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The human male reproductive tract antimicrobial peptides of the HE2 family exhibit potent synergy with standard antibiotics

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Reproductive tract infections pose a serious threat to health and fertility. Due to the emergence of antibiotic resistant pathogens, antimicrobial proteins and peptides of the reproductive tract are extensively characterized in recent years toward developing newer strategies to treat genital tract infections. Pathogen growth inhibition using a combination of naturally occurring male reproductive tract antimicrobial peptides and commonly used antibiotics has not been reported. Checker board analyses were carried out to determine the nature of interaction (synergistic, additive and antagonistic) between HE2α and HE2β2 peptides and the commonly used antibiotics. Using *Escherichia coli* as the target organism, the minimal inhibitory concentration and fractional inhibitory concentration indices were determined. We demonstrate for the first time that the human male reproductive tract antimicrobial peptides HE2α and HE2β2 act synergistically with the commonly used antibiotics to inhibit *E. coli* growth. A combination of HE2α and HE2β2 peptides resulted in an additive effect. Interestingly, the synergistic effects of HE2 peptides were highest with doxycycline and ciprofloxacin, antibiotics generally used to treat epididymitis. Results of this study demonstrate the potential of endogenous HE2 peptides to be pharmacologically important in designing novel strategies to treat reproductive tract infections. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: epididymis; antimicrobial; synergy; fractional inhibitory concentration index

Introduction

Antimicrobial proteins and peptides are widely expressed in both plants and animals. A variety of natural antibiotics belonging to different classes such as defensins, cathelicidins, cecropins and protease inhibitors [1] are found in epithelial tissues of organs that are exposed to the external environment. Among them, well characterized in humans are the defensins, which are broadly classified into three types, viz. alpha, beta and theta defensins depending on their disulfide bonding, tissue distribution and genomic organization. They exhibit broad spectrum antimicrobial activity [2–5], thus forming an important component of the innate immune system. Antimicrobial proteins and peptides including defensins are generally cationic in nature [6] and are believed to exert their bactericidal effect by permeabilizing the bacterial membranes [7], thinning the membrane [8] or by destabilizing the membrane bilayer [9]. In addition to these effects, antimicrobial proteins and peptides kill bacteria by inhibition of macromolecular biosynthesis [10–12] and/or interacting with specific vital components inside the bacteria [13,14].

In the epididymis, a major organ of the male reproductive tract, immature sperm released from the testis develop forward motility and fertilizing ability as a result of a series of sequential maturation steps. A wide variety of proteins including antimicrobial proteins released into the lumen of epididymis bind sperm and are thought to play an important role in epididymal immunity in addition to their role in sperm maturation [15]. Examples of antimicrobial proteins reported in the male reproductive tract include human cationic antimicrobial protein (hCAP18, a cathelicidin) [16], defensins [17–20], the epididymal β-defensin member Bin1b [21], cystatins [22,23], lactoferrin [24] seminalplasmin [25], seminogelin-derived peptides [26] and members of the HE2 family [27]. The HE2 gene located on chromosome 8p23 within the β-defensin gene cluster encodes a series of isoforms containing identical proregions joined to different *C*-terminal peptides [27]. Among them, HE2β1 conserves the characteristic β-defensin-like six-cysteine motif. Furthermore, like the β-defensins, HE2 *C*-terminal peptides are cleaved from their proregions by a furin-like proprotein convertase and these peptides are reported to exist in the epididymal epithelium, luminal fluid and the seminal plasma [28]. We previously identified and characterized an epididymis specific novel defensin, DEFB118, which also conserves the characteristic six-cysteine motif [29]. The antimicrobial activity of HE2α, HE2β1 and HE2β2 proteins and their *C*-terminal peptides against *E. coli* [30] and HE2α against *Neisseria gonorrhea, Staphylococcus aureus* and *Enterococcus faecalis* [31] was previously demonstrated. Their antimicrobial activities are structure dependent, salt tolerant and their mechanism of action involves interacting with and permeabilizing bacterial membranes and inhibition of macromolecular synthesis [30,32–34].

The ability of reproductive tract specific defensins and defensin-like proteins and peptides to display antimicrobial

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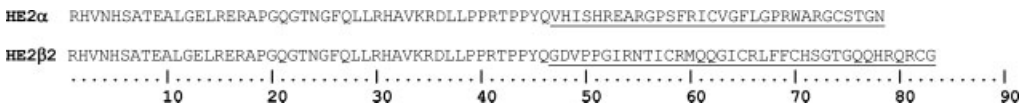

Figure 1. Amino acid sequences of epididymal HE2 proteins. The *C*-terminal amino acid sequences (underlined) of HE2α and β2 were synthesized and used in this study.

activity against *E. coli* and reproductive tract pathogens projects them as potential therapeutic agents to treat sexually transmitted diseases. Current regimens to treat sexually transmitted diseases such as epididymitis involve the administration of antibiotics. For example, when *Chlamydia trachomatis* and *N. gonorrhoeae* are the cause of infection, ceftriaxone and doxycycline are used, whereas when coliform bacterial infections are suspected, ofloxacin or levofloxacin is recommended [35]. Development of resistance by pathogens to conventionally used antibiotics has led to the identification and characterization of a variety of natural and synthetic peptide antibiotics that have the potential to be used to effectively treat infections. However, studies that demonstrate the effectiveness of microbial killing when antibiotics are used in combination with the natural reproductive tract antimicrobial peptides are unknown. In this study, for the first time, we demonstrate the synergistic antibacterial ability of reproductive tract antimicrobial peptides in combination with the commonly used antibiotics to treat genital infections. Results of this study provide vital information for the development of novel strategies to treat sexually transmitted diseases that involve using antibiotics in combination with reproductive tract specific antimicrobial peptides.

Methods

Antibiotics and Peptide Synthesis

Antibiotics used in this study – carbenicillin, amphicillin, ciprofloxacin, kanamycin, chloramphenicol, tetracycline, doxycycline, gentamicin, streptomycin and rifampicin – were obtained commercially (Sigma Aldrich, St Louis, MO). HE2α and HE2β2 *C*-terminal peptides (a kind gift from Dr Susan H. Hall and Dr Frank S. French, Laboratories for Reproductive Biology, University of North Carolina, Chapel Hill, NC) were individually tested in combination with each of the antibiotics. The amino acid sequences of the peptides used are shown in Figure 1. They were synthesized at the Peptide Synthesis Facility, University of North Carolina, Chapel Hill by standard fluoren-9-ylmethoxycarbonyl (f-moc) solid phase procedures using Rainin symphony multiple peptide synthesizer (Rainin Instrument, Woburn, MA). The purified peptides eluted as single peaks upon reverse phase HPLC and were further demonstrated to have the correct molecular weight by MALDI-TOF mass spectrometry.

Fractional Inhibitory Concentration Assay for Synergy

The synergistic antibacterial killing activity of HE2 peptides in combination with antibiotics was carried out by checker board analyses as described earlier [36] using *E. coli* XL-1 blue (Stratagene, La Jolla, CA) as the target organism. Though the incidence of epididymitis is lower with *E. coli* when compared with other reproductive tract pathogens, due to constraints in maintaining and culture of pathogenic organisms, *E. coli* was chosen as the target organism in this study. Initial dose dependent bacterial killing activity of HE2 peptides and antibiotics were analyzed by adding increasing amounts to the microtiter plate wells along with the bacteria to determine the MIC. Control wells were also maintained with no peptide or antibiotic added to the bacteria. Bacterial growth was measured by reading the absorbance at A_{600} 18 h after the addition of the peptide or antibiotic. The MIC is read as the minimal concentration necessary to inhibit growth by at least 90%, when compared to the no peptide or no antibiotic control well. To determine the fractional inhibitory concentrations (FICs), 50 µl of Luria-Bertani medium was added to each well in a 96 well microtiter plate followed by addition of 50 µl of antibiotic to the wells (A1 to A8) in the first row of the microtiter plate and double dilutions added from row 1 to row 7. Then the peptide was added to the wells (1A to 1H) of the first column and double dilutions added from column 1 to column 7. The concentration of each antibacterial agent added ranged between 4X MIC and 1/16X MIC. With these dilutions, row 8 and column 8 serve as antibiotic only treated and peptide only treated controls, respectively. The 64th well (H8) serves as no peptide or no antibiotic control. To each well, 10 µl of bacteria corresponding to 1×10^6 CFU/ml were added and incubated at 37 C for 18 h. The FIC index (FICI) was calculated by the following formula:

$$\text{FICI} = \text{FIC of peptide} + \text{FIC of antibiotic} = (\text{peptide})/(\text{MIC of peptide}) + (\text{antibiotic})/(\text{MIC of antibiotic}) \quad (1)$$

where (peptide) is the concentration of the peptide in the microtiter well that is the lowest inhibitory concentration of the peptide in its column or row and (MIC of peptide) is the MIC of the peptide alone; (antibiotic) and (MIC of antibiotic) are defined in the same way. An FICI of <0.5 indicates synergy, whereas it is considered additive when the index is >0.5 and <1.0. An FICI of >1.0 indicates antagonism. Assays were performed independently three times and the average FICI calculated.

Results

The MICs of the peptides and the antibiotics used in this study were initially determined. The MICs of HE2α and HE2β2 peptides were found to be 17.2 ± 0.6 and 6.4 ± 0.2 µM, respectively (Table 1). The MICs of the different antibiotics used in this study are also given in Table 1.

Our previous studies demonstrated the potent antibacterial killing ability of HE2α and HE2β2 peptides [30–33]. In order to determine whether these two peptides can interact and display enhanced bacterial killing, a checker board analysis was carried out. The average FICI was found to be 0.7 ± 0.1 when HE2α and HE2β2 peptides were used in combination, suggesting an additive nature of interaction (Table 2).

Development of synthetic or natural peptide antibiotics to treat diseases caused by antibiotic resistant pathogens has recently become a major area of investigation. Further, treating antibiotic resistant pathogens with antibiotics in combination with antibacterial peptides is an emerging strategy. To determine whether epididymal antimicrobial peptides can exhibit improved bacterial growth inhibition when used in combination with

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Table 1. MIC of the peptides/antibiotics tested

Peptide/antibiotic	MIC (μM)
HE2α	17.2 ± 0.6
HE2β2	6.4 ± 0.2
Ampicillin	14.3 ± 0.8
Chloramphenicol	7.7 ± 0.7
Carbenicillin	12.0 ± 0.3
Ciprofloxacin	1.8 ± 0.3
Doxycycline	10.4 ± 1.1
Gentamicin	1.3 ± 0.2
Kanamycin	0.9 ± 0.1
Rifampicin	6.4 ± 0.7
Streptomycin	0.8 ± 0.1
Tetracycline	1.1 ± 0.1

Table 2. FICI and the nature of interaction between HE2α peptide, HE2β2 peptide and antibiotics

	FICI (nature of interaction)
HE2β2	0.7 ± 0.1 (A)
Ampicillin	0.3 ± 0.06 (S)
Chloramphenicol	0.3 ± 0.04 (S)
Carbenicillin	0.3 ± 0.007 (S)
Ciprofloxacin	0.3 ± 0.04 (S)
Doxycycline	0.2 ± 0.01 (S)
Gentamicin	0.3 ± 0.008 (S)
Kanamycin	0.3 ± 0.001 (S)
Rifampicin	0.3 ± 0.003 (S)
Streptomycin	0.3 ± 0.03 (S)
Tetracycline	0.3 ± 0.02 (S)

(A) indicates additive; (S) indicates synergistic interaction.

Table 3. FICI and the nature of interaction between HE2β2 peptide and antibiotics

	FICI (nature of interaction)
Ampicillin	0.3 ± 0.04 (S)
Chloramphenicol	0.3 ± 0.02 (S)
Carbenicillin	0.2 ± 0.04 (S)
Ciprofloxacin	0.1 ± 0.04 (S)
Doxycycline	0.2 ± 0.007 (S)
Gentamicin	0.2 ± 0.003 (S)
Kanamycin	0.2 ± 0.04 (S)
Rifampicin	0.2 ± 0.002 (S)
Streptomycin	0.1 ± 0.04 (S)
Tetracycline	0.4 ± 0.03 (S)

(A) indicates additive; (S) indicates synergistic interaction.

antibiotics, checkerboard analyses were performed using HE2α or HE2β2 peptide and the commonly used antibiotics against *E. coli*. The nature of the interaction between HE2α peptide and the antibiotics seem to be synergistic as indicated by the average FICI (Table 2). Interestingly, a combination of ciprofloxacin or doxycycline (the most commonly used antibiotics to treat epididymitis) and HE2α peptide exhibited the best growth inhibition, with an FICI of about 0.26 ± 0.01. HE2β2 peptide when used in combination with various antibiotics exhibited synergistic effect (Table 3). Similar to HE2α peptide, its synergistic effect was best when used in combination with ciprofloxacin or doxycycline. The average FICIs of HE2β2 peptide in combination with various antibiotics (ranging from 0.38 to 0.1) seems to be much lower than that observed for HE2α peptide (0.36 to 0.2).

Discussion

Treatment of reproductive tract infections is a global challenge and current regimens involve the use of antibiotics. Prolonged use of antibiotics leads to the development of pathogen resistance, which necessitates the identification of a variety of peptide antibiotics that are promising in treating diseases caused by these antibiotic resistant pathogens. A strategy to circumvent the problem of the emergence of antibiotic resistant bacterial strains is to use new antimicrobial compounds and/or combination therapy. The combination therapy is generally used to increase the *in vivo* activity, to prevent the emergence of drug resistance and to broaden the antimicrobial spectrum. Recently, the increasing incidence of reproductive tract infections and the need to design novel therapeutic approaches to counteract them provided impetus to efforts to identify and characterize novel antimicrobial proteins and peptides of the reproductive tract. Earlier, we demonstrated that HE2 proteins and their *C*-terminal peptides exhibit salt tolerant and structure dependent antimicrobial activities utilizing mechanisms involving permeabilization of both outer and inner bacterial membranes [30] and inhibition of macromolecular synthesis [32]. Further, these peptides have been shown to exhibit antibacterial activity against reproductive pathogens, viz. *N. gonorrhea* and *S. aureus* [31]. There were earlier studies on the combined use of antimicrobial, antifungal and antiviral peptides to inhibit microbial growth in combination with conventionally used antibiotics or drugs [37–39]. However, to our knowledge this is the first report on the nature of interaction and ability of reproductive tract antimicrobial proteins and peptides to kill bacteria in combination with conventionally used antibiotics. Results of this study demonstrate that a combination of the synthetic HE2α and HE2β2 peptides exhibit an additive inhibitory effect on *E. coli* growth. Moreover, HE2α or HE2β2 peptide in combination with an antibiotic acts synergistically to inhibit bacterial growth. These results suggest that HE2α and HE2β2 peptides are potentially valuable for the treatment of reproductive tract infections in combination with antibiotics.

Cationic antimicrobial peptides can cross the outer membrane of Gram-negative bacteria by the self-promoted uptake pathway [40], which involves the high affinity binding of the peptide to surface lipopolysaccharide, resulting in the displacement of divalent cations that stabilize adjacent lipopolysaccharide molecules [41,42] leading to destabilization of the outer membrane. Our previous studies demonstrate that HE2 peptides bring about bacterial killing by membrane permeabilization and inhibition of macromolecular synthesis. It is possible that the synergistic effect observed in this study could be due to enhanced entry of antibiotic into the bacterial cell through the membrane pores created by the peptide. Synergistic action between antimicrobial peptides and antibiotics that involves membrane permeabilization was previously shown for a variety of peptides such as the α helical peptide p18 [38], menstrual hemocidin [43] and defensins [44]. The nature of interaction between the defensins and

antimicrobial proteins and peptides of the reproductive tract has been demonstrated earlier. For example, cathelicidins or the human CAP18/LL37 can act synergistically with defensins to bring about bacterial killing [45]. Though antimicrobial peptides that cause pores in the membrane are expected to increase the uptake of antibiotics when used in combination, this effect alone was found not to be sufficient to show synergistic effects. For example, synergy was not observed when synthetic peptides that have the ability to permeabilize the membranes of *E. coli* were used in combination with vancomycin or ampicillin [46], suggesting that increased access of intracellular targets to antibiotics due to membrane permeabilization by peptides as well as the secondary effects that the peptides can effect are important for synergy. The synergistic bacterial killing observed when HE2 peptides were used in combination with common antibiotics could be due to their ability to form pores in the membrane facilitating increased entry of antibiotics as well as the secondary effects of these peptides, i.e. inhibition of macromolecular synthesis.

In this study, we observed that a combination of HE2α and β2 peptides exhibited an additive effect. The inability of HE2α and β2 peptides to act synergistically with each other could be due to their similar mechanisms of action on a single target, the bacterial membrane. On the same lines, basing on previous studies it should be mentioned that synergy is not necessarily observed when antimicrobial peptides are used in combination with commonly used antibiotics. Absence of synergism has been attributed to various factors that govern the activity of lytic peptides. For example, no synergy was observed when synthetic antimicrobial peptides were used in combination with antibiotics against *S. aureus* [46]. Similar observation was made when bovine lactoferricin was used in combination with various antibiotics [47]. The absence of synergistic effects in these cases was due to the low MICs of the peptides used and it becomes experimentally difficult to assess synergy. On the same lines, it is also noteworthy to mention that depending on the chemical structures of antibiotics used in combination with polyethylenimine, a polycationic synthetic polymer, the effects were either synergistic or antagonistic or indifferent [48]. PGLa, a synthetic antimicrobial peptide, exhibits synergy with magainin (containing a 23 amino acid hydrophobic tail) but not with certain synthetic peptides that lack this tail [46]. Varying structural features of lytic peptides may allow aggregation or competition between the peptides to bind to the membranes of target organisms, thereby making it difficult to measure the synergistic actions.

In conclusion, we report that the antibacterial peptides of the male reproductive tract exhibit synergistic bacterial killing when used in combination with the conventionally used antibiotics. Results of this study may provide vital information to develop novel strategies to treat reproductive infections.

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