Amino Acids (2010) 39:1493–1505
DOI 10.1007/s00726-010-0618-z

ORIGINAL ARTICLE

SD-8, a novel therapeutic agent active against multidrug-resistant Gram positive cocci

Biswajit Mishra · Vipul Kumar Srivastava · Rama Chaudhry · Rishi Kumar Somvanshi · Abhay Kumar Singh · Kamaldeep Gill · Ramesh Somvanshi · Ishan Kumar Patro · Sharmistha Dey

Received: 28 January 2010 / Accepted: 28 April 2010 / Published online: 15 May 2010

Abstract Anti-bacterial drug resistance is one of the most critical concerns among the scientist worldwide. The novel antimicrobial decapeptide SD-8 is designed and its minimal inhibitory concentration and therapeutic index (TI) was found in the range of 1–8 μg/ml and 45–360, respectively, against major group of Gram positive pathogens (GPP). The peptide was also found to be least hemolytic at a concentration of 180 μg/ml, i.e., nearly 77 times higher than its average effective concentration. The kinetics assay showed that the killing time is 120 min for methicillin-sensitive *Staphylococcus aureus* (MSSA) and 90 min for methicillin-resistant *S. aureus* (MRSA). Membrane permeabilization is the cause of peptide antimicrobial activity as shown by the transmission electron microscopy studies. The peptide showed the anti-inflammatory property by inhibiting COX-2 with a K_D and K_i values of 2.36×10^{-9} and 4.8×10^{-8} M, respectively. The peptide was also found to be effective in vivo as derived from histopathological observations in a Staphylococcal skin infection rat model with MRSA as causative organism.

Keywords Multidrug resistant · Antimicrobial peptides · Anti-inflammatory · Surface plasmon resonance · Cyclooxygenase · Histopathology

B. Mishra · R. K. Somvanshi · A. K. Singh · K. Gill · S. Dey (✉)
Department of Biophysics, All India Institute of Medical Sciences, Ansari Nagar, New Delhi 110029, India
e-mail: sharmistha_d@hotmail.com

V. K. Srivastava · R. Chaudhry
Department of Microbiology, All India Institute of Medical Sciences, New Delhi, India

R. Somvanshi
Division of Pathology, Indian Veterinary Research Institute, Izatnagar, Bareilly, UP, India

I. K. Patro
School of Studies in Neuroscience, Jiwaji University, Gwalior, MP, India

Introduction

Gram positive cocci (GPC) are common cause of hospital-associated infections resulting in increased mortality, morbidity, hospital stay and cost of treatment. During last few decades, development of resistance has been noted in major human pathogens as exemplified by methicillin resistance in Staphylococci (MRSA), high level aminoglycoside resistant (HLAR) and vancomycin resistance Enterococci (VRE). The bacterial organism re-invents themselves, leading to re-emerging pathogens. The rapid spread of antibiotic resistance requires the development of alternative and innovative anti-infective agent.

Antimicrobial peptides (AMPs) are one of the key elements of the innate immunity against bacteria (Hancock and Sahl 2006). The knowledge acquired in the past two decades and discovery of a new group of AMP make them the basic element of a novel generation of drug for the treatment of bacterial infection. The innate immune system comprises the cells and mechanism that defend the host from infection by microbes, in a non-specific manner. AMPs are one of the most important elements of the innate immune system, they are ideal for the fast and efficient defense against the microbes (Nissen-Meyer and Nes 1997). The cationic AMP could represent a new class of antibiotics (Hancock 1997; Andreu and Rivas 1998; Sitaram et al. 2002). Cationic peptides exhibit a high

affinity for lipopolysaccharides (LPS) and lipoteiochoic-acid (LTA) (Hancock and Chapple 1999; Frecer et al. 2004; Zhu et al. 2006). Thus, cationic AMP causes permeabilization and lysis of microbes. These are also effective against the multidrug resistant bacterial strains (Schwab et al. 1999; Cirioni et al. 2007; Giacometti et al. 2005; Sader et al. 2007; Zhang et al. 2005; Cirioni et al. 2006). The two major classes of the cationic AMP exhibit α-helix and β-sheet (Hancock 1997; Andreu and Rivas 1998; van't Hof et al. 2001; Devine and Hancock 2002). The β-sheet consists of cyclic peptides. Unlike β-sheet peptides, α-helical peptides are linear molecules that mainly exist as disordered structure in aqueous and become amphipathic helices upon interaction with the hydrophobic membrane (Chen et al. 2005). The important factor for the antimicrobial activity is an amphipathic nature, which segregates basic and hydrophobic residues and form secondary structure like α-helix or β-sheet (Chen et al. 2007; Sigurdardottir et al. 2006). Further, an ideal AMP should also have an anti-inflammatory property as the infections are associated with inflammation (McInturff et al. 2005; Zaitsu et al. 1999). Bacterial infections interfere with cell signals leading to activated COX-2 and phospholipase A2 production, which are enzymes that trigger inflammation. The COX-2 is one of the major enzymes responsible for the prostanoid biosynthesis involved in inflammation and mitogenesis (Richards et al. 2002). In the present study, we have designed a decapeptide based on the sequence of Sapecin B, an anti-bacterial protein of *Sarcophaga peregrina* (flesh fly), effective against Gram positive bacteria (Alvarez-Bravo et al. 1994; Yamada and Natori 1994). The amino acids were systematically incorporated for an α-helical amphipathic structure effective for its high antimicrobial activity. This peptide was also modified N-terminally by dehydro-leucine residue effective for its structural stability as well as functional activity (Bisht et al. 2007; Mathur et al. 2007). The peptide also exhibited anti-inflammatory property by inhibiting COX-2. In addition to the in vitro activity of SD-8 alone, in vivo efficacy of this peptide was also tested in a rat model with epidermal lesions caused by MRSA.

Materials and methods

Organisms

The clinical isolates of all GPC including methicillin-sensitive *Staphylococcus aureus* (MSSA), MRSA, HLAR *Enterococcus faecalis* and *E. faecium*, Group A and B *Streptococcus*, *Micrococcus luteus* and Coagulase negative Staphylococci (CONS) from pus, blood, swab and bone marrow samples from hospitalized patients were tested. Identifications of the strains were performed using standard microbiological procedures (Mackie and McCartney 1996). *S. aureus* ATCC 25023 was used as quality control.

Synthesis of peptides

The peptides were synthesized by solid phase peptide synthesizer PS3 (Protein technology, USA) using Fmoc and Wang resin chemistry (Merrifield 1963). The solvent used for the synthesis was dimethylformamide (DMF). 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was used as an activator of the Fmoc amino acids (Chem Impex, USA). Fmoc was deprotected by 20% piperidine and wang resin was cleaved by trifluoroacetic acid (TFA). The peptides were precipitated from dry ether.

Analytical RP-HPLC of peptides

The purity of peptides was verified by analytical RP-HPLC, C18 reversed phase column (1.6 × 10 cm, Amersham Bioscience). The 1 mg/ml of peptide was loaded to the RPC column. The linear gradients were formed by passing two different solvents, where solvent A was 0.05% aqueous TFA, pH 2 and solvent B was 0.05% TFA in acetonitrile. The flow rate was 0.25 ml/min at room temperature. The molecular weights were confirmed by MALDI-TOF.

Solubility of peptides and stability of solutions

The peptide was found to be stable at room temperature. Neither precipitation nor decrease in the anti-bacterial capacity occurred even after storage for at least 1 month at room temperature, −20 and 4°C. A group of four aliquots of 200 µl (stock solution of 640 µg/ml) each was stored at room temperature, −20 and 4°C. Each aliquot was tested for its antimicrobial activity after 7, 14, 21 and 30 days, respectively (Jasir et al. 2003).

Measurement of minimal inhibitory concentration (MIC)

The MIC of cationic peptide in this study was determined by modified micro titer broth dilution method (Hancock, http://cmdr.ubc.ca/bobh/methods/methodsall.html). Serial twofold dilutions of each peptide solutions along with gentamycin as a control were prepared in polypropylene 96-well microtiter plates with Mueller–Hinton (MH) broth. A total of 100 µl of the adjusted inoculum (5×10^5 CFU/ml) organisms were added to each well and the plates were incubated at 37°C in ambient air overnight. In microtiter plate the positive control was bacteria without peptide and

negative control well was uninoculated MH broth. The MIC was taken as the lowest concentration in which growth was inhibited. This result was analyzed by spectrophotometer. Experiments were performed in triplicates and the average results were interpreted.

Measurement of minimal hemolytic concentration (MHC)

The effect of peptide on hemolysis was determined using human red blood cells (hRBC).The freshly collected hRBC were centrifuged for 10 min to remove the buffy coat and washed with phosphate buffered saline (PBS: 35 mM Na_2HPO_4, pH 7.0 and 150 mM NaCl). 100 µl of the hRBC [suspended in 1% (v/v) in PBS] and 10 µl peptide solution were added into the sterilized 96 well plates. The plates were incubated for 1 h at 37°C and centrifuged at 1,000×*g* for 5 min. The supernatant was transferred to fresh 96 well plates, where hemoglobin released was monitored by measuring the absorbance spectrophotometrically at 541 nm. Similar steps were carried out for gentamycin, as a control. The percentage of hemolysis (determined in PBS and 0.1% Triton X-100) was calculated from the standard graph which was plotted with % hemolysis of prepared RBCs versus optical densities.

Calculation of therapeutic index (TI)

The MHC and MIC values were carried out by twofold dilutions. The TI could vary as fourfold if the peptide is hemolytic and antimicrobial. If there is no detectable hemolytic activity, then the maximum possible error in the TI would be only twofold. The peptide, SD-8 was showing no detectable hemolysis at 180 mg/ml, so the MHC of 360 mg/ml was used to calculate the TI.

Time-kill assay

All the bacterial strains were grown overnight at 37°C in MH broth with ambient air supply. Aliquots of exponentially growing bacteria were resuspended in fresh MH broth at approximately 10^7 cells/ml and separately exposed to the peptide at two times the MIC for 0, 5, 10, 20, 40, 60, 90, 120, and 150 min at 37°C. After each observation, samples were serially diluted and plated into MH agar plates to obtain viable colonies.

Electron microscopic (EM) studies

The EM studies were conducted to evaluate the mechanism of action of the peptide SD-8 with MRSA and *S. aureus* ATCC 25023. Fresh inoculated liquid cultures of MRSA in MHB were grown up to mid-logarithmic phase and were washed well with sodium phosphate buffer (10 mM) and centrifuged for 3 min at 1,500×*g*. The pellet obtained was fixed in 2.5% glutaraldehyde in 0.1% phosphate buffer for 3 h at 4°C. The pellet was washed with 0.1% phosphate buffer three times. The samples were post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 2 h and were washed with phosphate buffer followed by dehydration with a series of acetone gradients. These samples were passed through propylene oxide and infiltrated in epoxy resin overnight. These were embedded in pure epoxy resin and cured at 60°C for 72 h. Ultracut Reichert Jung-Austria microtome was used to obtain golden color sections and was stained with 2% uranyl acetate and Reynold's lead citrate. The sections were observed under a Morgagni-268 electron microscope. Test samples were processed in the same way by pretreating with SD-8 (10^8 cells in 100 µl were treated with 8 µg of peptide) (Sitaram et al. 2002). The samples were analyzed for different lengths of time (5–30 min).

Kinetic studies of peptide SD-8

Initially, the activity assay for the enzyme, COX-2 was performed in the assay buffer (100 mM Tris–Cl; pH 8.0, 1 mM EDTA, 2 µM hematin) by the addition of arachidonic acid (substrate) and *N*,*N*,*N*′,*N*′-tetramethyl *p*-phenylenediamine (TMPD; co-substrate). TMPD oxidation was monitored spectrophotometrically at 610 nm (Gierse et al. 1999). The inhibition of peptide was determined by incubating with enzyme in 1:1 M ratio (Somvanshi et al. 2007). The enzyme concentration was fixed at 0.5 mm while substrate concentration was varied from 0.4 to 1.6 mM. The enzyme COX-2 was incubated separately with different concentrations of peptide ranging from 0.005 to 0.02 mM for 45 min. The reactions were initiated by the addition of substrate ranging from 0.4 to 1.6 mM, respectively, for each peptide concentration. The resulting products were estimated by the differences in the absorbance at 610 nm in spectrophotometer (T 60 UV, PG instruments). The competitive kinetic constants (K_i) were calculated graphically from double reciprocal plots of velocity versus substrate concentration (Lineweaver–Burk equation). The reciprocal of velocity was plotted on *Y*-axis that of substrate concentration on *X*-axis. The negative reciprocal of the *X* intersect gives K_m.

K_i was calculated as:

$$K'_m = K_m\left[1 + \frac{I}{K_i}\right],$$

where K'_m is the concentration of substrate that reduces half maximal velocity in the presence of competitive inhibitor and *I* is the concentration of peptide (inhibitor), and K_m is Michaelis Menton constant.

Surface plasmon resonance studies

The kinetic measurements of the interaction between COX-2 with SD-8 were performed using a biosensor based on SPR (Otto 1968; Kretschmann and Reather 1968; Nylander et al. 1982). Six Histidine-tag attached to the terminal position of COX-2 is an ideal tag for immobilization due to strong rebinding effect caused by the high surface density of immobilized Ni^{2+}-nitriloacetic acid (NTA) on the chip used here, the binding of analyte, i.e., the peptide in solution can be studied by monitoring the change in the resonance unit (RU) values of the sensorgram, where the progress of the interaction is plotted against time, revealing the binding characteristics.

The analysis of binding kinetics, i.e., the association (K_A) and dissociation (K_D) constant for the formation of multi-molecular complex and dissociation were achieved in a very short time and with small amount of samples. 60 ml of His-tag COX-2 (50 mg/ml) was injected to one of the flow cell at the flow rate of 5 ml/min. 900 RU of COX-2 were immobilized under these conditions, where 1 RU corresponds to immobilized protein concentration of $\sim$1 pg/mm^2. The analyte, i.e., SD-8 of concentration 9×10^{-6} M was passed over the immobilized COX-2 at a flow rate of 10 µl/min and the sensorgram was run for 4 min, likewise two different concentrations 45×10^{-6} M and 90×10^{-6} M of SD-8 of same volume were passed over the chip and the change in sensorgram was observed.

The rate constants K_A and K_D were obtained by fitting the primary sensorgram data using the BIA evaluation 3.0 software. The dissociation rate constant is derived using the equation:

$$R_t = R_{t_0} e^{-K_D(t-t_0)}$$

where R_t is the response at time t, R_{t_0} is the amplitudes of the initial response and K_D is the dissociation rate constant. The association rate constant K_A can be derived using equation given below from the measured K_D values.

$$R_t = R_{max}\left(1 - e^{-(KAC+K_D)(t-t_0)}\right)$$

where R_t is the response at time t, R_{max} is the maximum response, C is the concentration of the analyte in the solution. K_A and K_D are the association and dissociation rate constants, respectively.

In vivo experiment (Skin Infection Model)

A total of 15 adult Wistar laboratory rats were used in the experiment. The rats were anesthetized with Xylocain, the back skin was shaved without causing any injury and 1 cm circular superficial skin injury was created on each rat using a curved needle end. The skin surface wound was infected with topical application of 10 µl of saline containing 5×10^8 CFU MRSA. The bacterial-infected skin was treated tropically with 30 mg of 0.5% peptide SD-8 ointment with a cotton tipped applicator and was observed at 24, 48, 72 and 96 h post-infection with MRSA. Single dose of the peptide ointment was used. Skin biopsies of both the control and infected rats were collected in 10% formalin. These biopsies were processed for histopathological examinations as per conventional method. The sections were stained with H&E method (Culling 1995).

Results

Anti-bacterial activity of designed peptide

Ten peptides were designed on the basis of Sepacin B (Alvarez-Bravo et al. 1994; Yamada and Natori 1994). In the previous studies (Alvarez-Bravo et al. 1994; Yamada and Natori 1994), two peptides RLKLLLLLRLK-NH2 and KLKLLLLLKLK-NH2 designed on the basis of Sepacin B were found to have potent antimicrobial activity against wide range of microorganisms including the multidrug-resistant GPC. Natori et al. have reported that the motifs RLK and KLK at the terminals of the peptides are significant for antimicrobial properties as well as inducing the α-helical structure. The residues R and K are very important for antimicrobial properties.

In this study, ten peptides were designed by modifying the leading peptide L1 (RLKLLLLLRLK-NH2) to develop an AMP focusing primarily on GPC. In the first stage, SD-1 and SD-2 were synthesized (Table 1). In SD-1, the terminal K was removed and in SD-2, R and L positions of the leading peptide (L1) were interchanged in other terminal to see the effect of R at the terminal of the peptide. The MIC

Table 1 Primary screening of the peptides against standard strain of *S. aureus* (ATCC 25023)

Peptide	Sequence	MIC (µg/ml)
SD-1	RLKLLLLLRL-NH2	8
SD-2	LRKLLLLLRL-NH2	32
SD-3	RLKLLLLRL-NH2	16
SD-4	RLKLLLRL-NH2	128
SD-5	RLKLLRL-NH2	128
SD-6	RLKLLLLLRW-NH2	4
SD-7	RWKLLLLLRL-NH2	2
SD-8	RWKLLLLLRΔL-NH2	1
SD-9	RWKLLLLLRΔV-NH2	8
SD-10	RWKLLLLLRΔA-NH2	32

values of SD-1 and SD-2 were determined and found to be 8 and 32 μg/ml, respectively. The value showed that the presence of R at the terminal increases the anti-bacterial activity. In the second stage we have designed three peptides SD-3, SD-4, SD-5, the number of internal leucine residues were reduced to four, three and two, respectively. The MIC of SD-3 was higher than the other two (SD-4 and SD-5) peptides due to higher hydrophobicity. But MIC values of these three peptides were less than SD-1. So, five leucines are very important for the antimicrobial activity. In third stage two peptides, SD-6 and SD-7, were synthesized by modifying the terminal residues of SD-1. In SD-6, leucine at terminal of SD-1 was replaced by tryptophan and in SD-7 leucine attached with R was replaced by tryptophan. The MIC value of SD-6 and SD-7 was found to be higher than other peptides. The inclusion of tryptophan residue increases the hydrophobicity of peptides. In the hydrophobicity scale (Chen et al. 2005), the hydrophobicity of Trp (33.1) is higher than Leu (24.7). Among SD-1, SD-6 and SD-7, the peptide SD-7 showed to be the most active than other two. Finally, the peptide SD-7 was modified by introducing dehydro amino acid (ΔL, ΔV and ΔA) in N-terminal in three different peptide SD-8, SD-9 and SD-10.

The peptide, SD-8, contains dehydro-leucine at N-terminal position and was found to be α-helix (http://aps.unmc.edu/AP/prediction/prediction_main.php). This dehydro residue increases the stability of the peptide by making the stable α-helical conformation and facilitates structure function relationship. The purity of the peptides was >98% as assed by HPLC on C18 reversed-phase analytical column. Primary screening of the peptides were done by determining their MIC against standard strain of *S. aureus* (ATCC 25023) in which peptide SD-8 was showing minimum MIC value. The MIC of peptides with different bacterial strains is shown in Table 2. The ranges for the MIC were reported to be 1–8 μg/ml for differently isolated multi resistant strains.

Stability test

There was no change in the antimicrobial activity of the peptide SD-8 observed even after 30 days of storage in different temperature, when compared to freshly prepared solution. The result for the stability test is reported in Table 3 for the peptide SD-8 against standard strains of *S. aureus* ATCC-25023. The result showed that the peptide was stable in all the storage conditions of temperature and the strength of it was same.

Hemolytic experiment

The hemolytic activity was performed only with SD8 as its MIC values against Gram positive bacterial strains are minimum compared to the other peptides in this study. The result of the hemolytic experiment showed that the peptide is non-hemolytic even at a high concentration (up to 180 μg/ml) of 77 folds to its average MIC range. These doses infer that the peptide is non-toxic to human erythrocytes and hence can be administered intravenously. Figure 1 shows the hemolytic effect of SD-8 at very higher concentration and it was compared with gentamycin as a control.

Therapeutic index

The TI indicates the specificity of antimicrobial agent. The larger the value of TI, greater is the antimicrobial specificity. The TI of SD-8 for different Gram positive bacterial strains is shown in Table 2. The TI was found to be in a range of 45–360 for SD-8, which can be considered to be a highly specific AMP (Chen et al. 2005). The TI of peptide significantly increases against the bacterial strains whose MIC value is ≤1 μg/ml.

Time-kill assay

The kinetic activity of SD-8 against the MSSA was completed after 120 min exposure period and for MRSA it was slightly faster, i.e., 90 min at a concentration two times the MIC. Figure 2 showed the rapid killing of the standard strain *S. aureus* ATCC-25023, and MRSA by peptide SD-8.

EM studies

To understand the mechanism of action of SD-8, the morphologies of the MRSA were examined. As the kinetic experiments revealed that longer duration of incubation of MRSA cells with the peptide SD-8 causes total lysis of the cells, the shorter duration effects were analyzed after fixing and sectioning (Fig. 3). At 5 min of incubation, changes like invaginaton in the cytoplasmic membrane, blebbing and thickening of membrane and formation of vacuoles were prominent. After 15 min of incubation, membrane discontinuity and the leakage of cytoplasmic content were most prominent features. As a result, the less electron dense cytoplasm was observed. At 30 min of incubation, only the ghost cells without any cytoplasm were noticed.

Anti-inflammatory activity of SD-8

The anti-inflammatory property of peptide against COX-2 was determined both by spectrophotometer and BIAcore.

Table 2 Minimal inhibitory concentration (MIC), source of isolation and therapeutic index for the peptide SD-8 against different bacterial strains

Sample number	Organism	Cultured from	MIC (μg/ml)	Therapeutic index
ATCC 25023	*S. aureus*		≤1	360
P.S. 1	MSSA	Pus	≤2	180
P.S. 2	MSSA	Pus	≤1	360
P.S. 3	MRSA	Pus	≤2	180
P.S. 4	MSSA	Pus	≤2	180
P.S. 5	MRSA	Resp.	≤1	360
P.S. 6	MRSA	Pus	≤1	360
P.S. 7	MRSA	Pus	≤2	180
P.S. 8	MRSA	Bone marrow	≤4	90
P.S. 9	MSSA	Pus	≤2	180
P.S. 10	MRSA	Pus	≤1	360
P.S. 11	MRSA	Pus	≤2	180
P.S. 12	MRSA	Pus	≤1	360
P.S. 13	MSSA	Pus	≤8	45
P.S. 14	MSSA	Pus	≤1	360
P.S. 15	MRSA	Pus	≤2	180
P.S. 16	MRSA	Pus	≤2	180
P.S. 17	MRSA	Blood	≤2	180
P.S. 18	MSSA	Blood	≤1	360
P.S. 19	MRSA	Pus	≤2	180
P.S. 20	MRSA	Pus	≤1	360
P.S. 21	*Enterococcus* HLAR	Pus	≤4	90
P.S. 22	MRSA	Pus	≤4	90
P.S. 23	MRSA	Pus	≤4	90
P.S. 24	*Enterococcus* spp.	Pus	≤2	180
P.S. 25	*Enterococcus* spp.	Urine	≤1	360
P.S. 26	*Micrococcus leuteus*	Pus	≤2	180
P.S. 27	MSSA	Blood	≤2	180
P.S. 28	MRSA	Blood	≤1	360
P.S. 29	MRSA	Blood	≤2	180
P.S. 30	CONS	Blood	≤2	180
P.S. 31	CONS	Blood	≤4	90
P.S. 32	MSSA	Pus	≤1	360
P.S. 33	MRSA	Pus	≤2	180
P.S. 34	*Gp. A Streptococcus*	Pus	≤2	180
P.S. 35	*Gp. B Streptococcus*	Pus	≤2	180
P.S. 36	*Enterococcus* spp.	Pus	≤8	45

Kinetic studies of peptide SD-8 with COX-2 by spectrophotometer

The anti-inflammatory property of the peptide SD-8 was done by inhibition studies against COX-2 in the presence of substrate arachidonic acid. COX-2 mediates production of prostaglandins responsible for pain, inflammation and fever, and is induced by diverse inflammatory stimulations. This peptide showed 85% inhibition against COX-2 which proved to be effective anti-inflammatory agent. The K_i of SD-8 was calculated from the double reciprocal plot, i.e., the Lineweaver–Burk plot (Fig. 4) and was found to be 4.8×10^{-8} M.

Binding studies of SD-8 with COX-2 by BIAcore

Figure 5a shows sensogram for binding of SD-8 at varying concentrations with COX-2. The change in RU with varying concentrations of SD-8 indicated the change in mass on the COX-2 immobilized on chip with time and the dissociation constant (K_D) was found to be 2.36×10^{-9} M. The NSAIDs, i.e., diclofenac and nimesulide were used as

Table 3 Stability of the solution: MIC of standard *S. aureus* ATCC 25023 against different prepared and stored SD-8 solution

Days	Storage	MIC (μg/ml) *S. aureus* (ATCC 25023)
1	Freshly prepared	≤1
7	Room temp.	≤2
	4°C	≤2
	−20°C	≤4
14	Room temp.	≤1
	4°C	≤2
	−20°C	≤1
21	Room temp.	≤1
	4°C	≤2
	−20°C	≤2
30	Room temp.	≤1
	4°C	≤2
	−20°C	≤2

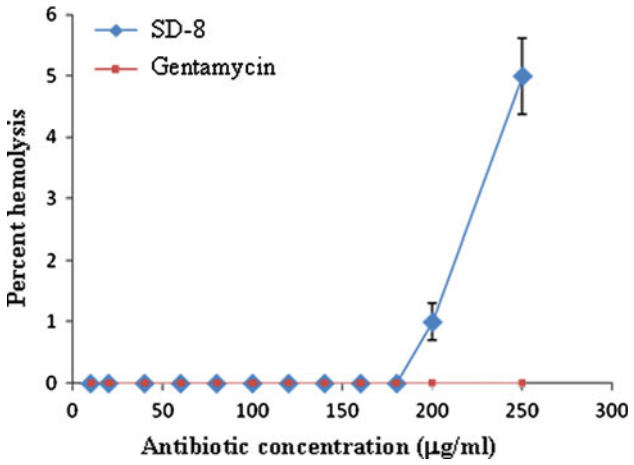

Fig. 1 Hemolytic activity of SD-8 on human RBCs. Each value shown is the mean ± standard error of the mean from three experiments

controls (Table 4). The K_D of diclofenac and nimesulide were found to be 1.45×10^{-7} M and 2.22×10^{-9} M, and are showed in Fig. 5b and c, respectively. These binding studies indicate the peptide to be more efficient anti-inflammatory agent.

In vivo studies

All results of in vivo efficacy of the peptide were based on gross histopathological observations. Figure 6 is the representative image obtained from in vivo studies with peptide SD-8. The observations after the post-infection and after effects with the peptide treatment are following (HPI, hours post-infection and HPIT, hours post-infection with treatment).

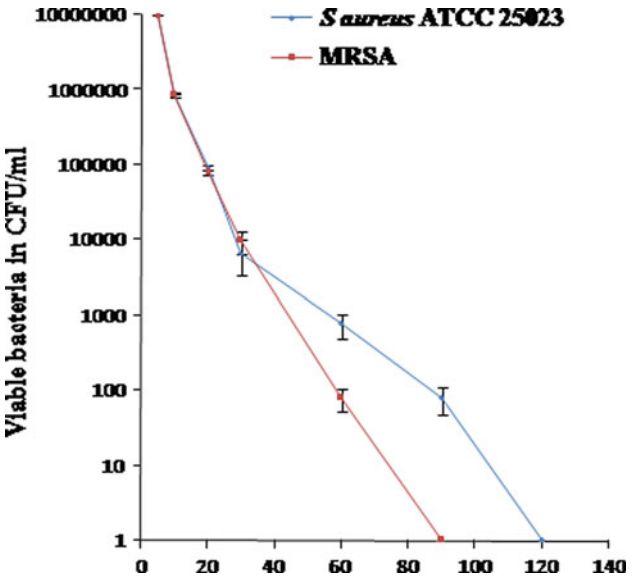

Fig. 2 Killing of *S. aureus* ATCC 25023 and MRSA by SD-8. Each value shown is the mean ± standard error of the mean from three experiments

Control

No change was observed. Skin showed keratin layers over epidermis while in hypoderms sections of hair follicles and sebaceous glands were seen. Reticulin fibres were loose and subcutaneous fat was observed. No vascular inflammatory cells were seen (Fig. 6a).

24 HPI

Focal area of suppuration and desquamation of epidermis were seen. Engorged blood capillaries, presence of fibrins or polymorphonuclear (PMN) and mononuclear cellular (MNC) infiltration were observed in subcutaneous fatty tissue (Fig. 6b).

24 HPIT

Keratin layer showed sloughing, focal suppuration and necrosis (Fig. 6c). Dermis showed pronounced edema and thickening. Hypodermis revealed ruptured, loosens reticulin fibres, hyperemia and folliculitis. Hair follicles showed enlargement, degeneration, necrosis and suppuration. Sebaceous gland revealed degenerative changes. Subcutaneous tissue/hypodermis showed both mild and severe engorged blood vessels, marked PMN + MNC infiltration and presence of fibrin. In adjoining areas of muscle

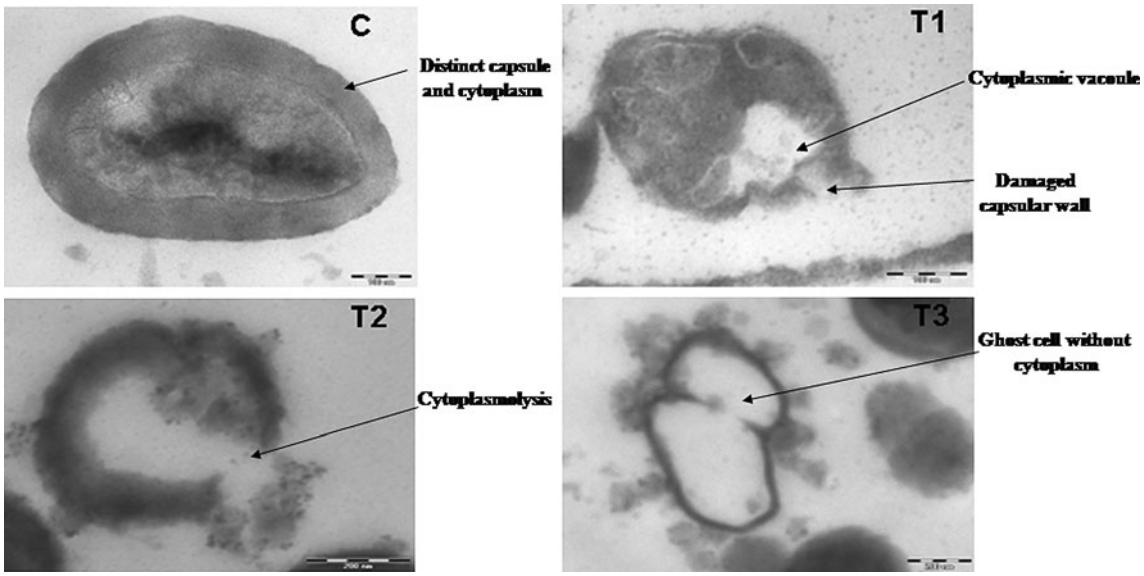

Fig. 3 **C** Transmission electron micrographs (TEM) of control MRSA, **T1** 5 min after incubation with SD-8, **T2** 15 min after incubation, **T3** 30 min after incubation

(Fig. 6d), fibres MNC and mast-like relatively large cells along with bluish granules were seen.

48 HPI

Epidermis showed break and marked sloughing of keratin layers (Fig. 6e). Hypodermis showed presence of RBC, fibrin, highly engorged blood capillaries, mixed cellular (PMN + MNC) infiltration and hemorrhages in one animal (Fig. 6f) while other showed suppuration and necrosis.

48 HPIT

Keratin layer over epidermis was seen. Sloughing and focal area of suppuration in epidermis was observed. Epidermis revealed markedly engorged blood vessels. Hair follicles revealed necrosis and enlargement (Fig. 6g). Acute inflammation was subsided.

72 HPI

Focal suppuration in epidermis was seen in one animal (Fig. 6h). In subcutaneous tissue engorged blood vessels and disrupted reticulin fibres were observed (Fig. 6i). Acute inflammatory reaction was subsided.

72 HPIT

Epidermis showed sloughing in one animal. Focal area of MNC was seen in subcutaneous tissue. Vascular changes were absent (Fig. 6j).

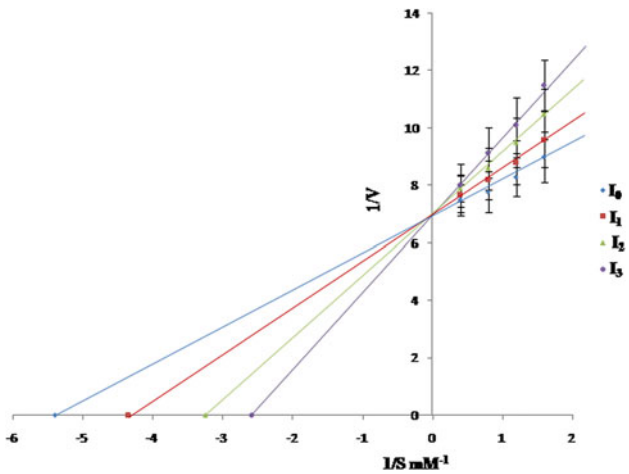

Fig. 4 Lineweaver–Burk plot for the kinetics of peptide SD-8 against COX-2. COX-2 was incubated separately with different concentration of peptides $I_1 = 0.005$ μM, $I_2 = 0.01$ μM and $I_3 = 0.02$ μM for 45 min. Each value shown is the mean ± standard error of the mean from three experiments

96 HPI

Focal suppuration in epidermis was seen. Hypodermis showed suppurative folliculitis, engorged blood vessels and MNC in subcutaneous tissue adjoining to muscle fibres.

The above observations showed no remarkable change in 24 and 48 h between infection and treatment groups. Prominent changes were observed in 72 h of treatment, the group showed more prominent MNC activity in the focal area that was absent in the infected group.

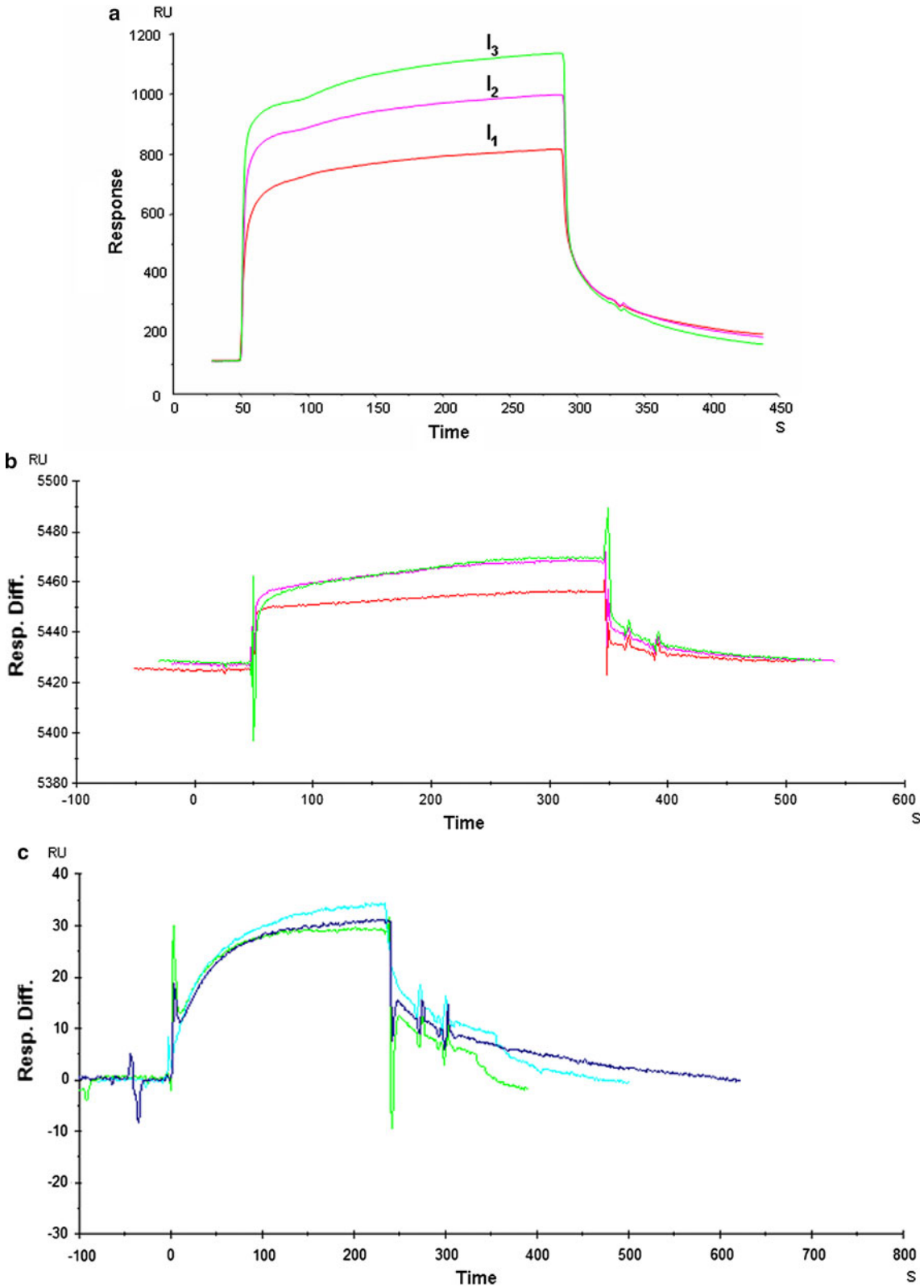

Fig. 5 **a** Sensorgram showing binding of different concentrations of peptide SD-8 ($I_1 = 9 \times 10^{-6}$ M, $I_2 = 45 \times 10^{-6}$ M, $I_3 = 90 \times 10^{-6}$ M) on the Ni^{2+}NTA chip immobilized with His-COX-2. **b** Sensogram showing binding of different concentrations of diclofenac ($I_1 = 0.15 \times 10^{-3}$ M, $I_2 = 0.31 \times 10^{-3}$ M and $I_3 = 0.37 \times 10^{-3}$ M) on the Ni^{+2} NTA chip immobilized with His-COX-2. **c** Sensogram showing binding of different concentrations of nimesulide ($I_1 = 0.24 \times 10^{-3}$ M, $I_2 = 0.40 \times 10^{-3}$ M and $I_3 = 0.48 \times 10^{-3}$ M) on the Ni^{+2} NTA chip immobilized with His-COX-2

Table 4 K_D values of SD-8 compared to other NSAIDs

Compound	K_D (M)
SD-8	2.36×10^{-9}
Diclofenac	1.45×10^{-7}
Nimesulide	2.22×10^{-9}

Discussion

The emergence of multidrug-resistant pathogens renders antibiotics ineffective in the treatment of various diseases. So, it is essential to develop synthetic alternatives to currently available microbicidal agents. Promising so far unexploited candidates are AMP. AMP represents elements of innate and induced defenses against invading pathogens (Hancock and Lehrer 1998; Duclohier et al. 1989). Gram positive bacteria especially *S. aureus* are extremely important pathogen causing infection in the hospital environments are resistant to antibiotic. Oxacillin resistance rates among *S. aureus* varied from 2.1% in Sweden, 42.5% in UK and 54.7% in Ireland and in All India institute of Medical Sciences, India MRSA was reported to be 44.7% (Sader et al. 2007; Gadepalli et al. 2006). The prevalence of the high level resistance to methicillin and ampicillin is of increasing concerns (Klein et al. 2007). The clinical efficacy of antimicrobials has also been reported to be limited. Vancomycin has been preferred over the different antimicrobial agents to treat MRSA infection. But more use of it can render the selection pressure for the development of vancomycin resistance (Schentag et al. 1998). Being a relatively non-virulent organism, the Enterococci have become increasingly common nosocomial pathogens because of their resistance to many antimicrobials including ampicillin and this has been reported to cause a failure in the treatment (Sakoulas et al. 2004; Jones 2006). The cationic AMP can represent a new class of antibiotics. It has been proposed that cationic peptide interact with membrane and system. The peptide SD-8 was found to be the best designed peptide with high hydrophobicity index and the minimum value of MIC compared to all other designed peptide. So, this peptide was further analyzed for its other in vitro activities. SD-8 is the decamer and it contains seven hydrophobic residues. The terminal amino acid was converted to α,β-dehydro amino acids to increase the activity and stability. The peptide SD-8 showed a broad spectrum antimicrobial activity against a wide variety of GPC including *Enterococcus,* HLAR, *Micrococcus leuteus*, *Gp. A Streptococcus, Gp. B Streptococcus,* HLAR and CONS. The MRSA infection is of critical concern among the strains tested. So, we have tried to test the efficacy of SD-8, focusing on MRSA. The results indicated that the peptide was very effective against MRSA. The other strains tested are also significant. The stability of the peptide SD-8 was also found to be high without any decrease in its antimicrobial capacity even after its storage for 1 month. The MHC of SD-8 was found to be 77 times its effective average MIC which shows that the peptide is nearly non-hemolytic against hRBCs. TI is the ratio of minimal hemolytic concentration to that of the MIC (MHC/MIC). As both the values were carried out by serial twofold dilutions, the therapeutic index could vary as much as fourfold for individual bacteria when the peptide is both hemolytic and antimicrobial. However, it may vary up to twofold when the peptide is non-hemolytic. It is reported that larger the value of TI, the more is the antimicrobial specificity (Chen et al. 2005). In this case the range was of 45–360 but most of the values are on the higher side, which suggest the peptide is of high microbial specificity. The time-kill assay results indicate that the killing of bacteria by SD-8 is rapid. It was an interesting outcome that the peptide is having a high kinetic rate for MRSA. Cytoplasmic membrane is the main target of some peptides, whereby peptide accumulation in the membrane causes increased permeability and loss of barrier function (Oren and Shai 1998; Friedrich et al. 2000). The α-helical peptides are very active antimicrobial agent due to linear in structure. In aqueous medium it becomes amphipathic upon interaction with hydrophobic membrane (Christensen et al. 1998; Zasloff 1987; Andreu et al. 1992). A number of factors have been identified like the presence of both hydrophobic and basic residues, an amphipathic nature that segregates basic, hydrophobic residues and an inducible or preformed secondary structure (α-helix or β-sheet). The mechanism of action of AMP has not been established yet but numerous studies indicate that AMP acts directly on the membrane of the target cells and increases their permeability, either by the effect of their positive charge with lipid or by membrane destabilization through lipid displacement due to the drastic change of the composed. EM studies suggest that the changes in the morphology of bacteria were clear evident for the membrane interaction of the peptide SD-8 against MRSA. All the observations of the EM experiment showed that SD-8 acts via membrane permeabilization like other reported cationic AMP. The binding study of SD-8 with COX-2 was tested by BIAcore analysis that showed it to be an interesting anti-inflammatory peptide. The dissociation constant (K_D) of SD-8 can be compared to that of known COX-2 inhibitors such as diclofenac and nimesulide (Table 4). The K_i of SD-8 was 4.8×10^{-8} M, which is comparable with the known NSAIDs. It clearly indicates that SD-8 exhibits effective anti-inflammatory property. Finally, the in vitro studies have also been correlated with the in vivo study by testing the peptide in a Staphylococcal skin infection rat model. It is the inherent property of the immune system to counter

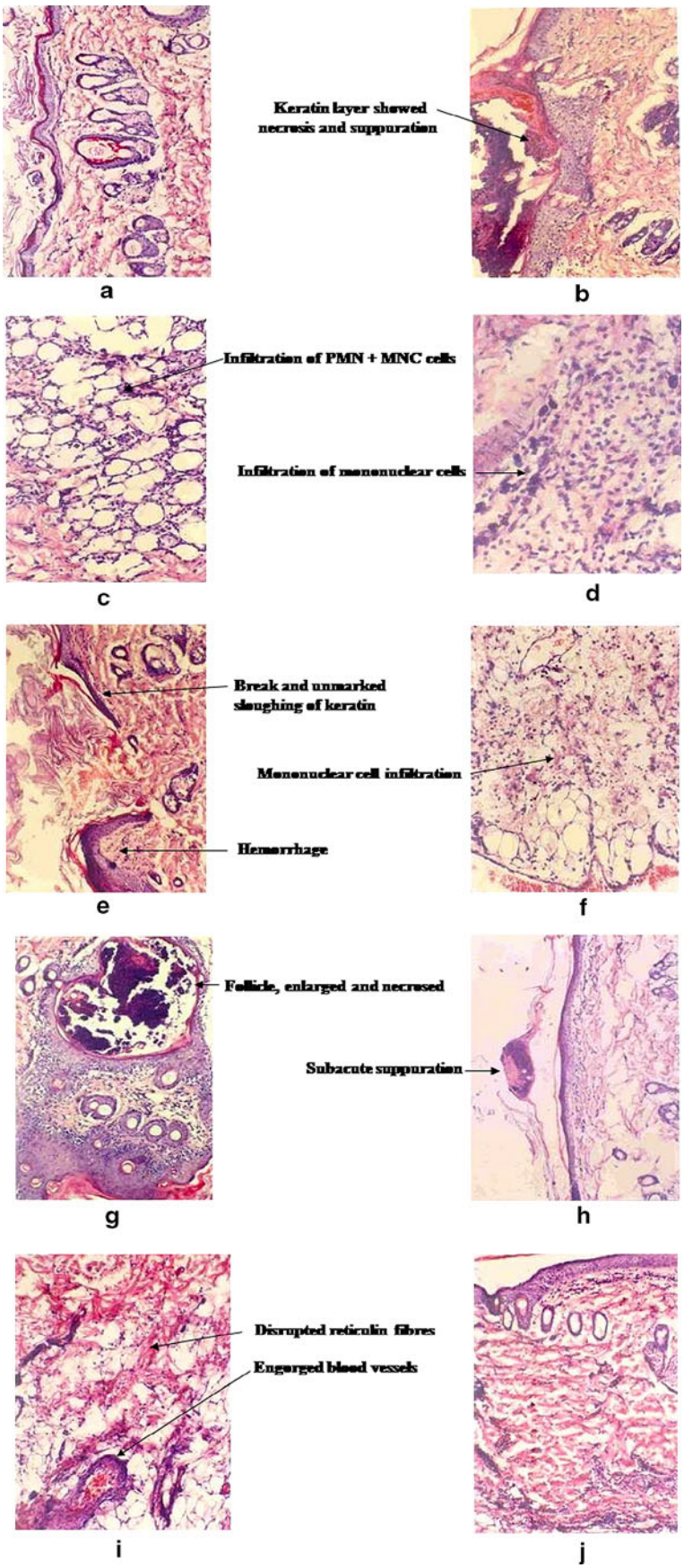

Fig. 6 **a**. Skin: control showing epidermis, dermis and hypodermis along with skin adenexae, HE ×40. **b** Skin: keratin layer showed necrosis and suppuration while epidermis revealed thickening, 24 HPIT, HE ×40. **c** Skin: hypodermis showed fatty cells and the infiltration of PMN + MNC cells, 24 HPIT, HE ×100. **d** Skin: hypodermis showed infiltration of mononuclear cells and mast-like large cells with bluish cytoplasm, 24 HPIT, HE ×150. **e** Skin: epidermis showed break and unmarked sloughing of keratin and hemorrhage, 48 HPI, HE ×40. **f** Skin: hypodermis showed presence of RBCs, fatty cells and mononuclear cell infiltration, 48 HPI, HE ×100. **g** Skin: epidermis revealed that the hair follicles were enlarged and necrosed, 48 HPIT, HE ×100. **h** Skin: subacute suppuration was seen in epidermis, 72 HPI, HE ×40. **i** Skin: subcutaneous tissue showed engorged blood vessels and somewhat disrupted reticulin fibres, 72 HPI, HE ×40. **j** Skin: mild MNC infiltration and absence of vascular changes, 72 HPIT, HE ×40

act any foreign body by its primary response, and inflammation is the earliest signs of wound healing process. In present investigation mild suppurative Staphylococcal infection characterized by the presence of pockets of neutrophils in epidermis, mild engorgements, suppurative folliculitis and infiltration of MNC was observed in a rat model infected by MRSA. Treatment groups showed almost similar inflammatory reaction which was pronounced on 24 and 48 HPIT. Moreover, in 72 h of treatment the group showed more prominent MNC activity in the focal area and which was absent in control group. This infers that treatment with peptide fastens the wound healing process by elevating the acute inflammatory response than normal healing process. It can be concluded that SD-8 is shown to have a wide spectrum of antimicrobial activity for major group of clinically relevant organisms specially MRSA with high specificity. The in vitro observations can also be correlated with the in vivo studies. The immunomodulatory property of SD-8 and analogs of it for higher efficacy are under experimental condition. This investigation showed first time the histopathological effects of AMP. The in vivo tests are rather preliminary in stage and it can be further confirmed by other immunological assays. In light of the above discussion we may state that SD-8 can surely set a platform for the development of a promising AMP drug.

Acknowledgments Authors acknowledge financial support from the Indian Council Medical Research, Department of Science and Technology, Council of Scientific and Industrial Research, Government of India and SAIF facility, All India Institute of Medical Sciences for performing TEM.

References

Alvarez-Bravo J, Kurata S, Natori S (1994) Novel synthetic antimicrobial peptides effective against methicillin-resistant *Staphylococcus aureus*. Biochem J 302:535–538

Andreu D, Rivas L (1998) Animal antimicrobial peptides: an overview. Biopolymers 47:415–433

Andreu D, Ubach J, Boman A, Wahlin B, Wade D, Merrifield RB, Boman HG (1992) Shortened cecropin A-melittin hybrids. Significant size reduction retains potent antibiotic activity. FEBS Lett 296:190–194

Bisht GS, Rawat DS, Kumar A, Kumar R, Pasha S (2007) Antimicrobial activity of rationally designed amino terminal modified peptides. Bioorg Med Chem Lett 17:4343–4346

Chen Y, Mant CT, Farmer SW, Hancock RE, Vasil ML, Hodges RS (2005) Rational design of alpha-helical antimicrobial peptides with enhanced activities and specificity/therapeutic index. J Biol Chem 280:12316–12329

Chen Y, Guarnieri MT, Vasil AI, Vasil ML, Mant CT, Hodges RS (2007) Role of peptide hydrophobicity in the mechanism of action of alpha-helical antimicrobial peptides. Antimicrob Agents Chemother 51:1398–1406

Christensen B, Fink J, Merrifield RB, Mauzerall D (1998) Channel-forming properties of cecropins and related model compounds incorporated into planar lipid membranes. Proc Natl Acad Sci USA 85:5072–5076

Cirioni O, Silvestri C, Ghiselli R, Giacometti A, Orlando F, Mocchegiani F, Chiodi L, Vittoria AD, Saba V, Scalise G (2006) Experimental study on the efficacy of combination of alpha-helical antimicrobial peptides and vancomycin against *Staphylococcus aureus* with intermediate resistance to glycopeptides. Peptides 27:2600–2606

Cirioni O, Giacometti A, Kamysz W, Silvestri C, Riva A, Della Vittoria A, Abbruzzetti A, Lukasiak J, Scalise G (2007) In vitro activities of tachyplesin III against *Pseudomonas aeruginosa*. Peptides 28:747–751

Culling CFA (1995) Handbook of histological techniques, 2nd edn. Butterworth & Co, London

Devine DA, Hancock RE (2002) Cationic peptides: distribution and mechanisms of resistance. Curr Pharm Des 8:703–714

Duclohier H, Molle G, Spach G (1989) Antimicrobial peptide magainin I from Xenopus skin forms anion-permeable channels in planar lipid bilayers. Biophys J 56:1017–1021

Frecer V, Ho B, Ding JL (2004) De novo design of potent antimicrobial peptides. Antimicrob Agents Chemother 48:3349–3357

Friedrich CL, Moyles D, Beveridge TJ, Hancock RE (2000) Antibacterial action of structurally diverse cationic peptides on Gram-positive bacteria. Antimicrob Agents Chemother 44:2086–2092

Gadepalli R, Dhawan B, Sreenivas V, Kapil A, Ammini AC, Chaudhry R (2006) A clinico-microbiological study of diabetic foot ulcers in an Indian tertiary care hospital. Diabetes Care 29:1727–1732

Giacometti A, Cirioni O, Kamysz W, D'Amato G, Silvestri C, Del Prete MS, Licci A, Riva A, Lukasiak J, Scalise G (2005) In vitro activity of the histatin derivative P-113 against multidrug-resistant pathogens responsible for pneumonia in immunocompromised patients. Antimicrob Agents Chemother 49:1249–1252

Gierse JK, Koboldt CM, Walker MC, Seibert K, Isakson PC (1999) Kinetic basis for selective inhibition of cyclo-oxygenases. Biochem J 339:607–614

Hancock RWE MIC determination for cationic antimicrobial peptide by modified micro titer broth dilution method. Hancock laboratory. http://cmdr.ubc.ca/bobh/methods/methodsall.html)

Hancock RE (1997) Peptide antibiotics. Lancet 349:418–422

Hancock RE, Chapple DS (1999) Peptide antibiotics. Antimicrob Agents Chemother 43:1317–1323

Hancock RE, Lehrer R (1998) Cationic peptides: a new source of antibiotics. Trends Biotechnol 16:82–88

Hancock RE, Sahl HG (2006) Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat Biotechnol 24:1551–1557

Jasir A, Kasprzykowski F, Kasprzykowska R, Lindstrom V, Schalen C, Grubb A (2003) New antimicrobial cystatin C-based peptide active against Gram-positive bacterial pathogens, including methicillin-resistant *Staphylococcus aureus* and multiresistant coagulase-negative staphylococci. APMIS 111:1004–1010

Jones RN (2006) Microbiological features of vancomycin in the 21st century: minimum inhibitory concentration creep, bactericidal/static activity, and applied breakpoints to predict clinical outcomes or detect resistant strains. Clin Infect Dis 42:13–24

Klein E, Smith DL, Laxminarayan R (2007) Hospitalizations and deaths caused by methicillin-resistant *Staphylococcus aureus*, United States, 1999–2005. Emerg Infect Dis 13:1840–1846

Kretschmann E, Reather H (1968) Radiative decay of nonradiative surface plasmon excited by light. Z Naturf A 23:2135–2136

Mackie TJ, McCartney JE (1996) Mackie & McCartney practical medical microbiology, 14th edn. Churchill Livingstone, New York

Mathur P, Jagannathan NR, Chauhan VS (2007) Alpha, beta-dehydrophenylalanine containing cecropin-melittin hybrid peptides: conformation and activity. J Pept Sci 13:253–262

McInturff JE, Wang SJ, Machleidt T, Lin TR, Oren A, Hertz CJ, Krutzik SR, Hart S, Zeh K, Anderson DH (2005) Granulysin-derived peptides demonstrate antimicrobial and anti-inflammatory effects against Propionibacterium acnes. J Invest Dermatol 125:256–263

Merrifield RB (1963) Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. J Am Chem Soc 85:2149–2154

Nissen-Meyer J, Nes IF (1997) Ribosomally synthesized antimicrobial peptides: their function, structure, biogenesis, and mechanism of action. Arch Microbiol 167:67–77

Nylander C, Leidberg B, Lind T (1982) Gas detection by means of surface plasmon resonance. Sens Actuators 3:79–88

Oren Z, Shai Y (1998) Mode of action of linear amphipathic alpha-helical antimicrobial peptides. Biopolymers 47:451–463

Otto A (1968) Excitation of nonradiative surface plasma waves in silver by the method of frustrated total reflection. Z Phys 216:398–410

Richards JA, Petrel TA, Brueggemeier RW (2002) Signaling pathways regulating aromatase and cyclooxygenases in normal and malignant breast cells. J Steroid Biochem Mol Biol 80:203–212

Sader HS, Watters AA, Fritsche TR, Jones RN (2007) Daptomycin antimicrobial activity tested against methicillin-resistant staphylococci and vancomycin-resistant enterococci isolated in European medical centers (2005). BMC Infect Dis 7:29

Sakoulas G, Moise-Broder PA, Schentag J, Forrest A, Moellering RC Jr, Eliopoulos GM (2004) Relationship of MIC and bactericidal activity to efficacy of vancomycin for treatment of methicillin-resistant *Staphylococcus aureus* bacteremia. J Clin Microbiol 42:2398–2402

Schentag JJ, Hyatt JM, Carr JR, Paladino JA, Birmingham MC, Zimmer GS, Cumbo TJ (1998) Genesis of methicillin-resistant *Staphylococcus aureus* (MRSA), how treatment of MRSA infections has selected for vancomycin-resistant *Enterococcus faecium*, and the importance of antibiotic management and infection control. Clin Infect Dis 26:1204–1214

Schwab U, Gilligan P, Jaynes J, Henke D (1999) In vitro activities of designed antimicrobial peptides against multidrug-resistant cystic fibrosis pathogens. Antimicrob Agents Chemother 43:1435–1440

Sigurdardottir T, Andersson P, Davoudi M, Malmsten M, Schmidtchen A, Bodelsson M (2006) In silico dentification and biological evaluation of antimicrobial peptides based on human cathelicidin LL-37. Antimicrob Agents Chemother 50:2983–2989

Sitaram N, Sai KP, Singh S, Sankaran K, Nagaraj R (2002) Structure-function relationship studies on the frog skin antimicrobial peptide tigerinin 1: design of analogs with improved activity and their action on clinical bacterial isolates. Antimicrob Agents Chemother 46:2279–2283

Somvanshi RK, Kumar A, Kant S, Gupta D, Singh SB, Das U, Shrinivashan A, Singh TP, Dey S (2007) Surface plasmon studies and biochemical evaluation of a potent peptide inhibitor against cyclooxygenase-2 as an anti-inflammatory agent. Biochem Biophy Res Commun 361:37–42

van't Hof W, Veerman EC, Helmerhorst EJ, Amerongen AV (2001) Antimicrobial peptides: properties and applicability. Biol Chem 382:597-619

Yamada K, Natori S (1994) Characterization of the antimicrobial peptide derived from sapecin B, an antibacterial protein of *Sarcophaga peregrina* (flesh fly). Biochem J 298:623–628

Zaitsu M, Hamasaki Y, Matsuo M, Miyazaki M, Hayasaki R, Muro E, Yamamoto S, Kobayashi I, Ichimaru T, Miyazaki S (1999) Induction of cytosolic phospholipase A2 and prostaglandin H2 synthase-2 by lipopolysaccharide in human polymorphonuclear leukocytes. Eur J Haematol 63:94–102

Zasloff M (1987) Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. Proc Natl Acad Sci USA 84:5449–5453

Zhang L, Parente J, Harris SM, Woods DE, Hancock RE, Falla TJ (2005) Antimicrobial peptide therapeutics for cystic fibrosis. Antimicrob Agents Chemother 49:2921–2927

Zhu WL, Lan H, Park IS, Kim JI, Jin HZ, Hahm KS, Shin SY (2006) Design and mechanism of action of a novel bacteria-selective antimicrobial peptide from the cell-penetrating peptide Pep-1. Biochem Biophys Res Commun 349:769–774