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

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CHAPTER 8

Summary and future perspectives

1. Challenges in treating burn wound infections

Infection of burn wounds remains the leading cause of death in burn patients [1, 2]. Treatment of such infections with conventional antibiotics is often unsuccessful, which is due to multiple reasons. Firstly, the presence of multidrug-resistant (MDR), extensively drug-resistant (XDR) and pandrug-resistant (PDR) bacteria (definitions in [3]) in the wound bed render conventional antibiotics ineffective. This problem has increased in recent years due to extensive and improper antibiotic usage that selects for antibiotic resistant microorganisms [4]. Secondly, formation of bacterial biofilms, an extracellular matrix mostly compost of exopolysaccharides, extracellular DNA and proteins, may prohibit entry of antibiotics into the biofilm, leading to failure to reach an adequate concentration around the pathogen or the compounds in the biofilm may antagonize or alter the activity of the antibiotic. Moreover, bacteria in such biofilms can be in a latent, metabolically dormant state, which prohibits antibiotic activity, [5-7]. Finally, systemic antibiotic treatment in burn wound patients may fail since an optimal antibiotic concentration may not be reached in the wound bed due to the presence of debris, clots and necrotic tissue in the wound bed.

Taken all this together, there is a clear need for new antimicrobial agents with modes of action different that of current antibiotics. Different approaches may be considered (figure 1), e.g. biofilm degradation by maggot secretions [8, 9], inhibition of quorum sensing that is essential for biofilm formation or can make bacteria more susceptible for antibiotics [10, 11], use of bacteriophages [12] or bacteriolysins derived from them [13-16]. Furthermore, the host immune defence can be boosted by, for example, boosting of the endogenous production of antimicrobial peptides (AMPs) by Vitamin D_3 or butyrate ([17], EM Haisma unpublished data)). Identification of new (peptide) antibiotics, e.g. the new antibiotic teixobacin from un-culturable bacteria [18] or the peptide antibiotic lugdunin from the commensal bacterium *Staphylococcus lugdunensis* [19, 20] holds another promising approach. In this thesis, assess the potential of synthetic AMPs as topical antimicrobials.

To advance the development of novel antimicrobial agents and identify new drug targets, it is essential to use an appropriate model system. In this thesis, we describe the development and use of different *in vitro* skin models and a mucosal membrane model. There are a couple of advantages to the use of skin models. Firstly, they pose similar properties as normal human skin, including the *stratum corneum* (SC) formation [21] and cell differentiation [22]. The SC of skin models have been demonstrated to resemble that of human skin to a great extent [21]. This is in contrast to studies in animals, especially murine models, where the skin structure,

i.e. epidermal thickness and hair follicle count, is considerably different from that of the human skin [23]. In addition, the immune system of mice and human differ, for example in the expression patterns of defensins as reviewed in [24]. The advantage over *ex vivo* skin is that skin models can be kept in culture longer than *ex vivo* skin biopsies, which stay viable for only a couple of days and experiments can be repeated with cells from the same donor. Finally, with the use of skin models we are able to modify gene expression and thereby mimic disease characteristics (**chapter 6**). Disadvantages of HSE in comparison to the use of animal models is the lack of immune cells such as macrophages and neutrophils; and the absence of some anatomical features such as sweat glands and hair follicles. Currently, investigators are working on skin models that incorporate an immune compartment, for example T-cells [25] or Langerhans-cells [26]. In this thesis, we used different *in vitro* models to study the effect of colonization and infection of healthy skin, (thermally) wounded skin (**chapter 2**), mucosal membranes (**chapter 3**) and skin with disease characteristics of AD (**chapter 6**). In addition, we studied the difference in bacterial colonization and biofilm formation on biotic (*in vitro* skin models) versus a-biotic surfaces (polystyrene) (**chapter 7**). Finally, we used the models to study the antimicrobial activity of (novel) topical synthetic AMPs (**chapter 3,4,5**).

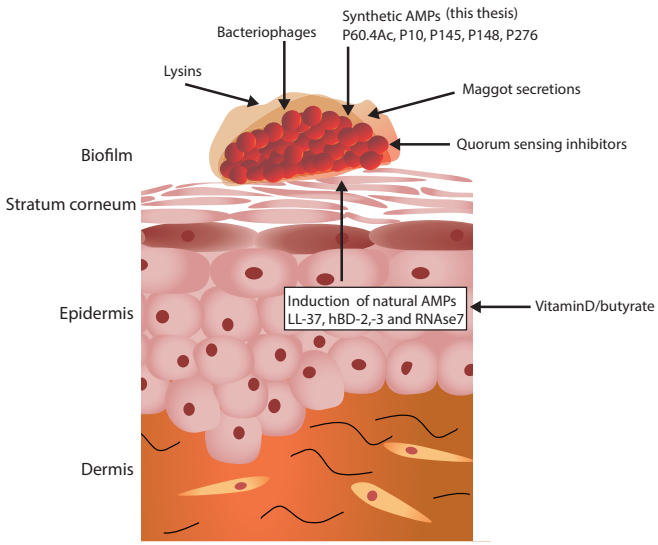

Figure 1: Strategies for the eradication of *S. aureus* and degradation of biofilms. Biofilm degradation by maggot secretions [8, 9], quorum sensing inhibitors [10, 11], bacterial products such as novel antibiotics teixobactin [18], AMP antibiotic lugdunin [19, 20], bacteriolysins [13, 14, 16], bacteriophages [12] or antimicrobial peptides P60.4Ac, P10, P145, P148, P276 (this thesis) or boosting of the endogenous AMP production of for example LL-37, hBD-2, HBD-3 or RNase7 by butyrate and/or Vitamin D_3 (unpublished data).

2. Development of in vitro models to study colonization and infection

To study colonization and infection on human skin or mucosae by bacteria we used three different models: (1) the human full thickness skin equivalent (HSE), composed of both a dermal and epidermal component; (2) the (Leiden) epidermal skin model (EM or LEM), composed of differentiated keratinocytes. In addition, we used a (3) mucosal membrane model using human bronchial epithelial models (BEM), composed of differentiated bronchial epithelial cells (explaining figure in introduction).

2.1 Skin models as models for wound healing

Skin models can be used to characterize aspects of the wound-healing process differently than the *in vitro* 'scratch' wound models using monolayer cultures. Monolayers are very limited for such studies, for example due to lack of cell differentiation and cross talk between the keratinocytes and fibroblasts [27]. Different studies have demonstrated similarities between *in vitro* wound-healing models and *in vivo* models, including proliferation, migration, expression of growth factors (e.g. Transforming growth factor beta 1 (TGF-β1), platelet-derived growth factor-β and keratinocyte growth factor [28, 29]) and cytokine secretion (e.g. Interleukin (IL)-1α, IL-6, tumor necrosis factor-α [27, 30, 31]), reviewed in [32, 33].

Since skin models consist only from a pre-defined cell population, they can be used as tools for the understanding of individual cellular processes that are involved in tissue repair. This brings also the downside of HSEs as research tools, since HSEs lack the complexity associated with wound-healing mechanisms in the *in vivo* situation. To study molecular mechanisms of tissue regeneration in a complex system, many researchers are now using rat and mouse wound models [34] regardless of the limitations as described above.

To overcome the clear differences between human and animal skin, researchers have used human *ex vivo* skin, HSEs or EMs grafted onto immunocompromised (athymic nude) mice as models. Using this approach, different models can be generated; e.g. to study hypertrophic scarring [35] or wound-healing in epidermolysis bullosa patients [36]. Also EMs composed of human embryonic stem cells have a structure consistent with that of mature human skin 12 weeks after grafting. This type of models could be used for long term research into wound healing, inflammation and skin irritation for example [37].

2.2 HSEs for thermal wounding and colonization

In this thesis we used HSEs to study colonization of (thermally) wounded skin by the Gram-positive bacterium *Staphylococcus aureus* (**chapter 2**). We observed that the course of *S. aureus* adherence and multiplication on thermally wounded and intact HSEs did not differ. Inoculation by MRSA induced the expression of various pro-inflammatory cytokines and chemokines (IL-6 and IL-8) and antimicrobial peptides (human beta-defensin (hBD)-2, hBD-3 and RNAse7) in HSEs. However, wounding of the HSEs did not further enhance the induction of these cytokines/AMPs as compared to colonization alone (**chapter 2**). This is in agreement with the findings of others who investigated responses of keratinocyte monolayers exposed to inflammatory stimuli, heat-killed *S. aureus* and skin commensals [38-40] and in HSE after exposure to viable *S. aureus* [41, 42]. Moreover, it is reported that hBD-2 and -3 expression can be induced by superficial barrier disruption [43] and that these are upregulated in the wounds of burn patients [44]. It has been demonstrated that hBDs can be induced by (heat-inactivated) bacteria, including *P. aeruginosa* and *S. aureus,* and toxins like LPS and cytokines like TNF-α [45, 46]. Finally, it has been shown that RNase7 can be induced by heat-killed *S. aureus* in primary keratinocytes [47] and human skin explants [48], and that RNAse7 has a role in killing skin colonizing *S. aureus* [48].

HBDs and RNase7 are proven to have antimicrobial activity against (MDR) *S. aureus*, but also Gram-negative bacteria and Candida. HBD-3 has a 90% lethal dose (LD_{90}), the concentration that kills 90% of colony forming units, of 2.5-4 µg/ml [46], RNAse7 a LD_{90} of 3-6 µg/ ml [48] and hBD-2 keeps the number of CFU static at 100 µg/ml [49]. We analyzed if supernatants produced by HSE were able to kill *S. aureus*/*P. aeruginosa*. However, we did not observe any killing activity (unpublished data). This may be due to low concentrations of the AMPs present in the supernatant.

2.2 Use of EMs to study colonization and biofilm formation

To investigate differences in biofilm formation on biotic and abiotic surfaces we used EMs. We studied the formation of biofilms of five different *S. aureus* strains on the SC compared to that on an abiotic surface, polystyrene. We screened for the presence of 52 proteins by *S. aureus* in biofilms, and found that six proteins in *S. aureus*, including alpha-toxin were differentially expressed between biofilm on abiotic and biotic surfaces. The detection of several toxins, gamma-hemolysin-B (HlgB), leukotoxins (LUK) -D and -E and alpha toxin in biofilms of multiple strains specifically on EMs suggests that the surface to which the bacteria adhere influences the expression of these bacterial proteins (**chapter 7**). This is confirmed

by others, who have also demonstrated a role for alpha-toxin in the promotion of biofilm development on mucosal membranes [50].

This implicates that currently used biofilm models, such as those using polystyrene plates, might not adequately reflect biofilm formation on a more complex surface such as EMs and, ultimately, the human skin. Others have started research on growth of bacteria on SC, by using human callus as a substrate for the growth of bacteria [51]. By creating an assay using bioluminescent bacteria in a 96-well plate, they created a high throughput screening assay that can be used for the screening of antimicrobial compounds. The results of this study could be used to specifically target biofilm components that are expressed on biotic surfaces like skin and mucosae, or to prevent biofilm development, e.g., the inhibition of quorum sensing to disturb formation of biofilms [10].

2.3 Use of EM to study effect of the atopic dermatitis characteristics on colonization

One of the advantages of using cell models is the ability to knock-down genes to investigate the effect of specific genes on barrier function, colonization or immune responses. Seventy to ninety percent of the AD patients are colonized with *S. aureus* and harbour biofilms [52], which is far more than the general population (where around 20% of people carry *S. aureus*). Although the exact causes of AD are still uncertain, filaggrin (FLG) mutations and overexpression of IL-31 [53] are associated with the occurrence of AD. To study the effect of *S. aureus* colonization on AD skin, we introduced FLG knockdown into our EMs, and/or supplemented the models with IL-31. We demonstrated that knockdown of FLG and/or supplementation with IL-31 leads to higher *S. aureus* colonization, which is in line with the association between AD and *S. aureus* colonization in patients. Moreover, both knockdown of FLG and IL-31 supplementation lead to a *S. aureus*-induced up-regulation of IL-8 mRNA. IL-31 prevented the up-regulation of *S. aureus*-induced AMP expression (hBD-2,-3 and RNAse7), whereas FLG knockdown did not, indicating a role for AMPs in the suppression of *S. Aureus* outgrowth. Furthermore, *S. aureus* colonization induced changes in mRNA expression of enzymes involved in cornification of the epidermis. For example in the expression of fatty acid elongase 4 (ELOVL4). ELOVL4 is involved in free fatty acid synthesis, and has been shown to be important for the skin barrier function. Absence of ELOVL4 resulted in impaired barrier function in mice [54]. We found that ELOVL4 expression was up-regulated in response to *S. aureus* colonization, irrespective of FLG-KD, but this was prevented by IL-31 (**chapter 6**). This together may demonstrate why for example extensive washing with soap,

which removes the upper layer of the SC (free fatty acids, AMPs, etc), is also associated with AD and eczema.

In a study where *S. aureus* colonization between AD patients and healthy volunteers were compared, genes like *lukD*, *lukE*, *splB*, *ssl8*, and *sasG* were more frequently found in *S. aureus* isolated from AD patients than from healthy volunteers [55]. All these genes are associated with *S. aureus* virulence. The correlation between AD and bacterial colonization can be further investigated using this model, for example by examining the presence of staphylococcal superantigens, or other virulence genes on AD skin.

3. AMPs as treatment for topical infections

3.1 AMP as alternatives for antibiotics

In 2016, 2235 antibacterial AMPs have been identified according to the antimicrobial peptide database (APD) (http://aps.unmc.edu/AP/main.php). There are more than 100 peptide-based drugs for various diseases on the market and almost 500-600 candidates in the preclinical trials, many of which are synthetic AMPs [56]. The first AMP used as a therapeutic agent was Gramicidin, a peptide isolated from a soil bacterium *Bacillus brevis* in 1939 [57]. Gramicidin is almost exclusively active against Gram-positive bacteria. Gramicidin D and Gramicidin S, the cyclic form, are approved by the FDA as a topical agents. Due to haemolytic activity at low concentrations they cannot be used as systemic agents. Secondly, polymyxins B and E (Colistin), both polypeptide antibiotics that were discovered in the 1940s, are used as topical and intravenous formulations against mainly Gram-negative bacteria. Although they were mostly abandoned in the 1980s due to reported nephrotoxicity and neurotoxicity [58, 59], in the past few years there has been more attention as a last resort antibiotic because of the emergence of MDR and XDR strains [60]. Colistin has been effective in treating infections, such as pneumonia or bacteraemia, caused by for example *Pseudomonas* or *Acinetobacter* species [61]. Moreover, in combination therapy it can be used to treat biofilms in cystic fibrosis (CF) patients [62]. Finally, Polymyxin B has been used to treat urinary tract infections and meningitis caused by *P. aeruginosa* and *Haemophilus influenza* [59, 60]. There are AMPs, such as omiganan, pexiganan and iseganan that successfully have completed phase III trials, but were denied approval by FDA for clinical use because of lack of advantage over the existing medications [63].

Table1: AMPs in clinical trials or used in the clinic.

AMP	Description	Indication	Development Phase	Clinical trail	Ref
Gramicidin	Gramicidin D and Gramicidin S, the cyclic form	Topical treatment of eye infections	Clinical use		[60]
Colistin	Cationic peptides polymixin B and E	Clinical use of diverse indications	Clinical use		[64]
Polymixin B	Polypeptide antibiotic	Last option antibiotic for MDR strains	Clinical use		[58]
Pexiganan	Synthetic analogue of magainin 2 derived from frog skin	Treatment of diabetic foot ulcers with 0.8% cream Locilex	PIII – not approved by FDA	NCT00563433 NCT00563394	[65-67]
Omiganan (Migenix MX-226)	Synthetic cationic peptide derived from indolicidin	Bactericidal, antifungal, catheter associated infections and rosacea 1% gel has been used as a topical antimicrobial	PIII PIIIIb	NCT01784133 NCT00608959	[68-70]
hLF1-11	Cationic peptide composed of amino-terminal amino acids 1–11 of human lactoferricin	Allogeneic bone marrow stem cell transplantation–associated infections	PII (terminated)	NCT00509938	[71] [72]
LL-37	Human cathelicidin	Venous leg ulcers	FIM	Eudract-2012-002100-41	[73]
OP-145 (P60.4Ac)	Synthetic 24-mer peptide derived from LL-37 for binding to lipopolysaccharides or lipoteichoic acid	Otitis media	PII		[74]
PMX-30063/ Brilacidin	Arylamide oligomer mimetic of a defensin, structure not disclosed	Acute bacterial skin infection caused by S. aureus	PIIb	NCT020388 NCT02324335	[75]

Table1: AMPs in clinical trials or used in the clinic. (*continued*)

AMP	Description	Indication	Development Phase	Clinical trail	Ref
Iseganan (IB-367)	Synthetic 17-mer peptide derived from protegrin 1	Oral mucositis as a mouth rinse against polymicrobial infections in patients treated with chemotherapy	PIII (failed)	NCT00022373	[76-78]
rBP121	Bactericidal/permeability-increasing protein (BPI) derivative	Burn Injury	PIIa	NCT00462904	[79]
LTX-109	Lytic broad spectrum peptide, protected for protease cleavage	Topical, nasal decolonization of methicillin-resistant and -sensitive S. aureus in humans	PI/IIa	NCT01158235	[80]
CZEN-002	derived from alpha-Melanocyte-Stimulating Hormone	Vulvovaginal candidiasis (vaginal)	PII		[81, 82]
XMP-629	BPI derivate	Acne (topical gel formulation)	PII (not continued)		[83]

Development phase (P), first in man (FIM). adapted and updated from [84].

3.2 The use of skin models for the testing of anti-infective drugs

Currently, the *in vivo* active concentrations of a novel antimicrobial compounds is often first associated with the minimal concentration that inhibits growth of a micro-organism *in vitro*, the MIC. The growth of the bacteria in such an assay depends among others on the type of culture medium (often a special bacterial growth agar, Muller-Hinton), which often differs considerably from the conditions in the patient [85]. Moreover, the presence of biofilm-associated infections is not taken into account in this assay. In addition, *in vitro* models involving biotic surfaces are required for studying bacterial biofilm formation. Therefore use of *in vitro* skin models (either HSEs or EMs) for the assessment of novel local topical therapies will have benefits over other *in vitro* systems, like medium or agar. It takes into account the local environment of the pathogen (instead of a standard culture medium or agar) and biofilm formation (table 2). Apart from the local environment of the pathogen, the pharmacokinetics of the drug should also be taken into account. The read-out for pharmacokinetic data generally use the blood (serum/plasma). However, for topical infections it is the local drug concentration in the skin that is relevant for the eradication of the pathogen. The use of skin models to screen for locally applied drugs may result in a more accurate determination of a dosing regimen to treat these type of infections clinically [86].

3.3 Use of skin models to test AMPs

In this thesis, we demonstrated the antimicrobial and anti-biofilm activity of 2nd and 3rd generation synthetic derivatives of the human AMP LL-37. LL-37 has been shown to have antimicrobial, immunomodulatory, anti-biofilm activities, acts as a growth factor during wound healing and has chemotactic ability (44,67-70) (figure 1). In former studies, P60.4Ac, a synthetic derivative of LL-37 has been described to poses higher antimicrobial activity towards Gram-negative bacteria than LL-37, while keeping immunomodulatory functions like toxin (LPS, LTA) neutralization [88]. Also, P60.4Ac has been shown to have additive value in the treatment of patients with chronic otitis media, showing increased efficacy compared to conventional antibiotics [74]. In this thesis, we demonstrated the antimicrobial activity of several new generations of AMPs derived from LL-37 against (MRD/PDR) *S. aureus*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa*. We showed that P10 was the most potent in eradicating MRSA, not only in an *in vitro* killing assay, but also from HSEs. Moreover, we demonstrated that P10 could eradicate established biofilms from EMs (**chapter 3**). Newer generation derivatives from LL-37, P145, P148 and P276 proved to be effective against colonization of EMs with XDR and PDR strains, including a *P. aeruginosa* strain (**chapter 4**). Moreover, we demonstrated that these peptides were also active

against *Mycobacterium tuberculosis* (data not included in this thesis). These results demonstrate the potential of these LL-37-derived peptides as novel treatments against topical infections.

Table 2: Advantages and disadvantages of the use of HSEs as screening tool for novel antimicrobial agents [86, 87].

	Skin models	In vitro MIC assay in 96-well format
Route of administration	Screen for local topical application	Used as model for systemic and local
Representation of bacterial state	Closely mimics the pathogens habitat, e.g. by letting the bacteria for a biofilm	Does not represent the habitat of the pathogen, screens while the bacteria is in suspension/ on agar plate. Medium for MIC tests is broth, which does not exist in any animal or human body.
Through-put	EMs most high throughput	High through-put
Dose-response	Mimics local topical concentrations of the anti-infective drug	Concentrations simulated are either the blood-drug concentrations or the drug-tissue concentrations. However, since these models do not simulate the host immune defense system (they only simulate the antibiotic concentration as a function of time) the end point is the eradication of bacteria.
Disease model	Can use disease models, e.g. for Atopic dermatitis or a wound model	-
Test combination therapies	Yes	Yes
Toxicity	Toxicity, irritation, sensitization and local immunity can also be tested	Give no information about toxicity for human cells
Costs	Expensive	Cheap

3.4.1 Formulation development for peptide antimicrobials

As described above, **chapter 4** demonstrates the activity of the synthetic antibacterial peptides P60.4Ac, P10, P145, P148 and P276 on HSEs. Moreover, these peptides may have additional effects like the parent peptide LL-37, such as

anti-inflammatory activities, pro-angiogenesis, chemotactic and enhancement of wound healing [89]. This makes these synthetic antimicrobial peptides promising candidates, either alone or in the combination with other antimicrobials [90], for the treatment of wound infections by (MDR) bacteria. To further assess the usage of these peptides as topical antimicrobials, we incorporated synthetic peptide P60.4Ac into different formulations and creams. In total two cream-based formulations and one gel was used. The antimicrobial activity of P60.4Ac in hypromellose-4000 gel proved to be the most potent. However, high peptide concentrations of this formulation (>0.5% wt/wt) also showed some toxic effects on the EMs, as mitochondrial activity was reduced and lactate dehydrogenase leakage was observed (**chapter 5**). Thus, it is important to improve safety of these peptides or search for novel peptides with reduced toxicity towards human cells.

3.4 Overcoming the limitations of AMPs

Compared to the number of papers and research efforts put into the therapeutic application of AMPs over the last decades, the number of successful clinical trials remains limited (table 1). There are a couple of reasons for this. First, the antimicrobial activity of natural AMPs under physiological conditions are limited. Many AMPs are sensitive to high salt concentrations [91], have short half lives *in vivo* (e.g., due to protease sensitivity) and limited bioavailability (binding to plasma proteins [71, 92]. While we find good antimicrobial effect of P60.4, P10 and P148 on the HSEs against a variety of (drug resistant) pathogens, we also find a reduced activity in plasma of P60.4 [93] and P10 (**chapter 4**). The 3rd generation peptides P145, P148 and P159, however, remained active in plasma (data not shown). Secondly, although AMPs have been described or claimed as molecules against which bacteria rarely develop resistance, some studies have described the development of resistance towards AMPs via several mechanisms, including modification of the bacterial surface, external trapping of AMPs, efflux pumps, proteolytic degradation, bacterial gene regulation and bacterial regulation of host AMP production [94-96] (table 3).

Table 3: Advantages and disadvantages of AMPs as (topical) drugs [56, 71, 97].

Advantages	Disadvantages
Broad spectrum activity	Costs of development / manufacturing
Rapid killing	Systemic and local toxicity
Suitable for topical application	Activity inhibition by salt, serum and pH
Anti-biofilm activity	Susceptible to proteolysis
Synergy with pharmaceuticals: antibiotics	Pharmacokinetic and pharmacodynamics issues, like a short half-life in vivo
	Sensitization to peptide
Biological other functions (wound healing, chemotaxis)	Natural resistance
	Biological other functions (chemotaxis, angiogenesis, wound healing)

Some of the limitations can be overcome by specific alterations to the peptides. This includes the addition of chemical groups like poly-ethylene glycol (PEG), which increases the stability of peptides *in vivo;* the use of retro-inverso peptides (stereochemical preparation of peptides) to prevent breakdown by enzymes [98]; different delivery systems like via liposomal delivery [99]. Wheat germ agglutinin (WGA)-modified liposomes encapsulating clarithromycin have been used to evaluate *in vitro* and *in vivo* efficacy against MRSA. The researchers found better bio-distribution of clarithromycin when formulated in WGA, and better *in vitro* and *in vivo* antibacterial efficacy against MRSA [99]. It has also been demonstrated that LL-37 can more efficiently protect against viral HPV infection in human epidermal model composed of immortalized keratinocytes when formulated into liposomes than when unformulated [100]. Moreover, multiple AMPs can be combined to get a synergistic effect (e.g., Gramicidin-S and Polymixin B against *P. aeruginosa*) [101]. Also, the combination of a conventional antibiotic and a peptide can be effective against biofilms, for example in the lungs of CF patients [62]. Finally, also the activity or expression of natural AMPs can be enhanced, as has been shown for butyrate [102]. Shigellosis can lead to a decreased LL-37 expression in the rectal epithelium. Butyrate treatment of Shigella-infected rabbits resulted in reduced clinical illness, reduced severity of inflammation in the colon, and reduced bacterial load in the stool, which was associated with a significant up-regulation of CAP-18 (the rabbit equivalent of LL-37) in the mucosal surface epithelium [17].

4. Conclusion

”For the first time, researchers have found a person in the United States carrying bacteria resistant to antibiotics of last resort, an alarming development that the top U.S. public health official says could mean “the end of the road” for antibiotics according to a recent article in the Washington post [103]. This paper emphasises that in the coming years the search for new treatment options to cope with infections caused by resistant bacteria is becoming more and more important.

HSEs can play an important role in the discovery and development of new drugs, as different aspects of these agents can be investigated, including antimicrobial effects, toxicity, effect on wound healing and initiation of host defense mechanisms. Currently, four reconstructed human EMs (Episkin, EpiDerm, SkinEthic, LabCyte EPI-MODEL) have been adopted by the Organization for Economic Cooperation and Development to provide alternative *in vitro* methods for classifying of irritants [104-107]. In the future, the use of HSEs could potentially be extended to that of a first line screening tool for new topical anti-infective agents. A start for this has been made by the use of the callus model [51] and the use of different models to assess the potency of AMPs as antimicrobials (**chapter 3,4,5**). Moreover, in this thesis we used one single pathogen to colonize the models, but also the interaction between different bacterial strains may be investigated, e.g. by the inclusion of commensal bacteria, or test for influence of the skin microbiome on disease phenotype, for example of AD skin [108]).

Although, the results of this thesis demonstrate the potential of the synthetic antibacterial peptides based on the sequence of LL-37, like P60.4Ac, P10, P145, P148 and P276, as novel anti-infective agents, the route from “bench to bedside” is a long one. As described in table 1, many AMPs that proved to be promising in the lab failed in clinical trials. Either due to effectiveness or added benefit from existing therapies. Moreover, these peptides have other roles in the human body like anti-inflammatory activities and enhancement of wound healing. Also the effectivity is depended on local the local environment, like salt concentrations. Therefore the question remains if the systemic use of AMPs will ever be possible. The in this thesis described local use of AMPs seems more obvious, this also in respect to their local expression pattern, e.g. in the skin. However, also in the development of synthetic peptides for local application a lot of knowledge needs to be gained.

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