Human skin equivalents to study the prevention and treatment of wound infections

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CHAPTER 4

Next generation peptides based on P60.4AC, a synthetic derivative of the human cathelicidin LL-37, are highly effective against extensively and pandrug-resistant bacteria

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Abstract

Background. Extensively- and pandrug-resistant (XDR and PDR) bacterial strains are associated with high morbidity and mortality as treatment options are limited. This stresses the need to develop novel antimicrobial agents. Antimicrobial peptides (AMPs) are considered promising candidates. In this study, we assessed the efficacy of synthetic AMPs based on P60.4AC, a derivative of the human AMP LL-37, against XDR and PDR isolates *in vitro* and *in vivo*.

Methods. The *in vitro* bactericidal activity of four AMPs was determined against three epidemic XDR strains of *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, against one XDR *K. pneumonia* isolate from a young patient that led to the rejection of donor organs and one *P. aeruginosa* isolate from a patient with a lethal necrotizing pancreatitis. The efficacy of these peptides against the PDR *P. aeruginosa* strain was assessed using a three-dimensional wounded human skin model *in vitro*.

Findings. P60.4AC-derived AMPs P10, P145, P148 and P276 were highly effective against all strains *in vitro*, killing >99.9% of bacteria at low micromolar concentrations. At 12.5-50 nmol/model, these peptides eradicated 74.4-99.5% of the PDR *P. aeruginosa* strain from wounded human skin models. At these amounts, the peptides were not toxic to the human cells.

Interpretation. Four synthetic AMPs killed XDR and PDR strains *in vitro*. These peptides successfully eradicated a PDR *P. aeruginosa* strain from three-dimensional wounded human skin models. We consider these peptides as attractive candidates for further development as novel agents in the fight against infections with resistant bacteria.

Introduction

A post-antibiotic era, in which common infections can kill substantial numbers of patients, might be a realistic scenario for the 21st century. The WHO's 2014 report on global surveillance of antibiotic resistance reveals that an increasing number of nosocomial infections is caused by multidrug-resistant (MDR) bacteria [1]. In the US alone over 2 million people get infected with MDR bacteria annually, which is associated with high morbidity, mortality [2] and up to 30% increased hospital costs [3]. Strains of *Enterococcus faecium*, *Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species (the so-called ESKAPE pathogens[4]) currently cause the majority of US hospital infections [5]. The emergence of extensively drug-resistant (XDR) and pandrug-resistant (PDR) pathogens, accelerated by the selective pressure exerted by extensive use and misuse of antimicrobials, has become a serious concern as treatment options become increasingly limited. This underscores the very pressing need for the discovery of novel treatment strategies to replace or complement the use of known antibiotics.

We consider naturally occurring human antimicrobial peptides (AMPs) as excellent templates for the development of novel synthetic antimicrobials that overcome the threats mentioned above. AMPs are mostly positively charged, amphipathic peptides that display antimicrobial activity against a wide variety of pathogens, including bacteria, fungi and viruses. In addition, many of them have immune-modulatory and wound healing activities [6]. Several AMPs have been tested in clinical trials, including the human cathelicidin LL-37 [7]. LL-37 is an α-helical amphipathic AMP with antimicrobial activity against *S. aureus* and *Escherichia coli* among others [8]. We have previously shown that the LL-37-derived synthetic peptide P60.4AC, also named OP145 [9], has bactericidal activity against *S. aureus* - including antibiotic-resistant strains - both *in vitro* and on colonized human skin equivalents [10]. Moreover, P60.4AC proved to be safe and successful in the treatment of patients with chronic otitis media that was resistant to conventional antibiotics [11].

In the present study, we assessed the *in vitro* efficacy of synthetic P60.4AC-derived AMPs against XDR and PDR isolates of *P. aeruginosa*, *K. pneumoniae* and *S. aureus* that had a major impact from a public health and individual patient care perspective. In addition, we used a three-dimensional *in vitro* wounded human skin infection model to determine the efficacy of these peptides against a PDR *P. aeruginosa* strain, which was isolated from a patient with a fatal necrotizing pancreatitis.

Methods

Peptides

N-terminal amidated and C-terminal acetylated synthetic peptides (shown in Table 1) were prepared by normal 9H-fluorenylmethyloxycarbonyl (Fmoc)-chemistry as described previously [12]. Couplings were performed for 60 min with 6-fold acylating species. After final Fmoc removal, peptides were cleaved with trifluoro acetic acid/H_2O 19/1 (v/v) containing ethanethiol for W-containing peptides. Peptides were isolated by ether/pentane 1/1 (v/v) precipitation and subsequent centrifugation. After air-drying at approximately 40°C, peptides were dissolved in acetic acid/water 1/10 (v/v) and lyophilized. Peptides were checked for purity and integrity using UPLC-MS and Maldi-Tof mass spectrometry, showing more than 90% purity and the expected molecular masses. Peptide stocks of 5.12 mM were prepared in 0.01% acetic acid or in phosphate buffered saline (PBS, pH 7.4) and stored in aliquots at -20°C. Peptides used in this study are given in table 1.

Table 1: N-terminal amidated and C-terminal acetylated synthetic peptides used in this study.

Peptide																							
P60.4Ac	I	G	K	E	F	K	R	I	V	E	R	I	K	R	F	L	R	E	L	V	R	P	L	R
P10	L	A	R	E	Y	K	K	I	V	E	K	L	K	R	W	L	R	Q	V	L	R	T	L	R
P145	L	K	R	L	Y	K	R	L	A	K	L	I	K	R	L	Y	R	Y	L	K	K	P	V	R
P148	L	K	R	V	W	K	R	V	F	K	L	L	K	R	Y	W	R	Q	L	K	K	P	V	R
P276	L	K	R	V	W	K	A	V	F	K	L	L	K	R	Y	W	R	Q	L	K	K	P	V	R

Clinical background of bacterial isolates

Clinical isolates of *P. aeruginosa*, *K. pneumoniae* and MRSA were selected that have (had) major impact on the individual-patient level or on a hospital-wide and national level. *P. aeruginosa* LUH15100 was isolated from a 69-year old male transferred to the Intensive Care Unit (ICU) of the Leiden University Medical Center because of severe necrotizing pancreatitis. He had been admitted to an affiliated hospital one month earlier, where he had received broad spectrum antibiotics (meropenem, vancomycin and caspofungin) as treatment for ventilator-associated pneumonia and fecal peritonitis, secondary to necrotizing pancreatitis. Furthermore, he was treated with prophylactic selective decontamination of the digestive tract consisting of intravenous ceftriaxon and oral administration of a solution containing colistin, tobramycin, and amphotericin B, via nasogastric tube and a paste in the oropharynx. Because of extensive ischemic colitis, a total colectomy was performed, after which the abdomen was left open and managed with intra-

abdominal vacuum-assisted closure and daily lavage. *P. aeruginosa* was isolated from sputum. Cultures of blood, abdominal pus and rectal swab were also positive for *P. aeruginosa*. The patient died ten days later from septic shock and multi-organ failure. Post mortem cultures of lung, liver and spleen tissue revealed the presence of *P. aeruginosa*, susceptibility testing on this strain was not performed. An Ambler class A carbapenemase producing *K. pneumoniae* (KPC, LUH15105) was isolated from a previously healthy 19-year old female, who died in the ICU of the University Hospital in Liege, Belgium because of cerebral haemorrhage. Consent for post mortem organ donation was obtained and the kidneys were offered for transplantation through Eurotransplant. Unfortunately, the culture taken from a urinary catheter in this patient was positive for KPC (>10^6 CFU/ml), which was MDR. As a consequence, the kidneys were considered unsuitable for transplantation by all consulted transplant centers. *P. aeruginosa* strain LUH15103 caused prolonged outbreaks in the ICU of the Leiden University Medical Center and another tertiary care hospital in the Netherlands [13,14]. This strain produced VIM-2 metallo-β-lactamase. *K. pneumoniae* strain LUH15102 producing OXA-48 oxacillinase and CTX-M-1 group extended-spectrum beta-lactamase has been responsible for a large outbreak in a secondary care hospital in the Netherlands [15]. MRSA strain LUH15093 belongs to clone USA300, which has emerged as the predominant MRSA strain in the US, reaching epidemic proportions [16].

Culture of microorganisms

Bacteria were preserved for prolonged periods in nutrient broth supplemented with 20% (v/v) glycerol at -80°C. Prior to each experiment, inocula from frozen cultures were grown overnight at 37°C on sheep blood agar plates. For experiments, fresh subcultures were made in tryptic soy broth (for MRSA) or brain heart infusion broth (for *P. aeruginosa* and *K. pneumoniae*).

Antibiotic susceptibility testing

Susceptibility to antibiotics was determined using the Vitek2 system (Biomerieux).

Antimicrobial activity *in vitro*

Bacteria were cultured to mid-logarithmic phase at 37°C under vigorous shaking and washed once with PBS. The bacterial suspension was diluted in PBS to 5 x 10^6 CFU/ml, as calculated from the absorbance of the suspension at 600 nm. In wells of a 96-wells polypropylene plate, 20 µl of bacterial suspension were added to 80 µl of peptide solution (with final concentrations ranging from 1.6-204.8 µM) in PBS or 50% PBS and 50% human plasma. As untreated control, bacteria were exposed to PBS without peptides. Two hours after incubation at 37°C under

shaking conditions, the number of viable cells was determined by plating samples on diagnostic sensitivity test (DST) agar plates. Antimicrobial activity is expressed as the 99.9% lethal concentration (LC99.9), i.e. the lowest peptide concentration that killed ≥99.9% of bacteria during two hours of incubation. The lower limit of detection was 50 CFU/ml.

Culture and wounding of human skin models

All primary human skin cells were isolated from surplus tissue collected according to article 467 of the Dutch Law on Medical Treatment Agreement and the Code for proper Use of Human Tissue of the Dutch Federation of Biomedical Scientific Societies. None of the authors were involved in the tissue sampling and only birth date, gender and skin type of the subjects was known. The Declaration of Helsinki principles were followed when working with human tissue. Epidermal skin models [10,17] and three-dimensional human skin equivalents (HSEs) [10] were prepared as described before. Normal human keratinocytes and fibroblasts were isolated from fresh human mamma or abdominal surplus skin. For epidermal skin models, approximately 2 x 10^5 human keratinocytes were cultured on filter inserts. After three days, cultures were lifted to the air-liquid interface and cultured for ten days at 37°C and 7.3% CO_2. Prior to the cell toxicity assay, the keratinocyte medium was replaced by keratinocyte medium without phenol red. For HSEs, rat-tail collagen was mixed with 1.25 x 10^5 fibroblasts and cultured onto a filter insert. After two days culture at 37°C and 7.3% CO_2, 5 x 10^5 keratinocytes were seeded on top of the collagen layer. The cells were cultured under submerged conditions for two days, after which the HSEs were lifted to the air-liquid interface and cultured for an additional ten days. Next, HSEs were thermally wounded as described earlier [10]. In brief, wounds were created using a 2 by 10 mm blunt metal bar, which was placed in liquid nitrogen for two minutes. Immediately thereafter, the metal bar was applied onto the HSEs for 15 sec without any pressure.

Antimicrobial activity against PDR P. aeruginosa on HSEs

One h after wounding, HSEs were inoculated with 50 µl of a mid-logarithmic culture of *P. aeruginosa* LUH15100, resulting in 2-5 x 10^4 CFU/HSE. After one h incubation at 37°C and 7.3% CO_2, the non-adherent and loosely adherent bacteria were removed by aspiration and the adherent bacteria were exposed to 50 µl of PBS containing peptide or ceftazidime (ranging from 12.5-50 nmol/HSE), or, as control, 50 µl of PBS without peptide/antibiotic for four h. In some experiments, treatment was repeated two hours after the first treatment. Thereafter, the number of viable bacteria/HSE was assessed. For this purpose, HSEs were homogenized in PBS using soft-tissue homogenizing beads by 3 x 30 s bead beating in a tissue

homogenizer. Ten-fold serial dilutions of the homogenates were plated onto DST plates. The lower limit of detection was 50 CFU/HSE.

Toxicity

One hundred microliters of peptide solution (ranging from 12.5-200 nmol/model) were applied apically on the epidermal skin models. As negative and positive control, models were exposed to 100 µl of PBS and 0.5% Triton-X, respectively. After four and 24 h incubation at 37°C and 7.3% CO_2, the level of lactate dehydrogenase (LDH) in the medium was determined using the Cytotoxicity Detection Kit (Roche), according to the manufacturer's instructions. The optical density at 490 nm (OD_{490}) was determined as a measure of LDH release, which corresponds to the level of cell lysis. Cell toxicity is expressed as the percentage of cell lysis relative to the positive control.

Statistical analysis

The ANOVA one-way analysis of variance and the Student's T-test were used to evaluate differences in distribution. Analyses were performed using Graphpad Prism 6.0. P-values of ≤0.05 were considered significant.

Results

Bacterial isolates

Clinical isolates of *P. aeruginosa*, *K. pneumoniae* and MRSA that have (had) major impact on the individual-patient level or on a hospital-wide and national level were selected for the current study. Table 2 shows the resistance profiles. MRSA USE300 (LUH15093) strain is considered to be very virulent [18], strains LUH15102, LUH15103 and LUH15105 were considered to be XDR. *P. aeruginosa* LUH15100, which caused a fatal pancreatitis, was XDR. Official definition of PDR and XDR in [19].

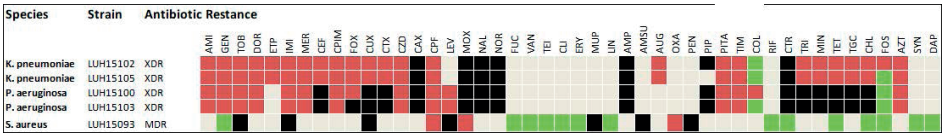

Table 2. Used clinical isolates are multiple or extensively drug resistant. Green and red boxes indicate that the isolate is susceptible or non-susceptible, respectively, to the antibiotic according to CLSI and EUCAST breakpoints (van der Bij et al. 2011). Gray boxes are shown if the susceptibility to agents in this class is not assessed. Black boxes indicate that the antibiotic is not relevant for that species.

Antimicrobial activity against XDR and PDR strains in vitro

In our laboratory, we have designed novel peptides based on the LL-37-derived peptide P60.4AC with improved antibacterial activities compared to P60.4AC, as shown by LC99.9 concentrations in table 2 and in [10] (A. de Breij et al, unpublished data). We assessed the antimicrobial activity of P60.4Ac, P10, P145, P148 and P276 against the strains described above. The peptides, except P60.4Ac, were highly effective against all strains *in vitro*, killing >99.9% of bacteria at concentrations of 1 - 6 µM (Table 3). The peptide concentrations that resulted in complete eradication of all viable bacteria, i.e. 100% killing, were only 1-1.5-fold higher than the LC99.9 values (data not shown). However, when tested in the presence of 50% plasma the effectivity of the peptides greatly diminished (with a median of 4-34 fold decrease in effectivity). In the next experiments we excluded P60.4Ac, due to the lower antimicrobial activity compared to the other peptides.

Table 3. Bactericidal activity of LL-37-derived AMPs against XDR and PDR strains.

A	**LC99.9 (µM)**					
Peptide	***P. aeruginosa***		***K. pneumoniae***			**MRSA**
	LUH15100	**LUH15103**	**LUH15102**	**LUH15104**	**LUH15105**	**LUH15093**
P60.4Ac	NT	25,6	12,8	25,6	6,4	6,4
P10	2.7 ± 0.9	6,4	4.3 ± 1.8	6,4	4.8 ± 2.3	3.7 ± 2.4
P145	1.3 ± 0.5	5.3 ± 1.8	3.7 ± 2.4	4.8 ± 2.8	2.7 ± 0.9	2.1 ± 0.9
P148	1.3 ± 0.5	5.3 ± 1.8	2.1 ± 0.9	5.3 ± 1.8	3,2	2.1 ± 0.9
P276	1.1 ± 0.5	4.3 ± 1.8	2.1 ± 0.9	2.7 ± 0.9	2.1 ± 0.9	1,6

B	**LC99.9 (µM) in 50% plasma**					
Peptide	***P. aeruginosa***		***K. pneumoniae***			**MRSA**
	LUH15100	**LUH15103**	**LUH15102**	**LUH15104**	**LUH15105**	**LUH15093**
P60.4Ac	NT	>204,8	204,8	>204,8	>204,8	>204,8
P10	>205	205	171 ± 59	NT	205	154 ± 89
P145	51	26	13	NT	26 ± 22	21 ± 7
P148	26	17 ± 7	13	NT	17 ± 7	13
P276	171 ± 59	85 ± 30	43 ± 15	NT	30 ± 20	13

A. LC99.9, lethal concentration 99.9%, i.e. the lowest peptide concentration that resulted in >99.9% killing of bacteria, when tested in PBS. **B.** LC99.9 when tested in 50% plasma. concentrations given Results are means ± standard deviations of three experiments. If no standard deviation is shown, the LC99.9 was identical in the three experiments. NT: not tested.

Antimicrobial activity against PDR *P. aeruginosa* on human skin models

We recently developed a three-dimensional wounded human skin infection model [10,20], which mimics the wounded human skin to a high degree and can therefore be used to study wound infection and the efficacy of treatments. The antimicrobial activity of the P60.4AC-derived AMPs against the PDR *P. aeruginosa* LUH15100 was assessed in this model. In PBS-exposed skin models, *P. aeruginosa* could multiply in four h to 2 x 10^5 ± 7 x 10^4 CFU/model. Treatment with P10, P145, P148 and P276 for 4 hrs resulted in a dose-dependent killing of *P. aeruginosa*. The number of bacteria on the skin was 86.7 - 98.8% lower after treatment with 12.5 nmol of these peptides as compared to the PBS-treated skin. Treatment with 25 and 50 nmol of these AMPs resulted in 98.0 - 99.9% and 97.5 - 99.8% reduction in the number of *P. aeruginosa*, respectively (Figure 1). Treatment with P148 resulted in the largest reduction in the number of bacteria on the skin. A second treatment two hours after the first treatment did not significantly improve the efficacy of the peptides (data not shown). As expected, exposure of this PDR strain to clinically acceptable doses of ceftazidime were not effective, although this antibiotic in equimolar concentrations (corresponding to 137-547 µg/ml), resulted in >90% lower numbers of *P. aeruginosa* than in the PBS-exposed skin models (data not shown).

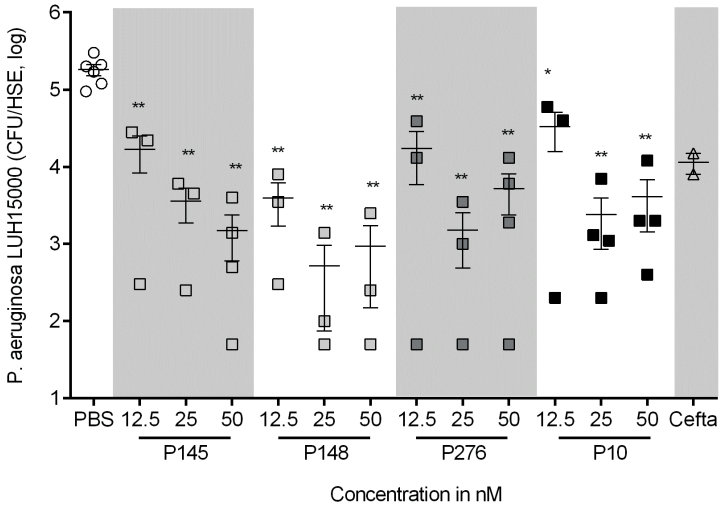

Figure 1. P60.4AC-derived AMPs eradicate PDR *P. aeruginosa* from (thermally) wounded human skin models. Wounded human skin equivalents (HSE) were inoculated with 2-5 x 10^4 CFU of *P. aeruginosa* LUH15100. Adherent bacteria were exposed to 0-50 nmol of P10, P145, P148, P276 in PBS or ceftazidime (12.5 nm which is equal to 137 µg/ml). After four hours of treatment, the number of viable bacteria was determined microbiologically. Results are expressed as median number of viable bacteria in colony forming units (CFU) per HSE with upper ranges. *p<0.05, **p< 0.001 as analysed with the student T-test. Lower limit of detection was 50 CFU.

To exclude the possibility of toxic effects of the AMPs on human skin, we assessed the safety of the peptides in a human epidermal skin model. Cell lysis, measured via LDH release, was <50% after 4 hrs exposure to ≤200 nmol/model of P10, P145, P148 and P276 (Figure 2). For the efficacy experiments described above, 12.5-50 nmol/HSE were used, which caused <10% cell lysis (Figure 2).

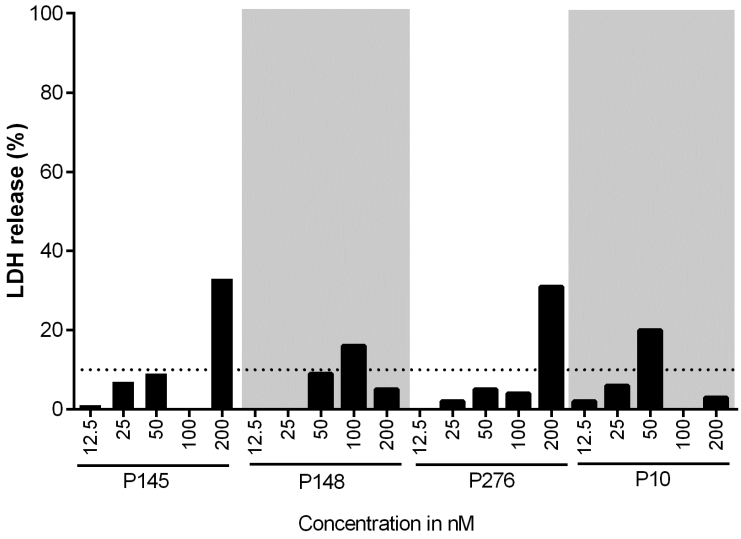

Figure 2. LDH toxicity testing of HSEs 4 inoculation with P145, P148, P276 and P10. Cell lysis was measured using human epidermal models (EM) via lactose dehydrogenase (LDH) release, 5 different concentrations of P10, P145, P148 and P276 were tested (12.5-200 nM/EM). The % cell lysis is given as the mean of 2 independent experiments.

Discussion

The occurrence of XDR and PDR strains such as described in this paper illustrate the clinical challenges that we will be facing in a post-antibiotic era. Antimicrobial resistance causing limited treatment options in critically ill patients or the rejection of otherwise perfectly well donor organs, stresses the importance of the development of new agents that can be used against MDR bacteria. In our laboratory, we have designed novel peptides based on the LL-37-derived peptide P60.4AC with improved antibacterial activities (de Breij et al in preparation, [10]) compared to P60.4AC. Here, we report that four of the P60.4AC-derived peptides - P10, P145, P148 and P276 – are highly effective against multiple XDR and PDR strains *in vitro* and *in vivo*.

First, we demonstrated that P10, P145, P148 and P276 were highly effective against XDR and PDR strains of *P. aeruginosa*, *K. pneumoniae* and MRSA *in vitro*, killing >99.9% of bacteria at low micromolar concentrations. These synthetic AMPs have a rapid mode of action, acting within minutes by disrupting the bacterial membrane [21] and/or by inhibiting RNA, DNA, and protein synthesis [22]. Probably due to the diversity in mechanisms of action that are different from those of conventional antibiotics, the P60.4AC-derived AMPs demonstrated potent activity in the low micromolar regardless of the antibiotic resistance phenotype of the strains. In this respect, it is important to note that it is generally believed that development of resistance to AMPs is unlikely [23] due to their rapid and diverse modes of action.

Secondly, we have shown that these peptides were effective against PDR *P. aeruginosa* in an *in vitro* human wound infection model, eradicating up to 99.9% of bacteria within four hours of treatment. Interestingly, there was a high degree of variability in the effect of the peptides among the different skin donors, e.g. 12.5 nmol of P276 completely eradicated *P. aeruginosa* in one donor, whereas it reduced the number of bacteria by 87.2% in another donor. Whether donor-specific characteristics like expression of naturally occurring AMPs, including human beta defensins and LL-37 may influence the efficacy of the P60.4AC-derived peptides needs further examination.

In the *in vitro* skin model, P148, which differs from P276 by only one amino acid (R_7àA_7), showed an improved antimicrobial activity compared to P276 (Table 1,3). In the *in vitro* killing assay in PBS, however, both P148 and P276 showed similar activity against *P. aeruginosa*. We have previously shown that human plasma affects the antimicrobial activity of AMPs. Indeed, both P148 and P276 showed a 4-16-fold decreased activity against a set of Gram-positive bacteria and Gram-negative bacteria (de Breij et al in preparation). Interestingly, the activity of P276 against the PDR *P. aeruginosa* in the presence of plasma was 171-fold reduced compared to a 17-fold reduced activity of P148. A possible explanation for these findings may be that P276 is more easily degraded by molecules produced by *P. aeruginosa* in the presence of human substrates than P148. *P. aeruginosa* is known to produce proteinases like elastase that can break down antimicrobial peptides, including LL-37 [24] and it needs to be investigated whether the P60.4AC-derived AMPs are susceptible to such proteinases.

We realize that these HSEs suffer from several limitations, including the lack of immune cells and blood circulation and that the obtained results may therefore not fully reflect the effects of the AMPs on patients with a wound infection.

In the present study, we have shown that the synthetic AMPs P10, P145, P148 and P276 rapidly and efficiently killed XDR and PDR *P. aeruginosa*, *K. pneumoniae* and MRSA strains responsible for fatal outcomes and major epidemics. These peptides were effective against PDR *P. aeruginosa* in an *in vitro* human wound infection model. Their potent and broad antimicrobial activities in combination with anti-biofilm [10,25] and potential immune-modulatory activities make these AMPs attractive candidates for further development as alternative agents in the fight against drug-resistant bacteria.

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