ANTIMICROBIAL AND ANTICANCER PEPTIDES

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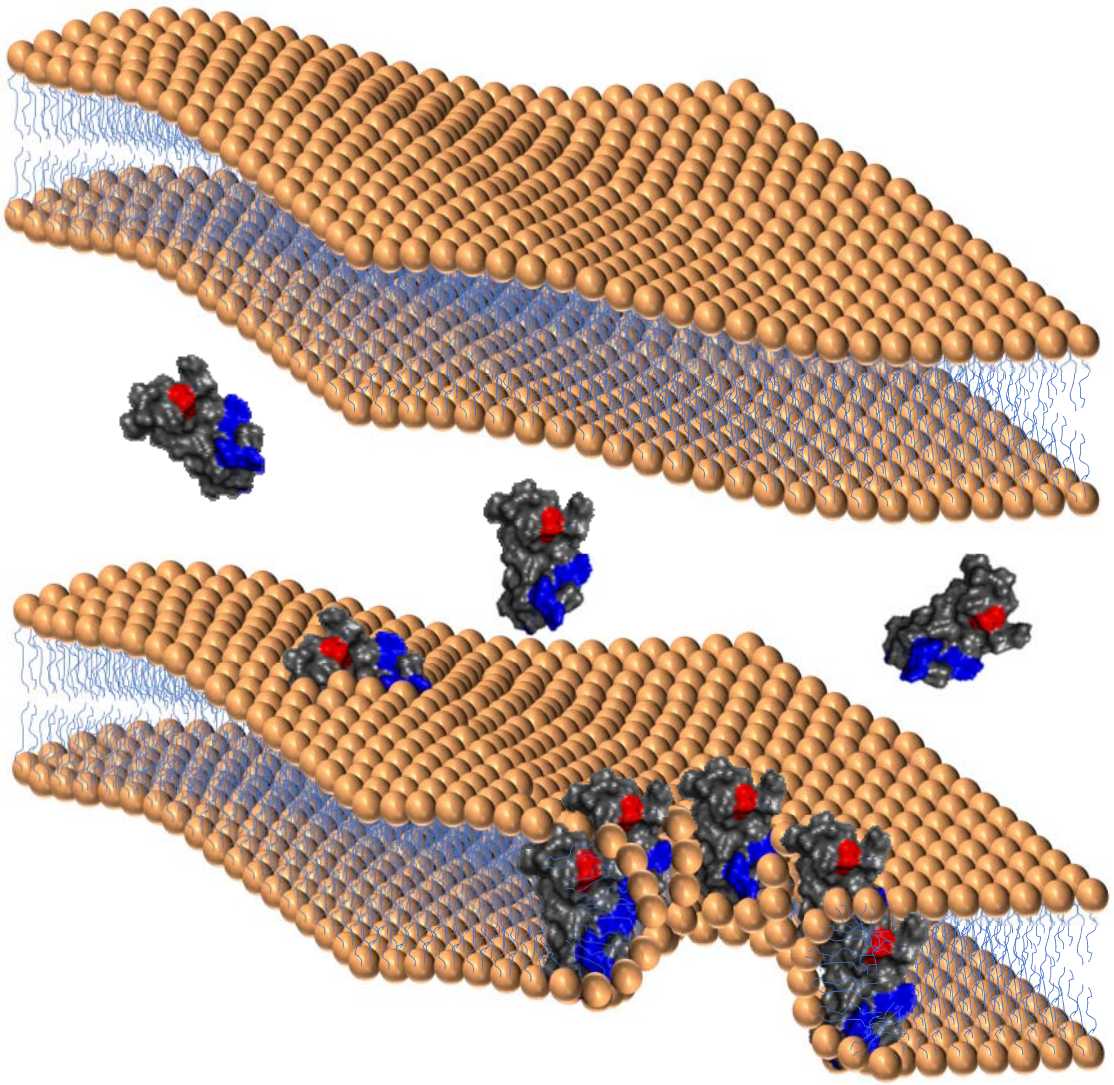

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ANTIMICROBIAL AND ANTICANCER PEPTIDES

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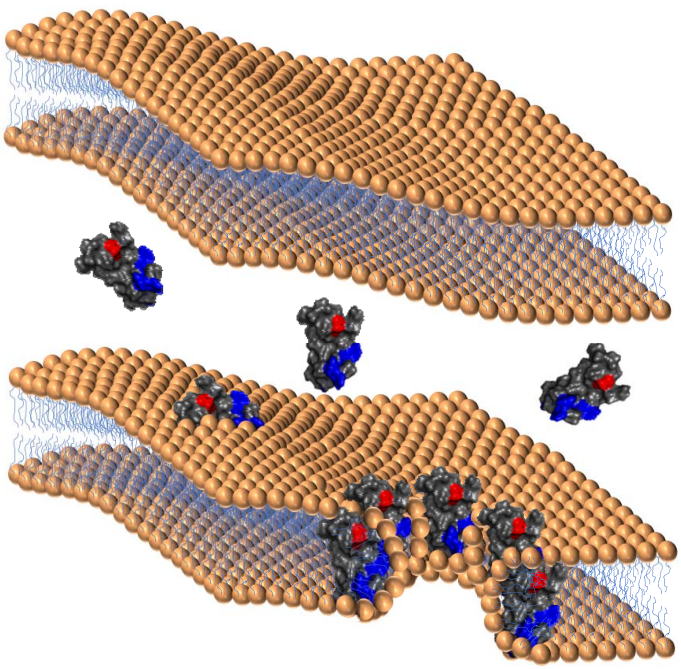

Schematic representation of insect defensin A peptide binding and forming a membrane pore. Insect defensin A structure provided by Dr Johannes Koehbach (Institute for Molecular Bioscience, The University of Queensland, Australia).

In 2014, the World Health Organization (WHO) listed cancer as the second leading cause of death and highlighted antimicrobial resistance as "a key global health challenge" that may, in a worst case scenario, lead to an annual death toll of 10 million by 2050, which would exceed predicted cancer deaths by 20%. Novel promising therapeutic options to reduce morbidity and mortality of both infectious microbial diseases and cancer are being developed based on antimicrobial peptides (AMPs), i.e., evolutionary proven antibiotics that also possess anticancer activities. Intriguingly, AMPs and anticancer peptides (ACPs) rely typically on novel mechanisms

and cellular targets not used by current antibiotics or chemotherapeutics. Initiated by presentations at the International Meeting of Antimicrobial Peptides in 2016 (IMAP 2016), hosted at Leipzig University, Germany, this book compiles the most recent strategies and promising lead compounds for treating multi- and pan-resistant microbes and chemo-resistant cancer cells in fourteen different chapters representing leading research groups from five different continents. In this respect, the book shall stimulate new avenues of thinking and strategies in tackling forthcoming antimicrobial and cancer resistance health threats with the hope that the scenarios recently reported by the WHO will never eventuate.

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EDITORIAL
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Editorial: Antimicrobial and Anticancer Peptides

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Keywords: antimmicrobial, anticancer, peptides, cancer, antibiotic resistance, bacteria, microbes, peptide chemistry

Editorial on the Research Topic

Antimicrobial and Anticancer Peptides

The World Health Organization (WHO) in their 2014 report describe cancer as "one of the leading causes of morbidity and mortality worldwide" and is the second leading cause of death globally (Stewart et al., 2014). In the same year, WHO recognized that antimicrobial resistance (AMR) as "a key global health challenge" and, if not addressed, estimated the annual number of deaths due to antibiotic-resistant bacteria could reach 10 million by 2050, exceeding that of cancer by 1.2 million (O'Neill, 2014; World-Health-Organisation, 2014). On the face of it, these reports may seem to be disconnected, however, surgery and chemotherapy, are two major therapies in cancer treatment that rely on the use of antibiotics/antimicrobials to prevent microbial infection post-surgery and when chemotherapy suppresses the immune system. Thus, the impact of a post-antibiotic era has far more reaching consequences than our ability to treat infections which will significantly impact the delivery and management of health care as a whole. According to WHO and the UK Prime Minister's Office reports (O'Neill, 2014; Stewart et al., 2014; World-Health-Organisation, 2014, 2017), there are urgent calls for more targeted approaches for cancer treatment and to address the global emergence of AMR. For both cancer and multi-drug resistant microbes, peptides are deemed as the next generation antimicrobial materials and strategies are being developed to design cell specific peptide-based therapeutics. In this Research Topic we present a salient collection of original research and review articles that show how multidisciplinary approaches to antimicrobial resistance and cancer are leading to the discovery and production of novel materials with specificity toward bacteria and/or cancer cells.

Intriguingly, it has been shown that cationic antimicrobial peptides (AMPs) have anticancer properties which may be due to both bacteria and cancer cell membranes having a net negative charge. This dual nature of peptides of having antimicrobial and anticancer properties is explored in the review by Felício et al. The discovery of bioactive peptides is an essential part of medicinal and peptide chemistry research and Díaz-Gómez et al. highlight this in the review article on anticancer peptides that have been discovered and isolated from maize. The importance of bioactive peptide discovery is further highlighted by Oyama et al. who designed an AMP that has potent antibacterial activity against *Enterococcus faecalis* that was identified from a rumen metagenomics study. A significant advantage of peptides as therapeutics is that they are straightforwardly modified by solid and solution phase chemical techniques, facilitating structure-relationship (SAR) studies on newly-discovered peptides. Insect defensins are a class of AMPs that have drawn considerable

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attention and SAR studies are being used to define bioactive and biotoxic sequences with the purpose of designing novel cysteine-rich highly effective AMPs (Koehbach).

Altering specific peptide residues with D- and unnatural amino acids can significantly impact on biological activity as shown by Li et al. on the activity of the AMP Chex1-Arg20. An approach taken by Mardirossian et al. is the synthesis of an all D-enantiomer of BMAP18 which showed improved *in vitro* protease stability and then demonstrated *in vivo* that other factors inhibited activity and these need to be considered in AMP design. In the review by Casciaro et al. how the use of D- and unnatural amino acids and conjugation to nanoparticles is impact on an AMPs bio-stability, cytotoxicity and delivery of the AMP to a specific target site is assessed. The use of stable isotope-labeled peptides of apidaecin analogs, pharmaocokinetics, and mass spectroscopy revealed how apidaecin AMPs were effective *in vivo* and demonstrates how these *in vivo* methods can be applied to other AMPs (Schmidt).

AMPs can be used as surface coatings on medical devices to prevent microbial colonization as reviewed by Riool et al. With an emphasis on how AMPs are conjugated on to biomedical devices and how effective they are López-Pérez et al. describe how SPOT synthesis can be employed to screen and optimize the activity of surface-bound AMPs. Colonization of surfaces by bacteria typically leads to the development of a biofilm and Grassi et al. demonstrate that AMP activity against biofilms can be enhanced by co-administration of adjuvant-like molecules that aid membrane disruption. Many AMPs exert their action directly on the cytoplasmic membrane of bacteria and various models of their mechanism of action have been proposed. However, Zeth and Sancho-Vaello question these models as they show that two well-studied AMPs, LL-37 and dermcidin, deviate from the traditional models of membrane-disruption. The novel nature of AMP modes of action differ significantly from traditional antibiotics and drugs and yet AMPs fall under the same drug approval regulations, hindering AMP development as highlighted by Otvos in a perspective article (Otvos). Finally, the complex nature of the mode of action of AMPs is emphasized by Del Cogliano et al. who show that cationic AMPs are able to inactivate Shiga toxin-encoding bacteriophages and thus reduce *Escherichia coli* virulence.

We believe that the Antimicrobial and Anticancer Peptide Research Topic exemplifies the multidisciplinary nature of peptide research and that the advancement of therapeutics that target cancer and/or microbes requires an interconnected research strategy as exemplified in this body of work. It has the objective of stimulating new avenues of thinking, approaches, and collaboration in tackling current and forthcoming cancer and antimicrobial resistance health threats.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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Peptides with Dual Antimicrobial and Anticancer Activities

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In recent years, the number of people suffering from cancer and multi-resistant infections has increased, such that both diseases are already seen as current and future major causes of death. Moreover, chronic infections are one of the main causes of cancer, due to the instability in the immune system that allows cancer cells to proliferate. Likewise, the physical debility associated with cancer or with anticancer therapy itself often paves the way for opportunistic infections. It is urgent to develop new therapeutic methods, with higher efficiency and lower side effects. Antimicrobial peptides (AMPs) are found in the innate immune system of a wide range of organisms. Identified as the most promising alternative to conventional molecules used nowadays against infections, some of them have been shown to have dual activity, both as antimicrobial and anticancer peptides (ACPs). Highly cationic and amphipathic, they have demonstrated efficacy against both conditions, with the number of nature-driven or synthetically designed peptides increasing year by year. With similar properties, AMPs that can also act as ACPs are viewed as future chemotherapeutic drugs, with the advantage of low propensity to resistance, which started this paradigm in the pharmaceutical market. These peptides have already been described as molecules presenting killing mechanisms at the membrane level, but also acting toward intracellular targets, which increases their success compartively to one-target specific drugs. This review will approach the desirable characteristics of small peptides that demonstrated dual activity against microbial infections and cancer, as well as the peptides engaged in clinical trials.

Keywords: anticancer peptides (ACPs), antimicrobial peptides (AMPs), cancer, multi-resistant infections, bacteria

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INTRODUCTION

At the beginning of the twenty-first century, the increased appearances of multi-resistant bacterial pathogens have become a worldwide problem (Arias and Murray, 2009). The World Health Organization has already emphasized the urgency in designing new antimicrobial molecules, because conventional antibiotics are increasingly useless as therapeutics, especially against the so-called *ESKAPE* pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa,* and *Enterobacter* species), which showed a high propensity to develop antibiotic resistance (McKenna, 2013). Another global concern is the rise in the incidence of cancer. Recent data released revealed 12.7 million new cases and 7.6 million deaths, just in 2008 (Ferlay et al., 2010). In Europe alone, 3.45 million new cases were diagnosed and 1.75

million deaths occurred during 2012 (Ferlay et al., 2013). Nowadays, cancer is the second most common cause of death worldwide (Arnold et al., 2015), caused by an abnormal cellular growth, in a uncontrolled manner, with the ability to invade other tissues, leading to the formation of tumor masses, neovascularization (angiogenesis), and metastasis (Thundimadathil, 2012). Lung, colorectal, prostate, and breast cancer are the most diagnosed forms of this disease (Domalaon et al., 2016). Considering the numbers revealed, it is urgent to find new anticancer drugs able to control tumor growth with minimal side effects (Dennison et al., 2007). This situation has become worse due to DNA-alkylation, hormone agonists, and antimetabolites, which show insufficient selectivity and unspecific targeting on healthy cells (Smith and White, 1995; Gaspar et al., 2013), contributing to increased resistance to anticancer drugs (Wang K.-r. et al., 2009). Moreover, the intersection between infection and cancer is highlighted by the number of cancer deaths and new occurrences that are related to treatment or chronic infections. Approximately 2 million of the new cancer patients are due to infectious agents like bacteria and viruses (Parkin, 2006; Attiê, 2014; Vedham et al., 2014). Patients that suffer from a chronic infection are more susceptible to cancer due to the weakened immune system, which cannot fight both the pathogen, and the emergence of cancer cells (Rolston, 2001). This weakness can also occur due to cancer treatments that are too aggressive to patient health, such as chemotherapy, radiotherapy, and surgical resection, leaving patients susceptible to infection agents (Fishman, 2011; Xiao et al., 2015). Also, continuous exposure to infection leads to inflammation, contributing to the appearance of cancer (Vedham et al., 2014).

In recent years, a promising new class of molecules has arisen, and it has different types of advantages against both of the above major world health concerns. Antimicrobial peptides (AMPs) are small peptides essential for the innate immune response of organisms of all branches, presenting activity against a wide range of pathogens, like bacteria, fungi, and viruses (Hancock et al., 2016). More recently, anticancer activity was also described for some of these peptides, termed anticancer peptides (ACPs) (Dennison et al., 2006). Properties like their short time-frame of interaction (which decreases the probability of resistance), low toxicity (which reduces side effects), mode of action, specificity, good solubility, and finally, good tumor penetration, indicate ACPs as a future chemotherapy cancer drug with high potential (Riedl et al., 2011; Figueiredo et al., 2014; Wu et al., 2014; Gaspar et al., 2015; Domalaon et al., 2016).

PEPTIDES WITH ANTIMICROBIAL AND ANTICANCER ACTIVITY

Antimicrobial peptides were first identified due to their importance in the innate immunity of a broad number of organisms, gaining interest from the scientific community (Jenssen et al., 2006). From the first identification until today, hundreds of AMPs have been identified and studied, either from natural sources or from *in silico* designs (Hancock et al., 2016). These peptides are characterized by an amino acid sequence usually from 5 to 50 residues, high hydrophobicity and positive net charge (Melo et al., 2011; Gaspar et al., 2012). These physicochemical properties set the basis for the activity against pathogens (Dennison et al., 2010). Bacteria present negatively charged membranes, promoting AMPs' initial electrostatic interaction. Even knowing that not all AMPs are ACPs, the similarity in terms of action is obvious, due to the phenotype of the membrane surface in cancer cells. In the plasma membrane inner-leaflet of healthy cells there is phosphatidylserine (PS), a negatively charged phospholipid. This asymmetry between inner and outer membrane leaflets is lost in cancer cells, leading to the presence of PS in the outer-leaflet (Bevers et al., 1996). PS exposure, the presence of O-glycosylated mucins, sialylated gangliosides, and heparin sulfate, in conjugation with an increased transmembrane potential, surface area, and membrane fluidity (Schweizer, 2009; Hilchie et al., 2011), promote the specific activity of AMPs toward cancer cells (ACPs), without being affected by tumors' heterogeneity (Kelly et al., 2016).

The physicochemical parameters determining the activity of some AMPs toward cancer cells are still unclear, considering that the characteristics of AMPs/ACPs are very similar. Efforts are being made in order to understand these differences, which would enable an improved design of ACPs (Dennison et al., 2006). Some AMPs can also be ACPs independently of the source of identification or synthetic route of design (Mader and Hoskin, 2006). The number of AMPs encountered in nature that have anticancer activity has increased in recent years. Aurein 1.2 (GLFDIIKKIAESF), a peptide isolated from the frog *Litoria aurea*, is one example of an AMP with broad-range activity toward bacteria that showed to be highly active toward 55 different cancer cell lines *in vitro*, without any significant cytotoxic activity (Rozek et al., 2000; Dennison et al., 2007; Giacometti et al., 2007). Another example is the human neutrophil peptide-1 (HNP-1, ACYCRIPACIAGERRYGTCIYQGALWAFCC), an AMP that plays a fundamental role in the defense against pathogens in the innate immune system. Its antimicrobial activity has been fully explored, with a broad spectrum activity against bacteria, but it is the possibility of using this AMP in cancer therapies that attracted attention in recent years (Nishimura et al., 2004; Varkey and Nagaraj, 2005). The full mechanism of action of this peptide against cancer cells has not yet been established, but activity was already confirmed for different cancer cell lines, with very low cytotoxicity against healthy cells (McKeown et al., 2006; Gaspar et al., 2015). Peptides pleuricidin 03 (GRRKRKWLRRIGKGVKIIGGAALDHL) and pleuricidin 07 (RWGKWFKKATHVGKHVGKAALTAYL), AMPs isolated from Atlantic flatfishes, were showed to be highly effective in killing different bacterial strains (Patrzykat et al., 2003). Recently, their anticancer activity was explored and their effectiveness against drug-resistant breast cancer cells confirmed, without toxicity against fibroblasts or erythrocytes, either in *in vitro* and *in vivo* models (Hilchie et al., 2011). These are just examples of ACPs that were studied after isolation from different natural sources, like animals, plants, and bacteria. Natural ACPs, even having a high anticancer activity, have

normally 30–40 amino acids in their sequence, which increases production costs. Therefore, synthetic routes for ACP design have gained attention. There are different possible approaches available, such as the improvement of natural ACP sequences or the use of *in silico* methods (Park et al., 1998; Lee et al., 2008). Both strategies take into consideration the improvement of the physicochemical properties, like amphipathicity, hydrophobicity, and overall positive charge, with the objective of better activity toward the target cells (Huang et al., 2011; Melo et al., 2011; Sinthuvanich et al., 2012). Furthermore, other strategies such as hybridizing different ACPs (Hoskin and Ramamoorthy, 2008) or changing the amino acids used for unnatural ones (D-enantiomers or cyclic tetra-substitution of C^{α} are examples; Hicks, 2016) have also been tested. The possibilities are endless, and depend on what the focus of the improvement is for each case. Bioinformatic algorithms integrated with machine learning, where the design is automatic through the properties chosen, taking into consideration AMP/ACP libraries of existing molecules, are considered the future method for their rational design (Tyagi et al., 2013; Lin et al., 2015).

AMPs and ACPs share most of the characteristics, like the physicochemical properties already described. Structure plays a central role in their activity. It is commonly accepted that most AMPs/ACPs do not fold in a well-defined structure when free in solution, but adopt α-helix or β-sheet structure when electrostatic interactions with membranes occur (Hoskin and Ramamoorthy, 2008). Differences in terms of structure were the first method for the classification of ACPs. Examples of some AMPs lately defined as α-ACPs are cecropin, magainin, melittin, and buforin II, with lactoferricin B, HNP-1/3, and gamesin being classified as β-ACPs (Papo and Shai, 2005). More recently, it was noticed that independently of the secondary structure that the peptide adopts, a classification considering the mechanisms of action in the target cancer cells was more suitable (Wu et al., 2014). AMPs were considered membrane-active peptides regarding their primary activity, but over the years, it was clarified that they can also target different processes of the pathogen (namely, metabolism, and cell division) and of the immune system (recruitment of immune cells; Hancock et al., 2016). These aspects were also studied for ACPs, with the identification of cell membrane lytic activity (necrosis), mitochondrial membrane lytic activity (apoptosis), and non-membrane activities (**Figure 1**; Wu et al., 2014). The first one is the most common anticancer method of targeting, with the electrostatic interactions promoting membrane disruption, leading to necrosis. Polybia-MPI, a natural ACP, and the synthetic BTM-P1 are just two examples (Segura et al., 2007; Wang K.-r. et al., 2009). These ACPs have high selectivity toward cancer cell membranes and develop low resistance, when compared to conventional chemotherapeutic drugs. Activity toward mitochondrial membrane, activating apoptosis signaling, was also observed for some ACPs, such as lactoferricin B and different β-ACPs (Furlong et al., 2006; Paredes-Gamero et al., 2012). After the activity at the membrane level, ACPs can also present other activities, either targeting essential cell proteins, inhibiting angiogenesis, or recruiting immune cells to attack cancer cells (**Figure 1**; Wu et al., 2014). HNP-1 was shown to be an ACP that recruits and activates dendritic cells in terms of immunomodulatory activity (Wang Y.-s. et al., 2009), but also inhibits angiogenesis, which is essential to the growth and development of tumors (Xu et al., 2008).

POTENTIAL CLINICAL APPROACHES USING ACPs

Although a wide variety of drugs are commercially available, treatments for infections, and cancer have one thing in common: the emergence of resistance against multiple drugs (Baguley, 2010; Theuretzbacher, 2012). Another associated problem is the lack of selectivity of the available drugs, and their consequent undesirable side effects for the patients (Mandell et al., 2001; Baguley, 2010). Thus, there is a need for the development of new antineoplastic and antimicrobial therapies, with higher selectivity, leading to fewer side effects than current ones. It is desirable that these new compounds present different mechanisms of action, without dependence on activity toward a single specific molecule in the target cells, like the ones used nowadays in therapeutics. The main goal is resistance prevention, overcoming the existing mechanisms that cancer and bacterial cells use, being active and diminishing the side effects (Lincke et al., 1990; Arias and Murray, 2009; Kakde et al., 2011).

As described earlier, several AMPs and/or ACPs have become the focus of research by different groups, mainly due to their ability to kill or inhibit the growth of a variety of microorganisms and tumor cells (Wu et al., 2014; Hancock et al., 2016). There are thousands of natural peptides and millions of synthetic peptides obtained by rational design, with a large number presenting antimicrobial and anticancer activity, but only a few being tested (Gordon et al., 2005). Furthermore, from these, unfortunately, only a small number are currently in clinical trials (**Table 1**). This is mostly due to the numerous challenges associated with the development of these peptides as pharmaceutical drugs, such as synthesis costs, which are higher than the synthesis of organic antibiotic small molecules. Due to this, peptide design has focused on primary structure shortening, accomplishing a lower production cost, and allowing physicochemical properties to be easily changed, which is important for the activity of AMPs/ACPs (Tørfoss et al., 2012a; Domalaon et al., 2016).

In addition, the adverse effects presented by some peptides (high toxicity to mammalian healthy cells and low immune response modification) increase the number of obstacles to applying these molecules to therapy (Hancock, 1997; Andreu and Rivas, 1998; Xiao et al., 2015; Kao et al., 2016). This is not surprising, since the activity of AMPs/ACPs usually depends on membrane-peptide interaction. However, to be commercially useful, it would be necessary to dissociate the toxicity to the mammalian cells from antimicrobial/antitumor activity, which can be achieved by increasing antimicrobial activity, reducing haemolytic activity, or both (Chen et al., 2005; Uggerhøj et al., 2015).

Another obstacle to the applicability of peptides is their susceptibility to proteolysis. Oral administration remains

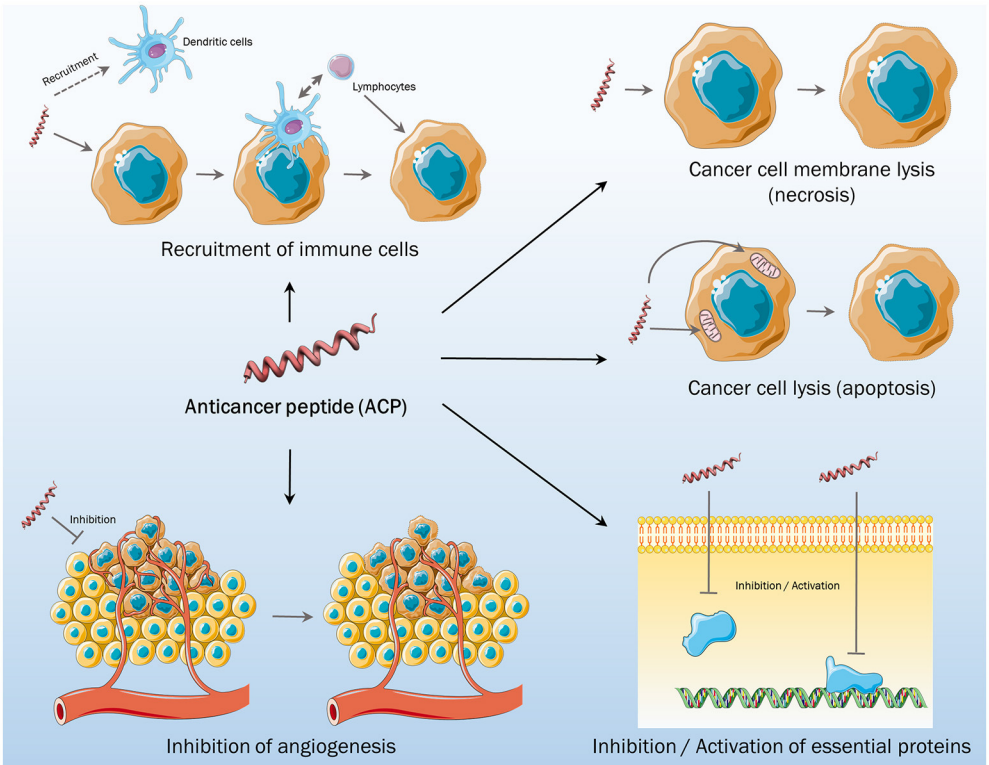

FIGURE 1 | Different mechanisms of action of anticancer peptides.

the preferred mode for drug delivery, corresponding to approximately 60% of the administration routes used for drugs (Renukuntla et al., 2013). This occurs due to the advantages that these drugs present, including low production cost and patient compliance in the administration. Even so, peptide drugs usually follow the traditional route of administration, like intramuscular (i.m.) or intravenous (i.v.) injection, due to their poor oral bioavailability, which is expressed by a low resistance to proteases and poor penetration through the intestinal membrane (Hamman et al., 2005). Sensitivity to proteolytic degradation can be mitigated by using rational design to replace naturally occurring amino acids with unnatural ones (Gordon et al., 2005; Uggerhøj et al., 2015). An example is the synthetic design of D-enantiomeric peptides, like DJK-5/6, which show improved activity against bacterial infections in *in vivo* models, comparable to that of the L-enantiomeric peptides, without showing any cytotoxic activity (de la Fuente-Núñez et al., 2015; Mansour et al., 2016). This type of peptide were also shown to be more actively effective against drug-resistant tuberculosis pathogens, and have already been tested with inhalable spray-dried formulations (Lan et al., 2014; Kwok et al., 2015). In terms of ACPs, SVS-1 was seen to be more effective, compared to its L-isomeric peptide form (Sinthuvanich et al., 2012). $\beta^{2,2}$ amino acids, also unnatural ones, can be another strategy to design AMPs/ACPs that are resistant to proteolysis, with a high effectiveness against the target cells and low toxicity toward healthy cells (Tørfoss et al., 2012a,b).

Together with proteolysis comes the limitation of pharmacokinetics and pharmacodynamics, because it is difficult to evaluate the direct action of the peptide against the pathogen *in vivo* and relate to a specific mode of action (Drusano, 2004). Moreover, the time of circulation, which is essential for a drug to be efficient, is not easy to determine (Kelly et al., 2016). Different strategies have been proposed for this problem, like the use of drug carriers, such as bacteriophages (Dąbrowska et al., 2014). Using a natural bacterial phage, displaying ACPs on their surface, increases the targeting (dynamics of action) and allows for improved dual activity. Conjugating the peptide with cell-penetrating peptides (CPPs) can be another interesting strategy to improve the specificity of the targeting. Some authors have used TAT protein from HIV virus as the CPP, conjugated to an AMP/ACP (HPRP-A1) in order to increase the specificity toward cancer cells (Hao et al., 2015). Coating or conjugation of peptides with polymers, like polyethylene glycol (PEG), can also increase circulation and improve pharmacokinetics/dynamics, independently of the polymer used, by allowing a higher time of circulation and improving their penetration toward the target cancer cells (Kelly et al., 2016).

In conclusion, these modifications may promote changes in amphipathic/hydrophobic properties, leading to the reduced cytotoxicity of peptides toward mammalian cells, without jeopardizing antimicrobial/anticancer efficiency, rendering peptides more impervious to proteolysis, and thus bestowing on

TABLE 1 | Anticancer and antimicrobial peptides in clinical trials, with the indication of the highest phase and the therapeutic condition for which they are being tested.

Product name	Peptide	Company	Highest phase	Condition treated	Route of administration
ACPs					
ANG-4043	ANG-4043	AngioChem Co.	Preclinical	Brain metastases	IV
CLS-001	MBI-226	Cadence Pharmaceuticals Inc.	II	Vulvar intraepithelial neoplasia	IV
		Carrus Capital Corp.			
		Cutanea Life Sciences Inc.			
		Migenix Inc.			
GRN-1201	GRN-1201	Green Peptide Co.	I	Solid tumors	IV
ICT01-2588	ICT01-2588	Incanthera Ltd.	I	Vascular disrupting agents	IV
		University of Bradford		Breast cancer (preclinical)	
				Colorectal cancer (preclinical)	
				Lung cancer (preclinical)	
				Prostate cancer (preclinical)	
ICT03-Es5	ICT03-Es5	Incanthera Ltd.	I	Solid tumors	IV
		University of Salford		Breast cancer (preclinical)	
				Liver cancer (preclinical)	
				Non-small cell lung cancer (preclinical)	
ICT04-CYP	ICT04-CYP	Incanthera Ltd.	Preclinical	Bladder cancer	IV
		University of Bradford		Colorectal cancer	
ITK-1	ITK-1	FUJIFILM Co.	III	Glioblastoma	IV
		Green Peptide Co.		Prostate cancer	
		Kurume University			
Oncopore™	LTX-315	Lytix Biopharma AS	I	Solid tumors	IV
Paclitaxeltrevatide	ANG-1005	AngioChem Co.	II	Brain metastases	IV
				Glioblastoma	
				Glioma	
WT-2725	WT-2725	Sumitomo Dainippon Pharma Co.	I	Hematological malignancies	IV
		Sunovion Pharmaceuticals Inc.		Solid tumors	
AMPs					
C16G2	C16G2	Chengdu Sen Nuo Wei Biotechnology Co.	II	Dental caries	Topical
		C3 Jian Inc			
Cefilavancin®	TD-1792	GlaxoSmithKline Co.	III	Gram-positive infections	Topical
		Theravance Biopharma Inc.		Skin and soft tissue infections	
		R-Pharm			
CLS-001	MBI-226	Cadence Pharmaceuticals Inc.	III	Rosacea	Topical
		Carrus Capital Corp.		*Acne vulgaris* (II)	
		Cutanea Life Sciences Inc.		Genital warts (II)	
		Migenix Inc.			
Dalvance™	MDL-63,397	Durata Therapeutics Inc.	II	Osteomyelitis	IV
		Pfizer Inc.		Osteomyelitis (I)	
		Vicuron Pharmaceuticals Inc.		Pneumonia (preclinical)	

(Continued)

TABLE 1 | Continued

Product name	Peptide	Company	Highest phase	Condition treated	Route of administration
DPK-060	DPK-060	DermaGen AB	II	Atopic dermatitis	Topical
		Pergamum AB		Otitis externa	
		Karolinska Development AB			
LL-37	LL-37	Pergamum AB	II	Leg ulcer	Topical
		Karolinska Development AB			
Locilex®	MSI-78	Dipexium Pharmaceuticals Inc.	III	Diabetic foot ulcer	Topical
		Genaera Corp.		Skin and soft tissue infections (I)	
		GlaxoSmithKline Plc.			
		RRD International Inc.			
Luminaderm®	NP108	NovaBiotics Ltd.	II	Bovine mastitis	Topical
Lytixar™	LTX109	Lytix Biopharma AS	II	Impetigo	Topical
				Staphylococcus aureus infections	
Murepavadin®	POL-7080	Polyphor Ltd.	II	*Pseudomonas aeruginosa* infections	IV
		University of Zurich		Gram-negative infections (I)	
Novamycin®	NP-339	NovaBiotics Ltd.	I	Cystic fibrosis	IV
				Invasive fungal disease	
				Oropharyngeal candidiasis	
Novarifyn®	NP-432	NovaBiotics Ltd.	Preclinical	Methicillin-resistant *Staphylococcus* aureus (MRSA) *P. aeruginosa*	IV
				C. difficile infections	
Novexatin®	NP-213	NovaBiotics Ltd.	II	Onychomycosis	Topical
		Taro Pharmaceutical Industries Ltd.			
NVB302	NVB302	Novacta Biosystems Ltd.	I	*C. difficile* infections	Topical
PXL-01	Lactoferrin	DermaGen AB	III	Post-surgical adhesions	Topical
		Karolinska Development AB			
		PharmaSurgics AB			
		Promore Pharma			
		Pergamum AB			
SGX-942	Dusquetide	Inimex Pharmaceuticals Inc.	Preclinical	Melioidosis	IV
		SciClone Pharmaceuticals Inc.			
		Soligenix Inc.			
		University of British Columbia			
Surotomycin	MK-4261	Cubist Pharmaceuticals Inc.	III	*Clostridium difficile* infections	IV
		Merck & Co. Inc.			
Telavancin®	TD-6424	Clinigen Group plc	III	Osteomyelitis	IV
		Innoviva Inc.		Bacterial infections (I)	
		Pendopharm			
		Theravance Biopharma Inc.			
		University of Illinois			

The search was carried out in the investigational drug databases Pharmaprojects (www.pharmaprojects.com), AdisInsight (www.adisinsight.com), Prous DDR (http://www.prous.com), and IDdb3 (http://science.thomsonreuters.com).

them improved therapeutic activity and pharmaceutic design (Chen et al., 2005; Uggerhøj et al., 2015; Kang et al., 2017).

CONCLUSION AND FUTURE DIRECTIONS

In conclusion, AMPs and ACPs have been known for several decades, but only in the last one an increasing number of publications on thier *in vivo* activities has arisen. Consequently, few peptides are used in medical practice. However, we believe that in the upcoming years peptides will have a major impact on the treatment of infectious diseases and cancer, two of the world's greatest healthcare concerns. As shown here, different microbial infections and/or cancer-targeting peptides are in clinical trials, with approval for clinical application expected for the next few years (at least 10 in the next 5 years). Moreover, that number should tend to increase due to advances in the rational design of peptides, minimizing or eliminating cytotoxic effects. In addition, advances in the large-scale synthesis of peptides has made this process cheaper, thus making peptide-based therapies likely to become more accessible to patients. Another strategy that has gained attention is the combined use of peptides with conventional drugs, which reduces costs per treatment, minimizing the problem of resistance and preventing recurrence. Thus, AMPs and ACPs have great potential, both alone and in combination with conventional drugs, to be used in infection and cancer therapies, mostly due to their effective mechanisms of action on the target cells.

AUTHOR CONTRIBUTIONS

MF, OS, SG, and NS wrote the article. SG, NS, and OF reviewed the article.

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Anti-Cancer Activity of Maize Bioactive Peptides

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Cancer is one of the main chronic degenerative diseases worldwide. In recent years, consumption of whole-grain cereals and their derivative food products has been associated with a reduced risk of various types of cancer. The main biomolecules in cereals include proteins, peptides, and amino acids, all of which are present in different quantities within the grain. Some of these peptides possess nutraceutical properties and exert biological effects that promote health and prevent cancer. In this review, we report the current status and advances in knowledge regarding the bioactive properties of maize peptides, such as antioxidant, antihypertensive, hepatoprotective, and anti-tumor activities. We also highlight the potential biological mechanisms through which maize bioactive peptides exert anti-cancer activity. Finally, we analyze and emphasize the potential applications of maize peptides.

Keywords: maize, disease, peptides, bioactive, antioxidant, anticancer, antihypertensive, hepatoprotective

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INTRODUCTION

According to the World Health Organization, chronic diseases are currently the major cause of morbidity worldwide, and will become one of the major causes of mortality by the next decade (WHO, 2017). In recent years, the consistent consumption of cereals and cereal-derived food products has been linked with a reduced risk of cancer and other chronic degenerative diseases. Several reports indicate that diets rich in whole-grain cereals are associated with lower cancer mortality rates, particularly colon, breast, and prostate cancers. (Jeong et al., 2003; Liu, 2007).

Cereals contain nutraceutical molecules that can exert specific biological effects and promote health and prevent diseases (Chaturvedi et al., 2011). These biomolecules are proteins and their derivatives, peptides, and amino acids present in different quantities in the grain. Legumes such as soybean are the most studied source of bioactive proteins and peptides due to their high (up to 40%) average protein content (Cavazos and Gonzalez de Mejia, 2013). Cereals such as wheat, rice, barley, rye, and maize have been recently identified as new sources of bioactive peptides. High quality cereal proteins are an important source of bioactive peptides, which consist of distinctive amino acid sequences, and which, once they are released, could display diverse functionalities (de Mejia et al., 2012; Zambrowicz et al., 2013). Cereal bioactive peptides have been a part of the human diet for centuries. In addition to their nutritional roles, these peptides could perform biological activities (Dia and Mejia, 2010).

The potential therapeutic use of peptides derived from food has been discussed previously (Malaguti et al., 2014; Ortiz-Martinez et al., 2014), and these peptides have displayed a broad range of effects in different models (McDermott, 2009). The main objective of this review was to report the current status and advances in knowledge regarding the bioactive properties of maize peptides, and

to review the evidence relating to the identification, characterization, and relevance of the biological activities through which these peptides exert an anti-cancer effect.

MAIZE AS A SOURCE OF BIOACTIVE PEPTIDES

Cereals, which are members of the grass family *Gramineae*, are the most important source of foods for the world population, and the main source of carbohydrates, proteins, vitamins, and minerals. Maize, rice, and wheat are the most important grains in the human diet, and with more than 1,000 million tons harvested in 2014, maize is the most popular crop worldwide (FAO, 2015).

A maize kernel consists of an embryo (or germ), an endosperm packed with starch grains, and bran (fiber). The most abundant kernel nutrient is starch, which is composed mainly of amylopectin and amylose (72–73% of the total kernel weight), followed by proteins that represent ∼8–12% of the total kernel weight (FAO, 1992). The essential amino acid content is ∼5–10% lower than the non-essential amino acid content, with glutamic acid being the most abundant amino acid (Tang et al., 2013). Four groups of storage proteins are present in maize kernels: albumins, globulins, prolamins, and glutelins. Albumins and globulins are found mainly in the germ, while prolamins and glutelins are found predominantly in the endosperm (Shukla and Cheryan, 2001). These four classes are also categorized according to their solubility: water-soluble albumins, globulins soluble in salt solution, prolamins soluble in alcoholic, and glutelins insoluble in neutral aqueous or saline solutions and ethanol. Globulins and albumins regulate and control grain metabolism, whereas prolamins and glutelins store the nitrogen necessary for seed germination (Anderson and Lamsal, 2011).

In terms of quantity, the protein content of maize kernels is composed mostly of prolamins or zeins (40%), followed by glutelins (30%), with globulins and albumins found in lesser quantities (5%) (Wang et al., 2008). Zeins are mainly found in protein bodies in the rough endoplasmic reticulum and constitute ∼44–79% of maize endosperm proteins (Giuberti et al., 2012). Zeins are devoid of lysine and tryptophan, amino acids that are essential for human survival (Huang et al., 2004). Zeins are composed of four fractions: α, γ, β, and δ; α-zeins represent 71–85% of the prolamins in the grain, whereas γ-, β-, and δ-zeins represent 20, 5, and 5% of the prolamins in the grain, respectively. Therefore, α-zein is the most important fraction because it stores most of the nitrogen (Momany et al., 2006). A structural representation and the amino acid sequence of α -zein are shown in **Figure 1**. The molecular weights of the four fractions are as follows: α, 19 and 22 kDa; γ, 18 and 27 kDa; β, 16 kDa; and δ, 10 kDa (Anderson and Lamsal, 2011). Another α zein fraction with a molecular weight of 24 kDa contains a defective signal peptide that induces the incorrect formation of zein bodies in the cell. This variant is present in the floury-2 (*fl2*) maize mutants that have higher content of the essential amino acid lysine in the endosperm, and this mutation leads to the synthesis of the α-zein variant and the defective signal peptide (Coleman et al., 1995). The most abundant amino acids found in these fractions are glutamic acid, leucine, proline, and alanine (Kong and Xiong, 2006).

Albumins are richer in aspartate, asparagine, threonine, glycine, alanine, proline, and half-cystine, but have low histidine, arginine, glutamic acid, glutamine, and phenylalanine content (Landry and Moureaux, 1987). Glutelins are alkali-soluble proteins with molecular weights of 10, 15, 18, and 27 kDa; they are present in protein bodies along with zeins, and their amino acid composition and function is similar to that of zeins (Wall et al., 1988).

Bioactive Peptides Properties

In general, cereal-derived peptides have been shown to possess opiate, antithrombotic, anticancer, antihypertensive, immunomodulatory, mineral-binding, antimicrobial, and antioxidant properties (de Mejia et al., 2012). Various bioactive peptides have been found and reported in maize (Tnani et al., 2013; Li et al., 2014). Maize bioactive peptides are obtained by the hydrolysis of kernels and sub-products. Among the different techniques for peptide generation, enzymatic hydrolysis is the most widely used method for maize. The main enzymes used in the hydrolysis of maize protein are listed in **Table 1**. For bioactivity evaluations, peptides have been fractionated by ultra-filtration (Wang Y. et al., 2014; Jin et al., 2016), and separated using chromatographic techniques with multiple purification steps (Puchalska et al., 2013; Wang X.-J. et al., 2014; Jin et al., 2016; Wang et al., 2016) to obtain low molecular weight peptides that have shown promise in further characterization. Various bioactive properties of maize peptides have been reported, including anticancer activity and other activities that have beneficial effects on health, such as antioxidant, antihypertensive, hepatoprotective, and alcohol protective activities. The different studies reporting the bioactive properties of maize peptides are listed in **Table 1**.

HEALTH EFFECTS OF MAIZE PEPTIDES

Anticancer

In the past few years, an important advance in cereal peptides research has been the finding that novel cereal-derived proteins and peptides exert preventive effects in different stages of cancer, including initiation, promotion, and progression. Because of the urgent need for effective cancer prevention therapy, chemoprevention has emerged as a viable anti-cancer approach. Chemo-preventive agents are expected to be safe, inexpensive, and abundant. Peptides fulfill these criteria, and are considered to be safer than synthetic compounds as they are present in the regular human diet and have a wide range of availability and acceptability (Li et al., 2014). Several studies have shown the anti-cancer potential of dietary proteins, peptides, and amino acids in the regulation of apoptosis and angiogenesis, important steps in controlling tumor metastasis; these molecules are naturally occurring or generated by fermentation, enzymatic hydrolysis, or gastrointestinal digestion (de Mejia and Dia, 2010).

Bioactive peptides exert anti-tumor activity via several key mechanisms: (a) Apoptosis induction, which involves an energy-dependent cascade mediated via specific proteases or caspases;

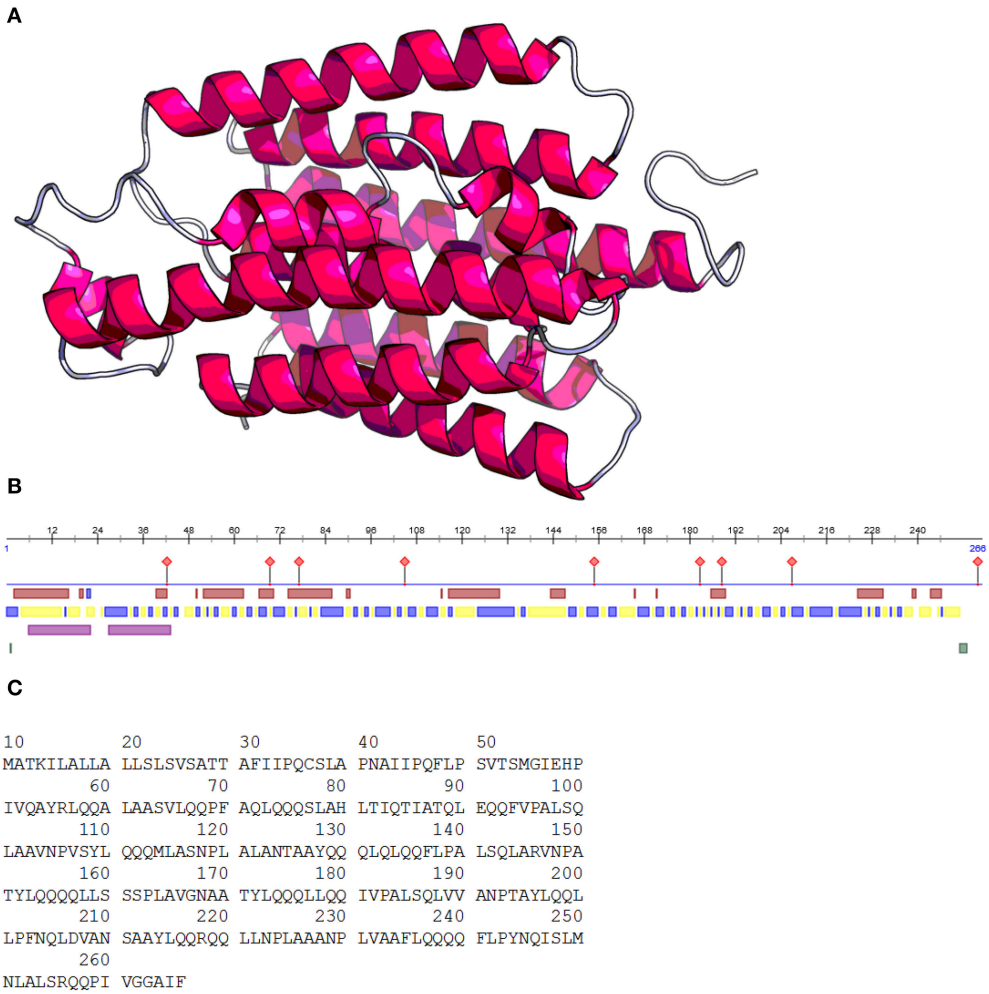

FIGURE 1 | Structure prediction of α-zein 22 kDa **(A)**. Zein regions **(B)**: protein binding regions (), helix (), exposed region (), buried region (), helical transmembrane region (), disordered region (). Zein amino acid sequence **(C)**. Images obtained using prediction web programs PredictProtein and RaptorX (Rost, 2003; Källberg et al., 2012).

strategies to overcome tumor resistance to apoptotic pathways include activation of pro-apoptotic receptors, restoration of p53 activity, caspase modulation, and proteasome inhibition (Burz et al., 2009). (b) Blockade of intermediate tumor generation by regulating cellular mechanisms associated with cell proliferation and survival, or biosynthetic pathways that control cell growth (Kornienko et al., 2013). (c) Regulation of immune system function by increasing the expression of tumor-associated antigens (antigenicity) in cancer cells, by triggering tumor cells to release danger signals that stimulate immune responses (immunogenicity), or by increasing the predisposition of tumor cells to be recognized and killed by the immune system (susceptibility) (Zitvogel et al., 2013).

The anticancer activities of different peptides have been characterized, with most of them showing pro-apoptotic activity (Dia and Mejia, 2010; Gonzalez de Mejia et al., 2010; Dia and Gonzalez, 2011; McConnell et al., 2015). Some mechanisms proposed for the anticancer activity of peptides are autophagy and apoptosis (de Mejia and Dia, 2010; Hernandez-Ledesma et al., 2013), and maize peptides may promote these processes in different cancer cells.

A primary anticancer effect of maize peptides has been demonstrated using *in vitro* models. HepG2 cells showed an increase in apoptotic activity when they were exposed to maize peptides obtained by enzymatic hydrolysis of protein extracted from corn gluten meal (Li et al., 2013). In that study, maize peptides were also evaluated in H22-tumor bearing mice. Treatment of animals with these peptides at a dose of 400 mg/kg resulted in an inhibition of tumor growth. Moreover, the administration of maize peptides enhanced immune system activity compared with that in the control group.

More recently, a study by Ortiz-Martinez et al. (2017) revealed important anti-cancer effects of maize peptides in HepG2 cells.

TABLE 1 | Main bioactivities of maize peptides, and methods used for their enzymatic hydrolysis fractionation.

Type of bioactivity	Bioactivity measured	Enzyme used	Use of ultrafiltration/ chromatografy	References
Anti-Oxidant	Inhibition of pyrogallol oxidation	Alcalase	N/Y	Zheng et al., 2006
	Inhibition of lipid peroxidation, reducing power, scavenging activity	Alcalase	N/Y	Li et al., 2008
	Radical scavenging, removal of superoxide anion, inhibition of lipid peroxidation, reducing power	Alcalase	N/Y	Li et al., 2010
	Radical scavenging	Alcalase	Y/Y	Tang et al., 2010
	Scavenging activity	None	N/N	Zhang et al., 2011
	Oxygen radical absorbance, scavenging activity, chelating activity, inhibition of lipid peroxidation	Neutral protease, alkaline protease, protease validase	Y/N	Zhou et al., 2012
	Scavenging activity of different radicals, chelating activity, inhibition of lipid peroxidation	Alkaline protease, flavourzyme	Y/Y	Zhuang et al., 2013
	Scavenging activity, chelating activity, reducing power,	Alkaline protease, trypsin, papain, flavourzyme	Y/Y	Tang and Zhuang, 2014
	Scavenging activity, chelating activity, reducing power	Alcalase	Y/Y	Wang X.-J. et al., 2014
	Scavenging activity, reducing power	Alkaline protease	Y/Y	Zhou et al., 2015
	Scavenging activity, oxygen radical absorbance, antioxidant effect in HepG2 and Caco2 cells	Alcalase	Y/N	Wang et al., 2015
	Scavenging activity, chelating activity, reducing power	Alcalase, flavourzyme	Y/Y	Jin et al., 2016
	Scavenging activity, inhibition of aggregation and oligomerization of Aβ peptides in *Caenorhabditis elegans*	Alcalase	N/Y	Zhang et al., 2016
Anti-Hypertensive	Inhibitory activity of ACE, decrease of blood pressure in an animal model	Trypsin	Y/Y	Yang et al., 2007
	Inhibitory activity of ACE	Trypsin, thermolysin, flavourzyme, fungal protease	Y/N	Parris et al., 2008
	Inhibitory activity of ACE, decrease of blood pressure in an animal model	Alcalase	Y/N	Huang et al., 2011
	Inhibitory activity of ACE, decrease of blood pressure in an animal model	Alcalase	Y/Y	Lin et al., 2011
	Inhibitory activity of ACE	Neutral protease	N/N	Zhou et al., 2013
	Inhibitory activity of ACE	Thermolysin	Y/Y	Puchalska et al., 2013
Hepato-Protective	Changes in hepatic enzyme levels, histopathological changes in hepatic tissue	Alcalase, neutral protease	N/N	Guo et al., 2009
	Changes in hepatic enzyme levels, histopathological changes in hepatic tissue	Alcalase	Y/N	Yu et al., 2012
	Decrease in alcohol blood concentration in an animal model	Alcalase	Y/Y	Ma et al., 2012
	Decrease in alcohol blood concentration in an animal model, changes in hepatic enzyme levels	Alcalase	Y/N	Yu et al., 2013
	Changes in hepatic enzyme levels, histopathological changes in hepatic tissue,	Alcalase	Y/Y	Lv et al., 2013
	Changes in hepatic enzyme levels in a clinical trial	Alcalase	Y/N	Wu et al., 2014
	Changes in hepatic enzyme levels, inhibition of apoptosis	Alcalase	Y/N	Ma et al., 2015
Anti-Cancer	Apoptosis induction in a HepG2 cell line, reduction of hepatic tumor growth in an animal model, stimulation of the immune system	Alcalase	Y/N	Li et al., 2013
	Antiprofilerative, modulation of cell cycle and apoptosis induction in a HepG2 cell line.	Alcalase	Y/Y	Ortiz-Martinez et al., 2017
Antimicrobial	Growth inhibition *in vitro* in bacetria and fungi (Inhibition of spore germination, hyphal elongation)	Trypsin, endoproteinase Glu-C, thermolysin	Y	Duvick et al., 1992

Peptide fractions isolated from albumin Alcalase hydrolysates from normal maize were stronger than the ones from quality protein maize. The treatment of HepG2 cells with this peptide fraction from the different maize varieties increased the apoptosis induction rates an average of 4-fold. These results suggested that the antiproliferative effect of peptide fractions isolated from both

varieties was based on the induction of apoptosis due to the decrease in antiapoptotic factor expression.

Antioxidant

Antioxidant capacity is the main reported biological activity of maize peptides. This activity is explained by the presence of specific amino acids with radical scavenging and reducing capacities, such as lysine, tyrosine, phenylalanine, proline, alanine, histidine, and leucine (Zhou et al., 2015). Several peptides with antioxidant activity have been identified; however, their antioxidant potential depends on the enzymatic process used to obtain them. Zheng et al. (2006) studied the antioxidant capacity of peptides obtained using an optimized enzymatic hydrolysis process. Peptides with low molecular weight (<5 kDa) exhibited higher antioxidant activity (Li et al., 2008; Zhou et al., 2012; Zhuang et al., 2013; Tang and Zhuang, 2014). The properties of these small peptides included hydroxyl radical and free radical scavenging activity, inhibition of lipid peroxidation, and ion chelating capacity. The antioxidant potential is not altered in peptides obtained from total protein or zein fraction (Li et al., 2010; Tang et al., 2010). Another factor affecting antioxidant activity is the pH: native α-zein has more antioxidant activity than α-zein extracted in less basic or acidic conditions (Zhang et al., 2011). Synthesized peptides also show similar antioxidant activities (Wang X.-J. et al., 2014; Jin et al., 2016).

Additionally, peptides smaller than 3 kDa have shown potent antioxidant activity in *in vitro* assays in HepG2 and Caco2 cells exposed to high oxidative stress (Wang et al., 2015). Maize peptides also enhance the activity of cellular enzymes, and therefore, may exert preventive effects against cell damage via their antioxidant activity (Wang et al., 2016). Furthermore, in studies conducted in macrophage models, maize peptides have shown anti-inflammatory effects (Hernández-Ledesma et al., 2009; Cam and Gonzalez, 2012), which could have been exerted via their antioxidant properties. Oxidative stress has been shown to induce lipid peroxidation, protein oxidation, and DNA damage, subsequently causing mutant cell proliferation and finally, carcinogenesis (Thanan et al., 2014). These properties are relevant because of the relationship between oxidative stress and degenerative processes like carcinogenesis. Therefore, the antioxidant activity of maize peptides could be beneficial for cancer treatment.

Antihypertensive

Maize peptides have shown antihypertensive effects. A single peptide with an Ala–Tyr sequence, obtained from corn gluten meal, which is a main by-product of maize wet milling, showed significant inhibitory activity against angiotensin I-converting enzyme (Yang et al., 2007; Lin et al., 2011). Moreover, the same peptide has shown antihypertensive activity in spontaneously hypertensive rats with a minimum effective oral dose of 50 mg/kg resulting in a diastolic blood pressure reduction of 9.5 mm Hg (Yang et al., 2007), and with an oral dose of 450 mg/kg resulting in a reduction of 40 mmHg (Lin et al., 2011). In another report, peptides smaller than 3 kDa showed greater blood pressure reductions (up to 34.45 mmHg) at a dose of 100 mg/kg in a spontaneously hypertensive rat model (Huang et al., 2011). These results show that molecular weight influences antihypertensive activity, with peptides smaller than 1 KDa being the most active. In addition, enzymes used for protein hydrolysis and peptide generation also influence the antioxidant activity of the resulting peptides, with peptides obtained using trypsin and thermolysin being the most potent (Parris et al., 2008). In addition, ultrasonic treatment applied to corn gluten meal proteins before hydrolysis appears to increase the antihypertensive activity of the resulting peptides (Zhou et al., 2013). Antihypertensive peptides have been isolated and identified as LRP (leucine–arginine–proline), LSP (leucine–serine–proline), and LQP (leucine–glutamine–proline) by using high-performance liquid chromatography (Puchalska et al., 2013). Hence, molecular interactions of peptides can affect enzymes responsible for vascular hemodynamics, suggesting further potential applications for these enzymes.

Hepatoprotective

Recent studies have shown that maize peptides exert a protective effect by reducing damage to hepatic tissue. Using rats with liver damage induced by exposure to lipopolysaccharides from bacillus Calmette-Guérin, Guo et al. (2009) showed that administration of 600 mg/kg peptide significantly lowered the level of cell necrosis, hepatic lesions, and enzymes indicative of liver damage compared to that in control rats. In this study, the peptides protective effect on liver cells could be attributable to their antioxidant capacity. Maize peptides smaller than 5 kDa exerted a hepatoprotective effect in a mouse model of carbon tetrachloride-induced hepatic damage. At a dose of 200 mg/kg, these peptides altered hepatic enzyme levels, lowering aspartate transaminase and alanine transaminase, and elevating superoxide dismutase and glutathione (Yu et al., 2012). Maize peptides may also exert a hepatoprotective effect by facilitating alcohol metabolism. Peptides as small as *five* amino acids have been shown to display this activity in mice (Ma et al., 2012). This effect is explained by the presence of leucine, which maintains the tricarboxylic acid cycle by supplying NAD+ (antioxidant effect). The same small peptide shown anti-apoptotic activity in mouse liver cells treated with alcohol to induce damage (Ma et al., 2015). A similar study found a hepatoprotective effect in mice at a dose of 200 mg/kg with peptides smaller than 5 kDa, which resulted in lower blood alcohol concentrations (Yu et al., 2013). Maize peptides have also shown protective activity against hepatic fibrosis. In rats exposed to thioacetamide, which induces hepatic fibrosis, the minimum protective dose was 100 mg/kg (Lv et al., 2013). These studies demonstrate the hepatoprotective effect and mechanisms of action of maize peptides in response to different types of stress, suggesting another possible field of therapeutic application.

Other Properties

In addition to the effects discussed so far, a few more bioactive properties of maize peptides have been identified. Maize basic peptide 1 (BMP-1), which was obtained through protein hydrolysis, identified, and later synthetized, was shown to exert antimicrobial activity, inhibiting the growth of fungi and bacteria (Duvick et al. 1992). In an *in vivo* study in *Caenorhabditis elegans*, Zhang et al. (2016) demonstrated that a bioactive maize tetrapeptide possessed scavenging activity against intracellular

reactive oxygen species. Additionally, this tetrapeptide inhibited the aggregation and oligomerization of β-amyloid peptide, which are involved in the development of Alzheimer's disease. These studies indicate the different bioactivities of maize peptides, which warrant further study and may be beneficial in the treatment of different diseases.

THERAPEUTIC PERSPECTIVES AND CONCLUSIONS

Peptides have attracted attention as drug candidates because they offer certain key advantages over alternative molecules. In contrast to traditional drugs, peptides have high affinity, strong specificity, low toxicity, and adequate tissue penetration. The therapeutic use of peptides has remained limited because of their high instability in biological environments, rapid depuration from the blood, poor membrane transportability, and effective digestion in the gastrointestinal tract (Sarmadi and Ismail, 2010). Peptide-based therapy depends on the ability of the peptide to remain intact until it reaches the target organ. Bioactive peptides must remain active and intact during gastrointestinal digestion and absorption to reach the cardiovascular system and potentially exert their physiological effects (Bhutia and Maiti, 2008).

Evidence supports the use of maize peptides as therapeutic molecules against a broad array of diseases linked to oxidative damage, such as cancer. *In vitro* models have been useful to investigate the antihypertensive and anticancer effects of maize peptides. However, in addition to *in vitro* evidence, *in vivo* experiments and clinical trials are needed to demonstrate the physiological effects of peptides. Few clinical trials have been conducted involving peptides and cancer (Gustafsson et al., 2004). Some synthetized peptides derived from seaproducts are being tested in phase II trials, these include: BioPep, Plitidepsin, Elisidepsin, and Tasidotin (Bouglé and Bouhallab, 2017). Even so this studies indicate that there is an enormous potential in the bioactivity of peptides derived from foods for the prevention and/or treatment of cancer. Also, there is an important opportunity for maize peptides with anticancer activity in diverse cancer cell lines as well as in different animal models that represent different carcinomas. In a future, the clinical efficacy will likely require intervention at several levels, and is necessary to test to evaluate their safety in short and long-term *in vivo* models and clinical trials on a large and heterogeneous populations.

AUTHOR CONTRIBUTIONS

All authors contributed equally to this work in terms of writing and conception. All authors wrote and reviewed the latest version of this manuscript.

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Buwchitin: A Ruminal Peptide with Antimicrobial Potential against *Enterococcus faecalis*

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Antimicrobial peptides (AMPs) are gaining popularity as alternatives for treatment of bacterial infections and recent advances in *omics* technologies provide new platforms for AMP discovery. We sought to determine the antibacterial activity of a novel antimicrobial peptide, buwchitin, against *Enterococcus faecalis.* Buwchitin was identified from a rumen bacterial metagenome library, cloned, expressed and purified. The antimicrobial activity of the recombinant peptide was assessed using a broth microdilution susceptibility assay to determine the peptide's killing kinetics against selected bacterial strains. The killing mechanism of buwchitin was investigated further by monitoring its ability to cause membrane depolarization ($diSC_3(5)$ method) and morphological changes in *E. faecalis* cells. Transmission electron micrographs of buwchitin treated *E. faecalis* cells showed intact outer membranes with blebbing, but no major damaging effects and cell morphology changes. Buwchitin had negligible cytotoxicity against defibrinated sheep erythrocytes. Although no significant membrane leakage and depolarization was observed, buwchitin at minimum inhibitory concentration (MIC) was bacteriostatic against *E. faecalis* cells and inhibited growth *in vitro* by 70% when compared to untreated cells. These findings suggest that buwchitin, a rumen derived peptide, has potential for antimicrobial activity against *E. faecalis*.

Keywords: microbiome, metagenomics, rumen bacteria, antibiotic resistance, antimicrobial peptides, antimicrobial activity, *Enterococcus faecalis*

INTRODUCTION

Enterococcus faecalis is a non-motile, Gram-positive, facultative anaerobic lactic acid bacterium of about 0.6–2.0 μm in size, that grows as individual cells, in pairs or as short multicellular filaments (Leavis et al., 2006; Ch. Schroder et al., 2015). It tolerates a wide variety of growth conditions, including temperatures between 10 and 45°C, hypotonic, hypertonic, acidic, or alkaline environments (Ch. Schroder et al., 2015). *E. faecalis* is normally a gut commensal found in many animals and in the environment (Gilmore et al., 2013). It is also a frequent cause of

many serious human infections, including urinary tract infections, endocarditis, bacteremia, and wound infections alongside *Enterococcus faecium* (Kau et al., 2005; Gilmore et al., 2013; Cahill and Prendergast, 2016). *E. faecalis* causes a variety of healthcare associated infections of which urinary tract infections are the most common (Kau et al., 2005; Hidron et al., 2008; Arias and Murray, 2012; Gilmore et al., 2013). Infections with *E. faecalis* can be especially challenging to treat because of their frequent resistance to multiple antibiotics, including aminoglycosides, and vancomycin, which is considered as drug of last resort for many Gram-positive infections (Baddour et al., 2005; Hollenbeck and Rice, 2012; Young et al., 2016). Vancomycin-resistant enterococci (VRE) are significant opportunistic pathogens in the hospital environment and often possess a multidrug-resistant phenotype (Chavers et al., 2003; van Harten et al., 2017) and their potential to spread enterococcal vancomycin resistance to other species remains a concern (Chang et al., 2003). VRE are also listed as priority pathogens by the World Health Organization for research and development of new antibiotics (WHO, 2017). It is therefore important to develop new drugs for the treatment of enterococcal infections.

Continued development of new drugs by the pharmaceutical industry, aided by genomics, high-throughput screening, rational drug design, and novel therapies offer a very promising prospect of effective bactericidal monotherapy for Enterococci and long-term solutions to VRE (Eliopoulos and Gold, 2001). Antimicrobial peptides (AMPs) are an integral part of the innate host defense system of many organisms including vertebrates, invertebrates, plants and bacteria (Wiesner and Vilcinskas, 2010), with broad spectrum activity against several groups of organisms including multidrug resistant bacteria, fungi, viruses and parasites (Jenssen et al., 2006). Due to this, AMPs represent one of the most promising alternatives to antibiotics, and future strategies for defeating the threat of antimicrobial resistance in bacterial infections might depend on peptide-based antimicrobial molecules (Czaplewski et al., 2016; O'Neill, 2016).

The rumen is one of the most diverse ecosystems in nature, harboring a microbial community, composed of a complex mixture of bacteria, protozoa, fungi, and viruses (Church, 1993; Sirohi et al., 2012) commonly referred to as the rumen microbiome, and enzymes isolated from this ecosystem have the potential to possess very unique biochemical properties (Hess et al., 2011; Ross et al., 2012). Several ruminal bacteriocins have been identified to date, but all of these bacteriocins are derived from bacteria that can be grown in the laboratory (Russell and Mantovani, 2002; Azevedo et al., 2015). Culture independent methods can be used to assess the rumen microbiome and increase the repertoire of bacteriocins, and other novel antimicrobials. It is possible to access and explore the total genetic information of this underexplored, uncultured fraction of the microbiome associated with any defined ecosystem through the application of metagenomics (Handelsman et al., 1998; Ekkers et al., 2012), which is the analysis of the DNA from a microbiome. Direct cloning of genomic or metagenomic DNA also offers the opportunity to capture genes encoding the synthesis of novel antimicrobials (Schloss and Handelsman, 2003), whether from species with already known antimicrobial properties (bacteriocin production), or from completely new species.

Previously, we prospected a 8,448 clone fosmid-based rumen bacterial metagenomic library generated from cow rumen solid attached bacteria (SAB) for novel antimicrobials, combining both functional and sequence based metagenomics and *in silico* mining (Oyama, 2015; Prive et al., 2015). From this work, we identified numerous AMPs and mini proteins. Results of the activity screens of the identified short AMPs ($\leq$25 AA) were reported elsewhere (Oyama, 2015). One of the longer proteins, buwchitin (71 AA) was selected for further characterization due to its potential activity against *E. faecalis*. In this study, we report the potential antimicrobial activity of buwchitin against *E. faecalis*.

MATERIALS AND METHODS

Bacterial Strains and Vectors

Bacterial strains used for antimicrobial activity testing were provided in-kind by Bath University. Strains include methicillin sensitive *Staphylococcus aureus* (MSSA) RN4220, *Escherichia coli* K12, *Salmonella enterica* serovar Typhimurium SL1344, *Listeria monocytogenes* NCTC 11994 (serovar 4b) and *Enterococcus faecalis* JH2-2. *E. coli* TOP10 (Invitrogen, Carlsbad CA, USA) was used for cloning (to host expression vectors for protein expression). The pTrcHis TOPO® vector (Invitrogen, Carlsbad, CA, USA) was used to clone polymerase chain reaction (PCR) products for protein expression.

Bacteriological Media and Culture Conditions

Mueller Hinton (MH) (Sigma-Aldrich UK) and Luria Bertani (LB) broth and agar (Fisher Scientific Leicestershire, UK) were used as growth media. When leakage assays were performed under buffered conditions, 5 mM HEPES (pH 7.2) supplemented with 5 mM glucose was used (Wu and Hancock, 1999). Media were prepared and sterilized according to the manufacturers' instructions. Bacterial strains were grown using standard conditions unless otherwise specified. Broth cultures were incubated at 37°C for 18–20 h with aeration and cultures on solid media were incubated at 37°C for 18–24 h.

Identification of Antimicrobial Genes from Fosmid Metagenomic Library by Agar Based Functional Screening and Sequencing Analysis

Antimicrobial genes were identified from the fosmid metagenomic library as previously described (Oyama, 2015). Briefly, sterile pin replicators (Molecular Devices Ltd., Berkshire UK) were used to transfer 2 μl metagenomic clones onto LB agar plates that had been plated before with 500 μl ($OD_{600\,nm} = 1$) of pathogens such as *S. aureus, E. coli, Sal. Typhimurium, E. faecalis* and *L. monocytogenes*. Plates were incubated at appropriate temperatures for 24 h and zones of clearing around the clones were used to identify clones with inserts encoding antimicrobials. Putative antimicrobial positive fosmid clones were sequenced

using Roche's 454 pyrosequencing platform. BLASTN (v2.2.28) on NCBI and BioEdit (version 7.1.11) (Hall, 1999) were used to edit and trim the vector sequence from the contigs. VecScreen on NCBI was used to search the sequences for vector contamination. Open reading frames (ORFs) were determined using the NCBI ORF finder program (Wheeler et al., 2003) and all ORFs with homology to antimicrobial genes and/or peptides were collated (**Table 1**). Based on expression levels and final protein yield, an ORF composed of 71 amino acids and named buwchitin was further investigated. Here, we report the activity of buwchitin.

Amplification of Antimicrobial Genes

Extracted fosmid DNA (1 μl) from a metagenomic clone containing the buwchitin insert was used as template for PCR amplification. The buwchitin sequence was deposited in the GenBank database with accession number KY823515 and predicted to contain a signal peptide, when analyzed on the SignalP 4.1 server (Petersen et al., 2011). Primers were designed to start and stop at the first predicted methionine and at the last stop codon, respectively in order to conserve the reading frame and take account of the entire gene of interest. The primers used for the amplification of buwchitin gene were 5′-ATGAGGCTGTCACACGTTTG-3′ (forward primer) and 5′-TCACCAATCTGTATGGCACCG-3′ (reverse primer). Primers were diluted to a stock concentration of 100 μM and a total volume of 50 μl PCR reaction was set up as follows: 2 μl DNA template, 1 μl each of the forward and reverse primers (2 μM final concentration), 39.5 μl molecular grade water and 1 μl Titanium® *Taq* DNA Polymerase (Clonetech- Takara Bio Europe/SAS, France). *Taq* polymerase was activated for 1 min at 95°C, followed by 30 cycles of 95°C for 30 s, 68°C for 1.5 min, followed by a final extension step at 68°C for 1.5 min. PCR products were verified by electrophoresis on a 1.5% agarose gel using a 1 kb DNA ladder. Gel image was taken after exposure to UV using the Gel Doc™ XR+ system (BIO-RAD Hertfordshire, UK). Subsequently, the band of interest was excised with a sterile scalpel under a Dark Reader blue transilluminator (Clare Chemical Research Inc. USA) and DNA was purified and eluted using the QIAquick Gel Extraction Kit (Qiagen, Crawley, UK) according to manufacturer guidelines.

Cloning of Buwchitin Gene and Confirmation of Cloning Reaction

Cloning of buwchitin was carried out using the pTrcHis TOPO® TA Expression kit as described by the manufacturer. Five positive colonies from the transformation were analyzed for correct size, sequence and orientation of the insert. Selected colonies were cultured overnight in LB medium containing 100 μg/ml ampicillin and 0.5% glucose, and analyzed by PCR. Briefly, aliquots (1 ml) were lysed by heating for 10 min at 95°C in sterile 1.5 ml microcentrifuge tubes. The cell debris was pelleted by centrifugation at 13,000 × *g* for 2 min. The supernatant was used as template for the subsequent PCR. The PCR was set up in a total volume of 50 μl as follows: 2 μl of template DNA, 1 μl of gene specific forward primer (5′-ATGAGGCTGTCACACGTTTG-3′) and vector specific reverse primer (5′-GATTTAATCTGTATCAGG-3′), 21 μl molecular grade water and 25 μl MyTaq™ Red Mix (Bioline, UK Ltd., London UK). Initial Taq activation was performed at 95°C for 1 min, followed by 35 cycles of 95°C for 15 s, at insert specific annealing temperature for 15 s with an extension step at 72°C for 10 s, and a final extension step at 72°C for 7 min and holding at 4°C. PCR products were verified by electrophoresis on a 1.5% agarose gel using a 500 bp DNA ladder. A positive PCR control was also prepared using the control PCR template (expected size of 750 bp) and primers provided with the pTrcHis-TOPO® expression kit. Positive transformants were further analyzed by Sanger sequencing using plasmid DNA from extracted positive transformants as templates. The Xpress™ Forward sequencing

TABLE 1 | ORFs with homology to antimicrobial (biosynthetic) protein coding genes in rumen metagenome fosmids. All ORFs are from contig 1 of each fosmid and are in the 5′-3′ direction.

Fosmid plate ID/ORF	Gene name	Protein size (AA)	Most similar homolog (*e*-value)	Putative function	Identity (overlapped AA)/% similarity
SABPL5 C17/11	Gene 6	184	*Prevotella ruminicola* 23 WP_013063463.1 (3e-104)	4′-phosphopantetheinyl transferase family protein Synthesis of unusual molecules including polyketides, atypical fatty acids, and antibiotics	140/184(76%)
			Butyrivibrio crossotus CAG:259 WP_021960962.1 (2e-33)	Putative biosurfactants production protein	58/161(36%)
SABPL12(1) C3/9	Gene 17A	350	*Prevotella* sp. CDD20257.1(0.0)	3-dehydroquinate synthase DHQS represents a potential target for the development of novel and selective antimicrobial agents	250/346(72%)
SABPL12(1) C3/50	Gene 17B	80	*Pseudomonas putida* S16 NP_744149.1 (1.4)	Colicin V production protein	19/61(31%)
SABPL27 L10/66	Buwchitin	71	*Streptomyces mobaraensis* WP_004942604.1 *e*-value 5.0	Penicillin amidase Penicillin biosynthesis and metabolism	16/43(37%)
SABPL27 L10/73	Gene 68	68	*Ornithinibacillus scapharcae* YP_004810705.1 *e*-value 8.4	beta-lactam antibiotic acylase Penicillin biosynthesis and metabolism	22/63(35%)

primer for pTrcHis-TOPO® (5′-TATGGCTAGCATGACTGGT-3′) was then used to sequence the insert and alignments to original sequence orientation was confirmed using BioEdit (Hall, 1999).

Expression and Purification of His-Tagged Buwchitin

A single recombinant *E. coli* colony from a clone confirmed as containing the buwchitin gene was inoculated into LB broth containing 100 μg/ml ampicillin and grown overnight at 37°C with aeration and agitation (225–250 rpm). The following day, 1 L of LB broth containing 100 μg/ml ampicillin was inoculated with 20 ml of the overnight culture and incubated at 37°C under aeration (225–250 rpm). Gene expression was induced at $OD_{600\,nm} = 0.6$ with 1 mM IPTG. Cells were harvested after 4 h by centrifugation (3,000 × *g* for 10 min at 4°C) and cell pellets were stored at −80°C for subsequent protein purification. Simultaneous purification and concentration of the buwchitin protein was carried out under native conditions using the Amicon® Pro Purification System (Merck Millipore Ltd Carrigtwohill, Ireland) following the manufacturer's protocol. Protein concentration was calculated as the ratio of absorbance at 280 nm [BioTek's Epoch™ Multi-Volume Spectrophotometer, (BioTek Instruments, Inc. Vermont, USA)] to the extinction coefficient absorbance (Abs 0.1% = 1 g/l calculated using the ExPASy ProtParam tool) (Gasteiger et al., 2005).

Determination of Minimum Inhibitory Concentration (MIC) of Buwchitin

Vancomycin, Polymyxin B sulfate and ciprofloxacin were purchased from Sigma-Aldrich (Poole, Dorset, United Kingdom). All stock solutions were dissolved in the appropriate solvent prior to dilution in sterile distilled water (Andrews, 2001). MICs of buwchitin was measured by broth microdilution method using two-fold serial dilutions of antimicrobial agents in MH broth (CLSI, 2012). Buwchitin or comparator agents, vancomycin hydrochloride, polymyxin B sulfate and ciprofloxacin were added to the wells of a 96-well plate containing bacteria from overnight culture (adjusted to 1×10^8 CFU/ml) to achieve a final inoculum concentration of 5×10^5 CFU/ml (Cherkasov et al., 2008; Wiegand et al., 2008). MIC was defined as the lowest concentration of test agent that inhibited visible growth of the organism after 18–24 h of incubation at 37°C.

Bactericidal/Bacteriostatic Activity of Buwchitin

The bactericidal or bacteriostatic activity of buwchitin against *E. faecalis* was measured at MIC concentration using optical density measurements. An increase in both cell mass and cell number can readily be estimated by measuring the turbidity of a cell suspension using a spectrophotometer, thereby offering a rapid and sensitive alternative to cell counting (Dalgaard and Koutsoumanis, 2001; Madrid and Felice, 2005). This method has been shown to produce comparable results to plate counting, flow cytometric and green fluorescence viability analyses methods (Lehtinen et al., 2006). In a 96 well plate, buwchitin was added to cells in mid-logarithmic phase (1×10^6 CFU/ml, $OD_{600\,nm}$ of ≤0.2) in MH broth and serially diluted as previously described. Plates were incubated at 37°C in a microplate incubator shaker. Wells without antimicrobial agents were used as growth control while wells with MH broth alone served as negative control. The rate of kill was calculated as a percentage ($OD_{600\,nm}$) of surviving cells over a 24 h period (Lehtinen et al., 2006; Hazan et al., 2012). The percentage of viable cells was normalized to 100% for the growth control (cells without antibiotic treatment).

Erythrocyte Leakage Assay

The ability of buwchitin to lyse red blood cells was assessed in a 96 well plate using defibrinated sheep blood (Oxoid Ltd Hampshire, UK). Sheep red blood cells (RBC) washed and diluted (4%) in phosphate buffered saline (35 mM PBS) (pH 7.3) were treated with buwchitin at different concentrations and incubated at 37°C for 1 h. Triton X-100 (0.1% causes 100% cell lysis) served as a positive control. Absorbance ($OD_{450\,nm}$) of the supernatant (70 μl) from each well of the plate was measured to detect hemoglobin leakage from the erythrocyte cytoplasm and obtained results were used to determine the percentage hemolysis given that the 0.1% Triton X-100 represented 100% lysis after normalizing auto-hemolysis (PBS only treatment).

Inner Membrane Depolarization Assay (diSC3(5) Method)

The ability of buwchitin to disrupt the electrochemical potential across the bacterial cytoplasmic membrane was measured by determining the amount of the membrane-associated probe, 3,3′-dipropylthiadicarbocyanine iodide [$diSC_3(5)$] released from the cytoplasm (Wu et al., 1999; Lee et al., 2004). Briefly, mid-logarithmic phase ($OD_{600\,nm} = 0.2$) *E. faecalis* cells were washed and resuspended to an $OD_{600\,nm}$ of 0.05 in 5 mM HEPES-glucose buffer, pH 7.2. In a 96-well plate, the cell suspension was incubated with 100 mM potassium chloride (KCl) and 0.4 mM 3,3′-dipropylthiadicarbocyanine iodide [$diSC_3(5)$] until a stable reduction of fluorescence (excitation λ 622 nm, emission λ 670 nm) was achieved (∼1 h). The KCl was added to equilibrate the cytoplasmic and external K^+. After 1 h, buwchitin, positive control agent (0.1% Triton X-100) or negative control agent (untreated cells) were added to the cells in the wells. The plate was further incubated at 37°C with shaking while fluorescence was continuously monitored (excitation λ 622 nm, emission λ 670 nm) upon addition of peptide at 2–5 min intervals for 2 h.

Transmission Electron Microscopy (TEM)

Exponential phase cultures of *E. faecalis* grown in MH broth were washed and resuspended to an $OD_{600\,nm}$ of 0.2 in 10 mM PBS. The cell suspensions (1 ml) were incubated at 37°C with buwchitin at 1 × MIC concentration in microcentrifuge tubes. To investigate possible changes in cell morphology following exposure to buwchitin, samples were removed at 1 and 24 h after exposure and prepared for TEM as previously described (Huws et al., 2013). Briefly, samples were fixed with 2.5% (v/v) glutaraldehyde, after which they were post-fixed with 1% (w/v) osmium tetroxide. Fixed samples were then stained with 2%

(w/v) uranyl acetate and Reynold's lead citrate and observed using a JEOL JEM1010 transmission electron microscope (JEOL Ltd, Tokyo, Japan) at 80 kV.

Molecular Modeling of Peptide 3D Structures

Structural modeling of buwchitin was completed using the PHYRE2 web portal (Kelley et al., 2015). Results were visualized using the PyMOL v1.7.6 program (Schrödinger, 2010). The biophysical properties of buwchitin were predicted on the antimicrobial peptide database (APD2) (Wang et al., 2009).

Statistical Analysis

Two-way analysis of variance (ANOVA) with factors "antimicrobial treatments" and "time" was performed to determine whether there were significant changes in cell viability and membrane depolarization before and after treatment (Harmon, 2011). This was followed by *post-hoc* multiple comparisons using Tukey's HSD (Honestly Significant Difference) test (Bender and Lange, 2001; Feise, 2002; Harmon, 2011). Alpha (α) levels were set at $P < 0.05$.

RESULTS

Sequencing, Cloning, Expression and Purification Using *In vivo* Expression Systems

The buwchitin gene was successfully PCR amplified using DNA from the fosmid clone, SAB PL27 L10/66. Bands of the correct size (expected size of 216 bp) were excised from the gel before proceeding to cloning. Electrophoresis results confirmed that the transformants carried the gene of the correct size, which was also confirmed by Sanger sequencing. The antimicrobial protein was expressed with an N-terminal 6xHis-Tag in *E. coli* to facilitate purification and investigation of its biochemical properties. Preliminary protein expression assay indicated that protein expression was optimal 4 h after induction (data not shown). SDS PAGE analysis of negative expression control (*E. coli* Top10 cells without plasmid) showed no protein expression bands while positive expression control (*E. coli* Top10 cells with pTrcHis-TOPO/lacZ) showed expression of the protein with a correct size of 40 kDa (data not shown). Cultivation of buwchitin transformants were scaled up to a total volume of 1 L to produce cell pellets for protein purification. Recombinant proteins were purified in their native conditions to preserve their activity (Karakus et al., 2016). **Figure 1** shows the SDS-PAGE analysis of the purification fractions for buwchitin. The purification protocol reproducibly yielded a total of ∼0.8 mg of purified protein per liter of culture.

Antimicrobial and Cytotoxic Activity of Buwchitin

Buwchitin was active against *E. faecalis* with an MIC of 100–200 μg/ml (**Table 2**). It also showed some inhibition of *E. coli* growth (observed in growth curves), but no detectable MIC at the highest concentration tested. This may account for the low level of expression of buwchitin in the *E. coli* expression host. The highest concentration of buwchitin tested was 400 μg/ml due to low levels of protein expression and/or yield of purified protein. The killing activity of buwchitin against *E. faecalis* was calculated as a percentage ($OD_{600\,nm}$) of surviving cells compared to the growth control. Only about 30 ± 1.4% surviving *E. faecalis* cells remained after a 24 h incubation period ($P < 0.05$). It would seem that buwchitin had a bacteriostatic effect against *E. faecalis* cells (**Figure 2**) as no change in *E. faecalis* cell density was observed over an incubation period of 24 h. Very little hemolytic effect (12.81 ± 0.02%) was observed when sheep red blood cells were treated with buwchitin at a concentration twice as high as the MIC determined for *E. faecalis* (**Table 3**).

Buwchitin did not induce membrane depolarization in *E. faecalis* in the first 2 h of treatment. To determine whether the loss of viability in *E. faecalis* following exposure to buwchitin was accompanied by or was a result of changes in cell morphology and cell wall ultrastructure, TEM was performed. Electron micrographs of untreated *E. faecalis* at 1 and 24 h reveal intact healthy cells. Electron micrographs of buwchitin treated *E. faecalis* cells at 1 h showed intact outer membranes with blebbing but no major damaging effects and cell morphology changes. In contrast, micrographs of buwchitin treated *E. faecalis* cells at 24 h revealed several changes in cell morphology including cell lysis and detachment of the cell interior from the cell envelope (**Figure 3**).

Structural Modeling of Buwchitin

Modeling and visualization of the 3D conformation of buwchitin using PHYRE2 (Kelley et al., 2015) and PyMOL v1.7.6 (Schrödinger, 2010), respectively, suggested that buwchitin is composed of a compact, all-helical, structure with major amphipathic helix connecting two smaller helices (**Figure 4**). The amphipathic helix agrees with a common structural feature of AMPs as the dual hydrophilic/hydrophobic nature allows the interaction and embedding of cellular membranes (Hancock and Sahl, 2006). As predicted by the APD2 database, buwchitin (71AA) is positively charged (+9), has a total hydrophobicity ratio of 29% and total Arginine and Lysine ratio of 19%.

DISCUSSION

Many currently used antibiotics were discovered by screening soil microorganisms that can be grown in the laboratory using standard microbial techniques for their antimicrobial activity (Ling et al., 2015). However, as natural product resources are practically inexhaustible, and approximately 99% of all species in external environments require more complex growth conditions than those provided using standard cultivation techniques, the majority of the world's microbial biodiversity remains to be explored (Harvey, 2007; Berdy, 2012; Lewis, 2013). Several recent studies already suggest that new organisms such as uncultured bacteria are likely to harbor new antimicrobials (Degen et al., 2014; Doroghazi et al., 2014; Gavrish et al., 2014; Wilson et al., 2014) and underexplored complex microbial communities, including the rumen, very likely represent rich sources of novel antimicrobials. These microbiomes have the potential to revive

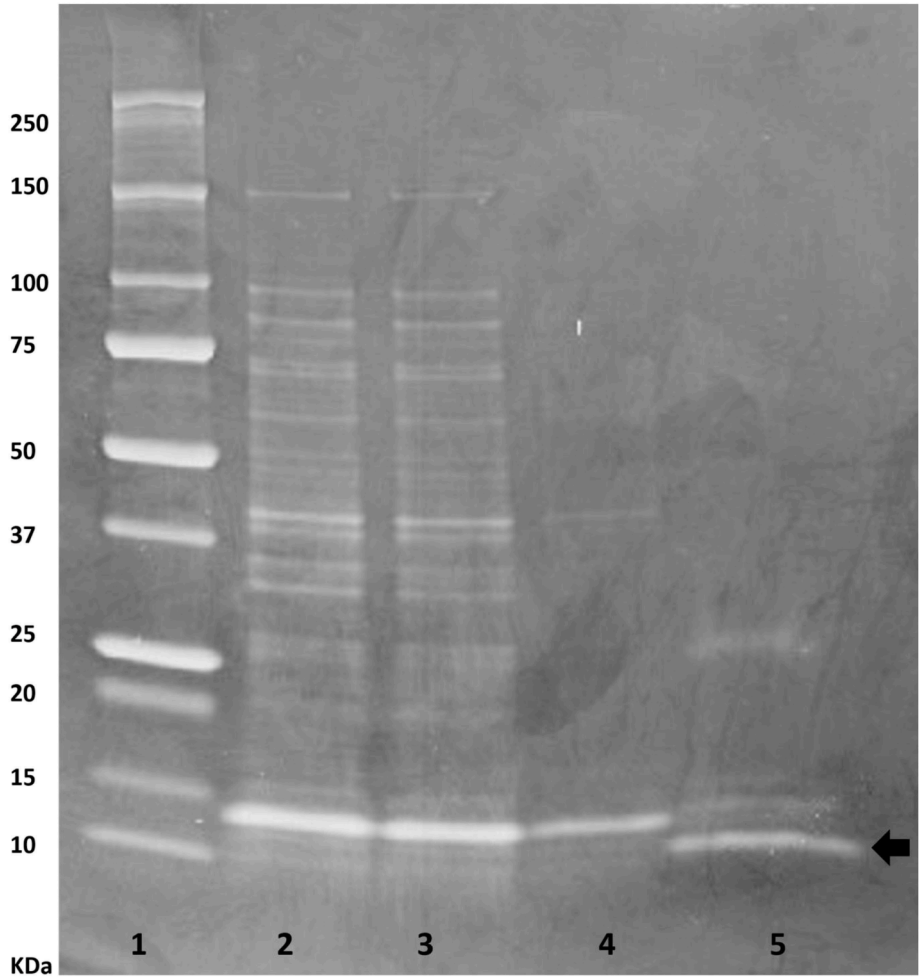

FIGURE 1 | SDS-PAGE analysis of purification steps of buwchitin protein expressed in *E. coli* TOP10 cells on a 20% denaturing polyacrylamide gel (4 h after induction with 1 mM IPTG). Lane 1, protein molecular weight marker; Lane 2, cell lysate; Lane 3, supernatant; Lane 4, Wash step; Lane 5, eluted buwchitin protein. The arrow indicates band of purified protein of interest. Expected size is 8.35 (±3–4 kDa from His-tag).

TABLE 2 | Minimum inhibitory concentration (MIC) of buwchitin and comparator antimicrobial agents ($n = 6$), > (precedes the highest concentration tested).

Peptide ID	MICs (μg/ml)			
	Sal. typhimurium	*E. coli*	*S. aureus*	*E. faecalis*
Polymyxin B sulfate	1.95	1.95	250	31.25
Ciprofloxacin	0.12	0.06	>250	62.5
Vancomycin hydrochloride	250	125	0.98	62.5
Buwchitin	>400	>400	>400	100–200

Highest concentration of buwchitin tested is 400 μg/ml due to low protein yield.

the platform of natural product discovery in a new culture-independent perspective, unbiased by the culturing aptitude of microbial species (Lewis, 2012; McCann et al., 2014; Kang et al., 2015). The potential for application of metagenomics to biotechnology seems endless as functional screens can be used to identify new enzymes, antibiotics and other biological agents in libraries from diverse environments (Gillespie et al., 2002; Lorenz and Schleper, 2002; Piel, 2002; Voget et al., 2003; Berdy, 2012).

In this study, we used a combination of functional and sequence based metagenomic screening strategies to prospect the rumen microbiome for novel antimicrobials as both strategies present advantages and limitations (Uchiyama and Miyazaki, 2009). Whereas, sequence homology based analysis allows for the identification of new enzymes from a range of environments, it requires a certain sequence similarity to members from known gene families, therefore limiting novelty. Functional screening of metagenomic libraries on the other hand, does not depend on previous sequence knowledge and therefore has the potential to discover novel classes of genes coding for desired functions without depending on their sequence similarity to already known genes (Ferrer et al., 2009; Simon and Daniel, 2009). We identified a novel antimicrobial gene, buwchitin, from the rumen microbiome and sought to express and characterize its

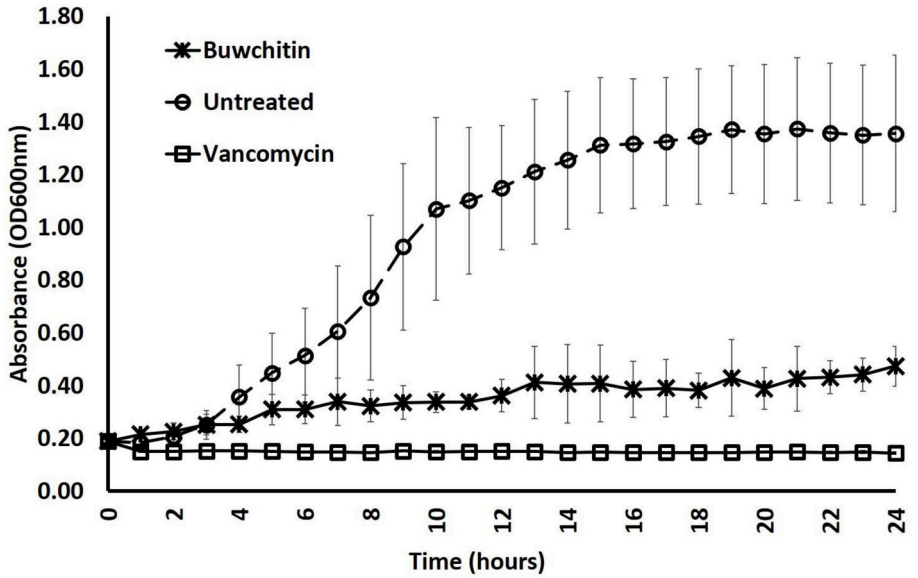

FIGURE 2 | Growth rate of *E. faecalis* in presence of antibacterial agents. Growth rate was determined by monitoring cell density at $OD_{600\,nm}$ in three independent measurements at 1 × MIC concentration. Error bars represent the standard deviation.

TABLE 3 | Hemolytic activity of buwchitin against sheep erythrocytes. Sheep erythrocytes resuspended and diluted (4%) in PBS were treated with buwchitin (at different concentrations) or 0.1% (v/v) Triton X-100 and hemolysis was monitored at $OD_{450\,nm}$ at 1 h after incubation at 37°C, (values from three independent replicates and showing the standard deviation).

Concentration (μg/ml)	% hemolysis
400	12.81 ± 0.02
200	9.69 ± 0.09
100	5.23 ± 0.08
50	4.12 ± 0.06
25	4.15 ± 0.06
12.5	3.08 ± 0.03
6.25	2.80 ± 0.02
3.125	3.11 ± 0.06

antimicrobial activity against *E. faecalis*. Firstly, a fosmid-based cow rumen metagenomic clone library created from the solid attached bacteria of rumen content was functionally screened for antimicrobial activity. Clones with antimicrobial activity were subsequently sequenced to identify genes potentially involved in the antimicrobial activity observed in functional screens. Buwchitin, which was identified as a potential antimicrobial gene, was then expressed and further tested for antimicrobial activity. Buwchitin is a cationic (charge of +9), α-helical peptide (as predicted by 3D modeling), 71 amino acids in length and has a molecular weight of 8.35 kDa. Expression of buwchitin yielded on average 0.8 mg of purified protein per liter of culture. This relatively low yield may be due to the inhibitory effects of buwchitin on *E. coli* growth. However, this yield falls in the range reported in literature where concentrations of 0.5–2.5 mg/ml (Guerreiro et al., 2008), and 0.8 mg/ml (Zorko et al., 2009; Pei et al., 2014) were retrieved from 1 L cultures by different approaches using Ni-NTA columns. It may be useful to explore alternative expression systems, such as *Pichia* sp. or *Aspergillus* sp. to improve the yield of the protein.

Buwchitin was active against *E. faecalis* JH2-2 with an MIC of 100–200 μg/ml. This MIC is high when compared to antimicrobial proteins isolated and expressed using similar methods in other studies (Zorko et al., 2009; Elhag et al., 2017). Buwchitin (at MIC concentration) inhibited growth of *E. faecalis* cells with no change in *E. faecalis* cell density over a 24 h incubation period and has a minimum bactericidal concentration (MBC) of 200–400 μg/ml, suggestive of a bacteriostatic killing activity. Although most antimicrobial peptides are bactericidal (Hancock, 2001; Reddy et al., 2004; Lohner, 2017), many examples of bacteriostatic antimicrobial peptides exist in literature (Mine et al., 2004; Choi et al., 2016). For example, the human β-defensin 2 (hBD-2) is bacteriostatic against *S. aureus* only at concentrations as high as 100 μg/ml (Harder et al., 1997; Jung et al., 2011). Another example of a bacteriostatic antimicrobial peptide is the human lactoferricin (LfcinH) (Gifford et al., 2005). Furthermore, most antibacterials are potentially both bactericidal and bacteriostatic depending on bacterial pathogen (Pankey and Sabath, 2004). Further investigations into the mechanism underlying the bacteriostatic action of buwchitin would be necessary to come to a final conclusion about its accurate classification. Buwchitin had minimal hemolytic activity against sheep erythrocytes, suggesting that buwchitin may have selective activity against microbial cells. Despite these encouraging results, it will be necessary to carry out cytotoxicity assays on human and other mammalian cell lines to determine whether buwchitin can induce apoptosis and

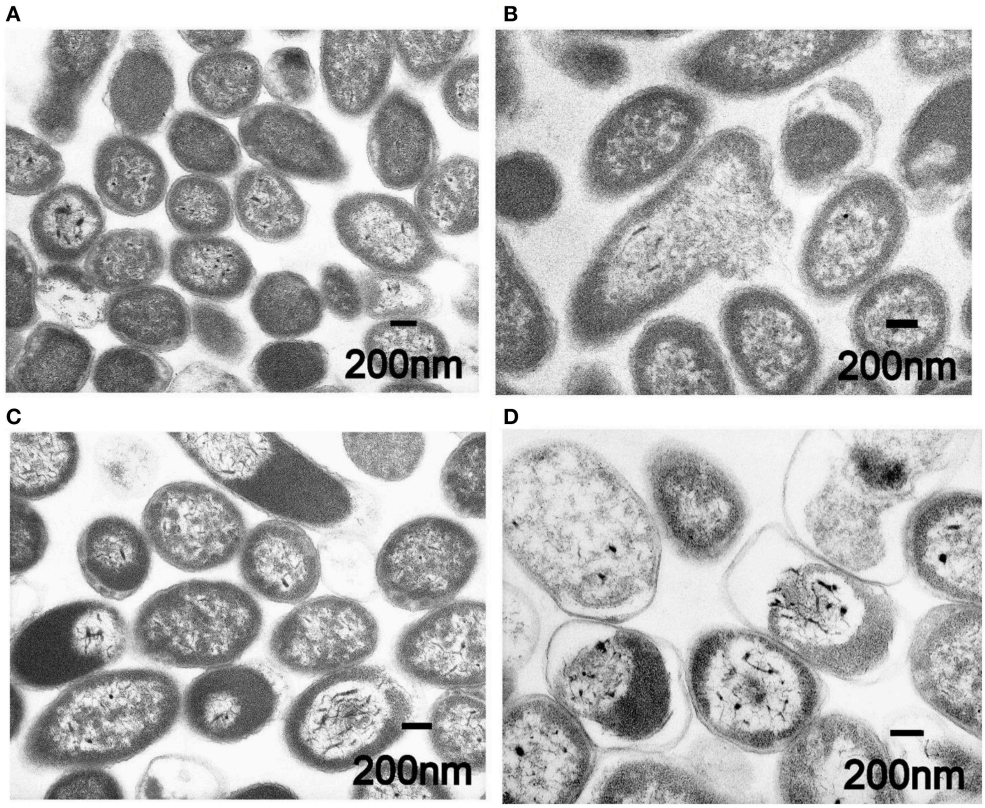

FIGURE 3 | Representative transmission electron micrographs of *E. faecalis*. **(A)** Untreated *E. faecalis* cells at 1 h. **(B)** Buwchitin treated cells (200 μg/ml) at 1 h. **(C)** Untreated *E. faecalis* at 24 h. **(D)** Buwchitin treated cells (200 μg/ml) at 24 h. Scale bars on micrographs.

necrosis in cells (Paredes-Gamero et al., 2012). Very little or no membrane depolarization was observed in *E. faecalis* cells treated with buwchitin and TEM images of buwchitin treated cells showed intact outer membrane and very little changes in cell morphology after 1 h of treatment. Only after 24 h of treatment were large vacuoles in the cytoplasm and separation of the cell envelop observed. Given the low depolarizing activity of buwchitin, it would seem that membrane-destabilizing activity alone does not explain the antimicrobial activity of buwchitin. It is known that poly-cationic AMPs bound to teichoic acids including lipoteichoic (LTA) and wall teichoic acids (WTA) build a poly-anionic ladder and may initiate bacterial killing by facilitating the entry of peptides into the cytoplasmic membrane without membrane depolarization (Schneewind and Missiakas, 2014; Malanovic and Lohner, 2016). Further investigation into buwchitin teichoic acid binding and other mode of action studies are required to gain insights into its mechanism of action and the events leading to cell death.

Buwchitin is positively charged and has an amphiphilic structure with 29% hydrophobic residues as has been observed for many antimicrobial peptides (Hancock and Sahl, 2006). This positive charge greatly facilitates the accumulation of AMPs at the polyanionic microbial cell surfaces and may be sufficient for antimicrobial action (Hancock and Sahl, 2006), thus perturbing the membrane integrity. Some cationic peptides have been shown to translocate or form multimeric transmembrane channels promoting the membrane depolarization, which seems to contribute to their activity (Shai, 1999; Bhattacharjya and Ramamoorthy, 2009) at higher concentrations. The amphipathic nature of the predicted peptide structure and the observations in the TEM images is in agreement with this type of interaction, indicating that although buwchitin is not membrane destructive, it may interact with components of the cell envelop such as the enterococcal polysaccharide antigen. The formation of vacuoles in the cytoplasm also appear to support this idea. Still, at the current stage, it remains difficult to say which of the known membrane interaction and disruption models (i.e., barrel stave, carpet models, or micellar aggregate model) explains the activity of this peptide without further experimental evidence.

Further studies remain to be performed to enhance the antimicrobial phenotype of buwchitin. One potential strategy to improve the antimicrobial activity of buwchitin is the pepscan technology, in which shorter active fragments and optimized amino acid substitutions and/or modifications are identified by a scanning approach. These active peptide fragments identified by pepscan can then be SPOT-synthesized on cellulose membranes and systematically screened for antimicrobial activity (Hilpert et al., 2007; Winkler et al., 2009). The use of pepscan

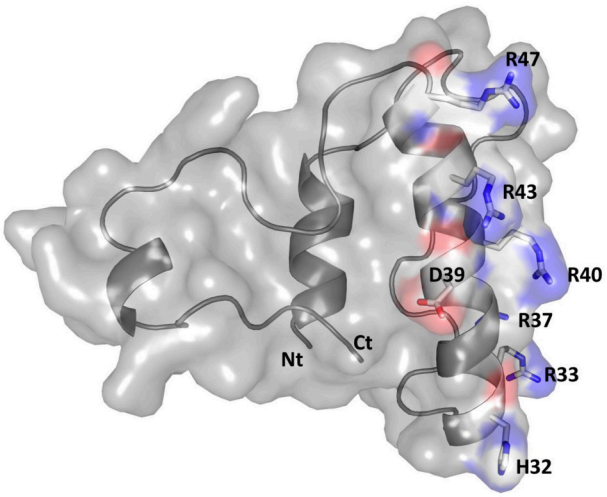

FIGURE 4 | Structural model of buwchitin (gray) in cartoon and surface representation. Side chains of selected amino-acid colored according to atom type (N: blue; C: white; O: red). The N- terminus (Nt) and C- terminus (Ct) is also shown. Figure prepared using PyMol (Schrödinger, 2010).

mapping and SPOT arrays has been shown to be useful for simultaneous optimization of peptides to generate new sequences that possess a variety of therapeutic and biological properties (Chico et al., 2010; Haney et al., 2015; Merino-Gracia et al., 2016; Ortega-Villaizan et al., 2016). Peptide improvements that might result from the pepscan technology might provide buwchitin derivatives with greater antimicrobial activity, similar to what has been achieved for other peptides in the literature (Knappe et al., 2016; Mikut et al., 2016). An evaluation of MICs against a panel of different bacterial species and *in vitro* stability studies in the presence of plasma or serum would also be beneficial. To explore the possible therapeutic relevance of buwchitin, further *in vitro* cytotoxicity studies and *in vivo* studies with acute toxicity in mice at concentrations above the MIC would be required.

In conclusion, the data we generated and present here suggest that we discovered a novel rumen protein, buwchitin, with potential antimicrobial properties. It is furthermore possible that with substantial modification, this AMP might qualify as a potential antimicrobial agent for the treatment of *E. faecalis* infections, which would favor further investigation of the protein. This study also highlights the enormous value of prospecting the rumen microbiome, and other microbial communities for novel compounds to expand our limited antimicrobial drug toolbox.

AUTHOR CONTRIBUTIONS

LO and SH conceived the project. LO and JC completed the laboratory work under supervision of SH, JE, and CC. SG and LO completed the sequencing and downstream analysis of the sequences, respectively. AC and NF helped LO with transmission electron microscopy and 3D structural modeling, respectively. FP, OG, and PG created the rumen fosmid metagenome library. MH, KH, CC, and HM have provided valuable ideas into the project from conception. LO wrote the paper with input from all co-authors.

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MINI REVIEW
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Structure-Activity Relationships of Insect Defensins

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Insects make up the largest and most diverse group of organisms on earth with several million species to exist in total. Considering the sheer number of insect species and the vast variety of ways they interact with their environment through chemistry, it is clear that they have significant potential as a source of new lead molecules. They have adapted to a range of ecological habitats and exhibit a symbiotic lifestyle with various microbes such as bacteria and fungi. Accordingly, numerous antimicrobial compounds have been identified including for example defensin peptides. Insect defensins were found to have broad-spectrum activity against various gram-positive/negative bacteria as well as fungi. They exhibit a unique structural topology involving the complex arrangement of three disulfide bonds as well as an alpha helix and beta sheets, which is known as cysteine-stabilized αβ motif. Their stability and amenability to peptide engineering make them promising candidates for the development of novel antibiotics lead molecules. This review highlights the current knowledge regarding the structure-activity relationships of insect defensin peptides and provides basis for future studies focusing on the rational design of novel cysteine-rich antimicrobial peptides.

Keywords: insect peptides, cysteine-stabilized, antimicrobial activity, drug design, antibiotic drug discovery

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INTRODUCTION

Peptides are known to play pivotal roles in many physiological functions and besides their action as signaling molecules they are crucial for the interaction with other organisms. This includes for example antimicrobial peptides (AMP) that represent an important part of the organism's defense machinery or peptide toxins as part of venom cocktails (Brogden et al., 2003; Favreau et al., 2006; Aili et al., 2014). AMPs are a diverse class of naturally occurring compounds that have been identified in a variety of organisms, from invertebrates to vertebrates including humans (Shafee et al., 2017). In particular insects are known for their immune system that has evolved a complex arrangement of constitutive and inducible AMPs that are used to defend against invading microorganisms (Kingsolver et al., 2013) and allow a symbiotic lifestyle with various microbes (Douglas, 2015). With regard to the quest for novel antimicrobial agents to target multidrug resistant pathogens such insect AMPs are promising starting points for antibiotic drug development approaches (Ageitos et al., 2016; Mahlapuu et al., 2016). The variety of these peptides, both in terms of structure as well as activity reflects the unique diversity of insect species. Insects are the largest and most diverse group of living organisms on earth (Hellmann and Sanders, 2007). Approximately, 950,000 species are described to date (Berenbaum and Eisner, 2008) and around 4,000,000 insect species are estimated to exist in total (May, 2000). Considering the sheer number of insect species and the vast variety of ways they interact with their environment through chemistry, it is clear that they have significant potential as a source of new lead molecules (Dossey, 2010).

In particular insect AMPs have recently attracted increased attention with regard to their possible medical as well as agrochemical applications (Yi et al., 2014; Tonk and Vilcinskas, 2017).

Bioactive peptides are promising novel drug leads that may fill the gap between small molecules and larger biologicals. This is reflected by a multitude of recent peptide discovery and development approaches (Craik et al., 2013). However, their use as therapeutic lead molecules is challenged by their typically poor stability and lack of oral bioavailability (Adessi and Soto, 2002; Otvos and Wade, 2014). This is often due to the linear nature of peptides that not only exhibit free ends but multiple cleavage sites that are readily recognized by enzymes that degrade peptide chains into inactive fragments or single amino acids. The presence of multiple disulfide bonds resulting in more compact structures typically increases stability and bioavailability. Several naturally-occurring disulfide-rich peptide families have been described such as plant cyclotides, knottins, conotoxins, or relaxin family peptide hormones. These cysteine-stabilized peptides are exhibiting a well-defined three-dimensional structure making them of particular interest for structure-activity relationship studies (Huang et al., 2010; Clark et al., 2011; Akondi et al., 2014; Patil et al., 2016). Due to their unique three-dimensional topologies, a wide range of activities and amenability to chemical synthesis they have been shown to represent valuable structural templates for peptide engineering (Carstens et al., 2011; Poth et al., 2013; Kintzing and Cochran, 2016). Within insects such a class of disulfide-rich peptides is known as "insect defensins" (Lambert et al., 1989). They exhibit a complex three-dimensional pattern, which is referred to as cysteine stabilized αβ motif (CSαβ). Importantly, as part of the immune system these peptides were found to have a broad spectrum of antimicrobial activities against bacteria, fungi and other parasites. This review will (i) highlight the current knowledge regarding structure-activity relationships of insect defensins and aims to (ii) provide a basis for future rational design of novel cysteine-rich AMPs.

EXPLORING THE DIVERSITY OF CYSTEINE-RICH INSECT ANTIMICROBIAL PEPTIDES

Given the sheer number of different insect species, it is not surprising that a variety of different AMPs can be found within these organisms. The ability of insects to adapt to almost every terrestrial ecosystem and their exposure to a variety of pathogens is reflected in the expression of different types and numbers of AMPs in individual species (Vilcinskas, 2013). However, the discovery of bioactive peptides from such small organisms by classical mass spectrometry based peptidomics approaches is facing serious challenges. This is due to highly complex samples that are limited in quantity and bioactive compounds present in trace amounts only that can easily be overlooked or only partially be identified (Wiese et al., 2006; Ueberheide et al., 2009). To fully unveil the diversity of peptides from such biological samples new advances in analytical chemistry, nucleotide sequencing and high-throughput drug screening are essential to aid in the discovery of novel AMPs. Indeed, refined methodologies that combine classical chemical analysis with bioinformatics workflows have proven useful to harness the variety of peptides and expand the knowledge of natural product peptidomes (Koehbach and Jackson, 2015). Though, to date the majority of identified defensin sequences was retrieved using either mass spectrometry based characterization of insect hemolymphs or cDNA cloning (see references in **Table 1**). Recent studies that describe the use of transcriptomes and genomes as valuable source of novel defensin sequences are significantly expanding the number of identified peptides per single study (Gruber and Muttenthaler, 2012; Poppel et al., 2015) and provide new insights into defensin diversity. Although nucleotide based peptide discovery provides additional information about the biosynthetic origin of peptides it lacks information regarding potential post-translational modification such as C-terminal amidation. Defensins are embedded in larger precursor molecules that consist of an ER signal peptide, a propeptide domain that precedes the mature peptide domain and ends with a conserved dibasic cleavage motif (Gruber and Muttenthaler, 2012). Mature peptides are typically around 40 residues long and carry an overall positive net charge with infrequent reports of anionic examples (**Figures 1B,C**; Wen et al., 2009; Xu et al., 2016).

STRUCTURE-ACTIVITY RELATIONSHIPS OF INSECT DEFENSINS

The variety of insect AMPs is also reflected within their secondary and tertiary structures. They can be broadly divided into three major groups, i.e., (i) primarily α-helical peptides, (ii) peptides rich in one particular amino acid (e.g., proline or glycine) and (iii) cysteine-rich peptides. Recent review articles have addressed current knowledge about groups (i) and (ii) (Huang et al., 2010; Li et al., 2014; Xhindoli et al., 2016). Based on additional secondary structure elements cysteine-rich insect AMPs can be subdivided into, (i) peptides exhibiting antiparallel triple-stranded β-sheets (e.g., Alo-3; Barbault et al., 2003), (ii) peptides that form a hairpin-like β-sheet structure (e.g., thanatin; Mandard et al., 1998) and (iii) defensins, i.e., peptides with a complex arrangement of α-helixes and β-sheets stabilized by disulfide-bonds (e.g., phormicin; Cornet et al., 1995), which are focus of this review. Insect defensins are defined to contain six conserved cysteines that form a typical arrangement of three disulfide bonds. However, peptides such as drosomycin contain eight cysteines, which is a conserved feature of plant defensins. Further, these peptides consist of a α-helix and antiparallel β-sheets (**Figures 1D–F**). Two disulfide bonds connect the C-terminal β-sheet and the α-helix and the third connects the N-terminal loop with the second β-sheet. Similar to peptides from plants or fungi they are hence classified as *cis*-defensins as opposed to *trans*-defensins found within vertebrate species (Shafee et al., 2017). The tight arrangement of secondary structural elements is reflected in high stability against heat or proteases. Accordingly, this structural topology is known as cysteine-stabilized αβ motif (CSαβ) and is common

TABLE 1 | Naturally occurring insect defensin peptides with reported antimicrobial activity.

Peptide[a] (Organism)	Length (AA)	Net charge	Activity[b]			References
			G+	G−	F	
Heliomicin[c] (*Heliothis virescens*)						Lamberty et al., 1999
DKLIGSCVWGAVNYTSDCNGECKRRGYKGGHCGSFANVNCWCET	44	+1	–	–	X	
ARD1[c] (*Archaeoprepona demophon*)						Landon et al., 2004
DKLIGSCVWGAVNYTSNCNAECKRRGYKGGHCGSFANVNCWCET	44	+2	n.d.	n.d.	X	
GmDefensin, GmDef-like (*Galleria mellonella*)						Lee et al., 2004; Cytrynska et al., 2007
DTLIGSCVWGATNYTSDCNAECKRRGYKGGHCGSFLNVNCWCE	43	0	–	–	X	
DKLIGSCVWGATNYTSDCNAECKRRGYKGGHCGSFWNVNCWCEE	44	0	X	–	X	
Gallerimycin (*Galleria mellonella*)						Schuhmann et al., 2003
GVTITVKPPFPGCVFYECIANCRSRGYKNGGYCTINGCQCLR	42	+4	–	–	X	
Drosomycin[c] (*Drosophila melanogaster*)						Fehlbaum et al., 1994
DCLSGRYKGPCAVWDNETCRRVCKEEGRSSGHCSPSLKCWCEGC	44	+1	–	–	X	
Termicin[c] (*Pseudocanthotermes spiniger*)						Lamberty et al., 2001b
ACNFQSCWATCQAQHSIYFRRAFCDRSQCKCVFVRG*[d]	36	+4	–	–	X	
Defensin (*Aeshna cyanea*)						Bulet et al., 1992
GFGCPLDQMQCHRHCQTITGRSGGYCSGPLKLTCTCYR	38	+3	X	–	n.d.	
Insect defensin A (phormicin)[c], B (*Phormia terranovae*)						Lambert et al., 1989
ATCDLLSGTGINHSACAAHCLLRGNRGGYCNGKGVCVCRN	40	+3	X	X	X	
Sapecin A[c], B, C (*Sarcophaga peregrina*)						Matsuyama and Natori, 1988; Yamada and Natori, 1993
ATCDLLSGTGINHSACAAHCLLRGNRGGYCNGKAVCVCRN	40	+3	X	–	–	
LTCEIDRSLCLLHCRLKGYLRAYCSQQKVCRCVQ	34	+4				
ATCDLLSGIGVQHSACALHCVFRGNRGGYCTGKGICVCRN	40	+3				
Tenecin 1 (*Tenebrio molitor*)						Moon et al., 1994
VTCDILSVEAKGVKLNDAACAAHCLFRGRSGGYCNGKRVCVCR	43	+4	X	–	n.d.	
Defensin (*Allomyrina dichotoma*)						Miyanoshita et al., 1996
VTCDLLSFEAKGFAANHSLCAAHCLAIGRRGGSCERGVCICRR	43	+3	X	–	n.d.	
AaeDef A (*Aedes aegypti*)						Lowenberger et al., 1995
ATCDLLSGFGVGDSACAAHCIARGNRGGYCNSKKVCVCRN	40	+3	X	X	n.d.	
AalDefD (*Aedes albopictus*)						Gao et al., 1999
ATCDLLSGFGVGDSACAAHCIARGNRGGYCNSKKVCVCPI	40	+2	–	X	n.d.	
AgaDef 1 (Def-AAA)[c], 2 (*Anopheles gambiae*)						Richman et al., 1996
ATCDLASGFGVGSSLCAAHCIARRYRGGYCNSKAVCVCRN	40	+4	X	–	–	
QLKNLACVTNEGPKWANTYCAAVCHMSGRGAGSCNAKDECVCSMT	45	+1	X	–	–	
Smd 1,2 (*Stomoxys calcitrans*)						Lehane et al., 1997
AAKPMGITCDLLSLWKVGHAACAAHCLVLGDVGGYCTKEGLCVCKE	46	0	n.d.	–	n.d.	
ATCDLLSMWNVNHSACAAHCLLLGKSGGRCNDDAVCVCRK	40	+1	n.d.	X	n.d.	
GmDef A[e] (*Glossina morsitans*)						Boulanger et al., 2002
VTCNIGEWVCVAHCNSKSKKSGYCSRGVCYCTN	33	+3	X	–	n.d.	
RprDef A, B, C (*Rhodnius prolixus*)						Lopez et al., 2003
ATCDLFSFRSKWVTPNHAACAAHCLLRGNRGGRCKGTICHCRK	43	+7				
ATCDLLSFRSKWVTPNHAGCAAHCLLRGNRGGHCKGTICHCRK	43	+6	X	–	–	
ATCDLFSFRSKWVTPNHAGCAAHCIFLGNRGGRCVGTVCHCRK	43	+5				
Lucifensin[c] [*Lucilia sericata, L. cuprina* (II)]						Cerovsky et al., 2010; El Shazely et al., 2013
ATCDLLSGTGVKHSACAAHCLLRGNRGGYCNGRAICVCRN	40	+4	X	n.d.	n.d.	
ATCDLLSGTGIKHSACAAHCLLRGNRGGYCNGRAICVCRN	40	+4	X	n.d.	n.d.	
LSerDef 3, 4, 6, 7 (*Lucilia sericata*)						Poppel et al., 2015
ATCDLLSGTGANHSACAAHCLLRGNRGGYCNSKAVCVCRN	40	+3	X	–	n.d.	

(Continued)

TABLE 1 | Continued

Peptide[a] (Organism)	Length (AA)	Net charge	Activity[b]			References
			G+	G−	F	
LTCNIDRSFCLAHCLLRGYKRGFCTVKKICVCRH	34	+6				
GTCSFSSALCVVHCRVRGYPDGYCSRKGICTCRR	34	+5				
FTCNSYACKAHCILQGHKSGSCARINLCKCQR	32	+5				
Defensin (*Drosophila melanogaster*)						Dimarcq et al., 1994
ATCDLLSKWNWNHTACAGHCIAKGFKGGYCNDKAVCVCRN	40	+3	X	–	n.d.	
Navidefensin2-2 (*Nasonia vitripennis*)						Gao and Zhu, 2010
FSCDVLSFQSKWVSPNHSACAVRCLAQRRKGGKCKNGDCVCR	42	+5	X	–	–	
Defensin NV (*Nasonia vitripennis*)						Ye et al., 2010
VTCELLMFGGVVGDSACAANCLSMGKAGGSCNGGLCDCRKTTFKELWDKRFG	52	+1	X	X	X	
Defensin (*Bombus pascuorum*)						Rees et al., 1997
VTCDLLSIKGVAEHSACAANCLSMGKAGGRCENGICLCRKTTFKELWDKRF*	51	+3	X	X	n.d.	
Royalisin (*Apis mellifera*)						Fujiwara et al., 1990
VTCDLLSFKGQVNDSACAANCLSLGKAGGHCEKGVCICRKTSFKDLWDKRF*	51	+3	X	–	n.d.	
SpliDefensin (*Spodoptera littoralis*)						Seufi et al., 2011
VSCDFEEANEDAVCQEHCLPKGYTYGICVSHTCSCIYIVELIKWYTNTYT	50	−5	X	X	–	
PxDef (*Plutella xylostella*)						Xu et al., 2016
RIPCQYEDATEDTICQQHCLPKGYSYGICVSYRCSCV	37	−1	X	X	X	
DLP4 (*Hermetia illucens*)						Park et al., 2015
ATCDLLSPFKVGHAACAAHCIARGKRGGWCDKRAVCNCRK	40	+6	X	–	n.d.	
Defensin 1 (*Tribolium castaneum*)						Rajamuthiah et al., 2015
VTCDLLSAEAKGVKVNHAACAAHCLLKRKRGGYCNKRRICVCRN	44	+8	X	n.d.	n.d.	
Defensin (*Simulium bannaense*)						Wei et al., 2015
ATCDLLSISTPWGSVNHAACAAHCLALNRGFRGGYCSSKAVCTCRK	46	+4	X	–	n.d.	
Defensin (*Cimex lectularius*)						Kaushal et al., 2016
ATCDLFSFQSKWVTPNHAACAAHCTARGNRGGRCKKAVCHCRK	43	+7	X	–	n.d.	
Psdefensin (*Protaetia brevitaris seulensis*)						Lee et al., 2016
VTCDLLSLQIKGIAINDSACAAHCLAMRRKGGSCKQGVCVCRN	43	+4	X	X	n.d.	
Defensin (*Oryctes rhinoceros*)						Ishibashi et al., 1999
LTCDLLSFEAKGFAANHSLCAAHCLAIGRKGGACQNGVCVCRR	43	+3	X	n.d.	n.d.	
Defensin A, B (*Anomala cuprea*)						Yamauchi, 2001
VTCDLLSFEAKGFAANHSICAAHCLAIGRKGGSCQNGVCVCRN	43	+2	X	–	n.d.	
VTCDLLSFEAKGFAANHSICAAHCLVIGRKGGACQNGVCVCRN	43	+2	X	X	n.d.	
Defensin (*Calliphora vicina*)						Chernysh et al., 2000
ATCDLLSGTGANHSACAAHCLLRGNRGGYCNGKAVCVCRN	40	+3	X	–	–	
Holotricin (*Holotrichia diomphalia*)						Lee et al., 1995
VTCDLLSLQIKGIAINDSACAAHCLAMRRKGGSCKQGVCVCRN	43	+4	X	–	n.d.	
PduDefensin[f] (*Phlebotomus duboscqi*)						Boulanger et al., 2004
ATCDLLSAFGVGHAACAAHCIGHGYRGGYCNSKAVCTCRR	40	+3	X	n.d.	X	
Defensin (*Pyrrhocoris apterus*)						Cociancich et al., 1994
ATCDILSFQSQWVTPNHAGCALHCVIKGYKGGQCKITVCHCRR	43	+4	X	X	n.d.	
Defensin (*Palomena prasina*)						Chernysh et al., 1996
ATCDALSFSSKWLTVNHSACAIHCLTKGYKGGRCVNTICNCRN	43	+4	X	X	–	
Coprisin[c] (*Copris tripartitus*)						Hwang et al., 2009; Lee et al., 2012
VTCDVLSFEAKGIAVNHSACALHCIALRKKGGSCQNGVCVCRN	43	+3	X	n.d.	X	
Defensin B, C (*Zophobas atratus*)						Bulet et al., 1991
FTCDVLGFEIAGTKLNSAACGAHCLALGRRGGYCNSKSVCVCR	43	+3	X	X	n.d.	
FTCDVLGFEIAGTKLNSAACGAHCLALGRTGGYCNSKSVCVCR	43	+2	X	X	n.d.	
Defensin 1 (*Acalolepta luxuriosa*)						Ueda et al., 2011

(Continued)

TABLE 1 | Continued

Peptide[a] (Organism)	Length (AA)	Net charge	Activity[b]			References
			G+	G−	F	
FTCDVLSVEAKGVKLNHAACGIHCLFRRRTGGYCNKKRVCICR	43	+7	X	X	n.d.	
Defensin A (*Chironomus plumosus*)						Lauth et al., 1998
LTCDILGSTPACAAHCIARGYRGGWCDGQSVCNCRR	36	+2	X	–	n.d.	
Mdde (*Musca domestica*)						Wang et al., 2006
ATCDLLSGTGVGHSACAAHCLLRGNRGGYCNGKGVCVCRN	40	+3	X	X	–	
Defensin (*Formica rufa*)						Taguchi et al., 1998
FTCDLLSGAGVDHSACAAHCILRGKTGGRCNSDRVCVCRA	40	+2	X	n.d.	n.d.	

[a]*Conserved cysteines are underlined in green for ease of comparison,* [b]*G+/G− gram-positive/gram-negative bacteria, F filamentous fungi, tested activity of peptides is indicated by X (active) or — (inactive) or n.d., if not determined, activities are extracted from given references as well as the Defensin Knowledgebase (Seebah et al., 2007),* [c]*NMR structure has been resolved,* [d]*Asterisk indicates C-terminal amidation,* [e]*Active against Trypanosoma brucei,* [f]*Active against Leishmania major.*

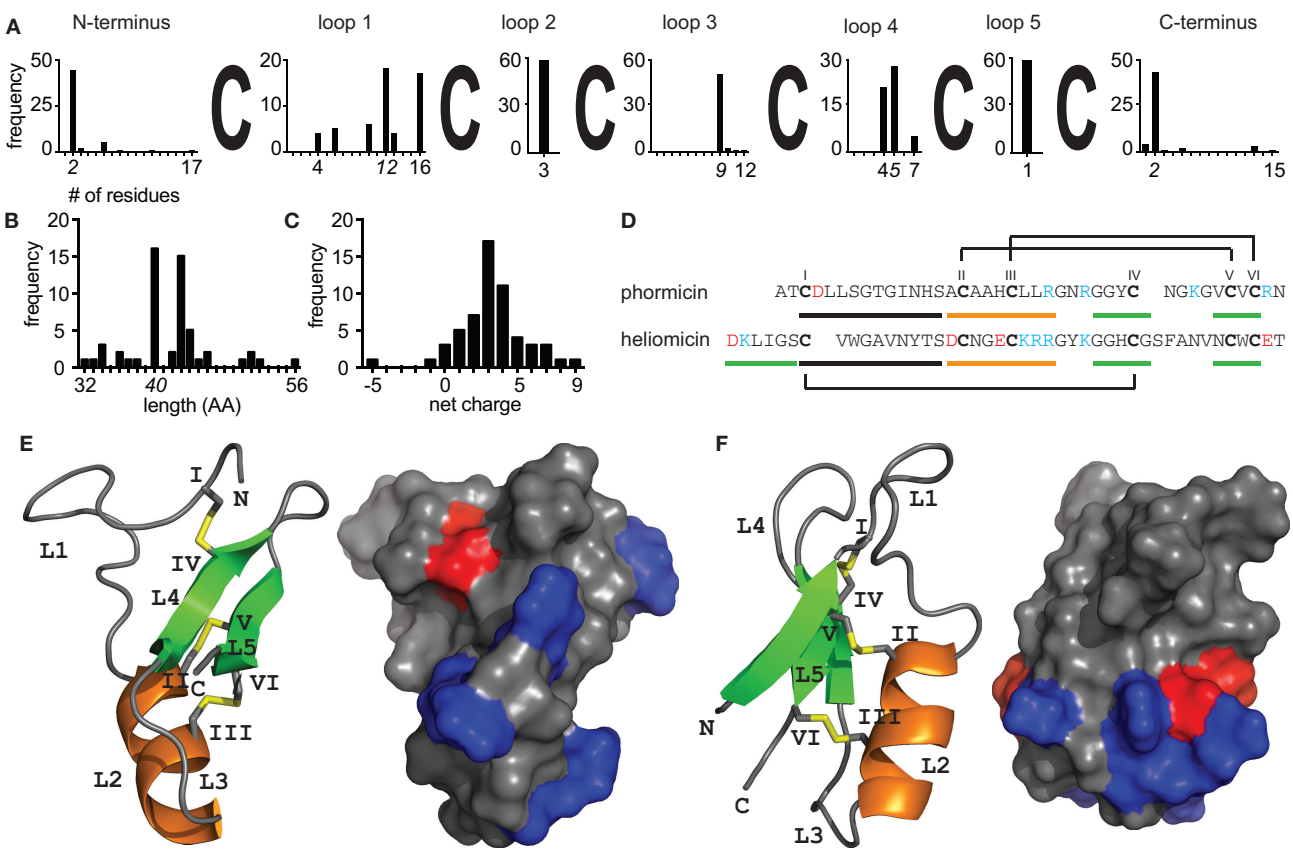

FIGURE 1 | Structural diversity of insect defensins. Sequence analysis of 57 peptides (**Table 1**) illustrating the diversity of insect defensins. **(A)** All sequences share a conserved pattern of six cysteine residues. Intercysteine loop 2 ($n = 3$) and loop 5 ($n = 1$) are fully conserved across all peptides and loop 1 shows the overall highest sequence variability ($n = 4$–16). The minimal, maximal, and most abundant (italic font) length of individual sequence stretches are indicated. **(B)** The length distribution shows that insect defensins are between 32 and 52 residues long, with the majority (54%) being 40 or 43 residues. **(C)** Insect defensins are typically cationic with reported charges varying between −5 and +8 and the majority (49%) of peptides holding a +3 or +4 net charge. **(D)** Sequences of the antibacterial phormicin and the antifungal heliomicin showing the disulfide connectivity of insect defensins. Stretches that form part of the α-helix (orange) and β-sheets (green) are indicated. Solution NMR structures of **(E)** phormicin (PDB: 1ICA) and **(F)** heliomicin (PDB: 1I2U) showing the cysteine-stabilized αβ motif. Secondary structural elements, i.e., α-helix (orange), β-sheets (green) and disulfide bonds (yellow) are highlighted, cysteines (roman numerals) and loops (L) are indicated. Surface representations show negatively (red) and positively (blue) charged residues, demonstrating the overall cationic character of the surface of the peptides.

among defensin peptides across different organisms, from plants to invertebrates to vertebrates (Dias Rde and Franco, 2015; Tarr, 2016; Shafee et al., 2017). Although all insect defensins share this common structural motif their primary sequence (**Figure 1A**) as well as their spectrum of antimicrobial activity varies considerably (**Table 1**). It is evident that the majority of

tested peptides exhibits activity against gram-positive bacteria, however several peptides exhibit potent activity against gram-negative bacteria or are primarily active against fungi (**Table 1**). Repositories such as Defensin Knowledgebase or ADP (Seebah et al., 2007; Wang et al., 2016) are useful resources for retrieving sequences for activity comparisons.

Yet, the attempt to accurately compare antimicrobial activities and relate them to the peptide sequences and secondary structures is challenging. Importantly, there is a large variety of different pathogens that have been selected for testing of defensin activity and some peptides have only been tested for individual pathogens, e.g., only one single strain of a gram-positive or gram-negative bacterium and it is also worth to mention that testing for antifungal activity was not carried out in a large number of studies (**Table 1**). Thus, activity spectra for these peptides need yet to be established. Further, assay conditions and concentration thresholds that are used to describe peptides as active or inactive can vary remarkably. For example in the initial study that identified the specific antifungal activity of drosomycin the highest concentration tested was 20 μM and the peptide thus referred as inactive against bacteria (Fehlbaum et al., 1994). In contrast the termite-specific termicin was reported to have weak activity against bacteria only at concentrations between 25 and 100 μM (Lamberty et al., 2001b). Additionally a range of different ways to describe activity is used such as μg/mL, molarity or the diameter of growth inhibition at a given concentration, thus making a quick and direct comparison not trivial. When characterizing defensin activity it also has to be noted that some studies use AMPs devoid of cysteines as control peptides and such studies are more difficult to use for comparison. Not at least experimental conditions such as the use of varying salt concentrations can change the activity of individual peptides dramatically and should also be considered in activity comparisons (Lee et al., 1997).

With regard to structure-activity relationships, a key limitation for insect defensins is the low number of resolved three-dimensional structures. Comparisons purely based on primary sequences are error-prone (Grishin, 2001) and conservation within secondary and tertiary structure is higher as compared to the primary sequences (Shafee et al., 2016). Currently only nine peptides have been characterized using solution NMR spectroscopy, including four antifungal, i.e., heliomicin (Lamberty et al., 2001a), drosomycin (Landon et al., 1997), termicin (Da Silva et al., 2003), and ARD1 (Landon et al., 2004) as well as five anti-gram-positive peptides, i.e., phormicin (Cornet et al., 1995), sapecin (Hanzawa et al., 1990), Def-AAA (Landon et al., 2008), lucifensin (Nygaard et al., 2012), and coprisin (Lee et al., 2013). Nevertheless, these studies provide valuable information about structure-activity relations for both antibacterial as well as antifungal insect defensins and shed light on structural determinants underlying biological activity.

For example mutation studies on the antifungal peptide ARD1 revealed subtle changes in hydrophobicity and cationicity to enhance the activity spectrum and increase potency (Landon et al., 2004). In an attempt to confer anti-bacterial activity onto the antifungal heliomicin which only differs from ARD1 in two positions changes within the N-terminal sequence led to a loss of antifungal activity highlighting its functional importance (Lamberty et al., 2001a). It should be noted here that peptides with antifungal activity have a longer N-terminus that forms an additional β-sheet and brings N- and C-terminal residues in close proximity as compared to antibacterial counterparts (**Figure 1F**, **Table 1**). The third antifungal peptide with the length of 44 amino acids is drosomycin. Interestingly, it has an additional disulfide-bond similar to the plant defensins RsAfp2 or NaD1 (Van Der Weerden et al., 2013). Compared to the other antifungal insect defensins it has an additional disulfide bond that connects the N-terminal loop to the C-terminus of the peptide. A modeling study comparing drosomycin to other plant antifungal defensins such as RsAFP2 suggested a hydrophobic patch in which a lysine residue is embedded as key determinant for antifungal activity (Landon et al., 2000). Indeed, experimental evidence verified this lysine residue while testing the functional role of charged residues for the antifungal activity of drosomycin (Zhang and Zhu, 2010). The fourth antifungal insect defensin for which a structure has been resolved is the termite-specific termicin. It carries an overall net charge of +4 and seems to be overall less different to antibacterial defensins. While exhibiting an amphiphilic character similar to drosomycin or heliomicin, the positions of hydrophilic and hydrophobic residues exposed on the surface are opposite. Several residues including for example the two arginine residues in loop 3 were proposed as possible interacting partners involved in antifungal activity (Da Silva et al., 2003), however experimental evidence for this hypothesis is still missing. The other three antifungal defensins known to date are Gallerimycin, Gm defensing, and Gm defensin-like peptide (Schuhmann et al., 2003; Cytrynska et al., 2007). Gm defensin and Gm defensin-like peptide show the highest sequence similarity (∼90% identity) to heliomicin and ARD1 including a conserved N-terminal sequence stretch, whereas Gallerimycin has a particular long N-terminus and shorter loop 1 and loop 5 sequences (**Table 1**). It appears that multiple factors contribute to specificity toward antifungal activity involving the N-terminal portion of the peptide as well as a subtle interplay between hydrophobic and charged residues.

For the primarily antibacterial defensins only five available structures represent a very limited number given the large number of different peptide sequences (**Table 1**). Additionally it is worth to mention that phormicin, sapecin, and lucifensin only differ by individual amino acids and thus it is not surprising that their three-dimensional topologies are highly similar (Hanzawa et al., 1990; Cornet et al., 1995; Nygaard et al., 2012). In an attempt to increase activity against *Staphylococcus aureus* a detailed study was reported using the *Anopheles* defensin as well as an alignment of 40 insect defensin sequences as basis for the design of 45 peptide mutants (Landon et al., 2008). A change in loop 1 (-GFGVGSSL- to -KWNWHTA-) resulted in a peptide with increased activity but also increased toxicity as compared to the native defensin. A second series of mutations further underpinned the importance of loop 1 for both, activity against *S. aureus* as well as toxicity yet fails to identify single residues that are responsible and highlighting the complexity of sequence-based approaches. Although sequence differences in loop 1 were clearly reflected by differences in the three-dimensional

structures, all but one peptide were found to have the CSαβ fold. This is in line with the sequence of the dung-beetle defensin coprisin. It has an elongated loop 1 sequence that shows a high degree of flexibility along the typical well-defined CSαβ portion (Lee et al., 2013) that is similar to all other insect defensins. A follow-up study that investigated the importance of the disulfide-bonds within coprisin clearly demonstrated that a removal of cysteine residues leads to dramatic loss of activity against bacteria, whereas antifungal activity was less affected (Lee et al., 2014). Notably also a series of non-apeptides showed significantly reduced activity and thus underpins the importance of the CSαβ motif for antimicrobial activity.

In addition to these factors relating to three-dimensional aspects of insect defensins, it is unclear whether posttranslational modifications such as amidation do play a role with regard to peptide activity. Amidation is common upon many bioactive peptides and often crucial for activity. It is also found within insect AMPs such as for example cecropins (Steiner et al., 1981). However, most of the insect defensins known to date are described as C-terminal acids and only few examples such as the bumblebee defensin or the termite defensin termicin have been identified by mass spectrometry to be C-terminally amidated (Rees et al., 1997; Lamberty et al., 2001b; Favreau et al., 2006). Possible implications regarding the biological activity and mechanism-of-action remain speculative.

MECHANISMS-OF-ACTION OF INSECT CSαβ PEPTIDES—FROM MEMBRANE EFFECTS TO ION CHANNEL PHARMACOLOGY

Similar to the broad range of microorganisms targeted by insect defensins their mechanisms-of-action seem equally complex and knowledge remains sparse. Their activity is primarily explained by the presence of positive charges exposed on the surface (**Figures 1E,F**) that can interact with negative charges on the outside of microbes leading to the insertion and perforation of membranes (Brogden, 2005). However, it is nowadays evident that antimicrobial peptides are also acting on specific target structures such as lipid II or sphingolipids (Wilmes et al., 2011). To date specific protein targets for insect defensins remain to be identified and structure-activity studies may prove useful in deciphering molecular mechanism underlying bioactivity. Further, reported activity of anionic antimicrobial peptides (Xu et al., 2016) clearly indicates that electrostatic interactions alone cannot explain the antimicrobial activity, and it remains to be shown if these peptides exhibit different mechanisms-of-actions. Interestingly there are several insect defensin-like CSαβ peptides from scorpions that potently inhibit voltage gated potassium channels. Indeed there seems to be an evolutionary link between the antimicrobial and (neuro)-toxic activity of peptides containing a CSαβ fold (Zhu et al., 2014). From an evolutionary perspective it seems evident that the presence of a conserved three-dimensional fold in both antimicrobial defense peptides and scorpion toxins reflects a common strategy to defend against invading organism or predators by means of membrane interaction. Within scorpion toxins a conserved lysine residue interacting with the channel's selectivity filter characterizes the pore-blocking activity (Garcia et al., 2001). Although this residue is also found in some insect defensins, it appears that a flexible N-terminal loop (=loop 1) within antimicrobial defensins impairs access to the channel pore as compared to scorpion type defensins that lack such a loop (Zhu et al., 2014). Notably this intercysteine loop sequence shows high variability (n = 4–16, **Figure 1A**) and thus it allows speculations regarding potential promiscuous activity of individual insect peptides. Though, to date no study attempted to accurately analyse this evolutionary link of antimicrobial insect peptides and scorpion K_v toxins and it is still unclear how peptides that share a such a high degree of structural similarity can exhibit such a diverse range of biological activities.

CONCLUSION

Given the number of insect species, the variety of defensin peptides and a range of activities it is evident that insect defensins are valuable structural templates for rational design of a novel class of "designer AMPs." Hence it is crucial to have a detailed understanding on how structural aspects are reflected in peptide activity both with regard to pathogen specificity as well as undesired side effects such as cell toxicity. Current literature provides a solid, yet incomplete basis for rational structure based drug design. The urgent need for the development of novel antibiotic lead molecules provides significant justification and new impetus for further detailed exploration of structure-activity relationships of antimicrobial insect CSαβ peptides.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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The Effect of Selective D- or N$^{\alpha}$-Methyl Arginine Substitution on the Activity of the Proline-Rich Antimicrobial Peptide, Chex1-Arg20

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In vivo pharmacokinetics studies have shown that the proline-rich antimicrobial peptide, A3-APO, which is a discontinuous dimer of the peptide, Chex1-Arg20, undergoes degradation to small fragments at positions Pro6-Arg7 and Val19-Arg20. With the aim of minimizing or abolishing this degradation, a series of Chex1-Arg20 analogs were prepared *via* Fmoc/tBu solid phase peptide synthesis with D-arginine or, in some cases, peptide backbone N$^{\alpha}$-methylated arginine, substitution at these sites. All the peptides were tested for antibacterial activity against the Gram-negative bacterium *Klebsiella pneumoniae*. The resulting activity of position-7 substitution of Chex1-Arg20 analogs showed that arginine-7 is a crucial residue for maintaining activity against *K. pneumoniae*. However, arginine-20 substitution had a much less deleterious effect on the antibacterial activity of the peptide. Moreover, none of these peptides displayed any cytotoxicity to HEK and H-4-II-E mammalian cells. These results will aid the development of more effective and stable PrAMPs *via* judicious amino acid substitutions.

Keywords: A3-APO, Chex1-Arg20, D-arginine, Gram-negative bacteria, *K. pneumoniae*, backbone N$^{\alpha}$-methylation, proline-rich antimicrobial peptide

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Li W, Sun Z, O'Brien-Simpson NM, Otvos L, Reynolds EC, Hossain MA, Separovic F and Wade JD (2017) The Effect of Selective D- or N$^{\alpha}$-Methyl Arginine Substitution on the Activity of the Proline-Rich Antimicrobial Peptide, Chex1-Arg20. Front. Chem. 5:1. doi: 10.3389/fchem.2017.00001

INTRODUCTION

The increasing widespread onset of bacterial multi-drug resistance, associated with major clinical pathogenic infections, has resulted in calls for the development new antimicrobial agents (Laxminarayan et al., 2013). Due to their broad-spectrum activities and multi-modal actions against pathogens, antimicrobial peptides (AMPs) (also known host-defense peptides), are considered as attractive potential candidates for new antibiotics (Hilchie et al., 2013; Lam et al., 2016). Importantly, these peptides have also attracted considerable attention as alternative means of plant disease control to conventional treatments that are polluting and hazardous to both human health and the environment (Datta et al., 2015, 2016). Among these peptides, the class of proline-rich AMPs (PrAMPs) possess a unique multi-modal mechanism of action against pathogens and display potent activity against Gram-negative bacteria (Otvos et al., 2005; Czihal et al., 2012; Guida et al., 2015). These actions include membrane rupture (Li et al., 2014), inhibition of the bacterial shock heat protein DnaK (Kragol et al., 2001; Scocchi et al., 2009), blockade of bacterial ribosomal protein

expression (Krizsan et al., 2014; Roy et al., 2015; Seefeldt et al., 2015, 2016; Goldbach et al., 2016), and immunostimulatory activity (Ostorhazi et al., 2011). Recently, a PrAMP and other AMPs were impregnated into nanofibers or hydrogels for the potential treatment of skin injuries in general and battlefield burns (Mateescu et al., 2015; Sebe et al., 2016).

The peptide, Chex1-Arg20, was *de novo* designed based on native PrAMPs with additional sequence optimization to enhance bacterial membrane penetration (Otvos et al., 2005; Noto et al., 2008; Rozgonyi et al., 2009). It has been shown that multimerization of Chex-Arg20 to a discontinuous dimer or tetramer results in an alteration of its mechanism of interaction with the *Escherichia coli* membrane (Li et al., 2015a). These observations were further confirmed on investigation of Chex1-Arg20 and its multimers with model membranes (Li et al., 2016). Additionally, specific *C*-terminal chemical modifications of the Chex1-Arg20 monomer were shown to expand both its activity and spectrum of Gram-negative bacterial action (Li et al., 2015b). These observations led to the development of a series of tetrameric Chex1-Arg20 bearing a C-terminal hydrazide that were shown to possess a more compact structure and potent and broadened activity against Gram-negative nosocomial pathogens (Li et al., 2017).

The discontinuous dimer of Chex1-Arg20, A3-APO, was shown in *in vivo* pharmacokinetic studies to undergo degradation at positions Pro6-Arg7 and Val19-Arg20, as well as to produce the major metabolite, Chex1-Arg20 (Noto et al., 2008). A key goal is to undertake chemical modifications at these labile sites to confer significant improvement in peptide stability in serum without undue effect on their activity (Otvos and Wade, 2014). D-amino acid substitution in AMPs has previously been shown to be a successful strategy (Hong et al., 1999). This suggests that partial D-amino acid substitutions within Chex1-Arg20 might be a useful means to improve its activity and stability. Furthermore, backbone N-methylation of peptide bonds can also confer high stability against proteases and improved pharmacological bioavailability (Di Gioia et al., 2016). Therefore, we undertook to incorporate the unnatural D-amino acid and N^{α}-methyl-amino acid into two key points within the peptide sequence to determine the effect on activity against Gram-negative bacterium *K. pneumoniae*.

MATERIALS AND METHODS

Materials

Nine-Fluorenylmethoxylcarbonyl (Fmoc)-L-amino acids, 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylamonium hexafluorophosphate (HCTU), and 1-[Bis(dimethylamino) methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid (HATU) were from GL Biochem (Shanghai, China). TentaGel-MB-RAM-resin was from Rapp Polymere (Tubingen, Germany). N^{α}-Fmoc-N^{α}-methyl-L-arginine(N^{ω}-Pbf), and N^{α}-Fmoc-D-arginine(D-Pbf) were purchased from Novabiochem (Sydney, Australia). N,N-Diisopropylethylamine (DIPEA), dimethylformamide (DMF), and trifluoroacetic acid (TFA) were obtained from Auspep (Melbourne, Australia). Piperidine, triisopropylsilane (TIPS), anisole, and acetonitrile (CH_3CN) were all obtained from Sigma (Sydney, Australia).

Peptide Synthesis

The peptides were synthesized by Fmoc/tBu solid-phase methods (Fields and Noble, 1990) using a CEM Liberty microwave-assisted synthesizer and TentaGel-MB-RAM-resin as previously described (Li et al., 2015a). Standard Fmoc-chemistry was used throughout with a 4-fold molar excess of the Fmoc-protected amino acids in the presence of 4-fold HCTU and 8-fold DIPEA. For the arginine derivative substitution, 1.5-fold of amino acid coupling was used together with 1.5 equivalents HATU and 3 equivalents of DIPEA. After synthesis, the peptides were cleaved from the solid support resin with TFA in the presence of anisole and TIPS as scavengers (95:3:2, v/v) for 2 h at room temperature. After filtration to remove the resin, the filtrate was concentrated under a stream of nitrogen and the peptide products were precipitated in ice-cold diethyl ether and washed three times. The peptides were then purified by reversed-phase high performance liquid chromatography (RP-HPLC) in water and acetonitrile containing 0.1% TFA using a gradient of 10–40% (acetonitrile) in 40 min. Due to the variation in hydrophobicity between the different analogs, the final products were characterized by RP-HPLC using a gradient of either 0–40% (acetonitrile) in 40 min or 10–40% (acetonitrile) in 30 min. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was also used for characterization.

Antibacterial Assay

An antibacterial assay was undertaken to determine the minimal inhibitory concentration (MIC) as described previously (Li et al., 2015b). The Gram-negative nosocomial bacterium, *K. pneumoniae* ATCC13883, was selected for testing the antibacterial activities of the Chex1-Arg20 analogs using 2.5×10^5 cells/ml in Mueller Hinton broth (MHB) at 37°C immediately prior to the determination of MIC.

Cell Proliferation Test

The proliferation of HEK-293 (ATCC® CRL-1573™) and H-4-II-E (ATCC® CRL-1548™) cells were tested with the Chex1-Arg20 analogs using the CellTiter 96 AQ_{ueous} Non-Radioactive

TABLE 1 | Primary structure of Chex1-Arg20 analogs used in this report.

No	Name	Sequence*	MW_{cal}	MW_{fd}
1	Chex1-Arg20	Chex-RPDKPRPYLPRPRPPRPVR-NH_2	2475.0	2476.8
2	DR7	Chex-RPDKP r PYLPRPRPPRPVR-NH_2	2474.9	2474.8
3	DR7(1–19)	RPDKP r PYLPRPRPPRPV-NH_2	2318.8	2319.3
4	DR7(7–19)	r PYLPRPRPPRPV-NH_2	1600.1	1603.0
5	Chex1-Val19	Chex-RPDKP r PYLPRPRPPRPV-NH_2	2318.8	2319.2
6	DR20	Chex-RPDKPRPYLPRPRPPRPV r-NH_2	2474.9	2475.2
7	mR20	Chex-RPDKPRPYLPRPRPPRPVmR-NH_2	2489.0	2488.9
8	reverse	Chex-RVPRPPRPRPLYPRPKDPR-NH_2	2475.0	2478.0

**Abbreviations: r, D-Arg; mR, N^{α}-methyl-arginine; MW_{cal}, calculated mass; MW_{fd}, found mass in MALDI.*

Cell Proliferation Assay (Promega) as described previously (Li et al., 2015b).

RESULTS AND DISCUSSION

Peptide Preparation

Peptide **1** was prepared as described in a previous report (Li et al., 2015b) and **2–8** were prepared on TentaGel-MB-RAM-resin *via* standard Fmoc/tBu solid-phase methods. Unnatural amino acid incorporation was achieved in presence of HATU instead of HCTU (**Table 1**) which produced better quality products. Each Chex1-Arg20 analog was obtained in an overall yield of *ca.* ∼15% relative to the crude cleaved starting material. Each analog was then subjected to comprehensive chemical characterization including analytical RP-HPLC and MALDI-TOF MS to confirm their purity (**Figure 1**).

Antibacterial Activity

Each Chex1-Arg20 analog was assayed against the nosocomial Gram-negative bacterium *K. pneumoniae* ATCC 13883. The

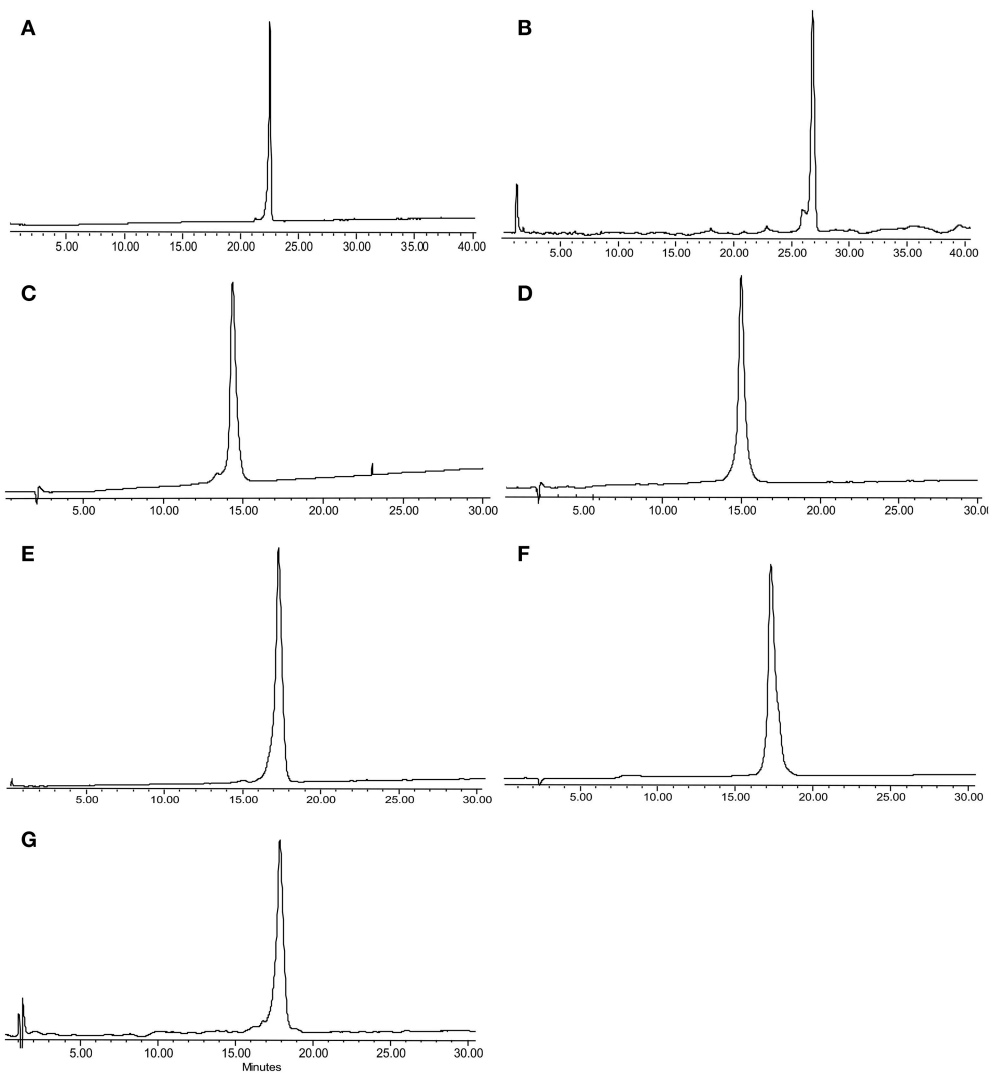

FIGURE 1 | RP-HPLC and MALDI-TOF/ESI MS for peptide analogs 2–8, respectively: (A) 2 , DR7; **(B) 3**, DR7(1–19); **(C) 4**, DR7(7–19); **(D) 5**, Chex1-Val19; **(E) 6**, DR20; **(F) 7**, mR20; **(G) 8**, reverse. Analysis condition: Phenomenex C18 column (WIDEPORE 3.6 μ XB-C18, 150 × 4.6 nm); buffer A, 0.1% aq. TFA; buffer B, 0.1% TFA in acetonitrile; gradient, buffer B 0–40% in 40 min for **(A) 2** and **(B) 3**, and 10–40% in 30 min for **(C) 4**–**(G) 8**.

TABLE 2 | Antibacterial activity, MIC (μM), of Chex1-Arg20 analogs against Gram-negative pathogen *K. pneumoniae* ATCC 13883.

Bacterium	1*	2	3	4	5	6	7	8
K. pneumoniae	0.8 ± 0.1*	>100	>100	>100	36.1 ± 0.6	11.8 ± 0.1	14.5 ± 0.1	>100

The activity of analog **1 was previously reported (Li et al., 2015b).*

TABLE 3 | Cytoxocity (μM) of Chex1-Arg20 analogs against mammalian cell lines, H-4-II-E (ATCC® CRL-1573™) and H-4-II-E (ATCC® CRL-1548™), in which >100 μM or >50 indicated there was no cytotoxicity at the highest tested concentration 100 μM or 50 μM.

Analogue	H-4-II-E cell	HEK cell
1	>100 μM	>100 μM
2	>100 μM	>100 μM
3	>100 μM	>100 μM
4	>100 μM	>100 μM
5	>100 μM	>100 μM
6	>100 μM	>100 μM
7	>100 μM	>100 μM
8	>100 μM	>100 μM

results are shown in **Table 2** in comparison with analog **1**, Chex1-Arg20. Replacement of arginine at position 7 with the D-form (analog **2**) resulted in substantial loss of activity. This highlighted the importance of arginine-7 and its native L-configuration for characteristic antimicrobial activity. Curiously, truncation of the C-terminal Arg20 from analog 2 to produce analog **5** partially restored activity. Compared with analog **5**, the N-terminal shortened analogs **2**–**4** containing a D-arginine substitution at position 7 showed a drastic loss of activity against this pathogen in MHB. In contrast, replacement of position Arg20 with either the D-arginine or N^{α}-methylated-arginine (analogs **6–7**) led to a maintenance of significant activity of the native Chex1-Arg20 which indicates that this residue is more tolerant to modification to improve its *in vivo* stability to degradation. Finally, the reverse sequence (analog **8**) was also evaluated and, as expected, it showed no activity against *K. pneumoniae* which confirmed the necessity of the native sequence for antibacterial action.

Cytotoxicity

In vitro cytotoxicity was also measured *via* the Promega CellTiter 96 AqueousNon-Radioactive Cell Proliferation Assay (Li et al., 2015a) using the mammalian cell lines HEK-293 (ATCC CRL 1573) and H-4-II-E (ATCC CRL-1548). None of the Chex1-Arg20 analogs showed any toxicity against either mammalian cell line at the highest tested concentration (100 μM) (**Table 3**).

CONCLUSIONS

In summary, a series of D-amino acid substituted analogs of the PrAMP, Chex1-Arg20, were prepared by standard Fmoc/tBu solid phase peptide synthesis. These analogs were tested against the Gram-negative bacterium *K. pneumoniae* for antibacterial activity. In this study, the activity of D-arginine Chex1-Arg20 showed the replacement of arginine at position seven led to drastic loss of activity. The short fragments, Arg2-Val19 and Arg7-Val19, also displayed no antibacterial activity. However, substitution at position 20 with either D-arginine or N^{α}-methyl-arginine did not greatly affect the activity against *K. pneumoniae*. Moreover, none of these peptides showed any cytotoxicity to HEK and H-4-II-E mammalian cells. Such findings will assist the development of more effective and stable Chex1-Arg20 and A3-APO analogs with further substitution at position 20.

AUTHOR CONTRIBUTIONS

WL performed chemical syntheses, antibacterial assay and drafted the manuscript; ZS performed cytotoxicity test; NO, LO, ER, MH, FS, and JW took part in experimental design. All authors worked on the manuscript.

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frontiers in Chemistry

ORIGINAL RESEARCH
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D-BMAP18 Antimicrobial Peptide Is Active *In vitro*, Resists to Pulmonary Proteases but Loses Its Activity in a Murine Model of *Pseudomonas aeruginosa* Lung Infection

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The spread of antibiotic resistant-pathogens is driving the search for new antimicrobial compounds. Pulmonary infections experienced by cystic fibrosis (CF) patients are a dramatic example of this health-care emergency. Antimicrobial peptides could answer the need for new antibiotics but translating them from basic research to the clinic is a challenge. We have previously evaluated the potential of the small membranolytic peptide BMAP-18 to treat CF-related infections, discovering that while this molecule had a good activity *in vitro* it was not active *in vivo* because of its rapid degradation by pulmonary proteases. In this study, we synthesized and tested the proteases-resistant all-D enantiomer. In spite of a good antimicrobial activity against *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* clinical isolates and of a tolerable cytotoxicity *in vitro*, *D*-BMAP18 was ineffective to treat *P. aeruginosa* pulmonary infection in mice, in comparison to tobramycin. We observed that different factors other than peptide degradation hampered its efficacy for pulmonary application. These results indicate that *D*-BMAP18 needs further optimization before being suitable for clinical application and this approach may represent a guide for optimization of other anti-infective peptides eligible for the treatment of pulmonary infections.

Keywords: antimicrobial peptide, BMAP18, cystic fibrosis, *Pseudomonas aeruginosa*, proteolytic degradation, bronchoalveolar lavage, lung

INTRODUCTION

Cystic fibrosis (CF) is a genetic disorder that significantly reduces the life expectancy. Most of CF-patients succumb to respiratory failure brought on by chronic bacterial infection and airway inflammation (Cribbs and Beck, 2017). CF lung disease begins early in life with inflammation and impaired muco-ciliary clearance, and gets worse due to the consequent chronic infection of the airways (Robinson and Bye, 2002). A progressive decline of lung function then follows, with episodes of acute aggravation of respiratory symptoms. CF has a peculiar set of bacterial pathogens that are frequently acquired in an age-dependent sequence (Gibson et al., 2003) and

Pseudomonas aeruginosa represents the most problematic-one, infecting 60–75% of the patients (Ahlgren et al., 2015). Moreover, physicians treating patients with CF are increasingly faced with infections by multidrug-resistant isolates of *P. aeruginosa* and other pathogens (Dasenbrook et al., 2010; Wu et al., 2016). The problem is also exacerbated by the high microbial adaptation to the CF pulmonary environment, resulting in an increased ability to form biofilms intrinsically resistant to antibiotics (Bhagirath et al., 2016). Multidrug-resistant infections may be treated successfully by using combinations of antibiotics with different mechanisms of action. Unfortunately, very few novel antimicrobials have come out in the last years to complement current therapies (Harbarth et al., 2015). Furthermore, potential new antibiotic drugs should be supported by other, non-classical, antimicrobial agents, in order to contain the diffusion of pathogens resistant even to these new compounds (Waters and Smyth, 2015). Anti-Microbial Peptides (AMPs) are under the spotlight as a promising class of antimicrobials for development as novel antibiotics (Mahlapuu et al., 2016). They are naturally occurring molecules of the innate immune system of animals with important roles in host defense (Lai and Gallo, 2009; Yeung et al., 2011). Most AMPs have a wide spectrum of activity also comprising multidrug-resistant pathogens; a relatively good selectivity toward bacteria and a rapid mechanism of action, most often based on the lysis/permeabilization of microbial membranes. This mode of action, in which no specific molecular targets are involved, is associated with a low frequency for selection of resistant strains (Brogden, 2005; Benincasa et al., 2006; Hancock and Sahl, 2006). The antimicrobial activity of AMPs has also been largely reported, with respect to CF pathogens (Saiman et al., 2001; Zhang et al., 2005; Kapoor et al., 2011; Bezzerri et al., 2014; Mangoni et al., 2015). Previously, we showed that some bovine alpha-helical AMPs [the BMAPs Skerlavaj et al., 1996] had a potent and rapid *in vitro* bactericidal and anti-biofilm activity against many *P. aeruginosa* and *S. maltophilia* strains from CF patients (Pompilio et al., 2011, 2012). We also demonstrated that some N-terminal shortened fragments of these peptides overall maintained their good antibacterial properties toward CF-related pathogens, but show a certain degree of acute toxicity when intra-tracheally administered to mice lungs. BMAP27(1–18) was selected for its good antimicrobial potential and reduced pulmonary toxicity, but its protective effect against *P. aeruginosa* lung infection in mice was scarce due to its rapid degradation in the pulmonary environment (Mardirossian et al., 2016). Proteolytic digestion is a problem that BMAP27(1–18) shared with many other native (Moncla et al., 2011; Mattiuzzo et al., 2014) and synthetic (Kim et al., 2014) antimicrobial peptides, reported also in the pulmonary environment (Sajjan et al., 2001; Morris et al., 2012). The use of enantiomeric all *D*-peptides represented a promising strategy to avoid proteolysis in lungs (Sajjan et al., 2001) and could possibly enhance BMAP27(1–18) antibacterial activity *in vivo*, reducing its degradation in the pulmonary environment. In this work, we synthesized the *D*-isomer of BMAP27(1–18), *D*-BMAP18. We tested *D*-BMAP18 it for stability in bronchoalveolar lavage (BAL) fluid, *in vitro* antibacterial activity, *in vivo* protective effect, and toxicity both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Bacterial Strains

P. aeruginosa, S. maltophilia, and *Staphylococcus aureus* strains were originally isolated from respiratory specimens of CF patients admitted to the "Bambino Gesù" Children Hospital in Rome and previously tested with AMPs (Pompilio et al., 2012). *P. aeruginosa* RP73, and PAO1 were tested as reference strains.

Design and Synthesis of *D*-BMAP18

The peptide *D*-BMAP18 (GRFKRFRKKFKKLFKKLS-am) was synthesized using the solid-phase Fmoc chemistry on a microwave peptide synthesizer Astra Initiator + (Biotage, USA). Protected amino acids and Fmoc-linker-AM champion resins were purchased from Advanced Biotech Italia (Milan, Italy) or Novabiochem (Merck, Darmstadt, Germany). For each coupling step, the Fmoc-protected amino acid and coupling reagents were added in a 5-fold molar excess with respect to resin substitution. Couplings were carried out with N-hydroxybenzotriazole (HOBt) and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoro-borate (TBTU) at 75°C. Cleavage from the resin and deprotection of the synthesized peptide were carried out with a solution of 85% trifluoroacetic acid (TFA), 2% water, 2% triisopropylsilane and 8% of phenol, 1,2-ethanedithiol and 3% of thioanisole. The peptide was purified by reversed phase HPLC on a C18 column (19 × 300 mm; Waters, MA, USA) using 0–40% acetronitrile-water linear gradients in 0.05% trifluoroacetic acid. Peptide's quality and purity was verified by ESI–MS (API 150 EX Applied Biosystems), (*D*-BMAP18 theoretical average mass = 2341.95 Da; measured average mass = 2342.2 Da). The peptide was lyophilized from 10 mM HCl solution to remove TFA and the concentration of the stock solution was evaluated by spectrophotometric determination of peptide bonds (ε_{214}) (Kuipers and Gruppen, 2007).

D-BMAP18 Degradation in Bronchoalveolar Lavage (BAL) Fluid

Bronchoalveolar fluid was collected from six C57/Bl6NCrl healthy male mice (2–3 months old, from the animal facility of the University of Trieste). Mice were killed by cervical dislocation, a blunt needle connected to a syringe was inserted into the mouth and trachea, and then lungs were washed with 1 ml of sterile, pre-warmed (37°C), 0.9% NaCl. Equal volumes of BAL samples from each mouse were pooled and stored in aliquots at −20 °C until further uses. The total protein concentration of BAL fluid (300 μg/ml) was determined by the BCA assay (Pierce, BCA Protein Assay Kit). To evaluate *D*-BMAP18 resistance to proteases, a very small volume of a highly concentrated solution of peptide was diluted in pooled BAL to a final concentration of 300 μg/ml, reaching a 1:1 (wt/wt) peptide/BAL total proteins ratio. *D*-BMAP18 in BAL was then incubated at 37°C. Thirty microliters of the mixture were sampled at indicated times, immediately cooled down on ice and frozen at −20°C. Subsequently, samples were heated for 5 min at 90°C in Laemmli Sample Buffer A, and 10 μl of each sample were loaded on a 16% tricine gel for SDS-PAGE (Schagger, 2006). The gel was stained

over-night with Coomassie Brilliant Blue and destained with 10% acetic acid in water (v/v). These experiments were carried out in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institute of Health and in accordance with the ethical standards of the Animal Care Committee of the University of Trieste. The protocol was approved by the Ethics Committee of the University of Trieste.

Evaluation of Antibacterial Activity of *D*-BMAP18

MIC values were determined using the broth microdilution method. Briefly, serial 2-fold dilutions of each peptide were prepared in Mueller-Hinton broth (MH; Difco) and aliquoted in round-bottom 96 well microtiter plates (Sarstedt). Each well was inoculated with a standardized inoculum to achieve a final test concentration of about 5×10^5 CFU/ml. The MIC was measured as the lowest concentration of the peptide that completely inhibited visible bacterial growth after incubation at 37°C for 24 h. To calculate the MBC, following the 24 h-incubation of the MIC assay, 25 μl of broth from clear wells were plated on MH agar plates, and incubated at 37°C for 24 h. MBC was defined as the lowest concentration of the peptide killing at least 99.9% of the original inoculum.

In vitro Toxicity of *D*-BMAP18 against Cell Lines

Cytotoxicity was determined by the MTT assay using human lung carcinoma A-549 cells (German DMSZ). Cells were grown, at 37°C and 5% CO_2, to sub-confluence in 100 μL of Dulbecco's MEM (Sigma-Aldrich) + 5% FBS (Euroclone) + 2.4 mM Glucose + 1% Pen/Strep, using a 96 wells flat-bottom microtiter plates. Serial 2-fold dilutions of the peptide were prepared in cell growth medium and 100 μL were added to the cells. After a 24 h-incubation the cells were washed using PBS (Sigma-Aldrich), 100 μL of PBS were added and 10 μL MTT were then added (5 mg/ml in PBS, Sigma-Aldrich). Following 4 h-incubation, 150 μl of IGEPAL 10% in 0.01N HCl were added (Sigma-Aldrich) and the plates were incubated at 37°C. After over-night incubation, the cytotoxicity was spectrophotometrically evaluated by measuring OD_{620nm}.

In vivo Toxicity of *D*-BMAP18

In vivo acute toxicity of *D*-BMAP18 was evaluated in C57BL/6NCrl mice ($n = 5$/group) (male; 22 g; 6 ± 2 week-old) obtained from Charles River Laboratories Italia S.r.l. (Calco, Milan, Italy). Mice were intra-tracheally challenged with increasing doses (1, 2, 4, and 8 mg/kg) of *D*-BMAP18 prepared in sterile distilled water. Control mice received vehicle only (sterile distilled water). General health and animal behavior (ruffled coats, huddled position, lack of retreat in handler's presence), weight loss, and survival were monitored daily over a 5-day period, comparatively to control mice. Mice were sacrificed 5 days post-exposure (p.e.) by intraperitoneal injection of tribromoethanol (Sigma-Aldrich), then lungs were observed *in situ* for macroscopic damage using the "four-point scoring system" (Johansen et al., 1993): 1, normal; 2, swollen lungs, hyperemia, and small atelectasis; 3, pleural adhesion, atelectasis, and multiple small abscesses; and 4, large abscesses, large atelectasis, and hemorrhages. Subsequently, lungs were removed *en bloc* from the chest via sterile excision and immediately weighed. These experiments were carried out in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institute of Health and in accordance with the ethical standards of the Animal Care Committee of the "G. d'Annunzio" University of Chieti-Pescara. The protocol was approved by the Animal Care Committee of the "G. d'Annunzio" University of Chieti-Pescara.

Activity of *D*-BMAP18 against Mouse Acute Lung Infection Caused by *P. aeruginosa*

C57/Bl6NCrl mice ($n = 8$/group) were intratracheally challenged with 1×10^7 cells *P. aeruginosa* RP73 clinical strain, and 5 min later a single dose of *D*-BMAP18 at different concentrations (0.5, 1, and 2 mg/kg) was intratracheally administrated. Sterile distilled water alone or tobramycin [10 mg/kg] (Sigma-Aldrich S.r.l.), were used respectively as negative and positive control. One day post-exposure, mice were sacrificed by intraperitoneal injection of tribromoethanol (Sigma-Aldrich S.r.l.), then lungs were observed *in situ* for macroscopic damage (Johansen et al., 1993), aseptically removed *en bloc* from the chest, and immediately weighed. Subsequently, lungs were homogenized (24,000 rpm) on ice in 1 ml of sterile PBS by using Ultra-Turrax T25-Basic homogenizer (IKA-Werke GmbH & Co. KG, Germany). Ten-fold serial dilutions of lung homogenates were plated on MH agar (Oxoid SpA), and the number of colony-forming units (CFU) was counted after incubation at 37°C for 24 h.

These experiments were carried out in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institute of Health and in accordance with the ethical standards of the Animal Care Committee of the "G. d'Annunzio" University of Chieti-Pescara. The protocol was approved by the Animal Care Committee of the "G. d'Annunzio" University of Chieti-Pescara.

Evaluation of Antibacterial Activity of *D*-BMAP18 in BAL

The antimicrobial activity of *D*-BMAP18 in the presence of BAL from healthy mice was evaluated against *P. aeruginosa* RP73 strain by a killing assay. Sterile 0.9% w/v NaCl alone was used as a control. Different concentrations of the peptide were prepared in 100 μL of BAL. To each concentration was then added 100 μL of bacterial suspension prepared in MH broth and diluted in sterile 0.9% w/v NaCl to a load of 2×10^6 CFU/ml. The final conditions were: 10^6 CFU/ml *P. aeruginosa* RP73 in 50% BAL in sterile 0.9% w/v NaCl (v/v). The final % of residual MH medium introduced in the assay with the diluted bacteria was below 5%. Samples were then incubated at 37°C for 1 h, serially 10-folds diluted in MH broth and plated on MH agar. Colonies were counted after overnight incubation at 37°C.

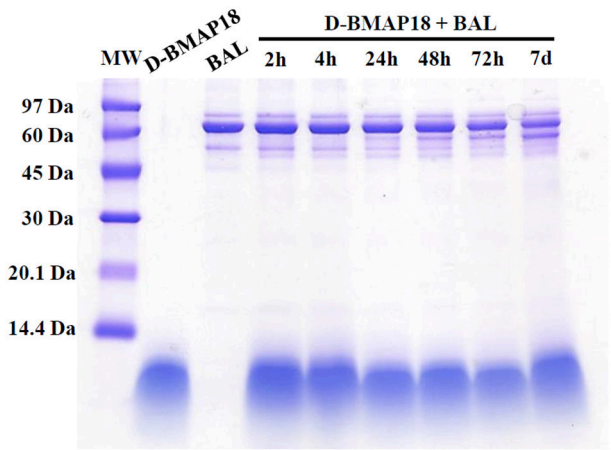

FIGURE 1 | Evaluation of the stability of *D*-BMAP18 in the presence of murine bronchoalveolar lavage (BAL) fluid at 37°C. Samples were collected at prefixed times and analyzed by SDS-PAGE (gel 16%, tricine) following staining with Coomassie Brilliant Blue. As control, 2.4 μg of *D*-BMAP18 and BAL alone were loaded, corresponding to the original concentration of both compounds at the beginning of the time-course.

Statistical Analysis

Statistical analysis of results was conducted with GraphPad Prism version 4.00 (GraphPad software Inc.; San Diego, CA, USA), considering as statistically significant a p-value < 0.05. Parametric (ANOVA-test followed by Student-Newmann-Keuls post-test) or non-parametric (Kruskal-Wallis test followed by Dunn's multiple comparison test) tests were performed when data were normally distributed or not, respectively. Differences in MIC values were considered statistically significant if > 2 $\log_2$.

RESULTS

D-BMAP18 Stability in Murine BAL Fluid

We recently showed that *L*-BMAP-18 peptide is degraded by pulmonary proteases in murine bronchoalveolar lavage fluids within 20 min of exposure, and already after 10 min most of the L-BMAP-18 was digested (see Mardirossian et al., 2016). *D*-BMAP18 was synthesized to provide a molecule more resistant to enzymatic cleavage. The stability of *D*-BMAP18 was tested in murine BAL fluid. No peptide degradation was in fact observed, even after 7 days of incubation at 37° C in undiluted BAL (**Figure 1**), indicating that *D*-BMAP18 is not a substrate for proteases in murine BAL.

D-BMAP18 *In vitro* Antibacterial Activity

We evaluated the *in vitro* antimicrobial activity of *D*-BMAP18 against multiresistant CF isolates of *P. aeruginosa, S. maltophilia* and *S. aureus,* previously tested with *L*-BMAP18 (Mardirossian et al., 2016). Overall, *D*-BMAP18 showed a relevant activity against both *P. aeruginosa* and *S. maltophilia* strains, whereas no effect was observed toward *S. aureus* (**Table 1**). Compared to the *L*-isomer, *D*-BMAP18 exhibited comparable activity against *P. aeruginosa* (MIC_{90}: 16 μg/mL, for both AMPs), but significantly higher activity toward *S. maltophilia* (MIC_{90}: >32 and 16 μg/ml, respectively). In particular, MIC values indicated that *D*-BMAP18 was significantly more active than L-isomer against *P. aeruginosa* RP73 and PA10, and *S. maltophilia* SM122. MBC/MIC ratio (killing quotient, KQ) was ≤ 4 for most of strains, indicating the bactericidal activity of both peptides.

TABLE 1 | Antibacterial activity of *L*-BMAP18 and *D*-BMAP18 against Gram-positive and Gram-negative strains from CF patients.

	L-BMAP17 (μg/ml)			*D*-BMAP18 (μg/ml)		
Strains	**MIC[a]***	**MBC[a]***	**KQ[b]**	**MIC[a]**	**MBC[a]**	**KQ[b]**
P. aeruginosa						
PA01	8	8	1	8	32	4
RP73	32	32	1	4	8	2
PA03	8	16	2	8	8	1
PA05	4	8	2	4	8	2
PA07	4	8	2	8	8	1
PA08	8	>32	>4	8	16	2
PA09	16	>32	>2	16	32	2
PA10	>32	>32	–	16	32	2
PA14	8	32	4	16	32	2
PA21	8	32	4	16	32	2
PA22	2	8	2	4	32	8
PA31	8	16	2	16	32	2
S. maltophilia						
SM103	4	8	2	4	4	1
SM105	8	>32	>4	8	8	1
SM106	>32	>32	–	32	32	1
SM110	4	8	2	4	4	1
SM120	16	>32	>2	8	16	2
SM122	>32	>32	–	16	16	1
SM123	32	32	1	16	16	1
SM126	32	>32	>1	16	32	2
SM130	8	8	1	8	16	2
SM136	8	8	1	8	16	2
SM139	8	16	2	8	8	1
SM143	4	16	4	8	8	1
S. aureus						
SA1	>32	–	–	>32	–	–
SA2	>32	–	–	>32	–	–
SA3	>32	–	–	>32	–	–
SA4	32	–	–	>32	–	–
SA5	32	–	–	>32	–	–
SA7	32	–	–	>32	–	–

Minimum inhibiting (MIC) and minimum bactericidal (MBC) concentrations are shown. Results are from three independent experiments performed as internal duplicates (n = 6).
**These data were already published (Mardirossian et al., 2016).*
[a] MIC and MBC values are expressed as μg/ml.
[b] KQ, killing quotient, measured as MBC/MIC ratio: KQ ≤ 4 is suggestive for bactericidal effect, KQ > 4 is suggestive for bacteriostatic effect.

In vitro D-BMAP18 Cytotoxicity

Cytotoxicity of *D*-BMAP18 and *L*-BMAP18 was evaluated, by the MTT assay against human pulmonary A-549 epithelial cells, to simulate the toxicity toward the host pulmonary cells.

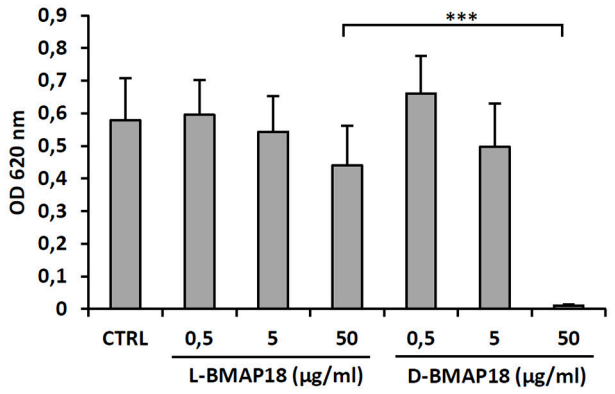

FIGURE 2 | Cytotoxicity of *L*- and *D*-BMAP18 toward A-549 human pulmonary cells, as assessed by MTT assay, cells following 24 h-exposure to *L*- and *D*-BMAP18. In control samples (CTRL), cells were exposed to the vehicle only. Results, from at least two independent experiments performed as internal triplicate ($n \geq 6$), are shown as mean + *SD*. ***$p < 0.001$, ANOVA + Student-Newmann-Keuls post-test.

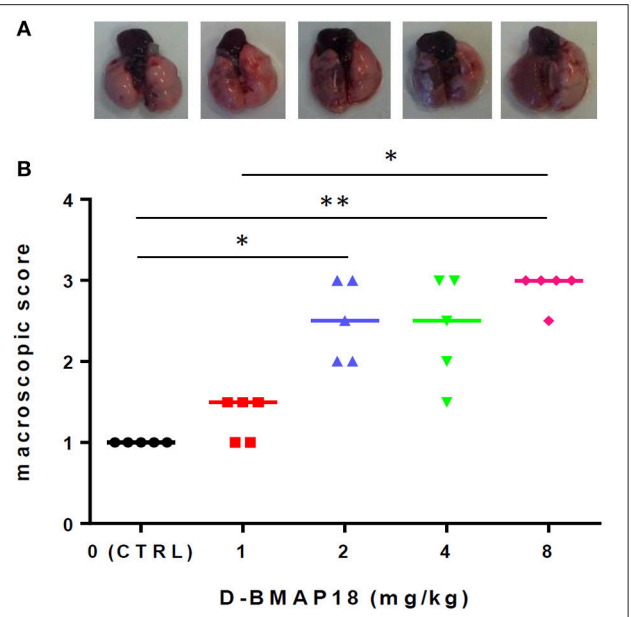

FIGURE 3 | *In vivo* toxicity study: macroscopic damage in C57BL/6NCrl mouse lungs ($n = 5$/group) following a single exposure to *D*-BMAP18 (1, 2, 4, and 8 mg/kg), or to vehicle (H_2O) only (CTRL). **(A)** Representative lung photograph for each condition are shown. **(B)** Macroscopic lung pathology was assessed on day 5 p.e. by using a "four-point scoring system" (Johansen et al., 1993): (1) normal; (2) swollen lungs, hyperemia, and small atelectasis; (3) pleural adhesion, atelectasis, and multiple small abscesses; and (4) large abscesses, large atelectasis, and hemorrhages. Horizontal bars are median values. Representative photographs for each condition are shown. *$p < 0.05$, **$p < 0.01$, Kruskal-Wallis + Dunn's multiple comparison post-test.

Both peptides did not significantly affect cell viability at a concentration of 5 µg/ml (**Figure 2**) and became cytotoxic only at 50 µg/ml. At this concentration, *D*-BMAP18 was, unexpectedly, more cytotoxic than the *L*-isomer (**Figure 2**), probably because the *L*-form of the peptide was more easily degraded by extracellular proteases secreted by cells, and therefore its cytotoxic effect was lower.

In vivo Acute Toxicity of *D*-BMAP18

We assessed the toxicity of *D*-BMAP18 in C57BL/6NCrl mice after a single intratracheal instillation of peptide at increasing concentrations. Macroscopic lung pathology was assessed on day 5 post exposure by using a "four-point scoring" system (**Figure 3**). No pulmonary damage was observed in unexposed or 1 mg/kg-treated mice, as assessed by the macroscopic score index evaluation. On the contrary, exposure to 2 and 8 mg/kg doses significantly damaged lungs (median score: 2.5 vs. 3; $p < 0.05$ and $p < 0.01$, respectively), as indicated by extensive hyperemia, atelectasis, and pleural adhesion.

Changes in mice and pulmonary weight (Figures S1A,B) were measured over 5 days p.e.. Exposure to the peptide caused significant reduction in body weight also at the lowest dose, and this effect is dose-dependent. Variations in pulmonary weight confirmed the same trend found for macroscopic score analysis.

In vivo Protective Effect of *D*-BMAP18

We assessed the *in vivo* antimicrobial potential of *D*-BMAP18 in a murine model of acute pulmonary infection caused by *P. aeruginosa*. On day 1 post-infection, mice and mice lungs weight, as well as macroscopic lung pathology and pulmonary bacterial load were assessed (Figure S2 and **Figure 4**).

No significant differences in mice body weight were observed on day 1 post-exposure, regardless of the group considered (not shown). A positive trend was found between *D*-BMAP18 dose and macroscopic damage, although it was not statistically significant because of the relevant data dispersion (**Figure 4B**). The lungs of mice treated with *D*-BMAP18 at 1 and 2 mg/kg were slightly more edematous, as suggested by the observed weight, compared to those administrated with tobramycin 10 mg/kg (Figure S2). The administration of *D*-BMAP18 was not protective against *P. aeruginosa* RP73 infection, regardless of the considered doses. The bacterial load measured in mice treated with 2 mg/kg *D*-BMAP18 was significantly higher than that observed in tobramycin-treated lungs (**Figure 4C**). Mortality was observed only in one tobramycin-treated mouse (1 out of 8; 12.5%).

Antibacterial Activity of *D*-BMAP18 in Presence of BAL Fluid

To explain the moderate *in vivo* antibacterial activity of *D*-BMAP18 despite its increased stability to proteolysis, to exclude procedural mistakes during *in vivo* assays, and to exhaustively evaluate its antimicrobial efficacy as a drug intended for pulmonary applications, we looked for conditions roughly approximating the lung environment used for *in vivo* experiments. Therefore, we performed a bacterial killing assay on *P. aeruginosa* RP73 incubating bacteria and peptide in presence of BAL fluid at 37°C. As a control, we used sterile 0.9% (w/v) NaCl, since it was used to wash the murine lung for collecting BAL. We observed that BAL fluid decreased the antimicrobial

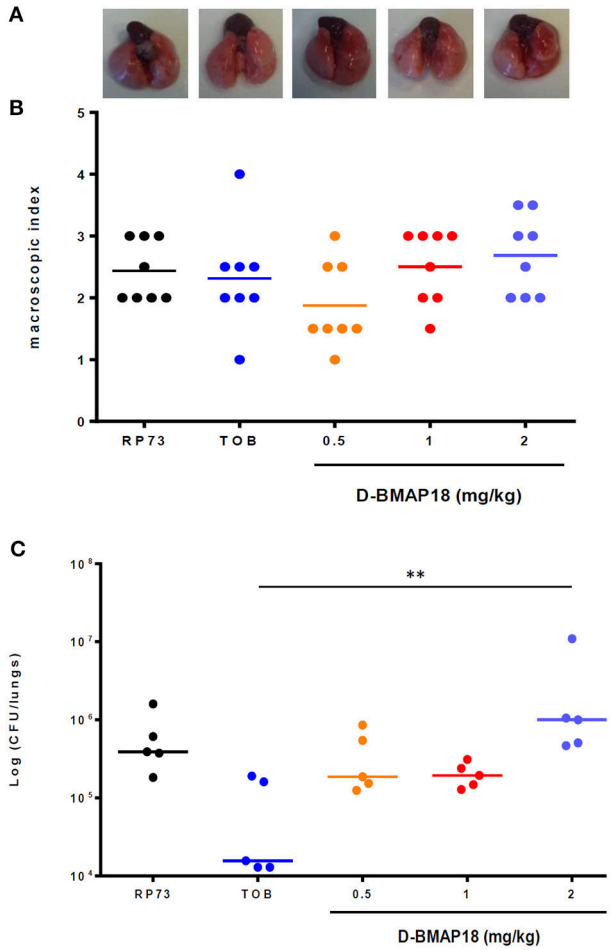

FIGURE 4 | *In vivo* protection study: macroscopic damage and bacterial load of C57BL/6NCrl mouse lungs measured on day 1 following *P. aeruginosa* RP73 infection. After infection, mice ($n = 8$/group) were administered with *D*-BMAP18 (0.5, 1, and 2 mg/kg) or Tobramycin (TOB) 10 mg/kg. Mice challenged with *P. aeruginosa* RP73, but not treated, were used as control group. **(A)** Representative lungs photographs for each condition are shown. **(B)** Macroscopic lung pathology was assessed on day 1 p.e. by using a "four-point scoring system" (Johansen et al., 1993) (see **Figure 3**). For each group of mice, the lung of 3 mice out of 8 were fixed to perform future histological analyses. Horizontal bars are median values ($n = 8$/group). Kruskal-Wallis + Dunn's multiple comparison test showed no significant differences. **(C)** Lungs of the remaining mice (5 out of 8) were homogenized and plated onto MH agar to assess viable bacteria. Bacterial colony counts from each mouse were expressed as CFU/lungs. Horizontal bars are median values. $^{**}p < 0.01$, Kruskal-Wallis + Dunn's multiple comparison post-test.

efficacy of 16 μg/ml *D*-BMAP18, and abolished its activity when used at 8 μg/ml (**Figure 5**). We hypothesized that this inhibition may be due to peptide binding/sequestration by BAL components, given its stability under the tested conditions. When the assay was repeated after adding 300 mM NaCl to the medium, the antimicrobial activity was partially restored (**Figure 5**) suggesting that electrostatic interaction between the components of BAL and the peptide may play a role in the inhibition.

DISCUSSION

The bacterial pathogens causing CF lung infections represent a life threat for patient because of their resistance to antibiotics, and because of their adaptation to the peculiar characteristic of CF-lungs. Antimicrobial peptides may potentially be effective compounds to combat pulmonary infections, but some aspects of their use need to be further analyzed and eventually optimized for future applications (Marr et al., 2006). In a previous study, we demonstrated that BMAP18 has a promising antibacterial activity against both *P. aeruginosa* and *S. maltophilia*. However, the peptide is rapidly degraded in murine pulmonary fluid, suggesting it may be unstable also in human lungs (Mardirossian et al., 2016). In this study, to overcome the degradation problems, we synthesized and tested an all-D isomer of BMAP18. The artificial introduction of *D*-amino acids in peptides has been widely used to avoid proteolysis in biological samples (Sajjan et al., 2001; Hamamoto et al., 2002). Moreover, the presence of single *D*-aminoacids into the structure of L-antimicrobial peptides has been shown to affect their structuring as α-helices. These modifications reduced on the one hand their activity on the cellular membrane, and therefore their cytotoxicity, but on the other hand, also their efficacy to bind to and insert into the LPS layer of the bacterial outer membrane, therefore decreasing their antimicrobial potency (Ghosh et al., 2016). We showed that *D*-BMAP18 is a possible alternative to the *L*-form to avoid proteolysis without losing its antibacterial potential. Our results in fact indicate that *D*-BMAP18 is stable when exposed to protease-rich BAL fluid from mice and, most importantly, its antimicrobial activity is comparable with that exhibited by the *L*-isomer. It is worth noting that often the MBC of both *D*-BMAP18 and *L*-BMAP18 is only twice the MIC, indicating that these AMPs possess a bactericidal activity. This makes these peptides even more attractive for the treatment of persistent infections. Interestingly, the *D*-isomer exhibited higher activity against *P. aeruginosa* RP73 and PA10 strains compared to the L-isomer. It is believed that *D*-analogs of membranolytic alpha helical AMPs are equipotent to the naturally occurring all-L peptides (Wade et al., 1990; Giangaspero et al., 2001). We therefore think that the different MICs we observed for RP73 and PA10 strains could be due to the different capacity of strains to inactivate or degrade the L-form. We also showed that the *D*-peptide, *in vitro,* is not toxic up to 5 μg/ml against human pulmonary A-549 epithelial cells. However, at higher concentrations (50 μg/ml), the *D*-BMAP18 becomes more toxic than the L-form. This additional toxicity may be linked to the failure of eukaryotic cells to selectively degrade *D*-peptide during the incubation, but we cannot exclude other reasons. A certain toxic effect of *D*-BMAP18 was confirmed also by acute toxicity test in lungs of mice, showing a concentration-dependent trend. We think that the route of administration could contribute to the toxicity. In fact the intra-tracheal administration route temporarily exposes narrow regions of the lung tissues to high, and presumably toxic, peptide concentrations. This effect could mask the beneficial antibacterial activity of the molecule. Microaerosol administration, or alternative devices, could be considered for further assays (Valenti et al., 2017). As a further strategy to

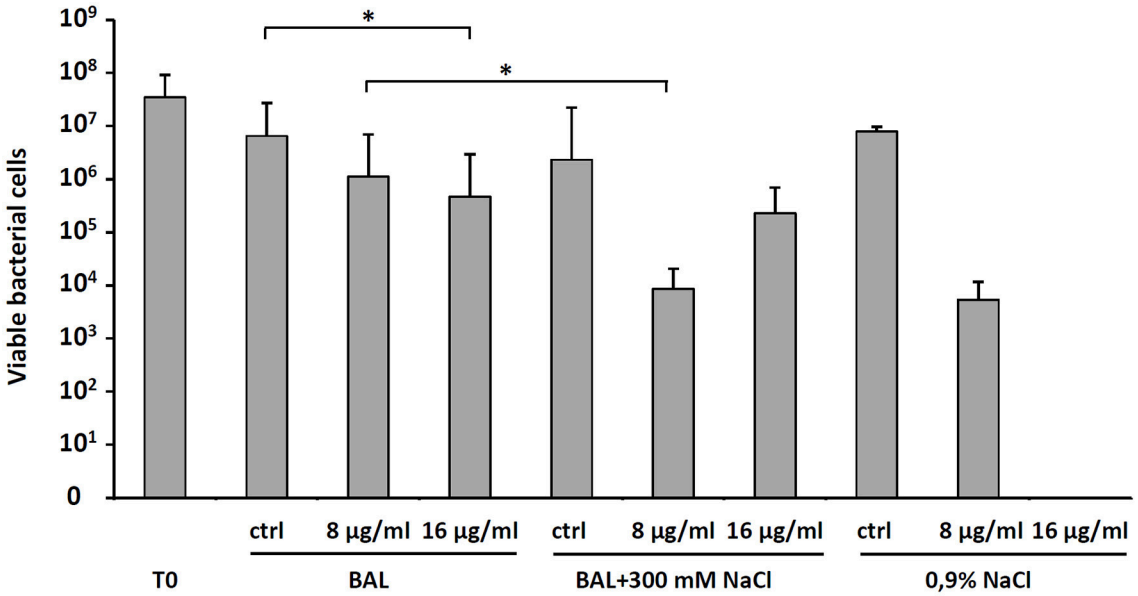

FIGURE 5 | Effect of bronchoalveolar lavage (BAL) without or with the addition of 300 mM NaCl, or 0.9% NaCl on the *D*-BMAP18 activity against *P. aeruginosa* RP73 strain. Peptide has been used at 8 and 16 ug/ml and bacterial killing was evaluated by viable cell count. In control samples, *P. aeruginosa* RP73 was exposed at H_2O only. Results are expressed as mean values + SDs ($n = 6$). *$p < 0.05$, ANOVA + Student-Newmann-Keuls post-test.

decrease the *D*-BMAP18 cytotoxicity, is now under investigation its administration as a cleavable pro-drug, in order to guarantee a controlled release of the drug. This stratagem could maintain the peptide's potent antimicrobial effect while mitigating the *D*-BMAP18 toxic effects on the pulmonary epithelium.

In light of the *in vitro* activity against *P. aeruginosa* and the acute *in vivo* toxicity, we assumed to have a sufficient therapeutic window to test the effectiveness of the peptide against a murine *P. aeruginosa* acute pulmonary infection. We considered concentrations around 1 mg/kg as safe to perform the *in vivo* efficacy tests. In any case, none of the peptide concentrations used, could significantly decrease the bacterial load in murine lungs. As weak effect was also observed with tobramycin, it is clear that factors other than proteolysis seem to inhibit the *in vivo* activity of the tested peptide. We indeed showed that the presence of murine BAL fluid markedly decreases, but does not abolish, the *in vitro* antimicrobial activity of *D*-BMAP18 against *P. aeruginosa* RP73. Murine BAL contains high amounts of lipids, mainly phospholipids, and a low amount of proteins (Goerke, 1998). It is known that lipids dispersed in aqueous solution induce α-helical AMPs to assume their amphipathic α-helix structures (Tossi et al., 2000). It is plausible that *D*-BMAP18 could be attracted mainly by surfactant phospholipids instead of bacterial lipopolysaccharide, forced to its active conformation by the lipids, and hence sequestered. Moreover, anionic mucin glycoproteins are also contained in BAL (Ballard and Inglis, 2004), which could potentially contribute to *D*-BMAP18 sequestration. In any case, the effects of BAL fluid on AMPs activity are still poorly understood and, in some cases, controversial. Some authors reported that the presence of BAL fluid did not interfere with antimicrobial activity of CaLL, an α-helical chimeric derivative of LL-37 and Cecropin A (Morris et al., 2012) with similar size to BMAP18. Apparently, this finding is in contrast with our observations, even though the different concentrations used (respectively 100 µg/ml vs. 8–16 µg/ml) do not allow a direct comparison between data. Conversely, Forde et al. showed that BAL fluid negatively interferes with bactericidal activity of different host defense peptides pro-drug (Forde et al., 2014), but the antimicrobial activity could be partially restored by increasing the ionic force of the medium. Interestingly, we observed a similar behavior using *D*-BMAP18. We previously observed that BMAP peptides, in contrast to most AMPs, continue to be effective also in hypertonic buffers (unpublished), and here demonstrated that the addition of 300 mM NaCl to BAL is favorable for the activity of *D*-BMAP18. High salts concentrations possibly counteracted the putative electrostatic sequestration of the peptide by BAL fluid components, as also previously suggested (Forde et al., 2014, 2016) and could open the possibility to use this molecule also in combination with hypertonic saline solutions, already used in the clinic (Reeves et al., 2012).

In conclusion, we showed that *D*-BMAP18 is an effective AMP against CF-related Gram-negative pathogens, being stable in biological fluid such as murine BAL, and not cytotoxic at low micromolar concentrations. However, we also demonstrated that, despite these desirable properties, *D*-BMAP18 is not yet suitable for *in vivo* applications, requiring additional studies for its optimization and lung delivery.

In this study we highlighted some critical points that should be addressed for designing AMPs suitable for pulmonary infections: (i) peptides are often prone to proteolytic digestion in the lungs, and this problem should not be underestimated regardless of

the structural diversity of the studied peptide from that of other peptides known to be degraded; (ii) the composition of pulmonary fluid plays an important role: under these conditions, the AMP should maintain its specificity for bacteria and its antimicrobial potential; (iii) the assessment of the protective activity in a pulmonary infection model is a crucial step to subsequently focus on other variables that could determine the success of the peptide. These considerations may help drawing a route to have more chances in obtaining effective compounds for the fight against antibiotic-resistant pathogens.

AUTHOR CONTRIBUTIONS

MM and MD synthesized the peptide, performed the microbiological and biochemical experiments *in vitro*, MD, GR and SP performed the cytotoxicity experiments. AP and GDB performed the *in vivo* toxicity assay and protective studies. MM, MS, AP, GDB and MD wrote and edited the manuscript. MM and MS designed the experiments. MS supervised the whole project.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fchem.2017.00040/full#supplementary-material

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Promising Approaches to Optimize the Biological Properties of the Antimicrobial Peptide Esculentin-1a(1–21)NH_2: Amino Acids Substitution and Conjugation to Nanoparticles

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Antimicrobial peptides (AMPs) represent an interesting class of molecules with expanding biological properties which make them a viable alternative for the development of future antibiotic drugs. However, for this purpose, some limitations must be overcome: (i) the poor biostability due to enzymatic degradation; (ii) the cytotoxicity at concentrations slightly higher than the therapeutic dosages; and (iii) the inefficient delivery to the target site at effective concentrations. Recently, a derivative of the frog skin AMP esculentin-1a, named esculentin-1a(1–21)NH_2, [Esc(1–21): GIFSKLAGKKIKNLLISGLKG-NH_2] has been found to have a potent activity against the Gram-negative bacterium *Pseudomonas aeruginosa*; a slightly weaker activity against Gram-positive bacteria and interesting immunomodulatory properties. With the aim to optimize the antimicrobial features of Esc(1–21) and to circumvent the limitations described above, two different approaches were followed: (i) substitutions by non-coded amino acids, i.e., α-aminoisobutyric acid or D-amino acids; and (ii) peptide conjugation to gold nanoparticles. In this mini-review, we summarized the structural and functional properties of the resulting Esc(1–21)-derived compounds. Overall, our data may assist researchers in the rational design and optimization of AMPs for the development of future drugs to fight the worldwide problem of antibiotic resistance.

Keywords: antimicrobial peptide, frog-skin, antibiotic-resistance, D-amino acids, gold nanoparticles, *Pseudomonas aeruginosa*

ANTIMICROBIAL PEPTIDES: GENERAL FEATURES

Gene-encoded antimicrobial peptides (AMPs) are evolutionally conserved molecules produced by almost all living organisms (e.g., bacteria, fungi, higher eukaryotes including humans, Ageitos et al., 2016). As part of key effectors of the innate immunity, they act as a sudden response against a multitude of microorganisms before the adaptive immune system comes into action (Boman, 1995; Hemshekhar et al., 2016). Despite their different length and secondary structure ranging from an

α-helix, a β-strand, a loop, or an extended conformation in hydrophobic environments, most of them share an amphipathic and cationic character at neutral pH (Powers and Hancock, 2003). These two properties are crucial factors, especially for the mechanism of action of α-helical AMPs, which is generally based on the perturbation of the target microbial membrane (Bechinger and Gorr, 2017). More specifically, it consists in an initial electrostatic interaction between the positively-charged AMP and the negatively-charged components of the microbial cell surface, such as lipoteichoic acids in Gram-positive bacteria, or lipopolysaccharides (LPS) in Gram-negatives, to finally reach the plasma-membrane. This is then destabilized by pores formation/local cracks or disintegration in a detergent-like manner, with consequent cell death (Hall and Aguilar, 2010). Peptide-membrane interaction is the most important step controlling the selectivity of AMPs toward microbial membranes, which are much richer in anionic phospholipids compared to those of mammalian cells mainly made of electrically-neutral (zwitterionic) lipids (Oren et al., 1999). However, peptide-membrane interaction is dictated not only by the peptide's cationicity, but also by other physiochemical parameters of AMPs encompassing their length, hydrophobicity, amphipathicity, and helicity (Marín-Medina et al., 2016). Remarkably, unlike conventional antibiotics, this non-specific mechanism of action of AMPs very rarely induces resistance (Bechinger and Gorr, 2017) and makes them an interesting class of molecules for the development of new antimicrobial compounds (Mazer-Amirshahi et al., 2016). To date, thousands of AMPs have been characterized from a variety of natural sources as well as their synthetic derivatives (Liu et al., 2016). Noteworthy, an increasing number of AMPs has already entered into advanced stages of clinical trials for topical treatment of different types of infections. Nevertheless, several limitations can hinder their development as new therapeutics (da Cunha et al., 2017). Among them: (i) the cytotoxicity at concentrations slightly higher than antimicrobial dosing; (ii) the low peptide biostability due to fast proteolytic degradation; and (iii) the inefficient delivery to the target site at effective concentrations (Fjell et al., 2012). Nowadays, thanks to the progress in computational studies and nanotechnologies, it is possible to circumvent these issues. In this mini-review article, after a brief overview on amphibian AMPs and the structural/functional relationships of the frog skin-derived AMP esculentin-1a(1–21)NH_2, Esc(1–21), we will mainly focus on the principal approaches that have been used to optimize the biological properties of Esc(1–21): (i) substitution by non-coded amino acids and (ii) conjugation to inorganic nanoparticles.

AMPHIBIAN SKIN ANTIMICROBIAL PEPTIDES

Among natural storehouses of AMPs, frog skin is one of the richest (Conlon, 2011b). The expression of genes encoding for these peptides in dermal serous glands is induced upon contact with microorganisms (Mangoni et al., 2001); and the produced AMPs are stored within granules that are released onto the skin surface in a holocrine mechanism after stress or tissue injury (König et al., 2015). It was first discovered that amphibian AMPs do not only protect the host from invading microbial pathogens, but also regulate the animal's natural flora (Simmaco et al., 1998); a trait which has then been confirmed also for human AMPs (Mangoni et al., 2016). Over the years, since the discovery of magainins from the skin of *Xenopus laevis* (Zasloff, 1987), an increasing number of AMPs has been identified from different Anuran species (Coccia et al., 2011; Conlon, 2011a). In particular, from various *Rana* genera, a large number of AMPs has been isolated and characterized. On the basis of their common structural features, they have been classified into several families encompassing brevinins-1, brevinins-2, nigrocins, temporins, esculentins-1, and esculentins-2 (Conlon et al., 2004).

Esculentin-1a(1–21)NH_2: Synthesis and Characterization

All members of the esculentin-1 family have a primary structure composed of 46 amino acids and contain a C-terminal hepta-membered ring stabilized by a disulfide bridge (Mangoni et al., 2015). They adopt an amphipathic α-helix structure in membrane mimicking environments and have a net charge of +5 at neutral pH (Wang et al., 2016). AMPs belonging to the esculentin-1 family were initially isolated and purified by reverse-phase high performance liquid chromatography (RP-HPLC) from the cutaneous secretions of *Pelophylax lessonae/ridibundus* (previously classified as *Rana esculenta*) specimen (Simmaco et al., 1993). They have a highly conserved C-terminal half and differ by only one or two residues in the N-terminal region (Simmaco et al., 1994). Interestingly, a fragment corresponding to the 19–46 portion of esculentin-1 peptides was isolated from one of the HPLC fractions but it was devoid of antimicrobial activity, probably due to its low net positive charge (+1 vs. +5 of the full-length AMP, Simmaco et al., 1994). It was then investigated whether the antimicrobial activity was retained in the N-terminal half of the molecule. For this reason, a peptide corresponding to the 1–18 fragment of esculentin-1b was chemically synthesized and amidated at its C-terminus (Mangoni et al., 2003). Note that the C-terminal amidation is a common post-translational modification in linear AMPs from frog skin (Nicolas and El Amri, 2009). This peptide, named Esc(1–18), had a comparable antimicrobial activity to that of the full-length parent esculentin-1, but a lower hemolytic capacity (Mangoni et al., 2003).

Structurally, Esc(1–18) was found to adopt an α-helix structure in lipid vesicles mimicking the anionic character of microbial membranes (Mangoni et al., 2003). It rapidly killed bacteria (e.g., *Escherichia coli*) within 15–20 min with concomitant leakage of cytosolic material, presumably due to the formation of transient membrane-breakages (Marcellini et al., 2009). Since the minimum length for a peptide in α-helix conformation to span a phospholipid bilayer (∼30 Å thick) is about 20 amino acids, a longer analog named esculentin-1a(1–21)NH_2, Esc(1–21), was further synthesized and characterized for its biological properties. Esc(1–21) shares the first 20 residues with esculentin-1a (**Figure 1**) followed by an amidated

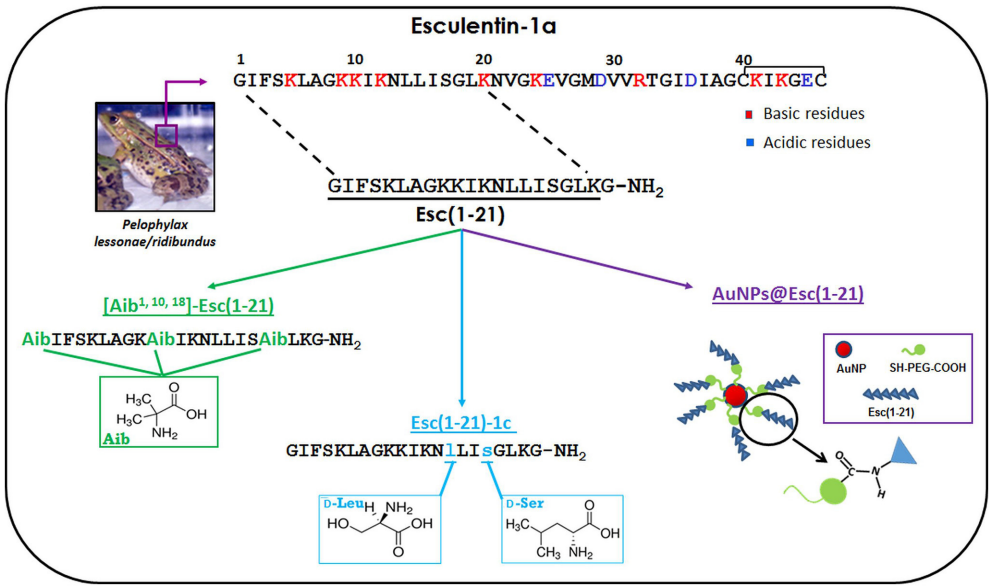

FIGURE 1 | Schematic representation of the amino acids substitutions used to design the Esc(1–21)-analogs and a representative image of the peptide-conjugated AuNPs.

glycine (Islas-Rodrìguez et al., 2009). It differs from Esc(1–18) for having (i) an Ile residue at position 11 instead of a Leu and (ii) three additional C-terminal residues (Leu-Lys-Gly) which confer it a higher net positive charge at neutral pH (+6). This should strengthen the electrostatic interaction of the peptide with the negatively-charged membrane of microbial cells. Indeed, Esc(1–21) exhibited a higher antimicrobial potency than Esc(1–18) (Mangoni et al., 2015), especially against Gram-negative bacteria, e.g., the opportunistic pathogen *Pseudomonas aeruginosa* (Gellatly and Hancock, 2013). Esc(1–21) displayed a quick bactericidal activity within 15 min against both reference and clinical isolates of *P. aeruginosa* with concentrations causing 99.9% killing between 0.5 and 1 μM in physiological solution (Luca et al., 2013). Differently, a weaker activity was detected against Gram-positive bacteria, as pointed out by the higher values of minimum inhibitory concentration (MIC) compared to those recorded toward Gram-negatives (Kolar et al., 2015).

Among other interesting biological features, Esc(1–21) was found to have the ability (i) to hinder the secretion of the pro-inflammatory cytokine TNF-α from *P. aeruginosa* LPS-stimulated immune cells and (ii) to induce re-epithelialization of a pseudo-"wound" area generated in a monolayer of keratinocytes, the most abundant cells in epidermis (Haslam et al., 2014), at a faster rate than the mammalian AMP LL-37 (Di Grazia et al., 2015b). This is a relevant matter, which is not shown by any traditional antibiotic. Note that the healing of an injured infected tissue does not only require elimination of microbial pathogens but also the recovery of the tissue integrity along with its barrier function to prevent pathogens penetration.

Nevertheless, Esc(1–21) is not free from the restrictions mentioned above. In the following sections, we summarize the outcome of two different rational approaches employed to increase the biostability of this peptide and to decrease its cytotoxicity without compromising its antimicrobial efficacy: modification of Esc(1–21) by non-coded amino acids i.e., α-aminoisobutyric acid (Aib) or D-amino acids, and (ii) its conjugation to gold nanoparticles (AuNPs). All these modifications are represented in **Figure 1**.

The Analog [Aib^{1,10,18}]-Esc(1–21)

The Aib residue is a non-natural amino acid mainly used to increase the stability of α-helix conformation (Bellanda et al., 2001). Due to their strong helicogenicity (De Zotti et al., 2012a), when Aib residues are inserted into the primary structure of peptides, they are expected to increase their helical content and to protect them from proteolytic attack (Rink et al., 2010). The usage of Aib residues is also expected to enlarge the spectrum of activity of Esc(1–21). This is because a stabilized α-helix structure is reported to be correlated to the AMPs' activity against Gram-positive bacteria (Giangaspero et al., 2001) toward which Esc(1–21) is not particularly active. The Aib-analog was synthesized by replacing three amino acids in positions 1, 10, and 18 with Aib residues (**Figure 1**). This replacement was rationally designed on the basis of the following considerations: (i) an Aib residue in position 1 should prevent enzymatic degradation by aminopeptidases, while the protection of the C-terminus from carboxypeptidases would not be necessary, due to the presence of an amidated glycine in Esc(1–21) (Rink et al., 2010); (ii) Aib residues in positions 10 and 18 should contribute to stabilize the α-helix structure, due to their stronger α-helix-promoting activity when placed internally to the primary structure of a peptide; (iii) according to an ideal α-helix folding of Esc(1–21), it was possible to identify an hydrophobic face and an hydrophilic one (Biondi et al., 2016). It is known that in naturally-occurring

Aib-rich peptides, such as peptaibiotics, Aib residues are located either within the hydrophobic face or at its boundary with the hydrophilic one (Toniolo et al., 1994; De Zotti et al., 2012b).

The secondary structure of both Esc(1–21) and its [Aib^{1,10,18}]-Esc(1–21) was initially investigated by circular dichroism (CD) (Biondi et al., 2016) in water and two different membrane-mimicking environments e.g., sodium dodecyl sulfate (SDS) aqueous solution and trifluoroethanol (TFE). The results confirmed that both peptides adopted an unordered conformation in water and an α-helix structure in both SDS and TFE. However, at increasing concentration of TFE (from 20 to 50%) the helical content in [Aib^{1,10,18}]-Esc(1–21) sharply increased with respect to the parent peptide. The helical and less flexible structure of [Aib^{1,10,18}]-Esc(1–21) compared to Esc(1–21) was also confirmed by 2D-NMR analysis in TFE solution (Biondi et al., 2016).

Overall, the greater stability and content of α-helix in the Aib-analog were found to influence the biological properties of the peptide. More precisely, the Aib-analog gained an overall higher activity against Gram-positive bacteria, especially those belonging to *Staphylococcus* genus [MIC of 2–4 vs. 16–64 μM of Esc(1–21)] without losing its efficacy against Gram-negative bacteria and *Candida* species (Biondi et al., 2016). It is possible that differences in the composition of the membrane or cell wall among these microorganisms account for the different activity of the two esc-peptides against them.

However, a higher α-helicity in [Aib^{1,10,18}]-Esc(1–21) resulted in increased cytotoxicity against mammalian cell lines (e.g., alveolar epithelial cells and keratinocytes). The experimental data showed that, at the antimicrobial concentrations, Esc(1–21) and [Aib^{1,10,18}]-Esc(1–21) were harmless to human cells, while at higher concentrations the cytotoxic effect of the Aib-analog became clearly evident in comparison with the parent Esc(1–21) (Biondi et al., 2016). This is consistent with the notion that both α-helix conformation and its stability are crucial parameters for mammalian membrane perturbation and cell lysis, likely assisting the peptide's penetration into the hydrophobic core of phospholipids bilayers (Shai and Oren, 1996).

The Analog Esc(1–21)-1c

With the aim to reduce the cytotoxicity and to protect Esc(1–21) from proteolytic degradation, another analog carrying two D-amino acids was synthesized: Esc(1–21)-1c. It was obtained by replacing two L-amino acids i.e., Leu^{14} and Ser^{17} with the corresponding D-amino acid enantiomers (**Figure 1**). This diastereomer was rationally designed on the basis of the following considerations: (i) D-amino acids are known to be "α-helix breakers" (Grieco et al., 2013) and a reduction in the α-helix content of the peptide should reduce its propensity to perturb mammalian membranes leading to cell death (Strahilevitz et al., 1994); (ii) previous studies on the shorter analog Esc(1–18) pointed out that in electrically-neutral lipid vesicles the peptide adopted an α-helix conformation at its C-terminal half. With the purpose to disrupt at least the first turn of the α-helix expected to be present in the C-terminal half of Esc(1–21) in mammalian cell membranes, analogously to what found for Esc(1–18), replacement of two L-amino acids with the corresponding D-enantiomers was carried out at position 14 and 17. Note that it was improbable that the C-terminal tail Gly18-Gly21 of Esc(1–21) folded in a stable helical conformation.

The stability of both isomers was initially examined in the presence of 10 and 30% fresh human serum after 24 h incubation at 37°C. The data revealed that in comparison with Esc(1–21) <50% of the diastereomer was degraded (Di Grazia et al., 2015a). Besides, the presence of these two D-amino acids made the peptide significantly more resistant to the proteolytic cleavage caused by both human and bacterial elastases (Cappiello et al., 2016).

When the structure of the two peptides was analyzed by CD in lysophosphatidylcholine (LPC), which simulates the zwitterionic nature of mammalian cell membranes, a loss of α-helix structure was clearly detected for Esc(1–21)-1c (Di Grazia et al., 2015a). In contrast with data obtained for the Aib-analog, this diastereomer was significantly less toxic than Esc(1–21) against mammalian cells, either circulating cells (e.g., erythrocytes, macrophages) or epithelial cells. More precisely, its LD_{50} was higher than 256 μM in comparison with a LD_{50} ranging from 64 to 150 μM for the all-L peptide toward macrophages and epithelial cells, respectively (Di Grazia et al., 2015a; Cappiello et al., 2016). Interestingly, the introduction of these two residues in the D-configuration also conferred the peptide: (i) a higher tendency than the all-L counterpart to kill *P. aeruginosa* biofilms at concentrations lower than 25 μM (despite the diastereomer had a slightly reduced bactericidal activity against the free-living form of this pathogen); and (ii) a higher "wound" healing activity *in vitro* (Di Grazia et al., 2015a).

AuNPs@Esc(1–21)

Amino acids replacement is not the only strategy to increase the stability of a peptide to proteolytic degradation. Moreover, this approach does not allow a peptide to overcome biological barriers (e.g., mucus, skin layers) before reaching the site of infection at high active concentrations (d'Angelo et al., 2015). A different biochemical approach to also assist drug delivery at effective concentrations is given by its conjugation to nanoparticles (NPs). This would enable not only to protect the drug from the external environment but also to increase its local concentration.

Among the various NPs produced in recent years, AuNPs have attracted most attention due to their small size, high solubility, stability, biocompatibility, and chemical inertness (Connor et al., 2005). They can diffuse through all layers of human skin (Williams et al., 2006) and because of their large surface area, they can be functionalized with a high number of molecules (Yih and Al-Fandi, 2006; Pietro et al., 2016; Soica et al., 2016). Nevertheless, only a limited number of studies has been reported to date on the effects of conjugation of AMPs to AuNPs (Rai et al., 2016). By using Esc(1–21) as a model peptide, it was demonstrated for the first time how a chemical conjugation of an AMP via polyethylene glycol (PEG) linker to AuNPs increases its antimicrobial activity while retaining its mode of action without becoming toxic to human keratinocytes. AuNPs were synthetized by the citrate reduction of gold and stabilized with a bifunctional PEG bearing a thiol and a carboxylic group. The PEG was

TABLE 1 | Structural properties and biological features of the designed Esc(1–21)-derived compounds.

Compound	Structural Properties*	Biological Features*
[Aib^{1,10,18}]-Esc(1–21)	• Higher α-helical content in the secondary structure	• Same activity against Gram-negative bacteria and yeasts • Higher activity against Gram-positive bacteria • Higher cytotoxicity against mammalian cells
Esc(1–21)-1c	• Lower α-helical content in the secondary structure	• Higher resistance to proteolytic degradation • Slightly lower activity against the planktonic form of *P. aeruginosa* • Higher activity against the sessile form of *P. aeruginosa* • Lower cytotoxicity against mammalian cells • Greater efficacy in promoting migration of human lung epithelial cells
AuNPs@Esc(1–21)	• Conjugation to AuNPs *via* PEG linker (∼16 peptide molecules per AuNP@PEG)	• Higher activity against both planktonic and sessile forms of *P. aeruginosa* • Higher resistance to trypsin degradation • Invariant membrane-perturbing activity • Negligible cytotoxicity on human keratinocytes • Similar ≪wound≫ healing effect

**with respect to Esc(1–21).*

attached to the AuNPs via a gold-thiol bond (AuNPs@PEG), while the carboxylic group was used for further derivatization with the peptide via carbodiimide-mediated coupling (Casciaro et al., 2017).

Remarkably, the obtained AuNPs@Esc(1–21) resulted to be more active than the free peptide against both planktonic and sessile forms of *P. aeruginosa*. This was indicated by the corresponding minimal concentrations causing 50% killing of both bacterial phenotypes which were found to be ∼15-fold lower than those of the free Esc(1–21). This is presumably due to the higher concentration of peptide molecules at the site of bacterium-NP contact, as visualized by electron microscopy images which evidenced how these AuNPs@Esc(1–21) form clusters at various points on the bacterial surface with disruption of the membrane and leakage of cytosolic material. Otherwise, our unconjugated bare-AuNPs did not show any anti-pseudomonal activity and were not detected around bacterial cells (Casciaro et al., 2017). This is in line with the findings that non-functionalized AuNPs are harmless also to other bacterial pathogens (Williams et al., 2006) and suggests that the cationic AMP represents the driving force allowing AuNPs@Esc(1–21) to reach the target site at high concentration. In addition, AuNPs@Esc(1–21) were resistant to proteolytic degradation preserving their antibacterial activity 2 h after treatment with trypsin (Casciaro et al., 2017). Finally, AuNPs@Esc(1–21) were harmless to keratinocytes and retained the peptide's capability to stimulate migration of keratinocytes in a pseudo-"wound" healing assay. Altogether these findings make AuNPs@Esc(1–21) an attractive nano-formulation for topical treatment of skin infections (Casciaro et al., 2017).

CONCLUSIONS

Antibiotic-resistant microbial infections cause thousands of deaths per year worldwide and this necessitates the discovery of new compounds to counter them. In this scenario, AMPs represent promising anti-infective molecules with expanding properties. However, their low biostability, cytotoxic effect at concentrations higher than therapeutic dosages and the difficulty in reaching target sites at active concentration, remain disadvantages that must be overcome. In this mini-review, by using Esc(1–21) as a reference, we have summarized how (i) substitution of natural amino acids by non-coded residues as well as (ii) peptide conjugation to AuNPs represent encouraging methodologies to optimize the biological properties of an AMP. Each synthetized analog/compound showed its own peculiarities according to its structural features (**Table 1**). Overall, the two different approaches should serve as an example to assist and to ameliorate the development of new peptide-based formulation for an efficient treatment of different types of infectious diseases.

AUTHOR CONTRIBUTIONS

BC wrote the review article; FC prepared and assembled the figure/table; MC and MM critically revised the manuscript.

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In vivo Efficacy and Pharmacokinetics of Optimized Apidaecin Analogs

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Proline-rich antimicrobial peptides (PrAMPs) represent promising alternative therapeutic options for the treatment of multidrug-resistant bacterial infections. PrAMPs are predominantly active against Gram-negative bacteria by inhibiting protein expression via at least two different modes of action, i.e., blocking the ribosomal exit tunnel of 70S ribosomes (oncocin-type binding) or inhibiting the assembly of the 50S ribosomal subunit (apidaecin-type binding). The *in vivo* efficacy and favorable biodistribution of oncocins confirmed the therapeutic potential of short PrAMPs for the first time, whereas the *in vivo* evaluation of apidaecins is still limited despite the promising efficacy of apidaecin-analog Api88 in an intraperitoneal murine infection model. Here, the *in vivo* efficacy of apidaecin-analog Api137 was studied, which rescued all NMRI mice from a lethal intraperitoneal infection with *E. coli* ATCC 25922 when administered three times intraperitoneal at doses of 0.6 mg/kg starting 1 h after infection. When Api88 and Api137 were administered intravenous or intraperitoneal at doses of 5 and 20 mg/kg, their plasma levels were similarly low (<3 μg/mL) and four-fold lower than for oncocin-analog Onc72. This contradicted earlier expectation based on the very low serum stability of Api88 with a half-life time of only ~5 min compared to ~6 and ~3 h for Api137 and Onc72, respectively. Pharmacokinetic data relying on a sensitive mass spectrometry method utilizing multiple reaction monitoring and isotope-labeled peptides revealed that Api88 and Api137 were present in blood, urine, and kidney, and liver homogenates at similar levels accompanied by the same major metabolites comprising residues 1–16 and 1–17. The pretended discrepancy was solved, when all peptides were incubated in peritoneal lavage. Api137 was rapidly degraded at the C-terminus, while Api88 was rather stable despite releasing the same degradation products. Onc72 was very stable explaining its higher plasma levels compared to Api88 and Api137 after intraperitoneal administration illuminating its good *in vivo* efficacy. The data indicate that the degradation of therapeutic peptides should be studied in serum and further body fluids. Moreover, the high efficacy in murine infection models and the fast clearance of Api88 and Api137 within ~60 min after intravenous and ~90 min after intraperitoneal injections indicate that their *in vivo* efficacy relates to the maximal peptide concentration achieved in blood.

Keywords: Api88, Api137, *E. coli* ATCC 25922, intraperitoneal infection, intraperitoneal lavage, mass spectrometry, multiple reaction monitoring, organ homogenates

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INTRODUCTION

Antimicrobial peptides (AMPs) are considered as promising alternatives to common antibiotics that threateningly lose their therapeutic potentials against multidrug-resistant pathogens (Fox, 2013; Wang et al., 2015). In contrast to approved therapeutic peptide hormones, antimicrobial peptides have to be administered at much higher doses, which may amplify general drawbacks of peptide therapeutics. For example, the diabetes peptide drugs exenatide and pramlintide are given at daily doses of 20 μg (Byetta®; ∼0.5 μg/kg) and 120 μg (Symlin®; ∼1.5 μg/kg; Heine et al., 2005; Hay et al., 2015), while antimicrobial peptides are usually administered at 50- to 100-fold higher doses (>5 mg/kg) in order to achieve high *in vivo* efficacies (Benincasa et al., 2010; Szabo et al., 2010; Brunetti et al., 2016). This is also true for proline-rich AMPs (PrAMPs), such as insect-derived oncocin and apidaecin analogs that showed protective effects in murine intraperitoneal and intramuscular infections with *Escherichia coli* ATCC 25922 and antibiotic-susceptible and -resistant *Klebsiella pneumoniae* strains (Czihal et al., 2012; Knappe et al., 2012, 2015; Ostorhazi et al., 2014; Schmidt et al., 2016). Mechanistically, cationic AMPs first electrostatically interact with the negatively charged surface of bacteria and—depending on the secondary structure—act on the membrane or reach the periplasmic space possibly by spontaneous translocation (Hancock, 1997; Brogden, 2005; Scocchi et al., 2016). PrAMPs exhibit a therapeutically favorable intracellular mode of action after translocating into the bacterial cytoplasm by utilizing different transporter proteins/complexes, such as SbmA, YjiL/MdtM, and YgdD (Mattiuzzo et al., 2007; Runti et al., 2013; Krizsan et al., 2015a; Paulsen et al., 2016). Internalized PrAMPs bind to chaperone DnaK and 70S ribosomes. While DnaK is most likely a binding partner with possible transport functions (Otvos et al., 2000; Knappe et al., 2011b, 2016a; Czihal et al., 2012; Zahn et al., 2013), the 70S ribosome appears to be the major bacterial target. For example, oncocin analogs Onc72 and Onc112 bind with K_d values of 450 and 90 nmol/L, respectively, in a fluorescence polarization assay to the exit tunnel, as recently revealed by X-ray crystallography (Krizsan et al., 2014; Roy et al., 2015; Seefeldt et al., 2015). Apidaecin-derived analogs Api88 and Api137 possess the same sequence, i.e., gu-ONNRPVYIPRPRPPHPRL (O: ornithine, gu: *N*,*N*,*N*′,*N*′-tetramethylguanidino), and differ only by the C-terminal amide and acid groups, respectively. They bind to 70S ribosomes with considerably lower affinities (K_d = 1.2 and 0.56 μmol/L, respectively). Surprisingly, only oncocins significantly inhibit cell free protein expression with IC_{50} values of ∼0.2 μmol/L, whereas both apidaecins and oncocins inhibit protein translation in *E. coli* equally efficient with IC_{50} values of ∼2 μmol/L. This different *in vitro* behavior appears to be related to different inhibition mechanisms. Api137 disturbs the assembly of the 50S ribosomal subunit in *E. coli* leading to a protein complex size of around 42S (Krizsan et al., 2015b). This unique mode of action leads to high antimicrobial activities against *E. coli* ATCC 25922 (MIC = 4 μg/mL) and other Gram-negative bacteria. Both Api88 and Api137 possess *in vitro* very similar antibacterial activities, but Api88 is much faster degraded in mouse serum *in vitro* with a low half-life time of only 5 min compared to 6 h for Api137 (Berthold et al., 2013). Api88 is degraded C-terminally to virtually inactive Api1-17 and Api1-16. Despite its low serum stability *in vitro*, Api88 was highly efficient in a lethal NMRI mouse model of intraperitoneal sepsis providing 100% survival rates at doses of 1.25 mg/kg (Czihal et al., 2012).

In the present study, we investigated the efficacy of Api137 in the same NMRI mouse infection model, which indeed turned out to be even better than for Api88. Focusing on the pharmacokinetics, Api88 and Api137 were quantified by reversed-phase (RP-) HPLC coupled online to an electrospray ionization (ESI) mass spectrometer (MS) utilizing multiple reaction monitoring (MRM) relative to isotope-labeled peptide standards. Both peptides and their two major metabolites were studied in blood, urine, and homogenates of kidney, liver, and brain. Together with previous reports on the pharmacokinetics and *in vivo* efficacy of Onc72 and Onc112 (Knappe et al., 2012; Holfeld et al., 2015; Schmidt et al., 2016) the results presented here for Api88 and Api137 provide the first comprehensive representation of the therapeutic potential of insect-derived PrAMPs.

MATERIALS AND METHODS

Peptides

Apidaecin derivatives (Table S1) were synthesized on solid phase using the 9-fluorenylmethoxycarbonyl/*tert*-butyl (Fmoc/^{t}Bu)-strategy and *in situ* activation with *N*,*N*′-diisopropylcarbodiimide in the presence of *N*-hydroxybenzotriazole as described previously (Czihal et al., 2012; Berthold et al., 2013). Isotope-labeled Fmoc-Pro-OH [97–99% $^{13}C_5$, 97–99% ^{15}N] (Euriso-Top GmbH, Saarbrücken, Germany) was coupled manually overnight using a 1.5-fold excess of the amino acid derivative. Peptides were cleaved with TFA containing a scavenger mixture (12.5% v/v; ethandithiole, m-cresol, water, and thioanisole, 1/2/2/2 v/v/v/v) and afterwards precipitated and washed three times with cold diethyl ether. Peptides were purified by RP-HPLC on a Jupiter C_{18}-column (ID: 10 mm) using a linear aqueous acetonitrile gradient containing TFA (0.1% v/v) as ion pair reagent. Purities were judged by RP-HPLC and the masses were confirmed by MALDI- or ESI-MS.

Mass Spectrometry

A protocol to quantify Api88, Api137, and their major metabolites was established analog to that of oncocin derivatives, reported recently (Schmidt et al., 2016). Briefly, an Alliance® 2695 HPLC system was coupled online to an ESI-QqLIT-MS (4,000 QTRAP®) equipped with a TurboV™ ion-spray source and operated in positive ion MRM mode using the Analyst® 1.6 software. Separation was achieved on a Jupiter C_{18}-column (internal diameter 1 mm, length 150 mm, particle size 5 μm, pore size 300 Å) at 55 °C using a linear 5-min gradient from 2.7 to 20.7% aqueous acetonitrile containing ammonium formate (26 mmol/L, pH 3.0). Further details about instrumentation, MRM optimization, and MS settings are provided as Supplementary Material (Tables S1–S4, Method M1).

Metabolites of Api88 and Api137 were identified on a nanoACQUITY UPLC® System coupled online to an ESI-LTQ Orbitrap XL™-MS (for details see Tables S2, S5). Peptides enriched by solid phase extraction were separated on a BEH C_{18} nanoACQUITY UPLC® column (100 μm internal diameter, 100 mm length, 1.7 μm particle size, 13 nm pore size, 30°C) using an aqueous acetonitrile gradient from 3 to 30% (within 18 min) and 30 to 95% acetonitrile (1 min) in the presence of formic acid (0.1% v/v) at a flow rate of 0.4 μL/min. Differences in the peptide profiles were identified by comparing the extracted ion chromatograms (XICs, $m/z \pm 0.01$) of samples obtained from treated and untreated mice. MassLynx™ software was used to control the nanoACQUITY UPLC® and the Orbitrap MS and Xcalibur™ software to process MS data.

Animals and Housing

Female outbred NMRI mice (Janvier Labs, Saint Berthevin Cedex, France) were housed in an individually ventilated cage system (Ebeco, Castrop-Rauxel, Germany) under pathogen-free conditions with water and standard food (R/M-H, ssniff Spezialdiäten GmbH, Soest, Germany) given *ad libitum*. After acclimatization for at least one week, studies were performed according to the guidelines for the Care and Use of Laboratory Animals and the study was approved by the Animal Care and Usage Committee of the state agency Leipzig (Landesdirektion Leipzig, file numbers 24-9168.11/14/37 and 24-9168.11/17/43).

Female outbred CD-1 mice (Innovo, Gödöllő, Hungary) were acclimatized 1 week after arrival in a sterile plastic type 2 cage (Innovo Kft., Gödöllő) on softwood granules with free access to water and sterile pelleted rodent food (Szinbád Ltd., Gödöllő). All animals were cared for and experiments were performed in accordance with the recommendations of the Guidelines for the Care and Use of Laboratory Animals and the study was approved by the Animal Care Committees of Semmelweis University (permission No.: 001/2218-4/2012).

Systemic Septicemia Infection Mouse Model

The acute toxicity of Api137 was studied by administering doses of 20, 40, 80, and 120 mg/kg intraperitoneally (i.p.) four times daily (0, 3, 7, and 24 h) into female NMRI mice (24–32 g, ∼7 weeks old). For *in vivo* efficacy, female NMRI mice (high dose experiment: 24–27.1 g, 6 weeks; low dose experiment: 26.8–30.3 g, 7 weeks) were infected intraperitoneal with *E. coli* ATCC 25922, as described before (Czihal et al., 2012; Knappe et al., 2012). Briefly, each mouse was infected with 9×10^5 bacteria in the presence of 2.5% (w/v) mucin (0.3 mL in total). Mucin restricts acute macrophage activation in the intraperitoneal space and promotes establishment of a reproducible infection (Frimodt-Møller et al., 1999). Mice were observed three times daily for their health status for a total of 5 days post-infection and weighed 1 day before and 1 and 5 days after infection. In accordance with the guidelines of the Animal Care and Usage Committee of the Landesdirektion Leipzig, moribund mice were euthanized.

Apidaecin Api137 (high dose experiment: 2.5 and 1.25 mg/kg body weight; low dose experiment: 0.6 and 0.3 mg/kg body weight) was administered intraperitoneal (0.3 mL) in seven mice per group three times post-infection (1, 4, and 8 h) as antibacterial therapy. Each experiment included seven mice receiving ciprofloxacin (40 mg/kg body weight) and four mice receiving only the vehicle (5% glucose in water, w/v) as positive and negative controls, respectively.

Pharmacokinetics

Female NMRI mice (8–9 weeks, 25–35 g) were injected intraperitoneally (10 mL/kg) with peptides dissolved in sterile PBS (Gibco® Life Technologies™, Darmstadt, Germany) at concentrations of 0.5 or 2 g/L corresponding to doses of 5 or 20 mg/kg body weight, respectively. After 10, 20, 30, 60, or 90 min animals ($n = 7$) were euthanized by carbon dioxide inhalation and terminal bleeding via cardiac puncture. Lithium-heparin plasma ($n = 7$) was prepared and stored at −80°C. Immediately after the final bleed, mice were perfused through the left ventricle with saline (0.9%, 50 mL, ∼3 min) to remove blood from all organs controlled by discoloration of the liver. Organs were removed aseptically and directly frozen in liquid nitrogen. Spontaneously released urine was collected and frozen immediately at −80°C.

Female CD-1 mice (20–23 g weight, 7 weeks) were injected intravenously (10 mL/kg) in the tail vein with peptides (0.5 g/L) dissolved in sterile saline (0.9%, w/v) corresponding to doses of 5 mg/kg body weight. Animals ($n = 4$ per time point and peptide) were euthanized 5, 10, 20, 40, and 60 min post-injection by carbon dioxide inhalation and exsanguination. For further details of sample preparation and recovery experiments see Supplementary Material (Methods M2, M3).

Peptide Stability in Peritoneal Lavage

Peritoneal lavage (1.5–2 mL) were collected from three euthanized female CD-1 mice (14 weeks, 32–33 g) after injecting sterile PBS intraperitoneal (4 mL). Peptides were incubated with aliquots (95 μL) of fresh lavage (75 μg/mL, 37°C, 750 rpm). After 0, 30, and 60 min trichloroacetic acid (25 μL, 15% w/v) was added to the samples to precipitate proteins, incubated on ice (10 min), and centrifuged (5 min, 12.400 × g). Supernatants were neutralized with sodium hydroxide (1 mol/L) and analyzed by RP-HPLC using a Poroshell 120 SB-C_{18}-column (2.1 mm internal diameter, 100 mm length, 2.7 μm particle size, 12 nm pore size, Agilent Technologies Inc., Santa Clara, CA, USA) at 60°C and an aqueous acetonitrile gradient containing trifluoroacetic acid (0.1%, v/v). Absorbance was recorded at a wavelength of 214 nm.

RESULTS

Intraperitoneal Toxicity and Therapy

When Api137 was administered intraperitoneally four times (0, 3, 7, and 24 h) at doses of 20, 40, and 80 mg/kg per injection, none of the seven animals per dose group showed any signs of adverse side effects. Higher doses of 120 mg/kg induced signs of discomfort in some animals and decreased mobility for up to 20 min after administration, but they recovered completely prior to the next treatment and all mice survived the observation period of 5 days.

As reported previously, Api88 administered intraperitoneally rescued all NMRI mice in a lethal *E. coli* ATCC 25922 infection model at doses of 2.5 and 1.25 mg/kg (**Figure 1A**; Czihal et al., 2012). Lower doses of Api88 were not tested, as the scoring of at least four animals in the low dose group (1.25 mg/kg) indicated that a further reduction of the dose would have led to very serious signs of infection and reduced weight (>20%) demanding euthanasia according to the guidelines of the ethical committee. Api137 appeared more efficient in this septicemia model due to the better scores obtained at the same dose and by rescuing all animals at doses of 0.6 mg/kg and higher and still four out of seven mice at a dose of 0.3 mg/kg, which represented approximately the ED_{50} (**Figure 1B**). The higher efficacy of Api137 had no detectable effect on body weights (**Figure 1C**), as the body weight losses of the highest dose groups of Api137 and Api88 (2.5 and 1.25 mg/kg) were comparable. The weight gain after 5 days post-infection lead to values slightly above the pre-infection values. Interestingly, animals treated with 2.5 mg/kg Api88 or Api137 showed similar weight losses as mice treated with ciprofloxacin at the recommended dose of 40 mg/kg within the respective experiment. The comparison of both peptides considering the corresponding ciprofloxacin controls indicates a slightly better outcome regarding the body weights for Api88, which may rely on the biological variance.

Pharmacokinetics

A sensitive RP-HPLC-ESI-MS approach for targeted quantification of Api88, Api137, and their identical metabolites containing the N-terminal 16 (Api1-16) or 17 residues (Api1-17) was developed based on a protocol recently reported for oncocins (Schmidt et al., 2016). The MRM relied on triply protonated precursor ions and a neutral loss of two molecules of dimethylamine from the N-terminal *N*,*N*,*N*′,*N*′-tetramethylguanidine group, which was highly selective and sensitive providing limits of quantification (LOQ) from 19 to 59 ng/mL (∼9 to ∼25 nmol/L). Recovery rates were 35 ± 5–56 ± 16% after sample preparation with only weak matrix effects (−4.4 ± 0.2–13 ± 3%). Accuracies were 89 ± 6–116 ± 17% and intra- and interday precisions were 10% or better for all peptides and concentrations. Details of the method development and validation of the optimized method are provided as Supplementary Material (Tables S4, S6–S9, Figures S1–S3).

Api88 administered intravenously at a dose of 5 mg/kg was detected at a plasma concentration of 1 μg/mL at the first time point after 5 min (**Figure 2A**). Unexpectedly, Api137, which has a 70 times longer half-life time in mouse serum, injected at the same dose was detected at a lower concentration of only 0.4 μg/mL in plasma (**Figure 2A**). The plasma levels of both peptides further decreased at similar kinetics to concentrations around the LOQ after 10 min. After 60 min Api137 was still present at concentrations around the LOQ, while Api88 was not detected.

Intraperitoneal applications were initially used in the infection models due to the presumed depot effect providing slower releases and thus longer circulation times in blood. Indeed, when Api88 and Api137 were injected intraperitoneal at doses of 5 mg/kg, slightly lower plasma levels of 0.6 and 0.2 mg/mL, respectively, were observed after 10 min that decreased even further during the next 20 min. Api137 was still detected after 1.5 h, whereas Api88 was undetectable (**Figures 3A,B**, Figures S4A,B, S5). The calculated initial concentrations (c_0) were 0.2 and 1 μg/mL, respectively (**Table 1**), but the plasma levels decreased slower than for intravenous applications demonstrating a rather small depot effect. Again, Api88 was detected at the initial time points at higher plasma levels than Api137, but the elimination half-life time of Api137 ($t_{½}$ = 33.8 min) was almost two-fold longer than for Api88 ($t_{½}$ = 17.3 min), which could be explained by the higher serum stability of Api137.

Four-fold higher doses (20 mg/kg) provided maximal plasma levels of 4.1 (Api88) and 3.6 μg/mL (Api137) after 10 min, which was 7- and 17-fold higher than observed for the low dose (**Figures 3A,B**). Surprisingly, the c_0 values were similar for both peptides (6.2 and 7.8 μg/mL, respectively), while the elimination half-life times decreased to 11 and 17 min, respectively (**Table 1**). However, the data points indicated an almost stable plasma level for Api88 over 30 min, whereas

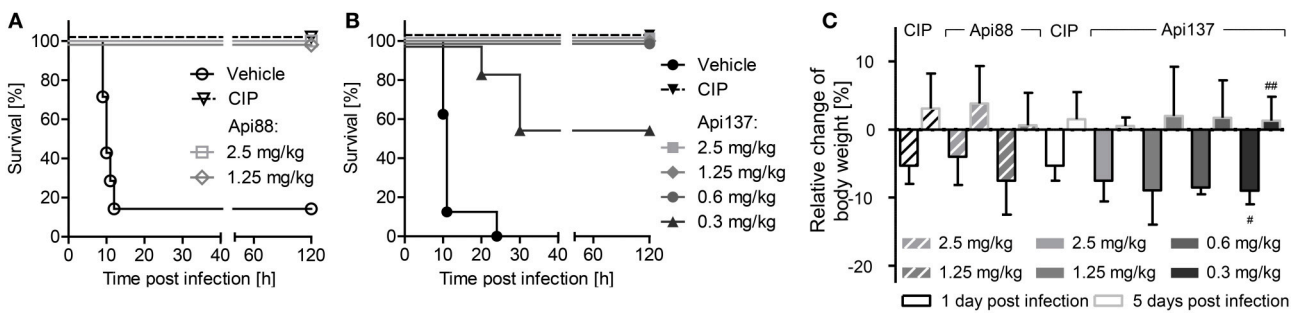

FIGURE 1 | Survival rates (A,B) and relative change of body weights **(C)** of NMRI mice (n = 7) infected intraperitoneally with *E. coli* ATCC 25922 (9×10^5 bacteria in the presence of mucin, 2.5% w/v, 300 μL in total). Api88 **(A)** and Api137 **(B)** were administered intraperitoneally (300 μL) at doses of 2.5 and 1.25 mg/kg and 2.5, 1.25, 0.6, and 0.3 mg/kg, respectively, three times post-infection (1, 4, and 8 h). Ciprofloxacin (CIP, 40 mg/kg body weight) and vehicle only (glucose in water, 5% w/v) were used as positive and negative control, respectively. Mice were controlled three times daily for their health status for 5 days post-infection and weighed 1 day before and 1 and 5 days after infection. Relative changes of body weights were calculated for Api88, Api137, and ciprofloxacin at 1 and 5 days post-infection (black and gray bordered bars, respectively). # and ## denote that 6 and 4 survivors were weighed, respectively.

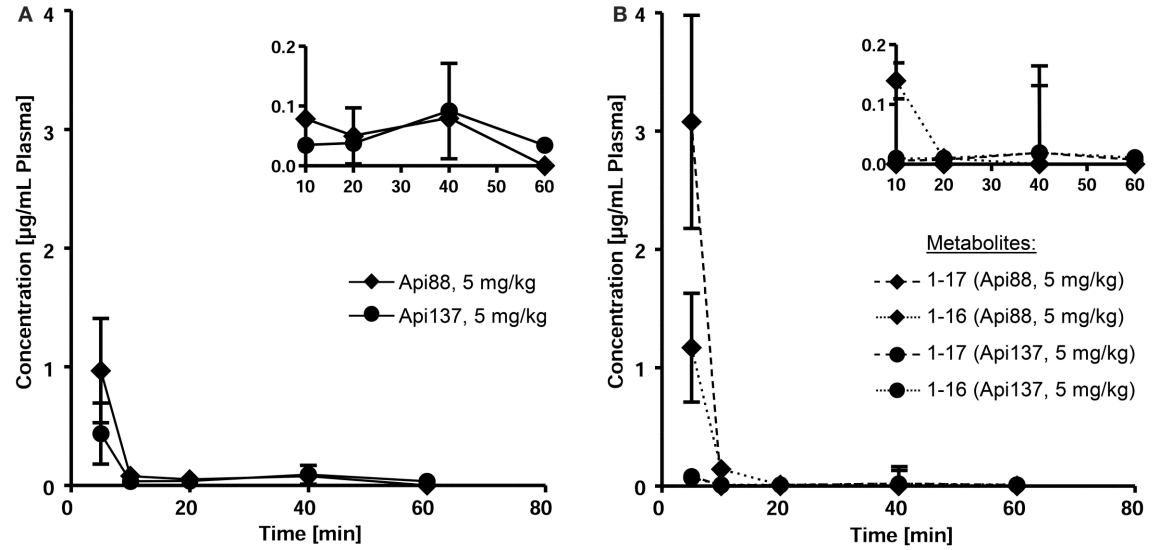

FIGURE 2 | Pharmacokinetic studies of Api88 and Api137 (A) after intravenous administration at doses of 5 mg/kg BW and the corresponding metabolites Api1-16 and Api1-17 **(B)**. Peptides were quantified by RPC-ESI-QqLIT-MS for blood samples collected 5, 10, 20, 40, and 60 min after peptide administration ($n = 5$). Inserts display the same data points using zoomed concentration ranges.

Api137 was cleared faster resulting in a lower plasma level at 30 min. Although difficult to interpret, a comparison of the pharmacokinetic parameters clearance and mean residence time of the high and low dose groups seemed to be similar for Api88 and different for Api137.

Metabolites in Blood

Metabolites Api1-16 and Api1-17, which were identified as major degradation products of both Api88 and Api137 in serum stability assays (Berthold et al., 2013), were quantified besides the full-length peptides by RP-HPLC-ESI-QqLIT-MS using the optimized MRM method. As expected from serum stability assays, metabolites Api1-17 and Api1-16 appeared at higher concentrations (1.2 and 2.9 µg/mL after 10 min, respectively) for Api88 than for Api137 (<0.1 µg/mL) after intravenous administration at a dose of 5 mg/kg (**Figure 2B**). Although, the low concentrations of both Api137 metabolites are in full agreement with the higher *in vitro* stability of Api137, the total quantities of intact peptide and metabolites was significantly lower than for Api88.

Intraperitoneal administration yielded Api88, Api1-16, and Api1-17 at similar plasma levels that remained relatively stable for the first 30 min (**Figure 3C**, Figure S4C) indicating again a depot effect in the peritoneum. In contrast, both metabolites of Api137 were detected at an equal level that was around six-fold higher than for the intact peptide after 10 min administered at a dose of 5 mg/kg (**Figure 3D**, Figure S4D). For the high dose group, the ratio was initially around 2. Interestingly, the plasma levels of the metabolites remained above the levels of Api137 for the first hour and the curves showed a more exponential shape than for Api137 indicative of a slower clearance rate. The two administration routes resulted in clearly different metabolite profiles for both apidaecins. The higher serum stability of Api137 was not reflected *in vivo* when it was injected in the peritoneum. Altogether, the plasma levels of both Api88 and Api137 were lower than assumed from previous studies on oncocins, even when considering the major metabolites Api1-16 and Api1-17. Thus, the plasma samples obtained 10, 30, and 60 min after intraperitoneal injections were screened for further degradation products of both peptides using nanoRP-UPLC-ESI-LTQ-Orbitrap XL^{TM}-MS (Figure S6). For Api88, five further metabolites with peak areas of 1 to 3% relative to the full length peptide were identified (Figure S6A), i.e., Api2-16, Api7-18, Api3-18, Api7-12, and Api1-6 in the order of decreasing peak areas. Surprisingly, besides Api1-16 and Api1-17, only three metabolites were detected for Api137 at low intensities (peak areas <1% relative to Api137), i.e., Api7-12, Api2-16, and Api2-18 in the order of decreasing peak areas (Figure S6B). However, numerous metabolites with very low intensities were observed indicating subsequent degradations, but their contents did not explain the unexpectedly fast clearance rates.

Organ Distribution

The low plasma levels and the corresponding high volumes of distribution (V_D) of Api88 and Api137 partially attributed to C-terminal degradation resulted in recovery rates below 1% in blood relative to the injected peptide amounts (Tables S10, S11). The strong accumulation in kidneys and renal excretion was expected for the short polar peptides, as already noted for oncocin derivatives (Holfeld et al., 2015; Schmidt et al., 2016). Therefore, the tissue distribution was further investigated using homogenates of kidney, liver, and brain besides the appearance in urine. Api88 and Api137 were detected in kidney

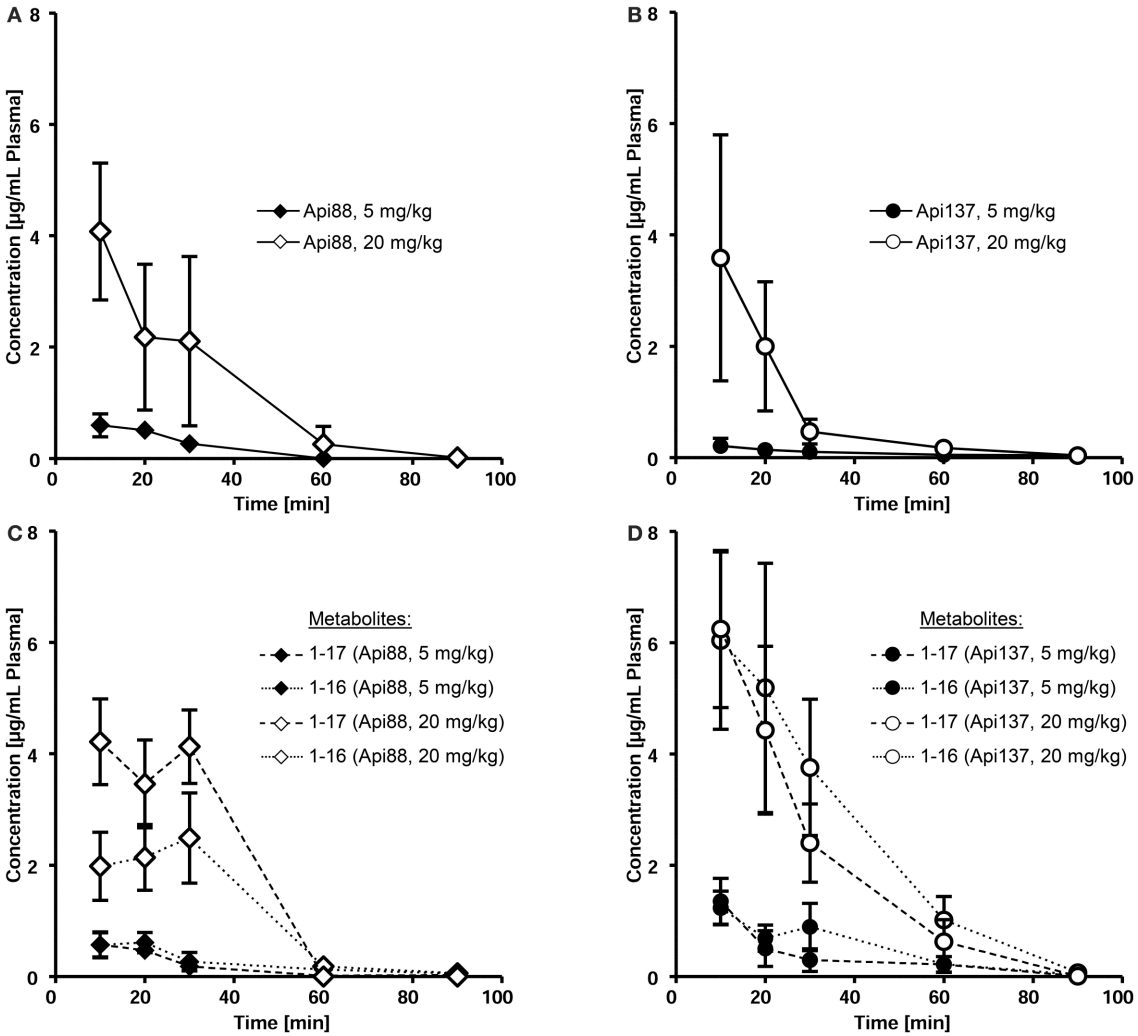

FIGURE 3 | Pharmacokinetics of Api88 (A) and Api137 **(B)** and metabolites 1–17 and 1–16 **(C,D)** after intraperitoneal administration at doses of 5 mg/kg BW and 20 mg/kg BW. Peptides were quantified by RPC-ESI-QqLIT-MS/MS in plasma samples collected at 10, 20, 30, 60, and 90 min post-injection ($n = 7$).

homogenates of the high dose group with almost stable peptide concentrations between 0.5 to 0.8 µg/g over the first 30 min post-injection (**Figures 4A,B**). The peptide concentrations decreased afterwards, but remained above the LOQ in some animals after 90 min. Four-fold lower doses reduced the kidney levels of Api137 around four-fold (0.18 to 0.11 µg/g), but only two-fold for Api88 (0.33 to 0.05 µg/g kidney). In contrast, the peptide levels in liver homogenates were dose-independent for both peptides and two-fold higher for Api88 (0.2–0.4 µg/g) compared to Api137 (0.1–0.2 µg/g) for the first three time points (**Figures 4C,D**). Metabolite Api1-16 was detected at slightly higher concentrations than Api1-17 in kidney and liver homogenates, but both were present at much lower levels than Api137 (**Figures 4A–D**). In contrast, Api88 and its metabolite Api1-17 were present in both homogenates at similar levels, while Api1-16 was lower concentrated (**Figures 4A,C**), which correlates to their plasma levels. Brain homogenates contained neither Api88 nor Api137, i.e., less than the LODs of 43 and 18 ng/mL homogenate, respectively, corresponding to ∼89 and ∼37 µg/g brain nor the two metabolites.

Three to seven mice of the studied groups spontaneously released urine during euthanasia. Api88 was present in all urine samples of the high-dose group at similar levels of ∼0.2 µg/mL (**Figure 5A**). Urine collected in the low-dose group contained Api88 at the same level at the first time point, but it decreased afterwards to concentrations below LOD. The Api137 levels were similar for both dose groups showing peak concentrations of ∼0.2 µg/mL in urine at 20 and 30 min, which decreased afterwards to levels below 0.1 µg/mL (**Figure 5B**). Metabolite Api1-17 was detected in the high-dose groups at intensities between the LOD and the LOQ and undetectable in the low dose group. The levels of Api1-16 were around the LOQ after 30 and 60 min in the low dose group, but similar to the concentrations of the full length peptides in the high dose groups.

TABLE 1 | Mean pharmacokinetic parameters of Api88 and Api137 in mice, after intraperitoneal administration, determined with PKSolver using the concentrations for the terminal elimination phase determined by the software.

Peptide	Api88		Api137	
Dosage [mg/kg BW]	5	20	5	20
Injected peptide amount [μg]	137	618	145	602
c_{max}[μg/mL]	0.6	4.1	0.2	3.6
c_0[μg/mL]	1.0	6.2	0.22	7.8
$t_{1/2}$[min]	17.3	11.1	33.8	16.9
k [min^{-1}]	0.040	0.062	0.020	0.041
Volume of distribution [mL]	137	100	659	77
$AUC_{0\rightarrow\infty}$ [μg*min*mL^{-1}]	21.3	114.2	9.6	72.0
$AUMC_{0\rightarrow\infty}$ [μg*min^2*mL^{-1}]	650	2730	1512	524
Clearance [mL/min]	6.4	5.4	15.1	8.4
Mean residence time [min]	30.5	23.9	54.9	21.0

c_{max}, c_0, $t_{1/2}$, k, AUC, and AUMC denote maximum concentration, initial concentration at time point 0 min, elimination half life time, elimination constant, area under the concentration time curve, and area under the moment curve, respectively.

Peptide Recovery in Blood and Organ Homogenates

Peptide recoveries were determined by spiking untreated blood and homogenization buffer with either Api88 or Api137 prior to heparin plasma preparation or homogenization of organs obtained from untreated mice. Recovery rates for plasma were 49 ± 5 and 78 ± 5% for Api88 and Api137, respectively (**Figure 6A**, Table S12), which was similar to the recovery rates determined for liver (Api88: 45 ± 5%, Api137: 49 ± 4%) and brain homogenates (Api88: 89 ± 24%, Api137: 42 ± 4%). In contrast, the recovery rates were much lower in kidney homogenates (17 ± 6 and 3.5 ± 0.4%, respectively), which was previously noted for oncocin peptides as well (Schmidt et al., 2016). Metabolites identified by nanoRP-UPLC-ESI-Orbitrap-MS in the kidney homogenates were detected with small signal intensities relative to the full-length peptides indicating small peptide quantities (Table S13) that do not explain the low peptide recoveries in the kidneys. The low recoveries might be related to (*i*) further degradation to small peptides missed by the analytics, (*ii*) different metabolic pathways (e.g., oxidation), or (*iii*) strong binding to insoluble cell compartments that were removed by centrifugation after cell lysis (analysis of cell debris indicated only small peptide quantities, data not shown).

The recovery rates obtained from the spiked samples, were used to calculate the total amounts of intact peptides in blood, liver, and kidneys after intraperitoneal administration (**Figures 6B–E**, Tables S10, S11). The relative recovery in the studied organs and blood was only around 1.5 ± 0.6–2.1 ± 0.7% of the injected peptide amounts. Considering the mean weight of 29 g per mouse in this study and the weights of blood, liver, two kidneys, and brain with 2.2, 1.5, 0.5, and 0.5 g, respectively (∼16% of the total weight), around 12% of the injected peptide quantities were detected in the samples collected at the first time point considering a homogeneous distribution in the whole animal. Notably, the recovery rates of Api88 and Api137 were similarly low and significantly lower than reported for oncocin analogs Onc72 and Onc112 administered intraperitoneal at a dose of 5 mg/kg (47 and 32%, respectively) in an equally performed pharmacokinetic study (Schmidt et al., 2016). In fact, C-terminal degradation of apidaecins could explain the difference, as metabolites of oncocins were detected at much lower amounts (Onc72) or not at all (Onc112). Nevertheless, the discrepancy between the bioavailability and peptide to metabolite ratios *in vivo* (especially for intraperitoneal administration) and the serum stability assay performed *in vitro* remained open.

Peptide Stability in Peritoneal Lavage

Peritoneal lavage was not collected during the pharmacokinetic study, as the main emphasis after euthanasia was the immediate collection of blood and perfused organs, whereas the discrepancy between *in vitro* degradation and *in vivo* metabolization was revealed only after completion of the animal studies. Thus, three untreated mice were euthanized and the peritoneum washed with PBS in order to obtain peritoneal lavage. Peptides Api88, Api137, Onc72, and Onc112 were incubated in aliquots of the lavage of each mouse *ex vivo* and analyzed for the added peptide and its degradation products after 0, 30, and 60 min. Surprisingly, Api137 was degraded very fast with a half-life time of ∼20 min yielding Api1-17 as major metabolite (**Figure 7A**). Api88 was cleaved at the same position, but the amidated C-terminus reduced the degradation rate with more than 60% of Api88 still present after 1 h. The total amounts of intact peptide and Api1-17 reached in both cases almost 100% indicating that no other metabolites were produced in significant quantities. Onc72 and Onc112 were even more stable with more than 89% intact peptide present after 1 h (**Figure 7B**). Only for Onc72 the metabolite 1–14 was detected at higher quantities (7% relative to the intact peptide) after 60 min. It should be noted that the degradation was studied in lavage diluted with PBS at an unknown ratio and that the degradation rates in the peritoneum are most likely even higher. This nicely explains the unexpectedly low concentrations of Api137 in the pharmacokinetic study compared to Api88 and to oncocin peptides and similarly the surprisingly high concentrations of metabolites.

DISCUSSION

Apidaecin analogs Api88 and Api137 are similarly active against Gram-negative bacteria with a tendency of higher activity of Api88 against *K. pneumoniae* (Czihal et al., 2012; Berthold et al., 2013). However, Api88 carries a C-terminal amide that is responsible for its very fast proteolytic cleavage in serum, the supernatant obtained after complete coagulation and centrifugation of blood. The *in vitro* serum stability assay, which is an important *in vitro* criterion to select therapeutic peptides for systemic applications, yielded very short half-life times of 5 min for Api88, which indicate that the peptide is not well-suited for *in vivo* applications, while the 6 h for Api137 are very favorable (Berthold et al., 2013; Czihal et al., 2012). Taking into account the comparable minimal inhibitory concentration of ∼4 μg/mL against *E. coli* ATCC 25922, it was predicted that Api137 exhibits a much better *in vivo* efficacy than Api88 in previous

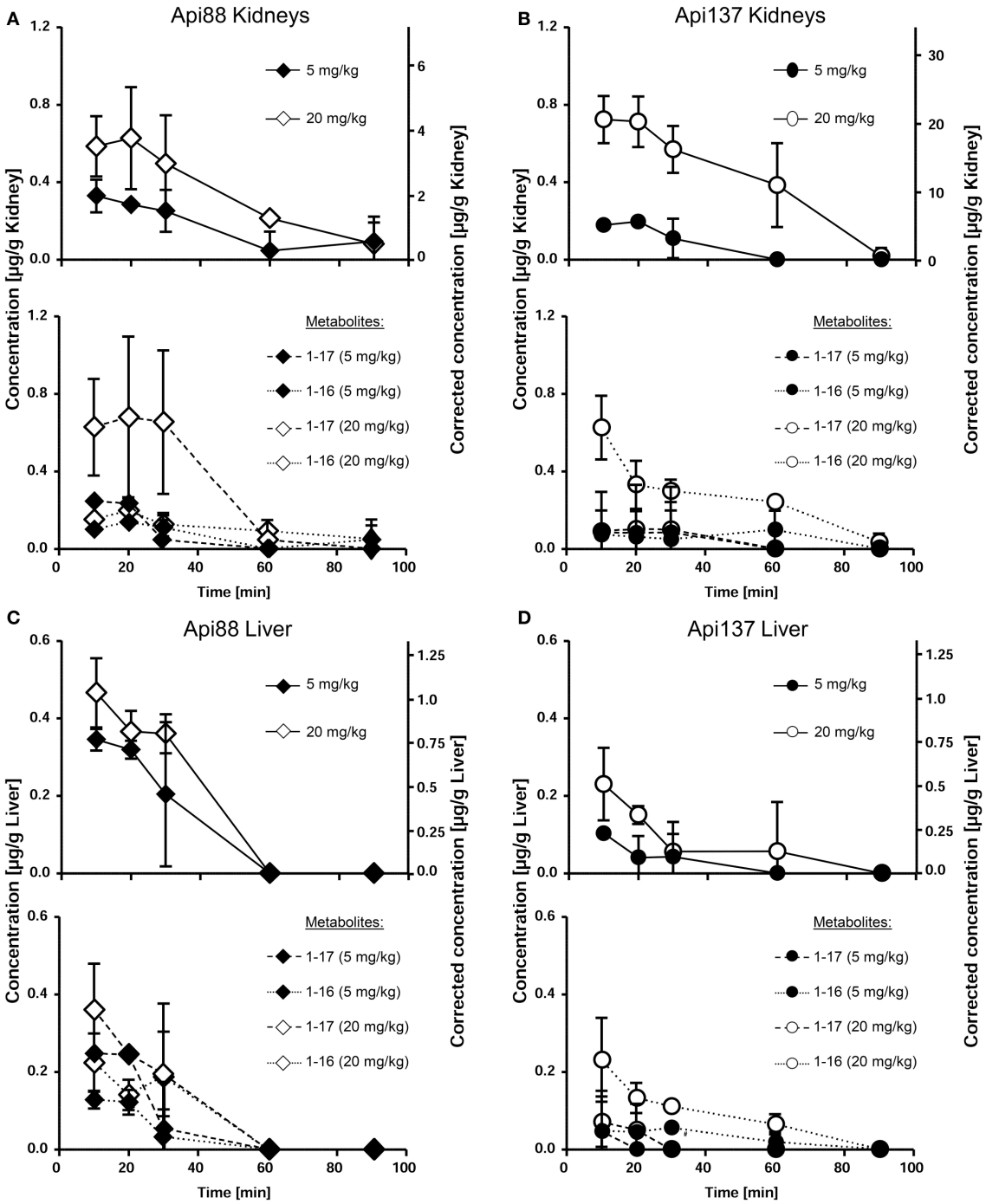

FIGURE 4 | Concentration profiles of Api88 (A,C) and Api137 **(B,D)** and the corresponding metabolites Api1-17 and Api1-16 in kidney **(A,B)** and liver **(C,D)** homogenates. Api88 and Api137 were administered intraperitoneal (5 mg/kg and 20 mg/kg) and quantified in homogenates prepared from organs ($n = 5$) isolated 10, 20, 30, 60, and 90 min after administration. The hash (#) indicates that the Api1-17 value of one mouse was removed as outlier.

studies, because Api88 would be degraded rapidly after injection. Surprisingly, the *in vivo* efficacy of Api137 was only two-fold better with doses of 0.6 mg/kg Api137 rescuing all mice compared to 1.25 mg/kg Api88. Oncocin analogs Onc72 and Onc112, which possess MIC values of 16 and 4 μg/mL, respectively, against *E. coli* ATCC 25922 (Knappe et al., 2016c), rescued in the same infection model all animals at doses of 5 and 2.5 mg/kg, respectively (Schmidt et al., 2016). While the MIC values correlate well to the *in vivo* efficacies, the pharmacokinetics differed completely from our expectations considering the serum

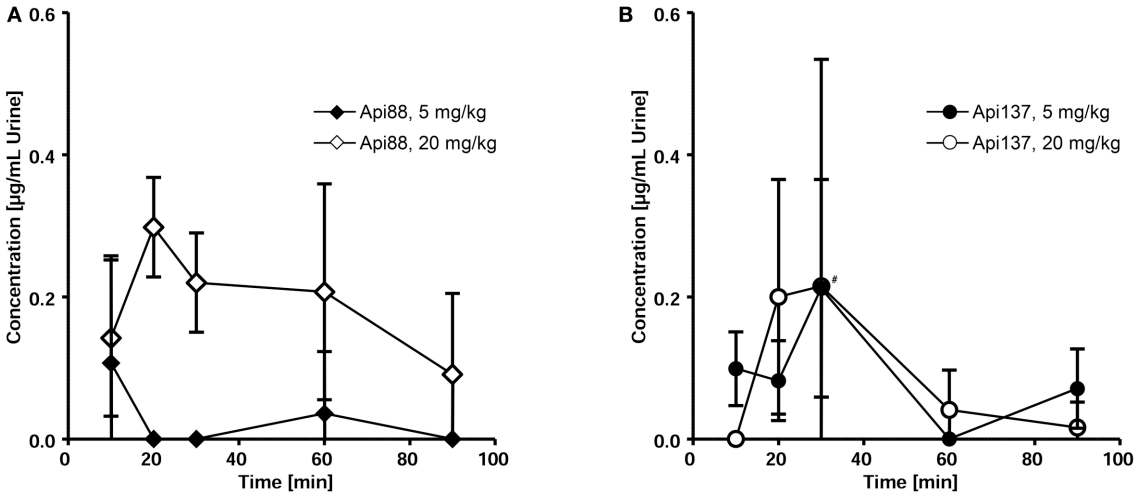

FIGURE 5 | Pharmacokinetics of Api88 (A) and Api137 **(B)** in urine after intraperitoneal administration at doses of 5 and 20 mg/kg. Both peptides were quantified in urine ($n = 3$–7) spontaneously released after anesthetization. Concentrations of metabolites were below LOQ. The hash (#) indicates that one mouse was removed at this time point from the low dose group as outlier.

stability assays. Maximum plasma levels of Onc72 and Onc112 were up to 10-fold higher than that of Api88 and Api137 after intraperitoneal administration. For the high dose groups, the concentrations of Api1-16, Api1-17, and the intact peptide corresponded in total to around 10 and 16 µg/mL, which matches well Onc72 (18 µg/mL). The lower dose groups showed total maximum plasma levels (intact peptides plus metabolites) of 1.7 and 2.8 µg/mL for Api88 and Api137, respectively, which was around three-fold lower than reported for Onc72 (6 µg/mL) and Onc112 (8 µg/mL). Metabolites Api1-17 and Api1-16 are virtually inactive *in vitro* with MIC values of 64 µg/mL and >128 µg/mL, respectively. This is most likely related to the reduced bacterial uptake, as both metabolites bind equally well to the 70S ribosome and DnaK as the intact peptide (Berthold and Hoffmann, 2014; Krizsan et al., 2014). Although speculative, the bacterial uptake of the truncated sequences might change *in vivo*, as bacteria might activate different transporter systems under less favorable growing conditions. Additionally, host peptides increasing the bacterial membrane permeability might assist shortened peptides to enter bacteria. All other pharmacokinetic values depicting biodistribution in liver, kidneys, brain, and urine were lower values for both apidaecin analogs indicating a reduced overall recovery compared to both oncocin analogs. Although, the serum half-life times of Onc112 exceeded 8 h *in vitro*, Onc72 (3 h) was only half as stable (Knappe et al., 2011a) as Api137 (6 h), which contradicts the faster *in vivo* metabolization of Api137. The apparent discrepancy was solved by a peritoneal lavage *ex vivo* stability assay that revealed a fast degradation of Api137 indicating its fast metabolization in the peritoneum. Contrastingly, the concentration of Api137 was also slightly lower after intravenous application than for Api88. As expected from the lower serum stability of Api88, its metabolites were more abundant in blood, whereas no metabolites were detected for Api137. As renal excretion of both peptides appeared to be similar, distribution of Api88 and Api137 might be inconsistent as indicated by their different volumes of distribution.

Considering the pharmacokinetic data, the high *in vivo* efficacies of Api88 and Api137 are surprising. Thus, a drawback of intraperitoneal sepsis models commonly applied in antibiotic research has to be discussed, as infection and treatment used the same route and direct bacterial killing in the peritoneum starting 1 h post-infection appears very likely. Nevertheless, the model is accepted for evaluation of antimicrobials and the bacteria were well-distributed in blood and even organs prior to treatment (Frimodt-Møller et al., 1999; Knappe et al., 2012). Additionally, if the *in vitro* activity would translate to the activity in the peritoneum and sterilization of the peritoneum would be the only *in vivo* effect, Onc112 should show a much higher *in vivo* efficacy than Api137 due to its higher peritoneal stability. However, the plasma concentrations do not substantiate efficient treatment with either of the four PrAMPs when considering their *in vitro* antimicrobial activities determined with standard MIC-testing methods, which are most likely not the proper conditions to predict *in vivo* efficacies of PrAMPs. Synergistic effects with intrinsic antimicrobial substances produced by the host, e.g., AMPs like CRAMP, and immunomodulatory effects could also explain this discrepancy (Ostorhazi et al., 2013; Otvos and Ostorhazi, 2015; Knappe et al., 2016b). The latter effect was studied for Onc72, but no immunomodulatory effects were observed on unstimulated and lipopolysaccharide (LPS)-stimulated murine dendritic cells or murine macrophages (Fritsche et al., 2012). Only human macrophages and monocytes showed a reduction of LPS-induced TNFα release after treatment with Api88 and Api137 indicating a mild anti-inflammatory effect (Tavano et al., 2011; Keitel et al., 2013). Unfortunately, further data on immunomodulatory effects of Api137 on murine cells are missing.

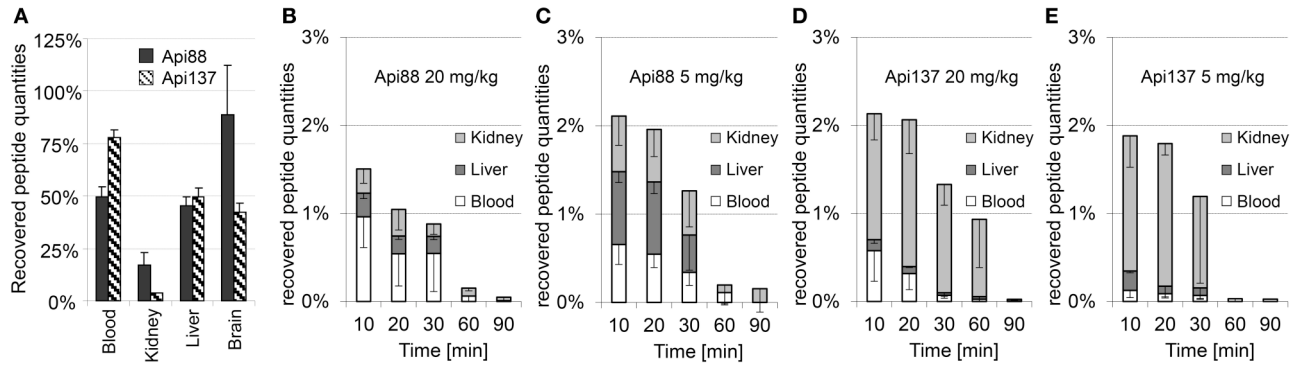

FIGURE 6 | Mean recovery rates ($n = 3$) of Api88 and Api137 spiked to heparin blood or organ homogenates (A) and mean percentage rates recovered in blood, liver, and kidneys of mice treated intraperitoneal with doses of 20 mg/kg **(B)** and 5 mg/kg of Api88 **(C)** or 20 mg/kg **(D)** and 5 mg/kg of Api137 **(E)**. Peptide distributions were calculated for plasma (one third of the whole mouse blood volume assumed to be 2.25 mL) and organs assuming that the peptides are present only in plasma and equally distributed in organ homogenates. Absolute quantities were calculated by assuming a total plasma volume of 25 mL/kg mouse weight and that organ volume in mL corresponds to the measured organ weight in g.

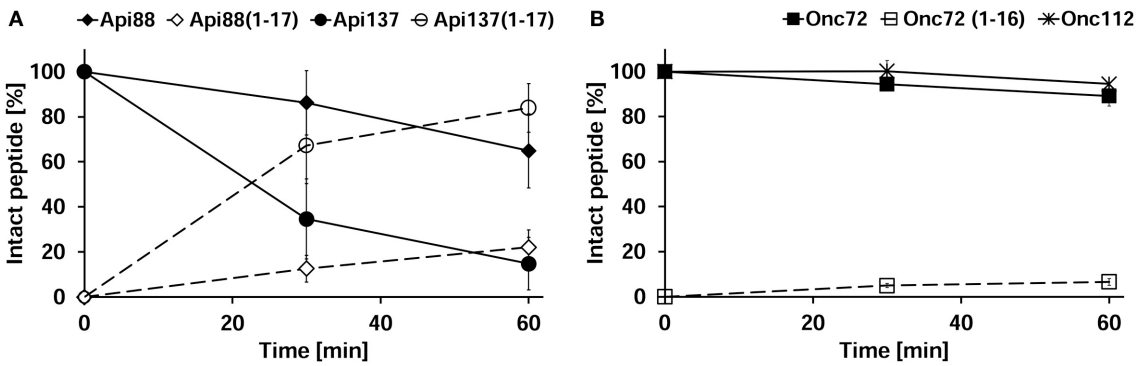

FIGURE 7 | Peritoneal lavage stability of apidaecins (A) and oncocins **(B)**. Api88 (full diamonds), Api137 (full circles), Onc72 (full squares), and Onc112 (full asterisks) were incubated in lavage *ex vivo* and quantified after 30 and 60 min by RP-HPLC. Shown are the quantities of the incubated peptides (full symbols) and their main metabolites Api1-17 and Onc1-16 (open symbols) normalized to the initial peptide amounts determined at 0 min. The considered monoisotopic masses of the quadruply protonated metabolites were m/z 545.5 (Api1-17) and m/z 479.5 (Onc1-16) matching very well the signals at m/z 545.6 and 479.5, respectively, recorded with an Esquire HCT ESI-Iontrap-MS.

Although speculative, the accumulation of the PrAMPs in the kidneys and possibly spleen (not tested) may lead to high local concentrations sufficient to kill bacteria in both organs that are responsible for clearance of bacteria in the blood stream. In this respect, the lower recovery rates of Api137 spiked to the buffer before kidney homogenization may indicate a strong binding to the organ structures, i.e., brush boarder membrane. Therefore, the recovered peptide amounts in kidneys of Api137 treated animals (2.4 μg/g) were slightly higher than in Api88 treated animals (1.8 μg/g). Notably, PrAMPs usually do not penetrate mammalian cells, but immune cells and HeLa cancer cells can internalize apidaecins and Bac7(1–35) after long incubation times (Tavano et al., 2011; Hansen et al., 2012; Pelillo et al., 2014; Bluhm et al., 2016). However, it is unlikely that PrAMPs internalized in kidney cells within 10 min (first time point of the pharmacokinetic study). More likely they bind to membrane structures or accumulate in the interstitial fluid of the kidney. Estimating the volume of both kidneys as 0.5 mL, the detected peptide amount would translate to kidney concentrations of 4.8 μg/mL, which is most likely higher in the interstitial volume and will easily kill bacteria surrounding kidney cells. In this respect, it has to be noted that the animals were carefully perfused with saline after euthanasia in order to remove blood from the organs and to measure only peptide amounts that are localized in the organs and not in blood. Considering all data presented above, it appears reasonable to assume that perfusion did also wash out peptides bound to the tissue or dissolved in the interstitial fluid. Therefore, the peptide amounts in the kidney with circulating blood might have been significantly higher.

Metabolization of therapeutic peptides in the peritoneum could be suppressed by formulating the peptides with EDTA or other biocompatible protease inhibitors providing maybe better *in vivo* efficacies and better pharmacokinetics. Alternatively, intramuscular and subcutaneous injections are valid alternative

routes with depot effects and were already successfully applied to treat animals (Ostorhazi et al., 2010; Knappe et al., 2015). Even though, the unexpectedly good pharmacokinetic profile of Api88 can be only partially explained by its higher peritoneal stability. Further studies have to explain why Api88 appears much more stable in intravascular blood than in serum.

Besides the partially unexpected effects, the pharmacokinetic data may indicate that their therapeutic effects are related to their maximal blood concentrations and not to the time period above a certain threshold or the AUC. Our most recent data indicate an irreversible uptake of Api137 by bacteria into the cytosol (unpublished data), which may provide an explanation for their high *in vivo* efficacy, i.e., the peptide concentration could fall below the MIC as soon as a certain amount of PrAMP entered the cells. Earlier studies on fluorophore-labeled peptides indicated that Api88 enters *E. coli* cells faster than Api137 at high quantities, but the data might be influence by the N-terminally attached dye that reduced also MIC values (Czihal et al., 2012; Berthold and Hoffmann, 2014). Thus, further studies using RP-HPLC-ESI-MS techniques to study the cellular uptake of unmodified Api88, Api137, Onc72, and Onc112 and to determine the post-antibiotic effect of these peptides are necessary to quantify their *in vitro* activities in the context of the *in vivo* studies presented here.

CONCLUSION

The data presented here together with literature data allowed comparing the *in vivo* efficacy of four PrAMPs, i.e., two apidaecin analogs Api88 and Api137 and two oncocin analogs Onc72 and Onc112 within and between both peptide families. The comparison relies on one infection model of peritoneal sepsis performed in one laboratory by the same persons revealing that the therapeutic efficacy increased in the following order: Api137 < Api88 < Onc112 < Onc72. This was not presumable from the order of stability determined *in vitro* in mouse serum (Onc112 > Api137 > Onc72 >> Api88) and the *in vitro* antimicrobial activities (Api88 > Api137 = Onc112 > Onc72). Both parameters are favoring Api137 and Onc112 well above Api88 and Onc72 with respect to their predicted *in vivo* efficacy. The stability in peritoneal lavage as third *in vitro* parameter ranked the peptides in a different order (Onc112 > Onc72 > Api88 > Api137) and explains partially the lower than expected performance of Api137 based on the initial *in vitro* data. There are a few possible explanations, e.g., organ accumulation, immunomodulatory effects, and irreversible bacterial uptake, to correlate the high *in vivo* efficacies with the relatively low plasma concentrations, which is commonly accepted as basis for antibiotic assessment. However, additional effects besides direct antimicrobial activity in the bloodstream have to be studied in more detail and the efficacy in different infection models will help to justify further clinical development of both apidaecin analogs and both oncocin analogs.

AUTHOR CONTRIBUTIONS

RS: peptide synthesis, LC-MS method development, organ, and sample preparation, data evaluation, manuscript preparation. DK: peptide synthesis, infection model, stability assay in intraperitoneal lavage, data interpretation, design of the work, manuscript preparation. EW and EO: pharmacokinetics, sample preparation, proof reading manuscript. RH: Project supervision, design of the work, manuscript preparation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fchem.2017.00015/full#supplementary-material

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Conflict of Interest Statement: RH is a cofounder of AMP Therapeutics GmbH (Leipzig, Germany) and a member of their scientific advisory board. EW and DK were temporarily co-workers at AMP Therapeutics GmbH. Peptide sequences of Api137 and Api88 are patented under PCT/EP2008059512.

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

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Antimicrobial Peptides in Biomedical Device Manufacturing

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Over the past decades the use of medical devices, such as catheters, artificial heart valves, prosthetic joints, and other implants, has grown significantly. Despite continuous improvements in device design, surgical procedures, and wound care, biomaterial-associated infections (BAI) are still a major problem in modern medicine. Conventional antibiotic treatment often fails due to the low levels of antibiotic at the site of infection. The presence of biofilms on the biomaterial and/or the multidrug-resistant phenotype of the bacteria further impair the efficacy of antibiotic treatment. Removal of the biomaterial is then the last option to control the infection. Clearly, there is a pressing need for alternative strategies to prevent and treat BAI. Synthetic antimicrobial peptides (AMPs) are considered promising candidates as they are active against a broad spectrum of (antibiotic-resistant) planktonic bacteria and biofilms. Moreover, bacteria are less likely to develop resistance to these rapidly-acting peptides. In this review we highlight the four main strategies, three of which applying AMPs, in biomedical device manufacturing to prevent BAI. The first involves modification of the physicochemical characteristics of the surface of implants. Immobilization of AMPs on surfaces of medical devices with a variety of chemical techniques is essential in the second strategy. The main disadvantage of these two strategies relates to the limited antibacterial effect in the tissue surrounding the implant. This limitation is addressed by the third strategy that releases AMPs from a coating in a controlled fashion. Lastly, AMPs can be integrated in the design and manufacturing of additively manufactured/3D-printed implants, owing to the physicochemical characteristics of the implant material and the versatile manufacturing technologies compatible with antimicrobials incorporation. These novel technologies utilizing AMPs will contribute to development of novel and safe antimicrobial medical devices, reducing complications and associated costs of device infection.

Keywords: antimicrobial peptide, biomaterial-associated infection, biofilm, antimicrobial resistance, implant, device manufacturing

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BIOMATERIAL-ASSOCIATED INFECTIONS

The use of medical devices, including catheters, artificial heart valves, prosthetic joints, and other implants, increased dramatically over the past century (Darouiche, 2004; Anderson and Patel, 2013; Kwakman and Zaat, 2013), and has become a major part of modern medicine and our daily life. With the aging society, the demand for medical devices to restore body functions and quality of life

increases, and so do the numbers of cases of biomaterial-associated infection (BAI). The risk for BAI may in part be explained by the reduced efficacy of the local immune defense induced by the foreign body. In agreement, the number of bacteria required to cause an infection is significantly lower in the presence of a foreign body, such as a stitch or an implant, than when such devices are not present (Elek and Conen, 1957; James and Macleod, 1961; Noble, 1965; Taubler and Kapral, 1966; Zimmerli et al., 1982; Southwood et al., 1987). Another contributing factor is that the bacteria—often derived from the commensal skin flora or the hospital environment—can adhere to the foreign body, replicate, and form a biofilm from which they can invade the peri-implant tissues and cause an infection. The most common causative microorganisms in BAI are *Staphylococcus aureus* and *Staphylococcus epidermidis* (Anderson and Marchant, 2000; O'Gara and Humphreys, 2001; Zimmerli et al., 2004). Depending on the type of device and location of application, other coagulase-negative staphylococci, enterococci, streptococci, *Propionibacterium acnes,* and yeasts such as *Candida* spp., can also cause BAI (Waldvogel and Bisno, 2000; Holmberg et al., 2009). Infections following primary implant surgery occur in 0.5–1% of the patients receiving an artificial hip or knee and in over 5% of those receiving a prosthetic elbow or ankle implant (Zimmerli et al., 2004; Krenek et al., 2011). As treatment of BAI is complex, combinations of antibiotics, such as vancomycin or ciprofloxacin with rifampicin, are recommended. Such combinations show some efficacy against biofilms, although much higher concentrations of antibiotics are required than effective against planktonic cells (Saginur et al., 2006). Nevertheless, treatments with antibiotic combinations often fail with the only option being removal of the medical device (Burns, 2006). Catheters suspected for infection are removed and replaced by a new device at a different location, as re-implantation at the original site is strongly discouraged because of the high re-infection risk (Safdar et al., 2002). Revision surgery of infected orthopedic devices in most cases involves removal of the implant, thorough debridement of the infected site and prolonged (4–8 weeks) antibiotic treatment before a new implant is placed (Zimmerli, 2006). Still, revision surgery is associated with high frequencies of infection due to extensive surgical procedures and more severe tissue damage.

Biofilm Formation

Bacterial biofilm formation is considered to play a major role in the pathogenesis of BAI (Costerton et al., 1999; Holmberg et al., 2009; Anderson and Patel, 2013). Biofilm formation is initiated by bacterial cells attaching to the surfaces of medical devices. Subsequently, bacteria replicate and produce extracellular matrix forming complex communities consisting of bacteria, bacterial exopolysaccharides, proteins, extracellular DNA, and host proteins (Costerton et al., 1999). Bacteria in biofilms are considerably more tolerant to antibiotics and less accessible to cells and molecules of the human immune defense system than their planktonic counterparts (Otto, 2009; Chen et al., 2013). This might be due to the extracellular polymeric matrix of the biofilm, making the bacteria less accessible for phagocytes and effector molecules, and to the persister state of the bacteria. Persisters are metabolically-inactive, antibiotic tolerant bacteria that maintain the ability to multiply after antibiotic treatment (Harms et al., 2016), thus explaining the recurrence of BAI (Gerdes and Semsey, 2016; Fisher et al., 2017).

Tissue Colonization

Another important element in the pathogenesis of BAI is bacterial colonization of the tissue surrounding the implant (Boelens et al., 2000a; Ciampolini and Harding, 2000). *In vivo* studies showed that *S. epidermidis* applied on the surface of titanium implants, both as adherent cells and as a pregrown biofilm, rapidly relocated from the implants to the surrounding tissue (Riool et al., 2014). Similarly, large numbers of *S. aureus* were cultured from mouse tissues around infected titanium (Riool et al., 2017a,b) and silicon elastomer implants (de Breij et al., 2016). In a murine model of chronic osteomyelitis, *S. aureus* was found in osteoblasts and osteocytes, as well as in canaliculi of live cortical bone (de Mesy Bentley et al., 2017).

Bacterial invasion of the peri-implant tissue and subsequent development of infection is facilitated by dysregulation of the local immune response resulting from the presence of a foreign body. The phagocytic and intracellular killing activities of neutrophils and macrophages are reduced due to altered cytokine tissue levels in the presence of a biomaterial (Boelens et al., 2000a,b,c; Broekhuizen et al., 2010; Zimmerli and Sendi, 2011). In agreement, microscopical examination has revealed that many of the bacteria reside within these inflammatory phagocytes (Broekhuizen et al., 2010). Interestingly, studies in mice infected with *S. epidermidis* as well as in infected peri-catheter tissue biopsies obtained from deceased intensive care unit patients showed that bacteria present in tissue surrounding the implants had incorporated bromodesoxyuridine, demonstrating that the bacteria can replicate in the peri-implant tissue (Broekhuizen et al., 2010). Furthermore, bacteria may adapt to the tissue and intracellular micro-environment by the formation of so-called small colony variants. The presence of such intracellular small colony variants further complicates treatment as they are more resistant to antimicrobial compounds (Tuchscherr et al., 2010; Zaat, 2013).

Antimicrobial Resistance

In addition to the limited activity of antibiotics against biofilm-encased bacteria, persisters, and intracellular bacteria, the emergence of resistance among staphylococci as well as other bacterial species causing BAI constitutes a major challenge to the efficacy of (combinations of) conventional antibiotics. The emergence of multidrug-resistant (resistant to at least one agent in three or more antimicrobial classes), extensively drug-resistant (resistant to at least one agent in all but one or two antimicrobial classes), and pan-drug-resistant (resistant to all agents in all antimicrobial classes) pathogens, is accelerated by the selective pressure exerted by extensive use and abuse of antimicrobials

Abbreviations: AMPs, antimicrobial peptides; BAI, biomaterial-associated infections; CM, cecropin melittin; PEG, polyethylene glycol; PET, polyethylene terephthalate; pHEMA, poly-hydroxyethylmethacrylate; PLEX, polymer-lipid encapsulation; matrixPS, polystyrene; PU, polyurethane; SAAP, synthetic antimicrobial and anti-biofilm peptide; TiO_2, titanium oxide.

(Magiorakos et al., 2012). Bacteria belonging to the so-called ESKAPE panel (*Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa,* and *Enterobacter* species) are increasingly prevalent and resistant and thereby a particularly dangerous group of bacteria (Rice, 2008). Currently, the majority of hospital infections in the United States is caused by multidrug-resistant ESKAPE bacterial strains (Boucher et al., 2009). The World Health Organization recently endorsed a global action plan to tackle antibiotic resistance to avoid the dark scenario of a "post-antibiotic era" (Chan, 2015). One of the key objectives of this plan is to develop novel antimicrobial drugs with a mode of action different from those of current antibiotics.

ANTIMICROBIAL PEPTIDES

Antimicrobial peptides (AMPs)—effector molecules of the innate defense of animals, plants, and microorganisms (Zasloff, 2002; Hancock and Sahl, 2006)—have recently attracted considerable interest as agents that may subvert many of the problems related to BAI, i.e., they display antimicrobial activity against bacteria resistant to antibiotics and residing within biofilms. A specialized biofilm-active AMPs database lists most of the published AMPs with anti-biofilm activity (Di Luca et al., 2015). AMPs are mostly amphipathic, cationic peptides that display antimicrobial activity against bacteria, fungi and (enveloped) viruses. They interact with specific constituents of the bacterial cell envelope resulting in depolarization, destabilization, and/or disruption of the bacterial plasma membrane leading to bacterial cell death within minutes (Pasupuleti et al., 2012). Due to the rapid and non-specific mechanisms of action, the risk of resistance development is generally thought to be low (Zasloff, 2002). Nonetheless, resistance to AMPs in bacteria does occur and several mechanisms of resistance have been described, including membrane and cell envelope structure alterations increasing positive charge, upregulation of efflux pumps, and proteolytic degradation of the peptides (Goytia et al., 2013; Ernst et al., 2015). For instance, resistance against the human cathelicidin LL-37 has been reported to involve degradation of the peptide by bacterial proteolytic enzymes, up-regulation of efflux pumps as well as bacterial-induced down-regulation of LL-37 expression in host cells (Bandurska et al., 2015). Under low calcium or magnesium ion concentrations, as in blood plasma, *P. aeruginosa* activates the *pmr* (polymyxin resistance) operon, which medicates the addition of N-arabinose to its lipopolysaccharide. This renders the outer surface of the bacterial cell more positively charged, repelling the cationic AMPs (Goytia et al., 2013). So, resistance of bacteria against AMPs is possible for several bacterial species, however development of such resistance against novel synthetic AMPs has not often been studied.

In addition to direct antimicrobial activity, AMPs display immunomodulatory activities. For example, they can prevent excessive activation of pro-inflammatory responses due to bacterial endotoxins such as lipopolysaccharide of Gram-negative bacteria, and peptidoglycan and lipoteichoic acid of Gram-positive bacteria. AMPs may improve clearance of bacterial biofilms by host defense systems (Mansour et al., 2014, 2015) as they may prevent derangement of immune responses after implantation of foreign bodies (Zaat et al., 2010; Heim et al., 2014, 2015). Other favorable characteristics of AMPs relate to wound healing (Nakatsuji and Gallo, 2012), angiogenesis (Salvado et al., 2013), and osteogenic activity (Kittaka et al., 2013; Zhang and Shively, 2013). Regarding the latter activity, it has been reported that in a trabecular bone growth *in vivo* study, cylindrical titanium implants coated with the antimicrobial peptide HHC36 had osteoconductive properties (Kazemzadeh-Narbat et al., 2012). Similarly, fusion peptide P15-CSP showed anti-biofilm activity and pro-osteogenic activity (Li et al., 2015) and LL-37 promoted bone regeneration in a rat calvarial bone defect model (Kittaka et al., 2013) and accelerated bone repair in NOD/SCID mice (Zhang and Shively, 2013).

Naturally occurring AMPs have been used as design templates for a large variety of synthetic AMPs, some of which have reached the stage of phase 2 and 3 clinical trials (Fox, 2013; Greber and Dawgul, 2016), such as OP-145 (Peek et al., 2009), LL-37 (Grönberg et al., 2014), Iseganan (IB-367; Mosca et al., 2000), Omiganan (MBI-226; Sader et al., 2004), and Pexiganan (MSI-78; Fuchs et al., 1998). With respect to the development of synthetic peptides for the treatment of BAI we will focus on a few of the most promising peptides. The synthetic peptide IDR-1018 prevented biofilm formation by *S. aureus* and various other species by blocking (p)ppGpp, which is a signal molecule in persister development (Harms et al., 2016) and biofilm formation (Mansour et al., 2015). In a murine model of *S. aureus* implant infection, IDR-1018 showed to be potentially useful in reducing orthopedic infections by recruiting macrophages to the infection site, blunting excess cytokine production and reducing osseointegration failures (Choe et al., 2015).

In an attempt to meet the requirements for the treatment of BAI as much as possible, a series of novel synthetic AMPs was recently developed based on two human AMPs, i.e., thrombocidin-1, the major antimicrobial protein of human blood platelets (Krijgsveld et al., 2000; Kwakman et al., 2011), and LL-37, a principal human AMP produced by mucosal epithelial cells and multiple immune cells. The LL-37-inspired peptide OP-145 (formerly designated as P60.4Ac; Nell et al., 2006) proved to be safe and efficacious for treatment of therapy-resistant otitis media patients (Peek et al., 2009). *In vitro*, OP-145 (de Breij et al., 2016), and the newer generation LL-37-inspired peptides SAAP-145 and SAAP-276 (Riool et al., 2017a) and the trombocidin-1-derived peptide TC19 (Zaat et al., 2014) inhibited biofilm formation by a clinical *S. aureus* BAI isolate in a dose-dependent fashion. The mode of action of these synthetic peptides may involve inhibition of adherence of bacteria to surfaces and/or reduction of expression of genes involved in biofilm formation, as has been reported for LL-37 (Overhage et al., 2008). These novel synthetic peptides all rapidly permeabilize the membrane of *S. aureus* bacteria (Riool et al., 2017a), explaining why they are highly effective against dividing as well as non-dividing, biofilm-encased bacteria whether or not resistant to antibiotics. Interestingly, these newer generation peptides display good bactericidal activity in the presence of human plasma, despite possible binding of the

peptides to plasma components (de Breij et al., 2016). In contrast, the first generation AMP OP-145 showed strong reduction of antimicrobial activity in plasma *in vitro*. Despite this OP-145 proved to be effective in preventing *S. aureus* colonization of subcutaneous implants in mice and protected rabbits from experimental intramedullary nail-associated osteomyelitis (de Breij et al., 2016). Apparently, *in vitro* activities in the presence of human plasma do not necessarily predict the *in vivo* potency of AMPs.

The physical properties of synthetic AMPs, i.e., cationic charge and peptidic nature, present challenges to their biological stability and balance between antimicrobial efficacy and host cell toxicity. Fortunately, several solutions can be considered to address these issues. For example, PEGylation is a well-accepted method for minimizing cytotoxicity while maintaining antimicrobial activity of AMPs and reducing elimination of the peptides by the liver and kidneys (Morris et al., 2012). D-enantiomers—peptides that are comprised of unnatural amino acids—and (retro-)inverso peptides are insensitive to most peptidase activity (Guichard et al., 1994; Feng and Xu, 2016). In this connection, a series of modified HHC10 peptides were synthesized, including inverso-CysHHC10 (i.e., different stereo isomer). Inverso-CysHHC10 was stable in human serum, showed microbicidal activities at low micromolar concentrations against *Escherichia coli*, *S. aureus,* and *S. epidermidis* and was active in a polyethylene glycol (PEG)-based hydrogel in serum (Cleophas et al., 2014). Of note, serum may be a worst-case scenario for peptides, since Fibrinopeptide A peptides were degraded in serum, but not in fresh blood (Böttger et al., 2017). Serum may have a level of proteolytic activity not encountered in blood or plasma, since preparation of serum involves blood coagulation, which leads to activation of coagulation pathway proteases (Chambers and Laurent, 2002). Therefore, inhibition/degradation is best studied in plasma or fresh blood.

Another area of potential improvement of synthetic AMPs is that of intracellular antimicrobial activity, required to treat intracellular infections. In general, AMPs do not effectively penetrate host cells due to their high positive charge. Several cell-penetrating peptides have been developed for intracellular "delivery" of peptides. Such peptides may be utilized to deliver AMPs and PEGylated peptides into host cells to facilitate elimination of intracellular pathogens, e.g., staphylococci, residing within inflammatory and other cells, such as osteoblasts (Iwase et al., 2016).

PREVENTIVE STRATEGIES

For prevention of BAI, various types of antimicrobial biomaterials have been developed, including (i) antifouling surfaces, (ii) contact-killing surfaces, and (iii) surfaces which incorporate and release antimicrobials (Busscher et al., 2012). These approaches all have their benefits and limitations, which need to be taken into account when designing an antimicrobial strategy for a particular device (Brooks et al., 2013). Importantly, both biofilm formation on the implant and colonization of the peri-implant tissue need to be taken into consideration when designing preventive strategies against BAI. Here, we will discuss various combinations of these strategies and AMPs to prevent BAI (summarized in **Figure 1**).

Antifouling Surfaces

Already in 1987, Gristina suggested that adhering tissue cells and bacteria compete for a spot on the implant's surface, the so-called "race for the surface" concept (Gristina, 1987). In case this race is won by the bacteria, this will result in infection instead of tissue integration. Gristina also realized that colonization of the tissue around implants was another possible mechanism of infection (Gristina, 1987). Bacterial adhesion and subsequent biofilm formation may be prevented by modifying the physicochemical surface properties of biomaterials such as the surface charge, hydrophobicity/hydrophilicity, and surface chemistry. One strategy is to use hydrophilic polymer coatings, e.g., immobilized PEG, as applied on contact lenses, shunts, endotracheal tubes, and urinary catheters (Banerjee et al., 2011; Busscher et al., 2012). Another approach is functionalization of the surface with a dense layer of polymer chains commonly known as polymer brush coatings (Nejadnik et al., 2008; Neoh et al., 2013; Keum et al., 2017). Large exclusion volumes of tethered polymer chains result in surfaces difficult to approach by proteins or bacteria.

Contact Killing Surfaces

Another approach to prevent implant colonization is the immobilization of AMPs on surfaces of medical devices, which can be performed with a variety of chemical techniques. An excellent overview of immobilization strategies has recently been published by Silva et al. (2016). There are several common "rules" for success. The structural characteristics important for the antimicrobial activity of the peptides should not be altered by the immobilization process. Length, flexibility, and kind of spacer connecting the peptide to the surface, orientation of the immobilized peptides, and the AMP surface density are additional important parameters (Costa et al., 2011). Interestingly, even short surface-attached peptides not likely to have a free interaction with the bacterial cytoplasmic membrane, have antimicrobial activity (Hilpert et al., 2009). This is thought to be due to destabilization of the bacterial membrane by displacement of positively charged counter-ions, disrupting the ionic balance, changing bacterial surface electrostatics, and activating autolytic enzymes (Hilpert et al., 2009). An example of a contact-killing surface is the hydrogel network with the covalently attached stabilized inverso-CysHHC10 peptide (Cleophas et al., 2014). This coating demonstrated high *in vitro* antimicrobial activity against *S. aureus*, *S. epidermidis,* and *E. coli*. Furthermore, brush coating molecules may also possess active functional groups with antimicrobial activity, e.g., by conjugation with the AMPs Tet20 (Gao et al., 2011a) and Tet213 (Gao et al., 2011b). Another example is polyurethane with a brush coating tethered with the AMP E6 for the prevention of catheter-associated infections (Yu et al., 2017). This surface coating reduced bacterial adhesion on the catheter surface in a mouse urinary catheter infection model. A variety of AMPs, like GZ3.27 (De Zoysa and Sarojini, 2017), GL13K

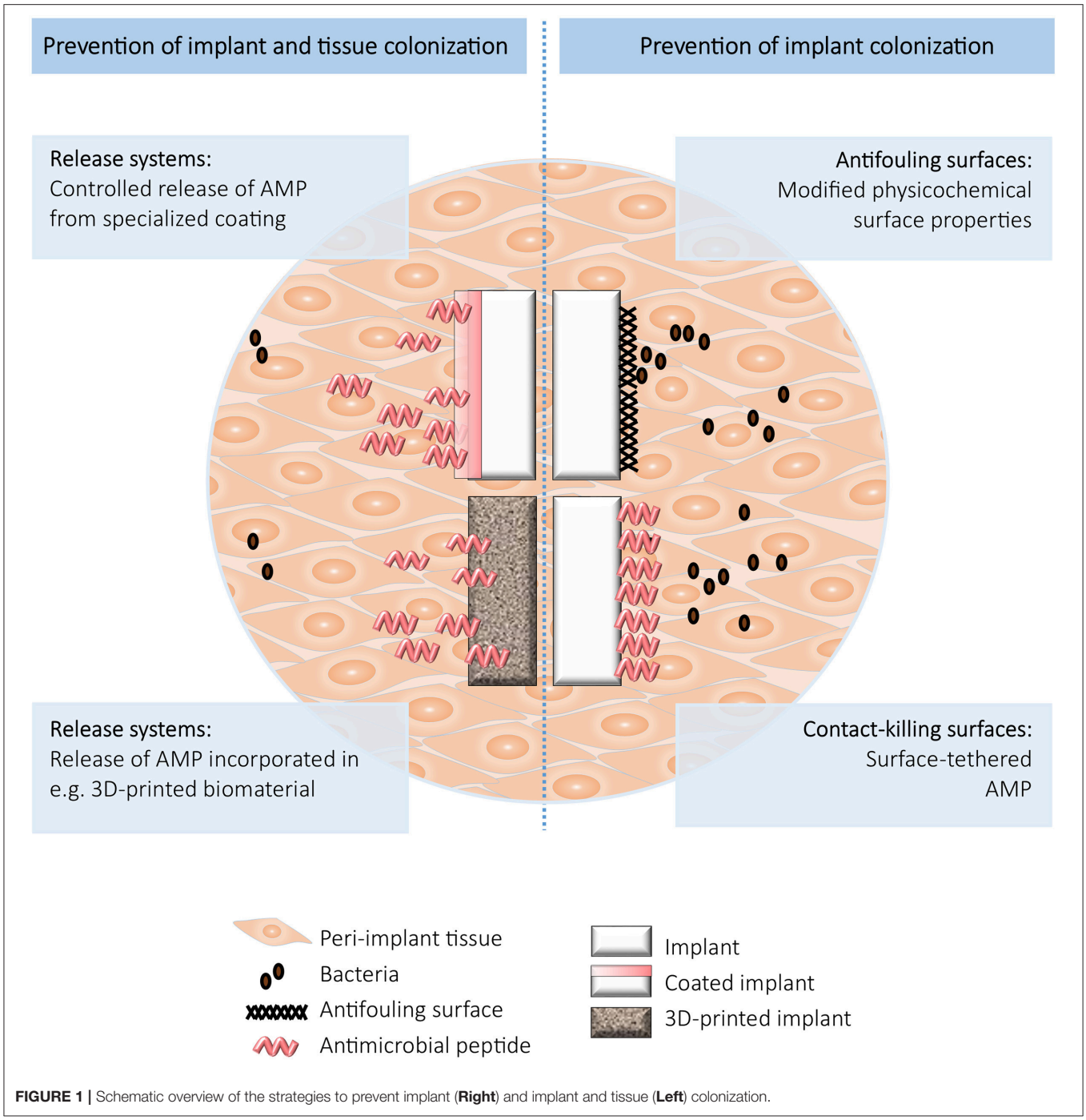

FIGURE 1 | Schematic overview of the strategies to prevent implant (**Right**) and implant and tissue (**Left**) colonization.

(Chen et al., 2014; Zhou et al., 2015), SESB2V (Tan et al., 2014), bacitracin (Nie et al., 2016, 2017), hLF1-11 (Costa et al., 2014; Godoy-Gallardo et al., 2015), LL-37, Melimine, lactoferricin, and Mel-4 (Chen et al., 2016; Dutta et al., 2016) have been covalently coupled onto various surfaces, such as glass, silicon, and titanium, with different degrees of success (summarized in **Table 1**). Chimeric peptides comprised of both a titanium binding domain and an antimicrobial motif are also used to create contact-killing surfaces (Yucesoy et al., 2015; Liu et al., 2016; Yazici et al., 2016). Due to their titanium-binding domain, the peptides preferentially bind the implant, while the freely exposed antimicrobial domain is available for combatting invading bacteria. Titanium surfaces modified with these chimeric peptides were found to significantly reduce adhesion of different *Streptococcus* species, *S. aureus*, *S. epidermidis*, *P. aeruginosa,* and *E. coli*, compared to bare titanium. Immobilization of GL13K onto titanium dental implants even enabled osseointegration when tested in rabbit

TABLE 1 | Overview AMP contact-killing surfaces.

AMP	Coating type[1]	Surface[2]	Antimicrobial activity	References
Bacitracin	Surface tethering	Titanium	Reduction surface adhesion by *S. aureus in vitro*, and reduction implant and tissue colonization by *S. aureus* in a rat femur implant infection model	Nie et al., 2016, 2017
Chimeric peptide[a]	Binding domain	Titanium	Surface bactericidal activity against *Streptococcus gordonii* and *S. sanguinis*[e] *in vitro*	Liu et al., 2016
Chimeric peptide[b]	Binding domain	Titanium	Reduction surface adhesion by *Streptococcus mutans*, *S. epidermidis,* and *E. coli in vitro*	Yucesoy et al., 2015; Yazici et al., 2016
E6	Polymer brushes	PU	Reduction catheter surface colonization by *P. aeruginosa*, *S. aureus,* and *Staphylococcus saprophyticus in vitro* and by *P. aeruginosa* in mouse urinary catheter infection model	Yu et al., 2017
GL13K	Surface tethering	Titanium	Surface bactericidal activity against *S. gordonii* and *Porphyromonas gingivalis in vitro*	Chen et al., 2014; Zhou et al., 2015
GZ3.27[c]	Surface tethering	Titanium, glass, silicon	Surface bactericidal activity against *P. aeruginosa* and *E. coli in vitro*	De Zoysa and Sarojini, 2017
hLF1-11	Polymer brushes	Titanium	Surface bactericidal activity against *S. sanguinis* and *Lactobacillus salivarius in vitro*	Godoy-Gallardo et al., 2015
hLF1-11	Surface tethering	Titanium, chitosan	Reduction surface colonization by *S. aureus* (both surfaces) and *S. sanguinis* (chitosan) *in vitro*	Costa et al., 2014; Hoyos-Nogués et al., 2017
Inverso-CysHHC10	Hydrogel	PET	Surface bactericidal activity against *S. aureus*, *S. epidermidis,* and *E. coli in vitro*	Cleophas et al., 2014
Magainin I	Self-assembling silk	PS	Reduction surface adhesion by *S. aureus in vitro*	Nilebäck et al., 2017
Melimine	Surface tethering	Titanium	Reduction surface adhesion by *P. aeruginosa in vitro*, and reduction implant and tissue colonization by *S. aureus* in mouse and rat subcutaneous implant infection	Chen et al., 2016
Melimine, Mel-4, LFc[d], LL-37	Surface tethering	pHEMA	Surface bactericidal activity against *P. aeruginosa* (LL-37, Mel-4, and Melimine) and *S. aureus* (Mel-4 and Melimine) *in vitro*	Dutta et al., 2016
SESB2V	Surface tethering	Titanium	Reduction tissue colonization by *S. aureus* and *P. aeruginosa* in a rabbit keratitis model	Tan et al., 2014
Tet20	Polymer brushes	Titanium	Surface bactericidal activity against *P. aeruginosa* and *S. aureus in vitro*, reduction surface adhesion by *S. aureus* in rat subcutaneous implant infection	Gao et al., 2011a
Tet213	Polymer brushes	Titanium	Surface bactericidal activity against *P. aeruginosa in vitro*	Gao et al., 2011b

[1] *Surface tethering by covalent immobilization of AMP to surface; Chimeric peptide consists of titanium-binding domain and antimicrobial motif.*
[2] *PET, polyethylene terephthalate; PU, polyurethane; pHEMA, poly-hydroxyethylmethacrylate; PS, polystyrene.*
[a] *Chimeric peptides consist of minTBP-1 and JPH8194 motifs.*
[b] *Chimeric peptides consist of TiBP(S)1–3 and E14LKK/H14LKK or KWKRWWWWR motifs.*
[c] *GZ3.27 with an added N-terminal cysteine is designated GZ3.163.*
[d] *LFc, lactoferricin.*
[e] *Formally known as Streptococcus sanguis, as mentioned in the reference.*

femurs (Chen et al., 2017). Another promising strategy is the development of multifunctional coatings by combining the well-known RGD cell adhesive sequence with the lactoferrin-derived AMP LF1-11, resulting in *in vitro* cell integration as well as inhibition of bacterial colonization by *S. aureus* and *Streptococcus sanguinis* (Hoyos-Nogués et al., 2017). Recently, others described a self-assembling coating of recombinant spider silk protein fused to the AMP Magainin I for different biomaterials, which reduced numbers of live bacteria on the coated surfaces (Nilebäck et al., 2017). It should be noted that not in all studies described above the absence of unbound peptide within the coating is verified. Thus, in those cases it cannot be excluded that the antimicrobial activity of the coating is caused by a combination of bound and released AMP.

It should be noted that surface attachment of peptides does suffer from some disadvantages. The antimicrobial activity of the surface with immobilized AMPs is critically dependent on the chemical tethering procedure and the orientation of the covalently attached AMPs. The antimicrobial activity of the resulting coating may be strongly reduced compared to the activity of the peptide in free form (Bagheri et al., 2009; Onaizi and Leong, 2011; Dutta et al., 2016). Apart from this reduction of activity due to the tethering process, proteins, blood platelets, and dead bacteria may block the antimicrobial groups on the surface. Moreover, since the antimicrobial activity is restricted to the surface of the implant, there is a lack of antimicrobial impact on bacteria in the tissue surrounding the implant. Contact-killing surfaces will only eradicate bacteria that are in direct contact with the active surface, meaning that clearance of any bacteria further

away from the surface will depend on efficient phagocytosis and systemic or local antibiotics. However, as mentioned before, due to the presence of a biomaterial the local host immune response is dysregulated, and therefore phagocytosed bacteria may not be killed and may even persist intracellularly (Boelens et al., 2000a,b).

Release Systems

As described above, the peri-implant tissue is an important niche for bacterial survival. Therefore, antimicrobial-releasing surfaces or coatings from which the antimicrobial agent also reaches this niche are preferred to prevent BAI. Antibiotic-releasing coatings are widely used for medical devices such as sutures and central venous catheters and urinary tract catheters. However, these coatings have two major disadvantages: (i) a patient may be infected with a bacterium resistant to the released antibiotic, and (ii) due to the local release a gradient of the antibiotic will be present near the implant thereby increasing the risk to select for resistant bacteria. Coatings releasing antibiotics for orthopedic devices remain mainly experimental (Lucke et al., 2003; Kälicke et al., 2006; Darouiche, 2007; Moojen et al., 2009; Alt et al., 2011). The first commercially available gentamicin-releasing intramedullary tibia nail has recently shown promising results in a first prospective study (Fuchs et al., 2011; Metsemakers et al., 2015; Alt, 2017). In view of the increasing development of antibiotic resistance among bacteria, the use of antibiotics in medical devices is discouraged by government regulatory agencies like the American Food and Drug Administration (FDA, 2007; Brooks et al., 2013). Obviously, coatings releasing antimicrobial agents that are less likely to induce resistance, such as AMPs, are preferred in view of both managing resistance development and compatibility with use of antibiotics for prophylaxis or treatment. To prevent the spread of bacteria from the implant surface to the surrounding tissue, and to eradicate bacteria contaminating tissue during surgery, a rapid initial release of antimicrobials is required. If this release is delayed, bacteria may "escape" into host cells before effective levels of the antimicrobial agent have been established. Subsequently, prolonged local release of the antimicrobial agent at sufficiently high concentrations will be required to eradicate any residual bacteria (Zilberman and Elsner, 2008; Emanuel et al., 2012).

Application of AMPs in antimicrobial surface coatings is a subject of increasing interest and different types of release-coatings have been described, including hydrogels, nanotubes, microporous calcium phosphate coatings, and polymer coatings (summarized in **Table 2**). Hydrogels with the AMP Cateslytin strongly adhere to dental implant surfaces. The hydrogels showed potent antimicrobial activities against *Porphyromonas gingivalis*, an important causative agent of peri-implantitis, without signs of toxicity (Mateescu et al., 2015). Another example is a gelatin-based hydrogel on titanium surfaces allowing for the controlled release of the short cationic AMP HHC36 preventing *S. aureus*, *S. epidermidis*, *E. coli,* and *P. aeruginosa* biofilm formation (Cheng et al., 2017).

Self-organized and vertically oriented titanium oxide nanotubes loaded with the broad spectrum AMP HHC36 showed *in vitro* bactericidal activity against *S. aureus* in liquid surrounding the nanotubular surface and reduced bacterial colonization on the surface ∼200-fold (Ma et al., 2012). GL13K-eluting coatings on these titanium oxide nanotubes prevented growth of *Fusobacterium nucleatum* and *P. gingivalis* in an *in vitro* disk-diffusion assay (Li et al., 2017). *In vitro* release of Tet213 from microporous calcium phosphate coatings applied on titanium showed bactericidal activity against *S. aureus* and *P. aeruginosa* (Kazemzadeh-Narbat et al., 2010). In a similar approach, release of PSI 10 from microporous calcium phosphate

TABLE 2 | Overview AMP release coatings.

AMP	Coating type[1]	Surface	Antimicrobial activity	References
Cateslytin (CTL)	Hydrogel	Titanium, gingiva[b]	Surface bactericidal activity against *P. gingivalis in vitro*	Mateescu et al., 2015
GL13K	TiO_2 nanotubes	Titanium	Antimicrobial activity against *F. nucleatum* and *P. gingivalis in vitro*	Li et al., 2017
HHC36	TiO_2 nanotubes	Titanium	Bactericidal activity against *S. aureus* in solution and on surface *in vitro*	Ma et al., 2012
HHC36	Hydrogel	Titanium	Surface bactericidal activity against *S. aureus*, *S. epidermidis*, *E. coli*, and *P. aeruginosa in vitro*	Cheng et al., 2017
OP-145	PLEX	Titanium	Bactericidal activity against planktonic *S. aureus in vitro*, prevention of *S. aureus* BAI in a rabbit intramedullary implant infection model	de Breij et al., 2016
PSI 10	Microporous calcium phosphate	Magnesium alloy	Bactericidal activity against *S. aureus* in solution *in vitro*	Tian et al., 2015
SAAP-145, SAAP-276	PLEX	Titanium	Reduction implant and tissue colonization by *S. aureus* in a subcutaneous mouse implant infection model	Riool et al., 2017a
Tet213	Microporous calcium phosphate	Titanium	Bactericidal activity against *S. aureus* and *P. aeruginosa* in solution *in vitro*	Kazemzadeh-Narbat et al., 2010
Tet213	Collagen[a]	Titanium	Antimicrobial activity against *P. gingivalis* and *S. aureus* in solution *in vitro*	Shi et al., 2015

[1] *PLEX, polymer-lipid encapsulation matrix; TiO_2, titanium oxide.*
[a] *Biodegradable coating of Tet213 linked to collagen.*
[b] *Hydrogel adheres upon injection.*

coated magnesium alloy inhibited *S. aureus* growth *in vitro* and promoted *in vivo* bone repair (Tian et al., 2015). Furthermore, controlled release of Tet213 linked to collagen IV inhibited *S. aureus* biofilm formation *in vitro* (Shi et al., 2015). However, these types of coatings have not yet been tested *in vivo*.

Injection of the LL-37-inspired AMPs OP-145 (de Breij et al., 2016), SAAP-145, and SAAP-276 (Riool et al., 2017a) along subcutaneous implants in mice did not reduce the numbers of *S. aureus* in the surrounding tissue. This might be because the AMPs did not effectively penetrate the tissue or were not taken up by the host cells and thereby not capable of killing internalized bacteria. However, when these AMPs were released from Polymer-Lipid Encapsulation Matrix (PLEX) coatings, the numbers of viable *S. aureus* bacteria were reduced in the peri-implant soft tissue in mice (Riool et al., 2017a) and even in bone in a rabbit humerus intramedullary nail infection model (de Breij et al., 2016). This clearly illustrates the benefit of the PLEX coating technology allowing controlled and prolonged release of the AMPs at the implant-tissue-interface. The SAAP-276-PLEX-coated implants were able to significantly, but not completely, reduce the number of doxycycline-resistant *S. aureus* in the peri-implant tissue, in contrast to the doxycycline-PLEX coated implants which failed to reduce their numbers in the tissue (Riool et al., 2017a). This underlines the potency of SAAP-276-PLEX coatings in the fight against BAI caused by multidrug-resistant staphylococci.

Although the AMPs mentioned above reduced the colonization of the peri-implant tissue *in vivo* when released from a coating, they might still not be able to act against intracellular bacteria. Apparently, the rapid initial release of the AMPs killed the vast majority of the infecting bacteria, preventing biofilm formation on the implant surface as well as colonization of the tissue, thereby protecting both these sites against colonization. Treatment of infections featuring intracellular bacteria remains difficult, as observed with the conventional antibiotic vancomycin (Broekhuizen et al., 2008), and likely with the novel AMPs as well. A possible way to improve the intracellular entry of AMPs is by adding a specific domain ("tag") to the peptides as a signal for uptake by the host cells (Splith and Neundorf, 2011; Ye et al., 2016). However, intracellular localization of bacteria does not seem to occur to a large extent when AMPs are used in BAI prevention, as shown for instance with the AMP-PLEX coatings described above. By directly killing the bacteria on the implant-tissue interface the AMPs prevented bacterial invasion into the tissue and internalization by and survival in host cells.

Novel Manufacturing Techniques for Biomaterials, a Role for AMPs?

Several novel technologies are arising for manufacturing implants with particular focus on the possibility of personalization. We will briefly address additive manufacturing and electrospinning with regards to the strategies of incorporation of antimicrobial agents and potential for AMPs.

Additive Manufacturing

Additive manufacturing (3D-printing) of medical devices is a major breakthrough that enables the production of implants customized in size and shape, and potentially with high porosity, thereby increasing the surface area. These aspects make this technique attractive for personalized implants. However, as with conventional implants, the 3D-printed implants are susceptible to infection. Therefore, different approaches are currently explored to develop 3D-printed medical devices with antimicrobial functionalities. For example, antimicrobials may be added by surface modification of the 3D-printed implants, using plasma electrolytic oxidation, also known as micro-arc oxidation (Fidan et al., 2017). In this process, a titanium oxide layer is generated and compounds or nanoparticles present in the electrolyte are incorporated in the growing surface oxide layer (Necula et al., 2009; Lara Rodriguez et al., 2014; Fidan et al., 2017). One antimicrobial agent often used for the implants is silver. Silver is used in numerous medical applications (Bach et al., 1999; Rupp et al., 2005; Osma et al., 2006; Kuehl et al., 2016) and has broad-spectrum antimicrobial activity (Bürgers et al., 2009; Sussman et al., 2015). In a recent study silver nanoparticles were embedded in the titanium oxide layer of 3D-printed titanium implants (van Hengel et al., 2017) using a plasma electrolytic oxidation protocol developed for conventional medical grade titanium implants (Necula et al., 2009, 2012). These 3D-printed implants released silver ions over time, and showed *in vitro* bactericidal activity against MRSA including prevention of biofilm formation, and eradicated MRSA in an *ex vivo* mouse femur implant infection model (van Hengel et al., 2017).

The antibiotics rifampin and vancomycin have been incorporated in 3D-printed calcium-phosphate scaffolds during manufacturing. Due to their local delivery these incorporated antibiotics rendered the scaffolds capable of controlling murine implant-associated bone infection (Inzana et al., 2015). A similar approach might very well be suitable to incorporate AMPs for local delivery, as an alternative for the use of conventional antibiotics. AMPs might also be incorporated in hydrogels to coat the 3D-printed implants, similar to approaches utilizing polymers to create antibiotic release systems (ter Boo et al., 2015, 2016).

Another novel approach currently explored for prevention or treatment of BAI is the use of gold nanoparticles with tethered AMPs to increase the *in vivo* stability of AMPs and decrease possible toxicity. This technology would be readily applicable to 3D-printed implants. Gold nanoparticles conjugated with the hydrophilic cationic peptide cecropin melittin (CM) demonstrated higher antimicrobial activity and stability in serum than the CM peptide in solution, The CM-gold nanoparticles had favorably low cytotoxicity for human cells and demonstrated high antimicrobial activity in mouse chronic wound infection and system infection models (Rai et al., 2016a,b).

Electrospinning

Electrospinning is an entirely different technique which offers many possibilities for manufacturing medical devices. An example is the electrospun prosthetic heart valve, which has reached the phase of advanced preclinical testing (Kluin et al., 2017). By electrospinning, biocompatible nanofibers can

be produced that have a large surface area mimicking the extracellular matrix of the body. The porosity of the matrices may however allow colonization by bacteria. To reduce the risk of infection of electrospun materials, antimicrobial agents have been incorporated in the polymers used for the electrospinning process. Examples include antibiotics such as vancomycin and/or rifampicin (Waeiss et al., 2014; Song et al., 2016), moxifloxacin (Song et al., 2016), silver nanoparticles (Tian et al., 2013; Almajhdi et al., 2014), or combinations of silver nitrate and chlorhexidine (Song et al., 2016). Recently, studies have also reported on the use of AMPs to render electrospun materials antimicrobial. Poly(E-caprolactone) nanofibers have been loaded with the synthetic AMP inverso-crabrolin (Eriksen et al., 2013), and poly(vinyl alcohol) nanofibers with pleurocidin (Wang et al., 2015) or the antifungal peptide Cm-p1 (Viana et al., 2015). In view of the increasing importance of electrospinning applications in the medical field, this is an area where additional studies on the use of AMPs will be highly relevant and novel forms of AMP structures may be highly desired. In this respect a novel class of antimicrobial agents is emerging. This is the class of structurally nanoengineered antimicrobial peptide polymers (SNAPPs). In the form of 16- or 32-arm star-shaped peptide polymer nanoparticles, these SNAPPs showed *in vitro* activity at sub-micromolar concentrations against a wide panel of Gram-negative bacteria, including multidrug-resistant pathogens. They were effective *in vivo* against an multidrug-resistant strain of *A. baumannii* and did not induce resistance (Lam et al., 2016).

CONCLUSIONS AND FUTURE PERSPECTIVE

Prevention and treatment of BAI is a major medical challenge, in particular due to the involvement of biofilm-encased and intracellular multidrug-resistant bacteria. Synthetic AMPs, displaying broad spectrum activity including activity against multidrug-resistant pathogens, anti-biofilm activities, little/no development of resistance, and *in vivo* activity in preventing BAI, are important candidates. Tethering of these AMPs to the biomaterial surfaces, and particularly combining AMPs with formulations to release the peptides in a controlled fashion is expected to protect both the implant and the surrounding tissue, both for conventional implants and biomedical devices manufactured by 3D-printing and electrospinning.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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frontiers in Chemistry

REVIEW
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Screening and Optimizing Antimicrobial Peptides by Using SPOT-Synthesis

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Peptide arrays on cellulose are a powerful tool to investigate peptide interactions with a number of different molecules, for examples antibodies, receptors or enzymes. Such peptide arrays can also be used to study interactions with whole cells. In this review, we focus on the interaction of small antimicrobial peptides with bacteria. Antimicrobial peptides (AMPs) can kill multidrug-resistant (MDR) human pathogenic bacteria and therefore could be next generation antibiotics targeting MDR bacteria. We describe the screen and the result of different optimization strategies of peptides cleaved from the membrane. In addition, screening of antibacterial activity of peptides that are tethered to the surface is discussed. Surface-active peptides can be used to protect surfaces from bacterial infections, for example implants.

Keywords: SPOT-synthesis, antimicrobial peptides, peptide synthesis, antimicrobial screening, peptide libraries, substitution analysis, multi-drug resistance, tethered peptides

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ANTIMICROBIALS AND MICROBIAL RESISTANCE

Since their introduction in the 1930's, antibiotics have been heralded as the wonder discovery of the twentieth century; in the 75 years since their initial introduction (Davies and Davies, 2010), the foundations surrounding the way physicians and health care practitioners care for patients has shifted from a focus on diagnostics, toward a more treatment focused approach that has saved millions of lives worldwide (Spellberg et al., 2011). Undoubtedly, access to efficient antibiotics is of critical importance to society, with numerous procedures including; organ transplants, orthopedic surgery and chemotherapy carrying high, if not prohibitive risk without the accessibility of these antimicrobial agents (Höjgård, 2012). In fact, in the aftermath of this indisputable success, in late 1960's US Surgeon General William H. Stewart stated: "it is time to close the book on infectious diseases and declare the war against pestilence won" (Spellberg et al., 2008). Unfortunately, these seven decades of medical advances and the effectiveness of any novel antibiotic is compromised by the relentless appearance of drug resistant organisms, exhibiting resistance against one or more antibiotic types (Spellberg et al., 2011). The (O'Neill, 2016) commissioned by the former UK Prime Minister David Cameron, estimates that as of 2014, 700,000 people die annually from antimicrobial resistance (AMR) bacterial infections, costing the US health care system alone, $20 billion (O'Neill, 2016). Even more worryingly, these numbers are set to rise, with an estimated 10 million people predicted to succumb to AMRs by 2050 at an increasing global cost of $100 trillion. While the trends of AMR are difficult to predict, it is estimated that the death toll could be as high as one person every 3 s if this issue is not immediately addressed (O'Neill, 2016). While these statistics are frightening, this fate could be avoided by implementing certain interventions including: global

public awareness campaigns, a reduction in the number of unnecessary prescriptions, dramatic reduction and restrictions of antibiotics in agriculture, development of rapid diagnostics and most importantly, the development of novel antimicrobial agents (Spellberg et al., 2011; O'Neill, 2016).

A large proportion of pharmaceutical companies have lost interest in the antibiotic market despite an overall increase in their research and development (R&D) budgets (A.A.D., 2004). In fact, as of 2013, only four big pharmaceutical companies have antibiotic programmes remaining (Fair and Tor, 2014). While the reasons for this are multifactorial, one main reason is that is has become substantially more difficult than it once was to develop a new antimicrobial, particularly against AMR Gram-negative bacteria. In addition, priced at a maximum of $1,000–$3,000 per course, antibiotics hold a very small profit margin in comparison to pharmaceuticals commonly used for long-term illness, which can reach in excess of $80,000 (Bartlett et al., 2013). Therefore, it is critical that pharmaceutical companies are provided an incentive to revitalize interest in antibiotic development.

ANTIMICROBIAL PEPTIDES

Antimicrobial peptides (AMPs) are a diverse family of short length molecules, between 5 and 50 amino acids in length and with most possessing an overall net positive charge to their structure (Hancock and Patrzykat, 2002). These peptides have been gaining attention over recent years as one of the more promising alternatives to traditional antimicrobial drugs–they can display broad spectrum killing properties to Gram-positive and Gram-negative bacteria, fungi, viruses, parasites and even cancerous cells, are fast acting and have a decreased likelihood to induce pathogenic resistance as compared to traditional antimicrobial drugs (Marr et al., 2006).

Over 2,000 AMPs, both natural and lab-synthesized, have been documented and submitted to the Antimicrobial Peptide Database (APD3) (Wang et al., 2009; Zhao et al., 2013). In 2016, the APD3 contained 2,169 antibacterial and 959 antifungal peptides. AMPs can be found naturally in a huge variety of organisms—in animals, plants and even in fungi and bacteria (Leippe, 1999; Radek and Gallo, 2007). It is now understood that these molecules are a major part of the innate immune system of animals, with a large quantity of them localized to the organs and tissues most at risk of coming into contact with pathogens, such as the skin, airways, digestive tract and other epithelial surfaces and mucous membranes (Wiesner and Vilcinskas, 2010). Through release by immune cells, such as phagocytes and T-cells, AMPs are able to rapidly target and kill pathogenic threats (Diamond et al., 2009). They are playing a role in host inflammatory response by their action in modulating cytokine response subsequent to infection, as was recently shown for example when the AMP LL-37 decreased the cytokine response of human neutrophils after infection with *S. aureus* and *P. aeruginosa* (Alalwani et al., 2010). Conversely, AMPs have also shown the ability to upregulate immune response, such as human α-defensins, produced in neutrophil cells, which have been shown to promote the production of pro-inflammatory cytokines (van Wetering et al., 1999).

The cationic nature of most AMPs means that they interact strongly with negatively charged molecules. Therefore, the selectivity of most AMPs relies on a fundamental difference in the architecture of mammalian and microbial membranes (Yeaman and Yount, 2003), allowing the peptide to selectively kill microorganisms (Matsuzaki, 2009). These include; differences in membrane composition, transmembrane potential and membrane polarization as well as other features including lipopolysaccharide (LPS), sterols, glycerides and peptidoglycan (Yeaman and Yount, 2003). The outside of bacterial membranes is rich in negatively charged phospholipids, such as: phosphatidylglycerol, phosphatidylethanolamine and cardiolipin (Zasloff, 2002). Further anionic molecules also exist in the bacterial cell wall, including lipoteichoic acids in the peptidoglycan of Gram-positive bacteria and LPS present in the outer membrane of Gram-negative bacteria (Matsuzaki, 2009; Guilhelmelli et al., 2013). In comparison, the negatively charged phospholipids of mammalian membranes usually reside in the inner leaflet of the bilayer (Zasloff, 2002), while the outermost leaflet, the layer exposed to the cytoplasm, is populated by the neutral phospholipids sphingomyelin, phosphatidylcholine, as well as sterols, such as cholesterol (Matsuzaki, 2009; Guilhelmelli et al., 2013). This difference in the charge of mammalian and microbial membranes means that AMPs show different affinities toward each respective membrane.

Another key variance between mammalian and microbial cells is the difference in the charge separation between the intra- and extracellular components of the cytoplasmic membrane. This transmembrane potential (Yeaman and Yount, 2003), dictated by the interactions between the negatively charged peptide and the phospholipid headgroups (Teixeira et al., 2012), is a result of the different rates and extents of proton flux over the cell membrane. In bacterial cells, this transmembrane potential is more negative, ranging from −130 mv to −150 mV in comparison to mammalian cells, with a range of −90 mv to −110 mV (Yeaman and Yount, 2003). Because cells with a more negative transmembrane potential facilitate membrane permeabilisation by encouraging cationic peptides to insert themselves into the membrane (Matsuzaki, 2009), cationic peptides can more easily permeabilise the bacterial cells, further providing AMPs with a means of selectivity toward microbial cells (Yeaman and Yount, 2003). A study performed by Matsuzaki supported this theory by demonstrating that the binding constant of the peptide tachyplesin is increased 200-fold when the membrane potential is as low as −120 mV (Matsuzaki et al., 1997; Yeaman and Yount, 2003).

Anionic AMPs can interact by amidation of the C-terminus (maxim H5) (Dennison et al., 2015), or through the formation of salt bridges by metal ions (Harris et al., 2009). Once this association has been completed, the AMP can be inserted into the membrane via the hydrophobic regions of its structure (Madani et al., 2011). AMPs can exert their antimicrobial effect by acting on the outside of their target at the cell membrane, or they can function by entering the cell and interacting with intracellular

proteins or disrupting key processes, such as RNA- and DNA-synthesis.

For some AMPs a combination of extra- and intracellular activity is required for the death of their target (Koo et al., 2001). A major mechanism by which certain AMPs carry out their function on an intracellular level is by the inhibition of DNA, RNA and protein synthesis (Hilpert et al., 2010). Buforin 2, an AMP found in the stomach of Bufo gargarizans, the Asian toad, is able to penetrate the cell membrane via the action of a proline hinge and bind to DNA and RNA (Kobayashi et al., 2000; Xie et al., 2011), likely due to the similarity in sequence between buforin 2 and histone H2A's N-terminus (Cho et al., 2009). Indolicidin, isolated from bovine neutrophil granules, has been shown to bind to DNA where it blocks DNA-dependent enzymes, such as integrase from binding (Marchand et al., 2006). The central PWWP amino acid motif, a feature common to proteins involved in DNA binding (Hale and Hancock, 2007) has also shown importance in stabilizing the DNA structure once the peptide has attached (Ghosh et al., 2014). Attacin is able to contribute to destabilization of the membrane of *E. coli* by preventing the transcription of the omp gene, thereby blocking the synthesis of important outer membrane proteins (Carlsson et al., 1991). It was also described that proline-rich peptides can bind the chaperon DnaK and the ribosome (Krizsan et al., 2015; Knappe et al., 2016a). Short AMPs can interact with ATP and inhibit ATP depended enzymes critical for the survival of the bacteria (Hilpert et al., 2010). AMPs are also capable of preventing cell wall synthesis. Peptidoglycan is a major structural component of bacteria, being the main constituent of the cell wall in Gram-positive organisms and linked with the outer cell membrane in Gram-negative bacteria, providing protection and support. An important precursor of its synthesis is lipid 2, which has been shown to be the target of several AMPs including HND-1 (de Leeuw et al., 2010) and Cg-Defh-1 (Schmitt et al., 2010).

PEPTIDE LIBRARIES

Screening of compounds libraries is well established as a high-throughput method for detecting and studying interactions in both biological and chemical systems. Libraries can be composed of various types of molecules, ranging from small organic compounds to peptides, proteins and RNA/DNA. Many important processes in life are regulated by peptides of different size and complexity. Well-known examples are peptide hormones, like insulin, peptide neurotransmitters (e.g., opioid peptides) that influence pain and mood and peptides that influence digestion and vascular functions. There are peptides that support the immune system like (AMPs). In many biological toxins, from plants (fungi, phalloidin) to animals (honey bee, apamin), peptides play an important role (Jakubke and Sewald, 2009). Proteins play key roles in many cellular processes and in some cases can be recapitulated by shorter peptides taken from the primary protein sequences (although often with partial loss of activity). Peptides have come into the focus of the pharmacological industry not only based on high potency, but also their high selectivity and safety. The global peptide drug market was US$14.1 billion in 2011 and is estimated to reach US$25.4 billion in 2018 (Fosgerau and Hoffmann, 2015). Hence, peptide libraries can serve as valuable tools for identifying and optimizing biologically active compounds. Biological synthesized peptide libraries, such as phage, yeast, bacterial or ribosomal mRNA displays (Ullman et al., 2011) rely on (i) creating a diverse genetic library in which the phenotype (binding to target) of each member of the library is linked to its genotype (the encoding DNA or RNA), and (ii) an iterative cycle in which library members are selected for binding to a target, and then amplified (by replication in a host cell, or by copying of the encoded nucleic acid *in vitro*). These techniques allow the selection of highly active peptides due to several rounds of enrichment and a huge number of different peptide sequences (over 10^9) that can be displayed.

However, even being nowadays possible (Tian et al., 2004), the use of non-natural amino acids is still difficult. Through optimization of the chemistry, automation and miniaturization of solid-phase peptide synthesis, chemical peptide libraries can be built using different solid supports (resin beads, pins, glass chips, tea bags, and cellulose membranes) not being restrictive to the use of gene-coded amino acids (Houghten, 1985; Weinberger et al., 1997; Tribbick, 2002; Weiser et al., 2005; Breitling et al., 2009, 2011; Diehnelt, 2013).

For the efficient analysis of large peptides libraries, an automated computer-based analysis of experimental results is crucial. It consists of a quantitative analysis of activity measurements (e.g., to compute activity-related values based on luminescence measurements of a dilution series Mikut, 2010) and the generation of new promising candidate sequences for a synthesis. The latter step can be done (i) based on a systematic substitution of single amino acids in a sequence or (ii) the computation of molecular descriptors of a sequence followed by a model-based evaluation of activity predictions (Cherkasov et al., 2009; Mikut and Hilpert, 2009; Torrent et al., 2011; Fernandes et al., 2012; Müller et al., 2016). Only few methods exist for the prediction of toxic effects to get an early insight in the therapeutic potential, see for example Cruz-Monteagudo et al. (2011). However, many relevant experimental procedures, e.g., for a detailed analysis of blood interactions (Yu et al., 2015), are not yet established for high-throughput screens. In this review, we will focus on screens for (AMPs) synthesized by the SPOT-synthesis technique.

PEPTIDE SPOT-SYNTHESIS

The basic concept of SPOT-synthesis is based on the capacity of reactions to run until completion in a porous and planar surface when enough reagents solution that the membrane is able to absorb is added. This observation was made separately for combinatorial nucleotides and peptide synthesis (Frank et al., 1983; Frank and Doring, 1988). Therefore, when a small droplet of liquid is dispensed on a porous membrane substrate and low volatility solvents containing reagents are used, the circular spot formed by the droplet absorption acts as an open reactor. Using

this principle a great number of reactions can be arranged as arrays on a larger surface as firstly described by Frank et. al. (Frank, 1992).

Peptides produced by the SPOT-synthesis can be used in surface- and solution-phase bioassays. Parallel peptide assembly on planar surfaces using the SPOT-technique comprises some general steps that will be described in further detail: (i) selection of a membrane and its functionalization to meet the chemical, biological, and technical requirements of the synthesis and screening method; (ii) attachment of spacers and/or linkers; (iii) peptide synthesis by conventional solid-phase Fmoc-chemistry (Merrifield, 1963); (iv) cleavage of side chain protecting groups; and optional (v) peptide cleavage from the membrane for analysis and liquid-phase bioassays.

Cellulose membranes are widely used as porous material for the SPOT-synthesis. Cellulose is an inexpensive flexible, hydrophilic material, resistant to the organic solvents and basic conditions used during peptide synthesis. Moreover, it is also stable in aqueous conditions and not toxic being appropriate to be used in biochemical and biological assays. Cellulose filter papers are available in almost all laboratories and different types of these commercially available filters have been described to be suitable for the use by SPOT-synthesis, namely Whatman Chr1, Whatman 50, or Whatman 540 (Hilpert et al., 2007a; Lacroix and Li-Chan, 2014). TFA-soluble cellulose membrane can be used to obtain soluble peptide-cellulose conjugates that can be subsequently spotted onto glass slides to produce CelluSpots microarrays (Chenggang and Li, 2009). A series of alternative materials have been proposed as substrates when non-compatible polysaccharide chemistry needs to be performed: Polyvinylidene difluoride (*PVDF*), nitrocellulose, polytetrafluoroethylene (PTFE/*Teflon*), acrylate-coated PTFE, polystyrene-grafted PTFE. Many types of optimized membranes are commercially available from AIMS Scientific Products. Deiss et.al. have recently reported patterned deposition of Teflon on paper allowing parallel flow-through peptide synthesis on paper that are not possible with standard membranes where the relationship between spot size and solution volume limits the volume that can be deposited onto the support (Deiss et al., 2014).

Cellulose membranes possess hydroxyl groups at the surface with low reactivity. Arrays of spot reactors providing suitable anchor functions for peptide coupling are often obtained by esterification of the hydroxyl groups. The most commonly utilized derivatization of cellulose consist in the coupling of beta-alanine-OH due to the molecules flexibility and linear structure (Weiser et al., 2005). Higher functionalization with subsequent potential higher yield has been described with glycine functionalization (Kamradt and Volkmer-Engert, 2004). Alternatives to cellulose esterification has been also proposed as the use of 2,6-dichlorobenzoyl chloride (Sieber, 1987), the activation of the amino acids with MSNT (1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole) (Blankemeyermenge et al., 1990) or the Mitsunobu-reaction (Barlos et al., 1987). Fmoc-amino acid fluorides have been shown to be highly reactive, but their synthesis is time-consuming and arginine cannot be produced (Wenschuh et al., 1999). Furthermore, they are not stable enough during SPOT-synthesis. P-hydroxymethyl-benzoic acid (HMBA) has also been proposed as a orthogonal safety-catch linkage suitable for peptides, peptoids and carbohydrate-peptide conjugates (VolkmerEngert et al., 1997).

Some protein domains require a free carboxyl-terminus (C-terminus) for ligand recognition. Peptides synthesized according to the standard SPOT-synthesis protocol lack a free C-terminus due to the coupling to the cellulose support. The approach proposed by Licha et al. to produce peptides with free C-terminus uses an Fmoc-amino acid 3-bromopropyl esters and mercapto-functionalized cellulose membranes (Licha et al., 2000). Alternatively, peptides with free C-terminus have been successfully produced by reversing the peptide orientation (inverted peptides) (Boisguerin et al., 2004). The pioneering work of Kania et al. (1994) for free C-terminal peptide synthesis on solid phase have been recently adapted to produce a 340-member library of peptides containing free C-termini on cellulose membranes (Wang and Distefano, 2012). A different strategy using 1,10-carbonyl-di-imidazole (CDI) or 1,10-carbonyl-di-(1,2,4-triazole) (CDT) activators has been also reported as an convenient approach to produce peptides with free C-terminal end by SPOT-synthesis (Ay et al., 2007a).

The size of the spots on the support is defined by the dispensed volume, the physical properties of the membrane surface and the solvent(s). The spot size together with the minimum distance between the spots limits the number of peptides that can be synthesized per membrane area (Tong et al., 2002; Tonikian et al., 2008). The SPOT-technique is particularly flexible regarding peptide number that can be synthesized. In addition, there is no special equipment needed although the laborious process of the manual synthesis (Hilpert et al., 2007b) make it only feasible when the aim is to produce a small number of peptides. Manual SPOT-synthesis kits with a membrane large enough to fit 96 spots are commercially available from Cambridge Research Biochemicals and Sigma-Genosys. Process automation allows the synthesis of up to 8000 different peptides on a 19*29 cm single sheet. Semi-automated and fully-automated synthesizers for SPOT-technique are commercially available, for example Intavis Bioanalytical Instruments and MultiSynTech.

Fmoc-chemistry is used for the SPOT-technique with two possibilities for the preparation of the coupling solution containing activated amino acids: *in-situ* activated or already pre-activated Fmoc-protected amino acids. For amino acids *in-situ* activation, an activator and a coupling reagent are added to the no activated Fmoc-protected amino acid derivative. The activations can be carried out by almost all known procedures: DIC/HOBT; HATU, HBTU, or TBTU with bases as DIPEA, PyBOP/DIC, or EEDQ. Pentafluorophenyl (OPfp) amino acids derivatives are normally used for the pre-activation strategy, which makes the preparation of coupling solutions very simple. However, only particular pre-activated amino acids (i.e., all common L-amino acids) are commercially available and the pre-activated derivatives of nonstandard amino acids would need to be synthesized or *in-situ* activation can be used instead. In both methods, 0.2–0.5 M solutions of Fmoc-protected derivatives are generally dissolved in NMP that are subsequently deprotected with 20% piperidine solutions. Capping steps by acetylation after each cycle are highly recommended to assure that inefficient

couplings only gives rise to truncated sequences. Bromophenol blue (BPB) staining as free amino indicator allows the visual monitoring of the proper performance of the synthesis steps, such as correct dispensing, coupling, capping and effective removal of piperidine from Fmoc-deprotection steps.

When using Fmoc/tBu strategy for the peptide synthesis, side chain deprotection by concentrated TFA treatment will be required. When cellulose membranes are used, the cleavage treatment regime for resin solid-phase peptide synthesis (i.e., around 90–95%TFA for 2–4 h) would not be feasible due to the cellulose degradation at high acid concentrations. Cellulose would last for about 3 h at 50% TFA concentration or around 1 h at 90% (Hilpert et al., 2007a). To overcome that problem, several cleavage cocktails and incubation times have been proposed and reviewed previously (Hilpert et al., 2007a). Short incubations at high TFA concentrations followed by longer reactions at around 50% TFA used to give good results even for the Pbf arginine protecting group that need normally higher TFA concentration to be cleaved (Hilpert et al., 2007b).

Peptide release from the membrane can be achieved by the cleavage of the peptide C-terminal bond and the cellulose membrane. Hydrolysis at high pH can be used for peptides coupled by ester bonds. Treatment of ester linkage from HMBA, glycolic acid or similar with 10 mM sodium hydroxide give the peptide free acids. The treatment of the dry membranes with ammonia vapors yields soluble peptide carboxamides. Reported cleavage strategies has been also extensively reviewed (Wenschuh et al., 2000; Frank, 2002; Hilpert et al., 2007a).

One of the main advantages of the SPOT-synthesis is the molecule versatility that can be produced. The chemical and technical performance has been optimized for the assembly of peptide structures including: linear, cyclic, branched molecules including D and L, coded and noncoded amino acids derivatives and commercially available building blocks even for non-peptidic compounds. Moreover, the peptides can be labeled for detection purposes or for surface immobilization (Toepert et al., 2003; Winkler and McGeer, 2008).

SPOT-synthesis is based on conventional solid-phase synthesis and the same problems are observed. The quality of SPOT-synthesized peptides has been extensively investigated and peptide purity between 50 and 96% were reported. HPLC analysis of SPOT-synthesized short peptides of up to 15 amino acids showed similar purities to those synthesized by solid-phase methods in reactors (Wenschuh et al., 2000). Peptide purity higher than 92% has been reported by Takahashi et al. (2000), while when a huge number of SPOT-synthesized cytomegalovirus deduced nonameric peptides where analyzed by HPLC/MS peptide purity in the range of 50–85% was found (Ay et al., 2007b). Purities of 74.4–91.3% has been reported by Molina and co-workers (Molina et al., 1996). Even longer peptides, such as the 34-meric FBP28 WW domain could be SPOT-synthesized with a high quality of 65% purity (Przezdziak et al., 2006) or 38mer peptides described by Toepert et al. (2001). Besides the high synthetic peptide quality, equivalent peptide array quality is achieved by applying identical chemical conditions during array synthesis. Therefore, taking peptide arrays from the same cellulose membrane (intra-membrane arrays) is highly recommended. As far as possible, this would ensure spots with similar peptide density (peptide concentration in a spot).

SCREENING OF PEPTIDES SYNTHESIZED BY SPOT-TECHNOLOGY FOR ANTIMICROBIAL ACTIVITY

Screening of Soluble Peptides

To our best knowledge the first publication combining peptide synthesis of AMPs on cellulose by SPOT-synthesis and antimicrobial screen was published in 2005 by Hilpert et al. (2005). Here the authors were investigating a linear variant (RLARIVVIRVAR) of the cyclic 12mer peptide bactenecin (RLCRIVVIRVCR). Bactenecin is a peptide discovered in bovine neutrophils (Marzari et al., 1988), active against Gram-negative and some Gram-positive bacteria. The linear variant Bac2A showed similar activity profile, but somewhat improved toward Gram-positive bacteria (Wu and Hancock, 1999). The minimal inhibitory concentration (MIC) against *Pseudomonas aeruginosa* was determined in Mueller-Hinton broth at 50 μg/ml (Wiegand et al., 2008). In order to gain a high yield the cellulose membrane was modified with glycine (Kamradt and Volkmer-Engert, 2004). The synthesis was performed via Fmoc-strategy. Side chain deprotection was performed by using 90% trifluoracetic acid, 3% tri-isobutylsilane, 2% water, 1% phenol in dichlormethan for 30 min, followed by a second treatment for 120 min with 50% trifluoracetic acid, 3% tri-isobutylsilane, 2% water, 1% phenol in dichlormethan. The cleavage of the peptide from the membrane was performed by ammonia gas atmosphere using an overnight incubation.

In order to make the screen as sensitive and fast as possible the authors developed an assay where a luminescent strain of *P. aeruginosa* (H1001) was used, containing the luciferase gene cassette luxCDABE that was incorporated into the bacterial chromosome (into the fliC gene) (Lewenza et al., 2005). The authors demonstrated that the traditional time kill assay and this new developed assay are very similar in their result. In recent years, we have demonstrated that luminescence is not required to use these cellulose-derived peptides. In several projects, we have used unmodified bacteria to perform this screen with other live/death stains.

The peptide Bac2A was systematically investigated by changing each single position with 20 most occurring amino acids in nature. This substitution matrix resulted in 228 unique peptides (12 positions x 19 alternative amino acids). As a negative control a peptide with no antimicrobial activity was used (GATPEDLNQKLS-NH_2). Each peptide was stepwise diluted seven times (1/2 the concentration each) and the activity against *P. aeruginosa* H1001 was determined. From this concentration curve, an IC_{50} was determined and in relation to the positive control (Bac2A) a proxy IC_{50} was calculated for each of the peptides. Based on this data, for each position of Bac2A, the effect of each amino acid substitution could be measured. For example, positions 1 (R), 4 (R), 8 (I), 9 (R), and 12 (R) are optimal occupied, since no other substitution, except for cysteine at some positions, improved activity, in fact most of the

substitutions drastically reduced the activity. It also confirm the importance of the positive charge and that the hydrophobicity can be achieved by different amino acids. In contrast, position 11 (A) showed many substitutions that strongly improved the activity. Overall, the substitution of C, W, R, K, and H often improved antibacterial activity, whereas A, D, E, and P never improved the activity. Peptides that are synthesized by the SPOT-technology are normally not purified, and the results need to be confirmed with purified peptides. The authors have, based on the substitution matrix, designed 11 single substitution variants of Bac2A, 4 multiple substitution variants and 5 Bac2A-derived 8mers with multiple substitutions. These peptides were synthesized on resin and HPLC-purified and then the MIC against three Gram-positive, three Gram-negative (including *P. aeruginosa*) and one yeast was determined. The MIC values of the purified peptides were compared to the IC_{50} values of the crude peptide (SPOT-technology) and a good correlation of $R = 0.895$, $P < 0.01$ by ANOVA was reported. That supports the observed IC_{50} data of the control peptide Bac2A that was synthesized and tested at several different syntheses and showed very robust data for 50 replicates with 0.13 ± 0.04. There were three peptides with single substitution found that improved the MIC against *P. aeruginosa* from 50 to 8 μg/ml and two of the multiple substitution peptides reduced the MIC to 2 μg/ml. It was also confirmed that introducing a proline in the sequence decreases activity to >250 μg/ml. One 8mer peptide also showed promise with an MIC of 8 μg/ml against *P. aeruginosa*. The detailed method for producing peptides on cellulose sheets and the use of luminescent bacteria to screen for (AMPs) synthesized on cellulose was published in Nature Protocols 2007 (Hilpert and Hancock, 2007; Hilpert et al., 2007b). Jenssen et al. used this data to evaluate different descriptors for the design of (AMPs) with enhanced activity (Jenssen et al., 2007). The best outcome was a correct predicted activity that reached 84%.

In 2006, a publication described the very same synthesis and screening approach to investigate possible optimization strategies in more detail (Hilpert et al., 2006). Peptides were synthesized on cellulose with a glycine linker to gain high yield, side chain deprotection and cleavage of the peptide from the membrane was performed as described before. In our opinion, there were three important observations described: First, substitution analysis as a tool to optimize an antimicrobial peptide was confirmed. Second, even in this very flexible 12mer peptide a substitution in one position of the peptide effects distant positions. In this example, the substitution of position three influences position 11. Third, neither the primary sequence of the peptides nor the composition of amino acids alone determines the antibacterial activity for these short (AMPs). This was shown by 49 scrambled variants of the 12mer Bac2A (RLARIVVIRVAR-NH_2) that indicates the whole bandwidth of activity from non-active to superior active. The data shows that there is one or more hidden features that also contribute to the antibacterial activity. In a quantitative sequence activity relationship study using a computational analysis and descriptors that translates sequence ordering and fragment-based hydrophobicity into meaningful numbers, a hydrophobic patch was discovered that was able to classify the peptides. In addition, circular dichroism (CD) revealed that the interaction with liposomes consisting of PPG/POPC 1:1 in 10 mM Tris buffer pH7.4 induced a strong structural change in the spectra compared to only buffer (random structure profile). These changes occurred in the active peptides but not in the less active peptides. Similar results obtained by a membrane depolarization assay using *E. coli* strengthened the data obtained by CD spectra, showing strong and fast depolarization with active peptides and only weak and slow depolarization with less active peptides. The hydrophobic patch, CD and depolarisation hint that the interaction with the membrane is a hidden feature that influences activity and is hard to predict based on the sequence. In this publication two peptides (VRLRIRVRVIRK-NH_2 and KRWRIRVRVIRK-NH_2) showed an MIC value of 3 μg/ml against *P. aeruginosa* and 0.8 μg/ml against *S. epidermidis* (in Mueller-Hinton-broth).

The synthesis of hundreds of peptides via the SPOT-technology and a direct cell based screen resulting in activities ranging from totally inactive to highly active provides an optimal training set for computational analysis and consequent peptide design. Another advantage is that newly computer designed peptides can be synthesized and screened in high numbers to provide confidence in the design rules. In 2009, two publications described that approach (Cherkasov et al., 2009; Fjell et al., 2009). Three 9mer libraries were synthesized and screened against *P. aeruginosa* in the previous described screening assay using the luminescent strain H1001. The peptide libraries were synthesized on Whatman 50 cellulose membranes using glycine as a linker. Side chain deprotection procedure and membrane cleavage protocol remained the same to what was reported before. The first library consisted of 200 computer designed totally random peptides, where each amino acid (except cysteine that was excluded) had the same chance to be incorporated. The screening result showed only inactive or weak active peptides. In consequence, a second library was designed based on occurrence of amino acids in short natural occurring (AMPs). This library contained 943 members and besides inactive and weak active peptides, 26% had similar activity to Bac2A and 2.3% were superior to Bac2A. Based on this data a third library with 500 members was designed using an optimized parameter for the probability of amino acids to be selected in the computer design of new AMPs. This library was synthesized and screened as described before and the antimicrobial performance improves, 48% were similar active to Bac2A and 5% are superior to Bac2A. Thus, the parameter of the third library was used to computer generate 100,000 peptide sequences. The data of library two and three were used to train a QSAR model using "inductive" chemical descriptors and an artificial intelligence approach based on artificial neural networks. These descriptors take into account all atoms of the peptides, including hydrogen and are sensitive to the three-dimensional structure of the peptides. Therefore, all 100,000 peptides needed to be modeled by estimating structural conformation based on energy minimization in the gas phase using MMFF94 force field. The QSAR model predicted the activity of all peptides, ranked them and grouped them into four quartiles, 25,000 in each. The first 50 of each quartile were then selected, synthesized on cellulose and screened against *P. aeruginosa*. The correlation coefficient between the measured and

TABLE 1 | Overview of eight peptide libraries.

Library	Number of peptides	Number without outliers	Superior active	Active	Weak active	Inactive
1	200	185	0 (0.0%)	0 (0.0%)	98 (53.0%)	87 (47.0%)
2	943	928	35 (3.8%)	163 (17.6%)	635 (68.4%)	95 (10.2%)
3	500	493	15 (3.0%)	132 (26.8%)	302 (61.3%)	44 (8.9%)
AA0	600	599	0 (0.0%)	274 (45.7%)	302 (50.4%)	23 (3.8%)
SR	600	598	9 (1.5%)	448 (74.9%)	141 (23.6%)	0 (0.0%)
BM	600	598	6 (1.0%)	360 (60.2%)	232 (38.8%)	0 (0.0%)
5BM	600	593	46 (7.8%)	533 (89.9%)	14 (2.4%)	0 (0.0%)
EP	600	597	1 (0.2%)	168 (28.1%)	283 (47.4%)	145 (24.3%)

Number of peptides analyzed and associated activity classes against Pseudomonas aeruginosa. Outliers–peptides with implausible luminescence measurement values–were excluded from subsequent analyses. All libraries were described before [(Cherkasov et al., 2009; Fjell et al., 2009; Mikut, 2010)].

predicted relative IC_{50} values was $r^2 = 0.986$ (linear regression) supporting the accuracy of the model. This correlation was also confirmed by 20 selected peptides that were HPLC purified (>95%) and MIC values against different pathogens were determined. Even though, the quartiles had extremely different antibacterial activity, peptides from each quartile showed similar hydrophobicity, charge and amphipathicity/hydrophobic moment, supporting previous results about a hidden parameter that also influences the activity (Hilpert et al., 2006). Two peptides were selected and tested against a series of multidrug-resistant (MDR) "superbugs," including MDR *P. aeruginosa*, methicillin-resistant *Staphylococcus aureus* (MRSA), extended spectrum β-lactamase producing *E. coli*, vancomycin-resistant *Enterococcus faecalis* and *faecium* (VRE) and *Enterobacter cloacea* with derepressed chromosomal β-lactamase. Most MIC values were between 0.8 and 12, especially peptide HHC-10 performed very well. Overall the peptides did outperform all tested conventional antibiotics. The highest MIC values were observed for 2 VRE isolates, with values of 99 and 49 μM, while other VRE isolates showed MIC values between 1.5 and 12 μM. The peptide HHC-10 performed also well in an intraperitoneally (IP) *S. aureus* infection in a murine model, significantly reducing the bacterial load after 24 h under both administration route, IP and IV at 4 mg kg^{-1}. While the QSAR model was extremely effective, its complexity was such that an understanding of the rules for activity was impossible. The data of the three libraries was therefore re-analyzed using simpler to understand descriptors in order to finally find understandable rules that define why a short peptide is antibacterial or not. Mikut et al. answered that question in a massive amount of synthesis and screening work (Mikut et al., 2016). With an unprecedented number of individual peptides synthesized and screened the authors have showed that even elusive rules can be discovered and used to improve antimicrobial activity. For that more than 3,500 individual peptides, that is more AMPs than stored in the APD3 data base, were synthesized and tested against *P. aeruginosa*, showing the power of the SPOT-synthesis technology. Library AA0 verifies that the right amino acid composition is important, but not enough to explain the activity. Library SR uses optimized amino acid composition from AA0 and was further restricted to contain at least three positive charges and at least two tryptophan. The results, see **Table 1**, show that about 75% of these peptides are active, indicating the importance of a balance of charge and hydrophobicity. Library BM showed that a computer model with only one descriptor is not enough to describe activity, but five models combined (library 5BM) achieve this with about 97% accuracy, using simple descriptors only. As an example, dilution series of 10 peptides in this library are shown in **Figure 1**.

Library EP looks at "exotic" peptides that were poorly described by the other models. Comparing 5BM and SR revealed that all weak active peptide had either too much or too little W or R/K, respectively. In 5BM, this balance was more enforced. In order to proof that the balance is an important feature, another library with all combination of W and R in a 9mer peptide (512 peptides) was synthesized and tested, verifying that the "right" balance leads to activity. It was shown that there is no positional preference for amino acids. In addition, these short AMPs differ from those that occur naturally. The most active three AMPs identified from these libraries showed MIC values of 2.2–2.7 μmol l^{-1} against *P. aeruginosa* (in Mueller-Hinton broth). They also showed broad spectrum activity as the other 9mer peptides described before.

Two publications describe the use of SPOT-technology to optimize proline rich antimicrobial peptides (PrAMPs) (Knappe et al., 2016b). The peptide oncocin, a peptide isolated from Oncopeltus fasciatus (large milkweed bug) is a 19mer peptide with rather weak activities against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, however it was successful in a systemic septicaemia infection model in mouse (Knappe et al., 2012). A substitution analysis of oncocin was synthesized on a cellulose membrane using the SPOT-technology, using glycine as a linker amino acid to improve the yield. The same side chain cleavage procedure and membrane cleavage procedure was applied as previously described. In total 361 variants of oncocin (VDKPPYLPRPRPPRRIYNR-NH2) were synthesized and screened against a luminescent *P. aeruginosa* strain (H1001). The screening was performed in 6.25% Mueller-Hinton-Broth (1.3 g/L) containing 40 mmol/L glucose, since oncocin is not active in full media. The MIC determination for selected peptides against *S. aureus* and *P. aeruginosa* was performed in 12.5% Mueller-Hinton-Broth. Analysis of the data showed that 25 substitutions at nine different amino acid positions increased

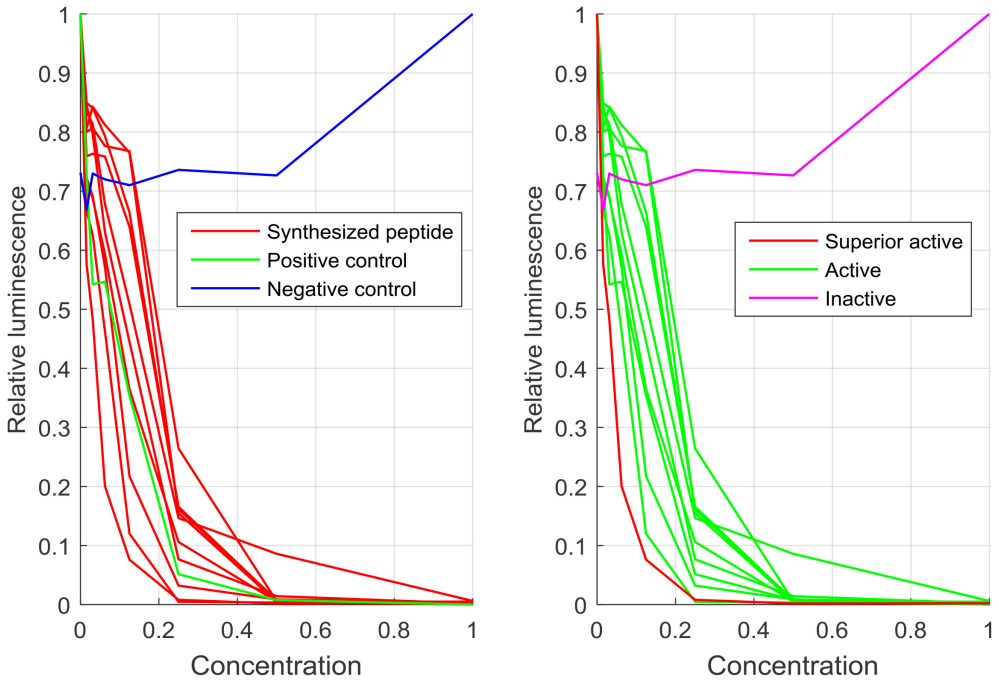

FIGURE 1 | Luciferase assay measurement of 10 peptides and two controls of library 5BM placed on plate 5 of the screen. In the left subfigure, the luminescence values of the positive and negative control on this plated are shown compared to the synthesized peptides. The right subfigure shows the same data with the color-coded activity classification. One peptide sequence with very low luminescence values even for low concentrations was marked as superior active. The concentration and the luminescence values were normalized to the maximum values of a dilution series. The classes were defined based on $RelIC_{75}$ values. As an example, the negative control that oscillates between relative luminescence values between 0.7 and 1 for different concentrations was classified as "inactive."

the activity, whereas 86 substitutions led to a complete loss of activity. The MIC data revealed that oncocin is very robust toward substitutions of single amino acids, no strong change in activity was observed. There was however double substitutions that indeed change the activity strongly, against *P. aeruginosa* a 10 times improvement was observed resulting in an MIC value of 4–8 μg/mL and against S. aureus a 100-fold more active variant than the original oncocin was discovered showing an MIC value of 0.5 μg/mL.

A second PrAMP was investigated using the same approach. Apidaecin is an 18mer peptide that was isolated from the honey be (*Apis mellifera*). Apidaecin and its variant were also successfully used in mouse models (Czihal et al., 2012). Apidaecin (GNNRPVYIPQPRPPHPRL-OH) is inactive against *S. aureus* and very weakly active against *P. aeruginosa* (500 μg/ml in 1/8 Mueller-Hinton broth). The aim of the project was to improve these activities (Hoffmann et al., 2012). For the substitution analysis, 341 unique peptides were synthesized via the SPOT-technology and tested against *P. aeruginosa* in 1/8 Mueller-Hinton broth. The result of this analysis is presented in **Figure 2**.

The substitution analysis reveals that an additional positive charge improves the activity nearby at each position. Such generalized results can be identified by looking for more active variants and the manual interpretation of similarities of the related amino acids, here caused by lysine and arginine indicating the positive charge. In accordance with this, a negative charge would decrease the activity dramatically, see columns for aspartic acid and glutamic acid substitutions. In addition, the N-terminal part can still be optimized, whereas the C-terminal part is quite sensitive to most substitutions. Based on this analysis, several peptides were re-synthesized on resin and HPLC purified. MIC values against three strains were determined in 1/8 Mueller-Hinton broth (MH, 2.5 g/L), results are given in **Table 2**.

By substituting a glycine on position one to a N,N,N0,N′-tetramethylguanidino-ornithine and substituting three amino acids that were identified by substitution analysis, the activity against *P. aeruginosa* could be increased by 125-fold. By the very same substitutions (without position 1), a peptide with no activity against *S. aureus* was now highly active showing an MIC value of 2 μg/ml. This example again demonstrates the power of the method.

Bluhm et al. reported the use of SPOT-synthesis to optimize an apidaecin variant Api137 (gu-ONNRPVYIPRPRPPHPRL-OH) (Bluhm et al., 2015). Api 137 showed only activity in diluted media and variants were required that were also active in ½ MH broth. The authors were concerned about a free C-terminus and therefore changed the linker strategy to the HMBA-linker, that was reported before (VolkmerEngert et al., 1997). The first coupling at the membrane was performed with a beta-alanine. HBTU in the presence of DIPEA as base (0.2 mol/L each, 10 mL DMF, RT, 1 h) was used to couple the HMBA-linker. Fmoc-Leu-OH (0.4 mol/L) was coupled using DIC (0.2 mol/L) and DMAP (8 mmol/L) in DMF (10 mL) overnight. Benzoic anhydride (0.2 mol/L) dissolved in a mixture of pyridine (40 mmol/L) and

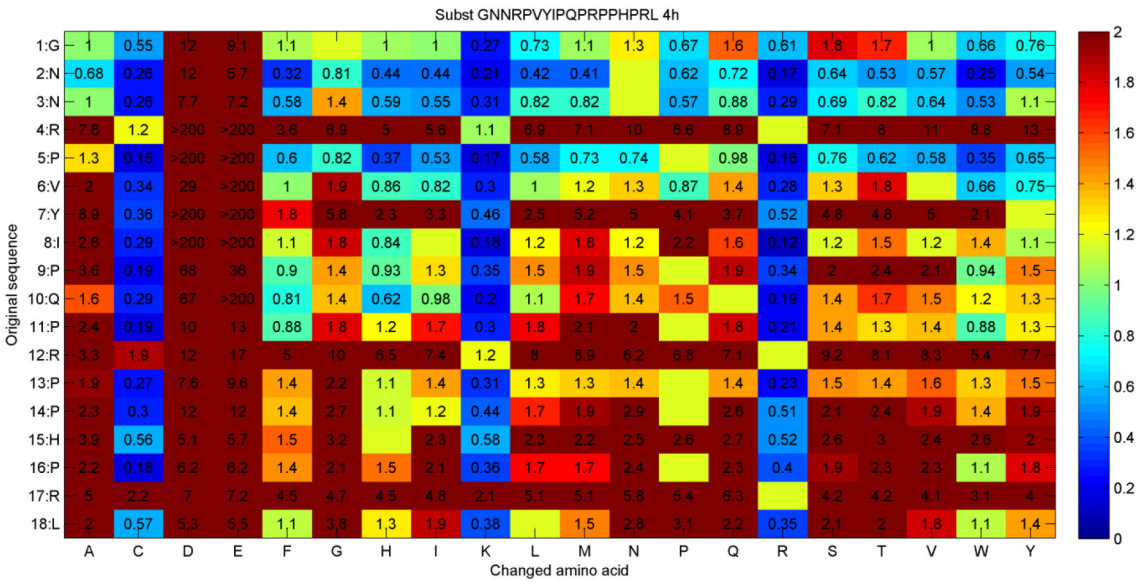

FIGURE 2 | Substitution analysis for Apidaecin (GNNRPVYIPQPRPPHPRL). The original sequence and amino acid positions are given in the first two columns. The other rows (A–Y) identifies the amino acid replacements at each position. Each box in the matrix corresponds to a single peptide containing an additional glycine at the C-terminus. The values within each box represent a $RelIC_{75}$ value, determined by treatment of the *Pseudomonas aeruginosa* reporter strain H1001 with any given peptide for 4 h. Boxes are color-coded by a dynamic range between blue and red: blue stands for improved activity compared to the parent peptide, green for similar activity, and red indicates no activity. Empty boxes represent the original sequence.

DMF (10 mL, 2 h) were applied to cap the remaining free anchors. HBTU and NMM (0.4 mol/L each) in DMF (10 mL, RT, 2 h) was used to obtain N,N,N′,N′-tetramethyl-guanidino-groups at the N-termini. Cleavage with aqueous ammonia resulted in a mixture of free-C-terminus and amidated C-terminus as well as peptide with beta-alanine. Changing to aqueous trimethylamine changed the cleavage product to free C-terminus, however a large part of the peptides still showed the beta alanine linker as undesired side product. Api 137 purity was determined by HPLC to be 57%. The impurities were not affecting the antimicrobial activity and the screen using the complete substitution analysis was performed. The authors identified four peptides, all single substitutions that were eight times more active in 50% MH broth compared to Api137. All multiple substitutions did not result in further improvements.

The aforementioned 12mer peptides that were optimized against antibacterial activity were also investigated for their immunological properties. Based on this work K. Hilpert designed peptide libraries HH1 to HH18 and to further improve the library he designed IDR-1001 to IDR-1048. Some of these peptides were very successful in several aspects, being potent innate defense regulators and also demonstrating potent anti-biofilm activity (Wieczorek et al., 2010; Rivas-Santiago et al., 2013; de la Fuente-Núñez et al., 2014). Haney et al. explored this further with SPOT-synthesis using a restricted set of amino acids and determining anti-biofilm properties but also immune-modulatory activities (Haney et al., 2015). Two peptides were investigated, IDR-1002 (VQRWLIVWRIRK-NH_2) and IDR-HH2 (VQLRIRVAVIRA-NH_2) and based on this results new peptides were designed.

Screening of Tethered Peptides

In 2009, a landmark publication showed that SPOT-synthesis can be used to screen and optimize surface-tethered (AMPs) (Hilpert et al., 2009). LaPorte et al. (1977) and Haynie et al. (1995) showed previously that (AMPs) can be active whilst tethered to a surface, however it was not followed up by the scientific community. The 2009 publication inspired directly and indirectly a lot of research on surface protection using (AMPs), now a field that has been reviewed on its own right. Crucial for the use of SPOT-synthesis and a screen for tethered peptides was the stability of the peptides on the membrane. An HPLC analysis of the supernatant of peptide spots, produced via the standard procedure resulting in an ester between glycine and the membrane, showed an almost completely release after 4 h incubation at 37°C in 100 mM Tris-buffer. The linker strategy was therefore changed to a N-CAPE linker, a strategy that allows with further modification the synthesis of peptides with free C-terminus (Licha et al., 2000; Bhargava et al., 2002). This N -modified cellulose-amino-hydroxypropyl ether provided very stable tethered peptides, showing no HPLC detectable traces after 4 h incubation at 37°C in 100 mM Tris-buffer. Peptides were synthesized at 50 nmol/spot and 200 nmol/spot. In total, 122 tethered peptides were screened and 23 highly active peptides were identified. These peptides were selected on their ability to kill bacteria in solution. There was no correlation observed between antimicrobial activities of tethered peptides compared to the MIC of the peptides in solution. There was however the observation that the 10 most active peptides on an MIC level were also highly active when tethered. It was also shown that the haemolytic activity of the peptides dropped once tethered to

TABLE 2 | Antibacterial activity of Apidaecin (GNNRPVYIPQPRPPHPRL) and analogs.

Peptide sequence	MIC in 1/8 of MH [μg/mL]		
	P. aeruginosa	*E. coli*	*S. aureus*
GNNRPVYIPQPRPPHPRL-OH	500	5	>125
GNNRPVYIPQPRPPHPRL-NH2	250	1.25	>125
GWNRPVYIPQPRPPHPRL-NH2	64	1.25	63
GRNRPVYIPQPRPPHPRL-NH2	64–128	0.625	32
GNNRCVYIPQPRPPHPRL-NH2	125	10	31
GNNRRVYIPQPRPPHPRL-NH2	64	5	32
GNNRPVYRPQPRPPHPRL-NH2	64	0.313	63
GNNRPVYIPQPRRPHPRL-NH2	125	10	31
GNNRPVYIPQPRPCHPRL-NH2	250	20	16
GNNRPVYIPQPRPPHCRL-NH2	125	20	32
GNNRPVYIPQPRPPHPRR-NH2	125	1.25–2.5	125
GWNRPVYIPRPRPPHPRL-NH2	16–32	0.63	16
GWNRPVYIPQPRRPHPRL-NH2	64	5	4–8
GNNRPVYIPRPRRPHPRL-NH2	64	2.5	4
GWNRPVYIPRPRRPHPRL-NH2	32	2.5	2
gu-OWNRPVYIPRPRRPHPRL-NH2	4	8	8–16

Minimal inhibitory concentrations (MIC) were determined 12.5 % Mueller-Hinton broth.
gu-O = (N,N,N0,N′-tetramethylguanidino-ornithine).
Changes that were introduced into the parent sequence are marked in color.

a surface. The activity of the antimicrobial activity of selected peptides were confirmed using other surface linking chemistry and other types of surfaces. Several experiments were performed to unravel the mode of action of these peptides. In a follow up study, several of these peptides were attached to a titanium surface using a copolymer brush (Gao et al., 2011). After the characterization of the surface the antimicrobial activity was tested and verified in a rat infection model.

SUMMARY AND OUTLOOK

Antimicrobial resistance is a natural phenomenon that is part of microbial surviving strategies to secure resources and ecological niches. Alexander Fleming already said: "It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body. The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant." Unfortunately, man became extremely ignorant and careless and started to misuse all the antibiotics on a large scale, accelerating the drive of resistant organism. Antibiotics are misused in a metric ton scale in animal farming, for example treating piglets to get fatter faster and less ill under the terrible condition they are kept in mass farming. A public awareness campaign is trying to change this mind-set, however the next generation faces a very difficult time with not much treatment options left for bacterial and fungal infections. Often it is referred to as the return to medieval medicine. This situation becomes more likely since the economic prospects to develop novel antibiotics for pharma are rather bleak and many companies dropped out from their antimicrobial drug development program, 36 of them last year. That leads to very little activity in this sector, for example last year 504 drug candidates entered clinical phase 2 and 3 studies for cancer treatment, but only 37 for antimicrobials.

Antimicrobial peptides (AMPs) are possible new candidates for the treatment of MDR bacterial infections since they are able to kill MDR microbes. It is a very diverse class and already different modes of actions were reported for different peptides, making them very interesting as drugs with novel mode of action. There is a substantial body of literature about antimicrobial action and their immunomodulatory activities. There is however a great lack of data about the translational aspect toward clinical phase and this is also reflected in the few peptides that entered clinical phase. In order to make detailed studies of these peptides, a method is needed that allows the synthesis of sufficient material to perform cell-based tests in a high throughput manner. SPOT synthesis is used for such studies, because it is fully automated, reasonable priced and produces enough material to perform a few cell-based studies. This technique is now more than 25 years old and many data is described that shows the impact, but also a lot of chemistry to adapt the protocols to different biological questions. Recent improvements in high density peptide arrays have outperformed the SPOT technology in the field of binding assays. However, for cell based assays, were more material is needed, SPOT technology remains the lead.

Antimicrobial peptides (AMPs) were investigated using the SPOT technology. It was shown that it is possible to systematically improve the antimicrobial activity by using substitution analysis. More than 100-fold improvements in activity were reported. In addition, peptide libraries can be designed and optimized to contain very potent antimicrobial compounds. These data can be used as a base for bioinformatics and powerful prediction algorithms were developed. In the future, this technology can support the process of moving these peptides toward clinical studies, for example peptide variants and modifications can be screened for stability and activity in serum/blood.

Unfortunately, (AMPs) are currently developed not only for treatment of MDR infections in humans but also for animals and plants. Mankind seems not to learn the lessons from their ignorance but intensify their behavior. At that large scale application resistant strains can develop. Since AMPs are a major compound of the innate immune system of many organisms, including plants and animals, bacterial and/or fungal strains that will develop resistance to AMPs might threaten the ecology of earth even further and can accelerate the dying of numerous species.

AUTHOR CONTRIBUTIONS

PL, EG, LB, RM, and KH were writing different sections to the paper. KH brought all parts together and wrote the paper in a uniform style. All authors have than proofread the manuscript and KH has finalized the manuscript.

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Analogs of the Frog-skin Antimicrobial Peptide Temporin 1Tb Exhibit a Wider Spectrum of Activity and a Stronger Antibiofilm Potential as Compared to the Parental Peptide

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The frog skin-derived peptide Temporin 1Tb (TB) has gained increasing attention as novel antimicrobial agent for the treatment of antibiotic-resistant and/or biofilm-mediated infections. Nevertheless, such a peptide possesses a preferential spectrum of action against Gram-positive bacteria. In order to improve the therapeutic potential of TB, the present study evaluated the antibacterial and antibiofilm activities of two TB analogs against medically relevant bacterial species. Of the two analogs, TB_KKG6A has been previously described in the literature, while TB_L1FK is a new analog designed by us through statistical-based computational strategies. Both TB analogs displayed a faster and stronger bactericidal activity than the parental peptide, especially against Gram-negative bacteria in planktonic form. Differently from the parental peptide, TB_KKG6A and TB_L1FK were able to inhibit the formation of *Staphylococcus aureus* biofilms by more than 50% at 12 μM, while only TB_KKG6A prevented the formation of *Pseudomonas aeruginosa* biofilms at 24 μM. A marked antibiofilm activity against preformed biofilms of both bacterial species was observed for the two TB analogs when used in combination with EDTA. Analysis of synergism at the cellular level suggested that the antibiofilm activity exerted by the peptide-EDTA combinations against mature biofilms might be due mainly to a disaggregating effect on the extracellular matrix in the case of *S. aureus*, and to a direct activity on biofilm-embedded cells in the case of *P. aeruginosa*. Both analogs displayed a low hemolytic effect at the active concentrations and, overall, TB_L1FK resulted less cytotoxic toward mammalian cells. Collectively, the results obtained demonstrated that subtle changes in the primary sequence of TB may provide TB analogs that, used alone or in combination with adjuvant molecules such as EDTA, exhibit promising features against both planktonic and biofilm cells of medically relevant bacteria.

Keywords: Temporin 1Tb, analogs, biofilm, peptide design, antimicrobial peptides

INTRODUCTION

The development and rapid spread of antibiotic resistance among clinically relevant bacteria has dramatically reduced the effectiveness of antimicrobial therapies, thereby emerging as a major challenge for modern medicine (Boucher et al., 2009; Högberg et al., 2010). The ability of bacteria to form biofilms, architecturally complex cell aggregates embedded in an extracellular polymeric substance (EPS) and intrinsically tolerant to conventional antibiotics, further exacerbates the problem of bacterial resistance and is responsible for the persistence and chronicization of many types of infections (Costerton et al., 1999). Biofilms can be up to 1,000-fold more resistant to antimicrobial agents than their planktonic counterparts thanks to unique phenotypic and metabolic properties that allow them to implement resistance mechanisms at the community level. These include the presence of the EPS that reduces the diffusion of antibacterial compounds into the biofilm structure, the overall low growth rate of biofilm-forming bacteria, the presence of subpopulations of cells in a dormant state ("persisters"), and the cell proximity that promotes the horizontal gene transfer and the acquisition of mobile genetic elements encoding resistance (Høiby et al., 2010; Batoni et al., 2016a).

Over the last years, antimicrobial peptides (AMPs) have gained increasing attention as novel antimicrobial drugs for the control of infections sustained by antibiotic-resistant bacteria and/or bacterial biofilms. Due to their main mechanism of action, which involves the disruption of cell membrane integrity, AMPs exert a strong antimicrobial activity against a broad spectrum of pathogens, including multidrug-resistant bacterial strains, and generally prove a low frequency in inducing resistance (Zasloff, 2002). Moreover, they are able to target metabolically inactive and even non-growing cells that are commonly found within microbial biofilms (Di Luca et al., 2014; Batoni et al., 2016a). To date, over 2500 AMPs have been identified and evaluated for their antimicrobial activity (Antimicrobial Peptide Database: aps.unmc.edu/AP/main.php) and a growing number of them have also been tested against biofilms (BaAMPs database: www.baamps.it) (Di Luca et al., 2015).

The frog skin-derived peptide temporin 1Tb (TB) is considered a promising template for the development of next-generation antibiotics (Di Grazia et al., 2014). It is a 13-amino acid, mildly cationic (net charge +2) and α-helical peptide endowed with a bacterial membrane-perturbing activity (Mangoni et al., 2000). The peptide has previously demonstrated a fast and potent bactericidal action particularly against Gram-positive bacterial species, such as multidrug-resistant nosocomial strains of *Staphylococcus aureus* and *Enterococcus faecium* (Mangoni et al., 2008). The antibiofilm properties of TB have been also investigated showing high activity against both forming and mature biofilms of *Staphylococcus epidermidis*, especially when the peptide was used in combination with EDTA (Maisetta et al., 2016). Interestingly, it has been recently reported that the peptide is able to penetrate eukaryotic cells, kill intracellular *S. aureus* and promote wound-healing, further important properties in view of a therapeutic development (Di Grazia et al., 2014). Despite the many favorable features of TB, the preferential spectrum of activity of the peptide against Gram-positive bacteria partially limits its translatability into a clinically useful agent. The rational *in silico* design of novel peptides with optimized structural properties and the chemical manipulation of existing ones represent valid approaches to overcome the limitations of native peptides (Maccari et al., 2013). The introduction of appropriate changes in the peptide primary sequence and, thus, the alteration of crucial physicochemical parameters of AMPs (e.g., cationicity, hydrophobicity and amphipaticity) may significantly influence their bactericidal, cytotoxic and antibiofilm potential allowing to obtain molecules with improved antimicrobial efficacy and broader spectrum of action (Conlon et al., 2007; Takahashi et al., 2010; Batoni et al., 2016b). The aim of the present study was the optimization of TB activity against both planktonic bacteria and biofilms of medically relevant bacterial species. In particular, the antibacterial, antibiofilm and cytotoxic properties of TB were compared with those of two recently developed TB analogs. The first one (TB_KKG6A), described by Avitabile and co-workers, was initially obtained by Ala scanning on TB sequence and further optimized by increasing its positive charge (Avitabile et al., 2013). TB_KKG6A was found to efficiently interact with the lipopolysaccharide (LPS) of the Gram-negative bacterium *Escherichia coli* and to fold upon binding into a bent helix (Malgieri et al., 2015). The second one (TB_L1FK), firstly described in this study, was designed by us through statistical-based computational strategies (Maccari et al., 2013). Overall, TB analogs displayed a faster and stronger bactericidal activity than the parental peptide, especially against Gram-negative bacterial species in planktonic form. In addition, a marked antibiofilm activity against preformed biofilms of *S. aureus* and *Pseudomonas aeruginosa* was observed for both TB_KKG6A and TB_L1FK used in combination with EDTA, highlighting the potential of combinatorial drug therapies in the management of biofilm-related infections. When assayed on mammalian cells, TB_L1FK showed a lower cytotoxic activity against human epithelial cells as compared to TB_KKG6A, emerging as a promising molecule for the topical treatment of biofilm-associated infections.

Abbreviations: AMP, antimicrobial peptide; BPM, biofilm promoting medium; BSA, bovine serum albumin; CCS, combined consensus scale; MCC, Mathews correlation coefficient; MOEA, multi-objective evolutional algorithms; CFU, colony-forming units; CV, crystal violet; EDTA, ethylenediaminetetraacetic acid; EPS, extracellular polymeric substance; FCS, fetal calf serum; FIC, fractional inhibitory concentration; LPS, lipopolysaccharide; MBC, minimal bactericidal concentration; MIC, minimal inhibitory concentration; PBMCs, peripheral blood mononuclear cells; PBS, phosphate buffered saline; PI, propidium iodide; RBCs, red blood cells; SPB, sodium-phosphate buffer; TB, temporin 1Tb; TSA, tryptone soy agar; TSB, tryptone soy broth.

MATERIALS AND METHODS

Peptides

TB, TB_L1FK (designed as reported in "Results") and TB_KKG6A were synthesized by Proteogenix (Schiltigheim, France). Analysis of the synthetic peptides by high performance chromatography (HPLC) and mass spectrometry revealed purity

over 98%. Peptides were diluted in milli-Q water to obtain a stock solution of 1 mM and stored at −80°C. The main features of the peptides are shown in **Table 1**.

EDTA

Disodium ethylenediaminetetraacetic acid (EDTA) was purchased from Sigma-Aldrich (St. Louis, USA). A stock solution of EDTA (0.5 M) was prepared in milli-Q water by adjusting the pH to 8.0 with NaOH. The working solution (50 mM) was obtained by diluting the stock solution in milli-Q water, sterile filtered and stored at 4°C.

Bacterial Strains and Culture Conditions

The reference laboratory strains *Klebsiella pneumoniae* (ATCC BAA-1706), *P. aeruginosa* (ATCC 27853), *S. aureus* (ATCC 33591), and *S. epidermidis* (ATCC 35984) were used for the study. For the preparation of stock cultures, bacterial strains were grown in Tryptone Soy Broth (TSB) (Oxoid, Basingstoke, UK) until mid-log phase, subdivided in aliquots and stored at −80°C. For the colony-forming units (CFU) count, serially diluted bacterial suspensions were plated on Tryptone Soy Agar (TSA) (Oxoid) and incubated for 24 h at 37°C.

Bactericidal Activity and Killing Kinetics in Sodium-Phosphate Buffer

The bactericidal activity of TB, TB_L1FK and TB_KKG6A against *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, and *S. epidermidis* was evaluated by the microdilution method in sodium-phosphate buffer (10 mM SPB, pH 7.4). Bacterial strains were grown in TSB until exponential phase and suspended in SPB to reach a density of 1×10^7 CFU/mL. A volume of 10 μL of the bacterial suspensions was added to 90 μL of SPB containing different concentrations of the peptides (from 1.5 to 48 μM). Bacteria suspended in SPB alone were used as cell viability control. Samples were incubated at 37°C with shaking for various times (5, 15, 30, 60, and 90 min), subsequently diluted 10-fold in TSB and plated on TSA to determine the number of CFU. The minimal bactericidal concentration (MBC) was defined as the minimal concentration of peptide causing a reduction of at least 3 Log_{10} in the number of viable bacteria after 90 min of incubation (Mangoni et al., 2008).

Biofilm Inhibition Assay

The ability of TB, TB_L1FK, and TB_KKG6A to prevent biofilm formation was evaluated against *S. aureus* and *P. aeruginosa*. Bacteria were grown overnight in TSB/Glc (TSB added with 0.25% (v/v) glucose) at 37°C. Stationary-phase cultures were diluted 1:1,000 in Biofilm Promoting Medium (BPM; TSB diluted 1:1 with 10 mM SPB at pH 7.4 and supplemented with 0.25% glucose). Bacterial suspensions were inoculated into flat-bottom polystyrene 96-well microplates (Corning Costar, Lowell, USA), in the absence (negative control) or in the presence of different concentrations of each peptide (from 12 to 48 μM). Microplates were incubated statically at 37°C for 24 h and biofilm biomass was estimated by crystal violet (CV) staining assay. To this aim, biofilms were rinsed three times with phosphate-buffer saline (PBS), air-dried for 15 min and incubated with 0.1% (w/v) CV (bioMérieux, Florence, Italy) for 15 min. The excess of CV was removed by washing the plates with PBS, while biofilm-associated CV was extracted with 98% ethanol (Sigma Aldrich) and quantified by measuring the optical density at 570 nm (OD_{570}) in a microplate reader (Model 550, Bio-Rad Laboratories Srl, Italy).

Biofilm Treatment Assay

The activity of TB, TB_L1FK, and TB_KKG6A against preformed (24-h old) biofilms of *S. aureus* and *P. aeruginosa* was also investigated. Briefly, biofilms were allowed to form for 24 h in flat-bottom 96-well microplates in the absence of antimicrobial compounds. Established biofilms were then washed three times with PBS in order to remove non-adherent cells and incubated in fresh BPM with different concentrations of the three peptides (from 15 to 120 μM). After 24 h of incubation, the viability of biofilm-associated cells was evaluated by CFU counting. For this purpose, biofilms were washed three times with PBS and bacterial cells were detached from the surface of the wells with a pipette tip, vigorously vortexed and plated in serial dilutions on TSA.

Evaluation of the Synergistic Effect between TB Analogs and EDTA on Preformed Biofilms

TB_L1FK and TB_KKG6A were combined with EDTA in order to enhance their activity against preformed biofilms of *S. aureus* and *P. aeruginosa*. To this aim, 24 h-old biofilms of the two bacterial species were exposed to different concentrations of the peptides (15 and 30 μM), alone and in combination with EDTA (1.25 and 2.5 mM). Microplates were incubated statically at 37°C for 24 h. Following incubation, the antibiofilm effect was evaluated in terms of number of biofilm-associated viable cells as previously described.

TABLE 1 | Main structural and physicochemical features of the peptides used in the study.

Peptide	Sequence	Molecular weight	Charge	Hydrophobicity[a]
TB	LLPIVGNLLKSLL-NH_2	1392.78	+2	3.62
TB_L1FK	**F**LPIVGLLKSLL**K**-NH_2	1440.86	+3	3.43
TB_KKG6A	**KK**LLPIV**A**NLLKSLL-NH_2	1663.15	+4	1.91

[a] *Hydrophobicity was calculated with the combined consensus scale (CCS) through the BaAMPs database (Di Luca et al., 2015).*

Evaluation of the Synergistic Effect Between TB Analogs and EDTA on Planktonic Bacteria in Biofilm-Like Conditions

The antibacterial activity of TB_L1FK and TB_KKG6A, used alone and in combination with EDTA, was also tested against planktonic cells of *S. aureus* and *P. aeruginosa*. The Minimal Inhibitory Concentration (MIC) of the peptides, EDTA and the peptide-EDTA combinations was determined by the microdilution method under the same experimental conditions used for the biofilm assay. Briefly, bacteria from overnight cultures were diluted 1:1,000 in BPM and incubated for 24 h at 37°C in propylene tubes in the presence of TB_L1FK and TB_KKG6A (from 3.75 to 120 μM), alone and combined with EDTA (from 0.3 to 10 mM). MIC was defined as the lowest concentration of the compounds resulting in the complete inhibition of visible growth. The effect of each combination on cell growth was studied using an adapted Fractional Inhibitory Concentration (FIC) index analysis. FIC index was calculated as follows: Σ ($FIC_A + FIC_B$), where FIC_A is the MIC of compound A in combination/MIC of compound A alone, and FIC_B is the MIC of compound B in combination/MIC of compound B alone. Synergism was defined as a FIC index ≤ 0.5, indifference as a FIC index > 0.5 and antagonism as a FIC index > 4 (Katragkou et al., 2015; Dosler et al., 2016).

Hemolysis Assay

Hemolytic activity of TB and its analogs was tested against human red blood cells (RBCs) as previously described (Tavanti et al., 2011). Briefly, peripheral blood obtained from healthy donors was centrifuged (1,000 × g for 10 min, 4°C) and washed three times with PBS (Euroclone, Milan, Italy). A suspension of RBCs (4%, v/v) was mixed with various concentrations of the peptides (from 12 to 96 μM) into a round-bottom polystyrene 96-well microplate (Corning Costar). RBCs suspended in PBS alone were used as negative control (0% hemolysis), while cells lysed with 0.1% Triton X-100 were taken as positive control (100% hemolysis). The microplate was incubated for 1 h at 37°C and then centrifuged at 1,000 × g for 20 min, 4°C. Supernatants were transferred to a new plate and the optical density at 450 nm (OD_{450}) was measured by means of a microplate reader. The hemolytic activity was quantified according to the following formula: hemolysis (%) = [($OD_{peptide} - OD_{negative\ control}$)/($OD_{positive\ control} - OD_{negative\ control}$)] × 100.

Cytotoxicity Assay

Cytotoxic activity of the peptides was assessed against human peripheral blood mononuclear cells (PBMCs) and human non-small-cell lung adenocarcinoma A549 cells (ATCC CCL-185).

PBMCs were isolated from buffy coats by conventional density gradient centrifugation. For this purpose, buffy coats were diluted 1:1 in PBS supplemented with 10% (v/v) sodium citrate (Sigma-Aldrich) and layered on Lympholyte-H gradient medium (Euroclone). Following centrifugation at 200 × g for 20 min at room temperature, the supernatant was eliminated in order to remove platelets. Buffy coats were further centrifuged at 800 × g for 20 min at room temperature and the lymphocyte/monocyte layer was harvested at the sample/medium interface. PBMCs were washed three times with PBS containing 0.5% (wt/v) bovine serum albumin (BSA; Sigma-Aldrich) and 10% sodium citrate, counted and re-suspended in RPMI 1640 (Euroclone) added with 10% (v/v) fetal calf serum (FCS; Euroclone) and 2 mM L-glutamine. Cells (1×10^5 per well) were seeded into round-bottom 96-well microplates (Corning Costar) and incubated with increasing concentrations of the peptides (from 12 to 96 μM) for 24 h at 37°C, 5% CO_2. PBMCs incubated with culture medium were used as negative (cell viability) control, while cells treated with cycloheximide (2 mg/mL) served as a positive (death) control.

A549 cells were grown in tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM; Euroclone) containing 10% FCS and 2 mM L-glutamine. Confluent monolayers of A549 cells were washed with PBS, treated with a trypsin-EDTA solution (Sigma-Aldrich), centrifuged at 300 × g for 10 min, counted and re-suspended in complete DMEM at a final density of 5×10^4 cells/mL. A volume of 200 μL of the cell suspension was seeded into flat-bottom 96-well microplates (Corning Costar) and cultured for 24 h at 37°C, 5% CO_2. Peptides at a final concentration of 12–96 μM were added to the cells and incubated for further 24 h at 37°C, 5% CO_2. A549 cells incubated with culture medium were used as negative (cell viability) control, while cells treated with cycloheximide (2 mg/mL) served as a positive (death) control.

Cytotoxic activity was evaluated by the propidium iodide (PI) flow cytometric assay. To this end, PBMCs were washed once in PBS, resuspended in 100 μL, and incubated with 5 μL of a PI solution (50 μg/mL) (Sigma-Aldrich) for 4 min in the dark. Similarly, A549 cells were harvested by trypsinization, rinsed once with PBS and exposed to PI. Counting of viable (PI-negative) and dead (PI-positive) cells was carried out with a BD Accuri C6 flow cytometer (BD Biosciences, Mountain View, CA) and data were analyzed using BD Accuri C6 software (BD Biosciences). Cytotoxic effect was determined according to the following formula: Cytotoxicity (%) = [(PI-positive cells $_{peptide}$ – PI-positive cells $_{negative\ control}$)/(100 – PI-positive cells $_{negative\ control}$)] × 100. The IC_{50} values (Inhibitory Concentration) were defined as the concentration of the peptides causing 50% cell death as compared to the untreated control.

Statistical Analysis

All the experiments were performed at least in triplicate, unless otherwise specified. Differences between mean values of groups were evaluated by one-way analysis of variance (ANOVA) followed by Tukey-Kramer *post-hoc* test, after normalization of the data. A p-value < 0.05 was considered statistically significant.

RESULTS

TB_L1FK design

In order to improve the therapeutic potential of TB, a novel peptide was computationally designed starting from TB

sequence. In a previous work, chemophysical analysis of known AMPs sequences was successfully employed to design a statistical model of membrane-disrupting peptides able to account for non-natural amino acids (Maccari et al., 2013). In this work, an additional statistical model was designed to account for peptides' cytotoxic effect. Together with the previously described models for the secondary structure and the antimicrobial activity, a forth constraint was imposed in order to retain as much as possible the sequence similarity with TB. A dataset of peptides with proved cytotoxic effect was appositely designed by collecting and combining data from different bioactive peptide databases (Gupta et al., 2013). Furthermore, another set of peptides was designed to represent non-cytotoxic peptides, allowing the statistical model to grasp the features that distinguish the two sets (see Section 1.1 in the Supplementary Material). A number of filters aimed to normalize and uniform the training set of peptides were applied and then, sequences were encoded into physicochemical variables representing global and topological properties of peptides. A machine learning algorithm was adopted to build a prediction engine able to discern between toxic and non-toxic peptides (see the Supplementary Material for details in model training and validation). Model performance was evaluated by the Mathews Correlation Coefficient (MCC), which assesses the prediction in terms of true and false positives and negatives. In the final configuration, a prediction model with an MCC value of 0.82 was obtained. The candidate sequence, named TB_L1FK, was designed by applying the statistical model to a particular class of Genetic Algorithms, called Multi-Objective Evolutional Algorithms (MOEA) that allows to screen for candidates that simultaneously satisfy different criterions.

As reported in **Figure 1**, that shows a predictive simulation of the structure of TB and its two analogs, TB_L1FK displays similar physicochemical characteristics to the parental peptide. Hydrophobicity and net charge of TB_L1FK are close to those of TB, while TB_KKG6A presents a different hydrophobic profile and an increased net charge, particularly localized at the C-terminus. One of the aims in the computational design of TB_L1FK was to retain all the features that could infer in the membrane interaction of the peptide with the target cells. Besides, molecular hydrophobicity and net charge, as well as size and molecular weight, represent important aspects for the loading and the controlled release of peptides such as TB from nanostructured delivery systems (Piras et al., 2015).

Bactericidal Activity and Killing Kinetics of Peptides in Sodium-Phosphate Buffer

The antimicrobial activity of TB, TB_KKG6A, and TB_L1FK was evaluated in terms of MBC values toward *S. aureus* and *S. epidermidis* as models of Gram-positive bacteria and against *K. pneumoniae* and *P. aeruginosa* as models of Gram-negative bacteria. As shown in **Table 2**, TB was mainly active against Gram-positive bacteria and exhibited a bactericidal effect against Gram-negative bacteria only at 48 μM. Both analogs displayed a markedly increased activity compared to the parental peptide against all the bacterial species tested, but especially against the Gram-negative ones. In particular, a 2- to 8-fold reduction in the MBC compared to TB was observed against the Gram-positive bacteria, while an up to 16-fold decrease in the MBC value was observed in the case of the Gram-negative bacteria.

Time-kill studies on two representative bacterial species, *S. aureus* and *P. aeruginosa*, were carried out using the peptides at concentrations equal to their MBC. TB exerted its bactericidal activity toward *S. aureus* after approximately 90 min of incubation (**Figure 2A**). Both TB_L1FK and TB_KKG6A exhibited a faster killing kinetics than TB against the same bacterial species causing a reduction of at least 3 Log_{10} in the number of viable bacteria within 30 and 60 min, respectively (**Figure 2A**). All three peptides showed a more rapid bactericidal effect against *P. aeruginosa* than against *S. aureus* (**Figure 2B**). In particular, TB and TB_KKG6A showed similar killing kinetics, being bactericidal after 15 min of incubation, while the most rapid bactericidal effect was exerted by TB_L1FK that determined the complete eradication of the starting bacterial inoculum within as little as 5 min of incubation (**Figure 2B**).

Effect of TB and TB Analogs on Forming and Preformed Biofilms

We first investigated the ability of TB, TB_L1FK and TB_KKG6A to inhibit the formation of biofilms of *S. aureus* and *P. aeruginosa*, two bacterial species often involved in the formation of biofilms particularly refractory to antimicrobial treatment. The inhibitory effect was assessed by CV staining (total biofilm biomass) evaluating the percentage of biofilm formation after 24 h of incubation with TB or the two TB analogs, as compared to the control biofilms (cells incubated in medium only). As shown in **Figure 3A**, differently from the parental peptide, TB_L1FK and TB_KKG6A reduced the ability of *S. aureus* to form biofilm of more than 50% as compared to the untreated control at 12 μM. All the peptides caused around 80% decrease of the biofilm biomass at the concentration of 24 μM. When the peptides were assayed against forming biofilms of *P. aeruginosa*, no inhibitory activity of TB and TB_L1FK was observed at concentrations up to 48 μM (**Figure 3B**). In contrast, TB_KKG6A displayed a considerable ability in reducing the biomass of *P. aeruginosa* biofilms, causing an 80% inhibition at the concentration of 24 μM (**Figure 3B**).

Secondly, the efficacy of TB and its analogs against preformed (24 h-old) biofilms of *S. aureus* and *P. aeruginosa* was evaluated by CFU counting after 24 h of incubation with the peptides. In the case of *S. aureus* biofilms, TB did not exert a considerable antibiofilm activity at concentrations up to 120 μM (data not shown), while TB_L1FK and TB_KKG6A caused a decrease of approximately 2 Log_{10} in the number of biofilm-associated viable cells as compared to untreated biofilms at 30 μM (**Figures 4A,B**). When tested against biofilms of *P. aeruginosa*, none of the three peptides displayed a significant ability to reduce the number of CFU at the highest tested concentration (120 μM) (data not shown).

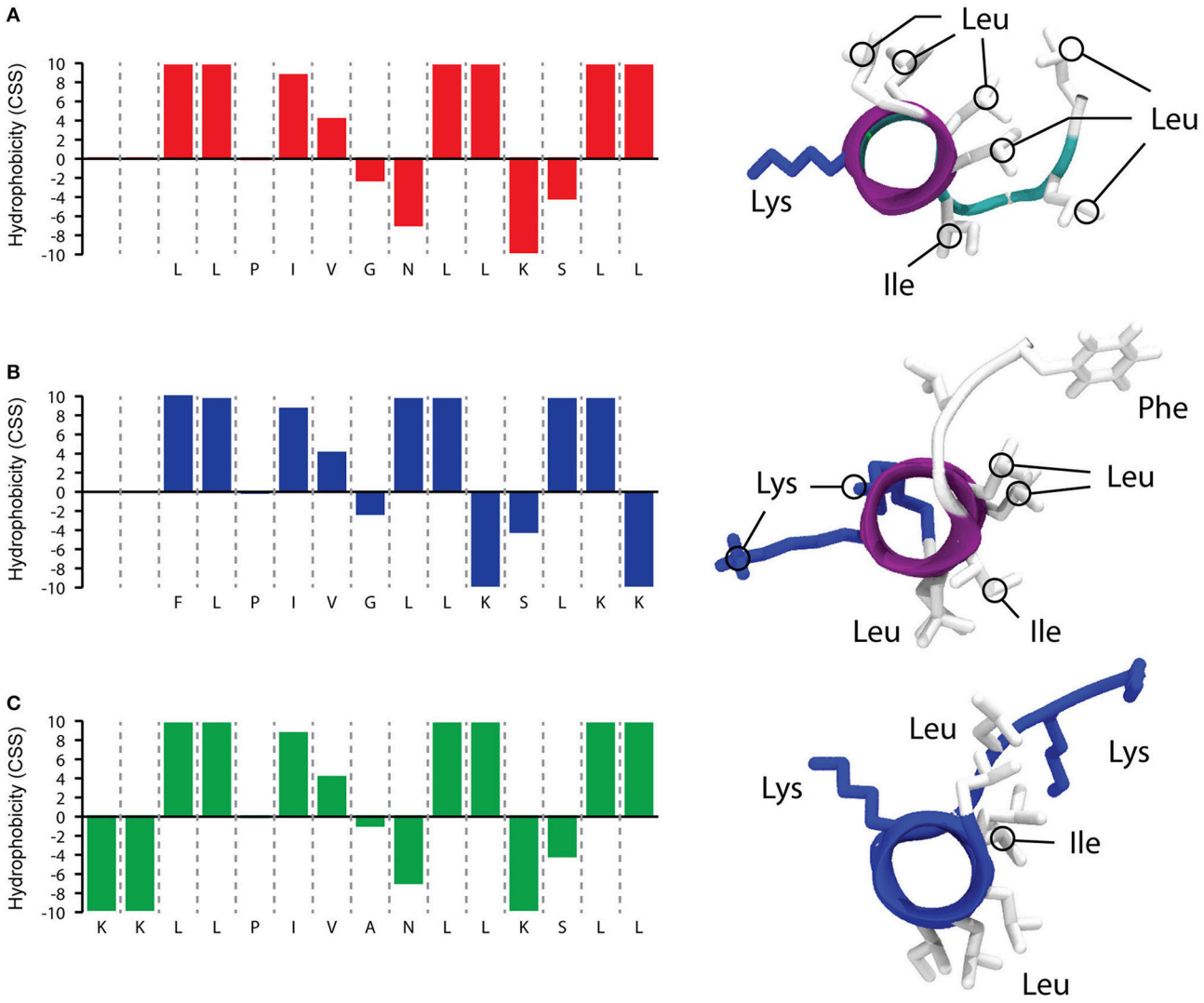

FIGURE 1 | Predictive simulations of hydrophobic profile and 3D structure of TB (A), TB_L1FK **(B)**, and TB_KKG6A **(C)**. The hydrophobic profile calculated with the combined consensus scale (CCS) is schematized on the left side (Maccari et al., 2013). 3D structures (right side of the figure) were calculated with PEP-FOLD3 (Lamiable et al., 2016); the distribution of hydrophobic and charged residues is highlighted.

Effect of TB Analogs, alone and in Combination with EDTA, on Preformed Biofilms

The possibility to improve the activity of TB analogs against preformed biofilms of *S. aureus* and *P. aeruginosa* was investigated combining the peptides with EDTA, a chelating agent previously reported to enhance the antibiofilm properties of TB (Maisetta et al., 2016). Indeed, the ability of EDTA to establish strong complexes with divalent cations essential for matrix stability could produce a matrix-disaggregating effect and promote the accessibility of peptides to biofilm-forming cells. The antibiofilm activity of various peptide-EDTA combinations was evaluated by CFU counting. Among all the tested combinations, the most powerful potentiating effect in terms of viable count reduction was obtained using both peptides at the concentration of 30 μM in combination with 1.25 mM (for *S. aureus*) or 2.5 mM EDTA (for *P. aeruginosa*). As regards *S. aureus* (**Figures 4A,B**), the combination of both TB_L1FK and TB_KKG6A with EDTA caused a reduction in the CFU number of approximately 1 Log_{10} (90%) compared to the peptides and EDTA used alone, and 3 Log_{10} (99.9%) compared to control biofilms after 24 h of incubation. Also in the case of *P. aeruginosa*, an enhanced ability of TB_L1FK and TB_KKG6A in biofilm reduction was demonstrated when peptides were used in combination with EDTA. Indeed, both peptide-EDTA combinations reduced the CFU number of

TABLE 2 | MBCs of TB, TB_L1FK, and TB_KKG6A against Gram-positive and Gram-negative bacteria in sodium-phosphate buffer (10 mM SPB, pH 7.4).

	Gram-positive		Gram-negative	
	S. aureus **ATCC 33591**	***S. epidermidis*** **ATCC 35984**	***K. pneumoniae*** **ATCC BAA-1706**	***P. aeruginosa*** **ATCC 27853**
TB	12[a]	6	48	48
TB_L1FK	6	1.5	6	6
TB_KKG6A	1.5	1.5	3	3

[a]*Numbers represent the MBC values expressed in μM.*

approximately 1 Log_{10} as compared to the peptide used alone (**Figures 4C,D**).

Effect of TB Analogs, alone and in Combination with EDTA, on Planktonic Bacteria in Biofilm-Like Conditions

In order to investigate whether the synergism between TB analogs and EDTA was due to a disaggregating effect on biofilm extracellular matrix and/or to a direct effect on bacterial cells, we assessed the activity of the combination on planktonic bacteria in biofilm-like conditions (i.e., stationary phase cells suspended in BPM) in terms of MIC values. As shown in **Table 3**, when tested alone, TB_L1FK displayed MICs of 15 and 120 μM against *S. aureus* and *P. aeruginosa*, respectively. In the case of TB_KKG6A, the growth-inhibiting effect was recorded at 7.5 μM for *S. aureus* and at 30 μM for *P. aeruginosa*. In order to identify any synergistic interaction, sub-inhibitory concentrations of each peptide and EDTA were combined and the FIC index for the different peptide-EDTA combinations was calculated. Differently to what observed for the biofilm mode of growth, EDTA was not able to potentiate the antibacterial activity of TB_L1FK and TB_KKG6A against planktonic cells of *S. aureus* (FIC index > 0.5, **Table 3**). Conversely, a synergistic effect between both TB analogs and EDTA was observed against *P. aeruginosa* planktonic cultures (FIC index $= 0.25$, **Table 3**). Interestingly, the combination with EDTA produced an 8-fold decrease in the MIC of both peptides against planktonic *P. aeruginosa* grown in biofilm-like conditions, suggesting a direct effect of EDTA in displacing divalent cations that are required for the integrity of the outer membrane of Gram-negative bacteria (Gray and Wilkinson, 1965; Asbell and Eagon, 1966).

Hemolytic Activity

The hemolytic activity of TB and TB analogs was evaluated toward human RBCs. As shown in **Figure 5**, no hemolytic effect of the parental peptide was assessed at concentrations up to 96 μM. An overall increase in hemolytic activity of both analogs was observed. Nevertheless, a hemolysis below 10%, commonly recognized as a safe cut-off (Amin and Dannenfelser, 2006), was observed at concentrations up to 24 μM of TB_KKG6A and up to 48 μM of TB_L1FK.

Cytotoxicity against PBMCs and A549 Cells

TB, TB_L1FK and TB_KKG6A were tested for cytotoxic activity on PBMCs and A549 cells by flow cytometric determination of PI incorporation in cells treated with different concentrations of the three peptides. As shown in **Figure 6**, TB did not exhibit a significant cytotoxic effect toward both PBMCs and A549 cells at any of the tested concentrations. Indeed, an approximately 90% viability was observed at 96 μM for both cell types. Both TB analogs displayed higher cytotoxicity against both cell types as compared to TB (**Figures 6A,B**). When the toxic effect was evaluated as IC_{50} value, TB_L1FK and TB_KKG6A showed comparable levels of cytotoxicity against PBMCs (IC_{50} values of 52 and 49 μM, respectively). In contrast, TB_L1FK displayed lower levels of cytotoxicity against A549 cells with an IC_{50} value of 59 vs. 16 μM of TB_KKG6A.

DISCUSSION

The use of AMPs as an alternative to conventional antimicrobial agents in the treatment of antibiotic-resistant and/or biofilm-associated infections represents a possibility that is increasingly taken into consideration. Over the last years, a growing body of research has focused on frog skin-derived AMPs with considerable attention being devoted to the antibacterial activity and the mechanism of action of TB (Conlon et al., 2014; Mangoni et al., 2016). It has emerged that such a peptide possesses significant membrane-perturbing properties and folds in a α-helix upon interaction with bacterial membranes (Mangoni et al., 2000). Like most of the members of the temporin family, TB is considerably effective against Gram-positive bacteria, including clinically important multidrug-resistant pathogens, but only poorly active against Gram-negative bacteria (Mangoni et al., 2008). The lower level of activity of TB against these bacteria is likely due to the presence of LPS that induces the oligomerization of the peptide, and hence prevents it to diffuse through the cell wall and reach the target cytoplasmic membrane (Rosenfeld et al., 2006; Mangoni and Shai, 2009). Design of TB analogs with modification of the peptide primary structure may provide peptides with stronger activity against Gram-negative bacterial species and increase the translational potential of TB. Computer-assisted design strategies have led us to obtain TB_L1FK, in which the leucine in position 1 has been replaced by a phenylalanine, the asparagine 7 has been eliminated and an extra lysine has been inserted at the C-terminus increasing the net charge of the peptide. Differently from the traditional optimization procedures, the computational method employed herein allowed to predict the effect of multiple amino acid positions on the antibacterial activity and cytotoxicity of TB, thereby enabling to improve different features of the peptide at the same time and to design a set of candidates for experimental validation. The other analog analyzed in this work, i.e., TB_KKG6A, has been designed by Avitabile and colleagues by replacing the glycine in position 6 with an alanine according to the Ala-scanning method and by adding two lysines at the N-terminus in order to produce a more cationic peptide (Avitabile et al., 2013). Circular dichroism and NMR studies have previously shown that TB_KKG6A strongly

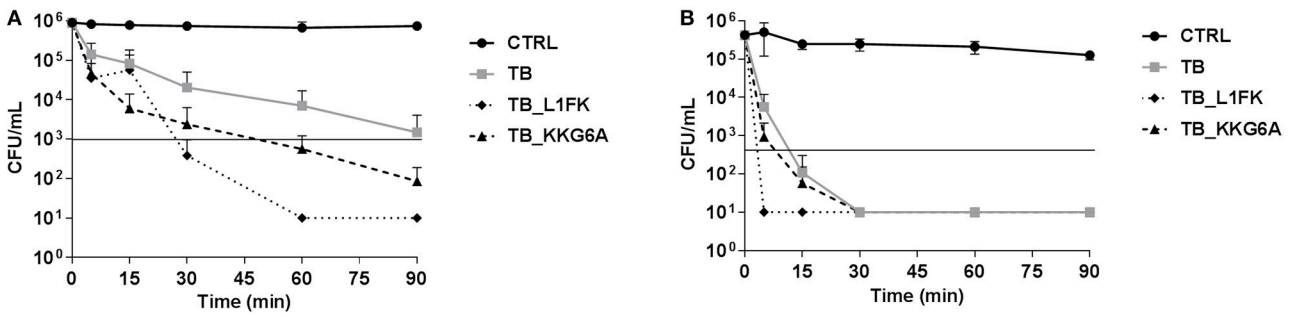

FIGURE 2 | Killing kinetics of TB, TB_L1FK, and TB_KKG6A against *S. aureus* ATCC 33591 (A) and *P. aeruginosa* ATCC 27853 **(B)**. Bacteria were incubated in sodium-phosphate buffer (10 mM SPB, pH 7.4) with the peptides at concentrations equal to their MBCs for various times. Control (CTRL) represents untreated bacteria. Solid line indicates a reduction of ≥3 Log_{10} in the number of control bacteria at each time of incubation. A number of 10 CFU/mL was taken as detection limit. Data are expressed as mean ± standard error of at least three independent experiments.

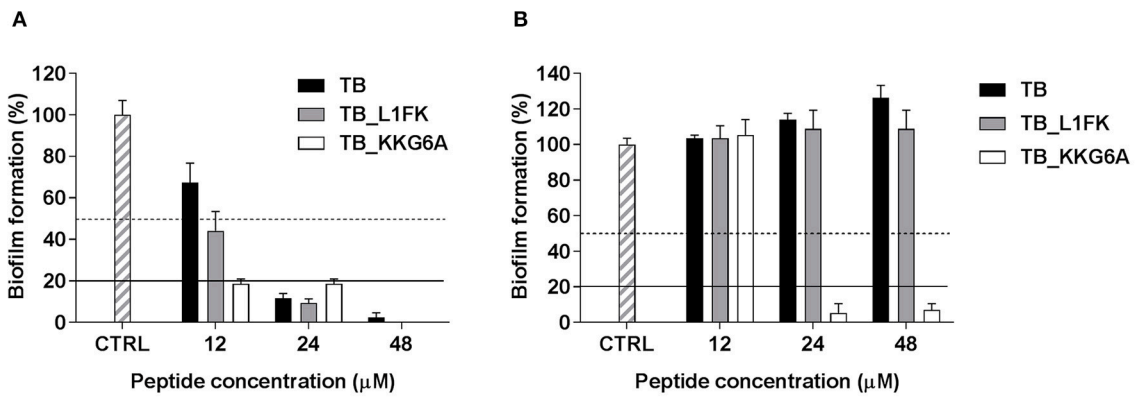

FIGURE 3 | Inhibitory effect of TB, TB_L1FK, and TB_KKG6A on biofilm formation of *S. aureus* ATCC 33591 (A) and *P. aeruginosa* ATCC 27853 **(B)**. The inhibitory effect was assessed by measuring the total biofilm biomass by crystal violet staining after 24 h of incubation with the peptides. Control (CTRL) represents untreated bacteria. Dashed and solid lines represent 50 and 80% reduction in biofilm biomass as compared to untreated controls, respectively. Data are reported as mean ± standard error of at least three independent experiments.

interacts with the LPS of the Gram-negative bacterium *E. coli* and assumes a bent helical conformation upon binding (Avitabile et al., 2013; Malgieri et al., 2015).

A comparative analysis of the properties of TB and these two analogs was performed starting from the evaluation of their bactericidal activity against multidrug-resistant bacteria in planktonic form. TB_L1FK and TB_KKG6A displayed an expanded spectrum of action as compared to the parental peptide, being active against all the tested Gram-positive and Gram-negative bacterial strains at very low concentrations. It is likely that the presence of additional positively charged amino acids in their sequence enhanced the affinity of the analogs toward Gram-negative bacteria. This observation is consistent with previous studies, in which optimized analogs of both TB and other temporins (Conlon et al., 2007; Capparelli et al., 2009; Srivastava and Ghosh, 2013) were obtained through the introduction of extra positive charges. Cationic amino acids, such as lysine, play a key role in the interaction of AMPs with the negatively charged components of the bacterial cell surface and the cytoplasmic membrane (Shai, 1999; Hancock and Sahl, 2006). Therefore, an increase in peptide cationicity can promote a more efficient interaction with bacteria, and hence a stronger antibacterial activity (Han et al., 2016). Moreover, faster killing kinetics were observed for the analogs compared to TB against both *S. aureus* and *P. aeruginosa*, selected as representative Gram-positive and Gram-negative bacterial species, respectively. The short time required for peptides to exert their bactericidal effect correlates with the bacterial membrane-permeabilizing activity of the temporin family (Mangoni et al., 2000; Saviello et al., 2010).

The three peptides were also compared regarding their antibiofilm properties using reference strains of *S. aureus* and *P. aeruginosa*. Biofilm-related infections currently represent a relevant clinical problem because of the intrinsic recalcitrance of biofilms to the antibiotic therapy. *S. aureus* and *P. aeruginosa* are common bacterial species involved in biofilm-associated infections, such as wound infections, lung infections in cystic fibrosis patients and implant-related infections (e.g., central venous catheters, endotracheal tubes, prostheses; Ciofu et al.,

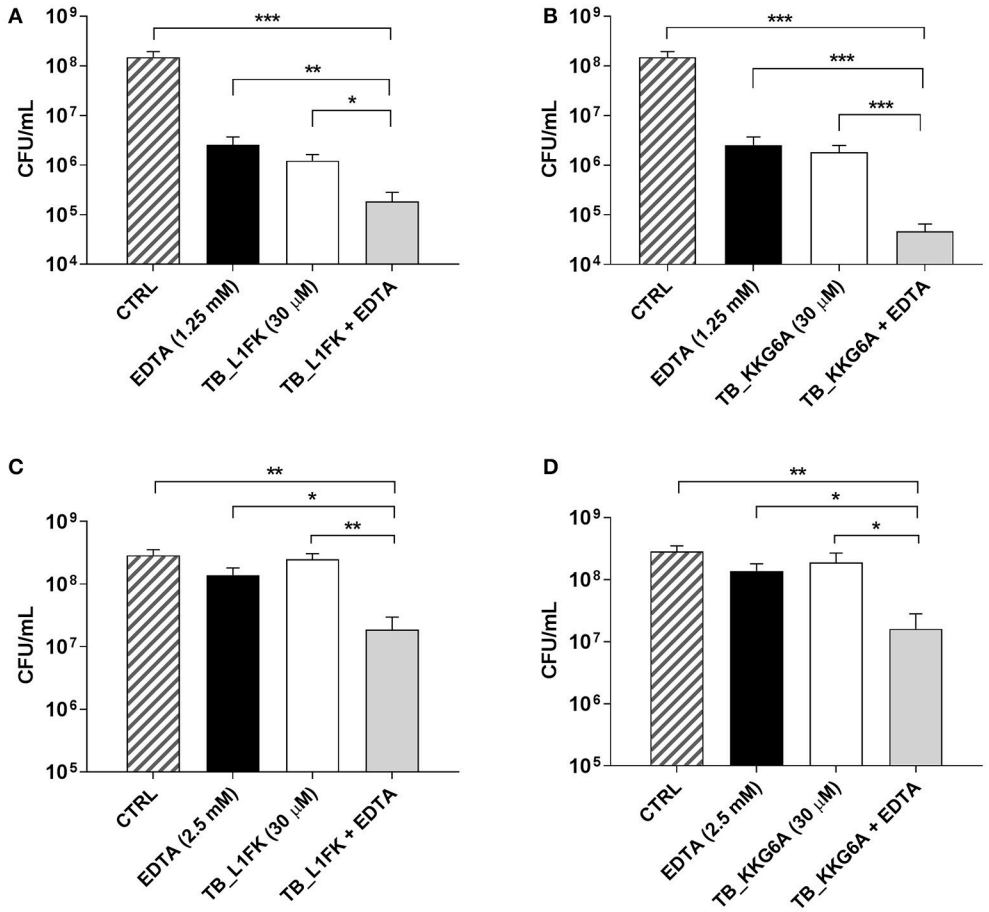

FIGURE 4 | Activity of TB_L1FK and TB_KKG6A, used alone and in combination with EDTA, against preformed (24-h old) biofilms of *S. aureus* ATCC 33591 (A,B) and *P. aeruginosa* ATCC 27853 **(C,D)**. The antibiofilm activity of the peptides, EDTA and the peptide-EDTA combinations was evaluated by CFU counting after 24 h of incubation. Control (CTRL) represents untreated biofilms. Data are reported as mean ± standard error of at least three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (one way ANOVA followed by Tukey-Kramer *post-hoc* test).

TABLE 3 | MICs of TB_L1FK and TB_KKG6A in biofilm-like conditions against *S. aureus* and *P. aeruginosa* and FIC index of the peptide-EDTA combinations.

	***S. aureus* ATCC 33591**		***P. aeruginosa* ATCC 27853**	
	TB_L1FK	**TB_KKG6A**	**TB_L1FK**	**TB_KKG6A**
MIC[a]	15	7.5	120	30
FIC index	>0.5	>0.5	0.25 (15 μM)[b]	0.25 (3.75 μM)

[a] *Concentrations are expressed in μM.*

[b] *Parentheses include the concentration of the peptide resulting in a synergistic effect.*

2015). The ability of these pathogens to produce biofilms is responsible for the establishment of chronic infections, thereby constituting a primary impediment to the complete recovery from infectious diseases (Costerton et al., 1999; Dean et al., 2011). Thus, the identification of new broad-spectrum antibiofilm agents and innovative therapeutic strategies appears as a growing need. To this aim, we explored the efficacy of TB and TB analogs both in preventing biofilm formation and in treating mature biofilms and attempted to enhance the antibiofilm activity of the peptides by combining them with adjuvant compounds. TB analogs showed an improved ability to inhibit the formation of *S. aureus* biofilms at 12 μM, while at 24 μM all three peptides were equally active, causing more than 80% reduction of the biofilm biomass. TB_KKG6A, but not TB_L1FK, showed also a marked activity in inhibiting biofilm formation of *P. aeruginosa* at the concentration of 24 μM. In all cases, the inhibitory activity of the peptides was observed at concentrations close to the MIC values determined in biofilm-like conditions (**Table 3**), suggesting that the antibiofilm effect was due to the direct killing of biofilm-forming bacteria at their planktonic stage rather than to biofilm-specific mechanisms (Segev-Zarko et al., 2015; Batoni et al., 2016a). When assayed against preformed biofilms, the two analogs, differently from TB, were able to significantly reduce the number of biofilm-associated cells of *S. aureus* at 30 μM, while none of the peptides was effective against *P. aeruginosa* even at 120 μM. It is commonly recognized that preformed biofilms are more challenging to target than the early stages of biofilm formation. The reduced susceptibility of mature biofilms

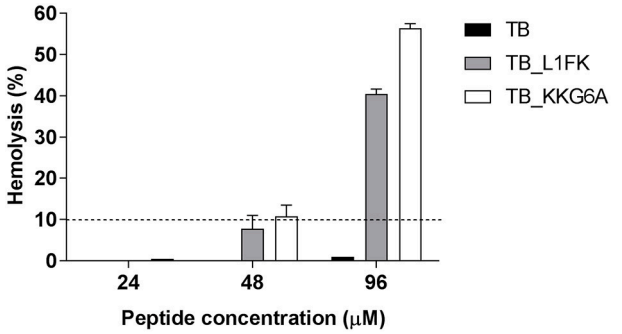

FIGURE 5 | Hemolytic activity of TB, TB_L1FK, and TB_KKG6A on human erythrocytes after 1 h of incubation at 37°C. The hemolytic activity was evaluated by the spectrophotometric determination of hemoglobin released from erythrocytes. PBS (0% hemolysis) and Triton X-100 (100% hemolysis) were used as controls. Hemolysis values ≤10% (dashed line) are considered to be non-hemolytic (Amin and Dannenfelser, 2006). Data are reported as mean ± standard error of three independent experiments.

to AMPs is mainly due to the presence of the extracellular matrix that surrounds the bacterial population and constitutes an actual impediment to peptide penetration into the biofilm structure (Otto, 2006; Batoni et al., 2016a). Cationic peptides can be repulsed or sequestrated by the biofilm extracellular polymeric molecules, especially exopolysaccharides and DNA, so that their interaction with bacterial cells can be significantly hampered (Batoni et al., 2016a). In particular, the polysaccharide intracellular adhesin (PIA) of staphylococcal biofilm matrix and alginate, Pel and Psl polysaccharides of *P. aeruginosa* biofilms have been demonstrated to play a major role in the protection from AMPs (Vuong et al., 2004; Chan et al., 2005). Thus, the use of AMPs in combination with compounds able to disaggregate the extracellular matrix could represent a promising strategy to increase their antibiofilm activity and therapeutic potential. In this regard, the chelator EDTA has been shown to reduce the structural integrity of the biofilm of several bacterial species by forming strong complexes with divalent cations (magnesium, calcium, iron) essential for matrix stability (Percival et al., 2005; Banin et al., 2006; Cavaliere et al., 2014; Maisetta et al., 2016). Herein, we combined TB analogs with EDTA in order to improve their efficacy against preformed biofilms of *S. aureus* and *P. aeruginosa*. The combination of TB_L1FK and TB_KKG6A with EDTA resulted in a potentiated antibiofilm effect that led to a statistically significant reduction in the viable count of both bacterial species at a peptide concentration of 30 μM. In order to prove that the enhancement of the antibiofilm activity of TB analogs was actually due to the destabilizing action of EDTA on the biofilm matrix, we also evaluated the effect of the combination peptide-EDTA on planktonic cells in biofilm-like conditions. Interestingly, the peptides exhibited synergy with EDTA against planktonic cultures of *P. aeruginosa*, but not against *S. aureus*. The combination treatment inhibited the growth of *P. aeruginosa* to a greater extent than the peptide used alone, suggesting a direct effect of EDTA also on planktonic bacteria. It is known that divalent cations are key elements in maintaining the integrity of the outer membrane of Gram-negative bacteria as they attenuate the electrostatic repulsive forces between adjacent LPS molecules by forming salt bridges (Gray and Wilkinson, 1965; Asbell and Eagon, 1966). Therefore, chelation of divalent cations by EDTA could enhance the action of the tested AMPs by destabilizing the outer membrane and thus facilitating the peptide access to the bacterial inner membrane. Furthermore, the chelating activity of EDTA may contribute to remove the cationic barrier that prevents the electrostatic interaction of cationic AMPs with the negatively charged bacterial surface (Walkenhorst et al., 2014). Thus, it is likely that EDTA mainly acted as an extracellular matrix-disaggregating agent in the case of *S. aureus* biofilms, facilitating the diffusion of the peptides through the biofilm layers. On the other hand, in the case of *P. aeruginosa* biofilms, the enhanced effect of the peptide-EDTA combinations could be very well due not only to the perturbing effect on the extracellular matrix, but also on a direct effect on biofilm-embedded cells.

The evaluation of the cytotoxicity of AMPs toward the host cells is an essential step to their development as therapeutics. It is generally accepted that there is a direct relationship between the antimicrobial potency of AMPs and their cytotoxic properties (Takahashi et al., 2010). A subtle balance of several physicochemical and structural parameters (cationicity, amphipathicity, hydrophobicity, and helicity) is necessary to ensure the maximum antibacterial efficacy and target cell selectivity of the peptides (Chen et al., 2005; Zelezetsky et al., 2005). Therefore, we evaluated the hemolytic effect of TB analogs on human erythrocytes and their cytotoxic activity on human PBMCs and the human-derived epithelial cell line A549. Along with the enhancement of the antimicrobial activity, modifications in TB sequence led to an overall increase of the hemolytic activity and cytotoxicity of the native peptide. Nevertheless, both TB_L1FK and TB_KKG6A were non-hemolytic at concentrations that resulted to be active against both planktonic and biofilm-growing bacteria. A percentage of hemolysis lower than 10% was assessed at peptide concentrations close to that used in combination with EDTA in treating mature biofilms of *S. aureus* and *P. aeruginosa*. When tested against mammalian cells, TB_L1FK resulted less cytotoxic than TB_KKG6A against human epithelial cells, suggesting that the computational method employed generated a sequence showing a good compromise between antibacterial and cytotoxic activity and promising features for topic applications. In the case of PBMCs, both TB analogs displayed comparable and quite high levels of cytotoxicity. A promising solution to reduce the toxicity of AMPs is the development of appropriate delivery systems for their controlled and/or targeted release. In this regard, our group has recently developed a chitosan-based nanostructured delivery system loaded with TB that ensured a considerable reduction of the cytotoxic activity of the peptide toward mammalian cells (Piras et al., 2015).

CONCLUSIONS

In the present study, we performed a detailed characterization of the bactericidal and antibiofilm activity of TB analogs in

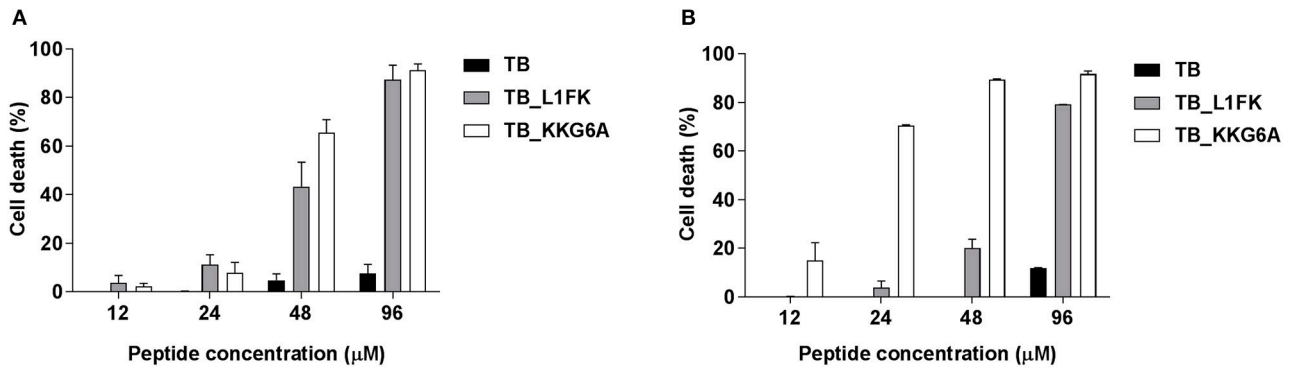

FIGURE 6 | Cytotoxicity of TB, TB_L1FK, and TB_KKG6A on human PBMCs (A) and A549 cells **(B)** after 24 h of incubation at 37°C, 5% CO_2. The cytotoxic activity was evaluated by the PI flow cytometric assay. Cells incubated with culture medium only (100% cell viability) and cells treated with cycloheximide (0% cell viability) were used as controls. Data are reported as mean ± standard error of three independent experiments.

order to demonstrate the potential of computational peptide design in the improvement of the antimicrobial properties of AMPs. The introduction of appropriate modifications in the primary sequence of TB led to optimized analogs with a stronger and faster bactericidal activity and a wider spectrum of action as compared to the parental peptide. Furthermore, TB analogs exhibited an improved ability both in preventing biofilm formation and in treating preformed biofilms of *S. aureus* and *P. aeruginosa*, especially when used in combination with EDTA. The antibiofilm action of the peptide-EDTA combination was likely due to a disaggregating effect on the biofilm extracellular matrix and/or to a direct effect on bacterial cells. Collectively, our results suggest that TB analogs represent a promising template for the development of novel antimicrobials for the treatment of antibiotic-resistant and/or biofilm-associated infections. In this regard, current work is devoted to the development of a nanostructured delivery system for TB analogs with the aim to reduce their toxicity and to control their pharmacokinetics, thus further improving the therapeutic potential of these molecules.

AUTHOR CONTRIBUTIONS

LG, GAM, SE, and GB: conception and design of the work; acquisition, analysis, and interpretation of the data for the work; GM: design and analysis of TB_L1FK; LG, GAM, GM, and GB: drafting of the work; LG, GAM, GM, SE, and GB: critical revision of the work; final approval.

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frontiers in Chemistry

MINI REVIEW
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The Human Antimicrobial Peptides Dermcidin and LL-37 Show Novel Distinct Pathways in Membrane Interactions

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Mammals protect themselves from inflammation triggered by microorganisms through secretion of antimicrobial peptides (AMPs). One mechanism by which AMPs kill bacterial cells is perforating their membranes. Membrane interactions and pore formation were investigated for α-helical AMPs leading to the formulation of three basic mechanistic models: the barrel stave, toroidal, and carpet model. One major drawback of these models is their simplicity. They do not reflect the real *in vitro* and *in vivo* conditions. To challenge and refine these models using a structure-based approach we set out to investigate how human cathelicidin (LL-37) and dermcidin (DCD) interact with membranes. Both peptides are α-helical and their structures have been solved at atomic resolution. DCD assembles in solution into a hexameric pre-channel complex before the actual membrane targeting and integration step can occur, and the complex follows a deviation of the barrel stave model. LL-37 interacts with lipids and shows the formation of oligomers generating fibril-like supramolecular structures on membranes. LL-37 further assembles into transmembrane pores with yet unknown structure expressing a deviation of the toroidal pore model. Both of their specific targeting mechanisms will be discussed in the context of the "old" models propagated in the literature.

Keywords: LL-37, structural biology, membranes, artificial, membranes, dermcidins

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HUMAN ANTIMICROBIAL PEPTIDES

Antimicrobial peptides evolved during an early stage of the mammalian evolution and represent ancient molecules optimized through their co-evolution with bacteria (Peschel and Sahl, 2006). AMPs are produced by virtually every organism and often comprise the majority of the broad-spectrum antimicrobial activity against fungi, bacteria and viruses. In humans they are an essential part of the innate immune system due to their pleiotropic functions in microbial killing, inflammation, angiogenesis, and wound healing (Nakatsuji and Gallo, 2012). They constantly protect the human body from microbes and inflammation, and their levels can be activated locally and in a timely manner (Zasloff, 2002; Ganz, 2003; Peschel and Sahl, 2006). While the functions of many of these peptides are not well-understood, it has been shown e.g., that a-defensin HD-6 can self-assemble on the bacterial cell surface into nanonets to entangle bacteria (Chu et al., 2012; Ouellette and Selsted, 2012; Chairatana and Nolan, 2017). Dermcidin is a peptide ion channel which

can integrate itself into bacterial cytoplasmic membranes to kill bacteria (Song et al., 2013; Zeth, 2013). Pore-like structures can also be formed by granulysin and LL-37 (Anderson et al., 2003; Lee et al., 2011).

In contrast to traditional antibiotics, AMPs often target the bacterial membrane—also known as "the Achilles heel of bacterial cells" (Zasloff, 2002). AMP-membrane interactions are described by three distinct models applicable only to amphipathic α-helical antimicrobial peptides (Zasloff, 2002; Brogden, 2005; Bechinger and Lohner, 2006). All these models are based on the assumption of an initial peptide-lipid interaction mediated through electrostatic properties, followed by free lateral diffusion and pre-assembly of peptides at the membrane surface (Brogden, 2005). The actual membrane insertion step divides the process into three divergent models depending on the particular mode of peptide assembly, the strength of peptide-lipid interactions, and the peptide concentration (Brogden, 2005). The barrel stave model describes the membrane induced assembly of amphipathic peptides into oligomeric transmembrane channels (Baumann and Mueller, 1974). The toroidal model delineates a pore architecture formed by peptide channels laterally stabilized via electrostatic lipid head group interactions (Ludtke et al., 1996; Matsuzaki et al., 1996). Finally, the carpet model describes severe membrane perturbation after the release of mixed peptide-lipid complexes, similar to detergent-induced membrane destruction (Bechinger and Lohner, 2006). To a variable extent, all processes lead to the formation of holes in membranes which—in cytoplasmic membranes—results in the breakdown of the transmembrane potential and cell death (Brogden, 2005). While these three models are frequently used in the literature, recent observations indicate a much greater complexity of AMP-membrane interactions and urge for the development of multistep models developed for each individual AMP. Among the various human AMPs there are two with a clear α-helical secondary structure: dermcidin and LL-37. Our approach aimed for the formulation of refined structure-function-based mechanisms using these peptides, followed by a comparison with the simple standard models.

HUMAN DERMCIDIN FORMS A HEXAMERIC CHANNEL AND FOLLOWS THE BARREL STAVE MODEL

Among the major AMPs detected on human skin, dermcidin is enriched as a constitutively expressed peptide (Schittek et al., 2001; Bardan et al., 2004; Paulmann et al., 2012). DCD is active against a broad spectrum of bacteria and fungi at concentrations of ∼10 μg/mL (Paulmann et al., 2012). Its antimicrobial activity is robust against changes in pH and ionic strength (Schittek et al., 2001; Paulmann et al., 2012). When isolated from sweat or after recombinant expression, DCD forms an equilibrium mixture of oligomers of varying size, both in solution and in membrane mimetics (Cipáková et al., 2006; Paulmann et al., 2012). Dermcidin is unique amongst AMPs for at least two reasons: it is significantly longer (49 residues) than most of the well-studied AMPs, and its net charge is negative which is in contrast to most of the known AMP molecules reported so far.

The structural analysis of DCD provided our group with an unexpected glimpse of a hexameric channel architecture (Song et al., 2013; **Figure 1A**). Trimers of dimers oriented along the channel axis form the 8 nm extended structure. Each monomer forms two different interfaces to neighboring monomers, one of which is hydrophobic and potentially more stable while the second is hydrophilic. The hexamer and hydrophilic interface formation is stabilized by the presence of divalent ions, in particular zinc ions (**Figure 1A**; Song et al., 2013). The channel is formed in the absence of lipophilic molecules such as detergents or lipids and is stable with a surplus of hydrophobic residues pointing outwards without being shielded—this is another unique feature of dermcidin (Song et al., 2013). DCD interacts with vesicles e.g., in a planar lipid membrane experiment leading to a channel with an approximate conductance of 100 pS at 1 M KCl but it does not normally insert, unless a voltage of more than 100 mV is applied (**Figures 1B,E**; Song et al., 2013).

DCD is currently the first antimicrobial peptide discovered at atomic resolution in the channel form (**Figures 1A,B**). In contrast to the barrel stave model, we show that DCD assembles into this hexameric structure already in solution and subsequently interacts with the bacterial membrane (Song et al., 2013). *In vitro* the channel can be translocated into the membrane by the application of a transmembrane potential. *In vivo* the physiological transmembrane potential formed over the bacterial cytoplasmic membranes may be sufficient to transfer DCD into the membrane. Once inserted in a membrane channel, nanopores destroy the transmembrane potential and this subsequently leads to bacterial cell death (Song et al., 2013). Channel structures such as those of magainin or alamethicin were modeled as oligomers but these models are based on monomeric or dimeric structures assembled on the basis of their transmembrane potential (**Figure 1C**; Terwilliger and Eisenberg, 1982; Zhu and Shin, 2009; Lorenzón et al., 2012; Hayouka et al., 2013). Their conductance, although defined, is significantly higher (300–600 pS) than for DCD pointing toward the formation of a channel with significantly larger diameter (**Figure 1E**).

LL-37 ASSEMBLES INTO FIBER-LIKE STRUCTURES AS AN INTERMEDIATE STEP BEFORE MEMBRANE PERFORATION

LL-37 is an intensively studied peptide with a broad variety of physiological functions, such as in host immunity and antimicrobial activity (Dürr et al., 2006; Vandamme et al., 2012). Its primary sequence clearly indicates amphipathicity, a hallmark of AMPs integrating into biological membranes. Structurally, the peptide was studied using circular dichroism, Fourier transform infrared, and NMR spectroscopy in various media (Johansson et al., 1998; Oren et al., 1999; Li et al., 2006; Wang, 2008). The combined studies indicate that the structure of LL-37 depends on pH, ion strength, and peptide concentrations (Johansson et al., 1998). High resolution studies by NMR were only performed in

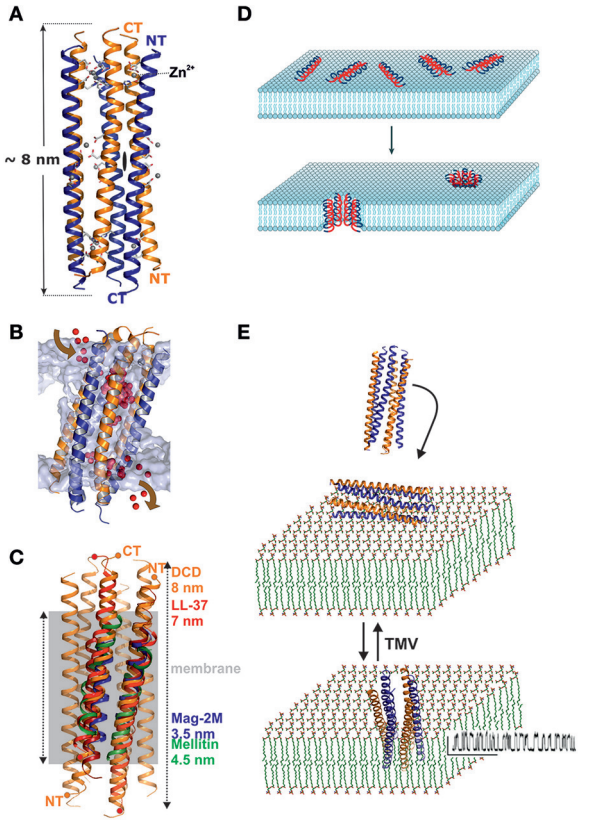

FIGURE 1 | Structure and functional mechanism of human dermcidin. **(A)** Side view of the hexameric structure of dermcidin shown in ribbon representation. The peptide forms regular helixes which are arranged in an anti-parallel manner (highlighted in blue and orange) so that the channel consists of a trimer of dimers which are aligned along the three-fold axis of the channel. The overall length of the channel is 8 nm and zinc binding (Zn^{2+}) sites are located at the end of the channel located between two helices. **(B)** Molecular dynamic studies of DCD in artificial membranes demonstrate an unexpected pathway of ion translocation. Ions enter the channel from the side of the membrane at the height of the membrane lipid head groups and leave the channel by the same mechanism. Due to the extension of the channel and the hydrophobic exterior, the energetically most favorable conformation is a tilted channel (20–30° relative to the membrane normal) in the membrane. **(C)** Mechanism of DCD interaction with membranes: the channel exists as a stable hexamer in solution. Interaction of the channel with the membrane does not lead to insertion unless a transmembrane voltage of >100 mV moves the channel into the membrane. Although small, the channel shows clear and defined conductivity steps with a high open probability [see also **(E)**]. **(D)** Simplified mechanism describing the carpet model which explain the activity of AMPs which are in a first step electrostatically attracted by membranes followed by an assembly of peptides and integration into lipid bilayers (Brogden, 2005). This figure is reprinted with permission from Brogden (2005). **(E)** By contrast, dermcidin is already oligomeric in solution and interacts with membranes via electrostatic interactions. Integration of the peptide cannot be detected in biophysical studies unless a transmembrane voltage (TMV) is applied which leads to the detection of a conductive channel (Song et al., 2013).

the presence of 1% SDS, so the structural transition from the solution into a putative membrane associated has not yet been characterized (Wang, 2008).

Because of the obvious lack of reliable experimental data, we crystallized the peptide in the presence and absence of detergents and achieved several structural states (Scientific reports in press). In the absence of detergents, LL-37 forms an anti-parallel dimer similar to the structure of magainin, mellitin, or the antiparallel dimer of DCD (**Figure 2A**; Terwilliger and Eisenberg, 1982; Hayouka et al., 2013; Song et al., 2013). One of the sides of this dimer is strongly hydrophobic while the opposite side is positively charged. Crystallization in the presence of detergents leads to the reorganization of the dimer, exposing aromatic residues for detergent interactions, and the formation of discrete peptide-detergent complexes (**Figures 1A,B**; Scientific reports in press). Detergents can bind at the N-terminal region and at the center of the dimeric peptide. Six detergent binding sites are observed per dimer, indicating potential lipid binding sites in the presence of natural or artificial membranes. N-terminally located detergents between two dimers are enclosed by nest-like architecture primarily lined up by aromatic residues (Scientific reports in press; **Figure 2B**). Furthermore, in the detergent-induced state the molecule forms unidimensional fiber-like chains in the crystal lattice (**Figure 2B**). These fiber-like structures could also be detected on vesicles using gold-labeled LL-37 and electron microscopy as imaging technique (Scientific reports in press). LL-37 has previously been shown to restructure lipid vesicles into elongated structures, possibly based on the formation of a similar supramolecular structure (Shahmiri et al., 2016). The formation of such fiber-like structures has been described previously for the synthetic peptides LAH4 and BTD-2 (Aisenbrey and Bechinger, 2014; Wang et al., 2016; **Figure 2E**).

Which model mechanism comes closest to the most recent (**Figure 2C**) LL-37 data? In the first step, LL-37 interacts with LPS and LTA and possibly removes part of these molecules from the cell wall (Neville et al., 2006). In the second step, according to our own data and the data of others implies that LL-37 can specifically interact with membranes or even specifically with individual lipid head groups via a multi-step mechanism (Scientific reports in press; Shahmiri et al., 2016; **Figure 2D**). This mechanism is more complicated than the simple toroidal model, where the monomeric peptide assembles on the membrane to form holes on lipid-peptide complexes (Ludtke et al., 1996; Matsuzaki et al., 1996). Oligomeric, fiber-like structures are possibly one intermediate state after potential lipid binding interactions are expressed. These interactions likely destabilize membranes and may also lead to the extraction of lipids from the outer membrane leaflet of the inner membrane. Finally, LL-37 forms channels or pores in membranes to destroy the transmembrane potential but it is unknown if these channels express a peptide-lipid stabilized architecture (Lee et al., 2011; **Figure 2D**).

SUMMARY

The growing number of AMPs from many sources forms a solid basis for the development of new antibiotics. This process can be enhanced once their individual mechanisms of action are understood in more detail. Here we show that for two human AMPs their membrane interactions are sophisticated multi-step pathways which deviate from the three simple model mechanisms. Although, our own work has mainly delivered indirect insights based on AMP interactions with detergents and

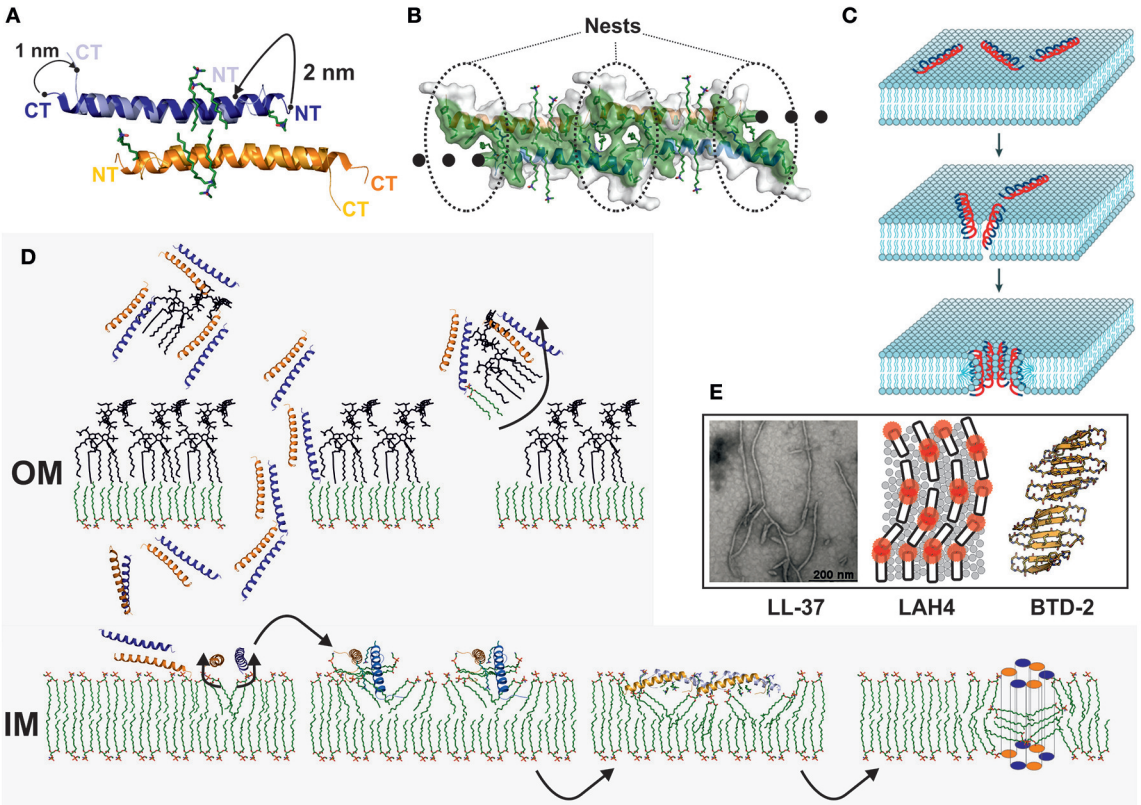

FIGURE 2 | Structure and membrane interaction mechanism of human cathelicidin. **(A)** Structure comparison of the LL-37 dimer in the presence and absence of detergents. Detergents induce a significant conformational change at the N- and C-terminus and discrete detergent binding sites are formed. **(B)** LL-37 tetramer in a surface representation. Hydrophobic residues on the side are marked in green. At the interface between two dimers, nest-like hydrophobic structures are formed to accommodate detergents *in vitro*. Lipid molecules *in vivo* may occupy these detergent positions, and lipid molecules or detergents may initiate the oligomerization of the channel. **(C)** Simplified mechanism describing the toroidal model which explain the activity of AMPs which are in a first step electrostatically attracted by membranes followed by their assembly and partial integration. In a final step the peptides form channels based on peptide-peptide and peptide-lipid interactions after full integration into lipid bilayers (Brogden, 2005). This figure is reprinted with permission from by Brogden (2005). **(D)** Model for the interaction of LL-37 with the cell wall of a Gram-negative bacterium. Significant interactions between LL-37 and LPS have been demonstrated, and, as a hypothesis, LPS may be translocated apart from the cell wall in order to build holes for the translocation of LL-37 into the periplasmic space (Scientific reports in press; Vandamme et al., 2012). Interactions of the peptide with lipid molecules will initiate the conformational changes, and fiber-like oligomers may form on the inner membrane. These fibers lead to an increased local concentration of the peptide and will interfere with the membrane stability. **(E)** Related models and experimental data which were recently published in the literature are based on fluorescence techniques applied to LAH4, crystallography and analysis of crystal packing of BTD-2 and electron microscopy of LL-37 mixed with lipid vesicles (Aisenbrey and Bechinger, 2014; Shahmiri et al., 2016; Wang et al., 2016). LL-37 TEM figure is reprinted from Shahmiri et al. (2016) published in open-access under CC BY 4.0 license. LAH4 figure is reprinted with permission from by Aisenbrey and Bechinger (2014). Copyright 2014 American Chemical Society. BTD-2 figure is reprinted with permission from Wang et al. (2016). Copyright 2016 American Chemical Society.

lipids it creates significant improvement of our understanding how DCD and LL-37 target artificial membranes. Together these data represent one critical step forward toward their full mechanistic understanding. However, there is no doubt that true mechanisms *in vivo* in the context of bacterial cell walls are even more complex, and future work needs to initiate studies on the direct interactions of AMPs with the bacterial cell.

OPEN QUESTIONS

DCD and LL-37 are only two selected examples of AMPs, and such do not represent the broadness of mechanisms of how AMPs perturb bacterial membranes. In spite of their improved understanding, general questions remain unanswered e.g.:

- Why has the long DCD channel version, with physical dimensions significantly longer than required for spanning an average membrane thickness been retained?
- How a negatively charged peptide like DCD would interact with an outermost LPS or LTA leaflet layer and how would it pass this layer?
- What is the mechanism by which DCD is translocated over the cell wall of Gram-negative bacteria?

LL-37 activity and killing mechanisms also harbors many secrets e.g.:

- How this peptide interacts with LPS and LTA, and if these molecules are extracted from the membrane to gain access to the cell?

- What is the reason for fiber formation of LL-37 and other AMPs on artificial membranes, and are these fibers also formed on natural membranes?
- Finally, it will be important to test if the detergent binding sites we see in our structures actually resemble lipid binding sites *in vivo*.
- The ultimate step of LL-37 forming pores in membranes and the putative involvement of lipids remains to be shown.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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Cationic Antimicrobial Peptides Inactivate Shiga Toxin-Encoding Bacteriophages

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Shiga toxin (Stx) is the principal virulence factor during Shiga toxin-producing *Escherichia coli* (STEC) infections. We have previously reported the inactivation of bacteriophage encoding Stx after treatment with chitosan, a linear polysaccharide polymer with cationic properties. Cationic antimicrobial peptides (cAMPs) are short linear aminoacidic sequences, with a positive net charge, which display bactericidal or bacteriostatic activity against a wide range of bacterial species. They are promising novel antibiotics since they have shown bactericidal effects against multiresistant bacteria. To evaluate whether cationic properties are responsible for bacteriophage inactivation, we tested seven cationic peptides with proven antimicrobial activity as anti-bacteriophage agents, and one random sequence cationic peptide with no antimicrobial activity as a control. We observed bacteriophage inactivation after incubation with five cAMPs, but no inactivating activity was observed with the random sequence cationic peptide or with the non-alpha helical cAMP Omiganan. Finally, to confirm peptide-bacteriophage interaction, zeta potential was analyzed by following changes on bacteriophage surface charges after peptide incubation. According to our results we could propose that: (1) direct interaction of peptides with phage is a necessary step for bacteriophage inactivation, (2) cationic properties are necessary but not sufficient for bacteriophage inactivation, and (3) inactivation by cationic peptides could be sequence (or structure) specific. Overall our data suggest that these peptides could be considered a new family of molecules potentially useful to decrease bacteriophage replication and Stx expression.

Keywords: bacteriophages (phages), antimicrobial peptides, *Escherichia coli* O157, hemolytic uremic syndrome (HUS), anti-infective agents

INTRODUCTION

Infections with Shiga toxin-producing *Escherichia coli* (STEC) strains are a serious public health problem. Children infected with STEC strains present diarrhea, hemorrhagic colitis, and a percentage of patients can develop Hemolytic Uremic Syndrome (HUS).

Shiga toxin (Stx) is the main virulence factor during STEC infections. Because the gene encoding for Stx is inside the prophage genome, these strains are also known as *Escherichia coli* Shiga

Toxin-Encoding Bacteriophages. During STEC infection, the bacteriophage is cleaved and the replication and Stx expression take place inside the gut. Then, free bacteriophages are able to infect other susceptible bacteria present in the gut, exacerbating Stx expression (Cornick et al., 2006). Currently, there are no effective treatments or vaccines available, and for this reason bacteriophage inactivation treatments are a promising strategy to prevent Stx expression after STEC infections.

Previously, we showed that chitosan has anti-bacteriophage activity *in vitro* and *in vivo* (Amorim et al., 2014). Chitosan is a cationic linear polysaccharide polymer obtained after the deacetylation of chitin; this polymer has been widely-used as an antimicrobial agent against several microorganisms (Kong et al., 2010). In a previous work Maffía and collaborators designed a group of new cationic antimicrobial peptides and tested them against a broad panel of multi-resistant clinical bacterial isolates (Faccone et al., 2014). In order to evaluate whether these sequences could affect bacteriophage infection we tested seven previously designed cAMPs (Faccone et al., 2014; Hollmann et al., 2016; Maturana et al., 2017) as potential anti-bacteriophage agents. To analyze the effect of these peptides, we used them to inactivate a previously reported mutant bacteriophage (ϕΔTOX:GFP) in which the *stx* operon has been replaced by a gene encoding for the green fluorescent protein (GFP) (Amorim et al., 2014). Five of these peptides were previously analyzed and displayed antimicrobial activity in different bacterial strains and structure as alpha helix in contact with lipid membranes (P5, P8, P8.1, P2, and P6.2). The other two peptides tested were Omiganan, a linear Beta-sheet cAMP derived from indolicidin that underwent clinical trials with activity against *S. aureus*; and a random sequence peptide with cationic charge but no antimicrobial activity (Faccone et al., 2014; Hollmann et al., 2016)

Therefore, the objective of this work was to evaluate if this group of cAMPs could inactivate Shiga toxin- encoding bacteriophages.

MATERIALS AND METHODS

Cationic Peptides

Each peptide was synthesized with C terminus amidation. Peptides were synthesized and obtained at a purity grade of >95% by HPLC (GenScript Co., Piscataway, NJ 08854, USA). Cationic alpha helical peptides P5, P8, P8.1, P2, and P6.2 were previously designed using a combined rational and computer assisted approach, identifying short putative active regions from AMP databases (Faccone et al., 2014; Maturana et al., 2017). Peptide sequences are: peptide 2: GLLKKWLKKWKEFKRIVG Y; peptide 8.1: RIVQRIAKWAKKWYKAGK, peptide 6.2: GLL RKWGKKWKEFLRRVWK; peptide 5: RIVQRIKKWLLKWKK LGY; peptide 8: RIVQRILKWLKKWYKLGK. Omiganan (MBI-226): ILRWPWWPWRRK; Random non-alpha helical peptide: MVVFSVPKFKSTVAKLLSSA.

Bacteriophage Induction

E. coli C600ΔTOX:GFP strain was obtained from Dr. Weiss, University of Cincinnati (Gamage et al., 2003). *E. coli* C600ΔTOX:GFP is a lysogenized C600 strain carrying the 933W bacteriophage in which the stx gene was replaced by the gfp sequence. Since this strain does not produce Shiga toxin, it represents a safety option to evaluate bacteriophage infection. *E. coli* C600ΔTOX:GFP strain was grown in Luria Broth (LB) plus 10 mM $CaCl_2$ and chloramphenicol (Sigma) (15 μg/ml final concentration) overnight (ON) at 37°C under agitation. The ON culture was diluted to OD 600 nm = 0.1 in LB plus 10 mM $CaCl_2$ and chloramphenicol (Sigma) (15 μg/ml final concentration). Induction was carried out by adding ciprofloxacin to a final concentration of 40 ng/ml (Ciprax 200, Roemmers). Bacteria were incubated for 6 h at 37°C under agitation and cultures were then centrifuged at 5,000 rpm for 15 min. The bacteriophage-containing supernatant was purified by sucrose ultracentrifugation. Briefly, supernatant containing bacteriophage was ultracentrifugated at 35.000 × g (Beckman XL-70, Rotor J-20), at 4°C during 2 h with 35% sucrose solution. The pellet was resuspended in 1 ml of PBS, filtered with 0.2 μm filters (MC-PES-02S, Microclar) and kept at 4°C until the titration assay was performed.

Titration Assay

E. coli strain (ATCC 37197) was grown in LB plus ampicillin (0.05 mg/ml final concentration) overnight at 37°C under agitation at 200 rpm. The culture was diluted 1:100 in LB plus ampicillin (0.05 mg/ml final concentration) and incubated for 2 additional hours at 37°C under agitation. At the end of the incubation, 1,000 μl samples of the *E. coli* strain were incubated with 100 μl of a suspension containing bacteriophages for 30 min at room temperature. At the end of this incubation, 3 ml of Top Agar (Tryptone 1%; NaCl 0.5%; Agar 0.7%) plus $CaCl_2$ (10 mM final concentration) was added, and plated on LB-Amp agar plates. Plates were incubated at 37°C and lysis plaques were counted after 24 h.

Anti-Bacteriophage Activity

Bacteriophage ΔTOX:GFP (ϕΔTOX:GFP) was incubated with peptides diluted in 400 μl of PBS at a final concentration of 0.1 μg/ml, 5 μg/ml, 10 μg/ml, or 50 μg /ml for 16 h at 37°C. After incubation, bacteriophage titers were measured as described above. *E. coli* strain (ATCC 37197), used for titration assay, was incubated with peptides alone as a control. During titration assay, the final concentration of peptides in the bacterial suspension was 1.4, 2.8, and 14 μg/ml, respectively.

Z Potential

The Zeta potential measured in volts (ξ) was calculated using the Smoluchowski equation: where η is the viscosity of the suspension at 20°C, D is the dielectric constant of the solution at 20°C and μ is the electrophoretic mobility of particles (micrometer/s per volt/cm). Bacteriophage was incubated with a solution containing peptide 5 or Omiganan at 5.7 μg/ml final concentration for 30 min at 25°C under gentle agitation. Measurements were conducted in a Nano Particle Analyzer SZ100 (Horiba) at 25°C, each value represents the average of two independents batches, and 100 individual determinations were obtained per batch.

Dynamic Light Scattering

Dynamic light scattering experiments were carried out on an Analyzer SZ100 (Horiba) with a backscattering detection at 173°, using disposable polystyrene cells. The bacteriophage suspensions (with or without peptide) were left equilibrating for 15 min at 25°C. Normalized intensity autocorrelation functions were analyzed using the CONTIN method (Provencher, 1982), yielding a distribution of diffusion coefficients (D). D is used to calculate the hydrodynamic diameter (D_H) through the Stokes-Einstein relationship: (3) where k is the Boltzmann constant, T the absolute temperature, and η the viscosity of the medium. The D_H value was calculated from a set of 15 measurements (∼13 runs each) for the bacteriophage in presence of peptide p5 or alone. The D_H of the sample was obtained from the peak with the highest scattered light intensity (i.e., the mode) in light scattering intensity distributions.

Statistical Analysis

The significance of the difference between concentrations was analyzed using Prism 5.0 software (GraphPad Software), and the P-value is indicated by asterisks in the figures. Data correspond to mean ± standard errors of the mean (SEM) for each concentration using triplicates. Statistical differences were determined using the one-way analysis of variance (ANOVA). Comparisons a posteriori between groups were performed using Tukey's Multiple Comparison Test analysis.

RESULTS

Anti-Bacteriophage Activity

Bacteriophages were incubated with 0.1, 5, 10, or 50 μg/ml of each peptide. After incubation, inactivation was evaluated analyzing the bacteriophage capacity to infect *E. coli*. The seven peptides displayed different inactivation activities against the bacteriophage. Peptides 6.2, 5, and 8 showed the highest inactivation activity, inactivating nearly the 100% of phages in the conditions tested (**Figures 1B**, **2A,B**).

On the other hand, Peptides 2 and 8.1 showed the lowest inactivation activity among the cAMP tested (**Figures 1A**, **2C**).

Random peptide and Omiganan showed no inactivation activity against bacteriophage under the conditions tested (**Figure 3**).

It is important to notice that, according to the protocol we used, after the incubation of each peptide with phages, the peptide-phage mixture is plated on the *E. coli* lawn. For that reason a possible antimicrobial activity of these cAMPS on this *E. coli* strain had to be previously assessed, and no visible activity of the peptides *per se* was observed. It is worth mentioning that the final peptide concentration affecting the bacterial lawn during the titration assay ranged from1.4 to 14 μg/ml, which is a much lower concentration than the effective minimal inhibitory activity (MIC) previously obtained for each peptide on *E. coli*. In addition we performed a MIC assay for these peptides at different concentrations between 54 and 3.3 μg/ml on the *E. coli* C600ΔTOX:GFP strain, and we found no antimicrobial activity below 27 μg/ml for all the peptides tested.

Bacteriophage-Peptide Interaction

The interaction between peptides and bacteriophages was analyzed using Zeta potential assays. We observed that peptide 5 was able to modify the surface charge of the bacteriophages, by decreasing the net negatively charge exposed by the bacteriophage. The results strongly suggest that positively charged peptides and bacteriophages are interacting through electrostatic charges (**Figure 4**). Interestingly, the same behavior was found with peptide Omiganan, although this peptide did not show anti-bacteriophage activity.

We used DLS to assess whether the size of the bacteriophage was altered as a consequence of its interaction with the peptides, or inducing any aggregation effect. The results suggest that peptides do not affect the hydrodynamic diameter of the bacteriophage (**Figure 5**).

DISCUSSION

It is well known that bacteriophage induction is required for HUS development (Tyler et al., 2013). Additionally, it was also reported that bacteriophage was able to infect bacteria *in vivo* and *in vitro* (Schmidt, 2001; Cornick et al., 2006).

We have previously observed a dramatical decrease of GFP expression *in vivo* in mice treated with chitosan after infection with a non-pathogenic *E. coli* strain containing a mutant bacteriophage in which the gene of *stx* was replaced by *gfp* sequence (Amorim et al., 2014).

In order to evaluate another anti-bacteriophage agent, we analyzed a group of cationic peptides. In this work, and for the first time ever reported, we observed that seven cAMPs were able to inactivate bacteriophage infection on bacteria. It is worth mentioning that in previous works we observed that all the peptides, except random peptide, displayed antimicrobial activity (Faccone et al., 2014; Hollmann et al., 2016; Maturana et al., 2017). Interestingly, even though they are both cationic sequences, Omiganan and random peptide did not display any bacteriophage inactivation activity. In regard to the structure, unlike the rest of the sequences tested, these two peptides did not structure as alpha helix in contact with lipidic membranes (Faccone et al., 2014).

It is interesting to note that for peptides 2 and 6.2 we observed a bell curve shaped behavior of the phage neutralizing activity vs. concentration curve. This kind of behavior is more or less common among some drugs, and in this case we could speculate that the tendency to aggregate that these particular peptides have at high doses, due to the high number of hydrophobic amino acids they harbor, could be responsible, at least in part, for this phenomenon. This tendency to aggregate, which depends on the amino acid composition and the structure the peptide sequence displays, is probably responsible for the inactivation of the peptide as we increase the concentration and more aggregates are formed.

In order to evaluate if a direct interaction between peptides and bacteriophage is involved in the inactivation activity, zeta potential experiments were conducted, using peptide 5 as a model. The result obtained (**Figure 4**) confirmed an electrostatic interaction between peptide and bacteriophage.

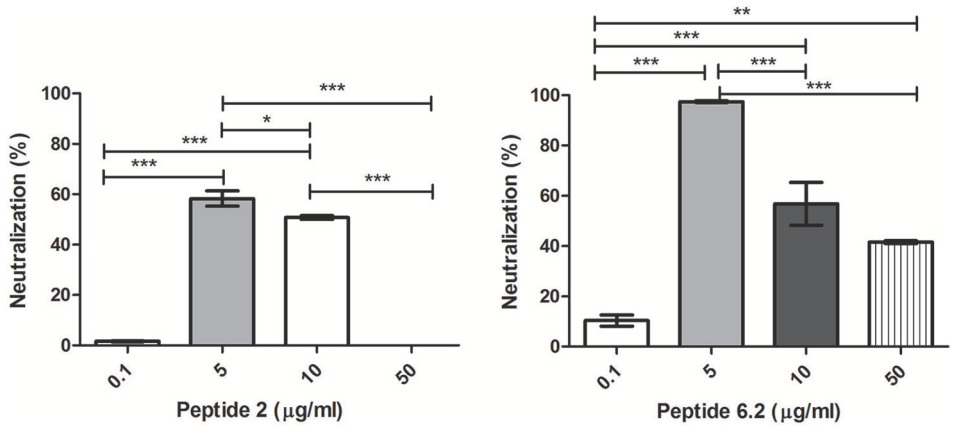

FIGURE 1 | Bacteriophage inactivation by cationic peptides. **(A)** Peptide P.2. **(B)** Peptide 6.2. After pre-incubation of bacteriophages with different concentrations of peptides the inactivation was measured by titration of *E. coli*, strain (ATCC 37197). Prism 5.0 software (GraphPad Software) was used to determine statistical significance between different samples. Peptide 2: $^{*}p < 0.05$, $^{***}p < 0.0001$. Peptide 6.2: $^{**}p < 0.05$, $^{***}p < 0.0001$.

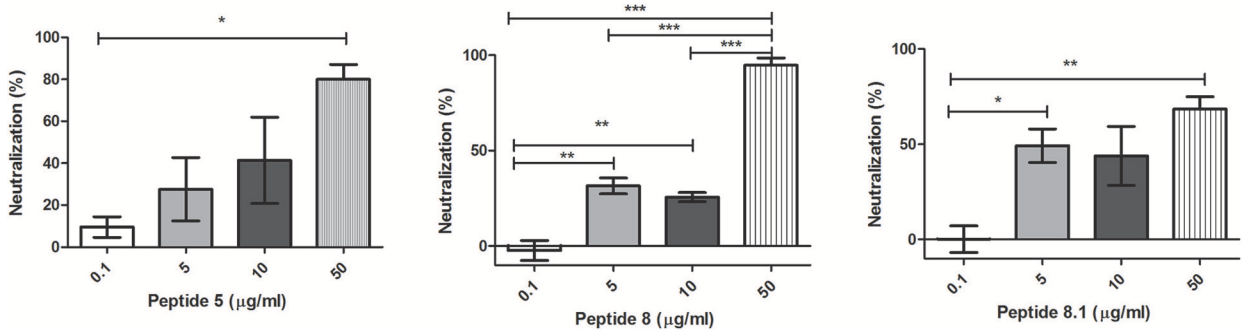

FIGURE 2 | Bacteriophage inactivation by cationic peptides. **(A)** Peptide 5 **(B)** Peptide 8 **(C)** Peptide 8.1. After pre-incubation of bacteriophages with different concentrations of peptides the inactivation was measured by titration of *E. coli*, strain (ATCC 37197). Prism 5.0 software (GraphPad Software) was used to determine statistical significance between different samples using one-way analysis of variance (ANOVA). Peptide 5: $^{*}p = 0.0313$. Peptide 8: $^{**}p < 0.005$, $^{***}p < 0.0001$. Peptide 8.1: $^{*}p < 0.05$, $^{**}p = 0.008$.

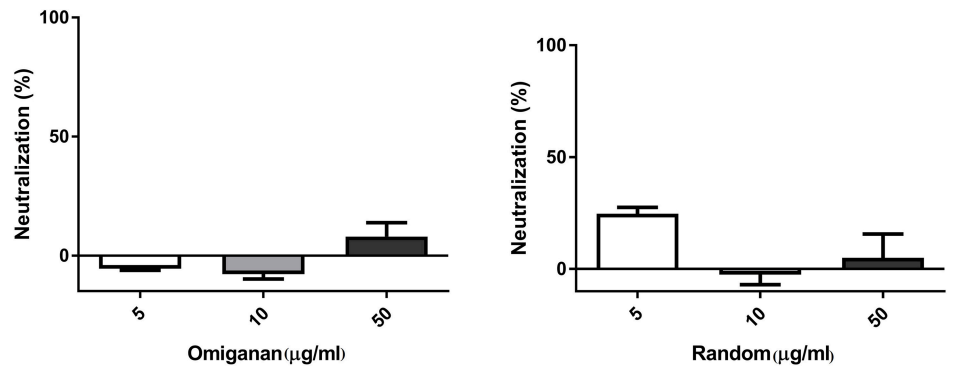

FIGURE 3 | Bacteriophage inactivation by control peptides. **(A)** Omiganan, a commercial cationic beta sheet peptide **(B)** Random sequence, a cationic non alpha helical peptide. Prism 5.0 software (GraphPad Software) was used to determine statistical significance between different samples using one-way analysis of variance (ANOVA). Omiganan: ns. Random: ns.

However, Omiganan, that displayed no inactivation activity, also showed direct interaction with the bacteriophage. These findings allow us to hypothesize that cationic properties, that are probably responsible for the interaction between peptides and bacteriophage, are necessary but not sufficient for achieving an inactivation activity.

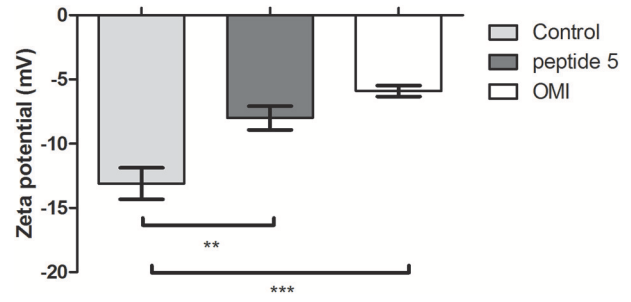

FIGURE 4 | Zeta potential measurements of bacteriophage incubated with peptides P5 or Omiganan (OMI), or in buffer solution (control). Each point represents the mean of 2 independent batches ± SEM. $^{**}P < 0.01$; $^{***}P < 0.001$, one-way ANNOVA followed by a Dunnett post-test for multiple comparisons vs. the control.

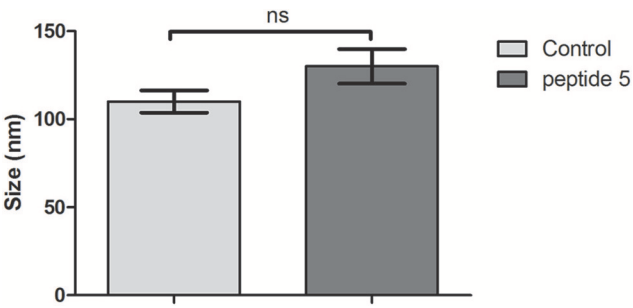

FIGURE 5 | Average hydrodynamic diameter of bacteriophage incubated with peptides 5 or in buffer solution (control). Ns, Not significant, *t*-test (unpaired).

Overall, our results lead us to hypothesize that specific interaction between cAMPs and bacteriophage proteins, after a first approach driven by electrostatic force, might be responsible for infection inhibition. We hypothesize that the complex generated after bacteriophage-peptide incubation could be responsible for preventing the bacteriophage adhesion on the bacterial cell wall (**Figure 6**). Further experiments analyzing bacteriophage proteins implicated on bacterial adhesion should be performed.

In the race to find a therapy to decrease the risk of HUS development, bacteriophage inactivation by cAMPs could be a promising new strategy for the inhibition of the bacteriophage replication and Stx expression. These peptides could be considered a new family of molecules potentially useful for a future HUS treatment.

The results obtained in this work, using novel designed cAMPS, open another relevant area of study related with the interactions between natural immune cAMPs and bacteriophages, for instance cAMPS produced by human leukocytes at the site of infection. If these results could be replicated with human cAMPs, a whole new perspective of SUH development could arise, in which human cationic peptides could

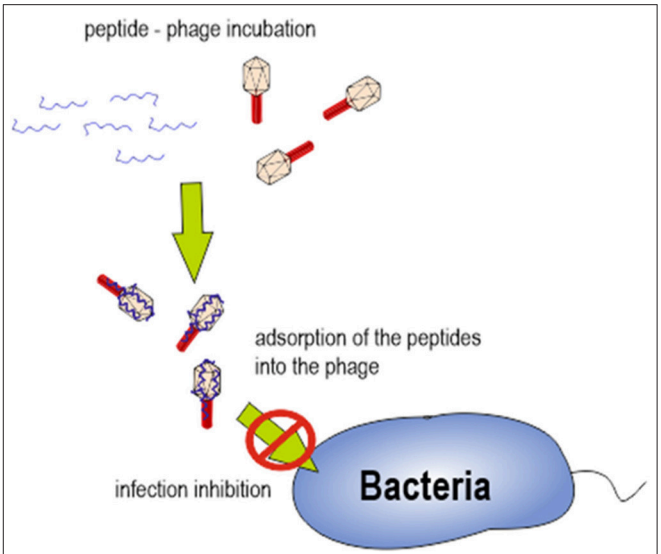

FIGURE 6 | Schematic representation of bacteriophage-peptide interaction and its role in bacterial infection.

play a crucial role in the natural control of phage replication. Altogether, these results highlight that cationic peptides are potential candidates for future research in alternative treatments for STEC infections.

AUTHOR CONTRIBUTIONS

LB designed, analyzed data and wrote the manuscript; PM provided advice on experimental design, analyzed data and wrote the manuscript; MD performed experiments and provided advice on experimental design, LS and PG provided substantial comments on the manuscript and on experimental design. MM and AH performed experiments and editing on the manuscript.

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Racing on the Wrong Track

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The preclinical *in vitro* and *in vivo* benchmark figures of cationic antimicrobial peptides have to be revisited based on the newly discovered alternative modes of action.

Keywords: immunostimulation, protein folding, protein synthesis inhibitor, resistance, toxicity

My Pubmed search of the keywords "antimicrobial peptide efficacy mouse" yielded >1,000 citations. Even when I included the further restrictive keywords "systemic infection" that should exclude cutaneous infection models, I still found almost 200 papers. These came from tens of laboratories all focusing on antimicrobial peptide efficacy in mouse models targeting Gram-negative and Gram-positive pathogens, fungi, as well as sepsis, and toxin models. In other words, essentially all aspects of clinical microbiology with the prevailing publication bias already understood, most reporting positive results. Given this robust preclinical interest by the research community one would think that development efforts would be a natural consequence, with clinical trials logically proceeding, in a pace faster than that we saw at the Kentucky Derby, in order to be the first antimicrobial peptide to reach NDA approval in the twenty-first century and establish market dominance. This is not the case, however. As of February, 2015 not a single clinical trial of an antimicrobial peptide against sepsis was registered (Martin et al., 2015), and, other than the decades-old polymyxins, no peptide is on the horizon against bacteremia. In fact, as a research community, we have started to unearth many roadblocks that concern clinical trialists. The more we characterize the mode of action, toxicity, resistance induction, pharmacokinetics and pharmacodynamics, the more exciting questions emerge to which we have little answer, if any at all.

Antimicrobial peptides (or as recently called host defense peptides) have reached NDA approval and late clinical trial stage against nail and skin conditions (Rabanal and Cajal, 2016). One of the reasons is clearly the role of host defense peptides in cutaneous biology and wound healing (Otvos and Ostorhazi, 2015, *vide infra*). Topical treatment therapy can mask systemic toxicity concerns (Bush et al., 2004), although as we very recently documented, cationic host defense peptides can enter the circulation after application to undamaged skin (Ostorhazi et al., 2017).

From the get-go, antimicrobial compounds are evaluated based on their ability to kill various bacterial strains. The desired *in vitro* minimal inhibitory concentration (MIC) threshold values of antimicrobials are strain-dependent and vary based dosage, pharmacokinetics and pharmacodynamics, just mention a few, as well as occasionally they differ in the USA and in Europe (Rodloff et al., 2008). Yet, it is safe to say that regulatory agencies expect MIC values below 2 mg/L (except against very hard to kill bacteria) measured under standard conditions, developed for small molecule drug screening. Almost all native and most designer antimicrobial peptides simply cannot do this. To explain why native peptides can protect insects and other animals from bacterial infection and to convince the industry that we are on to something good, we developed special low salt media in which peptide antibiotics perform better. Indeed, the *in vivo* microenvironment of bacterial growth might be completely different from that in Muller-Hinton broth. Nevertheless, the classic dogma says that cationic antimicrobial peptides kill bacteria by depolarizing of or simply by punching holes in the negatively charged bacterial membrane surface (Yang et al., 2001) and thus low ionic strength can not only influence the efficacy of membrane assembly, but also potentate ionic interactions. In any event, for regulatory approval peptide-friendly media have to be replaced with standard media.

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To demonstrate that antimicrobial peptides are as worthy as standard small molecules, we are racing to continuously improve the MIC figures; for cationic peptides this means enhancing a peptide's ability to destroy bacterial cell membranes. To continue the Kentucky Derby analogy, we might be racing on the wrong track. What if the cationic side-chains are just to promote entry into bacterial and host cells? Perhaps antimicrobial peptides have separate domains, one to penetrate cells and another to bind their intracellular target(s); the *in vitro* measure of an MIC would not be reflective of the actual mechanism of action and may falsely be read as activity in the micromolar (cell penetration) concentration rather than the pico- or nanomolar (intracellular targeting) concentration? In this case, we are quantifying something that is completely independent of the particular peptide, and cannot differentiate among peptides that inhibit bacterial nucleic acid or protein synthesis (Hale and Hancock, 2007; Krizsan et al., 2015), protein folding (Kragol et al., 2001) or lipid complexation (McCafferty et al., 1999) just to mention a few non-membrane related but still bacteria-related activities. Then we have to select prudent and universally acceptable measures and benchmarks for alternative modes of action. But if we want to accurately measure the extent of intracellular actions, our assays have little to do with whole bacterial cell survival or proliferation inhibition.

Up to this point, our working hypothesis has been that antimicrobial peptides inactivate something in bacteria. An ever-increasing body of evidence indicates however, that *in vivo*, antimicrobial peptides have stronger effects on host functions rather than bacterial survival with a primary mode host protection grounded in innate immunity activation, at least for peptides close to or under clinical development (Lai and Gallo, 2009; Brandenburg et al., 2012; Hilchie et al., 2013). Perhaps the best example is the remarkable efficacy of the peptide dimer A3-APO and its monomeric metabolite in several mouse infection models when the peptides have very limited bactericidal activity against *Staphylococcus aureus* or *Proteus mirabilis* strains *in vitro*, but significantly improve survival as well as reduce bacterial counts *in vivo* at the infection sites and in the circulation (Ostorhazi et al., 2011). Host responses to cationic peptides include complex immunomodulatory actions (Upton et al., 2012) such as immunostimulation (Wakabayashi et al., 2003), specifically macrophage activation (Welkos et al., 2011), chemotaxis (Radek and Gallo, 2010), or upregulation of anti- or pro-inflammatory cytokine production (Capparelli et al., 2012; Tang et al., 2014). Activation of angiogenesis and other processes instrumental to tissue repair represent the cornerstones of host defense peptide, as adjuvant, use in cutaneous conditions and wound healing with or without bacterial infection (Elsbach, 2003; Bardan et al., 2004; Ostorhazi et al., 2010). Showing efficacy of any given peptide *in vitro*, especially superiority to earlier analogs, requires comparison of a wide range of immune activities, much like comparing apples and oranges; certainly not MICs. *In vivo* we have to resort to improvement of survival and reduction of bacterial loads. Perhaps an accurate measure of pro- or anti-inflammatory cytokine production upon host-defense peptide administration *in vivo* can identify truly outstanding clinical peptide candidates. Luckily a wide range of easy to use ELISA kits are commercially available for this purpose.

The saving grace is that inhibition of bacterial protein folding can attenuate a major bacteria-related health concern, that is, activation of proteinaceous toxin production. A series of bacterial strains express life-threatening polyamide toxins, such as *S. aureus* (α-hemolysin, Berube and Bubeck Wardenburg, 2013), *Clostridium perfringens* (enterotoxin, Freedman et al., 2016), and *Burkholderia pseudomallei* (lethal factor, Cruz-Migoni et al., 2011). Our proline-rich antibacterial peptide dimer A3-APO inhibits *Bacillus cereus* enterotoxin production and *Bacillus anthracis* replication *in vitro*, and statistically significantly delays lethal toxin-induced mortality in a mouse model of anthrax (Otvos et al., 2014a). Luckily, highly accurate bacterial toxin detecting and quantifying kits are commercially available for many of the potential indications and the use of these kits can accelerate drug development. Worth noting, bacteria may still survive upon host defense peptide treatment but they would be unable to produce active proteinaceous toxins.

Along these lines, not only protein-based toxins can be inhibited but any bacterial enzyme that is resistant to conventional antibiotics. Proline-arginine rich antimicrobial peptides can recover the lost activity of legacy antibiotics including β-lactams, chloramphenicol, sulfonamides, or trimethoprim against multidrug resistant strains by inactivating the enzymes that provide resistance against the small molecule antibiotics (Cassone et al., 2008). Antimicrobial peptides may also potentate the *in vivo* effect of legacy antimicrobials through anti-inflammatory effects during classical antimicrobial chemotherapy (Li et al., 2014). Alternatively, synergy *in vivo* may arise between α-helical peptides (Cirioni et al., 2008) or peptides and other antibiotics (Hu et al., 2015) acting on cell wall synthesis through increased drug concentration locally on the target bacterial structures.

One of the perennial arguments for the use of antimicrobial peptides is the lack of resistance induction. This is based on *in vitro* assays in which bacteria are repeatedly incubated with sub-MIC concentrations of antibiotics and then changes in the MIC values are determined after 15–20 passages. This strictly microbiology measure can indeed be useful if the mode of action is only membrane disruption. Host defense peptide resistance is clearly dependent upon membrane activity (Tzeng et al., 2005; Kindrachuk et al., 2007). However, for peptides with alternative modes of action, the sublethal passage assay has little positive predictive value. The major microbiological difference between A3-APO and its monomeric analog, Chex1-Arg20, is the improved membrane-disruptive activity of the dimeric prodrug. Only the monomer induces microbiological resistance, and only against one strain. The intracellular target, however, DnaK, remains preserved after multiple passages with no genetic alterations; the DnaK multihelical lid region, where the peptides bind and the putative transport protein SbmA are unchanged (Cassone et al., 2009). More concerning, *in vitro S. aureus* develops resistance to magainin, with cross-resistance to human-neutrophil-defensin-1, a key component of the innate immune system (Habets and Brockhurst, 2012) projecting potential risks of host defense peptide therapies.

Finally, investigational new drug applications (IND) typically require pharmacokinetic parameters as detailed in published FDA Guidance documents. These parameters, however, may be problematic for antimicrobial peptides. In the classical view, to protect mammals from bacteremia, we need to maintain a sustained (multiple hours) circulating antimicrobial concentration of 1.3 × MIC (Otvos et al., 2005); this may not, as currently measured, be supportive of an antimicrobial peptide IND application. First, positively charged antimicrobial peptides avidly bind negatively charged components of not only bacteria, but also the mammalian body, including serum albumin. To measure the blood level of both bound and free antimicrobials, special chromatography/mass spectroscopy protocols should be used (Schmidt et al., 2016). Second, for alternative modes of action, e.g., to trigger a host immune response, the required circulation levels can be 1,000 times less than that for bactericidal activity and frequently below current detection limits (Otvos et al., 2014b). Third, when peptides bind their targets, the ligand residence time is very long, and the targets remain engaged considerably longer than the time period of typical renal elimination. For peptide drugs, pharmacodynamics (what the drug does to the body) is a more practical measure of biological activity than pharmacokinetics (what the body does to the drug, Otvos and Wade, 2014).

So my fellow host defense peptide riders please take off the blinkers and ride your horses on the correct tracks, sometimes not frequented by other contestants, to win the race. For one, I have not bet for the favorite at the Preakness.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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