A hybrid cationic peptide composed of human beta-defensin-1 and humanized theta-defensin sequences exhibits salt-resistant antimicrobial activity

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Running Title: Antimicrobial activity of a hybrid cationic peptide.

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ABSTRACT

We have designed a hybrid peptide by combining sequences of human β-defensin-1 and θ-defensin, in an attempt to generate a molecule that combines the diversity in structure and biological activity of two different peptides to yield a promising therapeutic candidate. HBD-1 was chosen as it is a natural defensin of humans that is constitutively expressed but its antibacterial activity is considerably impaired by elevated ionic strength. θ-defensins are expressed in the human bone marrow as a pseudogene that are homologous to rhesus monkey circular minidefensins. Retrocyclins are synthetic human θ-defensins. The cyclic nature of the θ-defensin peptides makes them salt resistant, non-hemolytic, and virtually non-cytotoxic in vitro. However, developing a non-human circular molecule for clinical use would be less viable as compared to a linear molecule. In this study, we have fused the C-terminal region of HBD-1 to the nonapeptide sequence of a synthetic retrocyclin. Cyclization was achieved by joining the terminal ends of the hybrid peptide by a disulfide bridge. The hybrid peptide exhibited enhanced antimicrobial activity with or without the disulfide bridge against both Gram-negative and Gram-positive bacteria as well as against fungi, including clinical bacterial isolates from eye infections. The peptide retained activity in the presence of NaCl and serum and was non-hemolytic in vitro. Thus, the hybrid peptide generated holds potential as a new class of antibiotics.

Keywords: antimicrobial activity; beta-defensin; retrocyclin; bacteria; fungus; clinical strains; salt sensitivity; serum; non-hemolytic
INTRODUCTION

Defensins are a group of peptides that are important components of host defense. They have the ability to recognize and neutralize invading microorganisms quickly and specifically (1-3). These peptides are diverse members of a large family of cationic host defense peptides (HDP) that are widely distributed throughout the animal kingdom (2, 4, 5). These cysteine-rich peptides vary in their length, the spacing of their cysteine residues, and their disulfide connectivities (4, 5). Defensins show antimicrobial activities against Gram-negative and Gram-positive bacterial strains, fungi, as well as some parasites and enveloped viruses (1, 2, 4, 6). Mammalian defensins can be classified into α-, β- and θ-defensins based on disulfide connectivities (2, 4, 6-8). Only α- and β-defensins are expressed in humans. θ-defensin (found in rhesus monkey) displays the same connectivity associated with that of the α-defensins but is not functionally present in humans (7). θ-defensins are much smaller (18 amino acid residues) than α- and β-defensins (29-45 residues), and their antiviral properties are considerably more pronounced than their antibacterial and antifungal effects (9, 10).

β-defensins are found in epithelial cells that line mucosal surfaces, which provide the first line of defense between an organism and the environment (2, 6). To date, four human β-defensins (HBD-1 to -4) have been characterized (5, 11-19). HBD-1 is expressed in epithelia that are directly exposed to the environment or microbial flora (e.g. in the eye, lung, mammary gland, salivary gland, kidney, pancreas and prostate (16, 17, 20, 21). HBD-1 shows anti-bacterial activity at micromolar concentrations against some Gram-negative bacteria (i.e. Escherichia coli, Pseudomonas aeruginosa, and Klebsiella pneumoniae), as well as the yeast Candida albicans (22). When tested in vitro, HBD-1 is relatively less potent against the Gram-positive Staphylococcus aureus (12, 17, 23), but these microbicidal activities are inhibited at high concentrations of NaCl (23).

θ-defensins have been purified only from the leukocytes and bone marrow of rhesus macaques (7, 8, 24). These are circular octadecapeptides with six cysteines pairing to form a ladder-like disulfide array that connects their two antiparallel β-sheets (7). Human bone marrow expresses θ-defensin homologues but the human retrocyclin gene does not produce a functional product owing to a premature stop codon (7, 9). Six rhesus macaque θ-defensin isoforms (RTDs 1–6) are expressed in neutrophils where they are packaged in cytoplasmic granules. Retrocyclins...
are synthetic humanized θ-defensins, whose structures are based on human multiple θ-defensin pseudogenes (7). They are octadecapeptides that contain two linked nonapeptides that may be identical or different. The N-terminus of one nonapeptide forms a peptide bond with the C-terminus of another nonapeptide resulting in a cyclic 18 residue peptide with three intramolecular disulfide bonds (7, 8). θ-Defensins have both Gram-positive and Gram-negative antibacterial activity (8, 25) as well as anti-fungal (8) and anti-viral (9, 26) activities. Retrocyclins have been shown to protect human cells from infection by HIV-1 (9) and have been evaluated as a topical anti-HIV agent for the prevention of HIV transmission (10, 27). θ-defensins have also been shown to inactivate germinating anthrax spores and act as a competitive inhibitor of anthrax lethal factor protease (28).

The emergence of resistance to antibiotics (29, 30) has led to a growing interest in exploiting the biological activities of host defense peptides as a new class of therapeutic agents to target multidrug-resistant pathogens. The main advantage of these peptides as factors of innate immunity is that they can function without either high specificity or memory (2).

The C-terminal cationic segment of HBD-1(Phd-1) with a single disulfide bond exhibits antimicrobial activity (31). However, the peptide is inactive in the presence of high concentrations of NaCl. With a view to generate a peptide with improved antimicrobial activity, we have designed a hybrid peptide composed of the C-terminal segment of HBD-1 and the RIGRRIC segment of θ-defensin. The hybrid peptide has greater number of R residues as compared to K which could enhance binding to the microbial cell surface. We have observed that the hybrid peptide shows antimicrobial activity against Gram-negative and Gram-positive bacteria, fungi, as well as multidrug resistant bacterial strains isolated from patients with eye infections. Antimicrobial activity was not attenuated in the presence of physiological concentrations of NaCl. Antimicrobial activity was also observed in the presence of serum except against E. coli.

MATERIALS:

9-Fluorenylmethoxycarbonyl (Fmoc) protected amino acids were purchased from Novabiochem (Merck). Fmoc-L-NovaSyn-Cysteine-TGA resin was obtained from Novabiochem (Merck). N-Hydroxybenzotriazole hydrate (HOBT) and 2-(1H-benzotriazole-1-yl)-1,1,3,3-
tetramethyluronium hexafluorophosphate (HBTU) were from Advanced Chemtech (Louisville, KY). Piperidine was from Loba-Chemie Pvt. Ltd (India). Reagents for deprotection of peptides were purchased from Sigma Chemical Co. (St. Louis, MO), 5(6)-Carboxyfluorescein (CF) was from Molecular Probes, Life Technologies.

METHODS:

Peptide synthesis:

The hybrid peptide hBTD-1 was synthesized by solid-phase methods manually, using Fmoc-L-Cys- (Trt)-Novasyn TGA resin and 9-fluorenlymethoxy carbonyl chemistry as described earlier (31). Peptides were cleaved from the resin using trifluoroacetic acid containing thioanisole, meta-cresol and ethanedithiol (10:1:1:0.5, v/v). Formation of disulfide bonds was accomplished by air oxidation in 20% (v/v) aqueous dimethyl sulfoxide (32) at a concentration of 0.5 mg/ml for 24 h at room temperature. Peptides were purified by HPLC on a reversed phase C-18 (Hi-pore reversed phase column 4.6 mm 250 mm) column using gradients of solvents: A; 0.1% (v/v) TFA in H$_2$O, B; 0.1% (v/v) TFA in CH$_3$CN. Purified peptide was characterized by Matrix-assisted laser desorption ionization time-of-flight mass spectrometry on Applied Biosystems 4800 instrument in the Proteomics facility of CSIR-CCMB using recrystallized α–cyano-4-hydroxycinnamic acid as matrix. The theoretical mass calculated was 2737.32 and the observed m/z was 2736.67.

Labeling of hBTD-1 with carboxyfluorescein (CF) at the free amino group of the N-terminal amino acid was carried out by treating 10 mg of resin-bound peptide with 0.8 ml of dimethylformamide containing CF and activating agents as described earlier (33). The deprotection of CF-labeled peptide from the resin, purification, and characterization by mass spectrometry were carried out as described earlier (31).

Antibacterial activity:

Bacterial strains used were *Escherichia coli* (MG 1655), *Staphylococcus aureus* (ATCC 8530), and *Pseudomonas aeruginosa* (NCTC 6751). Clinical strains tested were obtained from Jhaveri Microbiology Centre, L. V. Prasad Eye Institute, Hyderabad. All strains were isolated from clinical samples from patients with ocular infection. The bacterial isolates tested included...
multi drug resistant (MDR) *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Burkholderia cepacia*, *Corynebacterium amycolatum* and *Staphylococcus epidermidis*. All isolates were tested for antibiotic susceptibility to a large number of antibiotics by Kirby Bauer disc diffusion method and interpreted as per CLSI guidelines.

Antibacterial activity of the peptide was examined in sterile 96 well plates at a final volume of 100 µl as follows: Bacteria were grown in nutrient broth (Bacto Difco nutrient broth) to mid-log phase and diluted to $10^6$ colony forming units (cfu)/ml in 10 mM sodium phosphate buffer (pH-7.4). Bacteria were incubated with different concentration of peptide for 2 h at 37°C and suitably diluted aliquots were spread on nutrient agar plates. After the plates were incubated at 37°C for 18 h, colonies formed were counted. Lethal concentration is the concentration of the peptides at which no viable colonies were formed. Cell survival is expressed as a percentage of the control. Percentage killing was calculated as: \[ \frac{\text{colonies from control cells} - \text{colonies from treated cells}}{\text{colonies from control cells}} \times 100 \]. The LC determined was average of three independent experiments done in duplicate. In control experiments, cells were incubated with only buffer.

Activity of the peptide analogs was also tested in the presence of 1mM DTT. The disulfide bridges were broken by incubation with DTT at 37°C for 1h. Antimicrobial assay was performed with reduced peptide in the presence of 1 mM DTT. In all the experiments, untreated peptide and cells in the presence of 1mM DTT were used as controls.

To determine the effect of salt on antibacterial activity, different concentrations of NaCl were included in the incubation buffer at their LC. Different concentrations of divalent cations Ca$^{2+}$ and Mg$^{2+}$ (as their chloride salts), were included in the buffer to determine their effect on activity at lethal concentration of the peptides. In control experiments, cells were incubated with only buffer. Data are presented as mean ± standard deviation computed for three independent replicates.

**Antifungal activity:**

Fungal strains used were *Candida albicans* and *Saccharomyces cerevisiae*. Minimum fungicidal concentration (MFC) of the peptide was determined by growing the fungi aerobically.
in yeast extract-peptone-dextrose (YEPD) medium at 30°C. After 20 h, 0.5 ml from this suspension was subcultured for 2 h in 20 ml of YEPD broth to obtain a mid-log-phase culture. Cells were harvested by centrifugation, washed with 10 mM phosphate buffer (PB), pH 7.4 and resuspended in the same buffer, and the concentration was adjusted to 10^6 cells/ml. Aliquots of diluted cells were incubated with peptide in 100 μl volumes at 30°C for 2 h. Cell suspensions were diluted and spread onto YEPD agar plates, and the plates were incubated for 24 h at 30°C.

Colonies were counted, and the concentrations of the peptides at which no viable colonies were formed were taken as the Minimum Fungicidal Concentration (MFC). Cell survival was expressed as a percentage of the control. Percentage killing was calculated as [(colonies from control cells - colonies from treated cells)/colonies from control cells x 100]. The average of results from three independent experiments done with duplicate samples was taken for the calculation of MFC. In control experiments, cells were incubated with only buffer.

To determine the effect of salt on antifungal activity, different concentrations of NaCl was included in the incubation buffer at their LC. Different concentrations of divalent cations Ca^{2+} and Mg^{2+} (as their chloride salt, were included in the buffer to determine their effect on activity at lethal concentration of the peptides. In control experiments, cells were incubated with only buffer. Data are presented as mean ± standard deviation computed for three independent replicates.

**Kinetics of Killing:**

The kinetics of microbial killing were determined against Gram-negative, Gram-positive bacteria and fungi. Mid-log-phase bacteria (10^6 CFU/ml) were incubated with LC of the peptide in 10 mM sodium phosphate buffer (pH 7.4) in a final volume of 100 μl. Aliquots of 20 μl were removed at fixed intervals and spread on nutrient agar plates. The CFU were counted after an incubation of 18 h at 37°C and 28°C in case of bacteria and fungi respectively. Data are presented as mean ± standard deviation computed for three independent replicates.

**Serum sensitivity:**

To study the effect of serum components on the antimicrobial activity of the peptide, serum was isolated from human blood after the removal of erythrocytes by centrifugation. Serum
isolated was used for the study without heat inactivation. For the antimicrobial activity, 20% serum was included in the buffer before the addition of peptides. Cells with only serum were used as a control.

Circular Dichroism (CD):
Spectra were recorded in 10 mM phosphate buffer (pH 7.4), Trifluoroethanol (TFE), and 10 mM sodium dodecyl sulphate (SDS) micelles on a JASCO J-715 automatic recording spectropolarimeter at 25°C using quartz cell of 1 mm path length. Each spectrum (185-250 nm) was an average of six scans. Data are represented as mean residue ellipticities.

Hemolysis:
The hemolytic activity of the analogs was determined using human erythrocytes as described earlier (34). Briefly, erythrocytes were obtained by the centrifugation (800 x g) of heparinized blood and were washed thrice with 5 mM HEPES (pH 7.4) containing 150 mM NaCl. Aliquots containing 10⁷ red blood cells/ml were incubated in the presence of different peptide concentrations in 0.5-ml tubes containing a fewer volume of 100 μl for 30 min at 37°C with gentle mixing. The samples were centrifuged, and the absorbance of the supernatants at 540 nm was measured. Erythrocyte lysis occurring in deionized water was taken as maximal lysis.

MTT assay:
The toxicity effect of hBTD-1 on the human cell line HEK 293 was analyzed at enzymatic level through MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. In 96-well cell culture plates (Costar) 1 X 10⁵ cells were seeded and allowed to adhere to the surface for 12-14 hours. After settling of the cells, a fresh serum-free medium containing various concentrations of hBTD-1 was added to the wells. In the control, no test solution was added. The cells were allowed to grow for 24 hours and to each well 10 μl of MTT reagent (5 mg/ml stock concentration) was added to give final concentrations of 0.5 mg/ml. The reaction was stopped after observing purple precipitates in the control wells. The media were carefully removed without disturbing the cell layers and dried. In the dried wells, DMSO was added and incubated overnight to solubilize the precipitates. The absorbance of the solubilized material was
taken at 550 nm using Versa Max Microplate Reader (Molecular Devices, Sunnyvale, California). The experiments were performed in triplicate.

**Outer membrane permeabilization assay:**

The ability of peptide to permeabilize the outer membranes of *E. coli* MG 1655 cells was investigated using an NPN uptake assay (35, 36). Briefly, cells at an optical density at 600 nm (OD$_{600}$) of 0.1 were suspended in 5 mM HEPES (pH-7.4) with 10 mM NPN. After 15 min of incubation, peptide was added, and the fluorescence of NPN was monitored. The excitation wavelength used was 350 nm, and the emission wavelength was 420 nm. The experiment was carried out at 25°C.

**Inner membrane permeabilization assay:**

The ability of peptide to permeabilize the inner membranes of bacteria was studied using *E. coli* GJ 2455 (lac lac$^+$ strain derived from *E. coli* GJ2544 at Centre for Cellular and Molecular Biology), which constitutively expresses β-galactosidase in its cytoplasm. O-Nitrophenyl β-D-galactopyranoside (ONPG) was used as the substrate for β-galactosidase in the assay (37). Late-logarithmic-phase (OD$_{600}$, 0.5 to 0.6) cells were washed and diluted to an OD$_{600}$ of 0.03 in 10 mM sodium phosphate buffer (pH-7.4), and 0.53 mM ONPG was added. After the addition of different peptide concentrations, OD measurements were made every 5 min at 550 nm and 420 nm, and absorbance calculations [$A_{420} - (1.75 \times A_{550})$] were taken as a measure of the β-galactosidase activity. The production of o-nitrophenol was monitored at 420 nm. The value 1.75 x $A_{550}$ represents the light scattering by cell debris at 420 nm. Bacterial cells without any peptide and without ONPG were used as a control.

**Confocal Microscopy:**

Localization of the peptide was analyzed by treating *E. coli*, *C. albicans* and *S. cerevisiae* with CF- labeled hBTD-1 and FM4-64 and propidium iodide (PI) for 15 min, respectively. Organisms were grown overnight in nutrient broth and yeast extract-peptone-dextrose medium, respectively, washed with 10 mM phosphate buffer (pH-7.4) and the concentration were adjusted to 1 x $10^7$ cells/ml in 10 mM phosphate buffer. These cells were treated with sub-lethal concentration of peptide for different time intervals. Peptides were incubated for 5 min, 10 min,
20 min and 30 min at 37°C, to capture different stages of peptide entry, localization on the membrane and killing. The cells were examined with a Zeiss LSM 510 META confocal microscope. Optical sectioning was done at 1 airy unit by using the 488- and 543-nm-wavelength laser lines with a 100x oil lens objective. Emission data were collected using 500- to 530-nm band-pass and 565- to 615-nm band-pass filters for CF and PI, respectively, in the multitrack mode. Z-sections were acquired at 0.35-µm intervals and projected using the LSM-FCS software version 3.2. The bright-field images were obtained simultaneously using the transmitted-light detector. Images shown are representative of the different events (Initial interaction with the membrane and subsequent translocation into the cells) that could be captured.

RESULTS AND DISCUSSION:

Peptide design:

All mammalian defensins are cationic (2, 4, 38, 39). However, the R/K ratio is variable as also the number of R and K residues. R residues play an important role in modulating the activity of defensins (40-43). Hence, a hybrid peptide containing the C-terminal K-rich segment of HBD-1 and R-rich domain of θ-defensin was designed and synthesized. Sequences of peptides are shown in Table 1 (25, 44, 45). Since defensin analogs with a single disulfide bridge do show activity, only two cysteines were incorporated so that a disulfide bond can be formed. The hybrid peptide has a R/K ratio > 1. We designed the hybrid peptide to improve the activity spectrum and stability of HBD-1, which is active against certain Gram-negative bacteria and Candida albicans (22). The antibacterial activity of HBD-1 is attenuated in the presence of physiological concentrations of NaCl (23). The antimicrobial activities of HBD-1 and RTD-1-3 are shown in Table 2. RTD-1-3 has been shown to be active against the reported microorganisms at very low concentrations (25). Hence, we attempted to design an analog of HBD-1 with improved activity and stability by fusing its C-terminal sequence with the sequence from Human Retrocyclin. Cyclization was achieved by joining the terminal ends of the hybrid peptide by a disulfide bridge.

Antimicrobial activity:
We have examined antimicrobial activity of the hybrid peptide against Gram-negative, Gram-positive bacteria and fungi such as *C. albicans* and *S. cerevisiae*. HBD-1 is constitutively expressed by corneal and conjunctival epithelial cells (20, 46). It is rendered inactive by high salt concentrations present in tear fluid (47). Retrocyclin-2 has been explored as an anti-viral therapeutic agent in a murine model of HSV-1 keratitis (48). Hence, we have examined the activity of the hybrid peptide against clinical strains isolated from human eye.

The antimicrobial activity of the peptide against representative microorganisms is summarized in Table 3. The lethal concentration of the peptide was found to be 1 μM against the Gram-negative and 2 μM against Gram-positive bacterial strains and in case of fungal strains the minimum fungicidal concentration (MFC) was 2 μM. Activity was not lost when the disulfide bridge was broken by DTT (data not shown).

Reports of *in vitro* activity of θ-defensin against drug resistant strains have been reported. Lamers and co-workers (49) have characterized an analog of Retrocyclin (RC-101) that is preventative of *S. aureus* nasal carriage at a minimum concentration of 2.5 μM. Similarly another group has shown RTD-1 to be active against antibiotic resistant *S. aureus* and *P. aeruginosa* at 3 μM (50). However, there are no reported values of HBD-1 *in vitro*, against the multidrug resistant bacterial strains. Hence we checked the activity of the hybrid peptide against a few multidrug resistant bacteria. The data against clinical strains are summarized in Table 4. The peptide was found to be active against multidrug resistant (MDR) *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Corynebacterium anmycolatum*, *Staphylococcus epidermidis*, where the LC was 2 μM (Table 4). However, the hybrid peptide was not active when tested against *Burkholderia cepacia*. The resistance of the *B. cepacia* to the peptide is possibly due to the unusual structure of its LPS. It has been reported that the LPS of *B. cepacia* complex lowers the anionic charge of the cell surface, which inhibits the binding and subsequent effects of cationic antibiotics (51).

The hybrid peptide was active in the presence of serum against different microorganisms. However, a slight increase in the LC was observed (Table 3). Despite being rich in R and K, the peptide is active in the presence of serum suggesting that it is refractory to the action of proteases and binding to serum components. Vogel and co-workers (52), have shown that
peptide cyclization by disulfide formation results in increased serum stability and microbicidal activity of the peptides.

The hybrid peptide was inactive in the presence of serum when tested against *E. coli*. Similar result was observed in case of RTD-3, which exhibited activity against *S. aureus* whereas, against *E. coli* its activity was lost (25). In certain *E. coli* strains, a high molecular weight polysaccharide, O-antigen is produced which are not agglutinated by the serum unless heated (53). It is possible that this O-antigen binds to the peptide and inactivates it.

**Kinetics of Killing:**

Killing of bacteria and fungi (reduction in log CFU/ml) as a function of time at the LC is shown in Fig 1. Rates of killing of the microbes tested were comparable. Killing was observed at earlier time points. However, complete killing (100 %) was observed after 30 min.

**Salt sensitivity:**

In the presence of NaCl, in case of Gram-negative bacteria, peptide activity is only marginally attenuated at 100 and 150 mM concentrations of NaCl (Fig 2A). While, in the case of Gram-positive bacteria and fungi, the peptide exhibited decrease in activity (Fig 2A & 2B). In contrast, in the case of Gram-negative bacteria, the peptide showed complete loss of activity even at low concentrations of the divalent cations, Ca$^{2+}$ and Mg$^{2+}$ (Fig 2A). Whereas, in the case of Gram-positive bacteria and fungi, activity of the peptide was only marginally attenuated (Fig 2A & 2B).

Our results suggest that a stretch of K residues followed by R results in favourable electrostatic interaction with Gram-negative bacteria even in the presence of NaCl. Loss of antibacterial activity against Gram-negative bacteria in the presence of Ca$^{2+}$ and Mg$^{2+}$ arises as these divalent cations stabilize the LPS network preventing binding of the peptide to the surface of Gram-negative bacteria.

**Cell lysis:**

The hemolytic activity of the peptide against human erythrocytes was determined as a major measure of peptide toxicity toward higher eukaryotic cells. The hybrid peptide hBTD-1
does not exhibit lysis even at 100 µM concentration (Table 5), which is 100 times more than its LC (Lethal concentration) against the microorganisms.

The viability of HEK 293 cells were analyzed through MTT assay (Table 5). The data indicated no significant difference among different concentrations of hBTD-1 (10, 50 and 100 µM) which is comparable with the control (without peptide). The result suggests that the peptide does not have any effect on the viability of human cell lines.

CD:

In order to examine whether any secondary structural feature is responsible for the antimicrobial activity of hBTD-1, CD spectra (Fig 3A) were recorded in buffer, SDS micelles and the structure promoting solvent trifluoroethanol (TFE) (54). The spectrum in buffer shows a minima ~198 nm and crossover < 190 nm suggesting predominantly unordered conformation (55). In 10 mM SDS and TFE, the spectra show a negative band ~208 nm, shoulder ~220 nm, cross-over ~200 nm and positive band ~195 nm. These features are characteristic of peptides in helical conformation (55). The lower negative ellipticity value suggests lower helical content in SDS as compared to TFE. However, it is clear that the peptide has the propensity to fold into helical conformation on encountering negatively charged hydrophobic molecules as in bacteria or fungi cell surfaces and also medium of low dielectric constant. The helical wheel diagram of hBTD-1 (AC^9PIFTKIQGTYRGKAKRIGRRC^5) is shown in Fig 3B. While, the peptide is not amphipathic, the positioning of cationic residues in a helical conformation could facilitate effective interaction with negatively charged surfaces. Since the peptide is not amphipathic, it does not lyse erythrocytes and cultured cells, a characteristic feature of several cationic helical amphipathic peptides (56, 57). Specificity towards the microbial membranes and having no hemolytic property highlights the potential of the peptide to be used as a therapeutic agent or a drug scaffold.

Outer and Inner Membrane Permeabilization:

NPN, a neutral hydrophobic probe, has been used to examine the permeabilization of the outer membranes of Gram-negative bacteria. The peptide permeabilized the outer membrane of E. coli in a concentration-dependent manner, as observed by an increase in NPN fluorescence
When the ability of the peptide to permeabilize the inner membrane of *E. coli* was examined by the ONPG assay, influx of ONPG was observed, suggesting that the peptide was effective in permeabilizing the inner membrane (Fig 4B).

Preferential binding of the hybrid peptide to anionic membranes, compared with zwitterionic membranes, correlates with high activity against microbes and low hemolytic properties. It also indicates that electrostatic attractions between the cationic peptide and the anionic bacterial membrane are important for the antibacterial activity.

**Confocal Microscopy:**

Interaction of peptide with *E. coli*, *C. albicans* and *S. cerevisiae* was examined using CF-labeled hBTD-1 by confocal microscopy. Antimicrobial activity of the CF-labeled peptide was comparable to that of the unlabeled peptide. The cellular localization of the peptide was investigated using CF-hBTD-1 and confocal microscopy analysis.

In the case of bacteria, the labeled peptide was seen uniformly localized on the membrane as indicated by the ring like green fluorescence all along the membrane (Panel A of Fig 5). Subsequently, a diffused intracellular green fluorescence was observed (Panel B of Fig 5), which indicates the translocation of the peptides into the cells. The images show localization of the peptide on the cell surface followed by internalization. The images do not indicate co-localization with the FM 4-64 stained inner membrane. The peptides appear to be bound to the outer membrane. After initial localization on the outer membrane, the peptides translocate into the cytosol. Though the peptides traverse through the inner membrane the lack of diffuse staining indicates that they are rendered leaky but not solubilized. All the cells in the frame are not permeabilized as sub-lethal concentration of peptide was used for the study.

In the case of *C. albicans*, the peptide initially binds to the fungal cell surface (Panel A of Fig 6 and Fig 7). This is followed by entry into the cytoplasm (Panel B of Fig 6). The peptide then completely translocates into the cells as indicated by the increased green fluorescence (Panel C of Fig 6). The staining by PI and the peptide indicates extensive intracellular damage (Panel D of Fig 6). The membrane compromised cells take up PI and the diffused red staining is due to the spreading of the nuclear material in the dead cells. Control cells showed negative
staining for PI. *S. cerevisiae* is killed by a similar mechanism. Permeabilization and entry is more rapid in the case of *S. cerevisiae* (Fig 7).

The mechanism of bacterial and fungal killing involves initial binding to the cell surface resulting in destabilization, thereby providing a pathway for entry into the cytoplasm where they interfere with metabolic process resulting in cell death. The confocal images exclude a detergent-type lysis. We propose that such a mechanism would render development of resistance difficult or impossible as the metabolic cost would be very high for the microorganisms as demonstrated recently in amphotericin B resistant *C. albicans* (58) Even in the case of magainin-resistant strains, it has been shown that resistance mechanism is complex involving several metabolic pathway, although the peptide presumably acts by permeabilizing membranes (59).

Linear peptides with minimal length and without multiple disulfide bridges would be attractive candidates as therapeutic candidates. Another approach that would reduce the costs of peptide therapeutics is, improving peptide stability and pharmacokinetics. In the present study, the hybrid peptide that we have designed is a molecule that has activity < 5 µM, kills fairly rapidly and exhibits stability under physiological conditions. Moreover, it is not toxic to human cells. Although cysteines were introduced, a disulfide bridge does not appear to be necessary for antimicrobial activity. The findings from the present work suggest that production of β- and θ-defensin in the form of hybrid peptide is a promising strategy for the development of clinically relevant molecules for therapeutic applications and may lead to a cost-effective solution for the large-scale production of antimicrobial peptides.

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REFERENCES:


Table 1: Primary structure of HBD-1, Human Retrocyclin and the hybrid peptide.

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<tr>
<th>Peptide</th>
<th>Sequence(^a)</th>
<th>Net Charge</th>
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<tr>
<td>HBD-1</td>
<td>DHYN(^1)C(^2)VSSGGQC(^2)LYSAC(^3)PIFTKIQGTC(^2)YRGKAKC(^3)C(^2)K</td>
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</tr>
<tr>
<td>θ-defensin (Retrocyclin)</td>
<td>c(RC(^1)IC(^2)GRRIC(^2)-RC(^1)IC(^3)GRRIC(^3))</td>
<td>+6</td>
</tr>
<tr>
<td>hBTD-1</td>
<td>AC(^1)PIFTKIQGTYRGKAKRIGRRI(^1)C(^1)</td>
<td>+7</td>
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</tbody>
</table>

\(^a\)Disulfide bridges are shown by superscript numbers adjacent to cysteines in bold.
Table 2: Antimicrobial activities of HBD-1 and RTD-1-3

<table>
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<th>Peptide</th>
<th>E.coli</th>
<th>S. aureus</th>
<th>C. albicans</th>
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<td></td>
<td>LC (μM)</td>
<td>MFC (μM)</td>
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<sup>ab</sup> Values are from (44, 45).
<sup>c</sup> Values are from (25).
ND–Not determined.
Table 3: Antimicrobial activity of hBTD-1.

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>LC (µM)</th>
<th>LC (µM) in 20 % serum</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>1</td>
<td>IA</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Organism</th>
<th>MFC (µM)</th>
<th>MFC (µM) in 20% serum</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. albicans</em></td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>2</td>
<td>5</td>
</tr>
</tbody>
</table>

The values reported are average of three independent experiments done in duplicates. Variations observed were ~2%. IA-Inactive.
Table 4: Antimicrobial activity of hBTD-1 against clinical strains.

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Clinical Diagnosis</th>
<th>Type of sample</th>
<th>Resistance Phenotype&lt;sup&gt;a&lt;/sup&gt;</th>
<th>LC (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Corynebacterium amycolatum</em></td>
<td>Microbial keratitis</td>
<td>Corneal scraping</td>
<td>CHL CIP MXF</td>
<td>2</td>
</tr>
<tr>
<td><em>Multi-drug resistant P. aeruginosa</em></td>
<td>Microbial keratitis</td>
<td>Corneal scraping</td>
<td>AMK AMX AMC CAZ CRO CXM CTX CHL CIP COT GAT GEN IPM MEM MXF OFX PIP TZP TIC TOB</td>
<td>2</td>
</tr>
<tr>
<td><em>Methicillin resistant S. aureus</em></td>
<td>Microbial keratitis</td>
<td>Corneal scraping</td>
<td>CFZ FOX CTX CXM CIP MXF OFX</td>
<td>2</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>Post operative endophthalmitis</td>
<td>Vitreous biopsy</td>
<td>None</td>
<td>2</td>
</tr>
<tr>
<td><em>Burkholderia cepacia</em></td>
<td>Microbial keratitis</td>
<td>Corneal scraping</td>
<td>AMK AMX AMC CFZ CXM CHL CIP CST GAT GEN MEM MXF OFX TIC TOB</td>
<td>IA</td>
</tr>
</tbody>
</table>

<sup>a</sup>AMK, amikacin; AMX, amoxicillin; AMC, amoxyclav; CAZ, ceftazidime; CXM, cefuroxime; CHL, chloramphenicol; CIP, ciprofloxacin; CST, colistin; CFZ, cefazolin; CTX, cefotaxim; COT, cotrimoxazole; CRO, ceftiraxone; FOX, cefoxitin; CAZ, ceftazidime; GAT, gatifloxacin; GEN, gentamicin; IPM, imipenem; MEM, meropenem; MXF, moxifloxacin; OFX, ofloxacin; PIP, piperacillin; TZP, piperacillin/tazobactam; TOB, tobramycin; TIC, ticarcillin;

IA-Inactive.
Table 5: Mean values of optical density values of MTT and Hemolytic assay.

<table>
<thead>
<tr>
<th>Assays</th>
<th>hBTD-1 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>MTT</td>
<td>0.9920 ±0.027</td>
</tr>
<tr>
<td>Hemolysis</td>
<td>0</td>
</tr>
</tbody>
</table>

The values reported are mean values (±SD) of optical density recorded for three independent experiments done in duplicates.
Figure Legends:

**Figure 1:** Kinetics of killing of the hybrid peptide hBTD-1. Mid-log phase of both bacteria and fungi (10^6 CFU/mL) were incubated with peptides at LC for different time points. Data are means of three independent experiments. Standard deviation values ranged between 0.02-0.3.

**Figure 2:** Effect of NaCl and divalent cations on antibacterial and antifungal activity of the hybrid peptide. Mid-log phase of both bacteria and fungi (10^6 CFU/mL) were incubated with peptides at LC in the absence and presence of the indicated concentrations of NaCl and divalent cations. (A) represents antibacterial activity where, *E. coli* (■), *P. aeruginosa* (□), *S. aureus* (■) (B) represents antifungal activity where, *C. albicans* (■), *S. cerevisiae* (□). The data are mean values of three independent experiments and the error bars represent standard deviation of the measurements. Standard deviation values ranged between 0.3-1.5.

**Figure 3:** (A) Circular dichroism spectra of hBTD-1. CD spectra were recorded in 10 mM phosphate buffer (■), 10 mM SDS (● ) and TFE (▲). (B) Helical wheel representation of hBTD-1. The hydrophilic residues are represented as circles, hydrophobic residues as diamonds, potentially negatively charged as triangles, and potentially positively charged as pentagons. Peptide does not show amphipathicity.

**Figure 4:** Membrane permeabilization assays: (A) Outer membrane permeabilization of *E. coli* MG-1655 by hBTD-1. Permeabilization of the outer membrane was monitored as an increase in NPN fluorescence intensity in the presence of increasing concentrations of peptide (0-5 µM), as indicated adjacent to the traces. (B) Inner membrane permeabilization of *E. coli* GJ2544 by hBTD-1 as a function of time at different concentrations (0-5 µM) at 37°C. Hydrolysis of ONPG by β-galactosidase was used to monitor inner membrane permeabilization by absorbance at 420 nm.

**Figure 5:** Confocal microscope images of *E. coli* in the presence of CF-labeled hBTD-1. Bacterial cells (1 x 10^7) were treated with CF labeled peptide and FM4-64 and were incubated for different time points. Panel A represents the initial time point where the peptide is found spread uniformly on the bacterial membrane. Panel B represents a subsequent time point wherein the peptide is found to be uniformly distributed inside the cell (increased green fluorescence is shown by the arrow). The bar represents 5 µm.
Figure 6: Confocal microscope images of *C. albicans* in the presence of CF-labeled hBTD-1. *C. albicans* cells (1 x 10^7) were treated with CF labeled peptide at sub-lethal concentration and with 2 μg/ml of PI. The treated cells were incubated for different time points to capture different events during the process of peptide entry into the cell. Panel A represents an early time point wherein the peptide is found uniformly distributed over the membrane. Panel B represents the stages wherein, the peptide diffuses into the cells (green) and the membrane compromised cells are seen taking up PI stain (red). Panel C represents increased amount of peptide diffusion and PI spread at a subsequent time point. Panel D represents dead cells (yellow in the merged panel). Green fluorescence represents the diffused peptide and red the diffused nuclear material. The bar represents 5 μm.

Figure 7: Confocal microscope images of *S. cerevisiae* in the presence of CF-labeled hBTD-1. *S. cerevisiae* cells (1 x 10^7) were treated with sub-lethal concentration of CF labeled peptide and with 2 μg/ml of PI. The treated cells were incubated for different time points to capture different events during the process of peptide entry into the cell. Panel A represents an early time point wherein the peptide is found uniformly spread over the membrane. Panel B represents the stages wherein, the peptide diffuses into the cells (green) and the membrane compromised cells are seen taking up PI stain (red). Panel C represents increased amount of peptide diffusion and PI spread at a subsequent time point. Panel D represents dead cells (yellow in the merged panel). Green fluorescence represents the diffused peptide and red the diffused nuclear material. The bar represents 5 μm.
Fig 1.
Fig 2:

A

NaCl (mM)

% killing

0 50 100 150

B

NaCl (mM)

% killing

0 50 100 150

CaCl₂ (mM)

% killing

0 1 2 5

CaCl₂ (mM)

% killing

0 1 2 5

MgCl₂ (mM)

% killing

0 1 2 5

MgCl₂ (mM)
Fig 5:

<table>
<thead>
<tr>
<th>CF-Peptide</th>
<th>FM 4-64</th>
<th>Merged</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image A" /></td>
<td><img src="image2.png" alt="Image B" /></td>
<td><img src="image3.png" alt="Image C" /></td>
</tr>
</tbody>
</table>

0 min

3 min 45 sec
Fig 6:

![Image of cell culture experiment](http://aac.asm.org/)

<table>
<thead>
<tr>
<th>CF-Peptide</th>
<th>PI</th>
<th>Merged</th>
<th>BF</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td>0 min</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td>1 min 20 sec</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td>2 min 45 sec</td>
</tr>
<tr>
<td>D</td>
<td></td>
<td></td>
<td>5 min 20 sec</td>
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