Rationale based, de novo design of dehydrophenylalanine containing antibiotic peptides and systematic modification in sequence for enhanced potency

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Abbreviations; AMPs, antimicrobial peptides; CD, circular dichroism; CFU, colony forming units; diSC3-5, 3,3’ - Dipropylthiadicarbocyanine iodide; MIC, minimum inhibitory concentration; NPN, 1-N-phenylnaphthylamine; PI, propidium iodide; SDS, sodium dodecyl sulphate.
Abstract

Increased microbial drug resistance has generated a global requirement for new anti infective agents. As part of an effort to develop new, low molecular mass peptide antibiotics we used a rationale based minimalist approach to design short, non hemolytic, potent and broad spectrum antibiotic peptides with increased serum stability. These peptides were designed to attain an amphipathic structure in helical conformations. VS1 was used as the lead compound and its properties were compared with three series of derivates obtained by 1) N-terminal amino acid addition, 2) systematic Trp substitution and 3) peptide dendrimerization. The Trp substitution approach underlined the optimized sequence of VS2 in terms of potency, faster membrane permeation and cost effectiveness. VS2 (two Trp substituted variant of VS1) was found to exhibit good antimicrobial activity against both, Gram negative *E. coli* and Gram positive bacteria *S. aureus*. It was also found to have non cytolytic activity and ability to permeate and depolarize the bacterial membrane. Lysis of bacterial cell wall and inner membrane by the peptide was confirmed by Transmission Electron Microscopy. A combination of small size, presence of unnatural amino acid, high antimicrobial activity, insignificant hemolysis and proteolytic resistance provides fundamental information towards the de novo design of an antimicrobial peptide useful for the management of infectious disease.
Introduction

The accelerated emergence of pathogenic bacteria, resistant towards conventional antibiotics is a major threat today (1). Therefore, the development of a new class of broad spectrum antibiotics is an urgent need. Cationic antimicrobial peptides (AMP) could be one of the best possible alternatives (31). Antimicrobial peptides (AMPs) are an important component of the natural defences of most living organisms against invading pathogens (12). Naturally occurring AMPs are generally 12-50 amino acids in length and are folded in to several structural groups, including helix, sheet, extended and looped structures (43). Although they show marked variability in length, amino acid composition and structure, a majority of them share two common features and functionally important characteristics, namely a net positive charge that facilitates interaction with negatively charged microbial surfaces and the ability to form an amphipathic secondary structure that permits incorporation into microbial membranes (38). Although the exact mechanism of action of AMPs is still not completely understood, it has been well established that AMPs interact with the cell membrane of the susceptible microorganism, where either their accumulation in the membrane causes increased permeability and loss of barrier function or they enter the membrane to access cytoplasmic targets (4). Development of resistance against peptides that act on microbial membrane is unlikely, as it would require substantial alterations in the lipid composition of the cell membrane of the microorganisms (28).

However, most natural AMPs are large in size, have low potency and show toxicity to host cells (21). Moreover, due to their peptidic nature, they suffer from poor bioavailability and poor proteolytic stability (25). These features have significantly
hampered their pharmaceutical development as therapeutic agents. Short designer AMPs with increased half life offer excellent templates for future antibiotic drug design.

Different approaches are being followed in efforts to increase the effectiveness of AMPs, including alteration of sequences, inclusion of unnatural or D-amino acids or beta-amino acids, cyclization of peptides, peptoid mimics, and synthesis of multivalent constructs of short peptides (33, 9, 40, 18, 27, 37). Short designer AMPs that are less likely to induce resistance, and minimize damage to host cells or tissues would appear to be the most promising candidates.

In the present work we have focused our study on the use of a non-proteinogenic amino acid, α, β dehydrophenylalanine (ΔPhe) in designing relatively short antimicrobial peptides. The presence of more than one ΔPhe in peptides has been shown to constrain the peptide in a $3_{10}$ or $\alpha$ helical conformation, along with providing enhanced resistance towards enzymatic degradation than their phenylalanine containing counterparts (23, 29).

We have used a de novo designed prototype undecapeptide peptide (VS1) incorporating ΔPhe as a lead in an optimization strategy to design three sets of peptides with the same basic motif where ΔPhe is placed at two residue spaces. In order to identify the most promising, cost effective antibiotic peptide as a drug candidate, the designed peptides were compared in terms of (i) spectrum of activity, (ii) specificity, (iii) microbicidal properties, and (iv) membrane permeabilization.
Materials and Methods

Materials-

Amino acid derivatives and resin for peptide synthesis were obtained from Nova Biochem; diisopropylcarbodiimide (DIPCDI), piperidine, dimethyl formamide (DMF), dichloromethane (DCM), hydroxybenzotriazole (HOBr), isobutylchloroformate (IBCF), trifluoroacetic acid (TFA), triisopropyl silane (TIS), DL-threo-β-phenylserine, sodium hydroxide, citric acid, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), DiSC3-5 (3,3’ - Dipropylthiadicarbocyanine) iodide, dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), and N-methyl morpholine (NMM) were from Sigma-Aldrich (St. Louis, MO); sodium chloride, acetic anhydride, and tetrahydrofuran (THF) were from Qualigens (Mumbai, India); ethyl acetate, diethyl ether, sodium acetate, and sodium sulfate were from Merck (Mumbai, India); silica gel thin-layer chromatography (TLC) plates (60F-254) were from Merck (Germany); acetic acid was from SD Fine Chem Limited (Mumbai, India); acetonitrile was from Burdick and Jackson (Muskegon, MI). RPMI 1640 and fetal bovine serum (FBS) were from Invitrogen (Carlsbad, CA).

Methods-

Preparation of Fmoc-X-DL-threo-β-phenylserine- Fmoc-X-DL-threo-β-phenylserine (where, Fmoc is 9-fluorenylmethoxy carbonyl and X is, Lys [Boc], Trp [Boc] or Ala) was synthesized by a method of salt coupling using mixed anhydride. Fmoc amino acid (15 mmol) dissolved in 15 ml of sodium-refluxed and distilled THF was activated at -15°C for 10 min with IBCF and NMM (15 mmol each). A solution of 15 mmol of DL-threo-β-phenylserine made in 1 equivalent of NaOH (15 ml) was added to the above mixed anhydride, and the reaction mixture was stirred at room temperature overnight. Following
evaporation of THF, citric acid was added to the aqueous solution to attain a solution of pH 2.0. The precipitate obtained was dissolved in 100 ml ethyl acetate and transferred to a separating funnel. Following the removal of the lower aqueous layer, the ethyl acetate layer was washed extensively with water to remove the citric acid. The complete removal of citric acid was confirmed by measuring the pH. The ethyl acetate layer was further washed with brine and allowed to pass through a bed of anhydrous sodium sulfate. Evaporation of ethyl acetate on a rotary evaporator resulted in solid dipeptide acids.

**Preparation of Fmoc-X-∆Phe azalactone**- Fmoc-X-DL-threo-β-phenylserine was mixed with recrystallized anhydrous sodium acetate (obtained by fusing the salt and allowing it to cool in a desiccator) in freshly distilled acetic anhydride and stirred overnight. The thick slurry obtained was mixed with ice and stirred at 8-10°C. Following trituration, the yellow dipeptide azalactone was filtered on a sinter funnel and dried to constant weight. The authenticity and purity of the azalactones were assessed by TLC, mass spectroscopy, and UV-visible spectroscopy.

**Peptide synthesis**- Peptides were synthesized as C-terminal amides using standard Fmoc chemistry on rink amide MBHA (4-methylbenzhydrylamine hydrochloride salt) resin in the manual mode, with DIPCDI and HOBt as coupling agents. Fmoc-Lys (Fmoc)–OH was used to make a branching core for the synthesis of the lysine-branched dimer VSD1. Piperidine treatment of the lysine derivative immobilized on the resin gave rise to two amino groups (α and ε), allowing the synthesis of two identical peptide chains, as shown in Table 1. The synthesis of the dendrimer VSD1 was accomplished on a K-K2 core generated by coupling of Fmoc-Lys (Fmoc)–OH to the two amino groups of the lysine resin synthesized as described above. The side-chain protections used were Boc (Lys,
Trp). Couplings were carried out using DMF at a 4-fold molar excess at final concentrations of ~500 mM. Removal of Fmoc was carried out using 20% piperidine in DMF. Both the coupling of amino acids and the Fmoc deprotection were monitored by the Kaiser test (16). ΔPhe was introduced into peptides as an Fmoc-X-ΔPhe azalactone (where X is Lys [Boc], Trp [Boc] or Ala) dipeptide block (23), which was allowed to couple overnight in DMF. At the completion of assembly of the peptides, following Fmoc removal, the amino termini were acetylated using 20% acetic anhydride in DCM. After acetylation of the peptides, the resin was washed extensively with DMF, DCM, and methanol and dried in a desiccator under vacuum.

**Synthesis of FITC labeled peptides** To the free amino terminus of peptide VS2 on resins, Fmoc-ε-Aminohexanoic (Ahx) acid-OH was coupled using HOBt and DIPCDI in DMF. Fmoc was removed by treatment with 20% piperidine/DMF. The resin was washed with DMF and equilibrated in pyridine/DMF/DCM (12:7:5 v/v). A 1.1 equivalent of FITC in pyridine/DMF/DCM (12:7:5 v/v) was added to the resin and allowed to couple overnight. An orange color on the resin and a negative Kaiser test indicated coupling.

**Cleavage of the peptides from resin**- Peptides were cleaved by stirring the resin in a cleavage mixture (95% TFA, 2.5% water, and 2.5% TIS) for 2 h at room temperature. The suspension was filtered using a sinter funnel, TFA was rotary evaporated, and the peptide was precipitated by adding cold dry ether. The ether was filtered through a sinter funnel, and the peptide on the funnel was dissolved in 10% acetic acid and lyophilized.

**Peptide purification and mass spectrometry**- Crude peptides were purified by reverse-phase high-performance liquid chromatography (RPHPLC) on a Deltapac, C18, column (15 µm, i.d. 300 x 19 mm) using acetonitrile-water linear gradient of 5-65% acetonitrile
(0.1% TFA)/water (0.1% TFA) with flow rate of 5 ml/min; for 60 min, with detection at 214 and 280 nm). Purified peptides were re-injected into an analytical reversed-phase C18 column (Phenomenex, C18, 5 µm, i.d. 250 x 4.6 mm) using acetonitrile-water linear gradient of 5-65% acetonitrile (0.1% TFA)/water (0.1% TFA) at a flow rate of 1 mL/min over 60 min and were found to be 98% pure. The identity of the purified (98%) peptides was confirmed by electrospray ionization mass spectrometry at ICGEB, New Delhi, India.

**Solubility measurements**—Water was added to the purified peptide powders to attain complete dissolution, and the concentration of the peptide in the spun supernatant (13,000 rpm, 10 min) was determined by measurement of the absorbance at 280 nm. Extinction coefficient (ε) for α, β-didehydrophenylalanine [ΔPhe] is 19,000 M\(^{-1}\)cm\(^{-1}\) (23) and for tryptophan is 5,050 M\(^{-1}\)cm\(^{-1}\).

**Antibiotic susceptibility testing**—MICs were determined against Gram negative bacteria *Escherichia coli* ML35p and Gram positive bacteria *S.aureus* (700699) according to a modified MIC method for cationic antimicrobial peptides (41). Bacterial cells grown overnight were diluted in Mueller-Hinton (MH) broth to a cell density of 10\(^5\) CFU/ml. 100 µl of this culture was aliquoted into the wells of a 96-well, flat-bottomed microtiter plate (Costar), and 11 µl of 10 stock of each peptide (in 0.2% BSA and 0.01% acetic acid) was added. This mixture was incubated at 37°C in a rotary shaker incubator (Kuhner, Switzerland) set at 200 rpm. After 18 h of incubation, the optical density at 600 nm (OD600) was measured using a microtiter plate reader (VERSA max tunable; Molecular Devices, Sunnyvale, CA). The MIC is defined as the lowest concentration of a drug that inhibits the measurable growth of an organism after overnight incubation. Peptide
concentrations were determined spectrophotometrically at 280 nm (ε_{280}, 19,000 M⁻¹cm⁻¹ for ΔPhe and 5,050 M⁻¹cm⁻¹ for tryptophan). Each experiment was done in triplicate and was repeated at least twice.

**Hemolytic-activity testing** - Human blood in 10% citrate phosphate dextrose was obtained from the Rotary Blood Bank, New Delhi, India. Red blood cells (RBCs) were harvested by spinning (1,000 g, 5 min and room temperature). They were washed three to five times with phosphate-buffered saline (PBS). The packed cell volume obtained was used to make a 0.8% (vol/vol) suspension in PBS. 100 µl of this RBC suspension was transferred to each well of a 96-well microtiter plate and mixed with 100 µl of peptide solution at twice the desired concentration. The microtiter plate was incubated (37°C, 60 min) and centrifuged (1,000 g, 5 min, room temperature). The supernatant (100 µl) was transferred to new wells, and the OD_{414} was measured with a microtiter plate reader (VERSA max tunable; Molecular Devices, Sunnyvale, CA) to monitor RBC lysis. Cells incubated with PBS alone served as the negative control, and RBCs lysed using 0.1% Triton X-100 was used to measure 100% lysis (Positive control).

**Mammalian-cell cytotoxicity** - Cytotoxicity of the antibiotic peptides was determined using MTT assay against HeLa cells. Briefly, cells (5X10⁴ cells/well) were cultured at 37°C overnight in RPMI 1640 containing 10% fetal bovine serum in 96-well microtiter plates. Next day, peptides (prepared in RPMI 1640) at concentrations 1X and 5X of MIC values were added to the cells and incubated for 18 hr at 37°C. 10% DMSO was taken as positive control and untreated cells served as the negative control. 20 µl of MTT solution (5 mg/ml) in PBS was added, and the cells were incubated (37°C, 3-4 h). Supernatant (120 µl) was removed, DMSO (100 µl) was added, and the resulting suspension was
mixed to dissolve the formazan crystals formed by MTT reduction. The ratio of OD570 for treated cells to OD570 for untreated cells was used to calculate percent viability.

Proteolytic stability of peptides to trypsin and chymotrypsin- Proteolytic stability of the peptide VS1 was compared with its phenylalanine analog (Ac-KAWKFWVKFVK-NH₂) using reversed-phase HPLC. Peptide VS1 and trypsin/ chymotrypsin, taken at a ratio of 100:0.5 (mol/mol) in 0.1M ammonium bicarbonate, 0.1mM CaCl₂, pH 8.3 were incubated in a rotary shaker incubator (37°C, 200 rpm, 30 minutes). An aliquot was injected into reverse phase analytical C₁₈ column (Phenomenex, C₁₈, 5 µm, i.d. 250 x 4.6 mm), using an acetonitrile- water linear gradient of 5-65% acetonitrile (0.1% TFA)/water (0.1% TFA) at a flow rate of 1 mL/min over 65 min. Identically treated control peptide (Phenylalanine analog) sample was also injected into reverse phase analytical C₁₈ column using an acetonitrile- water linear gradient of 5-65% acetonitrile (0.1% TFA)/water (0.1% TFA) at a flow rate of 1 mL/min over 65 min. For the variable time dependent assay, peptides were incubated with enzyme for 0, 1.5 and 2.5 hrs respectively.

Outer membrane permeabilization assay (NPN assay)- The membrane permeabilization activity of the peptides was determined by the 1-N-phenylnaphthylamine (NPN) assay of Loh et al (20). In overview, an overnight culture of E. coli ML35p was diluted in MH broth and was grown to an optical density at 600 nm (OD₆₀₀) of 0.5 to 0.6. The cells were harvested, washed, and resuspended in the same volume of buffer (5 mM HEPES [pH 7.2], 5 mM glucose). For the NPN assay, 2 ml of cells and 10 µM NPN were mixed, and the fluorescence was measured with a
fluorescence spectrophotometer (excitation wavelength, 350 nm; emission wavelength, 420 nm). The increase in fluorescence due to partitioning of NPN into the outer membrane was measured by the addition of various concentrations of peptide (0.25 µM-2 µM). Polymixin B (PMB) was taken as positive control. All experiments were performed three times, and the trends that were observed were reproducible.

**Inner membrane permeabilization assay (PI and Syto 9 uptake based assay) -**

Overnight grown *E. coli* ML 35p cells were sub-cultured till an OD$_{600}$ of 0.35. Cells were harvested (4000 rpm, 10 min, 4°C), washed and re-suspended in a buffer (5 mM glucose in 10 mM sodium phosphate buffer, pH 7.5) to get $10^8$ cfu/ml. 15 µL of *E. coli* suspension ($10^8$ cfu/ml) was incubated (37°C, 200 rpm) in 135 µL of buffer (5mM glucose, 5 mM HEPES pH 7.2) containing peptides at their 1X MIC for 90 min. The samples were then incubated with PI (2.7 µM) and Syto9 (6 µM) for 15 min. A smear was made, heat fixed and visualized under a Nikon fluorescence microscope. Cells without peptide treatment served as a control for the experiment.

**FACS based analysis for PI uptake-** Overnight grown cells were subcultured till an OD$_{600}$ of 0.35. Cells were harvested (4000 rpm, 10 min, 4°C), washed and re-suspended in buffer (5mM glucose, 5 mM HEPES pH 7.2) to get 10 cfu/ml. 25 µl of *E. coli* suspension ($10^8$ cfu/ml) was incubated in 480 µl of buffer containing peptide and 5 µl of PI (1mg/ml) for 15 minutes at room temperature and then subjected to FACS analysis. 25 µl of cells suspended in 470 µl of buffer with and without PI served as controls.

**Membrane depolarization assay (DiSC$_3$-5) assay-** The depolarization of the cytoplasmic membrane of *E. coli* ML35p by the peptides was determined using the membrane potential-sensitive cyanine dye DiSC$_3$-5 by a modified method of Wu et al.
In this experiment, *E. coli* cells, in mid-exponential phase (*OD₆₀₀* of 0.35), was collected by centrifugation, washed once with buffer (5 mM HEPES, pH 7.2, 5 mM glucose) and resuspended to an *OD₆₀₀* of 0.05. The cells were incubated with 1 µM DiSC₃-5 for 2 h, for maximal uptake of the dye, after which 100 mM KCl was added to equilibrate the cytoplasmic and external potassium ion concentrations. The cells were mixed with the desired concentration of peptide and the fluorescence was monitored at an excitation wavelength of 622 nm and an emission wavelength of 655 nm. Dye released with the addition of 1 % DMSO was monitored as control.

**Cellular uptake of peptide studied by confocal microscopy** - *E. coli* ML35p cells grown overnight were subcultured to an *OD₆₀₀* of 0.35 (1×10⁸ cfu/mL). Cells were harvested by centrifugation (4000 rpm for 10 min), washed, and resuspended in 5 mM glucose/10 mM sodium phosphate buffer (pH 7.5) to yield 10⁸ cfu/mL; 100 µL of *E. coli* suspensions (10⁸ cfu/mL) was mixed with 5 µL of FITC-labeled peptide VS2 aqueous stock solutions to yield a final peptide concentration of 5 µM (1XMIC) and incubated (37 °C and 200 rpm) for 30 min in Kuhner rotary shaker incubator. Cells were harvested by centrifugation (4000 rpm for 10 min) and washed twice with 5 mM glucose in 10 mM sodium phosphate buffer (pH 7.5). A smear was made on a poly L-lysine coated slide. For confocal microscopy, confocal laser scanning (Radiance 2100, Bio-Rad) under a Nikon microscope (objective plane Apo 60×/1.4 oil) was used to observe the slide. The excitation wavelength for FITC was 494 nm (argon laser), and fluorescence was detected through an HQ 515/30 emission filter (high-quality band-pass). Image processing was conducted with Lazer Sharp (Bio-Rad), and Photoshop 6.0 (Adobe Systems, San Jose, CA) was used for the final image assembly.
Examination of bacterial membrane damage by electron microscopy-

**Scanning Electron microscopy (SEM)** - Overnight grown cells were sub-cultured till an OD$_{600}$ of 0.35. Cells were harvested (4000 rpm, 10 min, 4°C), washed and re-suspended in HEPES buffer, pH 7.2 to obtain $10^6$ cfu/ml. 15 µl of *E. coli* suspension ($10^6$ cfu/ml) was incubated for 30 minutes and 60 minutes in 135 µl of 5mM HEPES buffer, pH 7.2 containing the peptide at its MIC (5µM). Cells were spun down (4000 rpm, 4°C, 10 min) and washed thrice in 0.1M phosphate buffer pH 7.4. Cells were fixed in 2.5% glutaraldehyde buffered with 0.1 M phosphate buffer pH 7.4 at 4°C for 2 hours. They were further washed in 0.1 M phosphate buffer, post fixed with 1%OsO$_4$ and dehydrated with graded ethanol. The sample was dried with HMDS (hexamethyl disilizane) and coated with gold (15 nm). Observations were made on Zeiss EV040 Scanning microscope at the J. N. U., New Delhi.

**Transmission electron microscopy (TEM)** - Sample containing *E. coli* ($1 \times 10^6$ cfu/ml) in Muller- Hinton medium was incubated for one hour with peptide (VS2). The peptide was taken at its MIC value (5µM). After centrifuging at 3,000g for 10 min, the pellets were washed with 0.1 M phosphate buffer and re-suspended in the same buffer. *E. coli* cells were fixed with 2% glutaraldehyde in 0.1M phosphate buffer for 1 h. at room temperature (20°C). Cells were washed with 0.1M phosphate buffer (pH 7.2) and post fixed with 1%OsO$_4$ in 0.1M phosphate buffer for 1 h at 4°C. For ultrastructure study, samples were dehydrated with graded acetone, cleared with toluene and infiltrated with toluene and araldite mixture at room temperature then finally in pure araldite at 50°C and imbeded in Eppendorff tube (1.5ml) with pure araldite mixture at 60°C. Ultrathin section
cutting was done with ultra-microtome (Ultramicotome Lecia EM UC6). Sections were
taken on the 3.05 mm diameter and 200 mess copper grid, stained with uranyl acetate and
lead acetate. The grids were examined under Transmission Electron microscope, JEOL,
(JEOL 2100F).

Results

Rationale based design of prototype peptide antibiotic

*ab initio and de novo* designing resulted in a short cationic, amphipathic undecapeptide
to make initial electrostatic interaction with the negatively charged bacterial membrane,
VS1 harboured four lysine residues on its hydrophilic face. An excessive charge has been
shown to have deleterious effect on activity (because it prevents structuring) and the
optimal charge for maximal antimicrobial activity has been shown to be +4 (10). Our idea
for keeping the +4 charge was derived from such studies. Since, valine is the most
commonly found amino acid in natural as well as in synthetic antimicrobial peptide two
valine residues were incorporated on hydrophobic face to maintain amphipathicity (3).
Single alanine, at N-terminus hydrophobic face was incorporated to reduce peptide
hydrophobicity while maintaining its helicity. Abiotic residues ΔPhe’s were placed two
residues apart. In peptides, such placements of ΔPhe have been shown to induce a helical
conformation (22). Taking a cue from the membrane active properties of Trp, a single
Trp was also incorporated in the prototype peptide VS1. The helical wheel representation
in Fig-1(A), shows the segregation of polar and apolar faces of a 3₁₀ helix.

Systematic modifications in the prototype peptide sequence
Using the basic template of VS1 (11 residue, +4), systematic variations in the sequence resulted in three sets of peptides. The first set included three peptides VSL1 (12 residue, +4), VSL2 (13 residue, +4) and VSL3 (14 residue, +5). These peptides were identical to VS1 up to 11 residues from C-terminus and differed only in their N-terminus extensions. The second set of peptides were Trp analogs of VS1; VS2 (two Trp), VS3 (three Trp) and VS4 (four Trp). They were identical to VS1 in length, charge and number of ΔPhe residues, and differed only in their Trp content. The third approach resulted in a single peptide dimer of VS1; Dendrimer, VSD1, consisted of two VS1 peptide sequences on a core lysine residue. Thus, the branched dimer had a net charge of +8 (+4 per chain).

Physicochemical properties such as length, charge, number of α, β-didehydrophenylalanine (Δ Phe) and Trp residues of the designed peptides are provided in Table 1(A).

Effect of increasing length, Trp substitution and dendrimerisation of VS1 on antibiotic activity and cell selectivity-

Antibacterial activity- The antibacterial activity of VS1 and its analogues, against Escherichia coli ML35p and Staphylococcus aureus is shown in Table 1(B). Prototype peptide VS1 showed moderate potency (MIC, E. coli, 25 μM), narrow spectrum of activity (MIC S. aureus > 50 μM) and no hemolysis to RBCs or toxicity to HeLa cells. Increase in the chain length of VS1 resulted in five-fold increase in potency against E. coli (MIC, 5μM for VSL1 VSL2 and VSL3) however there was no augmentation in activity against Gram positive bacteria, S. aureus. Moreover, in VSL3, even a combined effect of additional positive charge (+5) and an increased length of the peptide was
unable to broaden the spectrum of antibiotic activity. However, in the second series of peptides, sequential inclusion of Trp residues in VS1 resulted in substantial enhancement in the antimicrobial activity of the peptides. Replacement of Ala by Trp in VS1 led to VS2 that exhibited five times increase in activity against *E. coli* (MIC, 5 µM). This replacement also resulted in moderate activity against *S. aureus* (MIC, 50 µM). VS3, where Ala and Val in VS1 sequence were replaced with two Trp was equipotent to VS2 against *E. coli*, but showed enhanced activity against *S. aureus* (MIC, 25 µM). VS4 (four Trp substitution in VS1) was also equipotent to VS2 and VS3 against Gram negative *E. coli* (MIC, 5 µM) but showed further enhancement in activity against Gram positive *S. aureus* (MIC, 10 µM). The dendrimer VSD1, showed excellent antimicrobial activity against both, *E. coli* (MIC, 1 µM) and *S. aureus* (MIC, 5 µM).

**Cell selectivity (Hemolysis and toxicity to HeLa cells)** - None of the designed peptides were hemolytic or cytotoxic at their MICs, except VS3 (negligible hemolysis at 5 µM, MIC for *E. coli*), VS4 (~60% hemolysis at 5 µM, MIC for *E. coli*) and VSD1 (~10% at 1 µM, MIC for *E. coli*). At these concentrations, none of the peptides showed any toxicity to HeLa cells. However, we observed that at 5X MIC values, which are much higher than physiologically relevant concentrations, the peptides VSL2, VSL3, VS2 and VS3 showed some degree of hemolysis and cytotoxicity. VSL2 was ~15% hemolytic and 50% toxic to HeLa cells whereas VSL3 was ~13% hemolytic and 55% toxic at 5X MIC values. VS2 showed 12% hemolysis and 30% cytotoxicity and VS3 showed 19% hemolysis and 40% toxicity to HeLa cells at 5X MIC values. VSL1 was exceptional; it was non hemolytic and cytotoxic at MIC but showed 75% hemolysis and 60% cytotoxicity at 5X MIC values, Fig 2(A) and 2(B). These results indicated that out of all peptides, VS2 provided
the right balance between antimicrobial activity and hemolytic activity and was chosen for further studies.

**Circular Dichroism based Structures of peptides-**

CD studies show that all the peptides acquired helical structures in a membrane mimetic environment of 20mM SDS (critical micelle concentration for SDS is 8mM), Fig 3(A). All the peptides exhibited an excitonic couplet at 267 nm (+) and 298 nm (-) which is the signature for the formation of a right handed $3_{10}$ helix in $\Delta$Phe containing peptides (23). However, different degrees of helicity were observed for the different peptides and a particular trend was observed in each series of peptides, as shown in Fig 3(B). In the first series, increasing the length of the peptides resulted in increase in their helicity (measured in terms of intensity of +267 nm band) of the peptides, with the only exception being VSL2 that showed no increment in helicity upon increasing length. However, an interesting and reverse trend was observed in the second series of peptides where the helical intensity (intensity of +267 nm band) decreased with increasing Trp content. VSD1, from the third set showed the highest molar ellipticity value of the helix associated excitonic couplet. Helicity in VSD1 resulted from combined helicities of two helical peptides (VS1).

**Proteolytic stability of peptides-**

Peptide stability is one of the most important parameters in the development of peptide therapeutics (34). In order to compare the relative stability of $\Delta$Phe containing prototype peptide VS1, its analog containing Phenylalanine (Phe) instead of $\Delta$Phe was synthesized.
and both treated with Trypsin/Chymotrypsin under identical condition. Analysis by RPHPLC showed that after one hour the saturated (Phe) analog (peak at 38 min) disappeared, giving rise to two small peaks at 14 min and 25 min. On the other hand, VS1, containing ΔPhe’s was found to be stable to proteolytic degradation; there were no observed changes in the retention time (39 min) and peak intensity of VS1 after 2.5 hrs, as shown in Fig 4.

Increased bacterial membrane permeabilization and membrane depolarization caused by peptides

Outer membrane permeabilization assay (N- Napthylphenyl amine assay)- The ability of peptides to destabilize the outer membrane was judged by the permeabilization of the outer membrane. N- Napthylphenyl amine (NPN) is a hydrophobic fluorescent molecule, which fluoresces strongly in a hydrophobic environment like the interior of a membrane and weakly in an aqueous environment (20). Normally the outer membrane of a bacterial cell is impermeable to NPN. However, permeabilization of the outer membrane by cationic peptides allows NPN to enter into the hydrophobic environment of the membrane resulting in an enhanced fluorescence of NPN. Fig 5, shows that the cationic peptides were able to permeabilize the bacterial membrane at concentration of 0.25 µM to 2 µM which is much below their MICs. VS4 and VSD1 showed maximum increase in NPN fluorescence at 2µM. Indeed, for VS4 and VSD1, at 2µM concentration increase in the fluorescence was much higher than polymixin B at the same concentration (2µM) which was taken as the positive control. PMB was taken as control because it is a bactericidal antibiotic which kills almost all Gram –ve bacteria at rather low
concentrations. Moreover, it have been shown to permeate the outer membrane (OM) of *E. coli* changing the packing order of lipopolysaccharides (LPS) and increasing the permeability of the OM to a variety of molecules including its own uptake called as ‘self-promoted’ uptake (6). Besides, it’s a decapeptide antibiotic with a net positive charge +5 and a molecular mass 1200 Da (comparable to the size and charge of the designed peptides). Outer membrane permeabilization by these peptides was remarkable as only very few peptides are able to permeabilize membranes at such low concentrations (2).

**Cytoplasmic membrane permeation (PI and Syto9 uptake by cells)**-

Fluorescence microscopic studies on cytoplasmic membrane permeabilization by the designed peptides is shown in Fig 6(A). Propidium iodide (PI) is a membrane impermeable red fluorophore, which enters only those cells, that have disrupted cell membranes and shows enhanced fluorescence upon binding to DNA. The fluorescence emission maximum for DNA bound PI is about 615-620 nm when excited by a 488 nm laser. Thus membrane-disrupted cells appear red when excited by the PI filter, whereas the cells with intact membranes do not show any fluorescence when excited using a PI filter. On the other hand, Syto 9 is a cell-permeant nucleic acid stain. Its absorption and emission maxima are 485-486 nm and 498-501 nm respectively. It has permeability to virtually all cell membrane (living or dead) including bacterial and mammalian cells. By differential staining of cells by Syto 9 and PI we were able to compare membrane disrupted cells with total number of cells. Comparison of PI and Syto9 fluorescence shows that peptides were able to permeabilize almost all bacterial cells membrane at their respective MICs after 90 minutes. Fig 6(A), shows that there was no significant difference in the Syto 9 (green) and PI (red) fluorescence in the peptide treated cells.
However, cells without any peptide treatment showed only Syto 9 fluorescence and no PI fluorescence. Since, these cells have intact cell membrane PI was unable to permeate the cells.

FACS based PI uptake assay was used to study kinetics of inner membrane permeabilizations by four selected peptides (VS1, VS2, VS4 and VSD1). As shown in Fig 6(B), VS1 and VSL3 were very slow in inducing membrane permeabilization. At their respective MICs (25µM and 5 µM), VS1 and VSL1 were able to permeabilize only ≈10% *E. coli* cells. Whereas, VS2 showed very fast kinetics of inner membrane permeabilization. At its MIC, it permeabilized ≈96% cells in 10 min. However, VS4 and VSD1 were even faster in action; they permeabilized ≈98% cells in 5 minutes, but were hemolytic.

**Membrane depolarization assay (DiSC$_3$-5) assay**- Dye DiSC$_3$-5 (3,3'-Dipropylthiadicarbocyanine iodide) is known to distribute between bacterial cells and the surrounding medium, depending on the membrane potential gradient. Once inside the membrane, the dye aggregates and self-quenches. With the addition of a membrane permeabilizing agent, the dye is released and the increase of fluorescence can be monitored over time. Fig 7 shows, disruption of bacterial membrane potential by VS2 and DMSO (Positive control). DMSO increases the membrane permeability and exerts a marked inhibitory effect on a wide range of bacteria and fungi. The graph clearly demonstrates that similar to DMSO, VS2 also efficiently disturbed the potential gradient across the bacterial membrane in a dose dependent manner.
Confocal microscopic analysis of uptake of FITC labeled peptide VS2- The Z-sectioned confocal images of cells treated with FITC-VS2 for 30 minutes showed the presence of the peptide in intracellular milieu of *E. coli* (Fig. 8). This was an interesting observation as it suggested that although the primary target for activity of VS2 was the bacterial membrane, it might have some intracellular targets also and the bacterial killing by VS2 might be the result of combined effect of the two independent activities.

Examination of bacterial lysis by electron microscopy- The effect of VS2 on the morphology of peptide treated *E. coli* was investigated by scanning electron microscopy. A change in the cell morphology was observed. Increased roughness of the cell wall was observed after 30 min of incubation, while cell shrinkage was observed after 60 min of incubation with the peptides, Fig. 9 (left panel).

In TEM micrographs, VS2 appeared to have the most severe effect on the bacterial cell wall and cell membrane. Cell wall breakage and variability in wall thickness was observed after 30 min. Separation of cytoplasmic membrane from cell wall was also observed. More severe effects were observed after 60 min, where bacterial cell lysis due to membrane damage was clearly visible. *E. coli* cells, without any peptide served as control, Fig. 9 (right panel).

Discussion

The aim of our work was to identify candidates for developing novel potent and cost-effective antibiotics, starting with a short cationic AMP as a template and then systematically engineering its structure to enhance the degree and spectrum of activity
without imparting excessive hemolytic activity to the peptide. In this section, we describe our experimental results related to the effectiveness of the three series of peptides that were designed.

The key element in the design of the lead template 11 residue peptide were three ΔPhe residues, four lysine residues and one tryptophan along with valine and alanine residues. The three ΔPhe were separated by two amino acid residues, an arrangement that induces 3_{10} helical structures in peptides (22). Four lysine residues in such an arrangement were expected to form a polar face while ΔPhe align themselves to form a hydrophobic face, resulting in an amphipathic helical structure. A tryptophan residue was included in the design because of its known membrane active properties and association with antibacterial activity in peptides like indolicidin and tritrypticin (36, 24). Peptides varying from 10-12 residues have shown significant antimicrobial activity (15). Previous reports from our laboratory have demonstrated antimicrobial activity of ΔPhe containing decapeptides (8).

Although increased length is often associated with enhanced activity, there are no set rules that correlate length with the activity of antimicrobial peptides. We decided to synthesize three more peptides VSL1, VSL2, VSL3 containing 12, 13 and 14 residues respectively. We found that increase in length did not result in any significant enhancement in antibacterial activity. Next, we sequentially increased Trp content in VS1 resulting in VS2, VS3, VS4 with two, three and four Trp residues respectively. Thus VS4 was made up of only ΔPhe, Lys and Trp residues. We observed remarkable changes in the properties of these Trp containing peptides. Presence of two Trp in VS2 enhanced its
activity against *E. coli* (MIC, 5µM) and *S. aureus* (MIC, 50µM). Addition of another Trp in VS3 did not alter its activity against *E. coli* (MIC, 5µM) but enhanced its potency against *S. aureus* (MIC, 25µM). VS4 with four Trp was the most potent antibacterial peptide. However, results of hemolysis experiments showed that VS4 was most hemolytic at the 5X MIC values. High Trp content in the antimicrobial peptides is known to be associated with enhanced hemolysis, making them unsuitable for use as antimicrobial agents (7). These results indicate that the presence of two Trp in VS2 have provided the right balance of charge and hydrophobicity and appropriate membrane properties making it reasonably potent and broad spectrum antimicrobial peptides.

Dendrimerization has been used as a strategy to potentiate the activity of antimicrobial peptides as it allows multivalent binding (due to doubling of charge) to the bacterial membrane and generates a higher local concentration that results in membrane destabilization (19). In support of this, our results showed that branched dimeric peptide, VSD1 exhibited excellent activity against *E. coli* (MIC, 1µM) and methicillin resistant *S. aureus* (MIC, 5µM). Dewan *et al.*, 2008, their study on ∆Phe containing peptides have shown that along with increased antimicrobial properties, branched dimers show faster kill kinetics and increased serum stability than the linear dimeric analogs (8). Duplication of the vital antimicrobial structure in VSD1 resulted in very high antibacterial activity but simultaneously compromised its cell selectivity. Although, it showed very low hemolysis (≈ 7%) at its MIC, but it was considerably hemolytic (≈40%) at 5X MIC values.

Structure-activity relationship studies on helical antibacterial peptides have shown that most of the α helical peptides are unstructured in aqueous solution and acquire a helical structure in the presence of a lipid like environment (5). Peptide chain lengths have been
associated with increased potency of helical peptides as long helices can better span the bacterial lipid membrane (32). All ΔPhe containing peptides showed some degree of helicities in SDS. For the first series of peptides, where the length of the template was successively increased by one residue, it was observed that VSL1 (12 residue), showed increase in helicity and five-fold enhancement in activity against *E. coli*. VSL2 (13 residue), however, was equipotent to VSL1, showed, lower helicity than VSL2. In VSL3, with increased length and higher helical content there was no gain in antimicrobial activity. A gradual decrease in helicity was observed on incorporation of 2, 3 and 4 Trps respectively. However helicity taken as parameter did not correlate with the activity of peptides of both the series. The results indicate that helicity of a similar structured peptide alone may not correlate with their activity. Studies exist in literature that cites a similar lack of clear structure-function correlation for AMPs (13). In such studies, activity of AMPs is shown to be derived from a correct balance of charge, hydrophobicity, solubility and amphipathicity collectively called the ‘interfacial activity’ (30). It has been shown that peptide composition is also an important parameter for antimicrobial activity of short designer peptides (35).

The cell membrane is vital for any microorganisms and is the primary target for most antibacterial peptides. Microbial membrane is the primary target for most of the antibacterial peptides (39). However, lethal action of peptide could be from membrane disruption solely, from translocation through the membrane to target receptor inside the cell or it can be a concerted effect of both the activities (14). We determined the ability of these peptides to permeabilize the cytoplasmic membrane, which is the mechanism of killing of a large number of AMPs (11). In PI uptake assay, we observed that all the
peptides were able to permeabilize the bacterial membrane in 90 minutes. However, the results on kinetics of membrane permeabilization by selective peptides showed that they differed in the kinetics of membrane permeabilization. VS1 and VSL3 were slow in their action on the inner membrane of *E. coli* as they were able to permeabilize only 10% cells in 60 minutes. However, VS2 and VSD1 efficiently permeabilized the outer membrane of *E. coli* at MICs in ten minutes of incubation. Also in DiSC$_3$-5 assay, VS2 was able to initiate membrane depolarization immediately at 0.5X of its MIC, which is remarkable as some AMPs do not depolarize the membrane until they reach concentration as high as four to ten times the MIC (42). However, presence of FITC labeled peptide VS2 inside the cytoplasm of bacteria shows that the peptide translocates itself into the cell where it might act on some intracellular targets as well. Our finding that these peptides were able to permeabilize and depolarize the bacterial cell membrane at concentrations much lower than its MIC suggests that membrane permeabilization is not the sole cause of bacterial death and that the designed peptides might have a multimodal mechanism of action (26, 17). SEM images of the VS2 treated *E. coli* showed changes in the cell wall morphology. Peptide induced cell wall breakage was clearly visible and an increase in the coarseness of the cell surface as compared to the control was also observed. TEM images of the VS2 treated cells also confirms the cell wall and inner membrane damage leading to cell lysis, thereby providing additional evidence about a putative multimodal action of the peptides. These observations suggest that antibacterial activity of VS2 might be a multi-step process, involving initial permeabilization and depolarization of bacterial membrane leading to its destabilization and then action on intracellular components of cell ultimately leading to cell death.
Our study with a set of designed peptides demonstrated that synthetic peptides such as VS2 containing a helicogenic residue ΔPhe, which exhibited a broad spectrum of activity, increased protease resistance and faster killing kinetics could be a suitable candidate for further development.

Conclusion

Short abiotic antimicrobial peptides are promising candidates for overcoming the critical and accelerating problem of bacterial resistance to currently utilized antibiotics. To provide a road map for development of improved second generation therapeutics, we have investigated a rationale based approach for optimizing the peptide sequence to significantly enhance its antimicrobial activity. Increase in length did not dramatically alter antimicrobial activity of peptides suggesting that 11-12 residue length is optimum for activity. The resultant study adds significantly to the field, providing a lead peptide VS2 with high potency, broad spectrum of activity, enhanced proteolytic resistance and faster membrane permeabilization kinetics, based on which novel potent antimicrobials could be produced.

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   of different classes of cationic antimicrobial peptides with planar bilayers and 
   with the cytoplasmic membrane of Escherichia coli. *Biochemistry.* 38: 7235-42.

   389-95.

Table 1(A)- Amino acid sequence of designed peptides
<table>
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<tr>
<th>Peptide</th>
<th>Amino acid sequence</th>
<th>Length</th>
<th>Charge</th>
<th>ΔPhe</th>
<th>Trp</th>
<th>MW (Calculated)</th>
<th>MW (Observed)</th>
</tr>
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<tbody>
<tr>
<td>VS1</td>
<td>Ac-K-A-ΔF-W-K-ΔF-V-K-ΔF-V-K-NH₂</td>
<td>11</td>
<td>+4</td>
<td>3</td>
<td>1</td>
<td>1466.8</td>
<td>1468</td>
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<tr>
<td><strong>Series I (Analogs with variable length)</strong></td>
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<td></td>
</tr>
<tr>
<td><strong>Series II (Analogs with different Trp content)</strong></td>
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<td></td>
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<tr>
<td>VS2</td>
<td>Ac-K-W-ΔF-W-K-ΔF-V-K-ΔF-V-K-NH₂</td>
<td>11</td>
<td>+4</td>
<td>3</td>
<td>2</td>
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<tr>
<td><strong>Series III (Branched dimeric analog)</strong></td>
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</table>
Table 1(B) - Minimum Inhibitory concentration (MIC) of peptide analogs against Gram-negative bacteria (E.coli ML35p) and Gram positive bacteria (S.aureus 700699). Rifampicin and Kanamycin was taken as reference antibiotic. MIC is given as the geometric mean of three sets of determinations.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>MIC (μM)</th>
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<tbody>
<tr>
<td></td>
<td><em>E.coli</em> ML35p</td>
</tr>
<tr>
<td>Series I</td>
<td></td>
</tr>
<tr>
<td>VIL1</td>
<td>5</td>
</tr>
<tr>
<td>VIL2</td>
<td>5</td>
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<tr>
<td>VIL3</td>
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<td>Series II</td>
<td></td>
</tr>
<tr>
<td>VIL2</td>
<td>5</td>
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<tr>
<td>VIL3</td>
<td>5</td>
</tr>
<tr>
<td>VIL4</td>
<td>5</td>
</tr>
<tr>
<td>Series III</td>
<td></td>
</tr>
<tr>
<td>VILD1</td>
<td>1</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>5</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>4</td>
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</table>

Figure legends

**Fig 1(A)**- de novo design of antimicrobial peptides: Prototypic, amphipathic, cationic undecapeptide sequence in 3,10 helical-wheel configuration. (B) Chemical structure of ΔPhe.

**Fig 2(A)**- Shows % hemolysis caused by the peptides. Peptide antibiotics at MIC and 5X MIC (E.coli) were incubated with 0.4% RBCs in PBS. The results are expressed as percent hemolysis. RBCs incubated with 0.1% Triton X-100 were considered to be 100% lysed. Standard deviations from three observations are plotted.

**Fig 2(B)**- Mammalian-cell cytotoxicity (MTT assay) of peptide antibiotics at MIC and 5X MIC (E.coli), against HeLa cell lines. Test samples were incubated with cells for 24 h.
in RPMI 1640. Untreated cells served as negative control. The ratio of OD$_{570}$ for peptide-treated cells to OD$_{570}$ for untreated cells was used to calculate the percent viability of cells. Standard deviations from three observations are plotted.

**Fig 3(A) & 3(B)** - Circular dichroism based conformational analysis of designed peptides (25 µM each) in 20 mM SDS/10 mM sodium phosphate buffer pH 7.5. (B) - Histogram showing mean residue ellipticity (MRE) values for the peptides (for 267 nm band).

**Fig 4** - Represents HPLC profile of VS1 at 0 and 2.5 hrs of incubation with trypsin/chymotrypsin. C & D represents HPLC profile of Phe analog at 0 and 2.5 hrs of incubation with trypsin/chymotrypsin.

**Fig 5** - Outer membrane permeabilization (NPN assay) by peptides. Polymixin B was taken as positive control. Dose dependent increase in the florescence intensity was observed upon addition of peptides at concentrations much lower (0.25-2 µM) than their MICs.

**Fig 6 (A)** - Fluorescence microscopy of peptide induced permeability of *E. coli* ML35p cells visualized by Syto 9 and PI staining at 90 min. After 90 minutes, all the peptide treated cells have increased membrane permeability as seen by PI (red) florescence except control cells that were without peptide treatment. On the other hand, Syto 9, stained all the bacterial cells (live, dead and membrane permeabilized) nonspecifically to give green fluorescence. Peptides were taken at their respective MICs.

**Fig 6 (B)** - Shows the kinetics of PI uptake as studied by FACS for four peptides, VSL1, VS2 and VSD1, belonging to different series. Peptides were taken at their respective MICs. Cells without any peptide served as control.
Fig 7- Shows the *E.coli* membrane depolarization caused by the peptide VS2 (Δ). Disruption of potential gradient across the membrane was assessed by increase in the fluorescence of potential sensitive dye diSC3-5. 10% DMSO (●) served as positive control.

Fig 8 (A)- Confocal images of the cells incubated with FITC labelled VS2 for 30 min. (B)- Z section of an individual *E.coli* mL35p cell showing intracellular presence of peptide.

Fig 9- Left and right panel shows the SEM and TEM micrographs of *E.coli* cells respectively, treated with VS2 at its MIC (5µM) for 30 min and 60 min. (A), shows untreated cells with smooth and intact cell surface. (B) shows peptide induced breakage and roughness in the cell wall after 30 min. (C), shows increased damage to the bacterial cell wall in the form of cracks after 60 min. Cell shrinkage due to loss of turgor was also observed. Similarly, in TEM micrographs of ultrathin sections of negatively stained untreated *E. coli* cells showed a normal cell shape with an undamaged structure of inner membrane and intact outer membrane (A’). (B’) shows peptide induced breakage in the cell wall and cell membrane after 30 min. Leakage of the inner mass of cell was also visible. (C’) shows complete damaged to the cell wall and inner membrane and lysis of bacterial cell after 60 min.
Fig 1-
Fig 2(A)-

![Chart Title](image)

Fig 2(B)-

![Chart Title](image)
Fig 3(A)-

![Graph showing wavelength (nm) against angle of rotation (deg cm² mol⁻¹)].

Fig 3(B)-

![Bar chart showing angle of rotation (deg cm² mol⁻¹) for different peptides].

Peptides: VS1, VSL1, VSL2, VSL3, VS2, VS3, VS4, VSD1.
Fig 4
Fig 6(A)-
Fig 6(B)-

Fig 7-

Fig 8-
Fig 9.

Control

SEM

TEM

30 min

60 min

Control

30 min

60 min