017) with human red blood cells were used. We used 2% Triton-X-100 and 1.25 µM Melittin as positive controls. Data are presented as mean /- SEM of at least three independent experiments.



Supp. Fig. 5. Efficacy of octapeptides in an oral infection epithelia model. Reconstituted human oral epithelia were pre-incubated with PBS (control uninfected) or 100 µg/ml of octapeptides as indicated. Subsequently cells were infected with *C. albicans* SC5314. Epithelial damage was evaluated by four independent experts and the combined evaluation (mean +/- SEM, criteria described in methods) is shown. Data are presented as mean /- SEM of at least three independent experiments. The statistic was evaluated by using Kruskal-Wallis test with ns = not significant and *= $p < 0.05$.

4 Curbing gastrointestinal infections by defensin fragment modifications without harming commensal microbiota

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Abstract

The occurrence and spread of multidrug-resistant pathogens, especially bacteria from the ESKAPE panel, increases the risk to succumb to untreatable infections. We developed a novel antimicrobial peptide, Pam-3, with antibacterial and antibiofilm properties to counter this threat. The peptide is based on an eight-amino acid carboxyl-terminal fragment of human β-defensin 1. Pam-3 exhibited prominent antimicrobial activity against multidrug-resistant ESKAPE pathogens and additionally eradicated already established biofilms *in vitro*, primarily by disrupting membrane integrity of its target cell. Importantly, prolonged exposure did not result in drug-resistance to Pam-3. In mouse models, Pam-3 selectively reduced acute intestinal *Salmonella* and established *Citrobacter* infections, without compromising the core microbiota, hence displaying an added benefit to traditional broad-spectrum antibiotics. In conclusion, our data support the development of defensin-derived antimicrobial agents as a novel approach to fight multidrug-resistant bacteria, where Pam-3 appears as a particularly promising microbiota-preserving candidate.

4.1. Introduction

The spread of antibiotic-resistant bacteria is an urgent public health threat. Specifically, the so-called ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) pathogens account for the majority of nosocomial infections worldwide being attributed to ≥ 700,000 deaths annually (Kelly & Davies, 2017). The World Health Organization (WHO) has recently published a list of 12 bacteria against which new antibiotics are urgently needed, including the ESKAPE pathogens (Tacconelli *et al*, 2018). While traditional antibiotics fight pathogens, they also have wide-ranging consequences for the commensal gut microbiota (Maier *et al*, 2020). Administration disrupts the microbial composition and can result in a long-lasting dysbiosis, which is associated with mounting diseases (Jackson *et al*, 2018). Decreased diversity and -taxonomic richness, the spread of antimicrobial resistance as well as increased colonization of opportunistic pathogens, including secondary infections with Clostridioides difficile, are just a few of the many side-effects traditional antibiotics impose (Francino, 2016; Kim *et al*, 2017). The current antimicrobial crisis is a product of the long-term neglected development of new antibiotics by pharmaceutical companies and governments (The Lancet, 2020). Thus, new strategies more resilient to multidrug resistance are urgently warranted (Falagas *et al*, 2016).

Antimicrobial peptides (AMPs) are small, cationic peptides existing in all multicellular organisms and exhibit a broad range of antimicrobial and immunological properties (Zasloff,

2002). They are considered a promising treatment option and have the potential to be a new generation of antimicrobials against multidrug-resistant bacteria. Recently, de Breij *et al.* demonstrated the potential of novel antimicrobial peptides by developing SAAP-148, which showed promising effects against biofilm-associated skin infections in ex vivo human skin and murine skin *in vivo* (Breij *et al*, 2018). First preclinical trials with SAAP-148 against methicillin-resistant S. aureus infections have already been conducted according to AdisInsight – a database for drug development (SAAP 148 - AdisInsight).

Defensins, the most prominent class of AMPs in humans, are key effector molecules of innate immunity. These peptides protect the host from infectious microbes and shape the composition of microbiota at mucosal surfaces (Bevins, 2003; Ganz, 2003; Peschel & Sahl, 2006; Thaiss *et al*, 2016). To this end, the first identified human β-defensin, human β-defensin 1 (hBD-1), is constitutively expressed in surface epithelia by monocytes, plasmacyoid dendritic cells and platelets (Bensch *et al*, 1995; Ryan *et al*, 2003; Kraemer *et al*, 2011). Previously, the antimicrobial activity of hBD-1 was underestimated until it was analyzed under reduced conditions as found in the human intestine. Reduced hBD-1 has an increased antimicrobial activity, but can be degraded by intestinal proteases (Schroeder *et al*, 2011; Raschig *et al*, 2017). We have recently shown that this creates an eight-amino acid carboxyl-terminal fragment (called octapeptide) with retained antimicrobial activity, albeit low in vivo stability (Wendler *et al*, 2019).

Here, we leveraged those findings by developing novel synthetic peptides with improved antimicrobial activity and enhanced in vivo stability. We modified the hBD-1-derived octapeptide with palmitic acid and various spacers, such as sugars or amino acids, to create lipopeptides (Pams) with increased stability and bactericidal activity (Krishnakumari & Nagaraj, 2015; Mak *et al*, 2003). The most promising peptide was tested against multidrug-resistant pathogens and biofilms followed by exploratory safety assessment. Lastly, we determined its influence on the murine microbiota after oral application as well as its efficacy in murine gastrointestinal infection models.

4.2. Material and Methods

Microorganisms and culture

Clinical isolates of *A. baumannii* DSM30007, *E. faecium* DSM2918, *K. pneumoniae* DSM30104 and *P. aeruginosa* DSM1117 were provided by the Department for Laboratory Medicine at Robert-Bosch-Hospital Stuttgart, Germany. *C. rodentium* DSM16636 and *E. coli* DSM8695 were obtained from German Collection of Microorganisms and Cell Cultures GmbH Braunschweig, Germany. Clinical isolates of *A. baumannii* LMG944, *A. baumannii* ECII, *E. coli* 6940, *E. coli* DSM682,

E. faecium 11037 CHB, *E. faecium* 20218 CHB, *K. pneumoniae* 6727 and *K. pneumoniae* 6970 as well as *S. aureus* DSM20231, *S. aureus* ATCC25923, *S. aureus* ATCC33592, *S. aureus* ATCC43300, *S. enterica serovar* Typhimurium DSM554, *P. aeruginosa* ATCC27853, *P. aeruginosa* NRZ01677 and *P. aeruginosa* PAO1 were provided by the Institute of Medical Microbiology and Hygiene Tübingen, Germany. *B. subtilis* ypuA and *S. aureus* NCTC8325 was obtained from the Interfaculty Institute for Microbiology and Infection Medicine, Tübingen, Germany. The wild-type *S.* Typhimurium strain SL1344 harboring a chromosomally integrated luxCDABE cassette, which is confirmed by kanamycin resistance (Flentie *et al*, 2008) and the nalidixic acid and kanamycin-resistant, bioluminescent *C. rodentium* strain ICC180 (Wiles *et al*, 2004) were obtained from Helmholtz Centre for Infection Research Braunschweig, Germany.

All bacteria were stored in cryo vials (Roth) at -80°C. Before each experiment, inocula from the frozen stocks were grown overnight at 37°C on LB or Columbia blood agar plates (BD). For experiments, fresh cultures were prepared in tryptic soy broth (BD).

Peptides

All lipopeptides were chemically synthesized by EMC Microcollections GmbH (Tübingen, Germany) and purified by precipitation. In detail, peptide synthesis was performed by solid-phase Fmoc/tert-butyl chemistry on Chloro-(2'-chloro)trityl polystyrene resin (Rapp Polymere, Tübingen, Germany) using an automated peptide synthesizer for multiple peptide synthesis (Syro, MultiSynTech, Germany). Side chain-protecting groups of Fmoc-amino acids were 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Arg), trityl (Cys) and tert-butyloxycarbonyl (Lys). Fmoc-protected amino acids including Fmoc-8-amino-3,6-dioxaoctanoic acid (Fmoc-Ado-OH, IRIS Biotech GmbH, Germany) were coupled twice (double couplings, sevenfold molar excess of amino acids) by in situ activation using DIC/HOBt and TCTU with DIEA. The removal of the Fmoc-protecting group was carried out twice by treatment with piperidine/DMF (1:4, v/v). Resins were washed with DMF (6x) after each coupling and deprotection step. N-terminal acylation of the resin bound peptide was performed manually using palmitic acid (1.2 eq), DIC/HOBt (1.5 eq) with DIEA (2 eq) in NMP/DCM (4/1) for 16 hours. After washing with DMF (8x) and DCM (5x) completeness of acylation was confirmed by Kaiser-Test. The lipopeptide was cleaved off the resin and side-chain deprotected by treatment with TFA/reagent K/H2O (80/15/5, v/v/v) for 3 h and precipitated by adding diethyl ether. After centrifugation, the peptides were dissolved in tert-butyl alcohol/H2O (4:1, v/v) and lyophilized. The structure of the lipopeptide was confirmed by RP-HPLC-ESI-MS and was >> 90 % (Supplementary Fig. 4). All peptides were dissolved in 0.01 % acetic acid.

Antimicrobial activity

Antimicrobial activity was analyzed by radial diffusion assay (Lehrer *et al*, 1991). Briefly, log-phase bacteria were cultivated for up to 18 hours in TSB (TSB, Becton Dickinson, USA), washed and diluted to 4 x 10^6 CFU in 10 ml agar. Bacteria were incubated in 10 ml of 10 mM sodium phosphate, pH 7.4, containing 0.3 mg/ml of TSB powder and 1% (w/v) low EEO-agarose (AppliChem). 1 µg of each Lipopeptides was pipetted into punched wells in a final volume of 4 µl and diffused into the gel for 3 hours at 37°C. After that, a nutrient-rich gel with 6% TSB (w/v) and 1% agarose in 10 mM sodium phosphate buffer was poured on top of the first gel and incubated for up to 24 hours at 37°C. Then the diameter of inhibition zones was measured.

Bactericidal activity

Bactericidal activity was assessed by broth microdilution assay (Wiegand *et al*, 2008). Log-phase bacteria were collected by centrifugation (2500 rpm, 10 min, 4°C), washed twice with 10 mM sodium phosphate buffer containing 1% (w/v) TSB and the optical density at 600 nm was adjusted to 0.1. Approximately 5 x 10^5 CFU/ml bacteria were incubated with serial peptide concentrations (1.17 – 150 µM) in a final volume of 100 µl in 10 mM sodium phosphate buffer containing 1% (w/v) TSB for 2 hours at 37°C. After incubation, 100 µl of 6% TSB (w/v) were added and absorbance was measured at 600 nm (Tecan, Switzerland) and monitored for 18 hours. Afterwards, 100 µl per well were plated on LB agar to determine the numbers of viable bacteria microbiologically. Bactericidal activity is expressed as the LC99.9, the lowest concentration that killed ≥ 99.9% of bacteria.

For time-kill experiments, bacteria (5 x 10^5 CFU/ml) were incubated with 9.375 µM Pam-3 in 10 mM sodium phosphate buffer containing 1% (w/v) TSB in LoBind tubes (Eppendorf) in a total volume of 550 µl. As an untreated control, bacteria were incubated in 10 mM sodium phosphate buffer containing 1% (w/v) TSB. After incubation at 37°C and 150 rpm for 1 to 120 min, a sample of 50 µl was taken from the suspension and added to 50 µl of a 0.05% (v/v) sodium polyanethol sulfonate (Sigma-Aldrich) solution, which neutralizes remaining peptide activity, and plated on LB agar to determine the number of viable bacteria.

Resistance development

Development of resistance to the peptides was assessed with *S. aureus* and *S.* Typhimurium. For comparison, the development of resistance to the clinically relevant antibiotics rifampicin and ciprofloxacin (Sigma-Aldrich) was determined. Bacteria were cultured overnight at 37°C at 150 rpm in TSB. Bacteria were washed twice with 10 mM sodium phosphate buffer containing 1% (w/v) TSB. Washed bacteria were incubated with serial Pam-3 or antibiotic concentrations (with

final concentrations of 1.17 to 150 µM peptide or 0.0156 to 0.5 µg/ml rifampicin or ciprofloxacin) in a final volume of 100 µl in 10 mM sodium phosphate buffer containing 1% (w/v) TSB for 2 hours at 37°C. After incubation, 100 µl of 6% TSB (w/v) was added and plates incubated in a humidified atmosphere for 21 hours at 37°C and 150 rpm.

The MIC, the lowest concentration of peptide/antibiotic that caused a lack of visible bacterial growth, was determined for each bacterial species. Thereafter, 5 x 10^5 CFU/ml of the 0.5-fold MIC suspension was added to a fresh medium containing peptides/antibiotics and these mixtures were incubated as described above. This was repeated for 25 passages.

Treatment of established biofilms

A log-phase culture of *P. aeruginosa* was diluted in BM2 medium and of *S. aureus* in TSB to 5 x 10^5 CFU/ml. 100 µl of each bacterial suspension was added to a round-bottom polystyrene microtiter plate and incubated for 24 hours at 37°C in a humidified atmosphere. Then, planktonic bacteria were removed by two wash steps with PBS. Next, biofilms were exposed to serial peptide dilutions (9.375 – 300 µM) in a final volume of 100 µl in 10 mM sodium phosphate buffer containing 1% (w/v) TSB for 1 hour at 37°C in a humidified atmosphere. As a control, bacteria were exposed to 10 mM sodium phosphate buffer containing 1% (w/v) TSB without peptide. Afterwards, adherent bacteria in each well were resuspended, and the number of viable bacteria was determined microbiologically. To visualize the data on a logarithmic scale, a value of 1 CFU was assigned when no growth occurred. The biofilm degradation assay was performed in agreement with the original report describing this method first (Segev-Zarko et al, 2015).

Interaction with the bacterial membrane / Reporter Gene Assay

A specific bacterial reporter strain with the genetic background of *Bacillus subitilis* 1S34, carrying the promoter of the *ypuA* gene, fused to the firefly luciferase reporter gene, was used to identify cell envelope-related damage caused by treatment with antimicrobial compounds (Urban *et al*, 2007). The assay was carried out in agreement with former reports describing this method (Wenzel *et al*, 2014; Raschig *et al*, 2017). Briefly, bacteria were cultured to an OD600 of 0.9 in LB broth with 5 µg/ml erythromycin at 37°C and diluted to an OD_{600} of 0.02. Serial peptide dilutions (0.146 – 150 µM) were prepared in a microtiter plate and incubated with the adjusted bacterial suspension at 37°C for 1 hour. Subsequently, citrate buffer (0.1 M, pH 5) containing 2 mM luciferin (Iris Biotech, Germany) was added and luminescence was measured using a microplate reader (Tecan, Switzerland).

Bacterial membrane potential

Determination of membrane potential changes was performed as described earlier (Schilling *et al*, 2019). *S. aureus* NCTC8325 was grown to log-phase in LB + 0.1% glucose, harvested and the optical density at 600 nm (OD_{600}) was adjusted to 0.5. Bacteria were incubated with 30 µM 3,3'- diethyloxacarbocyanine iodide ($DiOC_2(3)$, Invitrogen™) for 15 min in the dark and treated with serial peptide concentrations for 30 min. The protonophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP, Sigma Aldrich) was used as a positive control and DMSO or 0.01% acetic acid as negative controls. Fluorescence was measured at an excitation wavelength of 485 nm and two emission wavelengths, 530 nm (green) and 630 (nm) red, using a microplate reader (Tecan, Switzerland).

Bacterial pore formation

Pore formation was monitored using the Live/Dead BacLight bacterial viability kit (Molecular Probes) as described previously (Schilling *et al*, 2019). S. aureus was grown in LB at 37°C to log-phase and, 100 µl aliquots were treated with 37.5 µM Pam-3 (4x MIC) or left untreated as a control. Samples were taken after 10 min of peptide treatment, then 0.2 µl of a 1:1 mixture of SYTO9 and propidium iodide (PI) was added and further incubated for 15 min at RT in the dark. Fluorescence microscopy was carried out using a Zeiss Axio Observer Z1 automated microscope. Images were acquired with an Orca Flash 4.0 V2 camera (Hamamatsu), C Plan-Apo 63x/1.4 Oil DIC and alpha Plan-Apochromat 100x/1.46 Oil Ph3 objectives (Zeiss) and processed using the Zen software package (Zeiss).

Transmission and scanning electron microscopy

Morphologic analysis of bacteria was characterized by electron microscopy (Wendler *et al*, 2019). Approximately 1.2 x 10^9 CFU/ml bacteria were incubated with 37.5 µM Pam-3 (4x MIC) in 10 mM sodium phosphate buffer containing 1% (w/v) TSB broth for 30 or 120 min at 37°C. As a control, bacteria were exposed to 0.01% acetic acid. Afterwards bacteria were fixed in Karnovsky's reagent.

For transmission electron microscopy (TEM), bacteria were high-pressure frozen (HPF Compact 03, Engineering Office M. Wohlwend GmbH) in capillaries, freeze-substituted (AFS2, Leica Microsystems) with 2 % OsO_4 and 0.4 % uranyl acetate in acetone as substitution medium and embedded in EPON. Ultrathin sections were stained with uranyl acetate and lead citrate and analyzed with a Tecnai Spirit (Thermo Fisher Scientific) operated at 120 kV.

For scanning electron microscopy (SEM), bacteria were washed in PBS and finally fixed with 1% OsO_4 on ice for 1 h. Next, samples were prepared on polylysin-coated coverslips,

dehydrated in a graduated series to 100% ethanol and critical point dried (Polaron) with CO_2. Finally, samples were sputter-coated with a 3 nm thick layer of platinum (Safematic CCu-010) and examined with a Hitachi Regulus 8230 field emission scanning electron microscope (Hitachi) at an accelerating voltage of 5 kV.

Mice

C57BL/6N mice were generated and maintained (including breeding and housing) at the animal facilities of the Helmholtz Centre for Infection Research (HZI) under enhanced specific pathogen-free (SPF) conditions (Stehr *et al*, 2009). Animals used in the experiments were gender and age matched. Female and male mice with an age of 8-12 weeks were used. Sterilized food and water ad libitum was provided. Mice were kept under a strict 12-hour light cycle (lights on at 7:00 am and off at 7:00 pm) and housed in groups of two to six mice per cage. All mice were euthanized by asphyxiation with CO2 and cervical dislocation. All animal experiments were performed in agreement with the local government of Lower Saxony, Germany (approved permission No. 33.19-42502-04-18/2499).

Safety

Age- and gender- matched mice received two doses of peptides (0, 125 µg or 250 µg) solved in 100 µl PBS orally per day. Bodyweight and appearance were recorded. The following day, mice were sacrificed and stomach, kidney, spleen, liver, small intestine, cecum and colon were removed for histological scoring. Around 1 ml of blood was taken from the heart to measure inflammatory markers including creatinine in the kidney and the enzyme levels of glutamate-oxalacetate-transaminase (GOT) in the liver.

Infection and treatment of mice

***Salmonella* Typhimurium infection.** For *S*. Typhimurium infections experiments, age- and sex-matched mice between 10 and 14 weeks of age were used. Both- female and male mice were used in experiments. Water and food were withdrawn for 4 hours before mice were treated with 20 mg/mouse of streptomycin by oral gavage. Afterwards, mice were supplied with water and food ad libitum. 20 h after streptomycin treatment, water and food were withdrawn again, 4 h before the mice were orally infected with 10^5 CFU of *S*. Typhimurium in 200 µl PBS. Drinking water ad libitum was supplied immediately and food 2 h post infection (p.i.). After 6 and 22 h p.i. mice received 250 µg peptide solved in 100 µl PBS or only PBS orally. 48 h after infection, mice were sacrificed, and intestinal organs were removed to assess the bacterial burden in the lumen and tissues. Mice were weighed every day to record potential body-weight loss.

***Citrobacter rodentium* infection.** Bioluminescence expressing *C. rodentium* strain ICC180 was used for all infection experiments (Wiles *et al*, 2004). *C. rodentium* inocula were prepared by culturing bacteria overnight at 37°C in LB broth with 50 µg/ml kanamycin. Subsequently, the culture was diluted 1:100 in fresh medium, and sub-cultured for 4 hours at 37°C in LB broth[54]. Bacteria were washed twice in phosphate-buffered saline (PBS). Mice were orally inoculated with 108 CFU of *C. rodentium* diluted in 200 µl PBS. Weight of the mice was monitored and feces were collected for measurements of the pathogen burden. After 5 days post infection mice received twice 250 µg peptide solved in 100 µl PBS or only PBS orally. The following day, mice were sacrificed to assess bacterial burden in the lumen and tissues of the cecum and the colon.

Analysis of bacterial loads in feces

Fresh fecal samples were collected and weighted. Samples were homogenized in 1 ml LB media by bead-beating with 1 mm zirconia/silica beads twice for 25 s using a Mini-Beadbeater-96 (BioSpec). To determine CFUs, dilutions of homogenized samples were plated on LB plates with 50 µg/ml kanamycin.

Analysis of bacterial loads in intestinal content and systemic organs

All mice were euthanized by asphyxiation with CO_2 at indicated time points. Intestinal tissues (small intestine, cecum, colon) were removed aseptically. To collect intestinal content, organs were flushed with PBS. Organs were opened longitudinally, cleaned thoroughly with PBS and weighted. Organs and content were homogenized in PBS using a Polytron homogenizer (Kinemtatica). Dilutions of homogenized samples were plated on LB plates containing 50 µg/ml kanamycin to determine CFUs.

Microbiota analysis

Feces samples were collected at different time points (before and after infection), and bacterial DNA was extracted from snap-frozen feces using a phenol-chloroform-based method previously described (Turnbaugh *et al*, 2009). In brief, 500 µl of extraction buffer (200 mM Tris (Roth), 20 mM EDTA (Roth), 200 mM NaCl (Roth), pH 8.0), 200 µl of 20% SDS (AppliChem), 500 µl of phenol:chloroform:isoamyl alcohol (PCI) (24:24:1) (Roth) and 100 µl of zirconia/silica beads (0.1 mm diameter) (Roth) were added per feces sample. Lysis of bacteria was performed by mechanical disruption using a Mini-BeadBeater-96 (BioSpec) for two times 2 min. After centrifugation, the aqueous phase was processed by another phenol:chloroform:isoamyl alcohol extraction before precipitation of DNA using 500 µl isopropanol (J.T. Baker) and 0.1 volume of 3 M sodium acetate (Applichem). Samples were incubated at - 20°C for at least several hours or overnight and centrifuged at 4°C at maximum speed for 20 min. The resulting DNA pellet was

washed, dried using a speed vacuum and resuspended in TE Buffer (Applichem) with 100 µg/ml RNase I (Applichem). Crude DNA was column purified (BioBasic Inc.) to remove PCR inhibitors.

16S rRNA gene amplification of the V4 region (F515/R806) was performed according to an established protocol previously described (Caporaso *et al*, 2011). Briefly, DNA was normalized to 25 ng/µl and used for sequencing PCR with unique 12-base Golary barcodes incorporated via specific primers (obtained from Sigma). PCR was performed using Q5 polymerase (NewEnglandBiolabs) in triplicates for each sample, using PCR conditions of initial denaturation for 30 s at 98°C, followed by 25 cycles (10 s at 98°C, 20 s at 55°C, and 20 s at 72°C). After pooling and normalization to 10 nM, PCR amplicons were sequenced on an Illumina MiSeq platform via 250 bp paired-end sequencing (PE250). Using Usearch8.1 software package (http://www.drive5.com/usearch/) the resulting reads were assembled, filtered and clustered. Sequences were filtered for low-quality reads and binned based on sample-specific barcodes using QIIME v1.8.0 (Caporaso *et al*, 2010). Merging was performed using -fastq_mergepairs –with fastq_maxdiffs 30. Quality filtering was conducted with fastq_filter (-fastq_maxee 1), using a minimum read length of 250 bp and a minimum number of reads per sample = 1000. Reads were clustered into 97% ID OTUs by open-reference OTU picking and representative sequences were determined by use of UPARSE algorithm (Edgar, 2010). Abundance filtering (OTUs cluster > 0.5%) and taxonomic classification were performed using the RDP Classifier executed at 80% bootstrap confidence cut off (Wang *et al*, 2007). Sequences without matching reference dataset were assembled as de novo using UCLUST. Phylogenetic relationships between OTUs were determined using FastTree to the PyNAST alignment (Price *et al*, 2010). Resulting OTU absolute abundance table and mapping file were used for statistical analyses and data visualization in the R statistical programming environment package phyloseq (McMurdie & Holmes, 2013).

Statistics and Reproducibility

Experimental results were analyzed for statistical significance using GraphPad Prism v8.2 (GraphPad Software Inc.). Apart from microbiota analysis, all data were analyzed by Mann-Whitney test or Kruskal-Wallis test. Here, differences were analyzed by Wilcoxon signed-rank test or Kruskal-Wallis test comparison of totals between groups (Segata *et al*, 2011). OTUs with Kruskal-Wallis test <0.05 were considered for analysis.

Data Availability Statement

16S rRNA gene sequencing data have been deposited in the NCBI (Bioproject Database) under the accession number: PRJEB37278.

4.3. Results

4.3.1. Design and screening of octapeptide based lipopeptides identifies candidate with improved antimicrobial activity

Lipopeptides are used as antibiotics which are highly active against multidrug-resistant bacteria and fungi (Avrahami & Shai, 2004; Mangoni & Shai, 2011). Previous studies have demonstrated an enhanced activity of HDPs after fatty acid modification at the N-terminal end (Chu-Kung *et al*, 2010). Thereby, C_{14}-C_{18} long chain fatty acids have proven to be ideal for this purpose (Makovitzki *et al*, 2006; Reinhardt & Neundorf, 2016). Within this work, we used a C_{16} long fatty acid, namely palmitic acid together with different spacers such as sugars or amino acids to improve stability and bactericidal activity of the carboxyl-terminal fragment of hBD-1. We designed 5 unique lipopeptides, Pam-1 to Pam-5, based on the recently discovered octapeptide (Fig. 1a). This set of peptides was screened for their antimicrobial activity against several pathogenic bacteria using a radial diffusion assay (Fig. 1b). Pam-1, Pam-4 and Pam-5 were generally inactive against tested strains, thus contrasting the potent inhibition of bacterial growth mediated by Pam-2 and -3. Bacterial growth was most strongly inhibited by Pam-3, either on par (*S. aureus*) or superior (*C. rodentium*, *P. aeruginosa* and *S.* Typhimurium) to the octapeptide, pointing towards modification-specific activities. Notably, both Pam-2 and Pam-3 consistently inhibited *S*. Typhimurium growth, a species the non-modified octapeptide failed to inhibit.

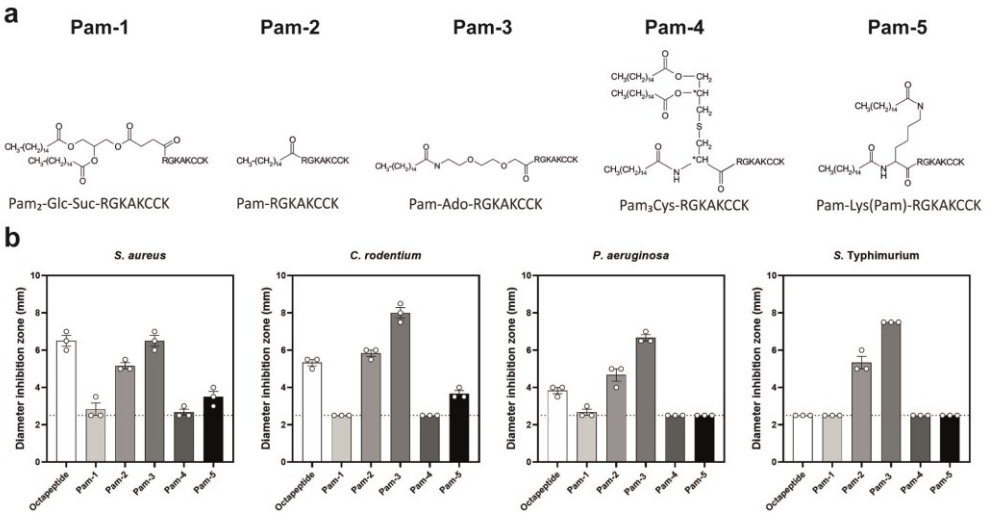

Figure 1. Screening of differently modified octapeptides reveals peptides with improved antimicrobial activity. (a) Octapeptide, the C-terminal eight amino acids of human β-defensin 1, was chemically modified with palmitic acid and different spacers such as sugars or amino acids or 8-amino-3.6-dioxaoctanoic acid (Ado) to generate lipopeptides. (b) Antimicrobial activity was measured by radial diffusion assay. The diameter of inhibition zones indicates antimicrobial activity; a diameter of 2.5 mm (dotted line) is the diameter of an empty well. Results are means ± SEM of three independent experiments.

4.3.2. Pam-3 displays bactericidal activity against multidrug-resistant bacteria and slow resistance selection

We used a broth microdilution assay to further analyze the potential of our lipopeptides to kill multidrug resistant bacteria belonging to the ESKAPE pathogen panel (Extended Data Fig. 1 & Table S1). Pam-1 and Pam-4 displayed no or low bactericidal activity, whereas Pam-5 showed moderate effects against these pathogens. Similar to the results of the radial diffusion assay, both Pam-2 and Pam-3 were highly effective. Remarkably, Pam-2 and Pam-3 inhibited the growth of an *A. baumannii* isolate (DSM30007), which is otherwise resistant to the last-resort antibiotics, colistin and tigecycline.

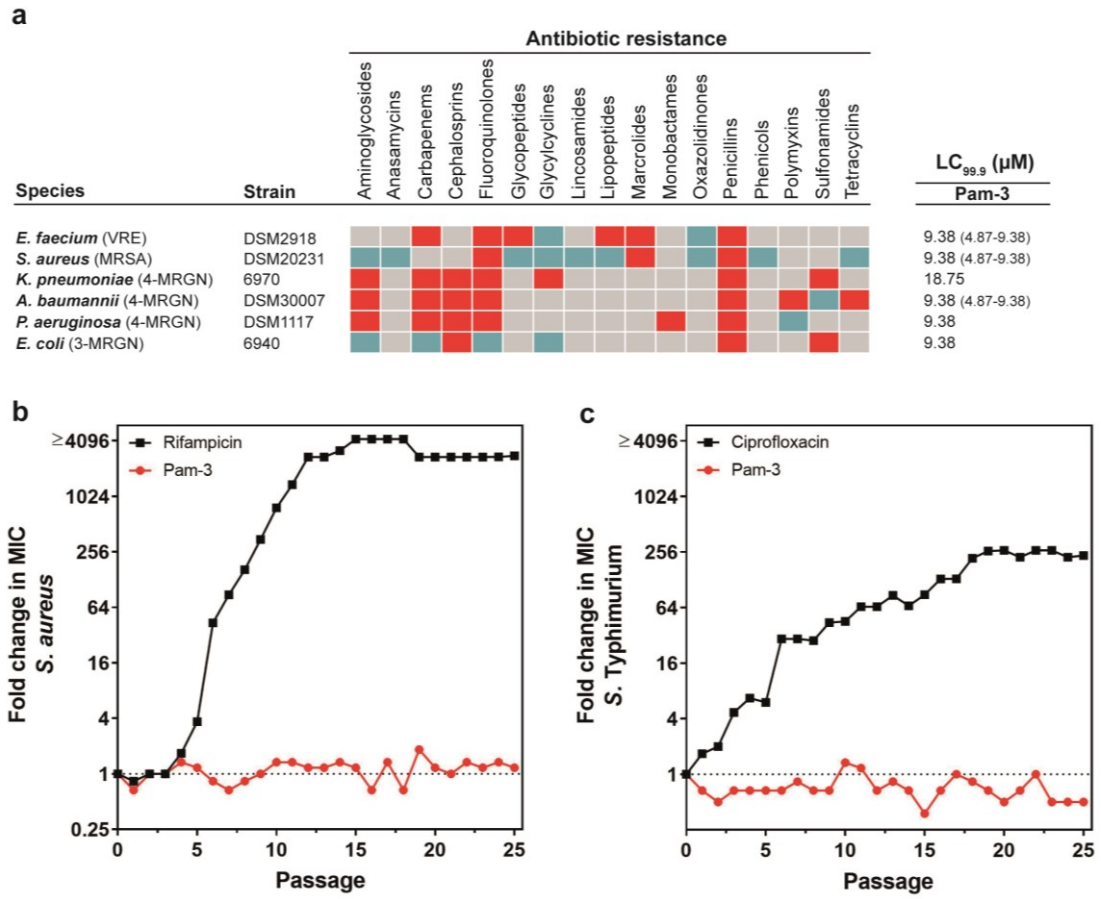

Figure 2. Pam-3 kills multidrug-resistant ESKAPE pathogens and resistance was not selected. **(a)** Susceptibility of multidrug-resistant ESKAPE (*E. faecium*, *S. aureus*, *K. pneumoniae*, *P. aeruginosa* and *E. coli*) pathogens to antibiotics and Pam-3. Bacteria susceptible to all (blue boxes) or intermediate/resistant to at least one (red boxes) of the antibiotics per class. Grey boxes are shown if the susceptibility to agents in that class was not assessed. Results are expressed as the $LC_{99.9}$, the lowest peptide concentration in micromolar that resulted in ≥ 99.9% killing. Results are medians (and ranges) of three independent experiments. If no range is indicated, then the $LC_{99.9}$ was identical in all experiments. **(b and c)** Resistance development of *S. aureus* ATCC 25923 (b) and *S.* Typhimurium DSM554 (c) to Pam-3 (red line) and the antibiotics rifampicin and ciprofloxacin (black line), respectively. Values are fold changes (in $\log_2$) in minimal inhibitory concentration (MIC) relative to the MIC of the first passage.

Despite some bactericidal similarities between Pam-2 and Pam-3, the latter proved superior to all other Pam's and was highly effective against these bacteria at concentrations of 4.69 to 18.75 µM (Fig 2a & Supplementary Fig. 1). We accordingly selected Pam-3 for further characterization as a potential therapeutic against multidrug resistant bacteria.

As the development and selection of antibiotic-resistant bacteria in response to new antibiotic candidates is a significant problem, we assessed the ability of S. aureus and *S*. Typhimurium to develop resistance against Pam-3. When cultured in the presence of sub-inhibitory concentrations of Pam-3 for 25 passages, no significantly increase in the minimal inhibition concentration (MIC) was observed for *S. aureus*. In contrast, the MIC for the standard antibiotic, rifampicin started to rapidly increase after five passages and had increased ≥ 4096-fold after 15 passages (Fig. 2b). Similarly, although exposed to Pam-3 for continuous serial passages, no resistant *S.* Typhimurium isolates emerged, whereas the presence of ciprofloxacin, resulted in an increased MIC already after 3 passages, and a ≥ 256-fold MIC increase after 19 passages (Fig. 2c).

4.3.3. Pam-3 eliminates established biofilms and causes rapid killing by permeabilizing the bacterial membrane

Bacterial biofilms are highly resistant to growth inhibitors and bactericidal treatment regimens. Apart from the hindered penetration of antibacterial agents, treatment is further complicated by 10 to 1000 times increased tolerance exhibited by biofilm protected bacteria compared to planktonic bacteria (Wu *et al*, 2015). Because of that, we assessed the ability of Pam-3 to eradicate established biofilms in a dose-depended manner. Within 1 hour, 300 µM of Pam-3 eliminated *P. aeruginosa* in biofilms (Kruskal-Wallis test, p = 0.0074, Fig 3a) and similarly eradicated ~99.99 % of *S. aureus* in biofilms (Kruskal-Wallis test, p = 0.0026, Fig 3a). A primary target of antimicrobial peptides is the bacterial cell envelope. Disturbing the integrity and function of the outer and/or inner membranes results in loss of the barrier function and dissipation of the membrane potential (Cole & Nizet, 2016). To clarify the mode of action of Pam-3, we used an ypuA promotor-based luciferase reporter strain of *B. subtilis* to identify cytoplasmic membrane-associated and cell envelope-related stress (Urban *et al*, 2007). The ypuA promoter was activated (2 fold) by Pam-3, indicating cell envelope impairment (Fig. 3b). Hence, to strengthen this result, we analyzed the influence of Pam-3 on the transmembrane potential of *S. aureus* NCTC8325. The protonophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was used as a positive control to depolarize bacteria, i.e. leading to a reduction of their membrane potential. Upon depolarization, DiOC2(3) shifts from green fluorescence towards red emission because of self-association of the dye molecules. Pam-3

treatment caused a breakdown of the membrane potential in a concentration-dependent manner (Fig. 3c). Both results emphasize that Pam-3 acts on bacterial membranes.

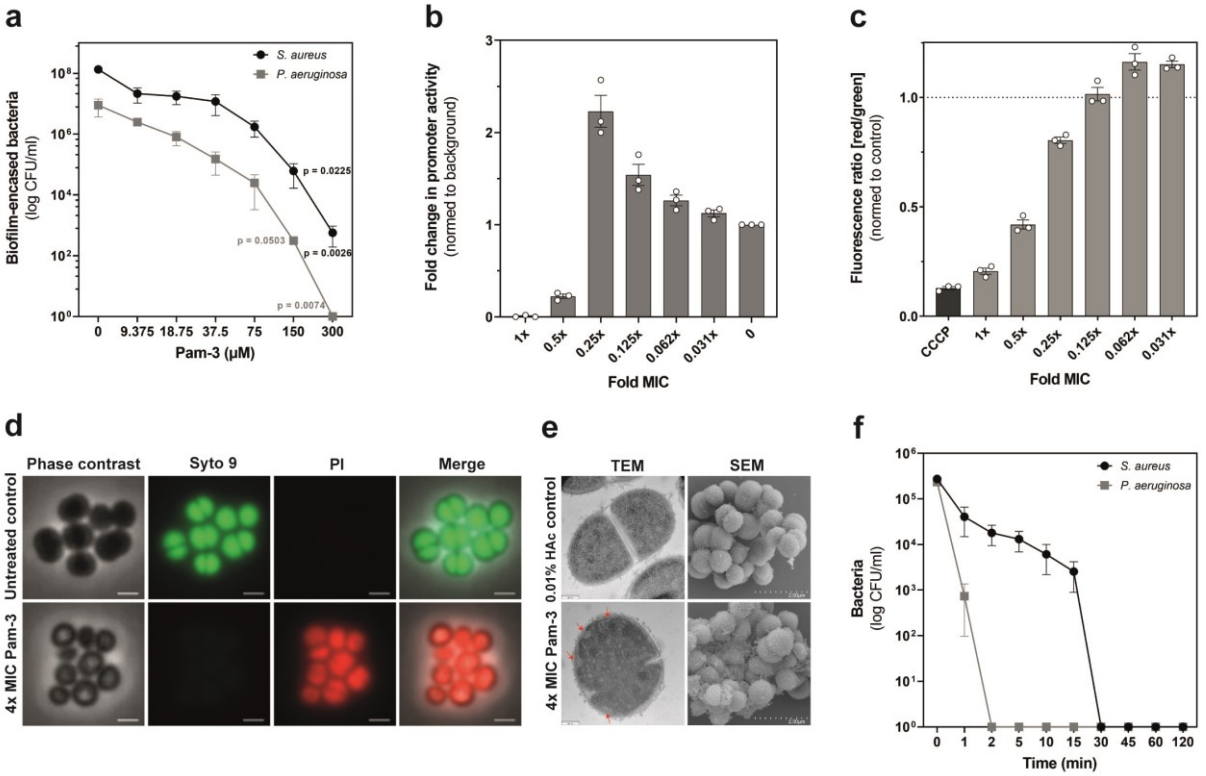

Figure 3. Pam-3 eliminates established biofilms, causing rapid killing by inducing large pores or lesions. **(a)** Bactericidal activity of Pam-3 against established biofilms of *S. aureus* ATCC25923 (black line) and *P. aeruginosa* PAO1 (grey line). Results are expressed as the number of viable bacteria (in $\log_{10}$ CFU) after 1 h treatment of 24 hour old biofilms with Pam-3. Values are means ± SEM of three replicates from three independent experiments. Statistics were evaluated by using the Kruskal-Wallis test. **(b)** Activation of the *B. subtilis* ypuA promoter by Pam-3 indicates cell envelope stress. Data are presented as mean ± SEM. Experiments were carried out three independent times **(c)** Effect of Pam-3 on *S. aureus* NCTC8325 membrane potential after 30 min of treatment. The protonophore CCCP was used as a positive control and 0.01% acetic acid as a negative control. Data are presented as mean ± SEM of three independent experiments. **(d)** Fluorescence microscopy of *S. aureus* NCTC8325 treated with Pam-3 (4x MIC) reveals pore formation by causing a strong influx of red-fluorescent propidium iodide. Scale bars: 1 µm. **(e)** Transmission electron microscopy of high-pressure frozen, freeze-substituted and embedded *S. aureus* NCTC8325 treated with Pam-3 or 0.01% acetic acid for 30 min to observe membrane disruption. Scale bars, 0.2 µm. *S. aureus* NCTC8325 was exposed to Pam-3 or 0.01% acetic acid for 60 min. The samples were fixed in Karnovsky's reagent, and morphology was analyzed by scanning electron microscopy. Scale bars, 2 µm. **(f)** Killing of *S. aureus* ATCC25923 (black line) and *P. aeruginosa* PAO1 (grey line) after 1 to 120 min exposure to 9.375 µM (1x MIC) Pam-3. Results are expressed as the number of viable bacteria (in $\log_{10}$ CFU) per milliliter. Values are means of three independent experiments. Data are presented as mean ± SEM of three independent experiments.

Since a large variety of AMPs target the membrane as pore formers (Brogden, 2005), we next analyzed the ability of Pam-3 to induce membrane lesions. To this end, we treated S. aureus NCTC8325 with Pam-3 at 4x MIC and added a mixture of Syto9 and propidium iodide (PI). The membrane-permeant Syto9 stains all living cells green, whereas the red-fluorescent PI can only enter cells through large membrane pores or lesions. Pam-3 led to a strong influx of PI (Fig. 3d).

Pore formation was associated with fast killing of these bacteria. To further assess the mechanism of killing, transmission electron microscopy (TEM) was performed to analyze changes in bacterial morphology to compare bacterial morphology before and after treatment with Pam-3. In agreement with the pore formation and the induction of cell envelope stress and depolarization, Pam-3 treatment resulted in strong cell envelope damage with disrupted membranes and pores in most of the cells. Furthermore, additional membranous structures could be observed in many cells similar to rhesus macaque θ-defensins (Wilmes et al, 2014). Additionally, scanning electron microscopy (SEM) was employed to observe cell morphological changes after Pam-3 treatment directly. Exposure to Pam-3 resulted in membrane surface disruption and lysed cells similar to hBD1 (Raschig et al, 2017), while control cells exhibited a bright and smooth surface (Fig. 3e). We assayed bactericidal kinetics to assess the rapidness of Pam-3 mediated killing. Pam-3 killed more than 90 % of P. aeruginosa within 1 min and S. aureus within 15 min (Fig. 3f). Eradication to the level of detection was observed 2 and 30 min after Pam-3 treatment, respectively.

4.3.4. Orally administered Pam-3 showed good acute tolerability in an animal model

Potential side effects of orally administered Pam-3 were assessed in mice. Histological analysis and determination of serum markers 24 hours after application of two doses of 250 µg Pam-3 did not reveal acute toxicity. Specifically, there were no alterations in bodyweight and no signs of systemic toxicity or distress (Fig. 4a). Moreover, measurement of serum levels of glutamic oxaloacetic transaminase and creatinine showed no significant differences between the groups suggesting no effect on kidney and liver metabolism (Fig. 4b & c).

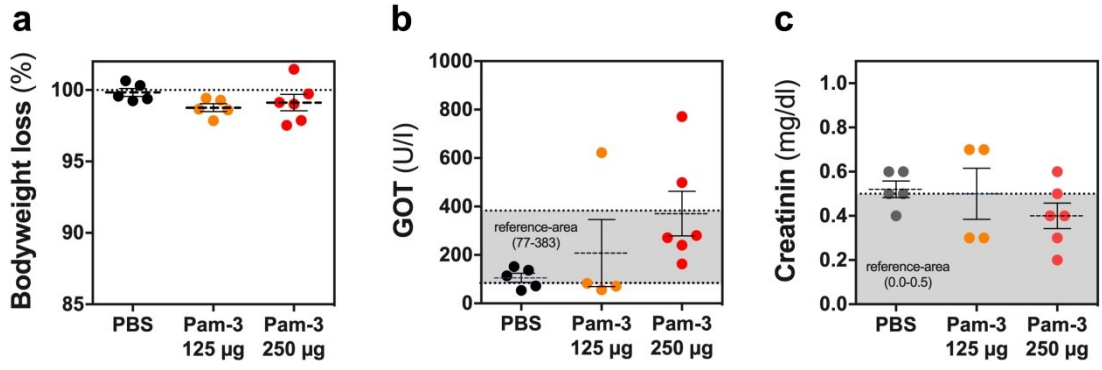

Figure 4. Safety of orally applied Pam-3 in mice. Dose depended oral tolerance test in mice. Animals were treated twice with 125 µg (orange circles) or 250 µg Pam-3 (red circles) or PBS (black circles). **(a)** Weight change of mice (125 µg Pam-3, N = 5; 250 µg Pam-3, N = 6 and PBS, N = 5), **(b)** glutamic oxaloacetic transaminase (GOT) levels of treated animals with 125 µg (N = 4) or 250 µg Pam-3 (N = 6) or PBS (N = 5) and **(c)** creatinine levels (125 µg Pam-3, N = 4; 250 µg Pam-3, N = 6 and PBS, N = 5) one day after Pam-3 application. Results are presented as mean ± SEM of biologically independent animals.

Finally, histological examination of gastrointestinal tissues, liver and kidney revealed no alterations, except a minor shortening of intestinal villi the jejunum-ileum of one control and two treated animals (Table 1). As no difference in these histopathological findings was observed between PBS and Pam-3 treated animals, they were regarded as background observations (Table 1 & Supplementary Fig. 2). Thus, we conclude that Pam-3 treatment was not associated with acute toxicity.

Table 1. Safety of orally application of Pam-3 in mice. Oral tolerance test in mice. Animals were treated twice with 250 µg Pam-3 or PBS. Results are expressed as the number of the total number of animals within the groups that showed signs of pathology within 24 h after treatment.

Gastrointestinal tract			
		PBS	Pam-3
Stomach			
	Glycogen	0/5	0/6
	Lymphocytic aggregates	0/5	0/6
Duodenum-Jejunum			
	Dysplasia	0/5	0/6
	Inflammation	0/5	0/6
Jejunum-Ileum			
	Dysplasia	1/5	2/6
	Inflammation	0/5	0/6
	Paneth cells	0/5	0/6
Cecum and **Colon**			
	Dysplasia	0/5	0/6
	Inflammation	0/5	0/6
Liver			
	Glycogen	0/5	0/6
	Lymphocytic aggregates	1/5	0/6
	Anisocaryosis	0/5	0/6
	Double nucleated cells	0/5	0/6
	Hematopoiesis	0/5	0/6
	Fatty change	0/5	0/6
Kidney			
	Glomeruli	0/4	0/6
	Tubuli	3/4	3/6
	Papilla	0/4	0/6
	Pelvis	0/4	0/6

4.3.6. Pam-3 preserves the core gut microbiota

To investigate the effect of Pam-3 on the intestinal microbiota, we treated mice twice at an 8-hour interval with Pam-3 (125 or 250 µg/each dose) or PBS orally and collected fresh fecal samples before and 24 hours after application. Microbiota composition was analyzed using 16S rRNA sequencing. Analysis of beta diversity and calculation of weighted Unifrac Distances demonstrated that changes in the microbiota between the before and after samples were similar between Pam-3 treated mice and PBS gavaged control mice (Fig. 5a and b). Similarly, while minor changes in the community structure were observed in both groups (i.e. treated and untreated), the number of detected species as well as the complexity (Fig. 5c and d; Wilcoxon-Test) remained comparable, thus contrasting treatment with traditional antibiotics, such as ampicillin (Supplementary Fig. 3). In line with the analysis of alpha and beta diversity, Pam-3 treatment did not affect the abundance of bacterial genera (Fig. 5e and f). Combined, these results demonstrate that, with the application regime conducted, Pam-3 treatment of healthy chow-fed mice does not affect the overall community structure or diversity of the microbiota.

4.3.7. Pam-3 treatment combats acute and established gastrointestinal infections of *S.* Typhimurium and *Citrobacter rodentium*

To assess the efficacy of Pam-3 on acute intestinal bacterial infections, mice were infected with *S*. Typhimurium and treated orally 6 and 22 hours post infection with 250 µg peptide or PBS (Fig. 6a) and showed significantly reduced Colony Forming Units (CFU) of *S*. Typhimurium in cecum content and tissue (Mann-Whitney test, $p < 0.0001$ and $p = 0.0409$, Fig. 6b). Furthermore, Pam-3 also lowered the bacterial load in the small intestine without affecting the small intestine tissue (Mann-Whitney test, $p = 0.0024$ and $p = 0.8621$, Fig. 6c) and tends to reduce weight loss (Fig. 6d). We subsequently assessed the therapeutic potential of Pam-3 against an already established intestinal infection. To this end, mice were infected with *C. rodentium* and received two doses of 250 µg Pam-3 or PBS 5 days post infection (Fig. 6a). Pam-3 treatment reduced the number of bacteria in the cecum content and cecum tissue (Mann-Whitney test, $p = 0.0104$ and $p = 0.0473$, Fig. 6e). Treatment also significantly reduced the number of viable *Citrobacter* in colon content and colon tissue (Mann-Whitney test, $p = 0.0010$ and $p = 0.0104$, Fig. 6f). Together these data corroborate the in vivo efficacy of Pam-3 against two different enteric pathogens.

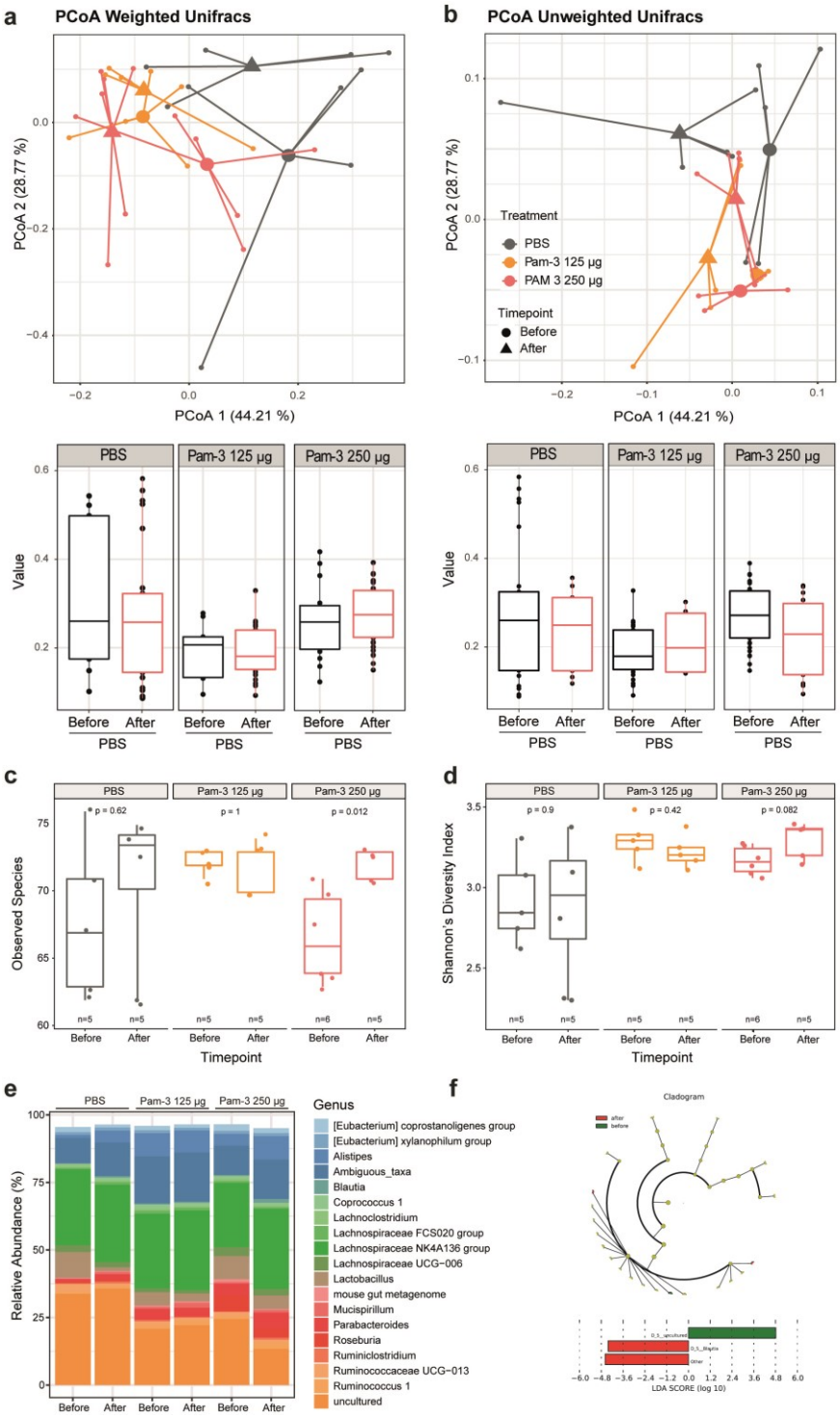

Figure 5. Pam-3 treatment does not affect microbiota diversity but has influence on certain bacteria phyla. Chow-fed mice were treated orally twice with 125 µg Pam-3 (N = 5), once with 250 µg Pam-3 (N = 6) or PBS (N = 5) as a control. Feces samples were collected before and after treatment to observe short term changes in the microbiome. **(a)** Principal coordinate analysis (PCoA including group mean) of fecal microbiota composition using Weighted UniFrac Distances before and after treatment, respectively. **(b)** PCoA including group mean of fecal microbiota composition using Unweighted UniFrac Distances, respectively before and after treatment. **(c)** Richness (observed species) before and after treatment. **(d)** Fecal microbiota was calculated by Shannon's Diversity index. The statistical significance was calculated by using Wilcoxon test. **(e)** Pam-3 treatment affects the abundance of bacterial genera. **(f)** Aggregated by genus. Statistical analysis performed by the LEfSe platform using default settings (https://galaxyproject.org/learn/visualization/custom/lefse/).

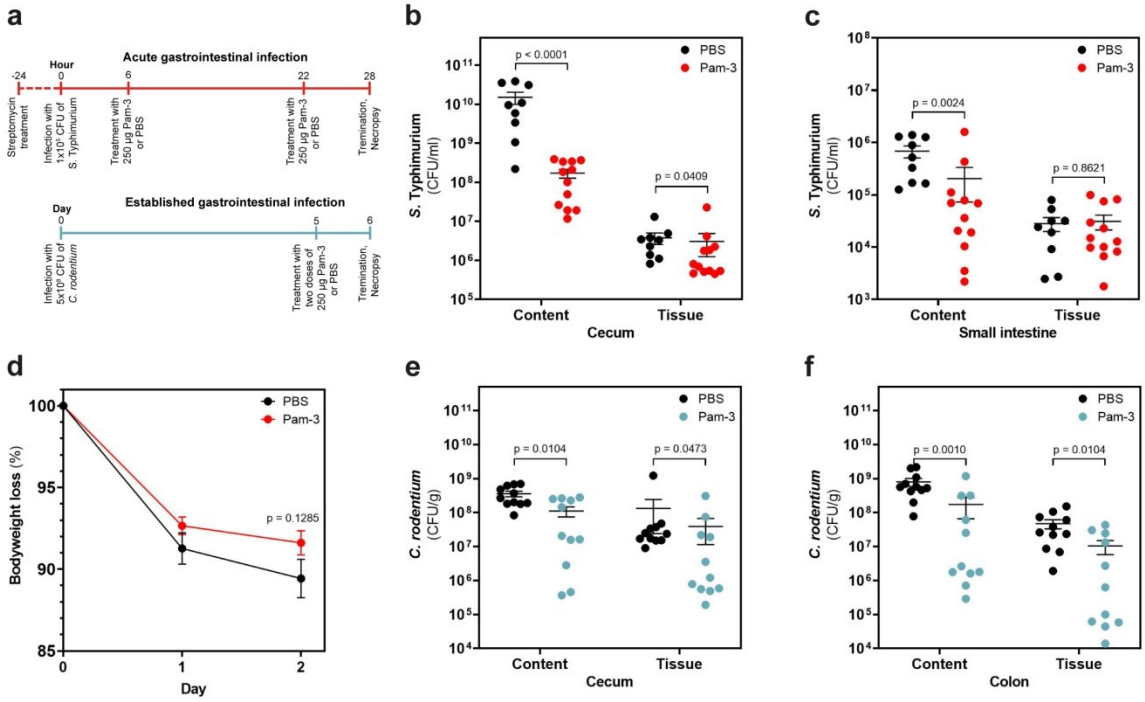

Figure 6. Oral application of Pam-3 combats acute and established infections of *S.* Typhimurium and *C. rodentium*. **(a)** Overview of experimental setup. Mice were infected with *S.* Typhimurium (red circles) and treated orally with either 250 µg Pam-3 (N = 12) or PBS (N = 9) after 6 h and 22 h post infection. Mice were infected with *C. rodentium* (blue circles) and treated orally with either 250 µg Pam-3 (N = 11) or PBS (N = 11) after 5 days post infection. **(b)** CFU/ml of *S.* Typhimurium in cecum content and tissue. **(c)** CFU/ml of S. Typhimurium in small intestine content and tissue. **(d)** Body weight change during acute *S.* Typhimurium infection. **(e)** CFU/g of *C. rodentium* in cecum content and tissue. **(f)** CFU/g of *C. rodentium* in colon content and tissue. Results are expressed as the number of viable bacteria (in $\log_{10}$ CFU) in the lumen and tissue and presented as mean ± SEM of biologically independent animals. Statistics were evaluated by using the Mann-Whitney test.

4.5. Discussion

The increasing number of multidrug resistant pathogens is one of the greatest challenges of our time (Nizet, 2015). The Centers for Disease Control and Prevention (CDC) estimates that the annual infection rate exceeds 2.8 million cases in USA alone, resulting in more than 35,000 deaths caused by multidrug-resistant bacteria and fungi (CDCP, 2019). Due to this alarming development, alternatives to conventional antibiotics are urgently needed (Ghosh *et al*, 2019; Theuretzbacher *et al*, 2019). Herein, we report that Pam-3, a palmitoleic acid-modified octapeptide fragment from hBD-1, is effective against multidrug-resistant ESKAPE pathogens *in vitro*. Moreover, Pam-3 is also highly effective *in vitro* against *A. baummanii* resistant to the last-resort antibiotics, colistin and tigecycline. Biofilm-encased bacteria are much less susceptible to conventional antibiotics than their identical planktonic counterparts complicating treatments (Hall & Mah, 2017). In contrast to most antibiotics, our experiments showed that Pam-3 was able to eradicate established S. aureus and P. aeruginosa biofilms *in vitro*.

Development of antibiotic resistance is increasing at an alarming rate (Frieri *et al*, 2017). Here, we demonstrated the lack of resistance development to Pam-3 in Gram-positive (*S. aureus*) and Gram-negative (*S.* Typhimurium) bacteria compared to conventional antibiotics, when cultured for 25 passages in the presence of sub-inhibitory concentrations. This result indicates that resistance development against Pam-3 is a rare event (Bechinger & Gorr, 2017).

One of the reasons for this observation could be the rapid killing of bacteria by Pam-3 and its associated mode of action. AMPs with bactericidal effects often interact with membranes as part of their mode(s) of action. Apart from membrane-disruptive mechanisms, like pore formation, AMPs can kill through electrostatics and localized perturbations or non-membrane disruptive mechanisms, which targets multiple microbial processes and/or physiological functions (Fjell *et al*, 2011; Andersson *et al*, 2016; Mwangi *et al*, 2019).

Our analysis revealed that Pam-3 causes cell envelope stress by breaking down the membrane potential and inducing pore formation. Taken together, the capacity to induce rapid killing of Gram-positive and Gram-negative bacteria, a low risk of resistance selection, combined with biofilm eradication underscores the value of Pam-3 for further drug development.

The rise in antibiotic resistance and the urgently search for potential alternatives shifted greater research focus on AMPs (Theuretzbacher *et al*, 2019; Wang *et al*, 2019). Most AMPs in pre- and clinical development are evaluated for topical rather than oral administration, for different reasons such as in vivo efficacy and stability (Koo & Seo, 2019). We demonstrated the efficiency of orally administered Pam-3 in two different in vivo models of gastrointestinal infections, namely *S.* Typhimurium and *C. rodentium*. In both models, Pam-3 treatment resulted in significantly reduced bacterial burden in the gastrointestinal tract. Further studies, including proper dose regimens, are necessary for full eradication of both infections.

Antibiotic treatment has non negligible consequences. Observational, clinical, and epidemiologic studies have demonstrated that antibiotic treatment affects the gut microbiota composition with immediate effects on health (Blaser, 2016; Francino, 2016). Changes in the microbiota composition, decreased diversity, reduced taxonomic richness and a so-called dysbiosis are the main consequences (Dethlefsen *et al*, 2008; Dethlefsen & Relman, 2011). Further, antibiotics can have long-term effects such as increased susceptibility to infections, obesity and obesity-associated metabolic diseases (Francino, 2016; Lange *et al*, 2016). In contrast to conventional antibiotics, Pam-3 treatment had unexpectedly no appreciable effects on commensal microbes. Although further studies are warranted to rigorously address this assumption, our data points towards pathogen specific killing by PAM-3 treatment, which would be a significant advantage over conventional antibiotics where disruptive effect on the resident microbiota as well as a rapid drop in diversity is commonly observed (Burdet *et al*, 2019). Furthermore, after antibiotic treatment, the intestine is often colonized by non-commensal bacteria, which can result in long-term environmental changes (de Lastours & Fantin, 2015). Instead of a loss of diversity, high-dose Pam-3 treated mice showed an unaffected bacterial diversity. Our data points towards an increased number of observed species and nominally increased diversity. This observation is in line with previous studies on other fragments from our group (Ehmann *et al*, 2019) suggesting that targeted attack on certain (rather high-abundant) bacterial strains frees up new niches for low-abundant taxa, which were previously below the limit of detection. Microbiota-modulating capabilities of AMPs could make a difference in the treatment of many gut microbiota associated diseases, including inflammatory bowel disease (Glassner *et al*, 2020).

In conclusion, the results presented here demonstrate that Pam-3 is a promising alternative to fight multidrug resistant infections in a post-antibiotic world because of its broad antimicrobial activity against Gram-positive and Gram-negative pathogens and its efficacy against gastrointestinal infections without disrupting the resident microbiota. Future studies are therefore warranted to examine the full potential of this and other biostable novel peptides. Pam-3 could open a new chapter of an effective, microbiota-saving treatment strategy of bacterial infections.

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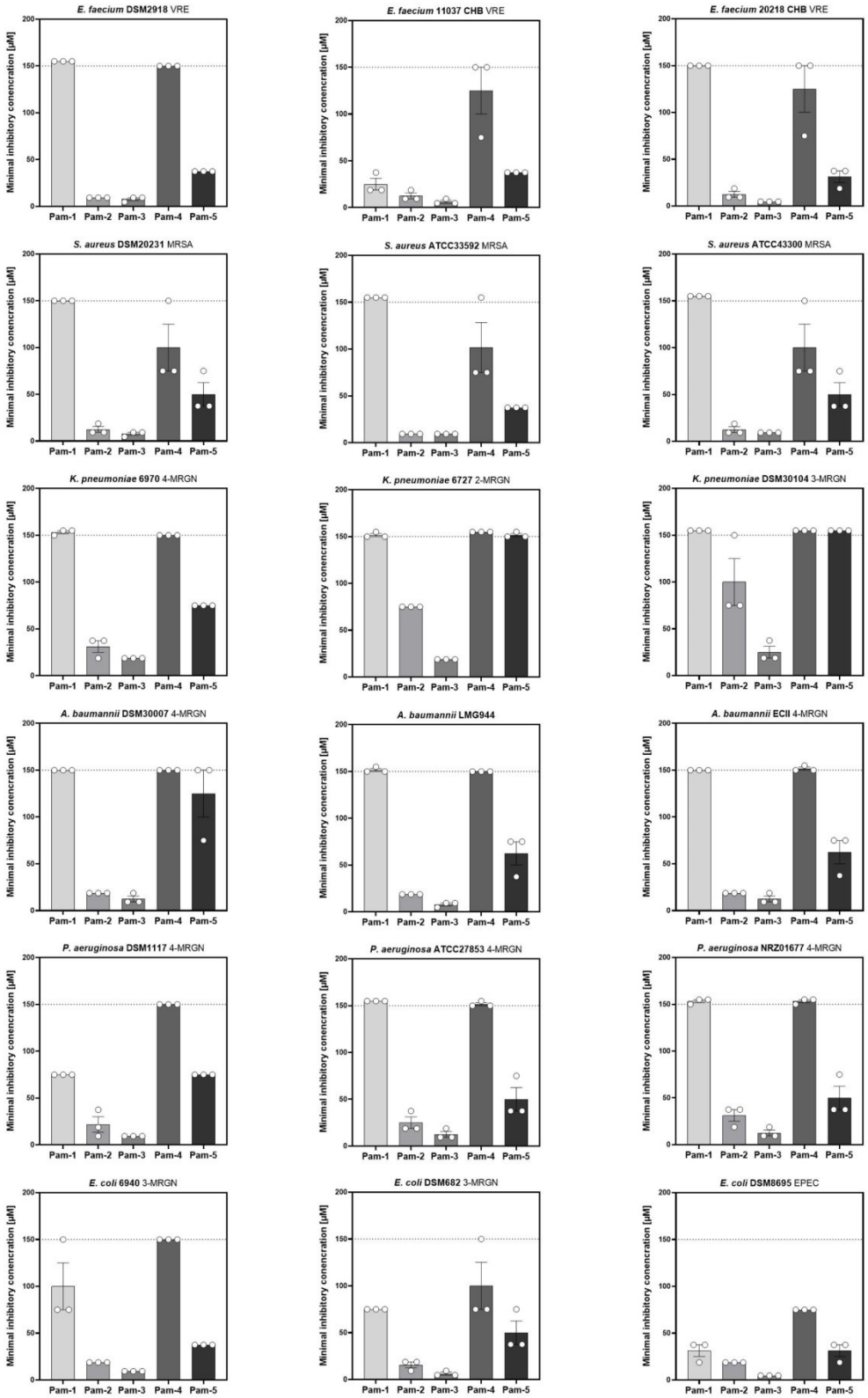
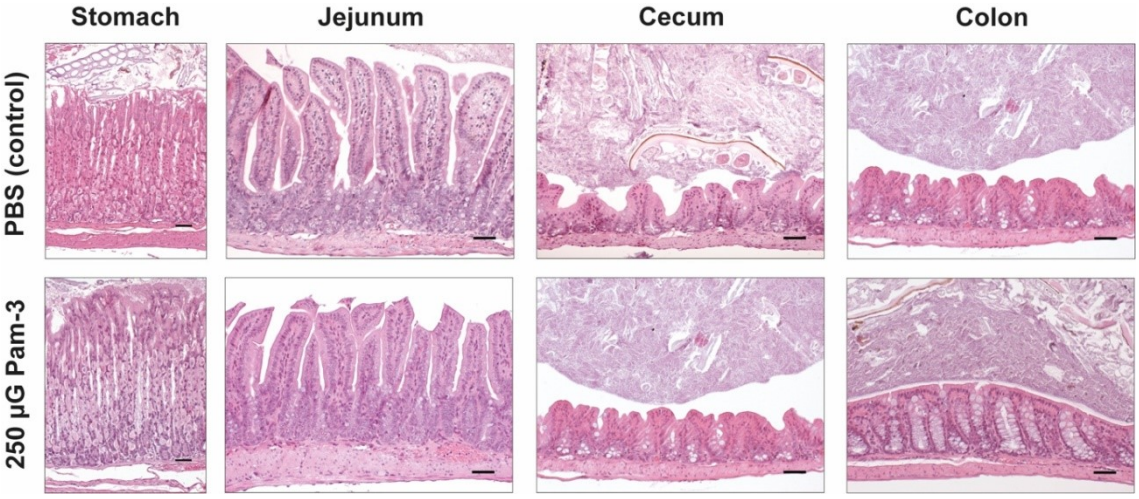
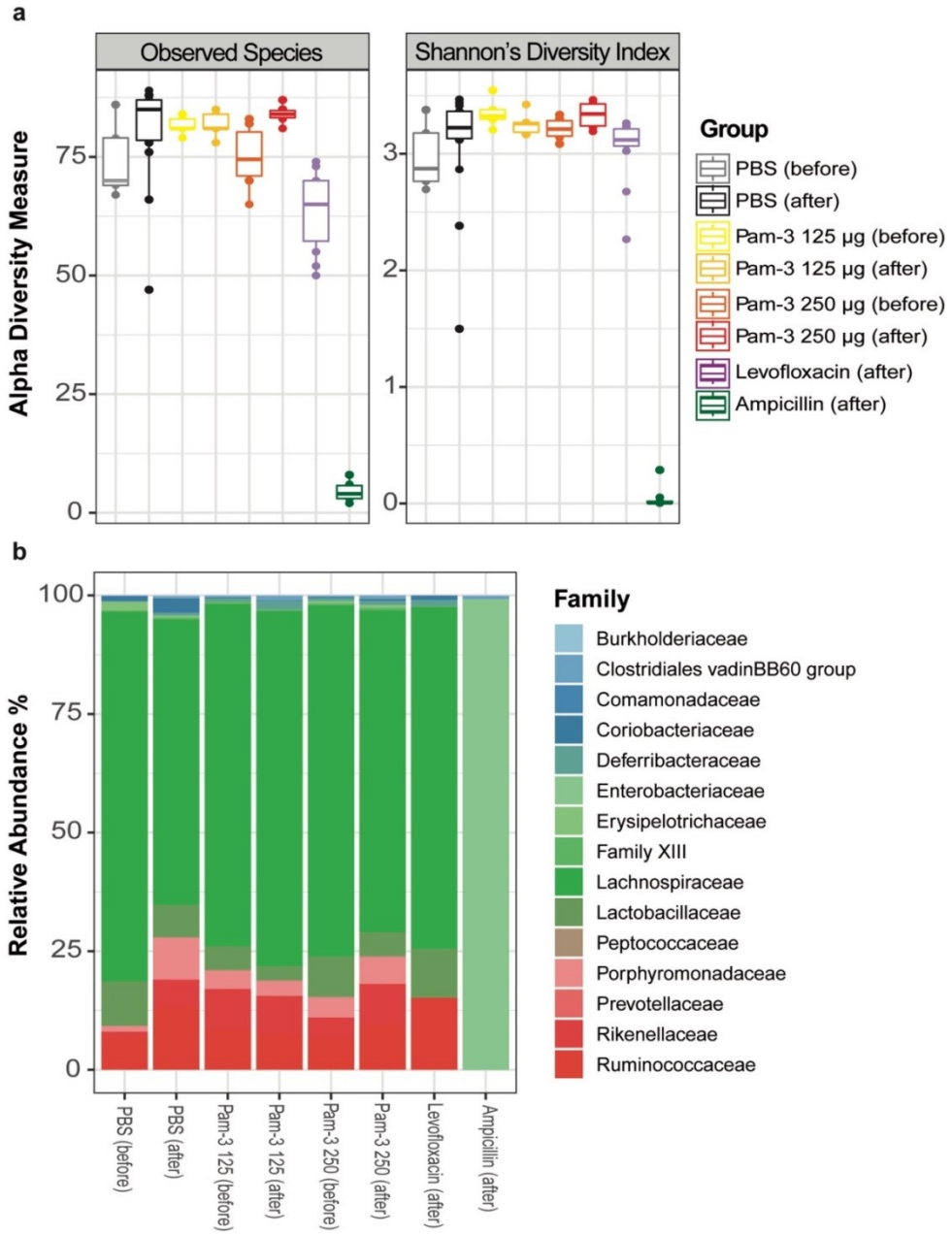
4.6. Supplementary material



Supplementary Fig. 1. Pam's kill multidrug-resistant ESKAPE pathogens. Broth microdilution assay with the Pam's against bacteria from the ESKAPE panel. Here we show the detailed results of the broth microdilution assay experiments. The dotted line marks the highest peptide concentration used in these experiments. Data are presented as mean ± SEM. Experiments were carried out three independent times.



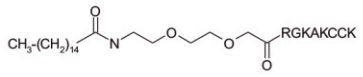
Supplementary Fig. 2 Images from the gastrointestinal tract of differentially treated mice. Oral tolerance test in mice. Animals were treated twice with 250 µg Pam-3 or PBS. Representative images from the stomach, small intestine (jejunum), cecum and colon are shown. Scale bars, 50 µm.



Supplementary Fig. 3 Ampicillin induces global alterations in the microbiota. Chow-fed mice were treated orally twice with 125 µg Pam-3 (N = 5), once with 250 µg Pam-3 (N = 6), once with Levofloxacin (N = 12), once with Ampicillin (N = 10) or PBS (N = 15). **(a)** Feces samples were collected before and after treatment to observe changes and recovery in the microbiome. Richness (observed species) and diversity (Shannon indices) were analyzed from fecal samples. **(b)** Pam-3 treatment affects the abundance of bacterial genera less compared to ampicillin.

Analytical Data Sheet

Name/Sequence Pam-Ado-RGKAKCCK:

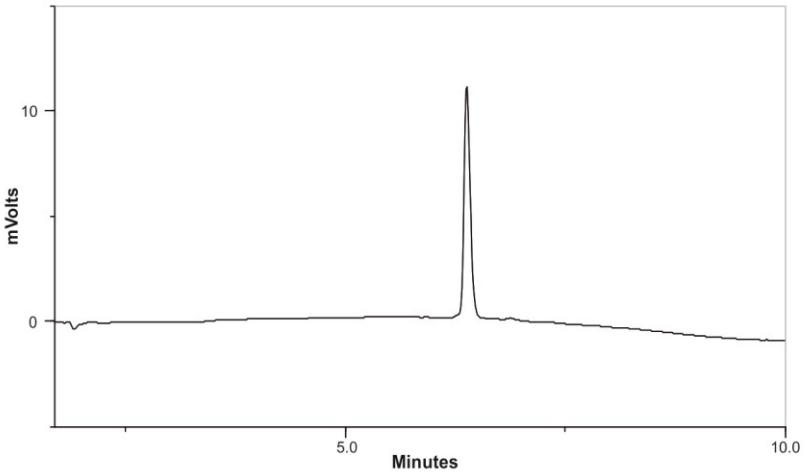


MW 1277

Counter ion TFA

HPLC

Purity
≥ 90 % (214 nm)



ESI mass spectrometry

MW (calc.)	1277
$[M+2H]^{2+}$	639
$[M+3H]^{3+}$	427
$[M+4H]^{4+}$	320

Supplementary Fig 4. Analytical data sheet of Pam-3. Here we show the detailed analysis of purity and characterization of Pam-3.

Supplementary Table 1. Antibiogram of ESKAPE pathogens used in this study. Bacteria susceptible to all (blue color) or intermediate/resistant to at least one (red color) of antibiotics per class following the EUCAST breakpoints. Black color is shown if the susceptibility to agents in that class is not assessed. Values are minimal inhibition concentrations (MIC) in µg/ml or for Chloramphenicol in diameter (mm).

	E. faecium			*S. aureus*			*K. pneumoniae*		
	DSM2918	11037 CHB	20218 CHB	DSM20231	ATCC33592	ATCC43300	6727	DSM30104	6970
Aminoglycosides				Gentamicin ≤ 0.5	Gentamicin ≤ 0.5	Gentamicin ≥ 16	Gentamicin ≤ 1 Tobramycin ≤ 1	Gentamicin ≤ 1 Tobramycin ≥ 16	Gentamicin ≤ 1 Tobramycin ≥ 16
Anasamycins				Rifampicin ≤ 0.5	Rifampicin ≥ 32	Rifampicin ≤ 0.5			
Carbapenems	Imipenem ≥ 16	Imipenem ≥ 16	Imipenem ≥ 16				Imipenem 2 Meropenem 2	Imipenem ≤ 0.25 Meropenem ≤ 0.25	Imipenem ≥ 16 Meropenem ≥ 16
Cephalosprins							Cefuroxim ≥ 32 Ceftazidim 4	Cefuroxim ≥ 64 Ceftazidim 16	Cefuroxim ≥ 64 Ceftazidim 16
Fluoroquinolones	Ciprofloxacin ≥ 8	Ciprofloxacin ≥ 8	Ciprofloxacin ≥ 8	Ciprofloxacin ≥ 8	Ciprofloxacin ≤ 0.5	Ciprofloxacin ≤ 0.5	Ciprofloxacin ≤ 0.25	Ciprofloxacin ≥ 4	Ciprofloxacin ≥ 4
Glycopeptides	Vancomycin ≥ 32 Teicoplanin ≥ 32	Vancomycin ≥ 32	Vancomycin ≥ 32	Vancomycin 1 Teicoplanin 0.75	Vancomycin 1	Vancomycin 1			
Glycylcyclines	Tigecycline ≤ 0.12	Tigecycline ≤ 0.12	Tigecycline ≤ 0.12	Tigecycline ≤ 0.12	Tigecycline 0.5	Tigecycline ≤ 0.12	Tigecycline ≤ 0.5	Tigecycline 4	Tigecycline 4
Lincosamides				Clindamycin ≤ 0.25	Clindamycin ≤ 0.5	Clindamycin ≥ 8			
Lipopeptides	Daptomycin 4			Daptomycin 0.5	Daptomycin 0.5	Daptomycin 0.25			
Marcrolides				Erythromycin ≥ 8	Erythromycin ≥ 8	Erythromycin ≥ 8			
Monobactames									
Oxazolidinones	Linezolid 2	Linezolid 2	Linezolid 1	Linezolid 2	Linezolid 2	Linezolid 2			
Penicillins	Ampicillin ≥ 32 Augmentin ≥ 256	Ampicillin ≥ 32	Ampicillin ≥ 32	Oxacillin ≥ 4 Augmentin ≥ 256	Oxacillin ≥ 4	Oxacillin ≥ 4	Ampicillin ≥ 32	Ampicillin ≥ 32	Ampicillin ≥ 32
Phenicols				Chloramphenicol 22mm					
Polymyxins								Colistin 0.25	
Sulfonamides	Trimethoprim/Sulfamethoxazol ≥ 320	Trimethoprim/Sulfamethoxazol 160	Trimethoprim/Sulfamethoxazol 20	Trimethoprim/Sulfamethoxazol ≤ 10	Trimethoprim/Sulfamethoxazol ≤ 10	Trimethoprim/Sulfamethoxazol ≤ 10	Trimethoprim/Sulfamethoxazol ≥ 320	Trimethoprim/Sulfamethoxazol ≥ 320	Trimethoprim/Sulfamethoxazol ≥ 320
Tetracyclins				Tetracyclin ≤ 1	Tetracyclin ≥ 16	Tetracyclin ≤ 1			

	A. baumannii			*P. aeruginosa*			*E. coli*		
	DSM30007	ECII	LMG944	DSM1117	ATCC27853	NRZ01677	6940	DSM682	DSM8695
Aminoglycosides	Gentamicin ≥ 16 Tobramycin ≥ 16	Gentamicin ≥ 16 Tobramycin ≥ 16	Gentamicin ≤ 1 Tobramycin ≤ 1	Gentamicin ≤ 1 Tobramycin ≥ 16	Gentamicin ≥ 16 Tobramycin ≥ 16	Gentamicin ≥ 16 Tobramycin ≥ 16	Gentamicin ≤ 1 Tobramycin ≤ 1	Gentamicin ≥ 16 Tobramycin ≥ 16	Gentamicin ≤ 1 Tobramycin ≤ 1
Anasamycins									
Carbapenems	Imipenem ≥ 16 Meropenem ≥ 16	Imipenem ≥ 16 Meropenem ≥ 16	Imipenem ≤ 0.25 Meropenem ≤ 0.25	Imipenem ≥ 16 Meropenem 8	Imipenem ≥ 16 Meropenem ≥ 16	Imipenem ≥ 16 Meropenem ≥ 16	Imipenem ≤ 0.25 Meropenem ≤ 0.25	Imipenem 2 Meropenem 8	Imipenem ≤ 0.25 Meropenem ≤ 0.25
Cephalosprins	Ceftazidim ≥ 64	Ceftazidim ≥ 64	Ceftazidim ≤ 1	Ceftazidim 16	Ceftazidim ≥ 64	Ceftazidim ≥ 64	Cefuroxim ≥ 64 Ceftazidim 16	Cefuroxim ≥ 64 Ceftazidim ≥ 64	Ceftazidim ≤ 1
Fluoroquinolones	Ciprofloxacin ≥ 4	Ciprofloxacin ≥ 4	Ciprofloxacin ≤ 0.25	Ciprofloxacin ≥ 4	Ciprofloxacin ≥ 4	Ciprofloxacin ≥ 4	Ciprofloxacin ≤ 0.25	Ciprofloxacin ≥ 4	Ciprofloxacin ≤ 0.25
Glycopeptides									
Glycylcyclines	Tigecycline 4	Tigecycline 4	Tigecycline ≤ 0.5				Tigecycline ≤ 0.5	Tigecycline ≤ 0.5	Tigecycline ≤ 0.5
Lincosamides									
Lipopeptides									
Marcrolides									
Monobactames				Aztreonam > 256					
Oxazolidinones									
Penicillins	Ampicillin/Sulbactam ≥ 32	Ampicillin/Sulbactam ≥ 32	Ampicillin/Sulbactam ≤ 2	Piperacillin ≥ 128	Piperacillin ≥ 128	Piperacillin ≥ 128	Ampicillin ≥ 32 Piperacillin ≥ 128	Ampicillin ≥ 32 Piperacillin ≥ 128	Ampicillin 8 Piperacillin ≤ 4
Phenicols									
Polymyxins	Colistin 16			Colistin 1					
Sulfonamides	Trimethoprim/Sulfamethoxazol ≤ 20	Trimethoprim/Sulfamethoxazol ≤ 20	Trimethoprim/Sulfamethoxazol ≤ 20				Trimethoprim/Sulfamethoxazol ≥ 320	Trimethoprim/Sulfamethoxazol ≥ 320	Trimethoprim/Sulfamethoxazol ≤ 20
Tetracyclins	Tetracyclin ≥ 256								

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5 General Discussion

5.1. Host defense peptides an alternative to conventional antibiotics

The causes of AMR as an emerging problem are manifold, but main reason is obvious: inappropriate use of antibiotics in the medical and agricultural sector (Ventola, 2015; Martens & Demain, 2017). Drug-resistant bacteria already cause at least 700,000 deaths annually (Kelly & Davies, 2017). The WHO has expressed deep concerns over the rise of multidrug-resistant pathogens (Tacconelli *et al*, 2018). The impact of uncontrolled spreading multidrug-resistant bacteria would be catastrophic with inestimably consequences. A scenario, not dissimilar the current one, caused by an unprecedented outbreak of a novel coronavirus leading to the disease COVID-19 (Sohrabi *et al*, 2020). Thus, it is urgent to develop new strategies and alternatives to conventional antibiotics.

HPDs are emerging as one solution to this impending medical crisis, due to their rapid and unique antimicrobial effects. Notably, despite their evolutionary conserved structure, resistance has not developed. Moreover, it has been reported that antibiotic-resistant bacteria showed an increased sensitivity against HDPs (Lázár *et al*, 2018). In addition to their antimicrobial activity (Jenssen *et al*, 2006; Hancock & Sahl, 2006), they have multiple non-antimicrobial attributes including antiviral, antifungal, immunomodulatory, anti-biofilm and wound healing properties (Mahlapuu *et al*, 2016; Mookherjee *et al*, 2020).

Despite their tremendous promise, the translation of novel HDP-based antibiotics from bench to beside has yet not been achieved. Although several candidates are in clinical trials, most were not approved because the lack of superiority to current treatments (Koo & Seo, 2019). There are many reasons for this sluggishness in developing this class of molecules as medicines: their complex structure, low stability in host environment, undesired toxicity, lack of correlation between *in vitro* and *in vivo* efficacies, high-cost-of-goods and modest interest from pharmaceutical industry (Li *et al*, 2017; Haney *et al*, 2019; Ting *et al*, 2020). Here we propose a way to overcome some of these hurdles and introduce potential new antimicrobials to fight AMR.

5.1.1. Generation of innovative peptide fragments

To-date, the antimicrobial peptide database has catalogued more than 3000 natural antimicrobial peptides from six kingdoms, most from vertebrates (Wang *et al*, 2016b; Mookherjee *et al*, 2020). This repertoire allows the development of novel therapeutic peptide

analogues, which represent the majority of peptides in clinical trials (Greber & Dawgul, 2017). Therefore, various strategies are being applied to design new drug candidates, such as residue substitution with amino acids, use of internal fragments to generate smaller peptides and a series of in silico methods to derive novel synthetic peptides (Mookherjee *et al*, 2020).

We have taken advantage of a naturally occurring phenomena, namely proteolytic digestion to generate new peptides (Schroeder *et al*, 2011a). The structure of intestinal defensins depends on environmental conditions and presence of specific enzymes like thioredoxin, which are capable to reduce defensins. This has already been demonstrated for the and the human β-defensin 1 (hBD1) and human α-defensin 5 (Schroeder *et al*, 2011b; Jaeger *et al*, 2013; Wang *et al*, 2016a). As a result, an opening of the disulfide bridges induces a conformational change to a linear form of hBD1 (Schroeder *et al*, 2011a). This reduced, linear structured peptide is more susceptible to proteolytic digestion, whereas the oxidized form is protease resistant. Proteases are strongly expressed in the intestine and involved in processing HDPs such as the cathelicidin LL-37 or α-defensins from propeptide to mature form (Sørensen *et al*, 2001; Ghosh *et al*, 2002). Using physiological intestinal proteases, linearized hBD1 was incubated with pepsin and chymotrypsin and a fragment corresponding to the eight carboxy-terminal amino acids of hBD1 have been detected, called octapeptide. While the *in vivo* presence of the octapeptide has to be proven, our *ex vivo* digestion with human duodenal secretion generated an identical fragment, supporting the hypothesis of antimicrobial active fragments in the human intestine. The newly identified octapeptide exhibits convincing activity against various bacteria and *Candida albicans*.

Although human neutrophil peptides 1-4 (HNP-1-4) are processed from propeptide to mature form during their trafficking activated by proteolytic digestion in polymorphonuclear neutrophils azurophilic granules (Valore & Ganz, 1992), no further fragmentation have been observed till now. Here, linearized HNP-4 served as precursor to identify new fragments by tryptic digestion. Using this unconventional approach we unleashed the antimicrobial potential of HNP-4. While the newly generated fragment, HNP-$4_{1\text{-}11}$, is considerably less toxic than the full-length peptide, it possesses equal or superior antimicrobial activity against bacteria on molar level. Remarkably, HNP-$4_{\text{-}1\text{-}11}$ inhibited growth of a multidrug-resistant *K. pneumoniae* isolate, while the naïve HNP-4 was ineffective.

5.1.2. Strategies to modify host defense peptides

HDPs are a naturally diverse group with different lengths, amino acid sequences and anionic or cationic charges. Despite this diversity, they share common features in their amphipathic and charged structures (Tossi *et al*, 1997). These characteristics are utilized as common traits for modified peptides. The design is generally based on amino acid residue substitution in divers

manner (Brogden & Brogden, 2011), hybridization, L-to-D heterochiral isomerization, C- and N-terminal modification and cyclization (Ting *et al*, 2020). Thus, some of the deficiencies of HDPs shall be overcome. A number of different factors need to be taken into consideration, i.e. production costs, antimicrobial activity in adverse environments, the appropriate range of antimicrobial activity and the host response. Another challenge with HDPs in therapeutic contexts is their susceptibility to proteolysis by host and bacterial proteolytic enzymes. (Reijmar *et al*, 2007; Haney & Hancock, 2013). Secreted proteases are the first bacterial defense mechanisms to encounter HDPs in reduced environments. This represents a simple, yet effective way of resistance to HDPs. Some representative members of outer membrane proteases, such as OmpT in *E. coli* or PgtE and PhoP in *S*. Typhimurium have been demonstrated to cleave HDPs (Guina *et al*, 2000; Joo *et al*, 2016).

L-to-D-heterochiral isomerization enables to counterpart this mechanism, albeit only the L-configuration can be utilized by cells and bacteria. (Carmona *et al*, 2013; Aliashkevich *et al*, 2018). Additionally, it has been reported that the D-form amino acids could enhance the antimicrobial efficacy of HDPs (Manabe & Kawasaki, 2017). Therefore, we generated a D-form of the octapeptide and evaluated the antimicrobial activity of both peptides against multiple antibiotic-resistant bacteria. Direct comparison revealed no superiority of the D-form over the naïve octapeptide, rather the naïve octapeptide inhibited growth of the most tested bacteria. Whether this is the case for HNP-$4_{1\text{-}11mod}$ was not clarified since we did not test the single D-form of HNP-$4_{1\text{-}11}$ in this work.

Further strategies haven been established in form of a series of C- and N-terminal modifications to enhance antimicrobial efficacy (Wang, 2012). Most common modifications are N-terminal acetylation (CH_3CO-) and C-terminal amidation (-NH_2) (Oliva *et al*, 2018). These findings are in line with our results for HNP-$4_{1\text{-}11mod}$ and modified octapeptide as we found increased antimicrobial activity compared to the naïve fragment. Interestingly, in our experiments the combination of isomerization and terminal modification results in enhanced efficacy, whereas terminal modification of the naïve octapeptide remains without any impact.

Another possibility of enhancing the activity of HDPs is to attach fatty acids at the N-terminal end, called lipidation (Chu-Kung *et al*, 2010). Lipopeptides are used as antibiotics which are highly active against multidrug-resistant bacteria and fungi (Avrahami & Shai, 2004; Mangoni & Shai, 2011). It was shown that C_{14}-C_{18} long fatty acids are the optimal length to improve antimicrobial activity (Reinhardt & Neundorf, 2016). Within this work, we modified the octapeptide N-terminal using a C_{16} long fatty acid, namely palmitic acid to improve stability and bactericidal activity. In addition to the palmitoylated N-terminus, we used various spacers such as sugars or amino acids to create a set of unique lipopeptides (Pam's). Pam-2 represents the precursor for all other Pam's, the N-terminal palmitoylated octapeptide. Pam-3 additionally contains 8-Amino-3.6-dioxaoctanoic acid as hydrophilic spacer. While Pam-1 contains glucose

and sucrose as spacer, Pam-4 contains cysteine and Pam-5 lysine as spacer. We screened this set of lipopeptides for their antimicrobial activity against Gram-positive and Gram-negative bacteria in direct comparison to the octapeptide. Pam-3 was superior (*P. aeruginosa* and *S.* Typhimurium) or equal (*S. aureus*) for all tested pathogens, while Pam-2 showed less activity against *S. aureus*. Notably, both Pam-2 and -3 consistently inhibited *S.* Typhimurium growth, a species the non-modified octapeptide failed to inhibit. Pam-1, -4 and -5 were generally inactive against tested strains. Reasons for that could be the greater space between palmitic acid and octapeptide resulting from the modification. Based on these findings, we analyzed the potential of Pam-3 killing clinical isolates of multidrug-resistant bacteria belonging to the ESKAPE panel (*E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* species). Yet, with Pam-3 we identified a promising candidate to combat multidrug-resistant bacteria.

5.2. Mechanisms of action of HDPs

HDPs are found in virtually all forms of life and play a fundamental role in innate immune defense (Jenssen *et al*, 2006). They are ubiquitous in nature and have been studied extensively since they were discovered. A major difference between most HDPs and conventional antibiotics is that antibiotics penetrate into the microorganism and act on specific targets, whereas HDPs can have receptor mediated or non-receptor mediated interactions (Shai, 2002). Although HDPs were initially considered as membrane targeting only, it is now widely accepted that there are several modes of actions. These can be divided into two groups: direct killing and immune modulation (Ulm *et al*, 2012). The fundamental difference in the lipid composition of bacterial membranes and eukaryotic cell membranes is essential for direct killing (Sohlenkamp & Geiger, 2016). Bacterial membranes contain negatively charged phospholipids such as phosphatidylglycerol and cardiolipin. In addition, the surface of Gram-positive bacteria contains teichoic acid and Gram-negative bacteria lipopolysaccharide (LPS), each further increases net negative charge (Shai, 2002). In contrast, eukaryotic cell membranes are rich in zwitterionic phospholipids such as cholesterol (Guilhelmelli *et al*, 2013). The negatively charged bacterial membrane allows the initial electrostatic interaction with positively charged HDPs, resulting in HDPs accumulation at the surface of the membrane (Yeaman & Yount, 2003). After reaching a certain concentration, HDPs self-assemble on the membrane and initiate pore formation or act without forming specific pores. Various models have been used to describe directed pore formation, namely the barrel-starve model, the toroidal pore model, the carpet mechanism and the detergent like model (Melo *et al*, 2009; Mukherjee *et al*, 2014; Kumar *et al*, 2018). When HDPs were discovered, nobody thought of specific membrane targets or even of intracellular targets. Preferred membrane targets are lipoteichoic acid (LTA), LPS and the peptidoglycan precursor lipid II (Münch & Sahl, 2015). HDPs such as human β-defensin 3 and human neutrophil

peptide 1 unleash their antimicrobial activity by binding selectively to lipid II and blocking bacterial cell wall biosynthesis (Sass *et al*, 2010; de Leeuw *et al*, 2010). HDPs with intracellular targets traverse the cell membrane without causing extensive membrane damage, but still cause bacterial death (Brogden, 2005). HNP-1, HD5 and hBD-4 have been shown to target intracellular components (Lehrer *et al*, 1989; Chileveru *et al*, 2015; Sharma & Nagaraj, 2015). Apart from direct killing of microorganisms, some HDPs possess immune modulatory abilities to enhance microbial killing and control inflammation (Hilchie *et al*, 2013). The human cathelicidin LL-37 and β-defensins were shown to chemoattract immune cells in an infection (Liu, 2001; Niyonsaba *et al*, 2002). In addition, HDPs can initiate activation, attraction and differentiation of white blood cells, stimulation of angiogenesis and control the expression of pro- and anti-inflammatory cytokines (Lai & Gallo, 2009; Nijnik & Hancock, 2009; Hancock *et al*, 2012). Because of the various potential mechanisms it is a challenging task to examine the mode of action(s).

Our data demonstrate that small peptide fragments cause a loss membrane potential and membrane integrity in bacteria. Interestingly, the octapeptide seems to have diverse antimicrobial strategies for different microorganisms. We observed strong membrane depolarization in *E. coli* after octapeptide treatment, whereas *C. albicans* displayed less membrane depolarization. These findings were supported by transmission electron microscopy (TEM). In *E. coli*, all peptides caused cell wall and membrane disruption, detachment of the cell membrane from the cytosol as well as disintegration of cytosolic structures. In contrast, *C. albicans* cells displayed a functional membrane but cytosolic defects. Because of their composition, lipopeptides are presumed to target and bind to the bacterial membrane directly and cause rapid depolarization of membrane potential (Mak *et al*, 2003; Straus & Hancock, 2006). Based on this hypothesis, we analyzed the mode of action of Pam-3. Indeed our data show similarities, Pam-3 treatment resulted in cytoplasmic membrane-associated and cell envelope-related stress in *B. subtilis*. Further, it caused a breakdown of the membrane potential in a concentration-dependent manner in *S. aureus*. Additionally, we observed pore formation and a rapid killing after Pam-3 treatment in *S. aureus*. These findings are in line with previous observations of lipopeptides. Nevertheless, further investigations are necessary for a better understanding of the mechanism of action, which will allow to continuously develop these promising fragments as therapeutic agents.

5.3. Risk of developing antimicrobial resistance to HDPs

A major concern for the use of HDPs as antimicrobials to treat infections is the development of resistance mechanisms to peptide-based therapies. It has been often argued that bacteria hardly displayed developing of resistance due to the rapid bactericidal effects and multiple potential targets (Zasloff, 2002; Bechinger & Gorr, 2017). Regrettably, the opposite was likewise

demonstrated in recent studies. Bacterial and fungal pathogens are capable of developing resistance in various ways (Figure. 3) (Sahl & Shai, 2015; Andersson *et al*, 2016). Examples include membrane modifications to reduce the overall negative charge by incorporation of positively charged molecules, blocking the anionic groups in LPS by attaching an aminoarabinose group or alanylating LTA or wall teichoic acid (WTA) (Band & Weiss, 2015; Andersson *et al*, 2016). Additional mechanisms of bacterial adaptions include lipid A acylation to alter membrane stability, lipid II modification, proteolytic degradation and expulsion by efflux systems (Sieprawska-Lupa *et al*, 2004; Joo *et al*, 2016).

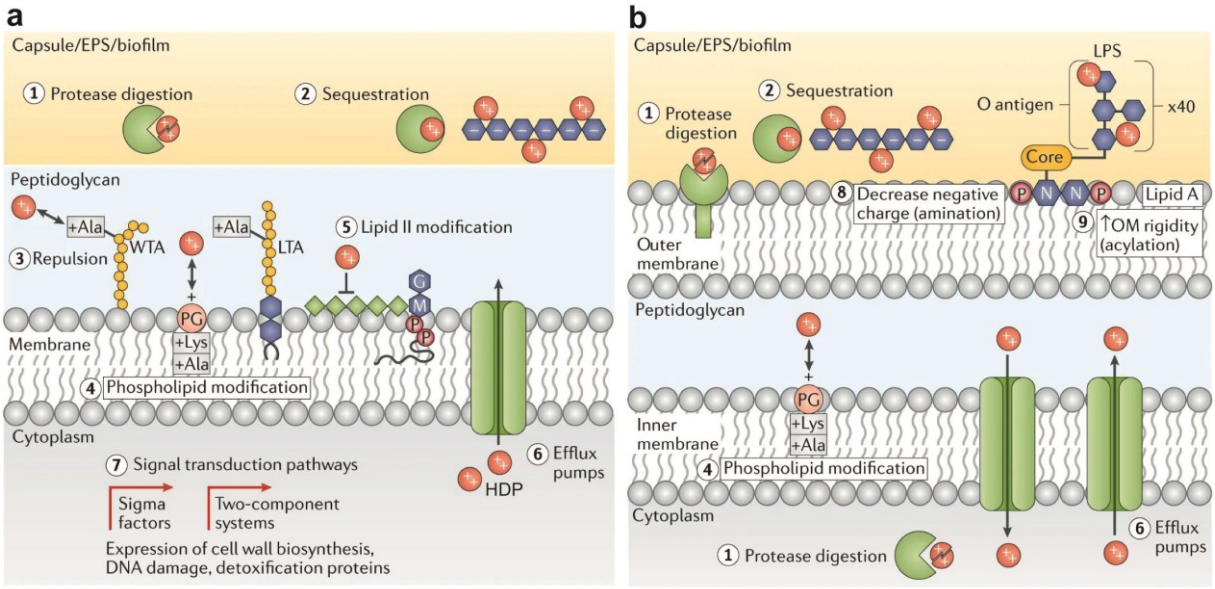

Figure 3 Summary of resistance mechanisms to HDPs in (a) Gram-positive and (b) Gram-negative bacteria. Mechanisms: **1** Degradation by secreted outer membrane or cytosolic proteases. **2** Sequestration by secreted proteins. **3** Electrostatic repulsion by anaylated LTA or WTA. **4** Electrostatic repulsion by aminoacylated phospatidylglycerol (PG). **5** Blocking peptide binding by altering lipid II. **6** Export by efflux pumps. **7** Activation of signal transduction pathways to reinforce the wall or detoxify products of HDPs. **8** Lipid A modification. **9** Enhancing membrane rigidity by lipid A acylation. Adapted with permission from Nature Reviews Drug Development (Mookherjee *et al*, 2020).

The frequency of arising resistance seems to be relatively low because of the multiple target sites of HDPs and their pharmacodynamics properties (Yu *et al*, 2018; Spohn *et al*, 2019). However, it must be taken into account that resistance is costly and the number of ways to resist is limited. This is in line with a recent study demonstrating that resistance against HDPs offers no survival benefits (El Shazely *et al*, 2019). To address the risk of resistance, we assessed the ability of *S. aureus* and *S.* Typhimurium to develop resistance against Pam-3. Although exposed to Pam-3 for continuous serial passages, no resistant isolates emerged. One reason for this is the obvious rapid and unique membrane mechanism of lipopeptides, which hardly allows the development of resistance. That makes HDPs superior to antibiotics as potential therapeutic agents. Regardless of our results, it is important to consider AMR mechanisms in the design and development of new therapeutically peptides.

5.4. Interplay between host microbiota and HDPs

The interplay between HDPs and intestinal microbiota is crucial for gut function and in maintaining health. One essential function of HDPs is fencing of commensal but also pathogen microorganisms (Ostaff *et al*, 2013). HD-5 is known to control homeostasis by adjusting the balance among bacterial populations (Menendez *et al*, 2010; Salzman *et al*, 2010). A recently published study indicates that fragmentation of HD-5 shifts microbiota composition and increases diversity (Ehmann *et al*, 2019). In addition, it has been demonstrated that HD-5 mediates resistance to *S*. Typhimurium in transgenic mice, which is consistent with the bactericidal activity of this α-defensin *in vitro* (Salzman *et al*, 2003). Conversely, the host microbiota also has a number of possibilities to influence HDP expression. Microbiota mediated metabolites such as SCFA promote the upregulation of HDPs in intestinal epithelial cells (Zhao *et al*, 2018), while also induced by probiotic bacteria (Schlee *et al*, 2007, 2008; Möndel *et al*, 2009). Taken together, this well-balanced interplay guarantees diversity, stability, resistance and resilience and last, but not least, health (Lozupone *et al*, 2012). Alterations in the microbiota can be a result from diet, toxins, drugs and pathogens. Besides enteric pathogens, antibiotics have the greatest potential to cause microbial dysbiosis (Tanoue *et al*, 2010; Carding *et al*, 2015). Although antibiotic treatment saves millions of lives, it also has non negligible consequences (Maier *et al*, 2020). Multiple studies have demonstrated the wide-ranging consequences for the commensal gut microbiota; changes in the microbiota composition, decreased diversity, reduced taxonomic richness and dysbiosis (Dethlefsen *et al*, 2008; Dethlefsen & Relman, 2011). In addition, antibiotics can have long-term effects such as increased susceptibility to secondary infections, obesity, obesity-associated metabolic diseases and a long-lasting dysbiosis, which is associated with multiple diseases (Francino, 2016; Lange *et al*, 2016).

In contrast to these downsides of conventional antibiotics and in line with recent findings (Ehmann *et al*, 2019), we demonstrated with Pam-3 that small peptide fragments have no disrupting effects on the resident microbiota. We treated healthy chow-fed mice twice at an 8-hour interval with Pam-3 (125 or 250 µg/each dose) and just minor changes in the community structure were observed in both groups (i.e. treated and untreated), the number of detected species as well as the complexity. Moreover, our data points towards an increased number of observed species and nominally increased diversity. Pam-3 seems to diminish certain high-abundant bacterial strains, which theoretically frees up new niches for low abundant taxa. Whether this is a common effect of HDPs or rather depended on specific HDPs has to be further investigated. However, the microbiota-modulating capabilities of HDPs could be used to treat many of the above mentioned diseases. The still unexploited potential of HDPs enables a wide range of therapeutic applications.

5.5. Conclusion and Outlook

The data obtained and presented in this work deliver new knowledge about the generation and modification of small peptide fragments with human α- and β-defensins as precursors. The linearized forms of the human neutrophil peptide-4 and human β-defensin 1 are susceptible to proteolytic degradation by proteases. This way, we discovered that the proteolytic digestion of HNP-4 led to an eleven amino acids short fragment (HNP-$4_{1\text{-}11}$) and detected a fragment corresponding to the eight carboxy-terminal amino acids of hBD1, named octapeptide.

While HNP-$4_{1\text{-}11}$ displayed a broad spectrum antimicrobial activity pattern against various multidrug-resistant bacteria, we did not focus on its mechanism of action. In contrast, the octapeptide was less active against antibiotic resistant bacteria, but ameliorated *C. albicans* in an *in vitro* infection model. Interestingly, our data demonstrate that the octapeptide has diverse antimicrobial strategies for bacteria and fungi. Modifications such as L-to-D-heterochiral isomerization and additionally N-terminal acetylation and C-terminal amidation enhanced the antimicrobial activity of HNP-$4_{1\text{-}11\text{mod}}$, whereas the activity of the modified octapeptide remained broadly unchanged. Next, we modified the octapeptide N-terminal with palmitic acid and created a set of unique lipopeptides (Pam's). One, namely Pam-3, showed an enormous increase in antimicrobial activity and was able to kill antibiotic-resistant bacteria from the ESKAPE panel. Further, we demonstrated the efficiency of orally applied Pam-3 in two different *in vivo* models of gastrointestinal infections, namely *S.* Typhimurium and *C. rodentium*. In both models, the bacterial burden was significantly reduced after Pam-3 treatment. In both models, the bacterial burden was significantly reduced after Pam-3 treatment. In principle, a topical or intranasal application would also be possible, but tolerability and efficiency have to been proven.

Taken together, the results presented here demonstrate that small peptide fragments derivate from human defensins have great potential to fight multidrug resistant infections. From this point of view, the strategy of proteolytic digestion of HDPs to generate new biologically active fragments can be therapeutically exploited. These novel peptide fragments can be optimized in various manners, to enhance activity, expand their function or type of application. Further studies are therefore warranted to examine the full potential of these and other biostable novel peptides. They open a new chapter of effective treatment strategy of bacterial infections.

Contribution to the publications in the thesis

1. Publication

Ehmann D, Koeninger L, Wendler J, Malek NP, Stange EF, Wehkamp J & Jensen BAH (2020) Fragmentation of human neutrophil α-defensin 4 to combat multidrug resistant bacteria. *Front. Microbiol.* 11, 1147.

Planning and/or execution of the following experiments as well as writing of the original draft were performed by me under the guidance of Dr. Benjamin A.H. Jensen and Prof. Jan Wehkamp. ; Screening for potential dimers of HNP-$4_{1\text{-}11}$ and HNP-$4_{1\text{-}11\text{mod}}$ using HPLC-MS was designed by me and performed by EMC Microcollections. I performed the Time-kill Assay, Reduction Assay and Protease Inhibitor Assay. Furthermore, I analyzed the data, prepared the figures and include corrections and editing's together with Dr. Dirk Ehmann under the supervision of Dr. Benjamin A.H. Jensen and Prof. Jan Wehkamp.

2. Publication

Wendler J, Schroeder BO, Ehmann D, Koeninger L, Mailänder-Sánchez D, Lemberg C, Wanner S, Schaller M, Stange EF, Malek NP, Weidenmaier C, LeibundGut-Landmann S & Wehkamp J (2019) Proteolytic Degradation of reduced Human Beta Defensin 1 generates a Novel Antibiotic Octapeptide. *Sci. Rep.* 9: 3640

All experiments for revision were performed by me, which include cytotoxicity assays with CaCo-2 cells and human red blood cells, analyzing the activity of the generated peptides in human blood serum and multiple Radial Diffusion Assays with low pH and without DTT to analyze environmental effects. Additionally, I analyzed the data and prepared the figures together with Dr. Judith Wendler.

3. Publication

Koeninger L, Osbelt L, Berscheid A, Wendler J, Berger J, Hipp K, Lesker TR, Pils MC, Malek NP, Jensen BAH, Brötz-Oesterhelt H, Strowig T & Wehkamp J. (in revision) Curbing gastrointestinal Infections by defensin fragment modifications without harming commensal microbiota. *Communications Biology*.

I conceived the research, designed and carried out experiments and data analysis, and wrote the manuscript under the guidance of Prof. Till Strowig and Prof. Jan Wehkamp. All Radial Diffusion Assays, Broth Microdilution Assays, Resistance Induction Assays, Biofilm Assays and Time-kill Assays as well as sample preparation for microscopy were performed by me. I conducted all mechanism of action experiments under the supervision of Dr. Anne Berscheid. All animal experiments and the associated microbiome analyses were performed by Lisa Osbelt, Till R. Lesker and Marina C. Pils under the supervision of Prof. Till Strowig.

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