Biochimica et Biophysica Acta 1828 (2013) 677–686

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

A novel antimicrobial peptide derived from modified N-terminal domain of bovine lactoferrin: Design, synthesis, activity against multidrug-resistant bacteria and *Candida*

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ARTICLE INFO

Article history:
Received 20 April 2012
Received in revised form 13 September 2012
Accepted 21 September 2012
Available online 29 September 2012

Keywords:
Antimicrobial peptide
Lactoferrin
Extended spectrum beta lactamase
Anti-inflammatory
Surface plasmon resonance
Cyclooxygenase

ABSTRACT

Lactoferrin (LF) is believed to contribute to the host's defense against microbial infections. This work focuses on the antibacterial and antifungal activities of a designed peptide, L10 (WFRKQLKW) by modifying the first eight N-terminal residues of bovine LF by selective homologous substitution of amino acids on the basis of hydrophobicity, L10 has shown potent antibacterial and antifungal properties against clinically isolated extended spectrum beta lactamases (ESBL), producing gram-negative bacteria as well as *Candida* strains with minimal inhibitory concentrations (MIC) ranging from 1 to 8 μg/mL and 6.5 μg/mL, respectively. The peptide was found to be least hemolytic at a concentration of 800 μg/mL. Interaction with lipopolysaccharide (LPS) and lipid A (LA) suggests that the peptide targets the membrane of gram-negative bacteria. The membrane interactive nature of the peptide, both antibacterial and antifungal, was further confirmed by visual observations employing electron microscopy. Further analyses, by means of propidium iodide based flow cytometry, also supported the membrane permeabilization of *Candida* cells. The peptide was also found to possess anti-inflammatory properties, by virtue of its ability to inhibit cyclooxygenase-2 (COX-2). L10 therefore emerges as a potential therapeutic remedial solution for infections caused by ESBL positive, gram-negative bacteria and multidrug-resistant (MDR) fungal strains, on account of its multifunctional activities. This study may open up new approach to develop and design novel antimicrobials.

1. Introduction

The emergence of epidemic causing drug resistant microbes has galvanized interest and research in the urgent task of developing peptide antibiotics. In recent times, infections caused by the community acquired extended spectrum beta lactamases (ESBL) producing *Escherichia coli*, *Klebsiella* sp. and potential epidemic causing fungal pathogens such as *Candida albicans* and *Candida tropicalis* have become an area of major interest, especially when one considers the threat they are likely to pose to hospitalized patients [1,2]. In an era where there is sufficient clinical evidence to indicate increasing microbial resistance to existing antibiotics, the task of developing alternate therapeutics assumes critical importance.

Antimicrobial peptides (AMPs) are currently under consideration as a potential alternative to conventional antibiotics, on account of their widespread occurrence throughout nature [3–5]. AMPs have already distinguished themselves, being one of the most important elements of the innate immune system. More than 1900 natural AMPs are registered in the Antimicrobial Peptide Database (APD: http://aps.unmc.edu/AP/main.html), emphasizing the importance of expanding AMP research [6]. Moreover, logic dictates that, given their capacity for broad spectrum activity, these molecules would also be unlikely to pose a serious cause, if any, for the development of antimicrobial resistance to these peptides. This is further enhanced by their ability to swiftly destroy infection causing microbes.

Being mostly cationic in nature, they exhibit a high affinity for the lipopolysaccharide (LPS) or endotoxin layer of the gram-negative strains that are associated with pathogenicity, a fact that is further reinforced by the critical role they play in the lethal endotoxic shock syndrome [7,8]. Moreover, microbial infections are known to interfere with cell signals, leading to the activation of cyclooxygenase-2 (COX-2) and phospholipase A_2 production — the very enzymes that trigger inflammation [9].

The fragments of lactoferrin (LF) are attractive candidates for pharmaceutical applications on account of a variety of reasons. First, they have proved to be effective within the problem scenario of accelerating

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http://dx.doi.org/10.1016/j.bbamem.2012.09.021

microbial resistance to currently used antibiotics [10,11]. Several antibacterial and antifungal peptides obtained from the N-terminal region of human and bovine LF have been shown to be highly active; most of them are membrane interactive [12–18]. Other possible targets of action are mitochondria and DNA of *C. albicans* [15,19]. Such antimicrobial peptides include the first 11 residues of N-terminal amino acids of human LF (hLF), which are highly effective against infections caused by multidrug-resistant (MDR) *Staphylococcus aureus* in rodents [20], besides also eradicating *Acinetobacter baumannii* from infected patients [12]. Secondly, the native LF molecule also acts as an anti-inflammatory factor by binding to the cell walls (LPS) of microbes and inhibiting the production of pro-inflammatory factors [21].

In this study, we focused on and synthesized the first 8 residues of N-terminal of bovine LF with sequence **APRKNVRW**, other peptides being designed from this template. Each amino acid was sequentially substituted by its homologs with varying hydrophobicity. Here, we have reported the antibacterial activity of modified N-terminal 8 residue peptide derived from bovine LF against gram-negative strains, the major focus being on ESBL positive *E. coli*, *K. pneumoniae* and *Acinetobacter* sp. Further screening was done for antifungal activity against clinically isolated *Candida* sp. related to blood stream infections, with a view to developing the best antimicrobial peptide L10 with sequence **WFRKQLKW**. Other *in vitro* properties related to haemolytic, anti-COX-2, and mechanisms involving their action, were also analyzed.

2. Materials and methods

2.1. Microorganisms

For antibacterial assay, clinical isolates of all ESBL positive gram-negative bacterial strains *i.e. E. coli*, *K. pneumoniae* and *Acinetobacter* sp. were isolated from sputum, urine and pus samples. *E. coli* (ATCC 25923) was used for quality control. The strains used for determining candidacidal activity were *C. albicans* (ATCC SC5314), *C. tropicalis* (ATCC 13803), *C. krusei* (ATCC 6258), *C. glabrata* (ATCC 15126) and *C. parapsilosis* (ATCC 22019) and their respective clinical isolates cultured from blood stream infection. All clinical isolates were obtained from hospitalized patients of All India Institute of Medical Sciences, New Delhi, India. Strains were properly identified by using standard biochemical tests.

2.2. Synthesis of peptides

The peptides were synthesized by solid phase peptide synthesizer PS3 (Protein technology, USA) using Fmoc and Wang resin (G.L. Biochem, China) chemistry [22]. The solvent used for synthesis was dimethylformamide (DMF). 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was used as an activator. Fmoc was deprotected by 20% piperidine, and Wang resin was cleaved using trifluoroacetic acid (TFA). The peptides were precipitated from dry ether.

2.3. Analytical RP-HPLC of peptide

The purity of the peptides (>95%) was verified by analytical RP-HPLC using C18 reversed phase column (1.6×10 cm, Amersham Bioscience). 1 mg/mL of peptide was loaded onto the RPC column. The linear gradient was formed by passing two different solvents: 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.

2.4. Measurement of minimal inhibitory concentration (MIC)

Cationic peptides tend to stick to the walls of polystyrene microtiter plates. Hence, the MIC of cationic peptide was determined using modified microtiter broth dilution method (http://cmdr.ubc.ca/bobh/methods/methodsall.html) in polypropylene 96 well microtiter plates. For antibacterial assay, serial two-fold dilutions of each peptide solution, along with Gentamycin as control antibiotic, were prepared in (COSTAR, catalogue no. 3790) Mueller Hinton (MH) broth. A total of 100 μL of the adjusted inoculum (5×10^5 CFU/mL) organisms were added to each well, after which the plates were incubated overnight at 37 °C in ambient air.

The antifungal activities of the peptides were determined by following the guidelines proposed by the Clinical and Laboratory Standards Institute (CLSI) (Clinical and Laboratory Standard Institute; formerly the National Committee for Clinical Laboratory Standards (NCCLS) [23]. Serial two-fold dilutions of peptide solutions (100 μL) were prepared in microtiter plate. A total of 100 μL of the adjusted inoculum (10^5 CFU/mL) organisms in RPMI medium buffered at pH 7.0 with MOPS buffer were added to each well. The plates were then incubated overnight at 37 °C in ambient air. Fluconazole was used as the control antibiotic.

In both tests, organisms without peptide were treated as the positive control, while uninoculated broth was used as the negative control. The MIC was taken as the lowest drug concentration at which the observable growth was inhibited. The results were confirmed by reading the plate in a 96 well Microplate reader (Biotech Instruments). Experiments were performed in triplicate and the mean values were interpreted.

2.5. Solubility of peptides and stability of solutions

To get an understanding of the self-degradation of the peptides during the study period, we checked the stability of the peptide under different conditions. Three aliquots each of 200 μL (stock solution of 640 μg/mL) of L10 were stored at room temperature, −20 °C and 4 °C, respectively. Each aliquot was tested for antimicrobial activity after time intervals of 7, 14, 21 and 30 min [24].

2.6. Measurement of minimal hemolytic concentration (MHC)

The hemolytic effect of the peptides was determined on human red blood cells (hRBC). The freshly collected blood was 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 4% (v/v) in PBS) and 100 μL of peptide solution were added into sterilized 96 well plate. The plate was then incubated for 1 h at 37 °C and centrifuged at 1000 ×*g* for 5 min. The supernatant in aliquots (100 μL) was transferred to fresh 96 well plates, where the hemoglobin released was monitored spectrophotometrically at 414 nm. Similar steps were carried out for Gentamycin as control. The percentage of hemolysis was calculated by using the following formula:

$$\%\ \text{Hemolysis} = \{(\text{Abs414 nm in the peptide solution} - \text{Abs414 nm in PBS}) / (\text{Abs414 nm in 0.1\% TritonX} - 100 - \text{Abs414 nm in PBS})\} \times 100$$

Zero percentage and 100% hemolysis were determined in PBS and 0.1% Triton X-100, respectively.

2.7. Calculation of therapeutic index (TI)

The value of TI refers to the property of cell selectivity of any drug molecule. It is a measurement of the capability of distinctions between any pathogen and its host cells. The ratio of MHC to MIC gives the value of TI. The MHC and MIC values were determined by 2-fold dilutions. There was no detectable hemolysis at 800 μg/mL of peptide concentration, so the value of 1600 μg/mL was used to calculate the TI.

2.8. Time-kill assay

The bacterial strains were grown overnight at 37 °C in MH broth in ambient air. Aliquots of exponentially growing bacteria were re-suspended in fresh MH broth at an approximate density of 10^7 cells/mL, then separately exposed to the peptide at a final concentration of two times the MIC, *i.e.*, 2 μg/mL, for 0, 5, 10, 20, 30, 40, 60, 90, 120, and 150 min at 37 °C. After each observation, samples were serially diluted and plated onto MH agar plates to obtain viable colonies.

A similar procedure was carried for the fungal strains, albeit with some modifications. The broth used was RPMI medium buffered to pH 7.0 with MOPS buffer, and the cell density used was approximately 2×10^5 cells/mL. Furthermore, Sabouraud Dextrose Agar (SDA) plates were used to obtain the viable colonies.

2.9. Electron microscopic studies

EM studies were conducted to evaluate the mechanism of action of L10. We used transmission electron microscopy (TEM) to see the interaction of the peptide with a clinical isolate of the ESBL positive *E. coli*. Freshly inoculated liquid cultures of the bacteria in MHB were grown, up to mid logarithmic phase. These were washed well with sodium phosphate buffer (10 mM) and centrifuged for 3 min at 1500 ×*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 three times with 0.1% phosphate buffer. The samples were post fixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 2 h. The fixed samples were washed with phosphate buffer followed by dehydrating with a series of acetone gradients. These samples were passed through propylene oxide and infiltrated in epoxy resin for 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 thereafter 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 L10 (10^8 cells in 100 μL were treated with 4 μg of peptide) [25]. The samples were analyzed for different lengths of time (5 to 30 min).

A similar study involving scanning electron microscopy (SEM) was conducted to evaluate the antifungal mechanism of action with quality control isolates of *C. albicans* (ATCC SC5314) and *C. tropicalis* (ATCC 13803). Similar steps as in TEM were followed to collect the fungal pellet. The test samples were pretreated with L10 (hundred folds MIC) for respective fungal strains for 2 h [15]. A high concentration of peptide was chosen in order to achieve killing of a high number of yeast cells (final concentration of 2×10^7 cells/mL). They were fixed in an aldehyde mixture (2% paraformaldehyde, 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.2) diluted in a ratio 1:1 with PBS. The suspension was then washed with PBS. The yeast cells (250 μL) were transferred to poly-L-lysine treated, gold coated Thermanox™ coverslips for 30 min to allow the cells to adhere to the surface. After rinsing with PBS, cells were prepared for SEM using OTOTO method comprising repeated treatment with osmium tetroxide and thiocarbohydrazide [26]. The coverslips were dehydrated with ethanol and were infiltrated with hexamethyldisilazane, and were then allowed to evaporate in a fume hood. The dried specimens were then mounted on aluminium SEM stubs and examined without thin film metal coating in a scanning electron microscope (Leo Imaging Systems).

2.10. In vitro tests supporting antibacterial activities

2.10.1. LPS-binding assay

The endotoxin binding assay was carried out by the quantitative chromogenic LAL kit. (Genscript, USA Inc.) Stock solution of L10 (64 μg/mL) was prepared in pyrogen-free water and serially diluted in the same solvent. Twenty-five microliter peptide solution and 25 μL of 1 U/mL of LPS were mixed in each well, and plates were incubated for 30 min at 37 °C to allow the peptide to bind with LPS. The mixture was incubated for 10 min in the presence of 50 μL of amebocyte lysate reagent, and 100 μL of the chromogenic substrate was added. Incubation was continued at 37 °C for 6 min and release of the product was monitored at 545 nm using Spectrophotometer (T 60 UV, PG instrument. The change in optical density (ΔOD) between 0 and 6 min was calculated for the control sample, which contained the peptide with no LPS, and this value was subtracted from the ΔOD between 0 and 6 min for samples containing both the peptide and LPS. The percentage of LPS binding with different concentrations of peptide was calculated from the quotient (Q) of the ΔOD, with peptide divided by the ΔOD of peptide-free control samples, using the formula $(1-Q)\times100$. Standard curves generated with increasing amounts of LPS were linear between 0.1 and 1.0 endotoxin units/assay. Colistin was used as positive control for its high binding property to LPS.

2.10.2. Bio-interaction between L10 peptide and Lipid A (LA)

The binding of L10 with LA was determined by surface plasmon resonance (SPR) [27–29] using BIAcore 2000 (Pharmacia, Sweden). HPA chip was washed with 25 μL of 40 μM n-octyl β-D-glucopyranoside (Sigma). LA (diphosphoryl from *E. coli* F583, Rd mutant) was sonicated for 15 min at 37 °C before immobilization. 100 μL of LA (0.1 mg/mL) was passed over washed flow cell of HPA chip at a flow rate of 1 μL/min for 100 min. The excessive LA was removed by 0.1 M NaOH to obtain a monolayer of LA. Around 1063 Resonance Unit (RU) of LA was immobilized under this condition. Three concentrations of L10 (4.19, 12.58 and 20.97 μM) were passed over LA at a flow rate of 10 μL/min, and changes in sensogram were observed. After passing each concentration of peptide, it was regenerated by 0.1 M NaOH. The graph shows the change in RU values with different concentrations of peptides. K_A and K_D were obtained by fitting the primary sensogram data using the BIAevaluation 3.0 software.

The dissociation constant is derived using the equation

$$R_t = R_{to}e^{-kD(t-to)}$$

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

$$R_t = R_{max}\left(1-e^{-(KAC+KD)(t-t0)}\right)$$

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

2.11. In vitro tests supporting antifungal activities

2.11.1. Candida membrane permeability

The permeabilization of the *Candida* sp. membrane was investigated by flow cytometric analysis by the DNA binding fluorescent probe propidium iodide (PI), as described previously [15]. Briefly, yeast cells were grown overnight and were transferred to fresh RPMI medium buffered to pH 7.0 with MOPS buffer for another 2 h of growth. Cells were washed and diluted in 10 mM phosphate buffer (pH 7.2). Yeast cells were exposed to the peptides at different concentrations (1, 10, 100 and 1000 μg/mL) and incubated for 2 h at 37 °C. The final density of the yeast cells was 2×10^6 cells/mL. The cells were then re-incubated at room temperature with 1 μg of PI for 5 min, followed by analysis on a FACScan Flow Cytometer.

2.11.2. Candida mitochondrial membrane permeability

The assessment of mitochondrial permeability was done by using the fluorescent probe rhodamine 123 (Sigma), since it accumulates in the cell compartment [19]. Briefly, the yeast cells in log phase were suspended in potassium phosphate buffer (1 mM, pH 7.0) and incubated with rhodamine 123 (5 μM) for 15 min at 37 °C in a water bath. After 3 washes with potassium phosphate buffer, the cells were treated with L10 at concentration two times their respective MICs for 10 min at 37 °C. The distribution of rhodamine 123 in the yeast cells was examined using a fluorescence microscope (*NIKON* Eclipse E600).

2.12. Anti-inflammatory assay

The inflammatory enzyme COX-2 used for binding assay with L10 by SPR and spectrophotometric methods.

2.12.1. SPR studies

The real time measurement of the interaction between COX-2 with L10 was performed using a biosensor method based on SPR [30]. Six Histidines-tags attached to the terminal position of COX-2 were ideal for immobilization due to the strong rebinding effect caused by the high surface density of immobilized Ni^{2+}–Nitriloacetic acid (NTA) on the chip. The binding of analyte, *i.e.*, the peptide in solution, can be studied by monitoring the change in the RU values of the sensorgram, where the progress of the interaction is plotted against time, thus revealing the binding characteristics. The parameters of binding kinetics, *i.e.*, the equilibrium association (K_A) and equilibrium dissociation (K_D) constant for the formation of multi-molecular complex and dissociation, were determined in a very short time and with small amounts of samples. 60 μL of His-tag COX-2 (50 μg/mL) was injected to one of the flow cells at a flow rate of 5 μL/min. 900 RU of COX-2 were immobilized under these conditions, where 1 RU corresponds to immobilized protein concentration of ~1 pg mm^2. The analyte, *i.e.*, L10 (8.39 μM) was passed over the immobilized COX-2 at a flow rate of 10 μL/min for 4 min. Likewise, two different concentrations 25.1 and 41.9 μM of L10 were passed over the chip and the change in RU was observed. The constants K_A and K_D were obtained from the primary sensogram data using the BIA evaluation 3.0 software.

2.12.2. Inhibition studies of peptide

The COX-2 inhibitory property of the peptides was assayed. Initially, the activity assay for the pure 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 [31]. The inhibition by the peptide was determined by pre-incubating it with enzyme in 1:1 molar ratio in the same conditions.

3. Results

3.1. Designing of peptide

Several studies have been conducted so far on the antimicrobial properties of LF in order to map the different antimicrobial regions of the LF molecule. In this study, we have focused on the role of the shortest possible N-terminal antimicrobial domain of the bovine LF prior to the disulphide bridge. The first 8 N-terminal residues of bovine LF, *i.e.*, **APRKNVRW** (named as L1) were chosen as a template for designing synthetic AMPs. Nine different peptides were designed by changing the amino acids of L1 by incorporating the homologous amino acid residues of different hydrophobicities by considering the hydrophobic scale [32], and were studied for the antimicrobial activity against *E. coli* (ATCC 25923).

We started the incorporation of the amino acids from the C-terminal region of L1 and proceeded towards the N-terminal (Table 1). In this respect, the first amino acid at position one was W in L1. Sticking to the hydrophobic scale and position specificity concept, W was replaced by F in L2 (W and F belong to the same structural class with different hydrophobicities). The MIC of L2 was found to be more than L1. In L3, the second residue, R of L2 was substituted by K. Similarly, L4 to L10 peptides were synthesized by replacement of amino acid and MICs were determined. The sequence and the activity data of all the peptides are provided in Table 1. Among all the peptides, L10 was the best-designed peptide showing MIC of 1 μg/mL, so it was further tested for its other *in vitro* activities.

3.2. Antimicrobial activity

Table 2A reports the MICs for the L10 with different ESBL positive bacterial strains cultured from different patient samples. The range for the MIC was reported to be 1–8 μg/mL (with an average of 4.06 μg/mL for 30 clinically isolated strains). L10 was also found to have promising antifungal activities. Table 2B shows the MICs for different *Candida* strains, ATCC and clinical isolates. The peptide was found to be most active against *C. albicans* (ATCC 4923) and *C. parapsilosis* (ATCC 22019) with significant MIC values.

3.3. Stability test

The stability of the peptide in the solution is one of the key factors in determining its antimicrobial efficiency within a given period of time. There was no detectable change in the antimicrobial activity of the L10 even after 30 days of storage, as compared to a freshly prepared solution (data not shown).

3.4. MHC measurements

The peptide was found to be nearly non hemolytic even at high concentrations. Less than 1.5% of 4% v/v hRBC were hemolyzed by 800 μg/mL of L10. Fig. 1 shows the hemolytic effect of L10. Gentamycin was used as positive control.

3.5. Therapeutic index

The peptide was found to be non-hemolytic to the mammalian erythrocyte at a concentration of 800 μg/mL, hence the TI value was calculated from 1600 μg/mL. The higher values obtained for the peptide reflect its being a highly specific AMP [32]. This factor indicates the ability of the drug to selectively target the pathogen instead of the patient's own cells.

Table 1
Primary screening of the peptides by the determination of MIC against standard strain of *E. coli* (ATCC- 25923). Gentamycin was used as control antibiotic.

Peptide	Sequence	MIC (μM)	MIC (μg mL^{-1})
L1	APRKNVRW	62.36	64
L2	APRKNVR**F**	129.66	128
L3	APRKNV**K***W*	64.11	64
L4	APRKN**LK***W*	31.61	32
L5	APRK***QLK****W*	7.79	8
L6	APR***RQLK****W*	15.17	16
L7	AP***KK****QLK****W*	16.02	16
L8	A**F***RK****QLK****W*	3.71	4
L9	**LF***RK****QLK****W*	1.78	2
L10	**WF***RK****QLK****W*	0.8	1
Gantamycin	–	0.52	0.25

Bold letters are for the substituted amino acids, italicized for the unfavorable substitution that are revert back to the parent amino acid and the bold and italized represents the favorable substitution of the amino acid that are carry forwarded. The final peptide L10 contains all the possible favorable substitutions.

Table 2A
Minimal inhibitory concentration (MIC), source of isolation and therapeutic index (TI) for the peptide L10 against different bacterial strains.

Sl No.	Sample number/Patient ID	Organism	Cultured from	MIC (μM)	MIC (μg mL^{-1})	Therapeutic Index[a]
1	ATCC (25923)	*E. coli*	–	0.8	1	1600
2	309/19.3.10	*K. pneumoniae*	Sputum	3.35	4	400
3	301/19.3.10	*Acinetobacter* sp.	Sputum	1.67	2	800
4	302/19.3.10	*Psudomonas aruginosa*	Sputum	6.71	8	200
5	305/19.3.10	*K. pneumoniae*	Sputum	3.35	4	400
6	312/19.3.10	*Acinetobacter* sp.	Sputum	3.35	4	400
7	224/18.3.10	*K. pneumoniae*	Pus	3.35	4	400
8	217/19.3.10	*E. coli*	Pus	1.67	2	800
9	202/19.3.10	*K. pneumoniae*	Pus	6.71	8	200
10	237/19.3.10	*E. coli*	Pus	0.8	1	1600
11	309/25.3.10	*K. pneumoniae*	Sputum	3.35	4	400
12	301/25.3.10	*Acinetobacter* sp.	Sputum	3.35	4	400
13	305/25.3.10	*K. pneumoniae*	Sputum	3.35	4	400
14	312/25.3.10	*Acinetobacter* sp.	Sputum	6.71	8	200
15	207/25.3.10	*E. coli*	Pus	1.67	2	800
16	302/25.3.10	*K. pneumoniae*	Sputum	6.71	8	200
17	287/25.3.10	*E. coli*	Pus	3.35	4	400
18	322/25.3.10	*Acinetobacter* sp.	Sputum	3.35	4	400
19	241/26.3.10	*E. coli*	Pus	6.71	8	200
20	301/26.3.10	*E. coli*	Sputum	3.35	4	400
21	35/26.3.10	*E. coli*	Urine	6.71	8	200
22	330/26.3.10	*K. pneumoniae*	Sputum	3.35	4	400
23	36/26.3.10	*E. coli*	Urine	6.71	8	200
24	214/26.3.10	*E. coli*	Pus	1.67	2	800
25	47/26.3.10	*E. coli*	Urine	3.35	4	400
26	225/26.3.10	*E. coli*	Pus	0.8	1	1600
27	322/19.3.10	*E. coli*	Sputum	3.35	4	400
28	341/27.3.10	*K. pneumoniae*	Sputum	1.67	2	800
29	43/27.3.10	*E. coli*	Urine	3.35	4	400
30	241/27.3.10	*K. pneumoniae*	Pus	3.35	4	400
31	291/27.3.10	*E. coli*	Pus	1.67	2	800

[a] The therapeutic index is the ratio of MHC to MIC.

3.6. Time-kill assay

As in many other AMPs, the bacterial killing by L10 is a very rapid process. Its kinetic activity against the *E. coli* (ATCC 25923) was complete after a mere 40 min exposure, at a concentration two times the MIC. However, it showed slightly slower (60 min) killing activity in ESBL positive *E. coli* isolated from patients. Fig. 2A shows the kinetics results obtained from the standard strain *E. coli* (ATCC 25923) and ESBL positive *E. coli*.

Similar rapid killing kinetics observed against fungi. *C. albicans* (ATCC SC5314) and *C. tropicalis* (ATCC 13803), these being killed within 140 min and 90 min exposure respectively, even at concentrations twice their respective MICs (Fig. 2B).

Table 2B
MIC and TI of the peptide L10 against different fungal strains.

Sl no.	Sample number/ patient ID	Organism	MIC (μM)	MIC (μg mL^{-1})	TI
1	ATCC SC5314	*C. albicans*	10.49	12.5	128
2	ATCC 13803	*C. tropicalis*	20.98	25	64
3	ATCC 6258	*C. krusei*	20.98	25	64
4	ATCC 15126	*C. glabrata*	20.98	25	64
5	ATCC 22019	*C. parapsilosis*	5.24	6.25	256
6	4762	*C. albicans*	83.93	100	16
7	4662	*C. albicans*	Not active	Not active	Not determined
8	4615	*C. albicans*	41.96	50	32
9	4748	*C. albicans*	20.98	25	64
10	4923	*C. albicans*	5.24	6.25	256
11	5527	*C. albicans*	20.98	25	64
12	4912	*C. tropicalis*	10.49	12.5	128
13	4345	*C. tropicalis*	20.98	25	64
14	4913	*C. tropicalis*	20.98	25	64
15	5552	*C. tropicalis*	83.93	100	16
16	4739	*C. glabrata*	Not active	Not active	Not determined

3.7. Electron microscopic studies

To further characterize the bactericidal and fungicidal effects of the peptides, we used TEM and SEM to examine ESBL positive *E. coli* *C. albicans* (ATCC SC5314) and *C. tropicalis* (ATCC 13803), respectively. We found that bacterial cells exposed to L10 at its MIC (4 μg/mL, which is the average MIC obtained when all strains were taken into account) showed altered cell membrane morphology, with the appearances of membrane blisters. Since the kinetic experiments revealed that longer duration of incubation of bacterial cells with the peptide causes total lysis of the cells, the shorter duration effects were analyzed after fixing and sectioning (Fig. 3A).

At 5 min of incubation, changes like invagination in the cytoplasmic membrane, blebbing and formation of vacuoles were observed. After 15 min, the majority of the cells displayed leakage of cytoplasm. At 30 min of incubation, a large amount of cell debris was present. Moreover, a number of the remaining cells appeared to have clumping or

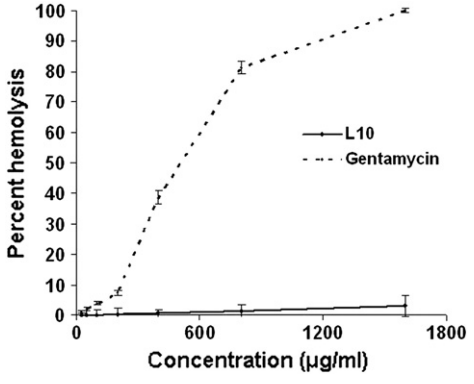

Fig. 1. Hemolytic activity of L10 on human RBCs. Each value shown is the mean ± standard error of the mean from three experiments. Amp B is positive control.

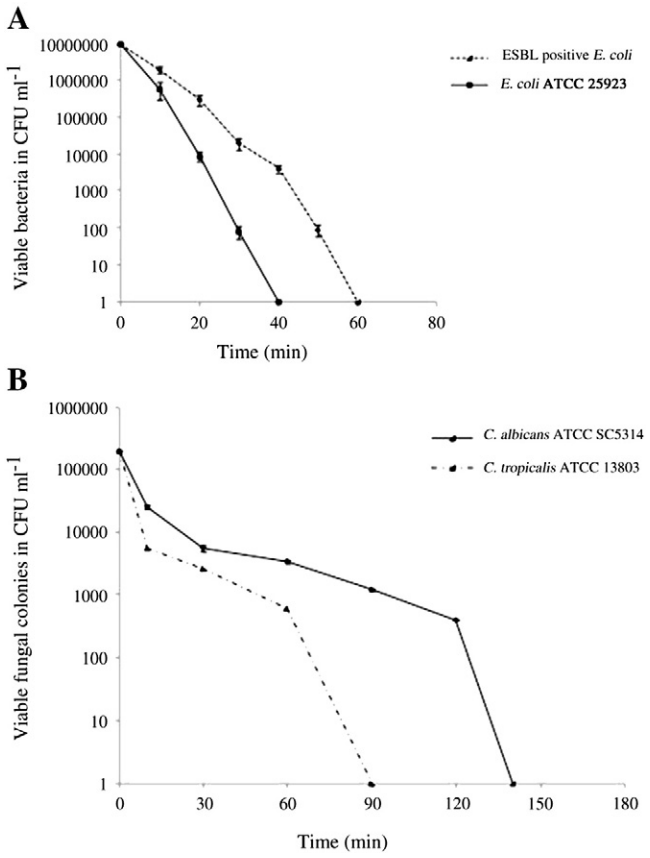

Fig. 2. Killing kinetics by the peptide L10. (A) *E. coli* (ATCC 25923) and ESBL positive *E. coli*, (B) *C. albicans* (ATCC SC5314) and *C. tropicalis* (ATCC 13803). Each value shown is the mean ± standard error of the mean from three experiments.

coagulation of cytoplasmic elements, in addition to membrane blistering. A similar kind of observation was recorded with the fungal cells' architecture, as assessed by the SEM on the cell walls of *C. albicans* (ATCC SC5314) and *C. tropicalis* (ATCC 13803), (Fig. 3B). The surface exposed to the peptide gave the appearance of being perforated. They also showed altered cell membrane morphology, leakages of cytoplasmic contents and appearances of membrane blisters, along with deep pits.

3.8. Properties supporting the antibacterial activity

3.8.1. LPS binding activity

Using LAL assay kit, L10 was analyzed for its capacity to bind with LPS. The binding of L10 to LPS was observed at low peptide concentrations, completely neutralizing the LPS at 24 μg/mL (Fig. 4). The factor has been related to the charges of AMPs. Since the L10 bears high cationicity, the LPS binding activity is predominant.

3.8.2. Binding of LA with peptide L10

The sensorgram (Fig. 5) shows the binding of the varying concentrations of the L10. The changes in RU with varying concentrations show the changes of mass on the LA immobilized on chips, over time. The dissociation constant (K_D) calculated by BIAevaluation software 3.0 was 1.52×10^{-8} M.

3.9. Properties supporting the anticandidal activity

3.9.1. Candida membrane permeability

The percentage of PI-positive cells demonstrated the permeabilization of the cytoplasmic membrane induced by the peptides after 2 h of incubation with the respective fungal strains (Fig. 6A). The percentage of PI-positive cells treated with L10 (1000 μg/mL) for *C. albicans* (ATCC SC5314) and *C. tropicalis* (ATCC 13803) were 97% and 89%, respectively. However, at lower concentrations of the peptide (10 μg/mL), there was indication of a high intake of dye. The data obtained can be correlated with MIC values.

3.9.2. Candida mitochondrial membrane permeability

The effect of the peptides on mitochondrial membrane integrity was studied by the mitochondrial fluorescent probe rhodamine 123 (Fig. 6B). It was found to be localized to sub-cellular compartments, presumed to be mitochondria, in the yeast cells. The typical granular appearance is indicative of mitochondrial localization. 10 min of exposure of the peptide L10 to *C. albicans* (ATCC SC5314) and *C. tropicalis* (ATCC 13803) changed the pattern of fluorescence in the cell, showing a uniform distribution all over the cells.

3.10. Anti-inflammatory assay

3.10.1. SPR studies

The changes in RU obtained with varying concentrations of peptide passed over immobilized inflammatory enzyme COX-2 on sensor chip indicated the change in bound mass on sensor surface, over time. Fig. 7A shows the sensogram of binding of three different concentrations of L10. The dissociation constant K_D was found to be 6.92 μM for the peptide. This can be considered as a potent anti-inflammatory peptide.

3.10.2. Inhibition studies of peptides with COX-2

The activity assay of COX-2 in the presence of the substrate (arachidonic acid) and L10 was performed using UV spectrophotometer. The activity profile of COX-2 showed an increase in absorbance with time, in presence of substrate (Fig. 7B). The rate of absorbance became stable much earlier in the presence of the peptide, indicating decline in the rate of activity of COX-2 an inhibition of 74%.

4. Discussion

The causes of the rapid emergence and dissemination of MDR strains in hospitals are multifactorial. One of the most important factors is the selective pressure of antimicrobials commonly used in treating hospitalized patients. Cross-transmission from patient to patient occurs owing to inconsistent application of appropriate infection control measures, and the inter hospital transfer of resistance. These factors lead to the development of the MDR strains. The most common pathogen, the *E. coli* responsible for bacterial infections in the urinary tract, and pneumonia, has been now characterized under the MDR strains.

The upcoming of ESBL producers has rendered the currently available antibiotics ineffective [33]. Similar is the status of the common *Candida* infection in human blood stream, a life threatening invasive disease causing significant mortality and morbidity. According to an Indian case report, the proportion of *C. tropicalis* was higher than that observed in US and Europe [34]. Flucanazole is widely used for treating fungal infections. Unfortunately, long term therapies have led to the emergence of flucanazole resistant *C. albicans* strains that are cross resistant to other azoles and also to amphotericin B [35].

As a consequence, AMPs represent an elite new class of antimicrobials with the capability of tackling these issues. The advantages of peptides as drugs include their high specificity, potency, and activity. Small peptides, as drugs, are very specific in nature. Other advantages over therapeutic proteins include higher solubility, better stability, more bioavailability and negligible immune response. In the present study, we have designed a peptide based on the N-terminal fragment of the parent bovine LF molecule because of its practical implications as regards many beneficial physiological activities. This domain

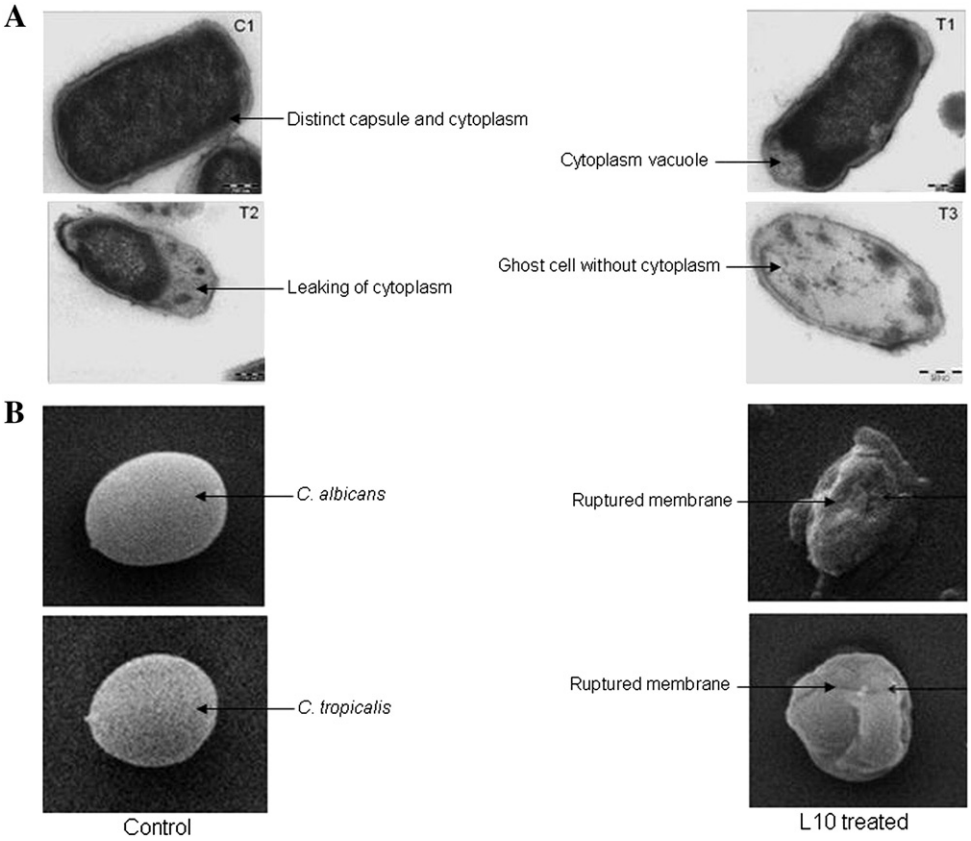

Fig. 3. Ultrastructural changes in bacteria and fungi, treated with peptide. (A) Transmission electron micrographs (TEM) of control ESBL positive *E. coli* (C1), 5 min after incubation with L10 (4 μg/mL) (T1), 15 min after incubation (T2) and 30 min after incubation (T3) (Scale: 200 nm in all images), (B) Scanning electron microscopy of *C. albicans* (ATCC SC5314) and *C. tropicalis* (ATCC 13803) exposed to 25 μg/mL and 100 μg/mL of L10 or buffer alone (control) for 2 h (magnification ×5000).

contains a high proportion (and asymmetric cluster) of basic amino acid residues that are important for antimicrobial activity. The cationic AMPs, derived from the N-terminal domain of the LF, have been shown to kill sensitive microorganisms by inducing cell membrane permeability, leading to the disruption of energy metabolism or other essential functions in the target organism [12]. The isolated peptides and the synthetic analogs of the identified peptide are more effective than the native LF, suggesting that the smaller size may facilitate access to target sites on the microbial surface [15,36].

This study revealed a short, potent AMP L10 which showed a broad-spectrum of antimicrobial activity against a wide variety of clinical isolates of ESBL producing gram-negative bacteria and MDR resistant *Candida* sp. isolated from hospitalized patients. The hemolytic studies showed that the peptide was almost non-toxic to human erythrocytes. This aspect of the peptide L10 showed that it can be administered by intravenous route. As both the values of MIC and MHC were carried out by serial two-fold dilutions, the therapeutic index could vary as much as four-fold for individual microorganisms, when the peptide is both hemolytic and antimicrobial. However, it may vary up to two-fold when the peptide is non hemolytic. It is reported that the larger the value of TI, the greater is the antimicrobial specificity [32]. L10 possesses high microbial specificity and was found to be highly stable in all conditions. The time-kill assay highlighted the rapid rate of bacterial mortality after administering this peptide. This property revealed that there are less chances of microbes developing resistance to it. Such resistance against a particular antibiotic is often encountered in the case of bacteria exhibiting a long annihilation time, thus giving strains enough time to revert themselves. Since cationic AMPs exhibit a high affinity for LPS and lipoteichoicacid (LTA) [37–39], permeabilization and lysis of microbes are the corollary.

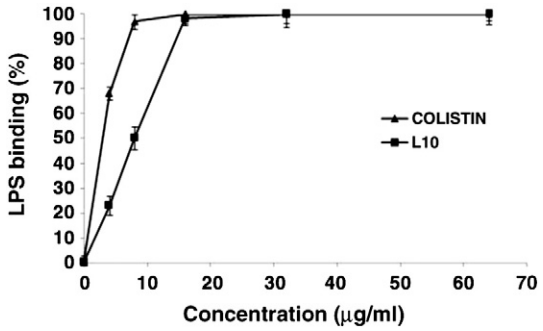

Fig. 4. LPS-binding activity (%) of L10 peptide. LPS was incubated with different concentrations of L10 and the binding was measured at 545 nm. Same concentrations of colistin were used as a positive control.

LPS is one of the most powerful stimulants of the immune system [40]. It activates toll like receptors on macrophages, monocytes and neutrophils, which then release prototypic pro-inflammatory cytokines like interleukin-1, interleukin-6 and tumor necrosis factor-alpha. These cytokines trigger an inflammatory cascade. This may lead to disseminated intravascular coagulation (mortality, 25–30%) as well as vascular instability and capillary leak contributing to the hypertension seen in septic shock [41,42]. By binding and neutralizing LPS, it would be possible to avoid these mechanisms, which contributes to the pathophysiology of sepsis. The LPS binding assay showed its potent and effective neutralization by L10. Further, it interacts with LA with a K_D value comparable to any of the known LA-peptide complexes (Table 3).

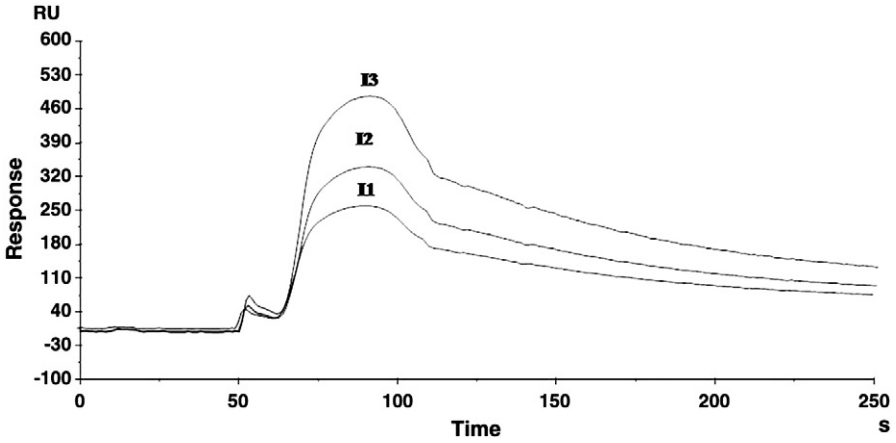

Fig. 5. Sensogram showing binding of different concentrations of peptide L10 ($I_1 = 4.19$ μM, $I_2 = 12.58$ μM and $I_3 = 20.97$ μM) on the LA immobilized surface over a HPA chip.

The mechanism of action of antimicrobial peptides is thought to involve an increase in membrane potential and permeability [43,44] and recently, the antimicrobial mechanism has been putatively associated with conductive ATP transport [45,46]. TEM and SEM studies also indicate that the peptide acts primarily on the cell membrane of *E. coli* and *Candida* sp. by disrupting the cell wall and mitochondrial membrane. The altered morphologies of the *E. coli* in TEM revealed the membrane as the target of action. Further, the result of SEM study showed the loss of fungal cytoplasmic contents by forming deep pits on the cell wall. However, the exact mode of action of AMP can be multiple, but here, the fast killing kinetics as well as the electron microscopy studies suggested that the major cause of peptide antimicrobial action is its membrane interactive nature. The effect of peptides from human LF on the cell surface of *C. albicans* as well as the membrane permeabilization activity has also been reported earlier [47,48].

Some AMPs appear to be antifungal molecules by virtue of their propensity to rupture the fungal membrane and suppress mitochondrial respiration [49]. The peptide also targets the mitochondria of the *Candida* as expressed by rhodamine 123, while the action of the fluorescence dye on the confined mitochondria indicated that the peptide accumulated in the cytoplasm. The permeabilization of cytoplasmic membrane was also confirmed by peptide stimulated PI positive fungal cells. In addition, L10 seems to be a potent competitive COX-2 inhibitory molecule. The binding affinity of L10 with COX-2

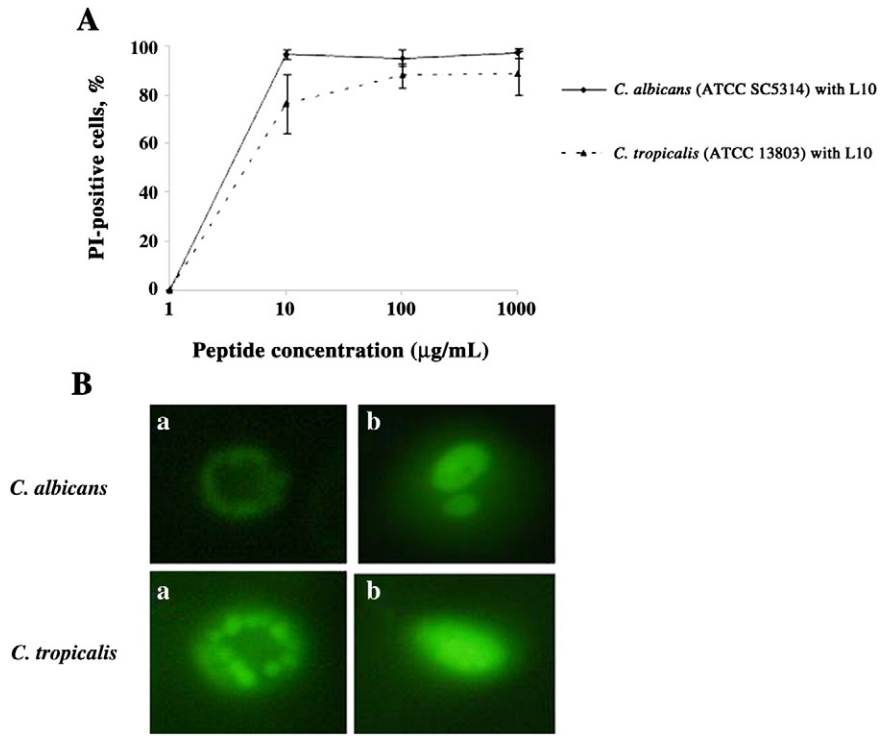

Fig. 6. Membrane permeabilization property of L10. (A) Propidium iodide (PI) staining and killing of *C. albicans* (ATCC SC5314) and *C. tropicalis* (ATCC 13803) incubated for 2 h at 37 °C with various concentrations (1, 10, 100 and 1000 μg/mL) of the peptide. Fluorescence microscopy studies of L10, (B) treatment of rhodamine 123-labeled *C. albicans* (ATCC SC5314) and *C. tropicalis* (ATCC 13803). Briefly, cells pre loaded with rhodamine 123 for 10 min at 37 °C were washed and treated with L10. (a) Pictures were made immediately after addition, (b) or after a 10 min incubation with the peptide (magnification ×100).

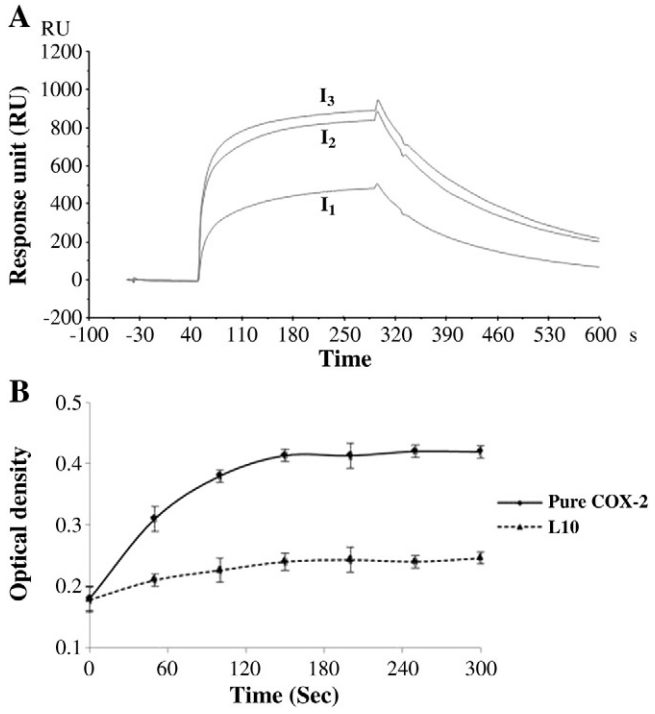

Fig. 7. COX-2 binding and inhibition by the peptide L10. Sensogram showing binding of different concentrations of peptide, (A) L10 ($I_1 = 8.39$ μM, $I_2 = 25.1$ μM and $I_3 = 41.9$ μM) on the Ni^{+2} NTA chip immobilized with His-COX-2. Inhibition kinetics assay of L10, (B) Activity profile of pure COX-2 alone and incubated with L10 in 1:1 molar for 45 min at room temperature. Each value shown is the mean ± standard error of the mean from three experiments.

was further confirmed by the BIAcore using the principle of real time analysis. This aspect of the peptide showed that it may exhibit anti-inflammatory property.

5. Conclusion

It can be concluded that L10 can be considered a potential therapeutic candidate against infections caused by ESBL positive *E. coli*, *K. pneumonia*, *Acinetobactor* sp. and *Candida* sp. due to its high antibacterial, antifungal, anti hemolytic, anti-inflammatory and antiendotoxin activities. Hence, it is a multifunctional molecule that may open up an alternative approach to the critical task of developing novel anti microbial agents, in view of the increasing problem of microbial resistance to conventional therapies.

Acknowledgements

Authors acknowledge the Indian Council Medical Research, Government of India and SAIF facility, all India Institute of Medical Sciences for performing electron microscopy.

Table 3
Comparison of K_D of different LA-peptide complexes.

Peptides	K_D(M)	References
V peptides	7.8×10^{-7}	Frecer et al. [38]
LA/11	3.7×10^{-7}	Zhu et al. [39]
Pm B	7.1×10^{-7}	Rustici et al. [50]
L10	1.46×10^{-8}	Present study

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