



Impact of Host Defense Peptides on Chronic Wounds and Infections

Evan F. Haney, Daniel Pletzer,
and Robert E. W. Hancock

1 Introduction

Bacterial infections are the most common cause of human infectious diseases and antibiotics, used to treat these infections, have saved millions of lives in the last century. Most antibiotics are effective against dividing bacteria, and their systemic application leads to a reduction in the bacterial burden, allowing for resolution of the infection. One of the most common indications for applying antimicrobial therapy in developed countries like the United States are skin and soft tissue infections [1]. In some cases, localized tissue damage can lead to chronic, non-healing wounds that fail to heal within 4–6 weeks and may persist for months or years, even with continuous treatment [2]. Chronic wound patients often suffer from other conditions such as diabetes or obesity [3], and this type of wound represents a growing concern in healthcare settings throughout the world. These chronic wounds include diabetic foot ulcers, pressure ulcers, and venous leg ulcers, all of which are painful and debilitating conditions that negatively impact the

quality of life of affected patients. It is estimated that up to 2% of the population will suffer from lower limb ulcerations [4], and direct hospital costs to treat skin ulcers and chronic wounds have been estimated to be as high as £5.3 billion in the United Kingdom [5] and \$25 billion in the United States [3]. As the global rates of obesity [6] and diabetes [7] rise coupled with an increasing elderly population who often have comorbidities that predispose them to the development of chronic wounds [8], there exists an urgent need to develop new treatment strategies to cope with this growing health issue.

It has been proposed that the pathogenesis of chronic wounds is a result of the interplay of multiple factors: aging, damaged or reduced blood flow to the wound site, and wound colonization by bacteria coupled with an inflammatory response [9]. There is increasing evidence that the bacteria in chronic wounds occur within an organized community known as a biofilm [9, 10]. Biofilms are generally considered to be the natural phenotype of bacteria, and they are intrinsically resistant to antibiotics and to the host immune response when found in a chronic wound. Debridement can be used to remove bacterial biofilms from the wound bed [11]; however, biofilms *in vivo* can be exceedingly small and difficult to identify [12] which further complicates their treatment, and biofilms frequently regrow after debridement [10]. Additionally, many biofilms within wounds are often polymicrobial [13, 14] which makes it difficult to isolate

E.F. Haney · D. Pletzer
R.E.W. Hancock, O.C., O.B.C. (✉)
2259 Lower Mall Research Station, University of
British Columbia, Vancouver, BC, Canada

Department of Microbiology and Immunology,
Centre for Microbial Diseases and Immunity
Research, University of British Columbia,
Vancouver, BC, Canada
e-mail: bob@hancocklab.com

and appropriately treat the pathogen(s) responsible for the chronic wound state.

Unfortunately, there are currently no pharmaceutical agents that specifically target the bacterial biofilms that contribute to chronic wounds. The European Society of Clinical Microbiology and Infectious Diseases released a guideline for the diagnosis and treatment of biofilm infections in 2014 [15], in which they outlined the various challenges associated with identifying and treating biofilm-associated infections. In this document, the urgent need for new anti-biofilm-specific antibiotic therapies is highlighted as is the use of anti-inflammatory approaches to reduce local tissue damage due to the host inflammatory response [15].

In this review, we highlight the emerging potential of naturally occurring host defense peptides (HDPs) and synthetic derivatives thereof as a possible treatment tool to address the growing problem of chronic wounds. HDPs have been shown to have potent immunomodulatory functions related to innate and subsequent adaptive immunity and wound repair. Indeed, several synthetic peptides have been identified that enhance wound healing *in vivo*, while their ability to dampen excessive inflammation (often associated with chronic wound infections) appears to be a very common property of such peptides. In addition, many reports are emerging of peptide sequences that selectively target a broad spectrum of bacteria growing within a biofilm. We propose that the sequence optimization of synthetic HDPs for an enhanced combination of wound healing properties, anti-inflammatory activity, and anti-biofilm potency could result in novel pharmaceuticals that will complement current chronic wound treatment strategies.

2 Prevalence of Biofilms in Chronic Wounds

Microorganisms can be found in any niche on earth, and they need to shelter themselves in these environments. Bacterial cells often cluster together to form communities of millions to billions of cells as an effective protection method. Within these groups, organisms are able to cross talk with each other, share genetic information, and encapsulate themselves in an extracellular polymeric matrix

containing exopolysaccharides and/or proteins and DNA [16]. These dynamic communities are called biofilms and form the major lifestyle of bacterial organisms. Of increasing concern is the rise of adaptive resistance where the biofilm growth state of the microorganism leads to non-mutational high-level resistance to most antibiotics [17]. Adaptive resistance is defined as resistance that is dependent on the growth state/environmental challenges of the organism, affects susceptibility to multiple antibiotics, and reverts when the organism leaves the growth situation, e.g., upon dispersal of bacteria from biofilms [18, 19]. For example, the growth of bacteria in biofilms leads to dramatic alterations in the transcriptome, including alterations to genes influencing antibiotic susceptibility [20]. Oxygen and nutrient limitation in the deeper biofilm mass also causes bacteria to slow down their metabolism and growth rate, affecting many antibiotics like most β -lactams and fluoroquinolones that only target growing cells. This anoxic environment causes cells to switch into a more dormant, sessile, non-growing state (including so-called antibiotic-tolerant “persister” cells). Consequently, bacteria within a nondividing growth state are protected against the host immune system and various antibiotics, rendering them extremely difficult to treat compared to their planktonic counterparts [21, 22].

There is increasing evidence that bacteria grow within biofilms in chronic wounds. These biofilms can serve as foci for the emergence of systemic infections and are often the underlying cause of recalcitrant, recurring, chronic disease. Examples of biofilm-related infections include medical device-related infections, osteomyelitis, infections accompanying cystic fibrosis and other chronic lung infections, and wound infections [23]. Currently, biofilm-related infections are often treated with aggressive and intensive application of antibiotics. However, these treatments are helpful only to control biofilm-related infections, and they often fail to eradicate mature biofilms [23]. Moreover, administration of high doses of antibiotics is often impossible due to toxicity or other serious side effects such as the impact of antibiotic use on renal and hepatic function [23]. Therefore, this situation can result in a cycle of antibiotic use in patients that creates ideal conditions to select for

antibiotic resistance in a bacterial population. The Centers for Disease Control and Prevention (CDC) estimated that about two million people become infected with antibiotic-resistant bacteria in the United States each year [24]. Alarming is the fact that 23,000 [24] individuals die as a result of antibiotic-resistant infections, and this number balloons to 159,000 deaths [25] when we consider patients who die of sepsis, another serious condition where conventional antibiotics fail.

In the clinic, biofilms form on virtually all surfaces, and such contamination represents one of the biggest threats in healthcare facilities. Although this has been known for years, routine microbiological examination only assists in the diagnosis of clinical infections and does not reveal whether a microbial biofilm has been established. Additionally, non-culturable microorganisms or small-colony variants often limit detection [26]. In a study by James et al. [13], biopsy samples were collected from patients suffering from acute (16 patients) or chronic wounds (77 patients), and it was demonstrated that 60% of chronic wound and 6% of acute wound specimens demonstrated biofilm-like structures when analyzed with light and scanning electron microscopy. A recent meta-analysis of chronic wound studies reported that biofilms were found in 78.2% of chronic wounds [27], and it is now generally accepted that biofilms play an important role in chronic wounds and contribute to their inability to heal.

3 Chronic Wounds and the Healing Processes

In general, wounds can either be described as superficial (i.e., break of the epithelium), partial thickness (involving the epidermis and dermis), or full-thickness (deep cuts that reach subcutaneous fat and sometimes bone) lesions. Injuries arising from cuts, scrapes, exposure to chemicals, extreme temperatures as well as surgery, and/or disease outcomes are all possible causes for wounding. The wound healing process is a complex and dynamic process that is essential for daily survival of knocks and cuts and it is absolutely required for anyone undergoing surgical intervention. The first step after initial wounding initiates coagulation to form

a blood clot in the wound bed (also known as hemostasis). The subsequent healing process can be divided into three overlapping phases: inflammation, proliferation, and maturation [28]. Since the healing process does not follow a linear order, wounds can progress both forward and backward through the different phases [29]. During the inflammation phase, blood vessels dilate to allow the entry of a variety of cells into the wound area. These include important cells of the immune system, including white blood cells such as macrophages and neutrophils, which produce a variety of enzymes, cytokines, and growth factors that are essential for the wound healing process. At this stage, the first clinical signs of healing become visible such as heat, erythema, edema, and pain. During the proliferation phase, the wound is rebuilt with the formation of new granulation tissue, the extracellular matrix reforms with collagen secreted by fibroblasts, and new blood vessels emerge (angiogenesis). Subsequently, reepithelialization occurs through the migration of keratinocytes at the surface of the wound to complete this phase. After complete wound closure, the final maturation phase remodels collagen and decreases blood vessels inside the scar tissue [28].

Chronic wounds are lesions that do not heal within a predictable amount of time and are often delayed in one (or more) of the aforementioned wound healing phases. The vast majority of chronic wounds can be categorized into three major classes: (1) leg ulcers, often associated with venous or arterial deficiencies, vasculitis, and skin malignancies; (2) pressure ulcers, which are localized skin and tissue damage as a result of constant pressure often seen in sedentary patients in hospitals and residential care homes; and (3) diabetic foot ulcers, which are a major complication of diabetes mellitus and are responsible for neuropathy and arterial damage [11, 30]. The normal wound healing processes is also often impeded by ischemia, a condition characterized by poor blood supply resulting in low oxygen levels in tissues [30].

The presence of biofilms in chronic wounds stimulates a chronic inflammatory response that attracts abundant numbers of neutrophils and macrophages to the infection site. These inflammatory cells secrete proteases to help break down injured tissue and generate reactive oxygen species (ROS)

[31]. However, this influx and retention of innate immune cells coupled with the excessive secretion of the aforementioned molecules can also damage normal and healing wound tissue [32]. Bacterial defense mechanisms, on the other hand, induce the production of biofilm matrices to protect against host defenses such as phagocytic activity, oxidative stress, and proteolytic degradation [33]. The combination of these factors contributes to a chronic inflammatory state that fails to successfully eradicate the biofilm from the wound tissue. Moreover, high densities of bacterial pathogens (and/or commensal bacterial species) inside the tissue negatively impact wound healing due to direct interactions of bacterial cells with keratinocytes and fibroblasts or through indirect modulation of the inflammatory response [9, 30].

Biofilms play a major role in bacterial infections and chronic inflammation. To clinically manage a biofilm-associated infection, necrotic and infected tissue must be physically removed (i.e., through debridement and/or vigorous cleansing) [34]. In extreme cases, limb amputation is a possibility. In fact, diabetic foot infections are the most common cause of non-traumatic amputations [35], and diabetic patients who undergo a limb amputation have high 5-year mortality rates similar to the levels seen for common cancers [36]. Unfortunately, biofilms in wound tissue are difficult to identify and often lack noticeable clinical signs. The lack of proper visualization methods to accurately identify biofilm within a wound bed makes it exceedingly difficult to remove all of the contaminated tissue. Incomplete removal of microorganisms from within the wound leads to the potential for regrowth, formation of new biofilm mass, or it could potentially promote bacterial dispersal and lead to a systemic infection. To manage such chronic, biofilm-related infections, multiple visits to a doctor are necessary to perform regular wound cleaning. In an attempt to prevent biofilm reconstitution, infections of this type are often treated with conventional antibiotics. Unfortunately, antimicrobials only prevent the growth and proliferation of planktonic bacteria and often have a minor impact on organisms still embedded in the biofilm matrix inside the wound. Another complicating factor is the polymicrobial nature of biofilms that requires patient-specific, broad-spectrum antibiotics.

The rise of antibiotic-resistant bacteria and the lack of antibiotics that efficiently work against bacterial biofilms necessitate the development of novel treatment options to help clinicians treat this type of infection in patients. In the next section, we will highlight the potential of natural host defense peptides as a potential alternative capable of fighting biofilm-related infections and promoting wound healing in chronic wound tissue.

4 Host Defense Peptides and Chronic Wounds

Host defense peptides are short (12–50 amino acids) cationic polypeptide sequences that possess antimicrobial and immunomodulatory properties and are produced by all complex life forms [37]. Larger proteins (>100 amino acid residues) are also sometimes included in the definition of HDPs including lactoferrin, calprotectin (also known as S100A8 and S100A9), psoriasin (S100A7), RNase 7, and lysozyme [38]. Originally appreciated for their direct antibacterial activity toward microbes, they are often referred to in the literature as antimicrobial peptides (AMPs). However, subsequent studies of these molecules have revealed that these peptides exert a diverse range of immunomodulatory functions, which might indeed be their major function in the body. These include cell recruitment/chemotaxis, antiendotoxin activity, modulation of chemokine and cytokine production, angiogenesis, leukocyte activation, and wound healing properties [37, 39]. It is for this reason that we typically use the term HDP to better encapsulate the breadth of biological functions mediated by these molecules. More recently it has been shown that a distinct subset of HDP also have preferential anti-biofilm activity [40].

In humans, HDPs are produced by various cell types throughout the body. Immune cells such as neutrophils, monocytes, lymphocytes, natural killer (NK) cells, and mast cells all produce and store various HDPs [37]. The innate immune response depends on the presence of these cells to release HDPs in response to an invading pathogen and to prevent the onset of an infection. Many HDPs are also produced by the epithelial cells of healthy skin, and it is thought that the

presence of these peptides on the skin surface helps maintain homeostasis with the skin microbiota and prevents colonization and/or infection by invading microbes [41]. Examples of HDPs present in healthy human skin include RNase 7 and psoriasin [42], hBD-1 [43], dermcidin [44], and lysozyme [45]. Importantly, the expression of many HDPs is upregulated upon skin wounding, indicating that they might play an important role in the wound healing process. For instance, the human cathelicidin HDP, LL-37, is upregulated in the skin in response to inflammation [46] and in response to sterile wounding, as well as during infection by group A *Streptococcus* [47]. A more recent study found that injury of the human epidermis of the skin alone was a major inducer of a wide range of HDPs, including human β -defensin 2 (hBD-2) and hBD-3, as well as various cytokines, such as interleukin (IL)-6 and IL-8 [48].

The role of natural HDPs in wound healing has been extensively summarized in other reviews [49–51], and we will only briefly describe it here. First, the upregulation of gene expression for these molecules leads to an increase in the local concentration of HDP which, if large enough, can directly kill bacteria and prevent infection. In addition, HDPs are known to interact with various cells of the immune system as well as epidermal keratinocytes to promote the wound healing process. These activities include modulation of cytokine production, promoting cell migration and/or proliferation, and blood vessel formation [51], all of which are involved in the wound healing process described above.

There is ample evidence that the dysregulation of endogenous HDP levels contributes to impaired wound healing and chronic infections. For instance, patients with atopic dermatitis have been shown to have reduced expression levels of hBD-2 and LL-37 in inflamed skin [52], and reduced hBD-2 expression has been found in burn wounds [53], both of which may account for the increased susceptibility to bacterial infections in these patient groups. In the context of chronic wounds, LL-37 levels have been found to be low near the wound edge of chronic ulcers [54], while hBD-2 levels are insufficiently upregulated in diabetic foot or venous calf ulcers [55].

The therapeutic use of natural HDPs to treat wounds has been explored for some time. Early animal studies revealed that exogenous LL-37 promoted angiogenesis in a rabbit ischemia model through the activation of endothelial cells by the formyl peptide receptor-like 1 protein [56]. The cutaneous adenoviral delivery of a gene encoding LL-37 significantly reduced bacterial burden in rats with cutaneous burn wounds [57]. A similar adenoviral vector approach was used to deliver hBD-3 to excision wounds on Yorkshire pigs that were subsequently infected with *S. aureus*. In this case, hBD-3 expression caused a tenfold reduction in bacterial burden and significantly promoted wound closure after 4 days of growth [58]. Recently, a non-viral gene delivery method was evaluated to deliver a plasmid encoding LL-37 to wounds in vivo. Using skin-targeted electroporation, an LL-37 encoding plasmid was efficiently delivered to skin wounds in mice resulting in enhanced expression of LL-37 which promoted reepithelialization of the wounded tissue [59]. Importantly, the use of HDPs to treat chronic wounds in humans has also been shown to be safe and effective. In a randomized, first-in-man placebo-controlled clinical trial, topical treatment with synthetic LL-37 promoted healing of hard-to-heal venous leg ulcers [60], demonstrating that natural HDP supplementation may be a viable strategy to improve the clinical outcomes of chronic wounds.

5 Synthetic HDPs as Novel Wound Healing Agents

Synthetic derivatives of natural HDPs have been shown to retain many of the biological properties of this class of peptides, and in some cases, peptides with enhanced activity or reduced cytotoxicity have been identified. Most of these optimization strategies have been aimed at identifying HDPs with improved antimicrobial activity [61]. This type of study has dramatically improved our understanding of the sequence requirements of AMP sequences and has expanded the breadth of sequences that are known to possess antibacterial activity. These sequence optimization approaches have also been extended to other HDP activity types suggesting that it may be possible to optimize synthetic peptides for specific biological

applications. For instance, fragments of LL-37 have been generated that retain the antibacterial potency and chemotactic activity of the parent peptide while exhibiting reduced cytotoxicity [62]. Of importance to chronic wounds, several synthetic peptides with anti-biofilm activity have also been identified by our group [40, 63–65] and others [66], and it appears that this activity is independent of direct antibacterial activity toward planktonic cells [65]. Therefore, an optimization strategy aimed at enhancing the anti-biofilm potency of synthetic peptides could potentially address the biofilm component of a chronic wound that is not specifically addressed by conventional antibiotics alone.

Notably, the wound healing properties of natural HDPs have also been recapitulated in synthetic peptides. For instance, a frog-derived AMP, Esculentin-1a, stimulated migration of keratinocytes more efficiently than LL-37 in vitro [67]. Promotion of wound healing has also been demonstrated in vivo by IDR-1018, a synthetic derivative of the bovine HDP bactenecin, which enhanced wound healing in *S. aureus*-infected porcine wounds (Fig. 1) [68]. A recent study described the wound healing properties of DRGN-1, a Komodo dragon histone-derived peptide, in a mixed species cutaneous infection model as well as in sterile wounds [72]. Emerging evidence suggests that optimizing synthetic peptides for specific wound healing properties could generate novel peptide sequences with therapeutic potential. Nakagami et al. [73] designed a series of derivatives based on a novel angiogenic peptide sequence, AG30, to try to improve both the antibacterial and angiogenic properties. One of these derivatives, AG30/5C, in which five residues in the parent sequence were replaced with Lys or Arg residues, accelerated wound healing and angiogenesis in a diabetic mouse wound model infected with MRSA [73]. This peptide has since been further optimized to peptide SR-0379 by identifying the minimal peptide sequence required for wound healing as well as incorporating a D-Lys residue near the C-terminus to improve the proteolytic stability and reduce toxicity (Fig. 1) [70]. The sequences and wound healing activities of a number of synthetic peptides are summarized in Table 1.

The wound healing properties of HDPs do not appear to be directly related to the antibacterial properties of a given peptide sequence. For

instance, HB-107 is a fragment of the insect AMP cecropin which lacks microbicidal activity but promotes wound healing in mice and enhances leukocyte migration and keratinocyte hyperplasia in wounds [75]. This observation suggests that many of the biological activities influenced by HDPs are sequence specific. Our group has demonstrated that the antibacterial properties of AMPs do not directly correlate with anti-biofilm activity. For instance, LL-37 and the synthetic peptides 1037 and IDR-1018 inhibit biofilm growth at concentrations well below their MICs against planktonic bacteria [40, 63, 65]. Moreover, IDR-1018 acts against biofilms of *Burkholderia cenocepacia*, which is completely resistant to AMP activity [65], while one of the more potent AMPs exhibited no anti-biofilm activity [63]. Furthermore, with the best anti-biofilm peptides, this activity is extremely broad in spectrum, preventing biofilm formation and destroying preformed in vitro biofilms caused by all of the major nosocomial antibiotic-resistant (so-called ESKAPE) pathogens, killing multispecies oral biofilm bacteria, and acting against in vivo biofilms [78]. Therefore, it is attractive to speculate that it may be possible to screen specifically for synthetic HDPs with enhanced wound healing properties, potent anti-biofilm activities, and anti-inflammatory activity to address three of the underlying concerns for chronic wounds. Interestingly, some synthetic peptides with wound healing properties have also been shown to exert anti-biofilm activity (Table 1), demonstrating that these activity types can overlap in a single synthetic HDP.

Various in vitro screening methods are used to evaluate synthetic peptides for wound healing activity. The most common are cell proliferation assays, looking primarily at sequences that promote fibroblast and epithelial cell growth [79], and migration assays to measure movement of epithelial cells, such as keratinocytes, across a surface [80]. Both of these activities are essential during the proliferative phase of wound healing as fibroblasts produce essential components of the extracellular matrix and keratinocytes migrate to reepithelialize wounds [81]. Many of the synthetic HDPs with wound healing properties described in Table 1 were characterized with these types of assays in vitro prior to in vivo studies. Some groups are already attempting to iden-

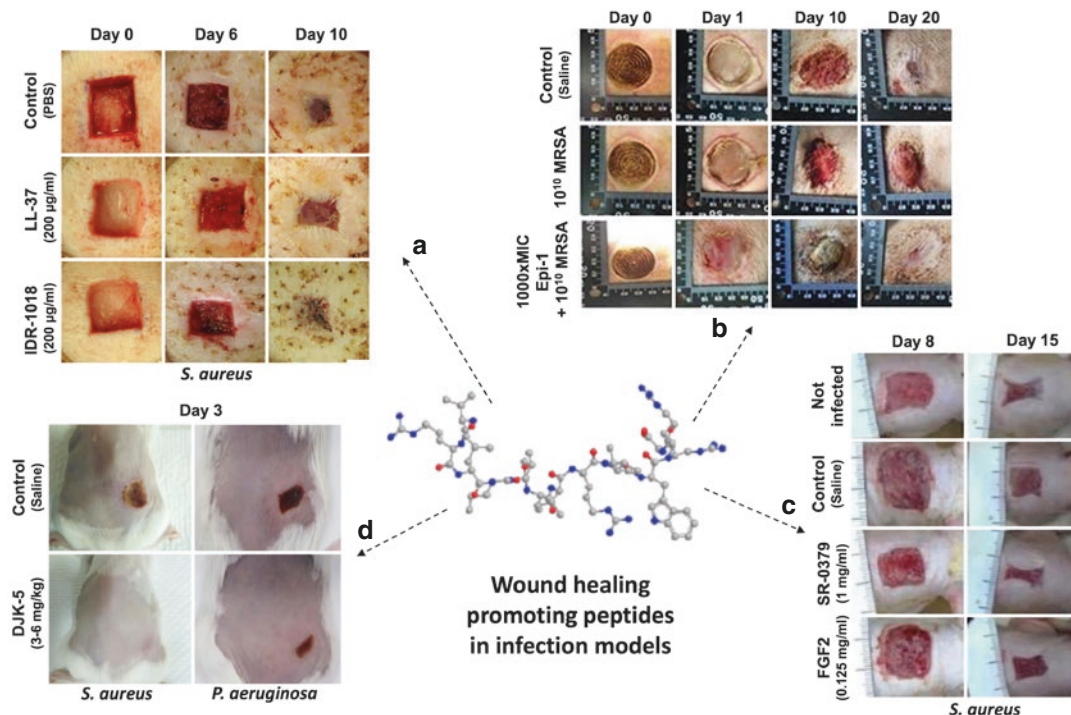


Fig. 1 Wound healing-promoting peptides in infection models. **(a)** Synthetic peptide IDR-1018 and natural peptide LL-37 compared to a PBS control in a *S. aureus*-infected porcine wound healing model. IDR-1018 demonstrated significantly accelerated wound healing when compared to LL-37 or PBS, while there was no observed change in the underlying bacterial colonization [68]. **(b)** Wound healing properties of antimicrobial peptide Epi-1 compared to a saline control and non-treated wounds in a *S. aureus* (MRSA)-infected burn wound swine model. Accelerated wound closure was observed when the infected heat-burned pig skin was treated with Epi-1 [69]. **(c)** Effects

of antimicrobial peptide SR-0379 and fibroblast growth factor 2 (FGF2) in an acutely infected wound with *S. aureus* compared to saline control treatment as well as uninfected wounds. SR-0379 treatment significantly accelerated wound healing when compared to FGF2 or saline [70]. **(d)** Anti-biofilm peptide DJK-5 in comparison to a saline control in a high-density bacterial infection model in CD-1 mice which were subcutaneously injected with *S. aureus* or *P. aeruginosa*. Infections were treated with 3 mg/kg (*P. aeruginosa*) or 6 mg/kg (*S. aureus*) DJK-5. The peptide significantly reduced dermonecrotic lesions with only a minor reduction in the underlying bacterial colonization [71]

Table 1 Synthetic HDPs with demonstrated wound healing activities in vivo or in vitro and any documented anti-biofilm activity

Peptide	Sequence	Wound healing	Anti-biofilm activity
IDR-1018	VRLIVAVRIWRR-NH ₂	Wound healing in pigs and mice [68]	Broad spectrum, including all ESKAPE pathogens [65]
DJK5	VQWRAIRVRVIR-NH ₂ (all D-amino acids)	Reduced MRSA abscess size and bacterial burden in mice [71]	Broad spectrum, including all ESKAPE pathogens [74]
HB-107	MPKEKVFLKIEKMGRNIRN	Wound repair in mice [75]	Unknown
DRGN-1	PSKKTTPVKPKKVA	Wound healing in mice [72]	<i>P. aeruginosa</i> and <i>S. aureus</i> [72]
SR-0379	MLKLIFLHRLKRMRLKRRK (K = D-amino acid)	Accelerates wound healing in mice [70]	Unknown
Epi-1	GFIFHIKGLFHAGKMIHGLV-NH ₂	Healed heat-burned MRSA-infected porcine skin [69]	Unknown
Tiger17	WC ₁ KPKPKPRC ₁ H-NH ₂	Accelerated healing in mice with full-thickness skin wounds [76]	Unknown
Esculentin-1a (1-21)NH ₂	GIFSKLAGKKIKNLLISGLKG-NH ₂	Stimulates keratinocyte migration [67]	<i>P. aeruginosa</i> [77]

tify novel wound healing peptides using these screening approaches. For example, Kosikowska et al. [82] sought to identify bifunctional AMP sequences with potent antimicrobial properties coupled with enhanced cell proliferation and migration properties. In this case, while the authors successfully identified peptides with either antimicrobial activity or wound healing potential, they unfortunately did not find a peptide that fulfilled their bifunctional objective. It is worth mentioning that this study was limited to only 15 sequences and future studies could dramatically expand the sequence space of the synthetic HDP sequences through the use of peptide arrays. We have successfully used such a strategy to simultaneously evaluate the immunomodulatory and anti-biofilm activity of hundreds of peptide sequences and generated optimized synthetic HDPs for multiple activity types [64]. As our understanding of the sequence requirements that govern the wound healing properties of HDPs improves, it should be possible to further enhance the therapeutic potential of these molecules and advance their progress to the clinic. In the next section, we will discuss various animal models that have improved our understanding of chronic wounds as well as serving an essential role in evaluating wound healing compounds in the context of a living organism.

6 Animal Models of Chronic and High-Density Bacterial Wound Infections

Molecular and cellular mechanisms underlying wound healing processes have been extensively studied in acute animal infection models. Unfortunately, most of these models fail to recapitulate the clinical features of chronic wounds and their pathology in humans. The host immune

response as well as the dissimilarity of the human skin architecture to that of common lab animals (e.g., mouse, rat, rabbit) brings additional complexity limiting their ability to fully capture the clinical scenario. The implementation of a clinically relevant chronic infection model still remains very challenging, and only a handful of chronic wound models have been described (Table 2). In the following section, we describe some of the currently available chronic infection models, including a new mouse model for high-density bacterial infections developed in our lab, and we briefly discuss the strengths and limitations of these models in the context of chronic wounds.

Currently, no single animal model is able to accurately and faithfully represent the diverse etiology and heterogeneous nature of chronic wounds, and this has hampered efforts to study and understand this complex biological process. The porcine model offers the closest anatomical comparison to human skin and is widely accepted as a preclinical model for human wounds [92], but is logistically difficult to implement and associated with far greater costs than regular laboratory rodents. Benefits and limitations (such as genetic tractability, reproducibility, costs, etc.) must be taken into account when choosing an animal model, and it is important to understand how well an animal model reflects a human outcome during the development of novel therapeutic treatments. Ultimately, animal models cannot and will not replace the verification of agents and mechanisms in human wounds, but are of critical importance in providing reliable, reproducible information on the response of wounds to therapeutic treatments.

Prolonged or chronic ischemia is a major contributing factor in impaired wound healing and often leads to ulcer formation and tissue necrosis [93]. Various ischemic animal models have been described that address the issue of reduced blood

Table 2 Animal models of chronic and high-density bacterial skin wound infections

Animal model	Clinical relevance	Test animals	References
Skin/ear ischemia	Ischemic ulcers	Rabbit, guinea pig	[83, 84]
Skin flap ischemia	Ischemic ulcers	Pig, rabbit, rodents	[85, 86]
Magnet ischemia-reperfusion	Pressure ulcers	Rat, mice	[87, 88]
Diabetic wounding	Diabetic ulcers	Mice	[89, 90]
Bacterial cutaneous wound infection	Infected ulcers/abscesses	Pig, mice	[68, 71, 91]

supply and fluid drainage. In the cutaneous ischemia model using guinea pigs [83], a plastic tip is subcutaneously inserted and further ligated with a nylon strap to cause a necrotic lesion suitable for wound debridement studies. Another often used ischemia model is the rabbit ear (ulcer) model where excisional wounds on the inner aspect of the ear are produced to the depth of the auricular cartilage [84]. Since the rabbit ear dermis is firmly attached to the cartilage, this creates a full-thickness excisional wound. The advantages of this model are that many wounds can be created on one animal, lesions can be therapeutically treated, and, unlike rodent models, there is no wound contraction, which reflects granulation-type healing in humans. The model can also be extended by slowing down the healing rate through ligation of two of three supplying arteries [84].

Skin flap ischemia models are used to recreate local tissue hypoxia, which usually involves the dorsal skin. In this model, a pedicle flap of the skin is surgically removed resulting in a compromised circulatory pattern (i.e., severed blood vessels) that creates an ischemic gradient. The flapped skin shows impaired growth mechanisms often associated with tissue necrosis [85]. The model has been used to study the repair of large wound defects and allows for the investigation of wound repair and potential wound therapies. Additionally, many humanlike chronic wound characteristics such as delayed healing, increased inflammatory cytokines (such as TNF- α , interleukin-1 β), and elevated proteases (e.g., metalloproteases) are observed in this model [86].

Pressure wound ulcer models use the ischemia-reperfusion model that requires surgical implantation of a metal plate under the skin followed by multiple tissue compressions using an external magnet [87]. This induces reduced blood flow, hypoxia, immune cell influx, and the release of free radicals of oxygen. Reperfusion (i.e., restoration of blood flow) of ischemic tissue is crucial for the tissue to survive, and periodic pressure application can replicate certain features of human chronic wounds where reperfusion has been restored.

Diabetes is a systemic disease that causes neuropathy and arterial damage thereby affecting various tissues and organs. Ulcers associated with

diabetes can lead to medical complications, including the most severe outcome of a limb amputation [94]. Diabetes in animals can either be chemically induced, using streptozotocin (a compound that kills pancreatic β cells) [89], or by using mice deficient in leptin (a hormone made by adipose cells to regulate energy balance) or the leptin-receptor protein [95]. These mice become obese 6 weeks after birth and subsequently develop type II diabetes. Macrophages have been shown to play an important role in this model. For instance, macrophages in healthy individuals show a balanced phenotype of classical pro-inflammatory/antimicrobial (M1) cells and alternative pro-repair/anti-inflammatory phenotype (M2) cells. However, this balance is disturbed in leptin-deficient mice, and studies have shown that recruited macrophages in diabetic mice fail to polarize toward M2 phenotypes, thereby increasing M1-associated metalloprotease secretion and reducing collagen deposition [96], factors that contribute to chronic diseases. Moreover, diabetic animal models can be used to study diabetic-impaired wound healing processes.

Recently it has become apparent that bacterial colonization in wound tissue interferes with the healing process and contributes to the development of chronic wounds. Therefore, a number of animal models have been described wherein bacteria are introduced to the wound site to try to better represent the conditions of a clinical chronic wound. Our laboratory recently developed a cutaneous wound infection (abscess) model using high-density bacterial pathogens [91]. A subcutaneous injection of appropriate doses of bacteria into the dorsum of mice caused the formation of an abscess and localized necrotic tissue. This model demonstrates the significance and persistence of bacterial invaders during abscess formation and could be a valuable tool to model hard-to-treat chronic bacterial and skin infections. Furthermore, it allows the establishment of chronic wounds for several days, is technically very easy to implement, is easily adapted to study therapeutic treatment, and improves animal welfare due to the possibility of using real-time in vivo imaging techniques that drastically reduce the numbers of animals needing to be sacrificed. Critically the abscesses formed

in this model are quite resistant to high-dose intravenous antibiotic therapy although local administration of antibiotics and especially synthetic anti-biofilm peptides demonstrates efficacy [91].

The presence of biofilm-producing bacteria in wound tissue has recently received more attention [9]. Biofilms in wounds can lead to post-closure complications (such as inhibition of tissue reepithelialization) as well as recurrence of skin breaks and infections [97]. While there is still a lack of adequate in vivo models that accurately address wound biofilm infections, some promising chronic infection models have recently been established. Examples include the chinchilla otitis media model where a *P. aeruginosa* c-di-GMP overproducing mutant showed greater persistence in chinchilla ears [98], as well as the mouse polymicrobial full-thickness wound model where preformed polymicrobial biofilms, transplanted onto the top of wounds, caused an impairment in wound healing [99]. Other mouse models specifically looking at the impact of biofilms on wound healing include the diabetic murine full-thickness wound model where 2-day-old *P. aeruginosa* biofilms in punch biopsy wounds caused delayed wound healing [100] and the splinted cutaneous wound model wherein biofilm forming *Staphylococcus* in a wound prevented reepithelialization while a biofilm-deficient mutant ameliorated wound closure [97]. In addition, the rabbit ear biofilm model has been described wherein mature *S. aureus* biofilms form in wounds as confirmed by epifluorescence and scanning electron microscopy [101, 102]. The effective use of these in vivo biofilm models offers the possibility to better understand chronic wounds and develop therapeutic treatments for biofilm-related infections with the ultimate goal to clinically translate the obtained results.

7 Application of HDPs in Chronic Wound Models

The use of synthetic HDPs to supplement the wound healing process represents a novel and promising future approach toward chronic wound therapy. Selective enhancement of innate immu-

nity with peptides, while suppressing excessive inflammation, has many advantages over direct antimicrobial compounds and has been shown to help protect against infection and inflammation in vivo [39, 103]. The following discussions will expand on some of the more recent developments and findings regarding HDPs in the context of in vivo bacterial animal models of infection and cutaneous wounds.

IDR-1018 (Table 1) is a synthetic peptide derived from the natural bovine HDP bactenecin with potent immunomodulatory and anti-biofilm properties [104]. Importantly, IDR-1018 has also demonstrated the ability to accelerate wound healing in a nondiabetic mouse splint model [68] highlighting its potential as a wound healing agent. Unfortunately, this peptide had no effect on wound healing in diabetic mice, possibly due to suppression of host immune pathways in diabetic wounds. In a cutaneous porcine infection model, where a methicillin-sensitive strain of *S. aureus* was inoculated into the dorsum of full-thickness wounds, the peptide showed superior activity by enhancing wound healing and reepithelialization, independent of antibacterial activity (Fig. 1) [68].

Epinecidin-1 (Epi-1) is a 21-amino acid antimicrobial peptide (Table 1) originally isolated from grouper fish with broad-spectrum antibacterial activity [105]. Interestingly, synthetic Epi-1 protected mice from lethal doses of MRSA administered to an excised region of the skin [106]. Recently, Huang et al. [69] demonstrated this property extended to porcine wound models revealing that treatment of heat-burned MRSA-infected wounds with Epi-1 improved healing (Fig. 1). The bacterial loads at the infection site were significantly reduced in animals treated with the peptide, and they confirmed that the peptide enhanced vascularization and extracellular collagen compound formation, as well as enhancing epithelial cell activities.

Accelerated wound healing was also demonstrated with the 20-residue AMP, SR-0379 (Table 1), in a skin ulcer model. Tomioka et al. [70] used rats that showed full-thickness defects under diabetic conditions (skin flap in streptozotocin-induced rats) as well as cyclophosphamide-

induced immunodeficient rats infected with *S. aureus*. In both animal models, treatment with SR-0379 led to significantly reduced wound areas within a week (Fig. 1). The authors explain the enhanced wound healing as being due to the beneficial effects of the peptide on angiogenesis, granulation tissue formation, and proliferation of endothelial cells and fibroblasts, as well as direct antimicrobial activity.

Bacterial infections can also lead to non-healing, recurring abscesses. Our research demonstrated that various pathogens (including *P. aeruginosa*, *S. aureus*, *A. baumannii*, etc.), when injected at high doses under the skin, were able to cause abscess formation and localized tissue necrosis in CD-1 and C57BL/6 mice. Interestingly, treatment of abscesses with the D-enantiomeric anti-biofilm peptide DJK-5 (Table 1) significantly improved visible dermonecrosis and resulted in a two- to threefold reduction in abscess size (Fig. 1) while also decreasing bacterial burden and dissemination [71, 91].

Evidently, the administration of HDPs in wound tissue to promote repair is a promising (alternative) approach in treating (chronic) wound infections. An in-depth understanding of molecular and cellular mechanisms in the future will help to deliver them into clinical trials.

8 Future Perspectives/Concerns with the Application of Peptides

In spite of their promise as alternatives to antibiotics as well as their immunomodulatory and wound healing properties, natural and synthetic HDPs have yet to fully realize their potential as pharmaceutical agents. A number of factors, such as undescribed systemic toxicities, tendency to aggregate under physiological conditions, and high production costs, might have contributed to their apparent lack of success as drug candidates [61]. Moreover, many cationic peptides tend to lose their antimicrobial activity under physiological conditions (e.g., presence of divalent ions, polyanionic glycosaminoglycan, etc.) [107],

although it is unclear if this effect extends to the anti-biofilm activity of synthetic HDPs. Importantly, many of the immunomodulatory functions of HDPs are preserved under physiological salt conditions, and therefore, future optimization strategies could focus on identifying synthetic peptides under conditions that are relevant to in vivo applications.

In the context of chronic wounds, the most pressing obstacle to overcome is the susceptibility of synthetic peptides to proteolytic degradation. Proteases, particularly matrix metalloproteases, are necessary components of the natural wound healing process that are required to degrade and remodel the extracellular matrix during tissue repair [108]. Chronic wounds are characterized by increased inflammation and elevated levels of serine proteases and matrix metalloproteases [109] that can lead to over-degradation of the ECM, ultimately preventing proper healing. Another source of proteases in chronic wounds are pathogenic bacteria which produce enzymes capable of degrading peptides present at the infection site [110]. Indeed, wound fluid from diabetic foot ulcers has been shown to degrade LL-37 likely arising from a combination of bacterial- and host-derived proteases [78], highlighting the necessity to overcome this issue as the development of synthetic wound healing HDPs progresses.

Various strategies have been employed to improve protease resistance in short polypeptide sequences such as the use of D-enantiomers or other peptidomimetics. The incorporation of non-proteinogenic amino acids has been proven effective in the context of improving stability of antimicrobial peptides while retaining antibacterial potency [61]. Both the SR-079 and DJK-5 peptides described above contain D-amino acids (one residue in SR-079 whereas DJK-5 is comprised of all D-amino acids) and both demonstrated activity in vivo (Table 1, Fig. 1). This demonstrates that these types of modifications can successfully be employed in the context of synthetic HDPs with improved wound healing and/or anti-biofilm properties.

Several synthetic HDPs have progressed through various stages of clinical trials with most

of them seeking approval for topical applications [111]. One of the earliest examples of a peptide taken to the clinic is that of pexiganan (also known as MSI-78 or Locilex), a synthetic analog of the frog AMP, magainin 2 [112]. In the late 1990s, pexiganan was evaluated in two separate phase III clinical trials to evaluate the effect of topical treatment on patient with mildly infected diabetic foot ulcers [113]. Promisingly, wounds treated with pexiganan cream closed at the same rate as those patients who received oral ofloxacin treatment, and there were fewer side effects in patients who received topical pexiganan treatment [113]. However, the FDA voted against approval and controversially requested a placebo-controlled trial to establish efficacy [114]. In October of 2016, Dipexium Pharmaceuticals completed these trials, but unfortunately they reported that treatment of diabetic foot ulcers with 0.8% pexiganan cream was not superior to treatment with cream lacking the active ingredient [115]. Another example of a peptide in the drug development pipeline is that of omiganan (MX-226), a 12-amino acid analog of indolicidin with broad-spectrum antimicrobial activity [116] that initially sought approval as a treatment for catheter-associated infections. In this case, the clinical trial of MX-226 failed to meet the primary endpoint, although secondary endpoints were achieved [111]. Interestingly, development of omiganan as a therapeutic agent has not completely ceased, and Cutanea Life Sciences, Inc. is currently involved in a phase III study to evaluate the long-term safety of topical omiganan in rosacea patients [117]. Many of the synthetic HDPs that have progressed through various stages of clinical development were likely identified and optimized for their direct bactericidal activity, and they were rarely pursued as antimicrobials for indications where antibiotic therapies fail, such as for biofilm infections. It is possible that an optimal synthetic HDP for wound healing applications might lack antimicrobial activity while still proving effective as a wound closure agent in patients with chronic wounds.

Current research has shown that the combined treatment of antibiotics with HDPs demonstrates synergy against both biofilms and infections aris-

ing from dispersed bacterial cells. In this context, synthetic HDPs could form the basis for novel adjunctive therapies to treat chronic wounds and biofilm-associated infections. Thus, Reffuveille et al. [118] showed that appropriate combinations of an anti-biofilm peptide and antibiotics showed strong synergy effects against biofilms grown in flow-cell chambers. While conventional antibiotics alone were unable to decrease biofilm thickness, disrupt biofilm structure, or cause cell death, the anti-biofilm peptide IDR-1018 alone was able to trigger all of these events. When the two therapies were combined, the effect resulted in significantly reduced or even completely eradicated biofilms at low concentrations of both antibiotic and peptide. It is important to highlight that increased dispersal of bacterial cells from biofilms, as is caused by anti-biofilm peptides, represents a potential danger in clinical settings, since dispersed cells may infect other organs or cause a septic shock [119]. Fortunately, Reffuveille et al. [118] also showed that the combined treatment of low concentrations of peptide IDR-1018 with only 40 ng/mL of the fluoroquinolone ciprofloxacin eliminated biofilms and drastically reduced the numbers of live dispersed cells, most likely due to the ability of antibiotics to eliminate dispersing cells. This provides further evidence that combinations of anti-biofilm peptides with conventional antibiotics represent a powerful new strategy to treat biofilm-related infections. In addition to the increased effectiveness due to synergy, when peptides are paired with antibiotics, they often decrease the required antibiotic dose which should reduce toxic side effects and costs, as well as slow the spread of antimicrobial resistance [19]. Since biofilm-related infections are often involved in chronic diseases that are frequently untreatable with antibiotics alone, the co-administration with alternative compounds such as peptides is highly relevant.

9 Future Perspectives

Understanding the underlying mechanism operating in non-healing, chronic wounds is crucial for developing appropriate treatment strategies.

It is well established that microorganisms, and particularly biofilm-producing organisms, found in wound tissues contribute to the development of chronic wounds and prevent wound closure. Non-healing wounds should be treated with care and proper antibiotics prescribed. Unfortunately, no biofilm-active compound has reached the clinic to date, and conventional treatment strategies for wound management often fail to address the underlying biofilm component of chronic wounds. Natural HDPs play an important role during wound repair, and they are involved in the activation of cells that either enhance or promote tissue repair, or they exert direct anti-biofilm effects. Various synthetic HDP derivatives have been identified that possess enhanced wound healing and anti-biofilm activities, and these molecules represent an exciting and novel treatment option that specifically addresses the underlying causes of chronic wounds.

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