Review

ANTIMICROBIAL PEPTIDES: A NEW DAWN FOR REGULATING FERTILITY AND REPRODUCTIVE TRACT INFECTIONS

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SUMMARY

Antimicrobial peptides are primarily concerned with the innate host defense mechanism and distributed in almost all forms of life. Over the years, several such peptides have been isolated, purified and characterized from the digestive, respiratory and reproductive tracts of mammals. These small peptides are coded by genes, the expression of which is regulated by several factors such as hormones, injury, chemical and microbial insults. Our understanding about these peptides has improved over the years and is now possible to commercially produce them in bioreactors on a mass scale. Until now, it has not been demonstrated that microbes acquire resistance to these peptides. Besides, being part of the innate immune system, problems of toxicity are rarely encountered making antimicrobial peptides potential substitutes for antibiotics. These unique peptides are now used as drugs in treatment of inflammatory diseases, as prophylaxis for neutropenic patients, and even in the treatment of septic shock. Due to their compatibility with the digestive tract of animals they have also been tried as food preservatives. Recently, there is an increasing body of evidence to show that peptides isolated from the reproductive tracts could serve dual roles of regulating fertility and preventing sexually transmitted infections. This review consolidates the advancements made in this area of research and development.

Key words: Antimicrobial peptides, microbicide, reproductive tract infections (RTI), spermicide, vaginal contraceptive

INTRODUCTION

Antimicrobial peptides (AMP), also called host defense peptides, are found among all classes of life and are active components of the innate immune response (1, 2). These peptides exhibit potent, broad-spectrum microbicidal activity and could be considered as novel therapeutic agents (3-6). AMPs have been demonstrated to kill Gram-negative and Gram-positive bacteria (including strains that are resistant to conventional antibiotics), mycobacteria (including *Mycobacterium tuberculosis*), enveloped viruses, fungi and even transformed or cancer cells (7-9). Unlike the majority of conventional antibiotics, it appears as though antimicrobial peptides may also have the ability to enhance immunity by functioning as immunomodulators (10). AMPs are an abundant and diverse group of molecules that are produced by many tissues and cell types. Their amino acid composition, amphipathicity, cationic charge and size allow them to attach to and insert into membrane bilayers to form pores (11-16). Although attempts have been made to explain the mechanism of action of these peptides through 'barrel-stave', 'carpet' or 'toroidal-pore' models (17-20), there is need to delve deeper to know how exactly the microorganisms are killed *in vivo*. Recently, there has been speculation that trans-membrane pore formation is not the only mechanism of microbial killing. In fact, several observations suggest that translocated peptides can alter cytoplasmic membrane septum formation and inhibit the synthesis of cell-walls, nucleic-acids, protein and enzymatic activity (21).

GENERAL STRUCTURE

Antimicrobial peptides of animal origin are short proteins generally comprising of 12 to 50 amino acids. These peptides include two or more positively charged residues provided by arginine, lysine or, in acidic environments, histidine, and a large proportion (generally >50%) of hydrophobic residues (22). The secondary structures of these molecules follow 4 themes, including i) alpha-helical, ii) beta-stranded due to the presence of two or more disulphide bonds, iii) beta-hairpin or loop due to the presence of a single disulphide bond and/or cyclization of the peptide chain, and iv) extended. Many of these peptides are unstructured in free solution, and fold into their final configuration upon partitioning into biological membranes. The ability to associate with membranes is a definitive feature of antimicrobial peptides although

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membrane permeabilization is not always the outcome (23-26). The characteristics that affect antimicrobial property and specificity are in Table 1.

Table1. Characteristics that affect antimicrobial activity and specificity

Size

The size of antimicrobial peptides varies from 6 amino acid residues for anionic peptides to greater than 59 amino acid residues for Bac7. Even di- and tripeptides with antimicrobial activity have been reported.

Sequence

Peptides often contain the basic amino acid residues lysine or arginine, the hydrophobic residues alanine, leucine, phenylalanine or tryptophan, and other residues such as isoleucine, tyrosine and valine. Some peptides contain amino acid repeats. Ratios of hydrophobic to charged residues can vary from 1:1 to 2:1.

Charge

Anionic peptides are rich in aspartic and glutamic acids and cationic peptides are rich in arginine and lysine. Anionic peptides that are complexed with zinc, or highly cationic peptides, are often more active than neutral peptides or those with a lower charge.

Conformation and structure

Antimicrobial peptides can assume a variety of secondary structures including: alpha-helices, relaxed coils and antiparallel beta-sheet structures. Amphipathic alpha-helical peptides are often more active than peptides with less-defined secondary structures. Peptides with a gamma-core motif (two anti-parallel beta-sheets with an interposed short turn) are often very active.

Hydrophobicity

This characteristic enables water-soluble antimicrobial peptides to partition into the membrane lipid bilayer.

Amphipathicity

A trait by which peptides contain hydrophilic amino acid residues aligned along one side and hydrophobic amino acid residues aligned along the opposite side of a helical molecule. For alpha-helical peptides, amphipathicity is often expressed as a hydrophobic moment, which is the vector sum of hydrophobicity indices, treated as vectors normal to the helical axis. Other peptides often show spatial separation of polar and hydrophobic residues that is less easy to quantify.

Table 2. Classes of antimicrobial peptides showing their distribution in the animal kingdom

Anionic peptides	· Maximin H5 from amphibians. · Small anionic peptides rich in glutamic and aspartic acids from sheep, cattle and humans.
Linear cationic alpha-helical peptides	· Cecropins (A), andropin, moricin, ceratotoxin and melittin from insects. · Cecropin P1 from Ascaris nematodes. · Magainin, dermaseptin, bombinin, brevinin-1, esculentins and buforin II from amphibians. · Pleurocidin from skin mucous secretions of the winter flounder. · LL37 from humans.
Cationic peptides enriched for specific amino acids	· Proline- and phenylalanine-containing peptides include prophenin from pigs. · Tryptophan-containing peptides include indolicidin from cattle. · Small histidine-rich salivary polypeptides, including the histatins from man and some higher primates
Anionic and cationic peptides that contain cysteine and form disulphide bonds	· Peptides with 1 disulphide bond include brevinins. · Peptides with 2 disulphide bonds include protegrin from pigs and tachyplesins from horseshoe crabs. · Insect defensins (defensin A). · Peptides with >3 disulphide bonds include drosomycin in fruit flies and plant antifungal defensins.
Anionic and cationic peptide fragments of larger proteins	· Lactoferricin from lactoferrin. · Casocidin I from human casein. · Antimicrobial domains from bovine alpha-lactalbumin, human haemoglobin, lysozymeand ovalbumin.

From proteomic analysis it is abundantly clear that several of the structurally diverse peptides conferred with antimicrobial activity show varying degrees of sequence homology (27, 28). The similarity is due to the functional convergence and the variations are largely due to the different sources from which they have been isolated and the additional roles played by these peptides (29). Several attempts have been made to establish structure-activity relationships (30-32). Detailed analysis shows that the epithelial cells belonging to the digestive, respiratory and reproductive tracts in the same species of animal produce variants of AMPs. Not only are the conditions (pH, temperature, humidity, etc.) at each of these sites special but also the resident microbes at these locations are unique. For instance, the microflora of the intestinal tract is not the same as the resident microbes of the genital tract. It must be emphasized that each AMP in isolation does not offer complete protection against all microbes. This implies that the same peptide may have different effects on different microbes. However, it is the abundance and multiplicity of the antimicrobial peptides that preferentially allow the commensal microbiota to survive while providing an intrinsic resistance to colonization by pathogens at different sites along the tract. The classes of antimicrobial peptides, showing their distribution in the animal kingdom, are in the table 2.

There are basically two views regarding the origin of AMPs. The first hypothesis suggests that the AMPs are coded by several special genes located on specific chromosomes (33). The expression of these genes is dependant upon several factors which include hormones, chemical, physical and microbial insult among others (34). The second hypothesis suggests that large proteins are first synthesized and their enzymatic cleavage by proteolysis result in bioactive fragments that exhibit antimicrobial activity (35). In general the antimicrobial activity of AMP is determined by measuring the minimal inhibitory concentration (MIC), which is the lowest concentration of a substance that reduces growth by more than 50%. The modes of action by which AMPs kill bacteria is varied and includes disrupting membranes, interfering with metabolism, and targeting cytoplasmic components (36-38). In many cases the exact mechanism of killing is not known.

THE QUEST FOR AMPs WITH MULTIPLE FUNCTIONS

Animal models indicate that host defence peptides are crucial for both prevention and clearance of infection (39,40). An emerging concept is that certain AMPs have multiple roles in host defense that supersede their bacteriostatic or bactericidal capacities. Many peptides, initially isolated as and termed “antimicrobial peptides” have now been shown to have more significant alternative functions *in vivo*. They not only kill bacteria directly but also have a number of immunomodulatory functions that may be involved in the clearance of infection, including the ability to alter host gene expression. At times, they may also act as chemokines and/or induce chemokine production, inhibit lipopolysaccharide-induced pro-inflammatory cytokine production, promote wound healing, and modulate the responses of dendritic cells and cells of the adaptive immune system. It is, therefore, time to explore the possibility of using natural peptides as drug leads for more than one purpose (41-44).

The multiple functions of AMPs have an important role to play in maintaining reproductive health in human subjects. As of today the number of deaths on account of unplanned pregnancies and reproductive tract infections is on the rise. At least in countries like India, there is an urgent need for developing options that allow women to prevent or delay pregnancy and to protect themselves from STDs. The acquired immunodeficiency syndrome (AIDS) is the latest of these maladies, and it appears to thrive and flourish in the midst of overpopulation, poverty and other sexually transmitted diseases (STDs). Therefore, the development of dual function microbicides is now recognized as an urgent global priority. Among various preventive strategies the development of microbicides with or without contraceptive properties, has recently gathered momentum, owing to better science, increased funding and political pressure. For women willing to prevent pregnancy and STDs, dually active contraceptive microbicides offer convenience as well as additional safety and sexual empowerment (45, 46).

THE FEMALE GENITAL TRACT: A POTENTIAL TARGET FOR THE DUAL ACTIONS OF AMP

The reproductive tract of mammals has a fairly large population of specific, transient and resident microorganisms in and around the genital opening, primarily due to its direct contact with the external environment. The conditions prevalent at this site also create a favorable ecological niche for microbial growth and colonization. In addition, sexual activity increases the chances of invasion by pathogenic microorganisms as well. Despite this, the upper reaches of the genital tract are relatively free from microorganisms. Obviously, there are effective mechanisms that restrict the movement of microbes into the upper genital tracts.

Table 3. Some antimicrobial peptides/proteins from the reproductive tracts of mammals

Peptide/protein	Localization	Mode of action
Lysozyme (muramidase)	Vaginal fluid, mucosal plug	Disrupt essential microbial structures, Hydrolyse peptidoglycan of bacterial cell wall
Lactoferrin	Vaginal fluid, mucosal plug	Bind essential nutrients in acid conditions Disrupt microbial membranes Inhibits viral fusion and entry
Secretory Leukocyte protease inhibitor (SLPI)	Cervical mucus,vaginal secretion	Inhibition of viral entry
Elafin	Cervix, endo metrium, vagina	Regulates proteolytic enzymes during menstruation
Secretory Phospholipase A2	Mucosal secretions	Destabilization of membrane
Cathelicidins	Seminal plasma, mucosal secretions, vaginal secretions	Prevents infection following sexual intercourse Binds and neutralizes LPS Protects against endotoxic shock
Calprotectin,	Neutrophils, monocytes, keratinocytes, vaginal fluid	Inhibits growth of microbes, sequesters zinc, stimulates transmission of HIV
Histones	Vaginal fluid	Cell permeabilization, Electrostatic attraction to anionic microbial surface
Defensins (alpha and beta)	Epithelial cells, ectocervix, vagina, keratinocytes, testis, epididymis, seminal plasma, sperm, germ cells	Penetrate microbial membranes, Impair metabolic processes

Evidence that cationic antimicrobial peptides and proteins have fundamental roles in the innate host defense of reproductive tracts has only recently become available (47-49). Table 3 shows a representative list of some host-derived antimicrobial peptides. Although these molecules are not solely confined to the reproductive tract, they all have therapeutic potentials. However, it would be best to focus on using the AMPs as therapeutics at the same site from where they are recovered since they would rarely invoke adverse immunological reactions. Thus, for tackling unwanted pregnancies and RTIs it is desirable that we concentrate on dual function vaginal AMPs isolated from the epithelial cells lining the genital tracts. This concept has clinical advantage over the currently marketed detergent or other types of spermicides since AMPs purified from inherent sources do not disturb the natural microflora and lack genital epithelial toxicity (50, 51). By the same logic, bacteriocins generated from the natural microflora of the reproductive tracts, which have generally regarded as safe (GRAS) status, have also proved useful. Nisin, a bacteriocin produced by *Lactococcus lactis* is one such peptide that has been demonstrated to have potent contraceptive properties in addition to its antimicrobial effect (52).

Recently, we conducted *in vitro* experiments to test the antimicrobial (Fig.1) and spermicidal activity of a 4.5 kDa cationic peptide isolated from the cervix of goats. We found that at concentrations of 35-200 µg/mL the peptide had dose- and time- dependent bactericidal activity against both Gram-positive and Gram-negative bacteria but not spermatozoa. We inferred that this could be a natural mechanism to favor conception and prevent reproductive tract infections. In comparable *in vitro* experiments conducted with Nisin it was reported that 10-20 fold higher concentrations are required to immobilize spermatozoa (Sander-Cramer test: 20 sec) than the minimal inhibitory concentrations (MIC: 10-50 µg/mL) required to inhibit growth of various pathogens (52, 53). Taken together, these results suggest biphasic effects of AMPs at low and high concentrations with respect to sperm and microbes making them ideal molecules for use as spermicidal microbicides. In fact two kinds of molecules, a) those that are spermicidal and antimicrobial, and b) those that are microbicidal but not spermicidal, would be of commercial interest.

It must be mentioned that most of the antimicrobial proteins/peptides are found in low levels in sub-clinical inflammation and healthy subjects. The expression of one or more of these peptides in the reproductive tracts is related to the hormonal profiles, the physiological state of the tissues involved and level of infection/injury. Further, the differential expression of these peptides has a significant effect on the natural population and the consortium of the microflora in the lower genital tracts. The situation is more

complicated than we can imagine since there are occasions when one AMP stimulates transmission of viruses (54) while another present at the same location inhibits the same (55, 56). At times, the AMP that has drastic effect against one microbe may have little or no impact on the other. Thus, it would be prudent to thoroughly check all the properties of isolated AMP before thinking of employing them for therapeutic purposes.

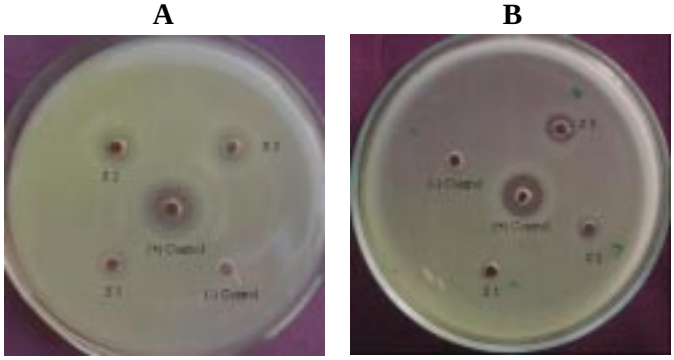

Fig.1. An *in vitro* radial diffusion assay conducted with a 4.5 kDa cationic antimicrobial peptide isolated from the cervical epithelium of goat. Clear zones are seen around the wells, the diameter of which reflects the intensity of antimicrobial activity. **A**) Results of tests conducted on Gram-positive (*B. subtilis*); **B**) Results of tests conducted on Gram- negative bacteria (*E. coli*).

Vaginal contraceptive products have been available for many years. However, the major drawback of using these products is the cytotoxic effect on vaginal cells (57, 58). Besides, some are also known to inactivate lactobacilli and other normal flora that are beneficial to vaginal tissues (50, 51, 59). Disturbance of the vaginal microflora can lead to vaginal infections, which in turn increase the chances of STI/HIV transmission. Therefore, development of vaginal spermicidal microbicides lacking toxicity may offer a considerable clinical advantage over the currently marketed spermicides. Since these compounds would probably be used repeatedly over decades, the safety aspect of the spermicidal microbiocide should be probed.

CONTRACEPTIVE EFFICACY OF ANTIMICROBIAL PEPTIDES

Unwanted pregnancies and prevention of sexually transmitted infections (STIs) is a predicament that threatens the present day world. Although we have separate remedies for treating infections and preventing conception, an option that can provide dual protection would be of great significance. For a contraceptive to be universally acceptable the main criteria are effectiveness, safety and reversibility. Varying degrees of success and satisfaction are provided by the range of contraceptives available in the market. Nevertheless, except for the barrier methods of contraception most of the devices do not protect against sexually transmitted diseases and opportunistic pathogens. Women prefer to use vaginal contraception since this option gives them privacy even from their partners (60-62). At the present time, spermicides incorporated into creams and gels are popular as vaginal contraceptives. But most of them do not protect against microbial infections (63).

Table 4. Antimicrobial contraceptive agents for vaginal prophylaxis

Compound	Action on microbes	Action on fertility process
Mandelic acid condensation polymer (SAMMA)	Non-toxic towards native resident microbes. Prevents infectivity of HIV, Herpes. Prevents multiplication of Chlamydia, Neisseria Not mutagenic	Non-toxic to host epithelial cells. Inhibits acrosin & hyaluronidase. Induces acrosome loss. Safe in viginal irritation assay
Nonoxynol-9	Broad-spectrum antibacterial activity in vitro. Not effective in vivo. Inactivate protective vaginal microbes. Promotes HIV entry	Potent cytotoxicants that destroy cervical and vaginal cells. Prolonged use causes irritation, inflammation and ulceration of lower genital tract
Gel microemulsions (GM4 and GM144)	Solubilizes lipophilic antiviral/ antimicrobial agents	Exhibit rapid spermicidal activity. Does not produce local inflammation or damage to the vaginal mucosa or epithelium. No local, systemic or reproductive toxicity
Nisin	Antibacterial	No local inflammation or damage to vaginal epithelium. Rate of absorption and clearance rapid. Block conception. Spermatozoa more susceptible than RBC or vaginal epithelium
Magainin A	Antibacterial, antifungal, antiviral	Inhibition of sperm motility. Spermicidal activities. No abnormalities in morphology of vaginal epithelial cells

Of late several molecules, both synthetic and natural, have been tried as contraceptive microbicides, a few of which are listed in table 4. Being part of the innate host defense, antimicrobial peptides have an advantage over synthetic peptides since they are not toxic to the vaginal epithelium and protect the resident microbes while averting the entry of invading microorganisms. Although the lower genital tract serves as the portal for the entry of infectious and sexually transmitted diseases (STDs), knowledge about immune mechanisms of the vagina in health and disease continues to be fragmentary. Nevertheless, microbicides capable of reducing the risk of STIs/HIV infection as well as controlling fertility are urgently needed (64-65). The combined spermicidal and antimicrobial properties of AMPs could make these molecules a safe, affordable and easy to use vaginal contraceptive for future therapeutic interventions.

CONCLUSION

The continued high rates of unintended pregnancies and the unrelenting expansion of sexually transmitted diseases including AIDS, especially in India, warrant the development of novel strategies to help individuals avoid these risks. Dually active compounds displaying contraceptive and microbicidal/anti-human immuno deficiency virus (anti-HIV) properties constitute one such strategy. There is mounting interest in exploring the role of inherent antimicrobial peptides and proteins in the innate host defense of the reproductive tract. Little emerging resistance to AMPs has been reported even upon repeat exposure of microbes under laboratory conditions. Evidence suggests that the overall protection can be best achieved from the combined/multiple effects of host-derived AMP molecules. Though microbes must have frequently encountered antimicrobial peptides through evolution, these natural antibiotics still remain highly effective against many microbial targets. It appears that a cocktail of AMPs could lead to the formulation of an effective contraceptive microbicide in the near future.

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