Controlling resistant bacteria with a novel class of β-lactamase inhibitor peptides: from rational design to in vivo analyses

Santi M. Mandal1*, Ludovico Migliolo2,3*, Osmar N. Silva2*, Isabel C. M. Fensterseifer2, Celio Faria-Junior4, Simoni C. Dias2, Amit Basak1, Tapas K. Hazra5 & Octávio L. Franco2,3

1Central Research Facility, Department of Chemistry, Indian Institute of Technology Kharagpur, Kharagpur 721302, WB, India,
2Programa de Pós-Graduação em Ciências Genômicas e Biotecnologia, Centro de Análises, Proteômicas e Bioquímicas, Universidade Católica de Brasília, Brazil, 3Pós-Graduação em Biotecnologia, Universidade Católica Dom Bosco, Campo Grande, MS, Brazil, 4Lacen, Laboratório Central de Saúde Publica do Distrito Federal, Brasília, DF, Brazil, 5Department of Internal Medicine, University of Texas Medical Branch, Galveston, TX 77555, USA.

Peptide rational design was used here to guide the creation of two novel short β-lactamase inhibitors, here named dBLIP-1 and -2, with length of five amino acid residues. Molecular modeling associated with peptide synthesis improved bactericidal efficacy in addition to amoxicillin, ampicillin and cefotaxime. Docked structures were consistent with calorimetric analyses against bacterial β-lactamases. These two compounds were further tested in mice. Whereas commercial antibiotics alone failed to cure mice infected with Staphylococcus aureus and Escherichia coli expressing β-lactamases, infection was cleared when treated with antibiotics in combination with dBLIPs, clearly suggesting that peptides were able to neutralize bacterial resistance. Moreover, immunological assays were also performed showing that dBLIPs were unable to modify mammalian immune response in both models, reducing the risks of collateral effects. In summary, the unusual peptides here described provide leads to overcome β-lactamase-based resistance, a remarkable clinical challenge.

Antimicrobial resistance is not a new problem, but the number of resistant organisms and lethal outbreaks is unprecedented1–3. Infectious agents that were once supposed to be controlled by antibiotics are returning in new forms resistant to conventional therapies, clearly making efficient and stable control of microorganisms difficult4–6. Among the antibacterial therapies, the most often used antibiotics commonly consist of lactam, including penicillins and cephalosporins6. Nevertheless, due to widespread use of lactam antimicrobials and also to genetic and biochemical factors, bacterial resistance represents a serious threat to the continuing use of antibiotic treatment7. The most conventional bacterial mechanism of resistance to lactam antibiotics is synthesis of β-lactamases, which are able to cleave the amide bond in the target β-lactam ring, rendering these antibiotics ineffective8. In this context, one logical strategy has been to pursue β-lactamase inhibitors as additives of lactams, to prevent or reduce cleavage of the β-lactam ring9,10.

Results and Discussion

Rational design for constructing synthetic peptides was first based on the pocket volume and distances between the amino acid residues that compose the β-lactamase catalytic triad (KSG) and amino acid residues adjacent to the catalytic triad. Evaluation by docking studies of inhibitor-enzymes was carried out by designing short and flexible synthetic peptide inhibitors that probably interact with the amino acid residues near the β-lactamase catalytic triad. Thus, two peptides named dBLIP-1 and -2 (designated β-lactamase inhibitor peptide 1 and 2) were rationally designed. The primary structures of dBLIP-1 and -2 were KKGEE and KQGQE, respectively. The relations between peptide and enzyme were highly coordinated and in silico guided via the side chains of amino acid residues.

In order to set out the peptide-enzyme interactions more clearly, theoretical models of dBLIP-1 and -2 were constructed. Procheck summary of dBLIP-1 and -2 showed that, for both peptides, 100% of amino acid residues are located in the most favorable regions in the Ramachandran plot. In addition, the general qualities for the models were reliable in accordance with values -0.04 and 0.36 for the g-factor, respectively. The RMSD values and variability observed among the experimental structure templates and the modeled structure demonstrated a fold
modification due to the post-modification carried out in the structure of dBLIP-1 and dBLIP-2. Moreover, neither peptide demonstrated any secondary structures. These data were confirmed by circular dichroism (data not shown). This fact was expected due to the short sequences, with only five amino acid residues in length.

Docking analyses suggested that both peptides are able to attach to the two different β-lactamase enzymes at the center of the catalytic site, being stabilized by hydrogen bond net in the case of dBLIP-1 (Figs. 1A and 1E). As well as hydrogen bonds, electrostatic and hydrophobic interactions were also observed for dBLIP-2 (Figs. 1B and 1F). The inhibitor dBLIP-1 presented more interactions and was complementary in comparison with dBLIP-2 in the *E. coli* β-lactamase *in silico* analysis (Figure 1C). The interactions observed for dBLIP-1 were between the backbone oxygen atom (O) of lysines (Lys¹ and Lys²) and the hydrogen (2HD2 and 1HD2) of Asn¹³² and Asn¹⁷⁰ forming, respectively, two hydrogen bonds with distance of 2.65 and 2.5 Å. Gly⁷ (N and O atom) participated in two hydrogen bond interactions between Ser⁷⁰ (HG) and Ala²³⁷ (HN), with distances of 2.6 and 3.1 Å, respectively. The last hydrogen bond observed was between Glu⁴ (OE1) and Tyr¹⁰⁵ (HH), with distances of 3.0 Å. In contrast, the inhibitor dBLIP-2 was less interactive, presenting a hydrogen bond between the hydrogen of N atom Lys¹ and oxygen atoms of Ser²³⁰ (OG) and Ala²⁵⁷ (O) with distance of 3.5 and 3.0 Å, respectively (Fig. 1d). Nevertheless, fewer interactions were observed in both peptides in docking analyses performed with *E. coli* β-lactamase (Fig. 1g). Additionally, the inhibitor dBLIP-2 showed low structural complementarity when compared to dBLIP-1 in two enzymes tested. Lysine also participates in a hydrophobic interaction, where the carbon side chain interacts with the aromatic ring of Tyr¹⁰⁵. Another interaction observed was between the oxygen atom (OE1) of Glu⁴ and the nitrogen atom (NH2) of Arg¹⁴⁴ with a distance of 3.2 Å, forming an electrostatic interaction. dBLIP-1 presented interactions between the backbone oxygen atom (O) of lysines Lys¹ and the nitrogen atom (ND2) of Asn¹³², forming a hydrogen bond with distance of 3.45 Å. The nitrogen atom of Lys also participated in hydrogen bond interaction between the oxygen atom of Ser⁷⁰ (OG), with a distance of 3.47 Å. The last interaction observed was between the oxygen atom of Glu⁴ (OE1) and Tyr¹⁰⁵ (HH), with distances of 3.4 Å. Two hydrogen bonds were observed: one between the oxygen atom (O) Lys¹ and hydrogen of nitrogen atom of Asn¹⁷⁰ (ND2), with a distance of 3.42, and the other between hydrogen of

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**Figure 1 | Docking studies of dBLIP-1 and dBLIP-2 and β-lactamases from two different bacterial sources.** Structural complementarity between dBLIP-1 (a) and dBLIP-2 (b) toward β-lactamase from *Escherichia coli*. In detail, the non-covalent interactions (dotted lines) of dBLIP-1 (c) and dBLIP-2 (D) and *E. coli* β-lactamase catalytic site. Structural complementarity between dBLIP-1 (a) and dBLIP-2 (b) with β-lactamase from *Staphylococcus aureus*. In detail, the non-covalent interactions (dotted lines) of dBLIP-1 (c) and dBLIP-2 (d) and *S. aureus* β-lactamase catalytic site.
nitrogen atom of Gly (N) and oxygen atom (OG2) of Ser with a
distance of 3.6 Å (Fig. 1h). Similar data were observed for other β-
lactam inhibitors in which the presence of cationic and hydro-
philic residues seems to be essential for the inhibition process.

Furthermore, a correlation between in silico and in vitro analyses
was performed in order to better understand the mechanism of
action of both peptides. In order to evaluate the in vitro results of
dBLIP-1 and -2, both were synthesized by solid phase method and
the identity with MALDI ToF MS analysis was checked after HPLC
separation (Fig. 2a and 2b). Peptides were also synthesized for
authenticity and we checked that the obtained results were the
same for the whole batch. dBLIP-1 was assayed against bacterial β-
lactamase from Bacillus cereus 569 (Calbiochem, UK). Both peptides
showed β-lactamase inhibition activity with a higher activity at
128 μg.mL−1 concentration (Fig. 2c). dBLIP-1 demonstrated greater
affinity for the β-lactamase enzyme by calorimetry assays when com-
pared to dBLIP-2. The binding affinity (K) and binding stoichi-
ometry of both dBLIP-1 and -2 towards β-lactamase was done
with isothermal titration calorimetry (ITC). The ITC results for
dBLIP-1 and -2 clearly show their affinity to β-lactamase. The titra-
cion curves for the two peptides and their stoichiometry at the end-
point are shown (Fig. 2d–2f). In both cases, complexation took place,
as indicated by their binding isotherm. The line through the simul-
ad data set corresponds to the theoretical heat produced by their
complex formation between β-lactamase and dBLIP-1 and -2, and
the best-fit values for the parameters K, AH and AS are listed in
Supplementary Table 1. The interaction processes are enthalpy-dri-
ven and based upon van der Waals interactions, along with H-bond-
ing, because the values of ΔH and ΔS were negative. This was also
corroborated by in silico studies comparing the output energy
encountered in the well-defined cluster generated after data mining
for the enzyme. In addition, to improve information about interac-
tions between dBLIPs and enzyme, the free energy values were eval-
uated through docking studies. It was observed that dBLIP-1 toward
the β-lactamase from E. coli interactions presented -5.8 Kcal.mol−1,
while the energy observed for dBLIP-2 toward the same β-lactamase
was -5.4 Kcal.mol−1, demonstrating a slightly lower interaction
affinity. In contrast, the energy value observed for dBLIP-1 toward
the β-lactamase from S. aureus was of -4.8 Kcal.mol−1, while the
energy observed for dBLIP-2 toward the same β-lactamase was -
4.8 Kcal.mol−1, demonstrating an identical affinity.

Since in vitro and in silico results showed that dBLIP-1 and -2 were
able to interact and inhibit β-lactamase activities, the next step was
obviously to challenge resistant bacteria with both peptides and see if
they were able to improve the activity of β-lactam antibiotics. MIC
values of amoxicillin, ampicillin and cefotaxime against β-lactamase
positive clinical isolates E. coli, P. aeruginosa, S. aureus and B. cereus
were varied from 128–512 μg.mL−1, showing that the isolates are
clearly resistant to β-lactam antibiotics (Table 1). Moreover, when
antibiotics were administered in combination with dBLIP-1 and -2,
the MIC values were reduced 8–16 fold (Table 2). It is also important
to note that dBLIP-1 and -2 are most active in combination with
cefotaxime in comparison to other antibiotics here evaluated. Interes-
tingly, considerable decreases in MIC values were observed
for strains that are able to produce the plasmid-mediated class A β-
lactamase CTX-M-14, offering initial evidence that dBLIPs may
inhibit both class C and class A β-lactamases.

Since a considerable reversal of bacterial resistance to β-lactams
was observed here for a broad spectrum of clinical isolates and no
cytotoxic effect was observed against erythrocytes and macrophages

![Figure 2](image_url)
In order to determine the in vivo efficacy of dBLIPs in a non-lethal infection by Gram-negative and -positive resistance models (Fig. 3), the CFU in the peritoneal cavity was determined. For *S. aureus*, the mice were divided into groups that received different treatments in combination with commercial antibiotics: penicillin, ampicillin and cefotaxime. Comparing the negative control (which did not receive any antibiotic treatment) with the group that received dBLIP-1 and dBLIP-2, it was observed that dBLIPs alone were unable to control any bacterial infection (Fig. 3a). In the case of *S. aureus*, penicillin and ampicillin alone at a standard dose of 100 mg kg\(^{-1}\) led to a similar CFU to the untreated control. When dose was increased to 200 mg kg\(^{-1}\) CFUs were slightly reduced. Furthermore, treatment with commercial antibiotics associated with dBLIP-1 and -2 led to a clear decrease in CFU count (Fig. 3b and 3c). Moreover, cefotaxime treatment led to an efficient reduction in CFU (Fig. 3d). However, when cefotaxime was associated with dBLIPs this effect clearly improved when compared to the untreated control, as previously observed in *in vitro* studies. For *E. coli*, the mice were divided into groups that received different treatments in combination with commercial antibiotics penicillin, ampicillin and gentamicin. Comparing the negative control with the group that received dBLIP-1 and -2, once more no direct bactericidal effect was observed for *E. coli* (Fig. 3e). Penicillin and ampicillin alone (Figs. 3f and 3g) at a standard dose of 100 mg kg\(^{-1}\) did not cause any bactericidal effect. When the dose was increased to 200 mg kg\(^{-1}\), a lower reduction in CFU was obtained. Treatment with commercial antibiotics associated with dBLIP-1 and -2 led to a CFU count reduction when animals were treated with ampicillin and penicillin in combination with dBLIP-1 and -2, in accordance with data obtained for Gram-positive bacteria. Gentamicine treatment, nonetheless, led to an efficient CFU decline (Fig. 3g), and both peptides only slightly improved the decrease. Similar data were obtained with other compounds and derivative fragments\(^{10,13}\), but dBLIPs seem to be the shortest peptides that were able to inhibit *S. aureus* lactamases when compared to longer proteins\(^{14}\). Based on these data, another question was proposed about the mechanism of action of dBLIPs. Since multifunctionality and promiscuity of short peptides have been commonly observed\(^{15}\), due to their ability to show different functions and to bind to different targets, are those peptides able to show a different function? Initially, dBLIPs were unable to kill bacteria by themselves, but are those peptides able to modify the mammalian immune response as host-defence peptides improving the resistance against bacteria? In order to evaluate this function, different cytokines were evaluated in mouse blood (Supplementary Fig. 1 and 2). The groups that showed a CFU increase also showed a related increase in IL-10 (Supplementary Fig. 1a and 2a), MCP-1 (Supplementary Fig. 1b and 2b) and IFN-γ (Supplementary Fig. 1c and 2c). Moreover, the values of IL-12 were different from other cytokines. For this one, the difference was that for penicillin 200 mg kg\(^{-1}\) and ampicillin 100 mg kg\(^{-1}\) values increased when combined with dBLIP-1. Additionally TNF-α, IL-12 and IL-6 did not show a significant difference compared to the untreated control (Supplementary Fig. 1d,e,f and 2d,e,f).

It is noteworthy that neither dBLIP showed any clear immunological response, which is extremely desirable in most designed drugs. These data suggest that dBLIPs probably do not show immune response side effects, and that deleterious activity is only related to lactamase inhibition\(^{16}\). In summary, dBLIP inhibitors show promise as tools to overcome resistant bacterial infections, a pervasive and growing threat to public health in several countries. They may help, as suggested in a review by Drawz et al.\(^{17}\), in resurrecting β-lactamase inhibitors in a world plagued by multidrug-resistant bacteria. These peptides could be useful as additives to commercial antibiotics.
Table 3 | In vitro evaluation of cytotoxic activity of dBLIPs in combination with antibiotics. Assays evaluating the cytotoxic activity of different treatments against mouse red blood cells (mRBCs). Mastoparan-L was used as positive control (100% haemolysis). The release of haemoglobin was measured at 550 nm and is expressed as % haemolysis. In assays evaluating the cytotoxic activity of the treatments against RAW 264.7 monocytes, cells were incubated for 24 h, cell viability was assessed by MTT assay. Data represent the mean of three experiments performed in triplicate and expressed as mean 

| Treatments (µg·mL⁻¹) | mRBCs | RAW 264.7 |
|------------------------|-------|-----------|
| dBLIP-1                | >200  | >200      |
| dBLIP-2                | >200  | >200      |
| Penicillin G           | 150   | 150       |
| Penicillin G + dBLIP-1 | >200  | >200      |
| Penicillin G + dBLIP-2 | >200  | >200      |
| Ampicillin             | 200   | 200       |
| Ampicillin + dBLIP-1   | >200  | >200      |
| Ampicillin + dBLIP-2   | >200  | >200      |
| Gentamicin             | 150   | 150       |
| Gentamicin + dBLIP-1   | >200  | >200      |
| Gentamicin + dBLIP-2   | >200  | >200      |
| Cefotaxime             | 100   | 100       |
| Cefotaxime + dBLIP-1   | >200  | >200      |
| Cefotaxime + dBLIP-2   | >200  | >200      |
| PBS                    | -     | -         |
| Lysis buffer           | >200  | >200      |
| Mastoparan-L           | 20    | 10        |

Methods

All methods described here were carried out in accordance with the approved guidelines.

Peptide designing and molecular modelling. Rational design for synthetic peptide construction was mainly based on pocket volume, which included distances between the amino acid residues that compose the β-lactamases conserved motif (KSG), Ser⁷⁶β (adjacent nucleophilic amino acid) and several adjacent amino acid residues around the catalytic triad (Lys⁶⁴β, Ser⁷⁶β and Glu⁸⁳β). Firstly, an isoelectric trapezoid was designed inside the catalytic enzyme pocket. The distances encountered inside this geometric form showed dimensions of approximately 12 (major base) x 9 (minor base) x 13 (sides) Å in a total of 13 Å (Supplementary Fig. 3). Peptides were designed with ideal lengths to fit and interact with the catalytic triad and neighbouring amino acid residues localized around the catalytic site inside the trapezoid. The peptides were designed with less than five amino acid residues, flexible (presence of glycines) and soluble (presence of hydrophobic amino acid residues such as lysine and glutamic acid) (Supplementary Table 3). Moreover, the output energy that could be correlated to affinity was also detected for each in silico docking simulation with β-lactamases from Gram-negative and -positive bacteria. The parameters used to rank the best peptides to be synthesized were the energy results, with values below -4.0 Kcal.mol⁻¹ being discarded. Five of seven constructed and analysed peptides were discarded, with four of them presenting moderate or no activity toward β-lactamases (data not shown).

β-Lactamase in vitro assays. The inhibition of β-lactamase activity degree by dBLIP-1 and dBLIP-2 was determined spectrophotometrically by the hydrolysis of nitrocefin (30 µg) and ceftazidime (30 µg) disks alone and in combination with clavulanic acid. Metallo-β-lactamase production was detected by imipenem-EDTA disk test. Two 10 µg imipenem disks were placed on the plate, and appropriate amounts of 10 µL of 0.5 M EDTA solution were added to one of them to obtain the described concentration (750 µg). The positive strain was determined if the increase in inhibition zone with imipenem and EDTA disk was ≥7 mm, and then the imipenem disk alone was considered as the MBL producer.

β-Lactamases among clinical isolates. The identification of different β-lactamase classes present in clinical isolates was carried out following the method described by Upadhyay and co-workers (2010) as observed in Supplementary Table 2. Screening for AmpC β-lactamase production was performed by cefoxitin disk test. Isolates that yielded a zone diameter less than 18 mm (screen positive) were further subjected to confirmatory testing. The disk antagonism test was used for detection of inducible AmpC β-lactamase; ceftoxime (30 µg) and cefotaxime (30 µg) disks were placed 30 mm apart from the centre to centre. Isolates showing blunting of the ceftoxime zone of inhibition adjacent to the cefotaxin disk were screened as positive for AmpC β-lactamase. The extended spectrum β-lactamase (ESBL) status of these strains was established by combined disk diffusion method per CLSI recommendations using cefotaxime (30 µg) and ceftaxime (30 µg) disks and were placed 20 mm apart from the centre. Isolates showing blunting of the ceftazidime zone of inhibition adjacent to the cefotaxin disk were screened as positive for AmpC β-lactamase. The extended spectrum β-lactamase (ESBL) status of these strains was established by combined disk diffusion method per CLSI recommendations using cefotaxime (30 µg) and ceftaxime (30 µg) disks alone and in combination with clavulanic acid. Metallo-β-lactamase production was detected by imipenem-EDTA disk test. Two 10 µg imipenem disks were placed on the plate, and appropriate amounts of 10 µL of 0.5 M EDTA solution were added to one of them to obtain the described concentration (750 µg). The positive strain was determined if the increase in inhibition zone with imipenem and EDTA disk was ≥7 mm, and then the imipenem disk alone was considered as the MBL producer.

β-Lactamase in vitro assays. The inhibition of β-lactamase activity degree by dBLIP-1 and dBLIP-2 was determined spectrophotometrically by the hydrolysis of nitrocefin as substrate. Assay mixture contained 83 mg of nitrocefin, 167 mg of BSA, 10% glycerol and 0.30 mL (0.5 µg·mL⁻¹) of β-lactamase, obtained from Bacillus cereus (569) in a final volume of 1.5 mL of 50 mM phosphate buffer. β-lactamase activity was checked by measuring the absorbance reduction at 340 nm. Inhibitors, dBLIP-1 and dBLIP-2 at various concentrations (10 to 500 µM) were pre-incubated with the enzyme for 10 min at 30°C before addition of the substrate. Percent inhibition was calculated as 100 x [(c-r)/c], where c is the remaining activity in samples incubated with inhibitor and r is the remaining activity in samples incubated without inhibitor. IC50 values are calculated to inhibit 50% of enzyme activity from the plot of percent inhibition versus the logarithm value of inhibitor concentration. Kinetic parameters were derived from the initial velocity using SIGMAPLOT version 10.0β.

Microorganisms. The β-lactamase positive clinical bacterial isolates used in this study (Table 2) were obtained from Priyamvada Birla Aravind Eye Hospital in Kolkata, India. The phenotypic characteristics and antibiotic sensitivity profiling of all isolates was used to identify the bacterial species. The extended spectrum β-lactamase production was determined by using the nitrocefin disk test with nitrocefin (30 µg) disks alone and in combination with clavulanic acid. The positive strain was determined if the increase in inhibition zone with nitrocefin and EDTA disk was ≥7 mm, and then the nitrocefin disk alone was considered as the MBL producer.

β-Lactamase in clinical isolates. Detection of β-lactamase in the clinical isolates was determined by using nitrocefin assaysα at different time intervals (1, 10, and 30 min).

Determination of minimum inhibitory concentration (MIC). The minimum inhibitory concentrations of selected antibiotics (ampicillin, ampicillin, cefotaxime, and gentamicin) and in combination with 128 µg·mL⁻¹ of dBLIPs against four clinical isolates were determined in Mueller Hinton Broth (MHB). Strains were characterized earlier and confirmed with 16S rDNA sequencing. Strains were pre
incubated in Tryptic Soy Broth (TSB) at 30°C to achieve an optical density equivalent to 0.5 McFarland standards and used as inoculum. The MICs were determined according to CLSI guidelines. The concentrations of each antibiotic used a range from 0.5 to 1024 μg.mL⁻¹. All sets of experiments were repeated three times.

**Cell Culture**. RAW 264.7 murine macrophage-like cells were purchased from the Rio de Janeiro Cell Bank and were maintained in supplemented Dulbecco’s modified eagle medium (DMEM) (Invitrogen) (4 mM L-glutamine, 10% FCS, 2 mM nonessential amino acids, 50 mg.mL⁻¹, gentamicin, and 100 units.mL⁻¹, penicillin/ streptomycin) in the presence of 5% CO₂ at 37°C.

**Cell Cytotoxicity Assays. MTT assay.** In order to determine the maximum non-toxic concentrations of dBLIPs and antibiotics free and in combination, cell viability was compared to the negative control (PBS buffer, pH 7.4) and positive control using RAW 264.7 cells. The results were expressed as the percentage of each sample from 0.5 to 1024 μg.mL⁻¹.

**Hemolytic assay.** The hemolytic activity of free dBLIPs and combined with antibiotics was determined by using fresh mouse red blood cells (mRBCs). Control samples included adult rat serum, heat-treated (56°C, 30 min) to inactivate the complement system serum from adult rats and from neonatal rats, and buffer without erythrocytes. The A415 resulting from 100% lysis was determined by analysing the supernatant of erythrocytes that had been incubated with mastoparan-L (1–100 μg.mL⁻¹).

**Animals.** C57BL/6 mice weighing 18 to 22 g were used in this study. Animals were provided by the Central Biotechnology of the USP Campus in Ribeirão Preto. Animals were housed in separate cages at a constant temperature (22°C) and humidity with a 12 h light/dark cycle with ad libitum food and water. The mice were euthanized by ether inhalation or CO₂ at the end of the experiments. All experiments, care, and handling of animals were approved by the Ethics Committee of the Catholic University of Brasilia.

**Murine non-lethal Staphylococcus aureus infection model.** Mice received an intraperitoneal injection of 100 μL S. aureus (ATCC33591) 2 × 10⁶ CFU.mL⁻¹, previously cultured as described by Steintraesser and co-workers. One hour after the S. aureus injection, mice (n=6/group) were intraperitoneally treated with dBLIP-1 and dBLIP-2 (1 mg.kg⁻¹) in combination with penicillin (100 and 200 mg.kg⁻¹), ampicillin (100 and 200 mg.kg⁻¹), or cefotaxime (35 mg.kg⁻¹). Treatments were performed every 12, 24, 48 and 72 h, for 7 days. Mice were euthanized at 7 days post infection, and blood and peritoneal fluid were collected. Serial dilutions of the samples were plated in triplicate on Mannitol salt agar (Himedia, India) and the results were expressed as CFU.mL⁻¹.

**Statistical analysis.** Data are presented as mean ± SD of all samples. Statistical significance of fatality rates between different groups was performed by Kaplan–Meier analysis. The Kaplan–Meier survival analysis was used to determine the survival rate in different treatment groups. The log-rank ( Mantel-Crowe) test was performed to identify significant differences between the survival curves of the various treatment groups. The survival curves were plotted using GraphPad Prism version 5.0 software.

**Cytokine assay.** Cytokines IL-6, IL-10, IL-12p70, MCP-1, IFN-γ and TNF-α were measured in plasma of mice subjected to the non-lethal S. aureus and E. coli infection model, using an ELISA kit (Peprotech, USA) according to the manufacturer’s instructions.

**Figure 3** dBLIP-1 and dBLIP-2 effects on mice in vivo model against infection of *Staphylococcus aureus* (top figure) and *Escherichia coli* (bottom figure). Determination of CFU in *S. aureus* groups treated with (a) dBLIP 1 and 2, (b) penicillin (PG), (c) ampicillin (AMP) and (d) cefotaxime (CTX). Determination of CFU in *E. coli* groups treated with (e) dBLIP 1 and 2, (f) penicillin (PG), (g) ampicillin (AMP), (h) gentamicin (GEM).
Meier test. The other data were submitted to one-way analysis of variance (ANOVA) followed by Bonferroni test. Values of p < 0.05 were considered statistically significant. GraphPad Prism software v5.0 (GraphPad Software, USA) was used for all statistical analyses.

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Acknowledgments

The authors would like to thank technican Tania Paula Garcez de Lucena Santana for bioassay facilities for animal care. The authors also thank Dr. Jayangshu Sengupta and Dr. Sunam Saha, Priyamvada Birla Aravind Eye Hospital, 10, Loudon Street, Kolkata-700 017, W B, India, for providing the necessary help to study clinical isolates. This work was supported by CNPq, CAPES, FAPDF and UCB.

Author contributions

S.M.M., L.M., O.N.S. and O.L.F. designed the experiments. L.M., performed in silico analyses. O.N.S., S.C.D. and C.E.Jr. performed anti-bacterial and immunological analyses. O.N.S. and I.C.M.F. performed in vivo analyses. S.M.M., A.B. and T.K.H. performed calorimetry and mass spectrometry analyses. S.M.M., L.M., O.N.S. and O.L.F. wrote the manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

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