ANTIMICROBIAL PEPTIDES

1. Introduction

1.1. Antibiotic Peptides versus Antimicrobial Peptides. The antibiotic armamentarium, which was developed mostly during the second half of the twentieth century, included a number of peptide-based compounds such as polymyxin, gramicidin and bacitracin. Historically, most such peptides were identified in spore-forming microorganisms. These proteinacious antibiotics differ in many respects from classical, ribosomally made peptides. Unlike ribosomal biosynthesis which use a limited repertoire of L-amino acids, most peptide antibiotics are synthesized by multienzymatic complexes and contain unusual fatty and amino acids (eg, D-amino acids, *N*-methyl amino acids, or imino acids). Moreover, peptide antibiotics usually lack methionine and histidine residues but often contain other non-amino acid moieties (eg, the chromophore of dactinomycin).

These peptide antibiotics however, were seldom the drugs of first choice for systemic therapy. Systemic use of peptide antibiotics was often limited because of nephrotoxicity and other toxicities. The therapeutic index (the ratio of toxic to effective dose) was smaller than for most non-peptide antibiotics. In addition, their complex structure added considerably to the problems of their synthesis. Still, the World Health Organization, which selects drugs based on efficacy, safety, quality, price, and availability, includes peptide antibiotics such as bleomycin, dactinomycin, and bacitracin in the list of essential drugs despite the forementioned drawbacks. This is not surprising considering that: (*1*) antibiotic resistance is an increasing problem worldwide, (*2*) the emergence and spread of multidrug resistant bacteria leads to treatment failure and is associated with severe outcomes (increased mortality, morbidity and expenditure). (*3*) for some of the responsible organisms, no treatment option is available; and (*4*) that in contrast, very few new antibiotics are being currently developed while no new classes of antibiotics that are active against gram-negative bacilli are under investigation.

In addition to the problems associated with multidrug-resistance, there is a growing worldwide population, of immunocompromised patients that present clinical conditions associated with impaired leukocyte function (eg, congenital hematopoietic defects, sepsis, cystic fibrosis, leukemia and exposure to chemotherapy (3). The ability to enhance hematopoietic cell defense has been limited. Granulocyte transfusions are associated with potential side effects, including alloimmune reactions and induction of inflammations (4), and administration of cytokines has not demonstrated improved clinical outcome (5). Thus, considerations relating to both microbe and host have generated the present enthusiasm for evaluating host-derived peptides as potential novel antibiotics. For the sake of differentiation between the peptides, the animal-derived ribosomally made peptides will be referred to as antimicrobial peptides as opposed to the former peptides, herein referred to as antibiotic peptides.

1.2. Animal-Derived Antimicrobial Peptides. At the dawn of the twenty-first century, the use of peptide-based antibiotics is envisioned with renewed optimism, due to the discovery of a large number of new peptides that are produced by ribosomes and that display promising properties including

toxicity. Thousands of consistent accounts in the scientific literature have gradually unfolded the secrets of an ancient and yet poorly understood peptide-based antimicrobial system. The identification of over 400 ribosomally made antimicrobial peptides in the past decade convinced the last skeptics that these peptides represent an essential defense component that controls cell proliferation, including the invading pathogens of both invertebrates and vertebrates (6–9). Indeed, an effective host defense against microbial invasion requires an innate immune system whose response is both rapid and independent of prior exposure (6–10). The innate immune system provides rapid and effective host defense against microbial invasion in a manner that is independent of prior exposure to a given pathogen. Phagocytosis represents a major component of this nonspecific defense system where circulating monocytes and neutrophils play an essential role by engulfing and digesting microorganisms using both oxygen-dependent and oxygen-independent mechanisms. Antimicrobial peptides play a decisive role in the nonoxidative microbicidal mechanisms of their producing cells by being delivered to phagocytic vacuoles containing ingested microorganisms (3). The oxygen-independent killing mechanisms of cells of the innate immunity were recognized based on the following observations: (*1*) neutrophils are capable of killing a variety of microorganisms (11), (*2*) neutrophils deprived of oxygen are still microbicidal (12), and (iii) crude extract of neutrophils is also microbicidal (12). These observations led to the isolation of a variety of proteins and peptides bactericidal/permeability increasing [eg, BPI defensins, and protegrins] that are responsible for the observed microbicidal activity (13–16). These animal derived host defense peptides have been shown to have broad spectrum antimicrobial activity, able to kill most strains of bacteria as well as some fungi, protozoa, and, many types of tumor cells. Structure–activity relationship studies have shown that in general, changes that increased the basicity and stabilized amphipathic structure have increased antimicrobial activity.

Most antibiotic peptides are synthesized (and often marketed) as groups of closely related structures, presumably reflecting a lack of specificity of the biosynthetic enzymes. Interestingly, the animal-derived antimicrobial peptides are also normally produced as closely related multimembered families (6,7). Examples are the mammalian peptides, defensins and protegrins, the amphibian peptides bombinins (17–19), magainins and dermaseptins (6,7) or insect peptides, cecropins and melittins (20,21). Nevertheless, dermaseptin members eg, were found to display dramatic synergy of action in various peptide combinations that have up to 100-fold more antibiotic potency than the individual peptides (7). Synergism has also been reported between other amphibian antimicrobial peptides, magainin-II and PGLa, as well as between a variety of similar mammalian antimicrobial peptides and proteins (22,23). In addition, despite the considerable structural similarities between the family members, they differ markedly in their ability to inhibit microbial proliferation. Hence, the biological significance for the occurrence of various antimicrobial peptides with similar sequences may in fact reflect nature's strategy to provide the producing organism with a maximum of coverage against a wider range of potential pathogens at a minimum metabolic cost.

1.3. A Mode of Action That Escapes Resistance Mechanisms. The mechanism of action of these peptides is not fully understood. The present

working hypothesis was formulated based on the following observations. (*1*) Antimicrobial peptides display a large heterogeneity in primary and secondary structures, but share a common feature of net positive charge. (*2*) The three-dimensional (3D) structures of most antimicrobial peptides present an amphipathic character. i.e, whether they adopt α-helical, β-sheet or some less defined structure, they are all organized in such a way that polar (and charged) residues are topologically separated from apolar residues. (*3*) Antimicrobial peptides are often active against a large spectrum of microorganisms that may include enveloped viruses, bacteria, protozoa, yeast, fungi, and cancer cells, although they often display a relative inactivity towards most mammalian cells. (*4*) Isomers composed only of D amino acids, display identical potency as the all L counterparts, which implies that the mechanism of action of their antimicrobial activity is not mediated by interaction with a chiral center (specific receptors, enzymes etc). Apparently, it is the physicochemical properties (eg, hydrophobicity and charge) of these structures that dictate the molecular events that lead to killing of target cells. Consistent with this view is the finding that at least two linear amphipathic helical peptides, magainin (24) and dermaseptin (unpublished data) derivatives have similar antibacterial activity as their respective reversed (retro) sequences; Moreover, the introduction of both L– and D-amino acids within amphipathic helical peptides preserved antimicrobial activity (25) thus emphasizing that even the specific structure of a particular molecule is not required (5). Despite the marked diversity in structure, size and spectrum of action, all antimicrobial peptides have manifest membranolytic properties.

Based on these observations and a host of concurring experimental data, a number of models for a membranolytic mechanism were described (24–34). A consensus model may be summarized in a basic two-step mechanism. Step 1: electrostatic interactions between the peptide's positive charges and the negatively charged head groups in the membrane lipids of the target cell promote peptide accumulation on the outer leaflet of the membrane (the peptide's long axis lies in parallel to the membrane surface). Step 2: with increasing concentrations, the membrane-bound peptide forms aggregates. Such aggregates could evolve to equilibrate with a multimeric water-filled pore in which the individual monomers have their hydrophilic residues facing inward and their apolar residues interacting with the acyl chains of the lipids. A transmembrane electric potential (negative inside) enhances the pore-forming activity of the peptide favoring insertion by interacting with the permanent dipole moment of the peptide that is oriented along the long axis. The solvent-filled pores cause the dissipation of the electric potential across the membrane thus permeabilizing the cell. Evidence for such antimicrobial pores crystallized in membranes was recently reported for magainin and protegrin (35). To form pores that span the membrane thickness, peptides need to be composed of at least 18 residues. Yet, membranolytic activity was observed with 10–13 residues antimicrobial peptides, which are significantly too short to span the membrane thickness (36). This observation argues in favor of a possible alternative mechanism, such as the "carpet" principle (28,29) which accounts for the activity of small peptides as well. Hence, regardless of their structure, antimicrobial peptides share two characteristic properties: they are polycationic and amphipathic. While the precise mechanism of action of these peptides remains to be fully understood, microbicidal effect is

believed to result from the capacity to permeabilize the cell and impair its ability to carry out anabolic processes.

1.4. Specificity Concerns. Antimicrobial peptides are potentially active against a large spectrum of microorganisms, yet they are generally less toxic to normal mammalian cells. The molecular basis for the relative inactivity of many of these peptides against mammalian cells is also poorly understood. Such selectivity might be due to the marked differences in the membrane compositions (eg, fluidity and negative charge density) of target- versus nontarget cells. This nonspecific (receptor-independent) cytotoxic mechanism of action carries certain advantages such as activity against a large variety of pathogens, and inability of pathogens to develop resistance as supported by the experimental data presented below. This receptor-independent mechanism however, also carries a major disadvantage since the task of conceiving antimicrobial drugs that act specifically against targeted organisms is rendered extremely difficult. Peptide-based antimicrobials are, nevertheless, widely believed to represent a promising solution to a variety of infectious diseases, including those caused by multidrug resistant organisms. Specificity is believed to be achieved by monitoring their physicochemical properties. Toward this goal, various natural antimicrobial peptides and proteins were optimized in terms of size, structure and activity as detailed below.

1.5. Indirect Antimicrobial Activity. Although the focus of this article is on the peptides direct antimicrobial properties, many of these agents manifest additional activities relating to immune modulation and wound healing (37). Thus, in addition to their membrane disrupting activity, cationic antimicrobial peptides are reportedly able to modulate certain responses of the innate immune system directly, by activation of macrophages, as well as indirectly, by neutralizing activators of macrophages. For example, the frog derived peptide dermaseptin S1 stimulates microbicidal activities of rat and human leukocytes (38). More recently, the insect-derived peptide cecropin-melittin hybrid (CEMA) was reported to be solely responsible for inducing the expression of some 35 genes in macrophages (39). Moreover, an increasing number of cationic peptides and proteins, such as lactoferrin (40,41), bactericidal/BPI protein (42), synthetic antiendotoxin peptides (SAEP) (43) and CEMA (44), are endowed with high binding affinity to lipopolysaccharides (LPS). LPS are potent activators of macrophages and are responsible for sepsis caused by gram-negative bacteria. By binding LPS, these peptides block the interaction of LPS with LPS-binding protein, suppress the ability of LPS to stimulate the production of inflammatory cytokins by macrophages and protect animals from lethal endotoxic shock. Various antibiotic treatments can cause the release of bacterial cell wall components such as peptidoglycan, lipoteichoic acid and LPS (44–47). Entry of these bacterial products to the bloodstream can induce septic shock due to an overwhelming inflammatory response whereby cells of the monocyte/macrophages lineage are stimulated to produced TNF-α, IL-1β and IL-6 as well as other proinflammatory cytokins (49–52). Thus, interfering with the ability of LPS to bind to macrophages might be an effective mechanism to prevent sepsis. As detailed below, a variety of cationic antimicrobial peptides bind to LPS, block the interaction of LPS with LBP, and suppress the ability of LPS to stimulate the production of inflammatory cytokins by macrophages (54–56). Hence, both naturally occurring

peptides as well as synthetic analogues maybe therapeutically useful for preventing sepsis and for suppressing inflammatory responses caused by LPS.

2. Description of Selected Proteins and Peptides

2.1. The BPI Protein. BPI is a 55-kDa protein found in the azurophilic granules of human neutrophils and eosinophils (57,58). BPI exerts multiple antiinfective activities against gram-negative bacteria: (*1*) cytotoxicity via disruption of bacterial membranes (59), (*2*) neutralization of bacterial LPS (60) and (*3*) opsonization of bacteria, which enhances phagocytosis by neutrophils (61). The crystal structure revealed a boomerang-like molecular organization composed of two structurally similar domains. A cationic N-terminal half that is responsible for antibacterial and antiendotoxic activities, and an anionic C-terminal half that is required for opsonic activity. An apolar lipid-binding pocket is present in each half of the molecule, believed to be important for interactions with LPS acyl chains (62,63).

BPI displays selective cytolytic activity against certain gram-negative bacteria (eg, the serum-resistant encapsulated clinical isolate *Escherichia coli* K1/r) but does not manifest cytotoxicity against other gram-negative bacteria such as *Klebsiella pneumoniae* (64,65), or against gram-positive bacteria, fungi and mammalian cells. Such selective action toward gram-negative bacteria was attributed to its high affinity for the lipid A moiety of LPS (66). LPS, which are a major component of the outer leaflet of gram-negative bacterial outer membrane, are normally stabilized by a regular array of divalent ions (calcium and magnesium) that serve to cross-link the negatively charged LPS molecules. Binding of BPI to the bacterial outer membrane is believed to displace the divalent cations, thereby perturbing the regular arrangement of LPS molecules, which leads to a change in trans-membrane potential and increased cell permeability (67,68). Although this mechanism may be independently responsible for rupture of the membrane, it is possible that BPI further facilitates the action of bacterial and host phospholipases, resulting in enhanced hydrolysis of bacterial phospholipids and acceleration of killing (69). BPIs action is further enhanced in the presence of antimicrobial peptides of the cathelicidin and defensin families (discussed below).

BPIs ability to neutralize LPS is opposite that of its structural homologue, the LPS-binding protein (LBP), which is a liver-produced acute-phase reactant, which amplifies LPS-mediated inflammatory signaling (70). The mechanism for opsonic activity of BPI toward gram-negative bacteria is unclear but requires both the N- and C-terminal domains of the protein (61). Opsonic activity is accompanied by mobilization of myeloperoxidase-mediated oxidative metabolism, suggesting possible collaboration between BPI and oxygen-dependent mechanisms of neutrophils.

Resistance to BPI is presumably low although there have been no published reports of the possible effect of serial passage of bacteria in media containing sublytic concentrations of BPI. In contrast, studies of isogenic strains of *Proteus mirabilis* suggest that expression of long-chain LPS might confer resistance to BPI (71,72).

A recombinant 21-kDa BPI fragment (rBPI21) expresses both the antibacterial and antiendotoxic activities of the parent molecule and has been demonstrated to have beneficial effects, either alone or in synergistic combination with conventional antibiotics, in animal models of sepsis, pneumonia, endotoxemia, and burns (73). Intravenous administration of rBPI21 reduced the mortality rate in gram-negative bacterial infection of animal models (mice, rats, and baboons). However, bacterial strains displayed variable susceptibility to rBPI21. Thus, BPI reduced the rate of mortality among mice injected with the rough strain *E. coli* J5 but was unable to reduce the rate of mortality *E. coli* O111:B4 and O7:K1 (74). Studies using rabbit model of *E. coli* O7:K1 bacteremia, suggest that BPI may also be a useful adjunct to conventional antibiotics, eg, cefamandole (75,76).

Human clinical trials indicated that rBPI21 is devoid of significant immunogenicity or toxicity. Although a biologics license application has not yet been submitted for rBPI21, evidence of clinical benefit has been noted for multiple indications, and other studies are being planned. Thus, in clinical studies (phase I), rBPI21 was well-tolerated and nonimmunogenic (77). Given intravenously to subjects who have received endotoxin, rBPI21 was able to inhibit LPS-induced cytokine release (78), coagulant responses (79) and pathophysiological changes such as alteration of cardiac index (80).

During phase II studies, open-label administration of rBPI21 to 26 children admitted to pediatric intensive care units with fulminant meningococcemia was associated with a reduced rate of mortality relative to that predicted by clinical prognostic scores, interleukin 6 levels, and the rate for historical controls (81). In addition, trauma patients with infectious complications associated with blood loss experienced a reduced incidence of pneumonia and adult respiratory distress syndrome following treatment with rBPI21 (82).

Two phase III double-blind placebo-controlled trials were conducted to evaluate rBPI21 in the treatment of hemorrhagic trauma and fulminant meningococcemia. The hemorrhagic trauma trial was discontinued due to insufficient activity. Analysis and speculation as to the reasons for the insufficient effect in that study must await publication of the study data. Results of a prospective, double-blinded, placebo-controlled phase III trial of rBPI21 involving 393 children (ages 2 weeks to 18 years old) presenting severe meningococcal sepsis have recently been published (83). The data suggest that rBPI21 is beneficial in reducing the complications of meningococcal sepsis. Treatment with rBPI21 reduced the number of multiple severe amputations (3.2 vs 7.4% in the placebo group, $P = 0.067$) and better functional outcome at day 60 (77.3 vs 66.3%, $P = 0.019$) but there was no significant difference in mortality (7.4% vs 9.9%, $P = 0.48$).

2.2. Lactoferrins. Native lactoferrin is an iron-binding 80-kDa protein, localized in the secondary granules of neutrophils as well as in tears, saliva, and breast milk (84). In addition to depriving microorganisms of an essential nutrient by binding iron, lactoferrin can exert a microbicidal effect via membrane disruption (85). The role(s) of lactoferrin in periodental, Parkinson and Alzheimer diseases were described (86).

Similarly, lactoferricins are naturally occurring non-iron-binding microbicidal peptides derived from the N-terminus of lactoferrin (87). Several studies have

documented its antiviral effects including eg, against the human immunodeficiency virus (HIV) (88). In addition to its antimicrobial properties, lactoferrin binds to the lipid A moiety of gram-negative bacterial LPS and neutralizes the endotoxic activity. The endotoxin-neutralizing activity of lactoferrin is reduced in the presence of the plasma LBP (70,89). Nevertheless, oral administration of lactoferrin reduced mortality in a porcine endotoxin shock model as well as suppressed tumor growth and metastasis in mice (90).

2.3. Serprocidins. The serprocidins are serine proteases of 25-kDa, localized in neutrophil primary granules and are structurally related to the granzymes of cytotoxic T cells (91). The family includes neutrophil elastase, cathepsin G, proteinase 3, and azurocidin, and were shown to act in synergy to kill bacteria (92). Unlike most antimicrobial proteins and peptides, azurocidin actually enhances cellular responses to endotoxin by a mechanism that has yet to be defined (93). The serprocidins display broad-spectrum cytotoxic activity namely against gram-negative and gram-positive bacteria, fungi and protozoa as well as mammalian cells. Cytotoxic activity is apparently due to combined action of both proteolysis and membrane perturbation.

2.4. Defensins. The defensins are a family of 4-kDa peptides, found in plants (94), insects (95) and mammals (96,97). The regulatory mechanisms involved in gene expression and biosynthesis of defensins in response to bacteria and inflammatory mediators, were described (98–105). Humans express defensins in neutrophils and intestinal Paneth cells, as well as pulmonary and reproductive epithelia (106,107). In each cDNA cloned, the mature defensin sequence constituted the carboxy terminus of a prepro-peptide (93–95) amino acids) containing a typical amino-terminal signal sequence followed by a 40–45 amino acid anionic propiece. This propiece may be responsible for masking the cytotoxic potential of defensins prior to their sequestration in lysosome-like organelles. Nuclear magnetic resorance (NMR) spectroscopy (108) and X-ray crystallography (109) showed that defensin molecules consist of a structurally rigid triple-stranded antiparallel β-sheet stabilized by three intramolecular disulfide bonds (Table 1). The activity of defensins depends on both their cationicity as well as their 3D structure. Defensins exhibit cytotoxic activity against a broad spectrum of microorganisms including bacteria, fungi, parasites, viruses, and host cells (110). Defensins form multimeric voltage-dependent pores that permeabilize cellular membranes. Activity of the defensins is inhibited by monovalent and divalent cations as well as by plasma proteins suggesting that the action of these peptides is limited in the extracellular environment to prevent indiscriminate cytotoxicity. Thus, defensins may be most active intracellularly in the phagolysosome.

2.5. CAP-18/LL-37. (CAPs) Cationic antimicrobial peptides) are linear members of the cathelicidins family of antimicrobial peptides. Cathelicidins are a large family of structurally diverse peptides found in mature neutrophils and possibly in other tissues of humans, pigs, cattle, sheep, goats, horses, mice, and guinea pigs, as identified either by prediction from cDNA clones or by direct isolation (111–117). This family includes the CAP-18 (118–122), protegrins (123–126), prophenins (123,127), PR-39 (128–131) and porcine myeloid antimicrobial peptides (132). Several genes of the cathelicidin family have been

characterized, and all encode a conserved prepro region, which is similar to that of cathelin, and a variable C-terminal antimicrobial domain (114,118–132).

In addition to their antimicrobial activities, these peptides have been implicated in wound healing, angiogenesis, and other innate immune mechanisms. Two members (whose study is most advanced) of the cathelicidins family, CAP-18 and protegrins, are further described.

CAP-18 is expressed by specific lymphocyte and monocyte populations as 10 to 20–kDa inactive proforms where the C-terminal fragment, termed LL-37, was found to be responsible for biological activity (133). Studies on its interaction with model membranes showed that presence of phosphorylcholine decreases bacterial susceptibility to the antimicrobial peptide (134). Both microbicidal and endotoxin neutralizing activities were observed for the CAP-18 derived from rabbit neutrophils (135). Carboxy-methylation leads to enhanced activity against gram-negative bacteria (136). Systemic administration of a CAP-18 derivative combined with aztreonam reduced mortality in a mouse model of *Pseudomonas aeruginosa* infection (137).

hCAP-18/LL-37, the only cathelicidin peptide expressed in humans, is induced in keratinocytes of inflamed skin and found in high concentrations in the lipoprotein fraction of plasma (119,138). The regulatory mechanisms involved in gene expression and biosynthesis of cathelicidins in response to bacteria and inflammatory mediators, were described (119). Adenovirus-mediated gene transfer of hCAP-18/LL-37 was found to restore bacterial killing in a cystic fibrosis xenograft model, raising the possibility that enhancing such innate immune mechanisms might someday be of clinical benefit (139).

2.6. Protegrins. The protegrins (PG) are a family of 2-kDa peptides expressed in porcine neutrophils (124). The regulatory mechanisms involved in gene expression and biosynthesis of protegrins in response to bacteria and inflammatory mediators were described (140). In both polar and apolar solvents, protegrins were shown to adopt an amphiphilic structure composed of an antiparallel β-sheet with a β-turn. The integrity of the β-sheet is maintained by the disulfide bonds (141–143) as shown in Table 2. Protegrins possess broad-spectrum microbicidal activity against gram-positive and gram-negative bacteria and yeast. Their activity requires intact disulfide bonds and is believed to be mediated by the formation of pores in the microbial membrane (144,145). Systemic administration of a representative protegrin reduced mortality of leukopenic mice injected with vancomycin-resistant *Enterococcus* faecium. Studies suggested that protegrins may be useful antimicrobial agents in therapy against gram-negative anaerobic bacteria which are believed to be involved in chronic, adult forms of periodontal infections (146).

An extensive structure–activity relationship (SAR) study was conducted on several hundred protegrin derivatives to identify a peptide for clinical development (147). Thus, PG-1 (IB-200) exhibited minimum inhibitory concentrations (MICs) in the range of 0.12–2.0 mg/mL against a range of gram-positive and gram-negative bacteria. In addition, PG-1 is rapidly bactericidal *in vitro*, reducing the number of viable colony forming units (CFUs) in cultures of methicillin resistant *Staphylococcus aureus* (MRSA) or P. aeruginosa by more than three log units in < 15min. Repeated subculturing of MRSA or *P. aeruginosa* in sublethal concentrations of PG-1 did not engender significant resistance.

The effect of sequence changes, size and the role of the stereochemistry have been addressed (148–150). A minimum core size of 12 amino acids appears necessary for protegrin antimicrobial activity and optimal activity was dependent on the presence of both disulfide bonds. Based on these studies, the protegrin analogue, IB–367 (Table 2), was selected for clinical development as a topical agent to prevent the oral mucositis associated with cancer therapy based on the acknowledgment that no effective and approved therapy exists for the prevention or treatment of oral mucositis (151). The possibility that a broad-spectrum antimicrobial agent might reduce the severity of oral mucositis has been supported by limited clinical studies using oral lozenges that contain a mixture of the antibacterial agents polymyxin and tobramycin, and the antifungal agent amphotericin B (152). Three properties of the protegrins (extent of spectrum, speed of kill, and lack of significant resistance induction) make them attractive candidates to use topically as a prophylaxis for the prevention of the polymicrobial infections that exacerbate oral mucositis. IB-367 was shown to be effective at reducing oral microflora and the incidence and severity of oral mucositis in the hamster cheek pouch model and was selected for further development (153).

In human studies, IB-367 was found to be safely delivered topically to patients that develop mucositis in the context of myeloablative chemotherapy. A recently completed phase II study of topical (oral) protegrin involving 177 patients undergoing bone marrow transplantation indicated that administration of this peptide is associated with a statistically significant reduction in mucositis after transplantation and a trend toward a reduced number of febrile days. In patients receiving myeloablative doses of chemotherapy in preparation for a bone marrow transplant IB-367 demonstrated a significant reduction in the severity of oral mucositis (154). IB-367 exhibited a broad-spectrum activity and was rapidly microbicidal in saliva at 1 mg/mL, a concentration readily achieved in the 9-mg dosing regimens used in clinical trials. No significant induction of resistance has been observed with IB-367 and the peptide was active against bacteria resistant to many conventional antibiotics. A phase III study of IB-367 for the prevention of mucositis associated with myeloablative chemotherapy is now underway. Protegrin peptides are also being evaluated as an aerosolized antimicrobial therapy for the chronic respiratory infections of patients with cystic fibrosis. Other clinical studies are underway (154).

2.7. Magainins. The granular glands of frog skin store secretory granules that contain a variety of short (2–3 kDa) and linear microbicidal peptides. Many of these peptides were reported to be potent killers of a broad array of pathogenic microorganisms as well as cancer cells. Two representative (most studied) multimembered families, magainins and dermaseptins, are described herein.

Magainins (I and II) are found in the skin of the African clawed frog, *Xenopus laevis* along with other analogues that are 21–27 residues in length (155). Magainins are broad-spectrum antimicrobial agents exhibiting cidal activity against gram-negative and gram-positive bacteria, fungi and protozoa. In addition, these peptides lyse many types of murine and human cancer cells at concentrations 10- to 20-fold lower than normal human cells (156). Because of their selectivity, broad-spectrum, low degree of bacterial resistance and ease of

chemical synthesis, magainins were developed as human therapeutic agents (157). Through a series of amino acid substitutions and deletions, a 22-residue derivative of magainin II was constructed, MSI-78 (Table 3). MSI-78 (also termed pexiganan) exhibits an enhanced potency relative to that of magainin II against both gram-positive and gram-negative bacteria. The *in vitro* activity of MSI-78 was compared with those of ofloxacin and other antibiotics against fresh clinical isolates. Of 411 aerobic fresh clinical isolates and 61 anaerobes tested, 91 and 97% respectively, were susceptible to MSI-78 (comparable to ofloxacin or ciprofloxacin). Of 10 isolates of *Candida albicans*, 3 were inhibited by MSI-78 (158).

MSI-78 exhibits properties *in vitro*, which make it an attractive candidate for development as a topical antimicrobial agent (158). MSI-78 exhibited broad-spectrum antibacterial activity when tested *in vitro* against 3109 clinical isolates of gram-positive and gram-negative, anaerobic and aerobic bacteria. The MIC at which 90% of isolates are inhibited by MSI-78 (MIC90) was 32 μg/ml or less for *Staphylococcus sp., Streptococcus sp., E. faecium, Corynebacterium sp., Pseudomonas sp., Acinetobacter sp., Stenotrophomonas sp.*, certain species of the family *Enterobacteriaceae, Bacteroides sp., Peptostreptococcus sp.*, and *Propionibacterium sp*.

Comparison of the MICs and minimum bactericidal concentrations (MBCs) of pexiganan for 143 isolates representing 32 species demonstrated that for 92% of the isolates tested, MBCs were the same or within one twofold difference of the MICs. At the concentration of 16 μg/mL, MSI-78 rapidly killed *P. aeruginosa*, reducing viable CFU by 6 log units/mL within 20 min of treatment. No evidence of cross-resistance to a number of other antibiotic classes (oxacillin, cefazolin, cefoxitin, imipenem, ofloxacin, ciprofloxacin, gentamicin, and clindamicin) was observed. No notable difference in potency was observed at pH value ranging from 5.0 to 8.0, indicating that the fundamental antimicrobial activity of MSI-78 does not notably decrease with changes in pH. Attempts to generate resistance in several bacterial species through repeated passage with subinhibitory concentrations of MSI-78 were unsuccessful (160). The antimicrobial spectrum of MSI-78 is broader than those of the current commercially available peptide antibiotics, such as polymyxin (B or E) since polymyxin exhibits no significant activity against gram-positive bacteria, such as staphylococci (161).

In two phase III multicentre randomized double-blind trials in diabetic patients with infected foot ulcers, topical pexiganan (MSI-78) acetate 1% was well tolerated and achieved clinical cure or improvement similar to oral ofloxacin in ~90% of patients. Eradication of pathogens in the two studies was achieved in 82% of ofloxacin recipients and 66% of pexiganan acetate recipients at the end of therapy. *In vitro* susceptibility to pexiganan of bacteria isolated from infected diabetic foot ulcers was assessed during two clinical trials involving the treatment of 835 outpatients with infected diabetic foot ulcers. For the 2515 bacterial isolates tested (2337 aerobes and 178 anaerobes) pexiganan had MIC90 values of 16 μg/mL or less. Pexiganan did not exhibit cross-resistance with other commonly used antibiotics, including β-lactams, quinolones, macrolides, and lincosamides, supporting its potential as a local therapy for infected diabetic foot ulcers (162).

Pexiganan has been developed as a therapeutic antimicrobial agent for the topical treatment of infected diabetic foot ulcers (163). Studies on the stability of a 1% pexiganan cream showed that after a year under stress conditions the

pexiganan cream lost – 15% of the active component to oxidative deamination of the N-terminal glycine residue (164). Preclinical studies have also demonstrated that magainin derivatives exhibit activity *in vivo* against malignant melanoma and ovarian cancer cells in mouse models. Intravenous administration of several magainin analogues has been shown to treat effectively systemic *E. coli* infections in the mouse (155).

2.8. Dermaseptins. The dermaseptins are a large family of linear peptides of $\sim$ 3-kDa, that were isolated from the skin of South American frogs, where they play a major host defense role (165). At nanomolar concentrations, dermaseptins are endowed with poorly understood activities involving the activation of immune functions (38). The overall data collected during a decade of investigation (*in vitro* and *in vivo*), indicates that native dermaseptins-and dermaseptin-based compounds could be useful in a variety of biomedical applications including the treatment of multidrug resistant infectious diseases and/or their prevention (166).

The amino acid sequences of prepro-dermaseptins are composed of three distinct typical domains. The amino terminal domain includes a 22-residue signal peptide that is followed by an acidic leader peptide domain containing 20–24 residues. The third domain consists of a progenitor sequence of variable lengths, coding for a dermaseptin peptide that is flanked by a pair of basic residues at its amino end (a cleavage signal for processing enzymes), and a tripeptide (Gly-Glu-Ala) at its C-terminus. This tripeptide is usually involved in the formation of the C-terminal carboxamide (165).

The structures of dermaseptin members are medium sensitive, readily switching from random coil to amphipathic α-helix in polar and apolar environments, respectively. Some dermaseptin members bind avidly to lipidic structures (168) and they exert rapid (within seconds) cytolytic activity *in vitro* against a variety of pathogenic microorganisms, including bacteria, protozoa, yeast and filamentous fungi (169). Dermaseptins are potent killers of nongrowing and slow-growing bacteria, suggesting a potential use in the eradication of bacteria placed in a dormant state and/or subject to low oxygen tension (170). By contrast, most conventional bactericidal or bacteristatic antibiotics are not effective against dormant bacteria, or those under conditions of low oxygen. Toward possible application as food preservatives, dermaseptins were also been demonstrated to effectively kill spoilage yeast (171).

Various structure–activity relationship studies showed that the activity of native dermaseptins improves in terms of potency and selectivity, while shortening the peptides sequence down to one-third of the original length (Table 4). Some synthetic versions composed of 12–14 residues are as potent as the parent peptide and single point mutations were shown to increase potency by up to 100-fold, and affect the selectivity by reducing toxicity toward mammalian cells (36). These studies showed that antimicrobial action of dermaseptins is readily altered by modifying their primary structure. For example, the range of minimal inhibitory concentrations of a 28 residues dermaseptin S4 derivative, K_4K_{20}-S4, against clinical isolates of *S. aureus* ($n = 23$), *P. aeruginosa* ($n = 17$) and *E. coli* ($n = 26$) were respectively, 0.3–1.5, 0.42–1.5 and 0.45–5 μM. Those of a shorter derivative, K_4-S_4(1–16), were rather similar or slightly higher. Compared with K_4-S_4(1–16), the yet shorter derivative, K_4-S_4(1–13), displayed a

range of MIC values that were generally about twofold higher . The three peptides were rapidly bactericidal *in vitro*, reducing the number of viable CFU of either E. coli or *S. aureus* by six log units in 30 min or less. Furthermore, bacterial resistance did not developed in either one of these dermaseptin derivatives under conditions of serial passage where bacterial resistance was shown in develop to commercial antibiotics. Compared with MSI-78 or PG-1, the short dermaseptin S4 derivative, K_4-$S_4(1-13)$, was at least as potent against bacteria but displayed less toxicity for human erythrocytes (166).

In vivo, naive mice exhibited 75% mortality in the vehicle control group compared to 18 or 36% mortality in mice that received a single intraperitoneal (i.p.) injection of 0.1 mg K_4-$S_4(1-16)$ or K_4-$S_4(1-13)$, respectively. *In vivo* bactericidal activity was confirmed in neutropenic mice where, i.p. administration of K_4-$S_4(1-16)$ reduced the number of viable CFU in a dose-dependent manner by >3 log units within 1 h exposure. Toxicity of these peptides emerged at much higher doses ($LD_{50} = 25$ mg/kg) 166). Overall, the data suggest that short dermaseptin the derivatives could be useful in treatment of infections, including infections caused by multidrug resistant bacteria.

A particularly interesting property of dermaseptins concerns their demonstrable ability to cross the plasma membrane of various mammalian cells in a rapid (instantaneous), energy-independent and receptor-independent manner. This property (which does not include normal erythrocytes) may be relevant with respect to a number of cases as, the need to reach intracellular targets (infected cells) or more generally, to carry inside the target cell a drug attached to the peptide. Thus, dermaseptin derivatives were directed to kill intracellular parasites without harming the host cells (172). Selective activity on intracellular parasites was also described for some insect-derived lytic peptides (173).

In a recent study, the affinity of some dermaseptins to the plasma membrane of red blood cells (RBC) was manipulated to demonstrate the potential use of RBC as a transport vehicle to deliver drugs to distant targets (174). This drug delivery system involved the transient "loading" of RBC with a dermaseptin-based "hook" molecule. Such a "hook" molecule has enough affinity for the RBCs plasma membrane to bind to the membrane and be transported in the blood circulation, but given the opportunity, the "hook" molecule will exit its position and transfer to another (target) cell for which it has a greater affinity. The efficacy of such an affinity driven molecular transfer (ADMT) system was demonstrated experimentally by the transfer of dermaseptin peptides from preloaded RBC to target cells (bacteria, yeast and protozoa). The principle of ADMT might therefore inspire methods for delivery of drugs, or other molecules, via the blood circulation of an animal for the treatment of, eg, infectious diseases or cancer. The "hooks" may contain intrinsic activity such as when the hook is an antimicrobial peptide. Alternatively, an inactive lipophilic hook molecule may be coupled to other drugs, in which case, the hook is used to anchor the drug to the RBC.

3. Uses

3.1. Food Preservatives. In the production of food, it is crucial to ensure the safety and stability of the products for the length of their shelf life. Presently, food manufacturers use one or a combination of a vast array of chemical (eg, weak organic acids) and biological (proteins and nonproteinaceous) compounds. However, pressures coming from both consumers and legislation makes the successful attainment of this objective by the food industry an ever increasing challenge, particularly when the use of some of currently admitted preservatives is restricted in different foods, and when the susceptibility of microorganisms to most currently used preservatives is failing (175).

Various types of peptide-based antimicrobials demonstrate potent activity against microorganisms of concern to the food industry, raising increasing interest as to their potential use with respect to the assurance of food safety and the prevention of spoilage. The only peptide-based antibiotics that are currently in use as food preservatives are nisin (176) and related compounds such as pediocin (177) which are secreted by lactic acid bacteria. The recognition that these peptides have no apparent adverse effects when ingested has led the U.S. Food and Drug Administration to accord them a generally recognized as safe (GRAS) status since 1988 (178). Peptide-based antimicrobials are perfectly suitable for such applications. They are active against a large variety of microorganisms under a wide range of conditions (such as pH, temperature and milieu) and their production is prone to a multitude of possibilities for structural modifications, whether produced by chemical or biological methods. By virtue of the considerable advantages that peptide-based antimicrobials offer, in the future it has been likely see increased uses of new and improved antimicrobial peptides as food preservatives.

3.2. Other Potential Uses. These properties could be put to advantage in a variety of other applications such as to avoid microbial contamination and infection of the host, which are major concerns in the area of therapeutic medical devices, such as catheters and orthopedic devices. For example, efficient antimicrobial activity was reported for various immobilized (polymer-bound) antimicrobial peptides (179). Similarly, the ability of cationic antimicrobial peptides and proteins to bind to LPS (endotoxins) suggests its usefulness, when immobilized on a solid support, for removing contaminating endotoxins from genetically engineered mammalian proteins made in bacteria such as *E. coli* (180).

4. Conclusion

The growing problem of microbial resistance is continually placing emphasis on the need to develop novel classes of antibiotics. Peptide-based antimicrobial agents are presently attracting considerable interest both to fundamental researchers [for their challenging mechanism(s) of action] and to pharmaceutical industries for their potential applications in the various antimicrobial fields. Also, the increasing appreciation of the role played by endotoxemia in the pathophysiology of bacterial infections suggests that optimal treatment requires the targeting of not only bacterial proliferation but the endotoxic properties as

well. In this respect too, antimicrobial peptides appear ideally suitable as they arrest bacterial growth and simultaneously neutralize bacterial endotoxic activity.

However, cationic antimicrobial peptides are still considered to have high potential for toxicity to humans and animals (181) and therefore are of limited suitability for systemic treatment. Based on these characteristics, various animal-derived antimicrobial peptides have been, so far, developed for external use (eg, the magainin-based peptide MSI-78 evaluated for treating diabetic foot ulcer or the protegrin-based peptide IB-367 evaluated for treating oral mucositis) much like their "cousins" the microbe-derived antibiotic peptides. Additional issues of concern include their present status with respect to lack of selectivity (inability to distinguish between pathogenic microorganisms and those of natural flora) and production cost (as long as they are produced by chemical synthesis).

It is nevertheless strongly believed that future developments of these peptides will result in new and improved generations of smaller, more potent, more selective and less toxic peptides. Dermaseptin-based peptide eg, show promising *in vitro* and *in vivo* activity against several pathogens. Their studies have also exhibited that both intravenous and i.p. injections of high concentrations (>10 mg/kg) of dermaseptin derivatives are well tolerated by animals. Thus, dermaseptin-based peptides may exemplify a new generation of peptides that appear to have improved tolerability as compared to the presently known antimicrobial peptides. Their therapeutic index makes them excellent candidates for systemic therapy.

Finally, antimicrobial peptides appear to have unique pharmacokinetics and pharmacodynamic properties; they have a short serum half-life, on the other hand, they are carried by RBCs and are released from the RBCs in the presence of microbes, for which they have a much higher affinity. These properties, combined with the rapid killing of pathogens on exposure to the antimicrobial peptides, may require the development of proper formulations as well as novel strategies of treatment.

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Table 1. **Amino Acid Sequence of Typical Defensins and Their C–C Bonds**

Peptide	Amino acid sequence (one letter code)
α-defensin (HNP-1)	ACYCRIPACIAGERRYGTCIYQGRLWAFCC
β-defensins (BNBD-1)	DFASCHTNGGICLPNRCPGHMIQIGICFRPRVKCCRSW
cryptidin-1	LRDLVCYCRSRGCKGRERMNGTCRKGHLLYTLCCR

Table 2. **Amino Acid Sequence of a Native Protegrin and Its Optimized Derivative** [a,b]

Peptide	Amino acid sequence (one letter code)
PG-1	RGGRLCYCRRRFCVCVGR
IB-367	---/-----G--------

[a]A dash designates identical residue to parent peptide and a slash designates a deleted residue.
[b]The C–C bond pattern is identical in both peptides.

Table 3. **Amino Acid Sequence of a Native Magainin and Its Optimized Derivative**

Peptide	Amino acid sequence (one letter code)[a]
Magainin II	GIGKFLHSAKKFGKAFVGEIMNS
MSI-78	----------KK----------KILKK

[a]A dash designates identical residue to parent peptide.

Table 4. **Amino Acid Sequence of a Native Dermaseptin and Its Optimized Derivatives**

Peptide	Amino acid sequence (one letter code)[a]
Dermaseptin S4	ALWMTLLKKVLKAAAKAALNAVLVGANA
K_4-S4 (1–13)	------K----------

[a]A dash designates identical residue to parent peptide.