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Modificação incremental de peptídeos: novas perspectivas para o tratamento de infecções e erradicação de biofilmes bacterianos

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RESUMO

Com o aumento na incidência de infecções resistentes a múltiplos antibióticos, existe hoje um grande interesse pelos peptídeos antimicrobianos (PAMs) como modelos para a produção de novos antibióticos. Os PAMs são mediadores multifuncionais da resposta imune inata, com atividade antibacteriana direta. O uso de PAMs como agentes terapêuticos tem algumas limitações, como a estabilidade, a toxicidade e alta massa molecular. Apesar dessas limitações, eles apresentam propriedades compensatórias, como imunomodulatória e antitumoral bem como a capacidade de inibir β-lactamases. O desenho racional de PAMs tem sido usado para gerar análogos com atividade melhorada. No presente trabalho avaliamos a atividade antibacteriana *in vitro* e *in vivo* da clavanina A e através da modificação incremental criamos dois análogos dos peptídeos mostoparano-L e clavanina A (clavanina-MO e mastoparano-MO),além disso, utilizamos o desenho racional de peptídeos para a criação de dois inibidores de β-lactamase (dBLIPs 1 e 2). A clavanina A mostrou se eficiente na eliminação de *S. aureus* em um modelo de infecção de ferida e impediu o início da sepse e, assim, reduziu a mortalidade de camundongos infectados em um modelo de infecção bacteriana sistêmica. A clavanina-MO e mastoparano-MO impediram o crescimento de bactérias planctônicas e levaram à erradicação de biofilmes bacterianos maduros. Os peptídeos modificados mostram-se promissores como agentes terapêuticos contra infecções bacterianas sistêmicas e biofilmes causadas por uma variedade de bactérias. dBLIP-1 e dBLIP-2 em combinação com antibióticos convencionais foram eficazes na eliminação de *Escherichia coli* e *Staphylococcus aureus* que expressam β-lactamases em um modelo murino de infecção sistêmica. dBLIPs 1 e 2 fornecem pistas para superar a resistência à base de β-lactamase.

Palavras-chave: peptídeos antimicrobianos, inibidores de β-lactamase, anti-biofilme, infecções bacterianas sistêmicas.

ABSTRACT

With the increased incidence of multiple antibiotic resistant infections, there is huge interesting in antimicrobial peptides (AMPs) as templates to produce novel antibiotics. The AMPs are multifunctional mediators of innate immune response with direct antibacterial activities. Nevertheless, the use of AMPs as therapeutic agents has certain limitations such as stability, toxicity and high molecular mass. Despite such limitations, they show additional properties such as antitumor and immunomodulatory as well as the ability to inhibit β-lactamases. Furthermore, the rational AMPs design has been used to produce analogues with improved activity. In the present study, we utilized the rational AMPs design for generation of two β-lactamase inhibitors (dBLIPs 1 and 2) and through two incremental modification created analogues of clavanin A and mostoparan-L (clavanin-MO and mastoparan-MO respectively) peptides. Both inhibitors in combination with conventional antibiotics were effective for control of *Staphylococcus aureus* and *Escherichia coli* expressing β-lactamase in a murine model of systemic infection. dBLIPs 1 and 2 provide clues to overcome resistance to β-lactamase base. The clavanin-MO and mastoparan-MO prevented the growth of planktonic bacteria, leading to the mature biofilm eradication of pathogenic Gram-negative and -positive. The clavanina-MO and mastoparan-MO are promising therapeutic agents against systemic infections and bacterial biofilms caused by a wide bacterial variety.

Keywords: antimicrobial peptides, β-lactamase inhibitors, anti-biofilm, systemic bacterial infections.

LISTA DE FIGURAS

Capítulo I

Capítulo II

Capítulo III

Capítulo IV

LISTA DE TABELAS

Capítulo I

Capítulo II

Capítulo III

Capítulo IV

LISTA DE ARTIGOS

A presente tese foi baseada nos seguintes artigos:

Artigo I

Silva, O. N., Fensterseifer, I.C.M., Rodrigues, E.A., Holanda, H.H. S., Novaes, N.R.F., Cunha, J.P.A., Rezende, T.M.B., Magalhães, K.G., Moreno, S.E., Jerônimo, M.S., Bocca, A.L., Franco, O.L. Clavanin A improves outcome of complications from different bacterial infections. ***Antimicrobial Agents and Chemotherapy***. 2015. 59:3, 1620-1626.

Artigo II

Silva, O.N., La Fuente-Nuñes, C., Fensterseifer, I.C.M., Ribeiro, S.M., Rezende, T.M.B., Faria-Júnior, C., Hancock, R.E.W., Franco, O.L. Improve the antibacterial activity of clavanin A by hydrophobic amino acids in the end-tag. Artigo submetido à revista ***Nature Communications***.

Artigo III

Silva, O.N., La Fuente-Nuñes, C., Fensterseifer, I.C.M., Ribeiro, S.M., Hancock, R.E.W., Franco, O.L. Turning a toxic peptide from wasp venom into a therapeutic agent that provides broad-spectrum protection against lethal bacterial infections. Artigo submetido à revista ***Plos Pathogens***.

Artigo IV

MANDAL, S.M., MIGLIOLO, L., **SILVA, O.N.,** FENSTERSEIFER, I.C.M., FARIA-JUNIOR, C., DIAS, S.C., BASAK, A., HAZRA, T.K., FRANCO, O.L. Controlling resistant bacteria with a novel class of β-lactamase inhibitor peptides: from rational design to *in vivo* analyses. ***Scientific Reports***. 2014. 4: 6015,1-12.

SUMÁRIO

1 INTRODUÇÃO

Ao longo da vida, o corpo humano interage continuamente com diferentes tipos de microrganismos, incluindo bactérias comensais, que colonizam pele e mucosas, e também agentes patogênicos.[1] Na maioria dos casos os patógenos invasores são eficazmente erradicados pelo nosso sistema imunitário, impedindo assim, o desenvolvimento de uma infecção. Infecções letais que antes eram incuráveis foram tratadas após a descoberta e subsequente comercialização dos antibióticos. No entanto, pouco tempo depois da introdução da penicilina no mercado foram relatadas cepas de *Staphylococcus aureus* resistentes. Atualmente várias bactérias são resistentes aos antimicrobianos, levando ao surgimento e propagação das chamadas "superbactérias", as quais são resistentes a praticamente todos os antibióticos disponíveis no mercado, sendo responsáveis por aproximadamente 18% das mortes em todo o mundo.[1,2]

No ambiente hospitalar pacientes que fazem uso de dispositivos médico-hospitalares, tais como, cateteres, tubos endotraqueais e diferentes próteses, apresentam maior incidência de infecções sistêmicas, uma vez que na superfície destes é comum o desenvolvimento de biofilmes bacterianos. Os biofilmes têm grande importância para a saúde pública, pois os microrganismos associados a biofilmes apresentam menor susceptibilidade aos antimicrobianos.[3] Além disso, os biofilmes diminuem a resposta inflamatória do hospedeiro,[4] diminuem a fagocitose e degranulação de neutrófilos[5] e inibem a ação de peptídeos antimicrobianos (PAMs)[5]. Em contextos clínicos, os biofilmes são particularmente problemáticos, uma vez que eles tendem a se formar em dispositivos de longa permanência e causar infecções persistentes e sepse.[6]

Uma infecção bacteriana em seu estágio mais avançado pode levar a sepse, a qual ocorre quando as bactérias invadem a corrente sanguínea do hospedeiro.[7-11] A sepse consiste em uma doença complexa e dinâmica caracterizada por uma resposta imune sistêmica a microrganismos patogênicos invasores ou suas toxinas.[12, 13] Esta resposta imune compreende o

reconhecimento do agente patogênico por células imunitárias, que liberam mediadores inflamatórios, tais como peptídeos e proteínas de defesa do hospedeiro, bem como a ativação sistêmica do complemento e a cascata de coagulação.[12] Apesar da melhoria nos procedimentos de cuidados a saúde, a incidência de sepse tem crescido nos últimos anos, aumentando a mortalidade e o tempo de internação.[7, 14] Não obstante os avanços na terapia e o desenvolvimento do tratamento com antibióticos de amplo espectro, a mortalidade é ainda considerável.

A investigação de novas possibilidades para o tratamento de infecções bacterianas[15, 16] tem sido de grande importância, e os peptídeos antimicrobianos (PAMs) se apresentam como excelentes candidatos. Dentre os estudos com PAMs a modificação incremental é um procedimento bem descrito na literatura científica. Tais modificações visam principalmente potencializar a atividade de peptídeos descritos anteriormente, bem como reduzir a atividade citotóxica preservando a atividade microbicida dos mesmos,[17] e em alguns casos aumentar a estabilidade dos PAMs no plasma.[18]

2 REVISÃO DE LITERATURA

2.1 Infecções bacterianas

As infecções bacterianas continuam a ser uma importante causa de morbidade e mortalidade no mundo ocidental, especialmente em pacientes hospitalizados. Os tratamentos longos, invasivos, e intensivos aos quais o paciente é submetido no ambiente hospitalar, tendem a reduzir as barreiras naturais que impedem à invasão microbiana de tecidos do hospedeiro, comprometendo o desenvolvimento de uma resposta imunológica adequada. Além disso, a resposta do hospedeiro pode ser enfraquecida por administração de imunossupressores, drogas citotóxicas ou como um resultado de uma patologia que afeta a defesa do hospedeiro. Associado a isso, infecções bacterianas que antes eram controladas facilmente com o uso de antibióticos estão retornando em novas cepas resistentes às terapias convencionais.[19]

As infecções causadas por bactérias Gram-positivas multirresistentes são as principais causadoras de morbidade e mortalidade, no ambiente hospitalar.[20] Nas duas últimas décadas, temos observado um aumento considerável no número e na gravidade de infecções relacionadas à assistência a saúde (IrAS) causadas por bactérias Gram-positivas (*Streptococcus pneumoniae, S. pyogenes, S. agalactiae, Staphylococcus aureus, Enterococcus faecalis, E. facium e Clostridium difficile*).[21]

O aumento considerável em IrAS causadas por bactérias Gram-positivas pode ser atribuída principalmente ao uso corrente e abusivo de cateteres e outros dispositivos intravasculares.[21] Em muitos hospitais, as bactérias Gram-positivas são responsáveis por mais de 50% de todas as infecções da corrente sanguínea. Além do aumento da frequência, devemos considerar a ampla disseminação de clones de estafilococos resistentes a todas as drogas β-lactâmicas (*S. aureus* (MRSA) e *S. epidermidis* (MRSE)).[22, 23] Os Enterococos são o segundo grupo de agentes etiológicos (depois de Estafilococos) causadores de IrAS, sendo *Enterococcus faecalis* e *Enterococcus faecium* os principais representantes deste gênero.[24]

Os casos de bactérias Gram-negativas multirresistentes têm aumentado drasticamente nas últimas décadas, causando aproximadamente 30% das IrAS.[25, 26] *Pseudomonas aeruginosa*, *Acinetobacter baumannii* e enterobactérias produtoras de β-lactamase de espectro estendido (ESBL) ou produtoras de carbapenemase, são as principais causadoras de IrAS.[25] Nestas circunstâncias, a terapia antimicrobiana falha com frequência, o que pode levar o hospedeiro a desenvolver infecções que podem evoluir rapidamente para um quadro mais grave denominado sepse.[27, 28]

2.1.1 Sepse

A sepse pode ser definida como uma resposta inflamatória sistêmica a microrganismos patogênicos em tecidos e fluídos normalmente estéreis (sangue, líquido cefalorraquidiano), onde ocorre uma produção exacerbada de uma série de mediadores inflamatórios, tais como citocinas, proteínas de fase aguda e ativação excessiva de células inflamatórias, podendo levar a disfunção ou falência de um ou vários órgãos.[29]

Bactérias multirresistentes Gram-positivas e Gram-negativas são os principais indutores de sepse, que também pode ser causada por fungos, vírus e parasitas. Dentre as bactérias causadoras de sepse mais comuns estão as bactérias Gram-positivas *S. aureus* e *S. pneumoniae*, e as bactérias Gram-negativas *Escherichia coli* e *Klebsiella pneumoniae*,[30] e os locais mais comuns de infecções são os pulmões, abdômen, o trato urinário, a pele e os tecidos moles.[27, 31] Durante o período inicial da sepse, acontece o reconhecimento dos microrganismos invasores desencadeando respostas sistêmicas do hospedeiro incluindo a liberação de citocinas pró-inflamatórias (ex.: TNF-α), quimiocinas, proteínas de fase aguda e outras moléculas (ex.: proteínas do grupo de alta mobilidade – HMGB-1).[27, 31]A liberação excessiva de mediadores pró-inflamatórios, a chamada “tempestade de citocinas”, medeia a progressão de uma resposta inicialmente benéfica, que posteriormente se torna danosa para o hospedeiro.[12] A presença de bactérias invasoras e produtos bacterianos no sangue ativam as cascatas de coagulação e complemento.[32] A formação do

coagulo induzida pelos fatores de coagulação pode ser considerada um dos primeiros sinais da sepse.[33] Os mecanismos anticoagulantes envolvendo antitrombina III (ATII), inibidor da via do fator tecidual (TFPI) ou proteína C são prejudicados, bem como a fibrinólise. Isto resulta num estado pró-coagulante que é refletido sistemicamente por níveis plasmáticos elevados de fragmentos de protrombina 1+2 (F1+2), complexos de protrombina-antitrombina (TATC) e diminuição do número de plaquetas. O consumo de fatores de coagulação resulta em prolongados tempos de coagulação da protrombina (PT) e um prolongamento do tempo da tromboplastina parcial ativada (TTPA).[34] Uma coagulação descontrolada induzida pela inflamação é a coagulação intravascular disseminada (DIC), que pode causar disfunção orgânica.[35] Assim, a coagulação descontrolada contribui significativamente para a falência de órgãos e morte.[36, 37] Outro fator determinante pode ser a acentuada ativação de células endoteliais que resulta no aumento da permeabilidade vascular, aumentando assim, a migração de neutrófilos e consequentemente o derrame vascular.[12] Notavelmente, os neutrófilos podem sofrer "paralisia imunológica" durante a sepse, que envolve um completo fechamento de importantes vias de sinalização intracelular e disfunção do sistema imune adaptativo, a qual é um fator importante que contribui para a imunossupressão que se observa nas fases posteriores da sepse.[12] O recrutamento de células do sistema imunológico, como neutrófilos liberam proteinases ou outras enzimas, bem como espécies reativas de oxigênio (ROS), que também contribuem para o dano tecidual e falência de órgãos.[12] O estado pró-inflamatório sustentado leva a um estado de imunossupressão devido à apoptose de células do sistema imunológico e o consumo de plaquetas, fatores de coagulação e outros mediadores. Pacientes nesta fase têm uma maior susceptibilidade a infecções secundárias.[12]

Dados consistentes sobre a incidência da sepse em unidades de terapia intensiva (UTI) são escassos. Em geral os registros locais consideram a sepse apenas como um diagnóstico de admissão, e não identificam os pacientes que desenvolvem sepse durante a sua permanência. Além disso, a

fisiopatologia da sepse nos países em desenvolvimento ainda não foi bem caracterizada.[38]

2.2 Resistência bacteriana aos antibióticos

Pouco tempo após a introdução da penicilina no mercado foram relatadas cepas de *S. aureus* resistentes. Atualmente várias bactérias são resistentes aos antimicrobianos, levando ao surgimento e propagação das chamadas "superbactérias", as quais são resistentes a praticamente todos os antibióticos disponíveis no mercado.[2] A causa para tal, foi a falta de experiência quanto a utilização dos antibióticos e, embora Alexander Fleming tenha previsto que doses muito baixas poderiam levar ao desenvolvimento da resistência à penicilina, e embora a resistência bacteriana a penicilina tenha sido descoberta antes mesmo da mesma ser disponibilizada no mercado, o uso da penicilina não foi acompanhado de qualquer medida restritiva.[39]

Meio século depois, em meados do século XX, foi finalmente reconhecido que era necessário um controle quanto ao uso dos antibióticos. No Brasil somente em 2010 o uso dos antibióticos passou a ser controlado, após a publicação da RDC 44/2010 (posteriormente substituída pela RDC 20/2011). Genes de resistência a antibióticos já existiam antes mesmo do uso generalizado dos antibióticos, uma vez que o ambiente não é uma monocultura, mas consiste em uma mistura complexa de organismos, e na luta pela busca de recursos.[39, 40] Alguns organismos produzem compostos tóxicos para as bactérias, e para superar a toxicidade, as bactérias desenvolveram contramedidas que garantiram ou podem garantir a sua sobrevivência. A maioria dos antibióticos foram isolados de estirpes de fungos e bactérias que ocorrem naturalmente em todos os ambientes, incluindo solo. Estas estirpes produtoras de antibióticos podem portar genes que codifiquem a resistência ao antibiótico que produzem como um processo de autoproteção,[41] Determinantes da resistência aos antibióticos existem naturalmente e provavelmente foram submetidos a transferência horizontal muito antes da pressão de seleção

extrema, a qual as bactérias foram submetidas na “era de ouro dos antibióticos".[39]

Além dos antibióticos presentes na natureza, toneladas de antimicrobianos são utilizados a cada ano para o tratamento de pessoas, animais e na agricultura.[42-44] O uso excessivo de antibióticos prescritos aparentemente é o principal fator para o aumento das taxas de resistência bacteriana.[45] Um terço das pessoas em todo o mundo acredita que os antibióticos são eficazes para o resfriado comum.[46] Mesmo quando prescrito corretamente para infecções bacterianas, grande parte dos pacientes não finaliza o tratamento o que pode induzir a resistência.[46] Além disso, cerca de 80% dos antibióticos que estão sendo produzidos a cada ano, são vendidos para uso animal, os quais são idênticos/quase aos medicamentos de uso humano. [42-44]

2.2.1 Resistência bacteriana aos ß-lactâmicos

A resistência aos antibióticos pode se desenvolver através de mutações genéticas espontâneas ou por transferência de genes de resistência.[39] O mecanismo mais comum e importante da resistência das bactérias Gram-negativas é a produção de β-lactamases, que inativam antibióticos β-lactâmicos por hidrólise do anel β-lactâmico.[47, 48]

Os antibióticos β-lactâmicos interrompem a síntese da parede celular bacteriana, tendo como alvo as proteínas de ligação da penicilina (PBP ou PLP), impedindo a reação de transpeptidação e a formação das ligações cruzadas bacterianas (as ligações cruzadas que conferem a rigidez à parede celular), interrompendo a síntese de peptideoglicanos e levando à morte da célula bacteriana devido à instabilidade osmótica ou autólise.[49, 50] Para neutralizar os efeitos dos β-lactâmicos, as bactérias produzem β-lactamases que hidrolisam o anel β-lactâmico do núcleo estrutural das penicilinas (ácido 6-aminopenicilâmico), levando a formação do ácido penicilóico, o qual é desprovido de atividade antimicrobiana.[24]

As β-lactamases tem sido tradicionalmente classificadas com base nas características funcionais das enzimas (perfil do seu substrato e sensibilidade aos inibidores)[51, 52] ou em sua estrutura primária.[53] Os dois método de classificação mais comum é baseado na sequência primária da enzima, onde as β-lactamases são classificadas em quatro classes: A, B, C, e D.[52, 54-57] As classes A, C, e D são as mais comuns e possuem um residuo de serina no centro ativo da enzima. Enquanto as β-lactamases da classe B são metaloenzimas que utilizam íons de zinco (Zn^{2+}). Embora a abordagem estrutural seja a maneira mais fácil e menos controversa para classificar um conjunto tão diverso de enzimas, a classificação funcional proporciona a oportunidade de relacionar essas enzimas com seu papel clínico, ou seja, fornecendo resistência seletiva para diferentes classes de antibióticos β-lactâmicos.[52] Assim, parece adequado continuar a agrupar essas diversas enzimas de acordo com suas propriedades hidrolíticas e de inibição.[57]

À medida que as bactérias desenvolveram resistência a um tipo de β-lactâmico, uma estratégia utilizada consistiu na criação de derivados dos antibióticos (cefalosporinas e carbapenêmicos e monobactâmicos). No entanto, as bactérias estão continuamente a desenvolver resistência, ao modificar ou substituir o alvo - PLPs - e adquirindo novas β-lactamases, provavelmente a partir de microrganismos do solo.[58, 59] As enzimas que podem quebrar os mais novos derivados são conhecidas como β-lactamases de espectro estendido (ESBLs) e foram observados pela primeira vez no início dos anos 1980.[51]

Um método alternativo para combater a resistência mediada β-lactamases tem sido a utilização de pequenas moléculas que sejam capazes de inibir β-lactamases, como, por exemplo, sulbactam, tazobactam e ácido clavulânico.[60] Estes inibidores protegem os fármacos da hidrólise por β-lactamases e restauram o potencial terapêutico do antibiótico.[60, 61] Entretanto, alguns anos após a introdução da combinações amoxicilina-clavulanato e ticarcilina-clavulanato para uso clínico, foram observado isolados de *E. coli* e *K. pneumoniae* resistentes.[62, 63] O grande número de bactérias resistentes a múltiplos antibióticos representa um desafio para o tratamento de infecções e

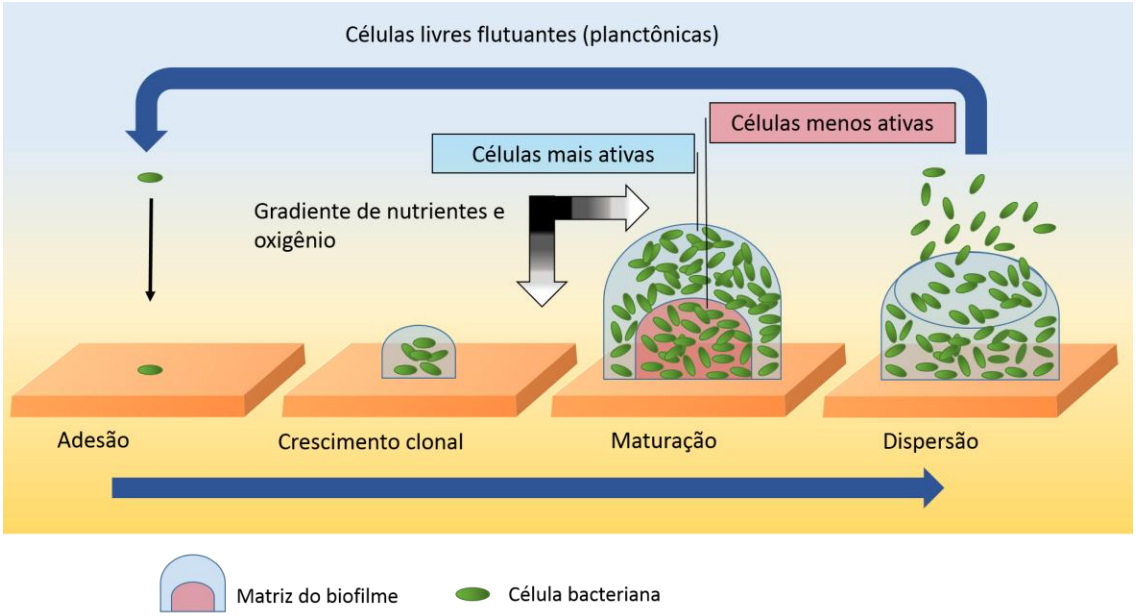
além disso, a taxa de obtenção de novos antibióticos não coincide com o número crescente de estirpes resistentes.

2.3 Biofilmes bacterianos

O crescimento bacteriano tem sido caracterizado por dois fenótipos, células livres e dispersas (planctônicas) ou agregados sésseis (biofilme), nos quais, células sésseis aderidas a um substrato e embebidas em uma matriz de polímeros extracelulares (EPS)[64, 65] Os estudos relacionados a busca e desenvolvimento de antibióticos estão tradicionalmente centrados em ensaios que utilizam como alvo bactérias planctônicas.[64, 65] No entanto, estima-se que <0,1% da biomassa microbiana total está presente como um fenótipo planctônico.[66, 67]

A formação de um biofilme tem origem com a aderência inicial das bactérias a uma superfície, as bactérias são incorporados dentro de uma matriz de substância polimérica extracelular, que contêm principalmente polissacarídeos, ácidos nucleicos, lipídios e proteínas.[68] Os biofilmes são constituídos por 90% de EPS, enquanto as células constituem os 10% restante.[69] A formação de um biofilme provê às bactérias várias vantagens, dentre elas: Imobiliza as células, mantendo uma arquitetura confortável na qual as células se comunicam, além de conferir maior resistência aos antibióticos. [36] O desenvolvimento de um biofilme foi exaustivamente estudado utilizando sistemas *in vitro*, a bactéria comumente estudada neste contexto é *P. aeruginosa* (Figura 1).

Figura 1. Formação in vitro de um biofilme de *Pseudomonas aeruginosa* em superfície sólida. Inicialmente ocorre a colonização primária de um substrato, as bactérias mudam a expressão de diversos genes, perdem os flagelos e iniciam a produção do exopolissacarídeos (EPS). As células se proliferam formando microcolônias e se comunicam com células adjacentes e, por ultimo ocorre a maturação e dispersão do biofilme maduro.[65] Figura gentilmente cedida pela Drª Suzana M. Ribeiro.



Vários estudos têm demonstrado que as infecções bacterianas persistentes são causadas por biofilmes.[70] Os biofilmes podem resistir a concentrações elevadas de antibióticos, que matariam facilmente as células planctônicas. Além disso, a sua tolerância a resposta imune do hospedeiro também é aumentada.[71] Embora o tratamento com antibióticos seja a medida mais importante e eficaz para o controle de infecções bacterianas, a antibioticoterapia convencional é pouco efetiva na eliminação do biofilme.[72] Ensaios *in vitro* e *in vivo* demonstraram que a concentração inibitória mínima (MIC) e a concentração bactericida mínima (MBC) para biofilmes é muito mais elevada (cerca de 10 a 1000 vezes) que as concentrações observadas para células bacterianas planctônicas.[72-74]

A diminuição da sensibilidade do biofilme aos antibióticos apresenta dois aspectos, a tolerância e a resistência. A tolerância significa que as bactérias não são mortas, embora elas não sejam capazes de crescer na presença do antibiótico, enquanto que a resistência permite que as bactérias

cresçam na presença de antibióticos.[75-77] Ambos os fenômenos são igualmente importantes e podem ocorrer simultaneamente, embora a perspectiva de tempo seja diferente. A tolerância surge quando um número de bactérias se agregam, a resistência por sua vez se desenvolve ao longo do tempo devido a fatores extrínsecos e intrínsecos, tais como mutações. A maioria das infecções crônicas implica em inúmeras divisões bacterianas, o que propicia o acúmulo de mutações que podem levar a resistência.[71]

Outra característica importante do biofilme bacteriano consiste na tolerância ou proteção contra os fagócitos. Para um biofilme bacteriano sobreviver em um ambiente hostil, como uma infecção, por exemplo, ele precisa de proteção contra os fagócitos.[78] Durante uma infecção, a primeira resposta à invasão de bactérias é a resposta imune inata, através de componentes celulares, especialmente as células polimorfonucleares (PMNs).[79] Bactérias planctônicas são facilmente fagocitadas,[80, 81] ao passo que os biofilmes maduros não são fagocitados.[80-82] O mecanismo exato deste fenômeno ainda não foi completamente elucidado. Inicialmente acredita-se que a matriz do biofilme atue como uma barreira física. Entretanto, um estudo recente acrescentou outros aspectos a esse fenômeno, pois mostrou que os fagócitos não entram em contato com as bactérias em biofilmes, mas em alguns casos eles chegam a penetrar os biofilmes.[75, 80, 83] No entanto, as bactérias nos biofilmes não são mortas. Deste modo, o mecanismo de defesa parece ser químico, pois as bactérias em biofilmes produzem compostos que desativam ou mesmo matam células eucarióticas como PMNs.[81, 84, 85]

Os biofilmes são os causadores de muitas infecções crônicas e recorrentes, sendo responsáveis por aproximadamente 65% das infecções bacterianas graves.[86, 87] Infecções da corrente sanguínea ou infecções do trato urinário podem ser causadas por biofilmes infecciosos originalmente formados na superfície de dispositivos médicos de permanência longa, como cateteres venosos centrais, válvulas cardíacas mecânicas, cateteres urinários, próteses articulares, cateteres de diálise peritoneal, marca-passos cardíacos, tubos endotraqueais, lentes de contato e dispositivos intrauterinos. Neste caso, as bactérias podem ser originárias da flora epitelial do hospedeiro, profissionais de

saúde ou outras fontes.[88] Infecções associadas aos biofilmes nativos são infecções crônicas, muitas vezes oportunistas, como por exemplo, infecções crônicas pulmonares de pacientes com fibrose cística, otite média, endocardite, osteomielite, prostatite, infecção urinária, feridas crônicas e periodontite.[88]

As infecções associadas a biofilmes podem ser causadas por uma única espécie microbiana ou por uma mistura de espécies, com interações entre múltiplas espécies aumentando a sua persistência.[89] Os patógenos frequentemente envolvidos em infecções associadas a biofilmes incluem bactérias Gram-positivas (especialmente *Streptococcus* sp. e *Staphylococcus* sp.), bactérias Gram-negativas (especialmente *P. aeruginosa*, *E. coli* e *K. pneumoniae*).[90]

Em suma, os biofilmes são um grave problema de saúde em todo o mundo devido à sua resistência aos mecanismos de defesa do hospedeiro e aos antimicrobianos convencionais, que geralmente tem como alvo bactérias planctônicas, além disso, os biofilmes que geralmente ocorrem em dispositivos e cateteres podem induzir a sepse grave.[65, 91] Deste modo, existe uma necessidade urgente de se identificar compostos que eliminem efetivamente os biofilmes bacterianos.

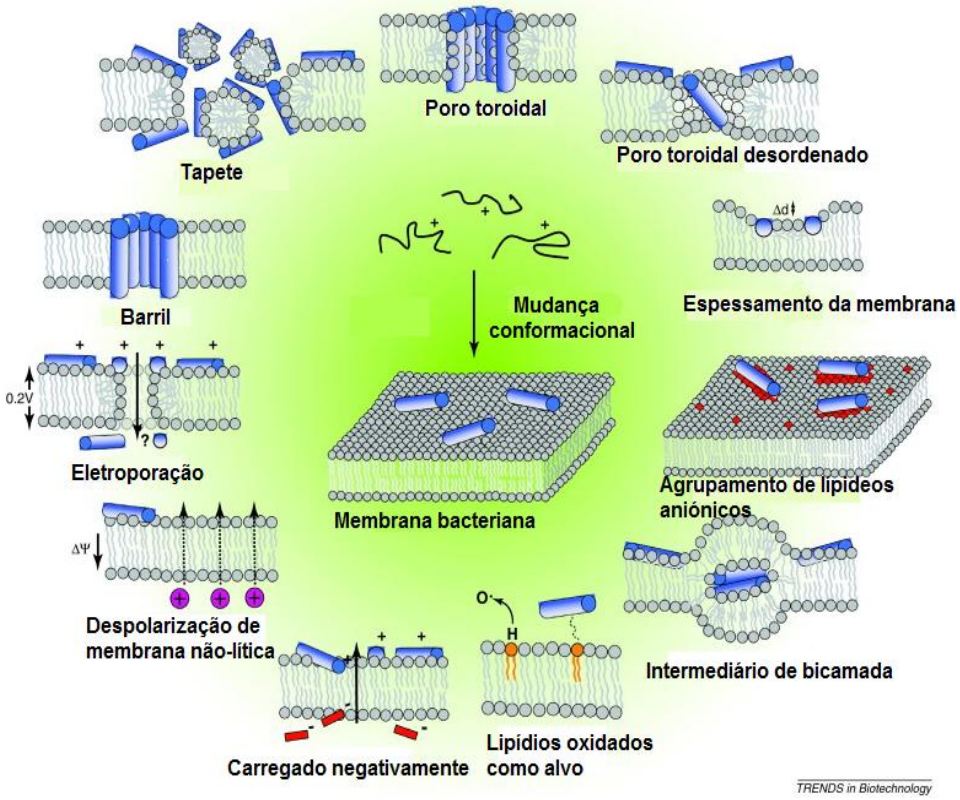
2.4 Peptídeos antimicrobianos

Os peptídeos antimicrobianos (PAMs) pertencem a um grupo diverso de moléculas que são importantes efetores da imunidade inata. Os PAMs são catiônicos, moléculas anfipáticas curtas (< 60 aa) com estruturas heterogêneas e múltiplos modos de ação em relação à morte bacteriana e atividade imunomodulatória. Estes componentes importantes da imunidade inata chamaram a atenção devido à sua atividade antimicrobiana rápida e amplo espectro de ação.[35] Os PAMs são produzidos por diversos organismos, incluindo bactérias, fungos, plantas, insetos, anfíbios, peixes ou mamíferos, e formam uma primeira linha de defesa não específica, contra patógenos invasores.[35]

Os PAMs mais estudados são as defensinas e catelecidinas. Esses peptídeos são expressos/armazenados como precursores e são então processados e liberados por vários estímulos.[92, 93] Schmidtchen e outros pesquisadores demonstraram que peptídeos endógenos humanos gerados por clivagem de outras proteínas que pertencem, por exemplo, ao sistema do complemento ou da cascata de coagulação são também importantes na defesa contra agentes patogênicos.[94-98] A principal característica destes peptídeos consiste na presença de uma região de ligação à heparina, região essa que tem sido utilizada para a busca de novos peptídeos endógenos.[99]

Existem vários mecanismos descritos mostrando como os PAMs matam as bactérias, sendo o mais atual proposto por Nguyen e colaboradores [100] (Figura 2). Os autores demonstraram que nos modelos clássicos de ruptura da membrana, os peptídeos encontram-se recobrindo a membrana até atingir um limiar de concentração e se inserem através da membrana, formando poros no modelo de barril (poros toroidais) e micelas no modelo de tapete. A espessura da bicamada lipídica também pode ser afetada pela presença de peptídeos, ou a própria membrana pode ser afetada de modo a formar domínios ricos em lipídios aniônicos em torno dos peptídeos. Em casos mais específicos, são formados intermediários de bicamada lipídica na membrana, ou seja, os peptídeos podem interagir com a membrana tendo como alvo fosfolipídios oxidados. Um peptídeo pode acoplar com ânions pequenos através da bicamada, resultando no seu efluxo e o potencial de membrana pode ser dissipado sem danos visíveis, ou, inversamente, no modelo molecular de eletroporação a acumulação de peptídeo na monocamada externa aumenta o potencial da membrana superior a um limiar que torna a membrana permeável a várias moléculas, incluindo os PAMs.[100]

Figura 2. Eventos que ocorrem na membrana citoplasmática bacteriana após contato inicial com peptídeos antimicrobianos (PAMs). Adaptado de Nguyen et al. (2011).[100]



Alguns PAMs são capazes de atravessar a membrana citoplasmática e inibir a síntese de ácidos nucleicos, síntese de proteínas e as funções enzimáticas ou a síntese da parede celular, matando assim a bactéria. Um exemplo é o peptídeo humano histatina 5, um peptídeo catiônico rico em histidina. A histatina 5 se liga a um receptor na superfície da célula e é absorvida para dentro da célula, onde atua sobre as mitocôndrias, induzindo efluxo de ATP e íons de potássio.[101]

Os PAMs exercem várias funções biológicas dentro do sistema imunitário. Estas funções incluem o recrutamento de células, como monócitos, neutrófilos e células dendríticas imaturas, a inibição das respostas pró inflamatórias, a estimulação da proliferação de células, a angiogênese, a promoção da cicatrização de feridas e também a modificação de células

eucarióticas, como, por exemplo, modificam a expressão gênica e proteica, além de matar células cancerígenas.[102-104]

O mecanismo mais conhecido de atividade dos PAMs sobre o sistema imune são os efeitos anti-lipopolissacarídeos. O lipopolissacarídeo (LPS) consiste em uma endotoxina que compõe a parede celular de bactérias Gram-negativas. O mesmo estimula a secreção de citocinas pró-inflamatórias e em casos extremos, leva ao choque endotóxico.[105] A inibição das respostas celulares induzidas pelo LPS é uma propriedade bem estabelecida de vários PAMs, mas tal mecanismo não está completamente elucidado. O cenário mais óbvio é a eliminação de LPSs extracelulares pelo peptídeo por meio de interações diretas. Vários estudos sugerem que a ligação do peptídeo ao LPS altera a estrutura agregada do LPS que é necessária para induzir as respostas celulares.[105] Alguns peptídeos, como por exemplo a foulicidina 3 se ligam ao LPS, evitando assim interações entre o LPS e a proteína de ligação ao LPS (LBP).[106, 107] Além disso, foi observado também que os PAMs bloqueiam a interação entre o grupamento de diferenciação 14 (*cluster of differentiation* 14 - CD14) e LPS inibindo assim a liberação de citocinas pro inflamatórias.[106, 107] Como observado anteriormente os mecanismos descritos visam a extinção extracelular do LPS. A LL-37 e a β-defensina 3 são exemplos de PAMs com tal atividade. Ambos os peptídeos inibiram as respostas induzidas por LPS, tendo como alvos componentes da via do fator nuclear *kappa* B (NF-κB).[108, 109] A LL-37 se liga ao gliceraldeído-3-fosfato desidrogenase (GAPDH), que inibe a sinalização proteína cinase ativada por mitógeno MAPK, resultando na diminuição da secreção de quimiocinas e citocinas pró inflamatórias.[110]

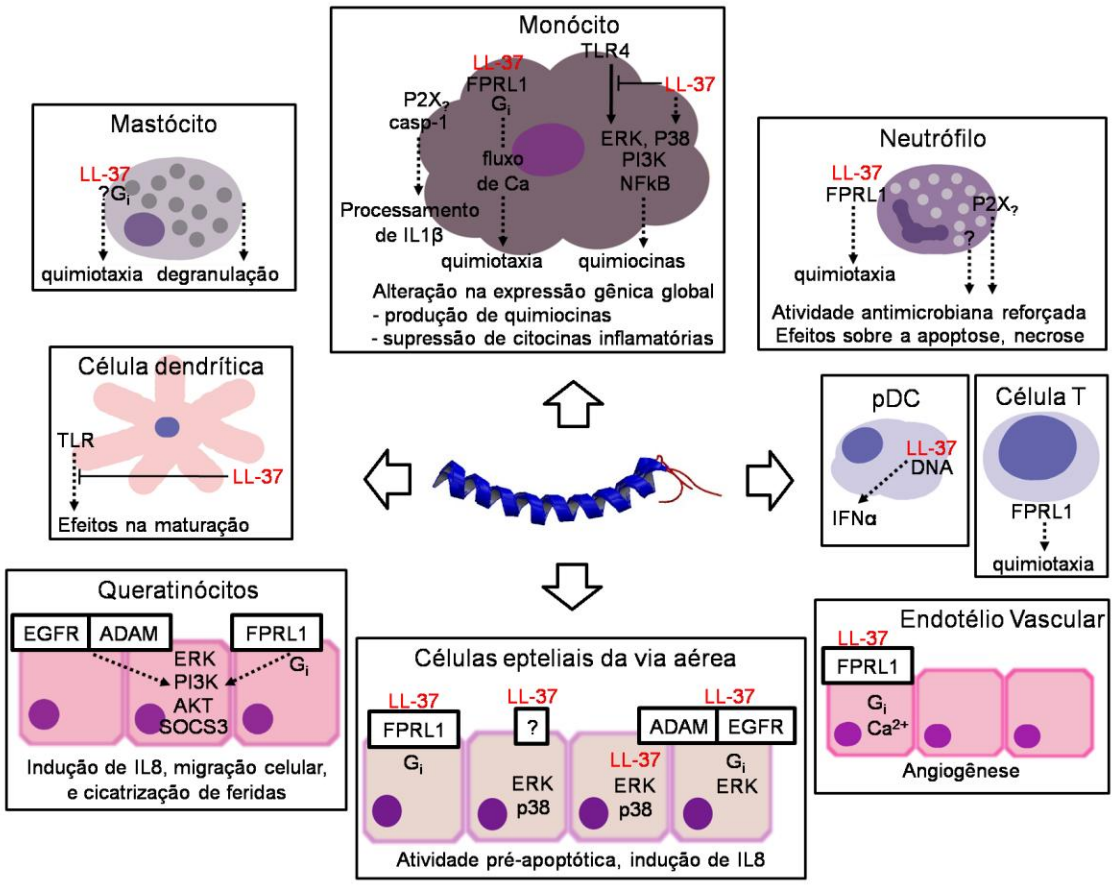
2.4.1 Catelecidina LL-37

As catelicidinas consistem em um importante grupo de PAMs produzidos por mamíferos. Semelhantemente as defensinas, as catelecidinas são expressas como pré/pro-peptídeos. Os precursores consistem em uma pré/pro -região altamente conservada, contendo o peptídeo sinal no N-terminal, uma pró-região conservada e uma extremidade C-terminal que contém a

sequência antimicrobiana.[111] O nome deste grupo de peptídeos foi denominado por Zanetti e colaboradores e baseia-se na semelhança da pró-sequência com a proteína catelina,[111] um inibidor de catepsina G isolada a partir de neutrófilos de porco.[112] A única proteína encontrada em humanos foi a proteína antimicrobiana catiônica humana (hCAP-18, 18kDa).[113] A hCAP-18 tem sido armazenada em grânulos específicos de neutrófilos[114] e expressa em subpopulações de linfócitos e monócitos,[115] várias células epiteliais,[116-118] no líquido seminal,[116] é produzida pelos queratinócitos durante a inflamação,[119] pelas células estaminais[120], bem como pelas células de cancro.[121]

A catelicidina LL-37 (hCAP-18) consiste em um peptídeo catiônico encontrado inicialmente em humanos no epitélio bucal e na saliva, sendo derivadas dos neutrófilos e das glândulas salivares.[93] Além da atividade microbicida, a LL-37 apresenta importantes funções adicionais na defesa do hospedeiro, incluindo propriedades quimiotáticas e modulação da resposta inflamatória, atraindo neutrófilos, monócitos, células T e mastócitos (Figura 3).[122, 123] Além da LL-37 outros peptídeos apresentam importante atividade sobre o sistema imune, onde estão envolvidos no recrutamento de neutrófilos e monócitos, como é o caso das α-defensinas, HNP1-3 e β-defensinas hBD3 e hBD4, já os mastócitos são atraídos para o local da infecção pela LL-37, HNP1-3 e hBD2.[124] Além disso, hBD1 e hBD3 são quimiotáticos para células dendríticas imaturas e células T de memória, enquanto que α-defensinas humanas induzem seletivamente a migração de células humanas $CD4^+$ $CD45^+$ e $CD8^+$ *naive*.[103]

Figura 3. Espectro de atividade da catelicidina LL-37. A LL-37 induz alterações na expressão gênica global dos monócitos, através da sinalização por p38, ERK, PI3K, e as vias de NF-kB, promovendo a expressão de quimiocinas e de outros genes envolvidos na comunicação celular. A LL-37 funciona como um quimioatraente direto para monócitos, neutrófilos, células T e mastócitos. A LL-37 inibe a produção de citocinas pró-inflamatórias de monócitos em resposta ao LPS e, a maturação de células dendríticas derivadas de monócitos. Monócitos tratados com LL-37 modulam o processo de diferenciação de células dendríticas. Outras atividades da LL-37 incluem a promoção de funções antimicrobiana dos neutrófilos e mastócitos. A LL-37 suprime a apoptose das células epitelial e neutrófilo, através da ativação do FPRL1 ou P2X7. Nos queratinócitos, a LL-37 induz a expressão de IL-8, a migração e cicatrização de feridas. Em células epiteliais das vias aéreas, LL-37 ativa p38 e ERK induzindo a secreção de quimiocinas, mediado por receptores: FPRL1 das metaloproteases da família ADAM e EGFR, através da internalização de peptídeos ativos. LL-37 também promove a angiogênese, mediada pelo receptor FPRL1.



*Adaptado de Nijnik & Hancock, 2009.[125]

Além de suas propriedades intrínsecas quimioatraentes, que promovem diretamente a locomoção e a chegada de diferentes grupos celulares até o local da lesão, os PAMs podem indiretamente favorecer a quimiotaxia induzindo ou aumentando a secreção de quimiocinas. A LL-37, por exemplo, induz a liberação de IL-8 por linhagens de células epiteliais pulmonares.[126, 127] As defensinas humanas HNP1-3 também favorecem o recrutamento de neutrófilos através da indução da ativação e degranulação de mastócitos, aumentando o influxo de neutrófilos e estimulando a transcrição e produção de IL-8 por células epiteliais brônquicas.[128]A LL-37 funciona como molécula anti-inflamatória, protegendo camundongos e ratos após administração de uma dose letal de LPS,[129] além disso, inibe a expressão de moléculas pró-inflamatórias como TNF-α e IL-6 e a translocação nuclear de NFκB p50/p65 induzida pelo TLR-2 e TLR-4 em resposta ao ácido lipóico e LPS, respectivamente.[109]

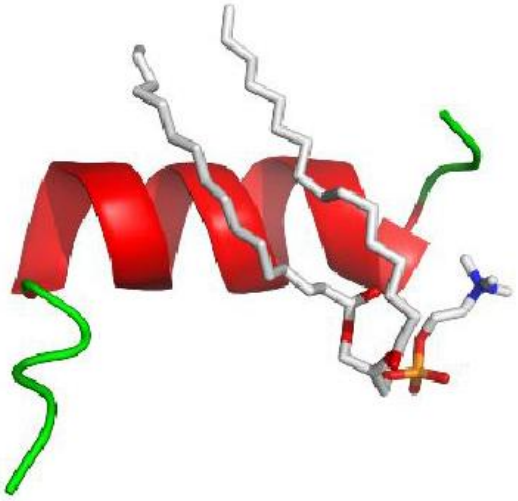
2.4.2 Clavanina A

As clavaninas são uma família de peptídeos com atividade antimicrobiana isolada dos hemócitos do tunicado marinho *Styela clava*.[130] Esses peptídeos estão presentes nos grânulos citoplasmáticos e/ou citoplasma de cinco diferentes tipos de granulócitos e também ocorrem em todo o citoplasma de macrófagos. Essa família é composta por quatro isoformas “A”, “B”, “C” e “D”, que contêm 23 resíduos de aminoácido, estrutura em α-hélice, são ricas em histidina, glicina e fenilalanina, possuem o C-terminal amidado, além da presença de oito dos vinte e três aminoácidos em posições idênticas para cada um dos peptídeos descritos. Estes peptídeos apresentam características catiônicas e anfipáticas muito comuns a peptídeos antimicrobianos.[130]

A clavanina “A” (VFQFL GKIIH HVGNF VHGFS HVF-NH_2) (Figura 4) além de apresentar as características catiônicas e anfipáticas compartilhadas com outros PAMs, apresenta uma sequência rica em resíduos de histidina, fenilalanina e glicina. Esse peptídeo atua em nível de membrana através de um

mecanismo dependente do pH. Em pH neutro, quando o peptídeo apresenta uma carga ligeiramente positiva, ele atua de forma inespecífica, desestabilizando a bicamada lipídica.[92, 131, 132] Estudos utilizando microscopia de força atômica mostraram que a clavanina A desestabilizou a bicamada lipídica, sugerindo que o mecanismo de ação é semelhante ao descrito para o modelo "*carpet-like*", dado esse que foi confirmado pela liberação de carboxifluoresceína, calceína e FITC-dextran, compostos com mais de 9 kDa.[49]

Figura 4. Estrutura tridimensional da clavanina A com DOPC. Em vermelho estrutura em α-hélice, em verde estrutura em *coil*.



Em condições ácidas (pH 5,5), quando os resíduos de histidina estão protonados, a Clavanina A apresenta atividade antimicrobiana superior (~10x) que aquela apresentada em pH neutro.[50] Este aumento da atividade pode ser acompanhado por um aumento da capacidade de colapso do potencial de membrana.[50] A clavanina A apresenta alta afinidade pelos lipídios de membrana em pH 5,5. No entanto, mostrou-se incapaz de liberar íons e calceína das vesículas lipídicas em condições ácidas.[50] Em tais condições, o peptídeo não mata as bactérias pela permeabilização da membrana, neste pH há fortes indícios de que o peptídeo utilize outro mecanismo de ação,de acordo com alguns autores, sendo o mais provável à interação com proteínas envolvidas na translocação de prótons.[50] Além disso, em ensaios *in vitro* a

clavanina A não apresentou atividade hemolítica para eritrócitos humanos (< 100 μM).[130] Esses resultados sugerem que a clavanina A atue sobre proteínas de membrana envolvidas no gradiente de prótons transmembrana ou gradientes de íons, tais como ATP sintases ou bombas de prótons, alvos mais prováveis da clavanina A. Todavia, o mecanismo exato das interações entre a clavanina A com componentes específicos das membranas biológicas ainda não foram descritos. Independentemente dos mecanismos apresentados pela clavanina A, os resíduos de glicina facilitam/promovem a flexibilidade conformacional do peptídeo.[50]

O nosso grupo, demonstrou recentemente que além de sua eficaz atividade antimicrobiana *in vitro*, a clavanina A apresenta uma potente atividade antimicrobiana *in vivo*, controlando a infecção bacteriana e prevenindo a mortalidade em um modelo experimental de sepse polimicrobiana. Os estudos *in vitro* mostraram que em concentrações nanomolares, os macrófagos murinos são estimulados a produzir IL-10, IL-12, TNF-α, NO, e em estudos *in vivo* este peptídeo atrai PMNs para o foco infeccioso.[133]

2.4.3 Mastoparano-L

Os mastoparanos são peptídeos catiônicos que apresentam estrutura tridimensional em conformação α-hélice. Suas sequências primárias possuem de 10 a 14 resíduos de aminoácidos (Tabela 2), apresentam a porção C-terminal amidada[134] e uma cadeia rica em lisina. Esse aminoácido auxilia na liberação de histamina dos mastócitos, além de conferir uma carga positiva ao peptídeo, aumentando assim sua afinidade com membranas biológicas.[98, 135]

Tabela 1. Sequência primária alguns peptídeos da família dos mastoparanos.[136]

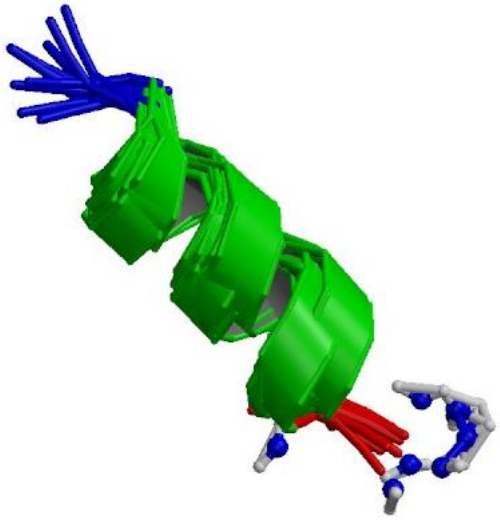
Peptídeo	Sequência	Origem
Agelaia-MP	INWLKLGKAIIDAL-NH_2	*Austropotamobius pallipes*
Mastoparano-A	IKWKAILDAVKKVL-NH2	*Vespa analis*
Mastoparano-M	INLKAIAALAKKVL-NH2	*V. mandarina*
Mastoparano-C	INWKALLAVAKKIL-NH2	*V. crabro*

Mastoparano-L	INLKALAALAKKIL-NH2	*V. lewisii*
Polybia-MPI	IDWKKLLDAAKQIL-NH2	*Polybia paulista*
Protonectarina-MP	INWKALLDAAKKVL-NH2	*P. sylveirae*
Protopolybia-MPI	INWLKLGKKVSAIL-NH2	*P. exígua*
Parapolybia-MP	INWKKMAATALKMI-NH2	*P. indica*
Protopolybia-MPII	INWKAIIEAAKQAL-NH2	*P. exígua*
Protopolybia-MPIII	INWLKLGKAVIDAL-NH2	*P. exígua*

Os mastoparanos são o grupo mais abundante de peptídeos presentes na peçonha de vespas sociais e solitárias. Apresentam inúmeras atividades biológicas como a ativação da fosfolipase A2 e fosfolipase C, que levam ao aumento da lesão tecidual e do edema; liberação de histamina por meio da degranulação de mastócitos (principal característica funcional; apresentam a capacidade de se ligar à calmodulina; aumentam a atividade da Guanosina trifosfato – GTP (regulador da proteína G, atuando como um receptor acoplado a essa); induzem a quimiotaxia de neutrófilos e células T helper resultando no recrutamento de outros leucócitos para o local; e apresentam ainda, atividade hemolítica e antimicrobiana.[137-141]

O mastoparano-L é um tetradecapeptídeo (INLKALAALAKKIL-NH_2) (Figura 5) que foi isolado inicialmente do veneno da vespa *Vespula lewisii*.[142] Esse peptídeo é um potente estimulador da exocitose de diversas células de mamíferos. Além disso, promove a secreção de histamina, serotonina, catecolaminas, prolactina[143] e insulina. Sua função biológica principal consiste na proteção contra predadores e defesa do ninho contra invasores.[143] Em geral, esse peptídeo apresenta atividade citolítica, hemolítica, além de promover a degranulação de mastócitos e quimiotaxia de leucócitos.[144]

Figura 5. Estrutura tridimensional do mastoparano-L com SDS.[145] Em verde estrutura em α-hélice, em azul e vermelho estrutura em *coil*.



Um dos mecanismos de ação do mastoparano se relaciona diretamente a liberação/exocitose de grânulos e/ou vesículas secretoras, como histamina, catecolaminas, prolactina e serotonina.[146, 147] Nas ilhotas pancreáticas, o mastoparano estimula a liberação de glucagon e insulina através de proteínas reguladoras ligadas à guanosina trifosfato (GTP) e acopladas à ativação das fosfolipases A e C, além da liberação de Ca^{++}.[148-151]

2.5 Desenho racional de PAMs

O desenho racional de PAMs consiste em uma abordagem moderna para o desenvolvimento de PAMs com potencial uso clinico. O desenho racional de PAMs tem sido utilizado a fim de reduzir ou eliminar os efeitos adversos, de acordo com o princípio da toxicidade seletiva, uma vez que o principal obstáculo à utilização de PAMs reside na sua toxicidade para células de mamífero. Isto não é surpreendente, pois a atividade dos PAMs depende significativamente da interação membrana-peptídeo. No entanto, para que sejam úteis comercialmente seria necessário dissociar a toxicidade para as células de mamífero da atividade antimicrobiana, o que pode ser alcançado

através do aumento da atividade antimicrobiana ou redução da atividade hemolítica – ou ambos.[152, 153]

Outro obstáculo quanto à aplicabilidade dos PAMs consiste em sua susceptibilidade à proteólise, uma vez que os PAMs formados por L-aminoácido são sensíveis à degradação proteolítica, o que pode ser amenizado com o uso do desenho racional de PAMs, mediante a substituição de aminoácidos, incluindo a substituição de L-aminoácidos por D-aminoácidos. Estas substituições podem promover alterações na anfipaticidade/ hidrofobicidade, levando a uma redução da citotoxicidade dos peptídeos, para células de mamífero, sem alterar a atividade antimicrobiana, além de deixar os PAMs menos susceptível à degradação proteolítica.[152, 153]

Os primeiros estudos que utilizaram o desenho racional de PAMs, geraram vários análogos de PAMs conhecidos – catelicidinas, defensinas, magaininas e cecropinas. No entanto, a maioria dos análogos gerados foram menos ativos que o protótipo original. Esses estudos desempenharam um papel importante na identificação das propriedades físico-químicas envolvidas na atividade antibacteriana dos PAMs. Estas propriedades serviram de base para o desenvolvimento de abordagens para previsão da atividade antibacteriana, através de vários métodos, como: máquina de vetor de suporte (MVS),[154-156] redes neurais artificiais (RNAs)[157, 158] e relações quantitativas entre a estrutura química e a atividade biológica ou alguma propriedade físico-química (QSAR/QSPR).[159] Os PAMs surgem como uma classe promissora, apesar de suas limitações. Deste modo, os métodos de previsão e planejamento racional desempenham um papel crucial na melhoria da atividade dos PAMs frente as 'superbactérias'.

2.5.1 Clavanina e mastoparano modificados (MO)

A clavanina-MO e o mastoparano-MO consistem em uma modificação da clavanina A e mastoparano-L, respectivamente. A clavanina A apresenta um amplo espectro de ação, no entanto, com atividade ótima em pH

ácido e altas concentrações de sal.[92, 131, 132, 160] O mastoparano-L por sua vez apresenta alta citotoxicidade para células eucarióticas (<10 μM).[161]

A modificação na sequencia primaria de PAMs consiste em um procedimento amplamente descrito na literatura cientifica.[17] Tais modificações visam principalmente potencializar a atividade de peptídeos, bem como reduzir a atividade citotóxica preservando a atividade microbicida dos mesmo[17], e em alguns casos aumentar a estabilidade dos PAMs no plasma.[18] Tal modificação consistiu na adição de cinco resíduos de aminoácidos apolares na região C-terminal do peptídeo (FLPII). Esta sequência curta foi obtidas após a comparação com peptídeos presentes no banco de dados AMPer database[245] (capítulo III, Tabela 1). O principal objetivo de tal modificação foi melhorar a atividade da clavanina A e reduzir a citotoxicidade do mastoparano-L. A clavanina MO foi testada anteriormente por nosso grupo, demonstrando um aumento atividade (~2x) antimicrobiana em comparação com a clavanina A frente a *E. coli*, *K. pneumoniae* e *S. aureus*.

2.5.2 Inibidores de β-lactamases

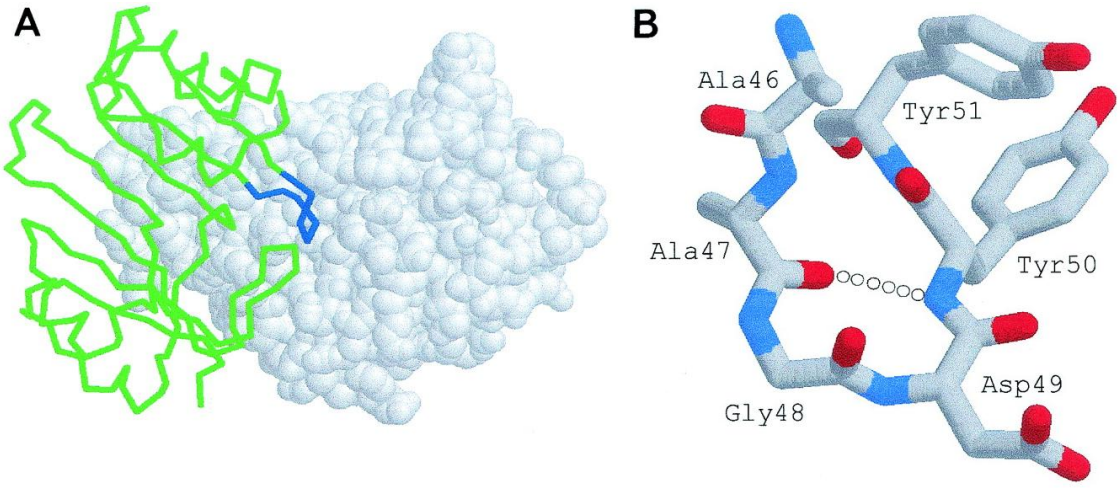
Como mencionado no tópico 1.2.1 os antibióticos β-lactâmicos, estão entre os agentes antimicrobianos mais utilizados. Tal como acontece com outros antibióticos, a resistência a estes agentes tem vindo a aumentar nos últimos anos. O mecanismo mais comum de resistência bacteriana aos β-lactâmicos é a produção de β-lactamases.[162] Bactérias da família Enterobacteriaceae expressam β-lactamases codificadas por plasmídeos que conferem resistência à penicilinas mas não às cefalosporinas de amplo-espectro.[51] Entretanto, em meados da década de 1980, um novo grupo de β –lactamases, posteriormente nomeadas de β -lactamases de espectro estendido (ESBLs) foi detectado em cepas de *Serratia marcescens* e *K. pneumoniae*.[163]

Um método alternativo para combater a resistência mediada por β-lactamases tem sido a utilização de pequenos inibidores como clavulanato, sulbactam e tazobactam.[60] As ESBLs, geralmente podem ser bloqueadas por inibidores de β-lactamases, como clavulanato, sulbactam e tazobactam.[164]

Estes inibidores são utilizados em conjunto com antibióticos β-lactâmicos existentes para tratar com sucesso infecções causadas por bactérias produtoras de β-lactamases.[165] Entretanto, as β-lactamases cromossomais induzidas (ESBLs classe C – AmpC), constituem um grupo de β-lactamases com resistência aos inibidores de β-lactamases.[166, 167]

Dentre os antibióticos naturais podem ser encontradas proteínas inibidoras de β-lactamases (BLIP). A BLIP é uma proteína de 165 resíduos de aminoácidos, produzida pela bactéria Gram-positiva do solo *Streptomyces clavuligerus*, que também produz as cefamicinas, uma classe de antibióticos β-lactâmicos, além do inibidor de β-lactamase, ácido clavulânico.[168] Com base na estrutura tridimensional da BLIP (Figura 6), foi criado um peptídeo inibidor de β-lactamase (BP46-51), uma vez que os resíduos 46-51 da BLIP se liga ao sítio ativo da β-lactamase TEM-1 (classe A). Esse peptídeo , além de inibir TEM-1, foi capaz de inibir β-lactamases da classe C que não são inibidas pela BLIP.[169] Além disso, vários outros peptídeos foram criados a partir dos resíduos de BLIP que se ligam ao sitio ativo da TEM-1, entretanto, nenhum apresentou atividade significativa.[165, 169-171]

Figura 6. Estrutura tridimensional da BLIP complexada com a β-lactamase TEM-1 e, do BP46-51. (A) Interação entre a BLIP (verde) e a β-lactamase TEM-1 (branco). A região de BLIP que interage com o sítio ativo da β-lactamase, são os resíduos 45-52 (azul). (B) Estrutura tridimensional do peptídeo obtido a parir da BLIP.[172]



3 OBJETIVOS

- ✓ Avaliação da atividade antibacteriana *in vitro* e *in vivo* da clavanina A;
- ✓ Avaliação da atividade biológica dos peptídeos modificados clavanina-MO e mastoparano-MO, concebidos através da modificação incremental de peptídeos (adição de aminoácidos apolares na região C-terminal);
- ✓ Avaliação da atividade biológica de inibidores de β-lactamase concebidos através do desenho racional de peptídeos.

4 CAPÍTULO I

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Clavanin A improves outcome of complications from different bacterial infections

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Abstract

The rapid increase in the incidence of multi-drug-resistant infections today has led to enormous interest in antimicrobial peptides (AMPs) as suitable compounds for developing unusual antibiotics. In this study clavanin A, an antimicrobial peptide, previously isolated from the marine tunicate *Styela clava*, was selected as a purposeful molecule that could be used in controlling infection and further synthesized. Clavanin A was *in vitro* evaluated against *Staphylococcus aureus and Escherichia coli* as well as toward L929 mice fibroblasts and SPCs cells. Moreover, this peptide was here challenged in an *in vivo* wound and sepsis model, and immune response was also analysed. Despite displaying clear *in vitro* antimicrobial activity toward Gram-positive and negative bacteria, clavanin A showed no cytotoxic activities against mammalian cells, and in acute toxicity tests no adverse reaction was observed at any of the concentrations. Moreover, clavanin A significantly reduced the *S. aureus* colony forming units in an experimental wound model. This same peptide reduced mice mortality caused by *E. coli* and *S. aureus* in 80 % when compared with PBS-treated control animals. These data suggest that clavanin A prevents sepsis formation and thereby reduces mortality. These data suggest that clavanin A is an AMP that could improve the development of novel peptide-based strategies for the treatment of wound and sepsis infections.

Keywords: antimicrobial peptide, *Staphylococcus aureus*, *Escherichia coli*, wound infection, systemic infection, clavanin A.

Introduction

Throughout life, the human body continuously interacts with different types of microorganisms, including commensal bacteria that colonize skin and mucous membranes.[1] In most cases, invading pathogens can be effectively eradicated by our immune system. Nowadays, infectious diseases remain a major health problem, accounting for around 18 % of deaths worldwide.[1] The skin has the largest surface area of all the body organs and is the most exposed of them. Although the skin provides effective protection from the external environment, skin infections can occur by bacteria, fungi or viruses. These infections are often the result of a breach of skin integrity, accompanied by the entry of pathogens into the dermis.[173]

Skin infections are quite common, but when treated quickly are not usually dangerous. However, in cases where the patient remains with a non-treated infection, it can evolve to a more serious condition, which can lead to sepsis and death.[7, 8] The most frequent bacterial sepsis-associated species are *Staphylococcus aureus* (30%, including 14% methicillin-resistant), *Pseudomonas* species spp. (14%) and *Escherichia coli* (13%).[174] Sepsis is a complex and dynamic disease characterized by an exacerbated and systemic immune response to invading pathogens or their related toxins.[12, 13] This immune response includes pathogen recognition, which triggers the release of inflammatory mediators such as peptides and proteins, as well as systemic activation of complement and coagulation cascade.[12] Despite improvements in health care procedures, the incidence of sepsis has increased in recent years, increasing mortality and the period of hospitalization.[7, 12]

Currently, several alternative approaches are used for the treatment of local and systemic bacterial infections, producing effective results in clinical trials.[15, 16] Therefore, the investigation of new and unusual therapies is vitally important. In this context, antimicrobial peptides (AMPs) appear to be excellent candidates. AMPs are major short cationic and amphipathic molecules (<60 amino acid residues) with heterogeneous and multiple action modes that may directly lead to bacterial death, besides having immunomodulatory activity.[35, 175] The search for novel antibiotics has become more intense in recent decades, in response to

increasingly frequent reports of multi-resistant pathogens, or the side effects caused by antibiotics on the market today.[175]

Clavanin A consists of an AMP isolated from the hemocytes of the marine tunicate *Styela clava*.[130] In addition to cationic amphipathic characteristics, this peptide also presents a sequence rich in histidine, phenylalanine and glycine residues.[160] Clavanin A is broadly effective against Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* and Gram-negative bacteria and fungi.[130, 176] These peptides are also active in high salt concentrations and in acidic pH, however, little is known about proteolytic and thermal stability of this peptide.[130, 176] Due to these special characteristics clavanin A was investigated in this study with regard to its biological properties such as its antibacterial activity (*in vitro* and *in vivo*) and toxicity.

Material and Methods

Peptide synthesis

All peptides used here (clavanin A and LL-37) were synthesized by Shanghai Hanhong Chemical (China) using the solid-phase with the N-9-fluorenylmethyloxycarbonyl (Fmoc) strategy and purified by high-performance liquid chromatography (HPLC).[130, 177] Peptide purity used in biologic assays was higher than 95%. Moreover, sequence was confirmed by MALDI-ToF analysis (Supplementary Figure 1).

Bactericidal microdilution assays

Clavanin A MICs against *Escherichia coli* ATCC8739 and *Staphylococcus aureus* ATCC29213 were determined using a standardized dilution method according to NCSLA guidelines.[178] A single colony of *E. coli* or *S. aureus* was inoculated in Mueller-Hinton broth (MH) (Himedia, India) and incubated for 12 h at 37 ºC. Overnight-cultured *E. coli* or *S. aureus* were transferred to MH medium and cultured to exponential phase (OD_{600} of ~0.6). The culture was centrifuged and resuspended in sterile PBS and adjusted to a final amount of 1×10^5colony-forming units (cfu).mL^{-1} by use of the equation (cfu.ml = OD_{600}nm$\times2.5\times10^8$).[179] For MIC determination, peptides were used at variable

concentrations (1–200 μM) from a stock solution. Ten microliters of each concentration of peptide solution was added to each corresponding well of a 96-well plate (Becton & Dickinson, USA) and 90 μL of bacteria (1×10^5) in MH medium was added. The polypropylene plates (TPP, Switzerland) were incubated at 37 °C for 24 h. MICs were determined as the lowest tested concentration that leads to complete inhibition (100%) in comparison to negative control group.[180]

Animals

C57BL/6 mice weighing 18 to 22 g were used in this study. Animals were provided by the Central Bioterium of the USP Campus in Ribeirão Preto. All animals were housed in individual cages under a constant temperature (22°C) and humidity with a 12-h light/dark cycle and had access to food and water *ad libitum* throughout the study. The mice were euthanized by CO_2 or ether inhalation at the end of the experiments. All procedures, care, and handling of the animals were approved by the Ethics Committee of the Catholic University of Brasilia number 005/13.

Isolation and culture cells

Newborn C57BL/6 mice were used to isolate skin primary cells (SPCs). First, mice were euthanized by decapitation and the entire trunk skin was removed. Biopsies (~5 mm) were incubated overnight with 0.1% trypsin (Sigma, USA), 0.02% EDTA in PBS, followed by mechanical separation of epidermis from dermis. The treatment solution was filtered through a 100 μm cell strainer, centrifuged and resuspended in supplemented DMEM (Dulbecco's modified eagle medium) (GIBCO-Invitrogen, USA) (4 mM L-glutamine, 10% foetal bovine serum (FBS) (GIBCO-Invitrogen, USA), 2 mM nonessential amino acids (GIBCO-Invitrogen, USA), 50 $mg.mL^{-1}$ gentamicin, and 100 units mL^{-1} penicillin/streptomycin) (GIBCO-Invitrogen, USA), seeded into tissue culture flasks (TPP, Switzerland) and incubated at 37 ºC, in 5% CO_2.[181] L929 mouse fibroblasts were also cultured in supplemented DMEM, into tissue culture flask (TPP, Switzerland) and incubated at 37 ºC, in 5% CO_2.[180, 182]

Cell Cytotoxicity Assay

L929 mice fibroblasts (Rio de Janeiro Cell Bank) and SPCs were seeded in 96-well microtiter plates in a concentration of 1.0 x 10^5 cells per well, in DMEM medium, supplemented with different concentrations of tested peptides (1-600 μM). After 48 h incubation, a 3-[4,5-dimethylthiazol-2-yl\-2,5-diphenyltetrazolium bromide (MTT) protocol was performed. Briefly, 60% of the medium was removed, and 10 μL of MTT (5 $mg.mL^{-1}$) (Sigma, USA) solution was added to each well and the plate was incubated for 4 h, in 5% CO_2, at 37 ºC. The blue formazan product generated was dissolved by the addition of 100 μL of 100% DMSO (Mallinckrodt, Germany) per well. Plates were then gently swirled for 5 min, at room temperature, to dissolve the precipitate. The absorbance was monitored at 575 nm using a microplate spectrophotometer (Bio-Tek, USA). Cytotoxicity was determined as a percentage of the maximum value after subtracting the background. The results were expressed as the percentage of each sample compared to the negative control (PBS buffer, pH 7.4) and cell culture was incubated in a lysis buffer (10 mM Tris, pH 7.4, 1 mM ethylenediamine tetraacetic acid (EDTA), and 0.1% Triton X-100).[178]

Acute toxicity

Acute toxicity was performed based on the work of Navon-Venezia and co-workers.[183] The experiment was examined by intraperitoneal (i.p.) injection of the tested clavanin A to groups of 10 C57BL/6 mice. Each mouse was injected with a 0.5-ml solution of freshly prepared clavanin A in PBS. The doses of peptide administered per mouse were 0, 5, 10, 20, 30, 40 and 50 $mg.kg^{-1}$ of body weight. Animals were directly inspected for adverse effects for 4 h, and mortality was monitored for 7 days thereafter. Differences between groups were analysed using the Fisher exact test (differences were considered to be statistically significant when the P value was <0.05).

Murine surgical wound infection model

The murine surgical wound infection model was performed as previously described by McRipley and Whitney [184] with minor modifications. Mice (n = 6 per group) were anaesthetized, their dorsal surface was shaved and the surgical area was disinfected with 70% ethanol. An incision was performed in the dorsal surface using 6-mm punch biopsy needles (Stiefel Laboratories, UK), and then 10 µL of *S. aureus* ATCC 29213 suspension (2×10^9.mL^{-1}; previously cultured as described in the section on bactericidal microdilution assays) was introduced into the incision and the skin was closed with one silk suture.[185] The wounds were treated every 24 h with 20 µL of clavanin A solution (solubilised in PBS buffer (GIBCO®, USA)) containing 1, 5 or 10 mg.kg^{-1}, as well as with a solution of LL-37 (solubilised in PBS buffer (GIBCO®, USA)) and gentamicin injection (as gentamicin sulphate USP, 100 mg.mL^{-1} -Merck, Germany) (10 mg.kg^{-1}) as positive controls and PBS (GIBCO®, USA) as negative control; for this, animals were mobilized manually, and treatments were applied with the aid of an automatic pipette. Mice were euthanized at 7 days post-surgery, the wounded muscle tissue was excised, weighed, and half of it was homogenized in 1 mL of PBS. Serial dilutions of the homogenates were plated in triplicate on mannitol salt agar (Himedia, India), and the results were expressed as CFU.gram^{-1} of tissue. [179, 186] The other half of the wounded tissue was stored for histological analysis as described previously by Lima and co-workers.[187]

Murine lethal infection model

Mice received an intraperitoneal injection of 100 µL *E. coli* ATCC8739 or *S. aureus* ATCC 29213 containing 2×10^9 CFU.mL^{-1}. One hour after the *S. aureus* injection, mice (n=6) were treated, intraperitoneally, with different concentrations of clavanin A (1, 5 or 10 mg.kg^{-1}), LL-37 and gentamicin 10 mg.kg^{-1} or PBS for 7 days.[188]

Cytokine assays

Proinflammatory cytokines IFN-γ, IL-6, IL-12p70, and TNF-α were measured in the plasma of mice subjected to the lethal *E. coli* and *S. aureus* infection model,

24 h after infection using an ELISA kit (Peprotech, USA) according to the manufacturer's instructions [180].

Statistical analysis

Data are presented as mean ± SD of all samples. Statistical significance of fatality rates between different groups was analysed by Kaplan–Meier test. The other data were submitted to one-way analysis of variance (ANOVA) followed by Tukey test. Values of $p < 0.05$ were considered statistically significant. GraphPad Prism software v5.0 (GraphPad Software, USA) was used for all statistical analyses.[180]

Results

In vitro antibacterial activity of clavanin A

The effects of clavanin A on *E. coli* ATCC8739 and *S. aureus* ATCC29213 were studied by the microdilution method. As shown in Table 1, the MICs showing that clavanin A was more effective against *E. coli* with MIC of 24 µM than *S. aureus* with MIC of 45 µM. Clavanin A was as potent as gentamicin against *S. aureus*. However, the MIC of LL-37 was approximately three times lower (12.5 µM) than clavanin A. LL-37 and gentamicin (1.5 and 0.7 µM respectively) were highly effective against *E. coli*, presenting greater overall potency than clavanin A in *in vitro* assays.

Table 1. Antibacterial activities of clavanin A, LL-37 and gentamicin against *E. coli* ATCC8739 and *S. aureus* ATCC29213[a]

Compounds	MIC (µM) for:	
	S. aureus	***E. coli***
Clavanin A	45.0	24.0
LL-37	12.5	1.5
Gentamicin	45.0	0.7

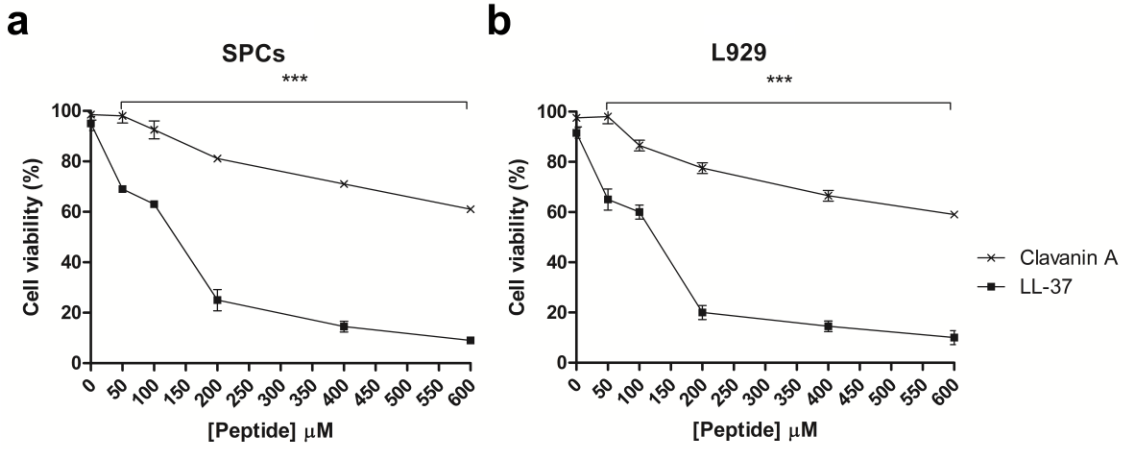
[a]Antibacterial activity was determined in three independent microtitre assays with a discrepancy of not more than one dilution.

Effects of clavanin A in cell viability/metabolism

Clavanin A cytotoxic effects on L929 mouse fibroblasts and mouse skin primary cells (SPCs) were determined. Cell viability (cell oxidative metabolism) was measured by MTT assay after 24 h treatment with different clavanin A concentrations.

Clavanin A cytotoxic effects on L929 mouse fibroblasts and mouse skin primary cells (SPCs) were determined. Cell viability (cell oxidative metabolism) was measured by MTT assay after 48 h treatment with different clavanin A concentrations. *Differences between groups were analysed using the Fisher exact test (differences were considered to be statistically significant when the P value was <0.05* LL-37 was used as control. Clavanin A showed low cytotoxicity (viability over 60%) for L929 and SPCs, even when the cells were treated with high concentrations of clavanin A (600 µM ($p<0.001$)) (see Fig. 1a and 1b). In contrast, LL-37 treatments reduced cell viability to approximately 10% of both cells at higher concentrations (600 µM ($p<0.001$)). These results demonstrate that clavanin A did not show significant cytotoxic effect on cultured cells, and peptide LL-37 did not exhibit cytotoxicity at the physiologically effective antibacterial concentrations.

Figure 1. Effects of clavanin A on cell viability/metabolic activity. The effects of clavanin A and LL-37 on viability/metabolic activity were determined using an MTT-Assay with L929 mouse fibroblasts (**a**) and mouse skin primary cells (SPCs) (**b**) at the indicated concentrations. Cells were incubated with peptides for 24 hours. Standard deviation was calculated out of three independent experiments and assessed as mean ± SD (Statistical significance clavanin A vs. LL-37: * = $p<0.05$; ** = $p<0.01$; *** = $p<0.005$).

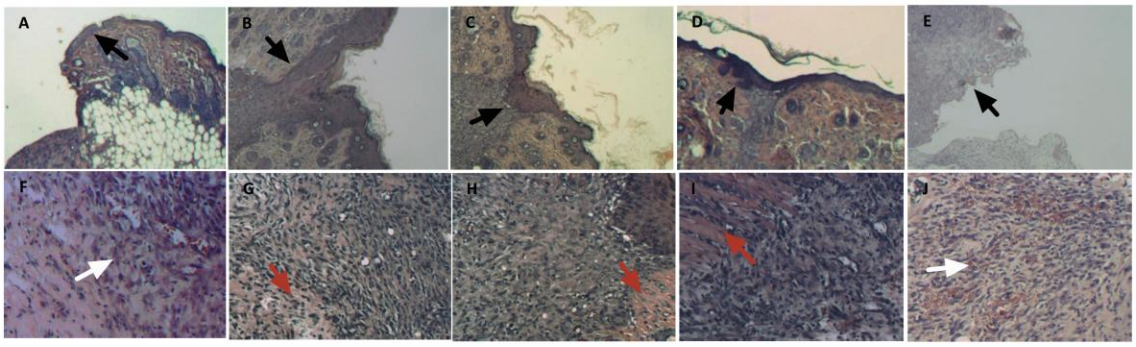


Effects of clavanin A on *S. aureus* infected wounds in mice

After confirming the *in vitro* antibacterial effects with the absence of cytotoxic activities of clavanin A on L929 mouse fibroblasts and primary mouse skin cells (SPCs), further *in vivo* experiments were performed. In order to evaluate the therapeutic potential of clavanin A, a model of surgical wound infection with *S. aureus* was used here. The animals were daily treated with clavanin solution containing 1, 5 or 10 $mg.kg^{-1}$. Furthermore, LL-37 and gentamicin were used at a standard concentration of 10 $mg.kg^{-1}$. All treatments were performed during a 7-day period.

Mice treated with PBS (Fig. 2A) showed a healing process of the infection site damaged due to the presence of the microorganism, including little epithelialization, focal inflammation with intense polymorphonuclear and mononuclear cell infiltration, loose irregular connective tissue and initial neovascularisation (Fig. 2F). Gentamicin treated animals (Fig. 2B) showed a complete absence of bacteria and a stratified and well developed epithelium with little keratinization. Moreover, gentamicin also caused diffuse inflammation with a clear predominance of polymorphonuclear cells and some fibroblasts, connective tissue with a well-developed collagen network and clear vascularisation indicating early healing (Fig. 2G). Animals treated with 5 $mg.kg^{-1}$ clavanin A showed lack of microorganisms, besides stratified and very well developed keratinized epithelial (Fig. 2C) connective tissue with a dense network of collagen, predominantly (Fig. 2H). Data obtained by clavanin at a lower concentration were very similar to gentamicin treated animals. The animal group that received 10 $mg.kg^{-1}$ clavanin also showed a well-developed keratinized epithelium (Fig. 2D) and scar contraction indicating tissue recovery (Fig. 2I). The animals that received 10 $mg.kg^{-1}$ LL 37 treatments were clear of bacterial infection, as observed with the uninfected animal group just treated with PBS (Fig. 2E). Furthermore, diffuse inflammation with predominantly polymorphonuclear cell infiltrate, intense neovascularisation and loose irregular connective tissue (Fig. 2J) were also observed.

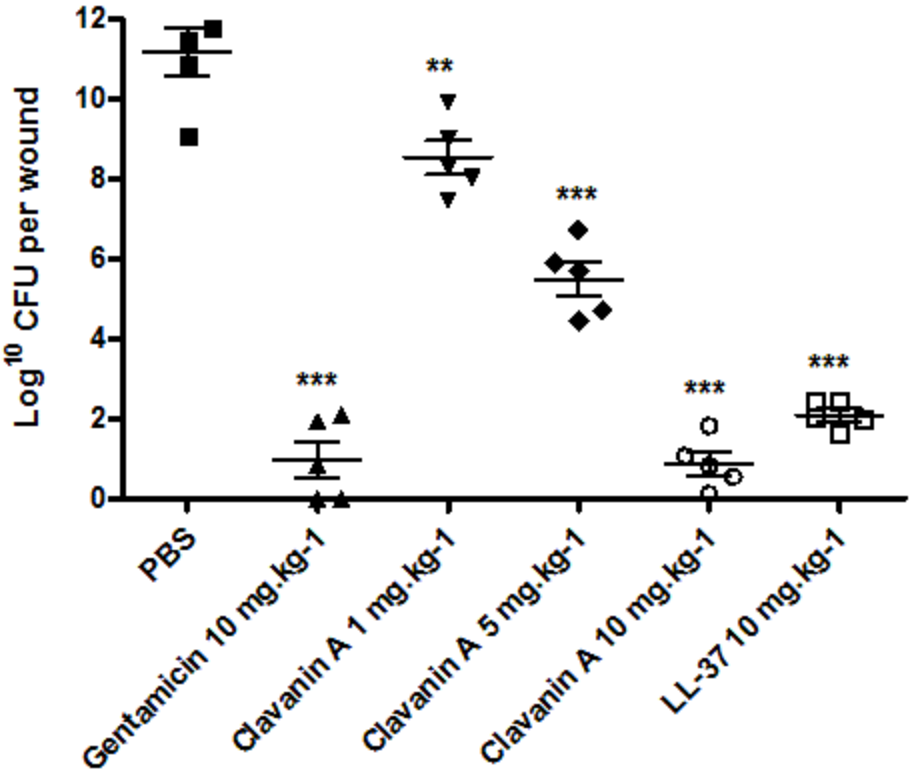
Figure 2. Histopathological sections of first surgical wounds after seven days on the back of mice infected with S. aureus and treated or not with different concentrations of clavanin A. Wounds were infected with S. aureus ($2\times10^{9}.mL^{-1}$), and then the mice were treated with clavanin A 5 $mg.kg^{-1}$ (**2C and 2H**) and 10 $mg.kg^{-1}$ (**2D and 2I**), LL-37 (**2E and 2J**) and gentamicin (10 $mg.kg^{-1}$) (**2B and 2G**) for 7 days or were left untreated (2A and 2F). Wounds were removed after experiment. The black arrow shows the epithelialisation area, the white arrow shows the intense polymorphonuclear infiltration and the red arrow shows connective tissue with a dense network of collagen.



Colony-forming units of *S. aureus* recovered from wounds

After 7 days of daily administration of clavanin A treatment, animals were euthanized and the wound tissue was removed, macerated, plated and seeded on mannitol salt agar (a selective medium for *S. aureus*) for evaluating the treatments' efficacy. Clavanin A administration resulted in a significant dose-dependent decrease in wound bacterial count ($P < 0.001$) when compared to PBS treatment. Mice treated with 10 $mg.kg^{-1}$ of clavanin A, gentamicin and LL-37 presented statistically similar results, being able to practically eradicate all microorganisms presented at the infection site (Fig. 3).

Figure 3. Efficacy of clavanin A in reducing bacterial (*S. aureus*) burden in infected skin wounds. CFU of *S. aureus* recovered from mice treated with clavanin A (1, 5 and 10 mg.kg^{-1}), LL-37 and gentamicin (10 mg.kg-1); wounds were removed 7 days into the experiment. * = p<0.05; ** = p<0.01; *** = p<0.005 compared to PBS. Horizontal bars represent means.

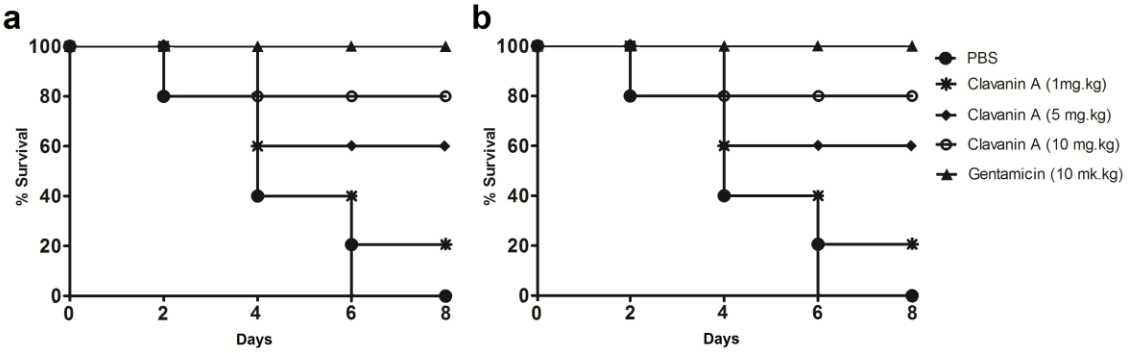


Effects of clavanin A on murine lethal *S. aureus* and *E. coli* infection model

In vivo protective activity of clavanin A was also evaluated by using an invasive infection in the *S. aureus* model. All treatments were daily performed for 8 days. *S. aureus* infected mice treated with clavanin A at a dose of 10 mg.kg^{-1} showed a survival rate of 80% after 8 days of treatment (Fig. 4a). Animals treated with 5 mg.kg^{-1} of clavanin A showed a survival rate of 60%, the same percentage presented for animals treated with 10 mg.kg^{-1} of cathelicidin LL-37 (data not shown). Infected mice treated with 1 mg.kg^{-1} of clavanin A showed a survival rate of 20%. Mice treated with 10 mg.kg^{-1} of gentamicin showed 100% survival at experiment endpoint. It is worth mentioning that clavanin A activity was dose-dependent.

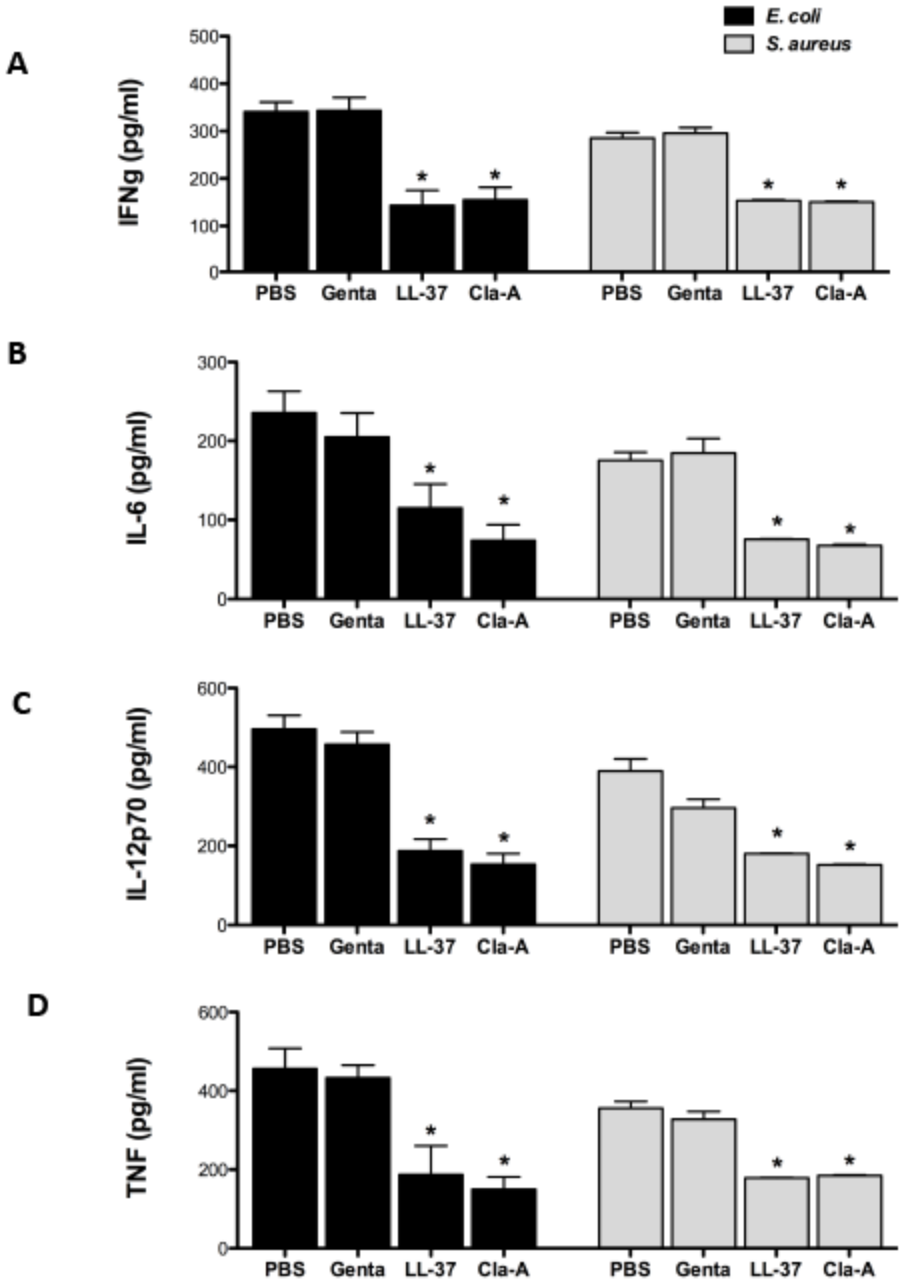
Efficacy of intraperitoneal administered clavanin A to counteract *E. coli*-induced infection in a mice model was also tested. In this case, the lethality rate in the PBS control group was 100% (Fig. 4b). All groups treated immediately after infection with clavanin A (1, 5 and 10 $mg.kg^{-1}$) showed a better outcome than control groups (PBS) ($p<0.05$). The survival rates in mice were 40%, 60%, and 80% in the groups treated with clavanin A (1, 5 and 10 $mg.kg^{-1}$ respectively) (Fig. 4b). Mice treated with gentamicin and LL-37 (10 $mg.kg^{-1}$) showed a survival rate of 100% and 60% respectively.

Figure 4. Antibacterial effects of clavanin A *in vivo*. The peptide clavanin A significantly increases survival. Mice were injected i.p. with *S. aureus* (**a**) or *E. coli* (**b**) bacteria (2×10^{9} $CFU.mL^{-1}$), followed by i.p. injection of clavanin A (1, 5 and 10 $mg.kg^{-1}$), LL-37 and gentamicin (10 $mg.kg^{-1}$) or PBS only, after 1 h. The injections were repeated after 24 h for 8 days. Treatment with the peptide significantly increased survival (n = 5 for controls and treated animal groups).



Analyses of cytokines 24 h after bacterial injection showed that mice infected by *E. coli* and treated with clavanin A, compared with the control group (PBS), and presented reductions in pro-inflammatory cytokines IFN-γ (55%) (Fig. 5A), IL-6 (69%) (Fig. 5B), IL-12p70 (69%) (Fig. 5C) and TNF-α (68%) (Fig. 5D). Similar data were obtained when *S. aureus* infected mice were treated with clavanin A, showing a reduction in pro-inflammatory IFN-γ (53%) (Fig. 5A), IL-6 (38%) (Fig. 5B) IL-12p70 (39%) (Fig. 5C) and TNF-α (52%) (Fig. 5D).

Figure 5. Effects of clavanin A on cytokines release (pg.mL^{-1}) during *E. coli* and *S. aureus* infection. Mice were infected with *E. coli* or *S. aureus* (2×10^{9} cfu.ml^{-1}) i.p. followed by administration 200 µL of PBS or clavanin A, LL-37 and gentamicin (10 mg.kg^{-1}) sc. after 1 h after infection. Mice were euthanized 24 h after infection and pro-inflammatory cytokine (**a**) IFNg, (**b**) IL-6, (**c**) TNF and (**d**) IL-12p70 levels were analyses in plasma (n = 5/group). Asterisks represent differences between treatments and PBS ($p<0.05$).



Discussion

In the present study, the *in vitro* antibacterial activities of clavanin A were determined against *S. aureus* and *E. coli.* The clavanin A MICs for *E. coli* and *S. aureus* shown here were higher than those described in the literature (<10 μM) [92, 132, 160]. Apparently this is due to the fact that clavanin A presents better activity in environments containing elevated NaCl concentrations and more acidic pH [176]. After confirming the *in vitro* antibacterial activity of clavanin A, the therapeutic potential was evaluated in two contrasting situations; firstly in an infected wound and secondly in a murine model of systemic infection. In our study a surgical wound infected by *S. aureus* was used due to its high frequency (30).

Here it was demonstrated that in addition to previous reports on *in vitro* antibacterial activities,[130] clavanin A also has potent activity in reducing *S. aureus* in an *in vivo* wound model, showing higher efficiency than LL-37 (Fig. 3). Furthermore, clavanin A treatment showed lower cytotoxicity on *in vitro* assays for L929 cells and SPCs, being significantly less toxic than LL-37, and in acute toxicity tests no adverse reactions were observed in any of the concentrations here tested (data not shown). These data corroborate the previous studies where clavanin A showed low cytotoxicity in human cell erythrocytes and HEp-2 as well as monkey cells Vero and MA104.[189] Normally, cathelicidins are highly toxic to eukaryotic cells [190]. Although some cathelicidin peptides have been shown to be effective in some *in vivo* studies, there is a very small pharmacological window available, because the effective dose is very near the toxic dose [191, 192]. In addition to cathelicidins, other HDPs such as defensins [193], magainins [194] and temporins[195] are also cytotoxic to mammalian cells. The data obtained showed that despite a higher MIC, clavanin A deserves special attention due to its low cytotoxicity. Furthermore, clavanin A indicated a dose-dependent effect on reducing bacterial colonies in infected wounds and on the increase of survival in a systemic bacterial infection model.

In the infected murine surgical wound model, it was observed that clavanin A showed potent activity in reducing bacteria in the surgical site, reducing by ~10x the bacterial load in wounds infected by *S. aureus*. Similar results were found

by Malmsten and co-workers [196], who proved the efficacy of a peptide derived from a sequence rich in arginine, leucine and proline by using an *ex vivo* surgical wound model in pig skin infected with *S. aureus* and *P. aeruginosa*. Furthermore, topical administration of AMPs derived from halocidin HG1 exerted strong antimicrobial activity in a surgical wound model infected with methicillin-resistant *S. aureus* in mice [197]. Cecropin peptide D2A21 also strongly inhibited *P. aeruginosa* growth in a wound model in rats [198]. Other unusual activities have also been reported. A trial using IDR-1018 showed that in spite of a complete absence of antibacterial activity against *S. aureus* in *in vivo* wound models.[179] Another interesting peptide evaluated was pexiganan. This peptide was evaluated as a medication for topical use, presenting antimicrobial activity against Gram-positive and Gram-negative organisms that normally infect skin and soft tissues [199]. LL-37 treatment (control group) demonstrated a significant bacterial reduction in the surgical site, showing high cytotoxicity towards skin cells *in vitro*. However, LL-37 treated wounds showed decreased organization of the newly formed epithelium and reduced re-epithelialisation compared to an equal dosage of clavanin A and gentamicin.

Given that sepsis may be caused by both Gram-negative and Gram-positive bacteria and can also start from a skin infection, clavanin A was also challenged in a systemic infection model against *E. coli* and *S. aureus*. Normally, Gram-negative bacterial sepsis is caused by the LPS component of the outer membrane, while sepsis caused by Gram-positive bacteria is associated with the production of exotoxin production, as well as cell wall components, such as LTA [200]. It is noteworthy that the antibacterial activity presented by clavanin A in the experimental model of systemic bacterial infection is peculiar, since the infected and treated animals showed a significant survival rate, which reached 80%. Comparing this finding with the literature, the same significance was observed, since animals receiving a lethal dose of *E. coli* and treated with 5 $mg.kg^{-1}$ of LL-37 showed only 28% survival.[129] The animals infected with *E. coli* and treated with clavanin A at standard concentration of 5 $mg.kg^{-1}$ showed 60% survival. In order to assess whether clavanin A and LL-37 act faster than gentamicin and if the reduction in cytokine response is merely a consequence

of infection decreasing the state health of these animals, the CFU count was evaluated just 24 h after infection. Groups treated with LL-37 and clavanin A 10 $mg.kg^{-1}$ showed a reduction in CFU with a significant difference from the control group (PBS) but no significant difference between these two groups and the group treated with gentamicin (Supplementary Fig. 2). These data suggest that modifications in the cytokine response probably occur due to reduction in bacterial growth.

In conclusion, clavanin A, a native AMP, has shown specific effects on the inhibition of *E. coli* and *S. aureus* proliferation and survival, and this peptide is as effective as LL-37, but significantly less toxic to the organism. These *in vitro* and *in vivo* antibacterial activities suggest that clavanin A will facilitate studies on the development of novel peptide-based strategies for the treatment of infected wounds and sepsis, opening new doors in the discovery of anti-infective drugs.

Acknowledgements

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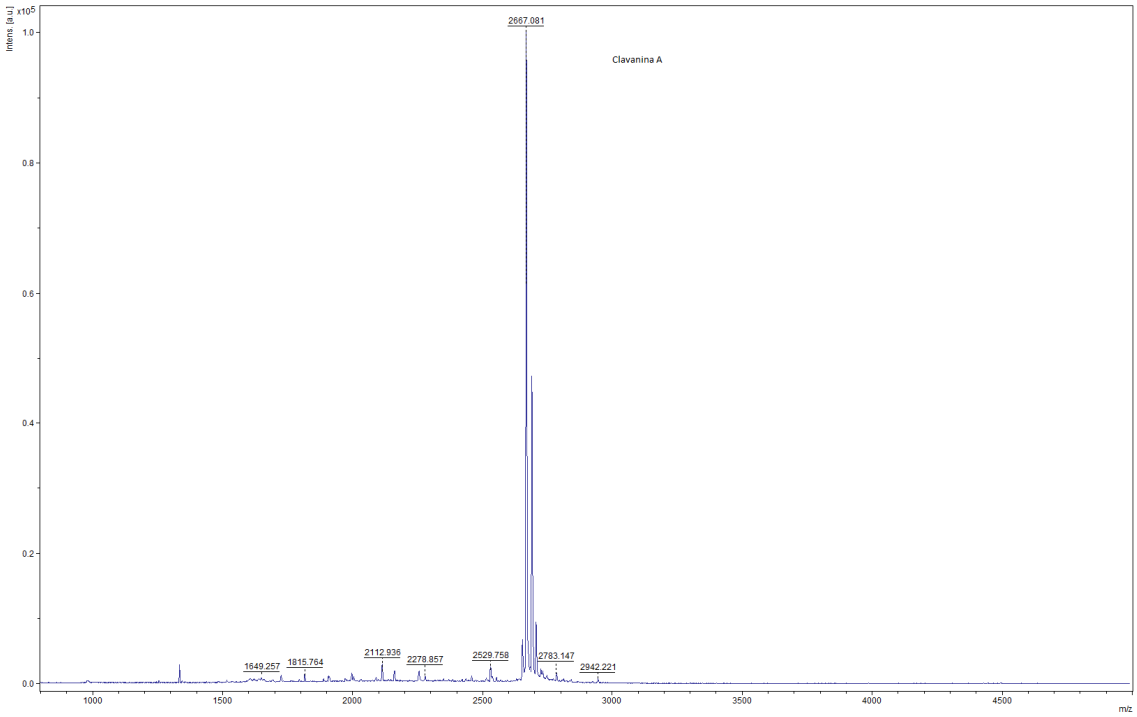
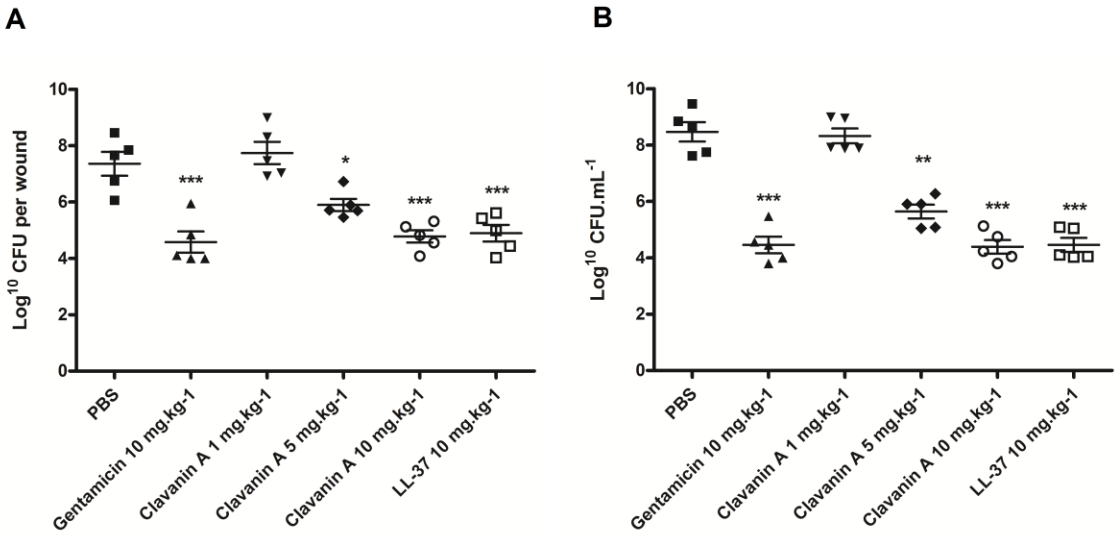
Supplementary figures

Supplementary figure 1. Synthesis and purification of Clavanin A. clavanin A (VFQFLGKIIHHVGNFVHGFSHVF-NH_2) was synthesized by China Peptides Co., Ltd., using solid phase method and F-moc strategies. Lyophilized peptide was re-suspended in 0.1% TFA (trifluoroacetic acid) and further purified using a reverse-phase analytical HPLC (high-performance liquid chromatography) Phenomenex C18 column (4.6 mm ×250 mm), with a linear acetonitrile gradient (5%–95%) at a flow rate of 1.0 mL·min^{-1}. Peptide elution was monitored at 216 and 280 nm absorbance. (b) Molecular mass and purity of synthetic peptide was confirmed by MALDI-TOF/TOF™ UltraFlex III (Bruker Daltonics, Billerica, MA, USA) analyses. Samples were solubilized in α-cyano-4-hydroxycinnamic acid saturated solution (1:3), and 1 µL of this solution was applied onto MALDI plate type MTP 384 and further air-dried. Mass spectrometer was calibrated with Peptide Calibration Standard II (Bruker Dal tonics).



Supplementary figure 2. Antibacterial effects of clavanin A 24h after bacterial infection. The peptide clavanin A significantly increases survival. Mice were injected i.p. with *S. aureus* (**a**) or *E. coli* (**b**) bacteria (2×10^9 CFU.mL^{-1}), followed by i.p. injection of clavanin A (1, 5 and 10 mg.kg^{-1}), LL-37 and gentamicin (10 mg.kg^{-1}) or PBS only, after 1 h. Treatment with the peptide significantly increased survival (n = 5 for controls and treated animal groups).



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5 CAPÍTULO II

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Improve the antibacterial activity of clavanin A by hydrophobic amino acids in the end-tag

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Abstract

Antimicrobial peptides (AMPs) are multifunctional mediators of innate immune response of living organisms; with direct and indirect antibacterial activity. The use of natural AMPs as therapeutic agents has certain limitations such as stability, toxicity and high molecular weight. In the current work a new peptide clavanin-MO designed peptide by incremental modification in the end-tagging of clavanin A. *In vitro* and *in vivo* antibacterial activities of clavanin-MO and clavanin A were tested against Gram-positive bacteria and Gram-negative bacteria as well as clinically isolated methicillin-resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli* KPC-positive. *In vitro* and *in vivo* cytotoxicity of the peptides against erythrocytes, monocytes and fibroblasts was evaluated to verify selective antimicrobial activity. In addition, immunomodulatory activity of these peptides was evaluated. In the present study, show that the addition of a small hydrophobic sequence (FLPII) at the C-terminus of clavanin A left the most active peptide. These results show that peptides end-tagging by hydrophobic amino acid are potential candidates for the development of molecules with more specific activity.

Key-words: hydrophobic amino acids, end-tag, clavanin-MO, multifunctional peptide.

Introduction

The search for new antibiotics has become more intense in recent decades, in response to increasingly frequent reports of multidrug-resistant pathogens, or by the side effects submitted by antibiotics on the market today[201]. In this configuration, antimicrobial peptides (AMPs) represent a promising concept. AMPs are multifunctional mediators of innate immune response of living organisms, with direct and indirect antibacterial activity[202, 175, 4]. The use of natural AMPs as therapeutic agents has certain limitations such as stability, toxicity and high molecular weight[203, 204] (>20 aa). These limitations may be remedied by increasing the hydrophobicity of AMPs[205-208]. One of the well-described methods for increasing the hydrophobicity of a peptide is by addition of hydrophobic amino acid residues in the end-tagging of peptide[209]. Since the labeled peptide-finals have limited toxicity combined with high antimicrobial activity of broad spectrum *in vitro* and *in vivo*.

Antimicrobial peptide clavanin A was previously characterized by Lee et al (1997)[130]. In view of the possibility of potentiating of clavanin A activity, an intensive search for a conserved region in peptides with antibacterial activity and immunomodulatory. As a result of this search, we have a hydrophobic region located in the C-terminal portion composed of five non-polar amino acid residues (FLPII)(Silva, O.N., la Fuente-Núñez, C., Ribeiro, S.M., Fensterseifer, I.C.M., Faria-Junior, Hancock, R.E.W., Franco, O.L. article submitted for publication), then, we added those the five residues in the C-terminal region of clavanin A, the new molecule is referred to herein as modified clavanin (clavanin- MO).

In the current work a new peptide clavanin-MO designed peptide by incremental modification in the end-tagging of clavanin A. *In vitro* and *in vivo* antibacterial activities of clavanin-MO and clavanin A were tested against Gram-positive bacteria and Gram-negative bacteria as well as clinically isolated methicillin-resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli* KPC-positive. *In vitro* and *in vivo* cytotoxicity of the peptides against erythrocytes, monocytes and fibroblasts was evaluated to verify selective antimicrobial activity. In addition, immunomodulatory activity of these peptides was evaluated.

Methods

Peptides

Clavanin A, clavanin-MO and cathelicidin LL-37of high quality were synthesized by Shanghai Hanhong Chemical (China) using the solid-phase with the N-9-fluorenylmethyloxycarbonyl (Fmoc) strategy and purified by high-performance liquid chromatography (HPLC)[130, 177]. Peptide purity used in biologic assays was higher than 95%.

Strains and growth condition

The bacteria strains useded in this work were: KPC-producing *Escherichia coli* ID Nº.1812446[210] (KPC-*E.coli*), *Escherichia coli* ID Nº.2101123[210] and KPC-producing *K. pneumoniae* 1825971 (both multi-drug resistant clinical isolates), *Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 12953, *Staphylococcus aureus* ATCC 29213, Methicillin-Resistant *Staphylococcus aureus* ATCC 33591, *Streptococcus pyogenes* ATCC 19615, *Escherichia coli* ATCC 8739, *Klebsiella pneumoniae* ATCC 13885, *Proteus mirabilis* ATCC 25933, *Pseudomonas aeruginosa* ATCC 15442 and *Salmonella enterica* ATCC 14028. Bacteria Isolates were preserved at -70 ºC with the addition of 20 % of glycerol.

MICs of peptides

Bacteria from frozing stock were plated on brain heart infusion (BHI) agar (Himedia, India) and incubated at 37 C for 24h. Three isolated colonies were incubated in 1 mL of BHI broth at 37°C for 12-16 h, on constant shaking (200 rpm). Minimum inhibitory concentration (MIC) of peptides were evaluated using the broth microdilution technique in Mueller-Hinton Broth medium (MHB) (5×10^5 cells) in Polystyrene microtiter plates (Corning, USA) in accordance with

Wiegand et al.[178]. The MIC was interpreted as the lowest concentration of peptide or antibiotic that completely inhibited the visible growth of bacteria after 12 h of incubation of the plates at 37°C in microplate reader (Victor X, Perkin-Elmer, Germany). Each agent was tested in triplicate in at least three independent experiments[211].

Flow cell assays

Flow cell assays were performed to evaluate the effect of antimicrobial treatments on pre-formed biofilms as described previously[212].

Hemolysis assay

Hemolytic activity of clavanin A and clavanin MO was determined by using fresh mouse red blood cells (mRBCs), by measuring the peptide-induced changes of the optical density (OD) at 540 nm (Victor X, Perkin-Elmer, Germany)[213], 100% lysis was determined by analyzing the supernatant of erythrocytes that had been incubated with Triton X-100 (1%)[214].

Cytotoxicity assay

Cellular cytotoxicity was measured by a colorimetric assay that makes use of the thiazolyl blue tetrazolium bromide (MTT). A subconfluent monolayer culture of mice fibroblasts L929 and RAW264.7 macrophage cell line were collected in complete Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 100 U/mL penicillin, 100 $\mu g.mL^{-1}$ streptomycin and 100 $\mu g.mL^{-1}$ gentamicin. Cells were seeded in 96-well microtiter plates in a concentration of 1.0×10^5 cells per well, with different concentrations of tested peptides (1-600 µM).Cells were allowed to incubate at 37 °C in the presence of 5% CO_2.Post 48 h

incubation in the presence of compound, cells was tested with MTT (10 µL at 5 $mg.mL^{-1}$–Sigma, USA). The plate was incubated for 4 h more, and the absorbance was monitored at 575 nm (Victor X, Perkin-Elmer, Germany). Cytotoxicity was determined as a percentage of the maximum value after subtracting the background. The results were expressed as the percentage of each sample compared to the negative control (PBS buffer, pH 7.4)[178].

In vitro anti-inflammatory effects

Murine macrophage-like cells RAW 264.7 were purchased from the Rio de Janeiro Cell Bank and were maintained in supplemented Dulbecco's modified eagle medium (DMEM) (Invitrogen, USA) (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% CO_2 at 37°C.Cells were stimulated by adding 0.1 ng/mL LPS into the medium specified above. The peptides (2 µM) were added 30 min after addition of LPS. After 6, 12, 24 and 48h of incubation, plates were centrifuged for six minutes at 400 × g and the supernatants were collected and kept frozen at −20°C until analysed for IL-1β, IL-1β, IL-12p70, IL-10, MCP-1, TNF-α and GM-CSF production. Cytokine levels were analysed using enzyme-linked immunosorbent assay (ELISA; Peprotech, USA) according to the manufacturer's instructions[210].

Animals

Six-week-old female C57BL/6 (Central Bioterium of the USP/RibeirãoPreto) mice were used for all studies. All procedures, care, and handling of the animals

were approved by the Ethics Committee of the Catholic University of Brasilia number 005/13.

In vivo toxicity

Before evaluating the antibacterial efficacy of clavanin-MO *in vivo*, it was essential to examine its toxic potential, which was performedby intraperitoneal (i.p.) injection of the tested clavanin A to groups of 10 C57BL/6 mice[183]. Each mouse was injected with a 0.5-ml solution of freshly prepared of peptides in PBS. Doses of peptide administered per mouse were 0, 10, 30, 50, 70 and 90 $mg.kg^{-1}$ of body weight. Animals were directly inspected for adverse effects for 6 h, and mortality was monitored for 7 days thereafter[211].

Murine systemic infection model

Based on preliminary experiments to determine a challenge dose of bacteria (*E. coli* ATCC 8739 and *E. coli* KPC-positive ID Nº. *1812446* or *S. aureus* ATCC 29213 and *S. aureus* (MRSA) ATCC 33591) that resulted in consistent systemic infection without rapidly killing the mice (data not shown), mid-log-phase bacteria were diluted to ~2 x 10^7 (cfu/mouse in phosphate buffered saline – PBS) for Gram-negative bacteria and 2×10^9 (cfu/mouse in PBS) for Gram-positive bacteria. Mice were challenged with either strain of *E. Coli* ATCC 8739 and *E. coli 1812446* or *S. aureus* ATCC 29213 and *S. aureus* (MRSA) ATCC 33591 in a final volume of 200 µL of PBS by intraperitoneal injection (i.p.). The day of challenge was designated as day 1 of the experiment. Three hour after bacterial injection, mice (n=5) were treated, intraperitoneal, with different concentrations of peptides (1, 5 or 10 $mg.kg^{-1}$), gentamicin and imipenem 10

mg.kg^{-1} or PBS for 8 days[211, 215].For bacterial load evaluation, 5 mice/group were humanely killed 24 h after bacterial administration. To detect bacterial colonization, peritoneal lavage was obtained by washing the cavity with 5 ml of sterile PBS. Samples were diluted serially, and 100 µl of each dilution was spread in duplicate on appropriate agar plates for the count of developed colonies[216]. Induction of cytokines GM-CSF, IFN-γ, IL-1β, IL-6, IL-12p70,IL-10, MCP-1 and TNF-α in the peritoneal lavage of the mice by peptides was measured.Cytokine levels were analysed by ELISA (Peprotech, USA) according to the manufacturer's instructions[210].

Isolation of leukocyte from peritoneal cavity

C57BL/6 mice were injected i.p. with 10 mg.kg-1 of peptide in sterile saline. The mice were euthanized, and peritoneal lavage was realized in different times post-injection. Leukocyte count present in the exudate of animals infected by *E. coli*ATCC 8739 was also determined in peritoneal cavity of mice. Animals infected and treated with peptides were euthanized 3 and 24 hours after treatment. Peritoneal lavage was collected for cell counting[217] and for determination of cytokines leves (IL-6, GM-CSF and MCP-1) . Cytokines were analysed by ELISA (Peprotech, USA) according to the manufacturer's instructions[210].

Statistical analysis

Data are present as mean ± SD of all samples. Statistical significance of fatality rates between different groups was analysed by Kaplan–Meier test. The other data were submitted to one-way analysis of variance (ANOVA) followed by

Bonferroni test. Differences between groups of *in vivo* toxicity assay were analysed using the Fisher exact test (differences were considered to be statistically significant when the P value was <0.05). Values of $p<0.05$ were considered statistically significant. GraphPad Prism software v5.0 (GraphPad Software, USA) was used for all statistical analyses[211].

Results

In vitro antibacterial activity

Antibacterial activities of clavanin A and clavanin-MO (Table 1) against clinical isolates and reference strains were assessed using the microdilution broth assay (Table 2). As reported in Table 2, clavanin A and clavanin-MO were active against all bacteria tested, with MIC values among 1.5 and45 µM. Interestingly, clavanin-MO showed MIC ~2 to 5 times lower than the clavanin A, including against resistant bacteria. Clavanin MO were less active against *S. aureus* ATCC 29213 and *E. coli* ATCC 8739 (MIC = 20 and 12 µM respectively) and highly active against *B. subtilis* ATCC 6633 and *E. faecalis* ATCC 12953 (MIC = 1.5 µM) (Table 2).

Table 1. Amino acid sequences and net charge of peptides used in this study.

Peptide	Sequence*	Net charge at neutral pH	Hydrophobicity
Clavanin A	VFQFLGKIIHHVGNFVHGFSHVF-NH_2	3.0	0.15
Clavanin-MO	FLPIIVFQFLGKIIHHVGNFVHGFSHVF-NH_2	3.0	0.21
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES-NH_2	6.0	-0.34

* Amino acids are indicated using the single-letter code.

Table 2. Antibacterial activity of clavanin A and modified peptide clavanin-MO. Bacteria were cultured in the presence of different peptide concentrations. Gentamicin and cathelicidin LL-37 were used as positive control. Data are representative of three independent experiments.

Microorganisms	MIC (μM)				
	Clavanin A	Clavanin-MO	LL-37	Gentamicin	imipenem
Gram-positive					
B. subtilis ATCC6633	6.0	1.5	6.0	6.0	0.5
E. faecalis ATCC12953	6.0	1.5	6.0	6.0	0.5
S. aureus ATCC29213	45.0	20.0	12.5	1.5	0.5
S. aureus (MRSA) ATCC33591	12.0	7.0	38.0	40.0	10.0
S. pyogenes ATCC19615	12.0	6.0	24.0	24.0	0.5
Gram-negative					
E. coli ATCC8739	24.0	12.0	1.5	0.7	1.0
E. coli KPC-positive (1812446)	30.0	6.0	25.0	46.0	6.7
E. coli (2101123)	12.0	6.0	20.5	53.0	7.5
K. pneumoniae ATCC13885	6.0	2.5	3.0	1.5	1.5
K. pneumoniae 1825971 (KPC971)	6.0	2.5	12.5	46.0	8.0
P. mirabilis ATCC25933	12.0	6.0	6.0	6.0	2.0
P. aeruginosa ATCC 15442	12.0	3.0	12.5	1.5	1.5
S. enterica ATCC14028	6.0	3.0	6.0	6.0	2.0

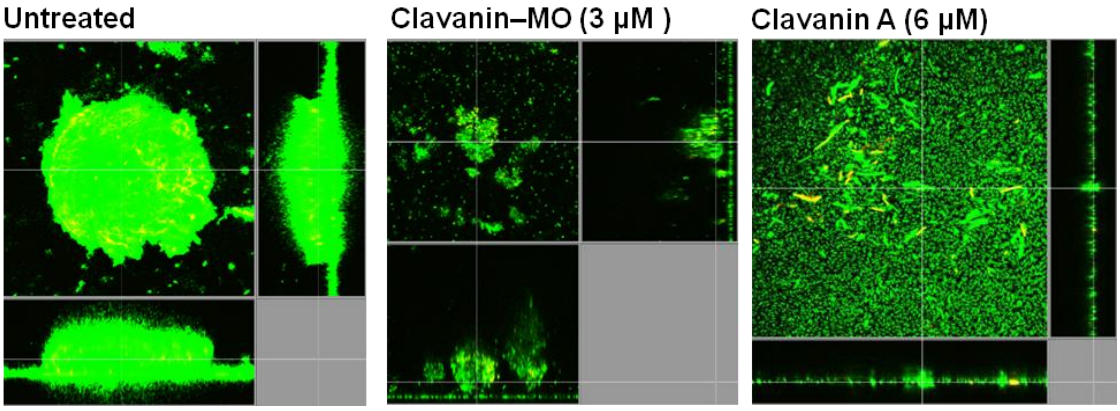
Anti-biofilm activity

We observed that 3 µM of clavanin-MO and 6 µM of clavanin A (Table 3), reduced the biofilm formed by the multidrug resistant KpC 1825971. However no filamentation was observed with clavanin-MO (Figure 1).

Table 3. Evaluation of anti-biofilm activity of the peptides Clavanin A and Clavanin-MO against KpC producing *K. pneumoniae*.

Peptides	Biofilm formation inhibition
Clavanin A	6.0 µM
Clavanin-MO	3. 0 µM

Figure 1. Effect of clavanin A and clavanin-MO on pre-formed biofilms of KpC strain 1825971. Biofilms were grown in the flow cell system. Treatments were applied for 24 h on 2-days old biofilms. After this time, bacteria were stained green with the all bacteria stain Syto-9 and red with the dead-bacteria stain propidium iodide (merge shows as yellow to red) prior to confocal imaging. Each panel shows reconstructions from the top in the 422 large panel and sides in the right and bottom panels (xy, yz and xz dimensions).



In vitro and *in vivo* toxicity studies

According to previous papers, clavanin A was not hemolytic peptide[218]. In this study we show that clavanin-MO despite having improved antibacterial activity compared to clavanin A (2 to 5 times), showed less than 10% cytotoxicity to erythrocytes, monocytes and fibroblasts even at concentrations greater than twenty times its lowest MIC (20 uM) (Table 4). Consistent with these results clavanin A and clavanin-MO did not caused toxicity in animals intraperitoneal treated with a dose of 90 $mg.kg^{-1}$ of peptide (Supplementary Table 1), 9 times the maximum reported in this article.

Table 4. *In vitro* cytotoxic activity of clavanin A and clavanin-MO. In assays evaluating cytotoxic activity of the peptides against human red blood cells (hRBCs) 1% Triton X-100 served as positive control (100% hemolysis). The release of hemoglobin was measured at 540 nm and is expressed as% hemolysis. In assays evaluating the cytotoxic activity of the peptides against L929 and 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.

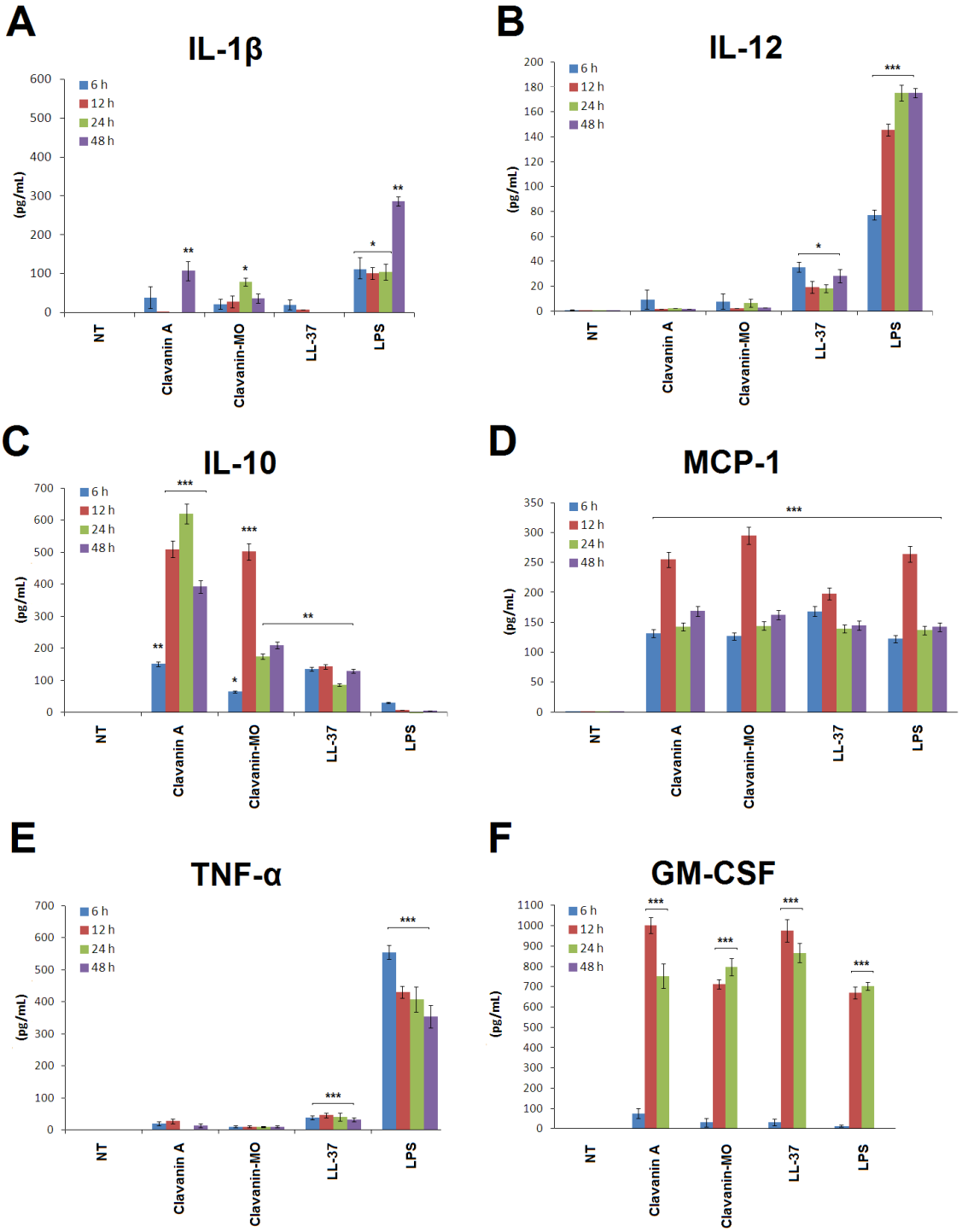
Cells type	[peptides] (µM)				
	Clavanin A	Clavanin-MO	LL-37	Gentamicin	Imipenem
hRBCs	> 400	> 400	87	> 400	> 400
L929	> 400	> 400	95	> 400	> 400
RAW 264.7	> 400	> 400	100	> 400	> 400

In vitro anti-inflammatory effects of peptides

The anti-inflammatory effect of clavanin A and clavanin-MO was studied in Murine macrophage-like cells RAW 264.7. Clavanin A and clavanin-MO demonstrated a significant reduction in the release of the pro-inflammatory cytokines IL-1β, IL-12, TNF-α(Figures 2A, 2B and 2E) in LPS-stimulated cells when compared with the untreated control (NT).The reduction in cytokine

production by cells treated with clavanin A and clavanin-MO is not related to decrease in cell viability peptide-induced, since the peptides stimulated the production of cytokine IL-10, monocyte chemoattractant protein-1 (MCP-1/CCL2) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Figures 2C, 2D and 2F). Moreover, as shown previously even at high concentrations (400 µM) these peptides caused less of 10% cytotoxicity (Table 3).

Figure 2. Effects of clavanin A and clavanin-MO on cytokines release (pg.mL-1) from RAW264.7 macrophages. RAW264.7 cells were stimulated with LPS (10 ng.mL^{-1}) and treated with 2 µM of peptides. Cathelicidin LL-37 (1 µM) was used as control. All experiments were preformed in triplicates. 6, 12, 24 and 24h after treatment pro-inflammatory cytokine (A) IL-1β, (B) IL-12p70, (C), IL-10, (D) MCP-1, (E) TNF-α, (F) GM-CSF levels were analyses. Asterisks represent differences between treatments and PBS ($p<0.05$).



In vivo activity of clavanin A and clavanin-MO against Gram-positive and Gram-negative bacterial infection prevented death in mice

We previously showed that clavanin A can protect mice from systemic bacterial ,infection[133, 218]. Thus, the *in vivo* antibacterial properties of clavanin-MO were similarly tested. Mice were treated i.p. with 1, 5 or 10 $mg.kg^{-1}$ in sterile saline and 3 h later infected with 2 x 10^7CFU of *E. Coli* ATCC 8739 and *E. coli* KPC-positive ID Nº.1812446, and 2×10^9 CFU of *S. aureus* ATCC 29213 and *S. aureus* (MRSA) ATCC 33591.As shown in Figure 3 clavanin A and clavanin-MO prolonged the survival of infected mice dose-dependently response. After 8 days of experiment, 80% survival was observed for animals treated with clavanin A (10 $mg.kg^{-1}$) and infected by *E. coli* ATCC 8739 (Figure 3A) and *S. aureus* ATCC 29213 (Figure 3E); and animals treated with clavanin-MO (10 $mg.kg^{-1}$) and infected by *E. coli* ATCC 8739 (Figure 3B),*E. coli* KPC-positive (Figure 3D) and *S. aureus* ATCC 29213 (Figure 3F). Animals infected with MRSA and treated with clavanin-MO showed 60% survival (Figure 3H), 20% survival rate higher than that of animals treated by clavanin A (Figure 3G). Twenty-four hours after infection, peritoneal fluid were collected and bacterial were counted, showing a significant decrease when compared to the untreated group, showing the highly protective activity of clavanin A and clavanin-MO (Figures 4A, 4B, 4C and 4D).

Figure 3. Protective activity of clavanin A and modified peptide clavanin-MO in mice infected. The mice were infected with 2 x 10^7 CFU of *E. coli* ATCC 8739 and *E. coli* KPC-positive ID Nº. 1812446 and 2×10^9 CFU of *S. aureus* ATCC 29213 and *S. aureus* (MRSA) ATCC 33591. Mice were treated I.P. with a single dose of peptide (1. 5 and 10 mg.kg^{-1}) 3h after infection. The bars represent the standard deviation. Statistical significance of differences between experimental groups of animals was determined using the Bonferroni test. Survival of mice was evaluated over time during 8 days. Survival data were compared with the PBS group. **P, 0.01; ***P, 0.001.

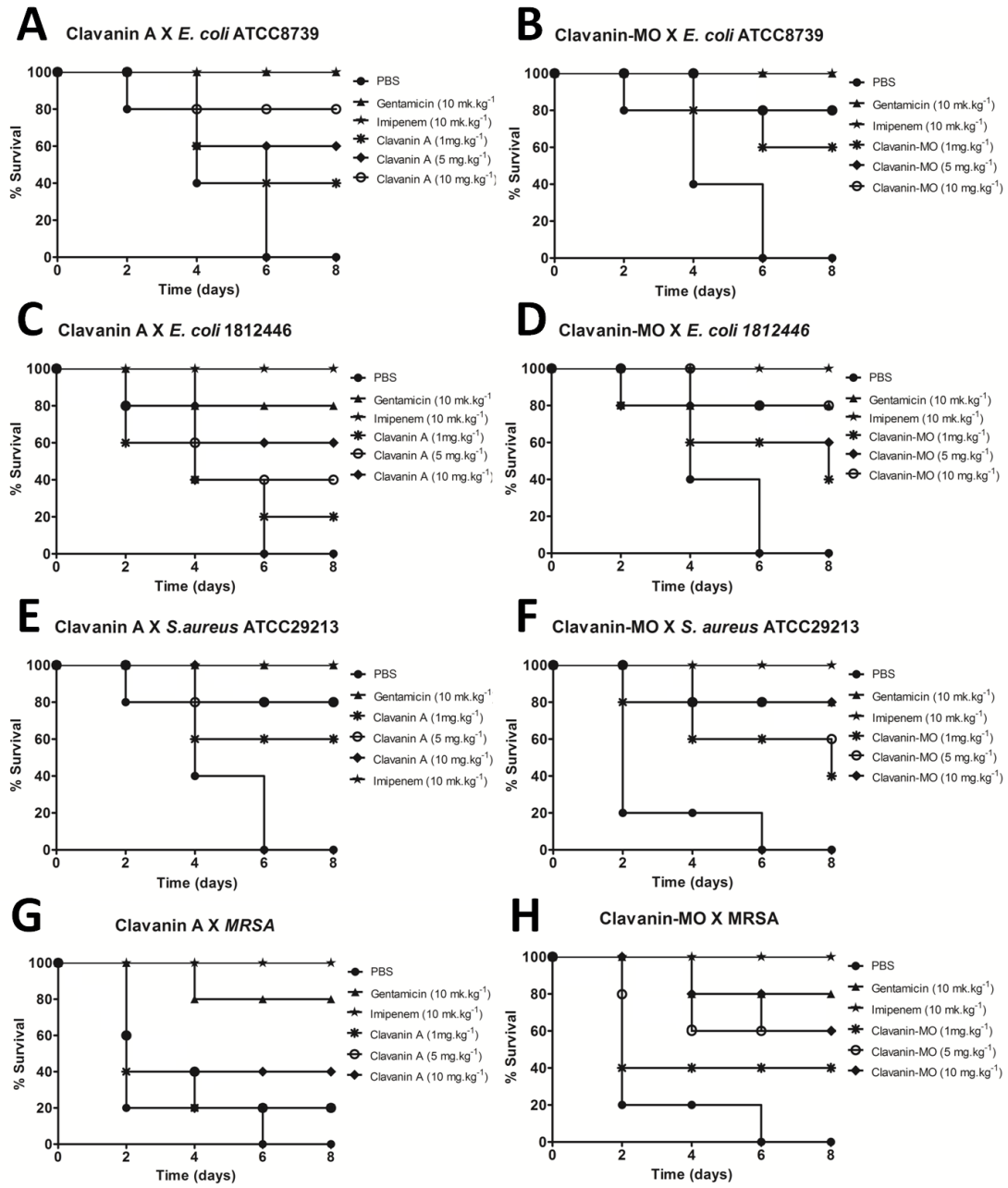
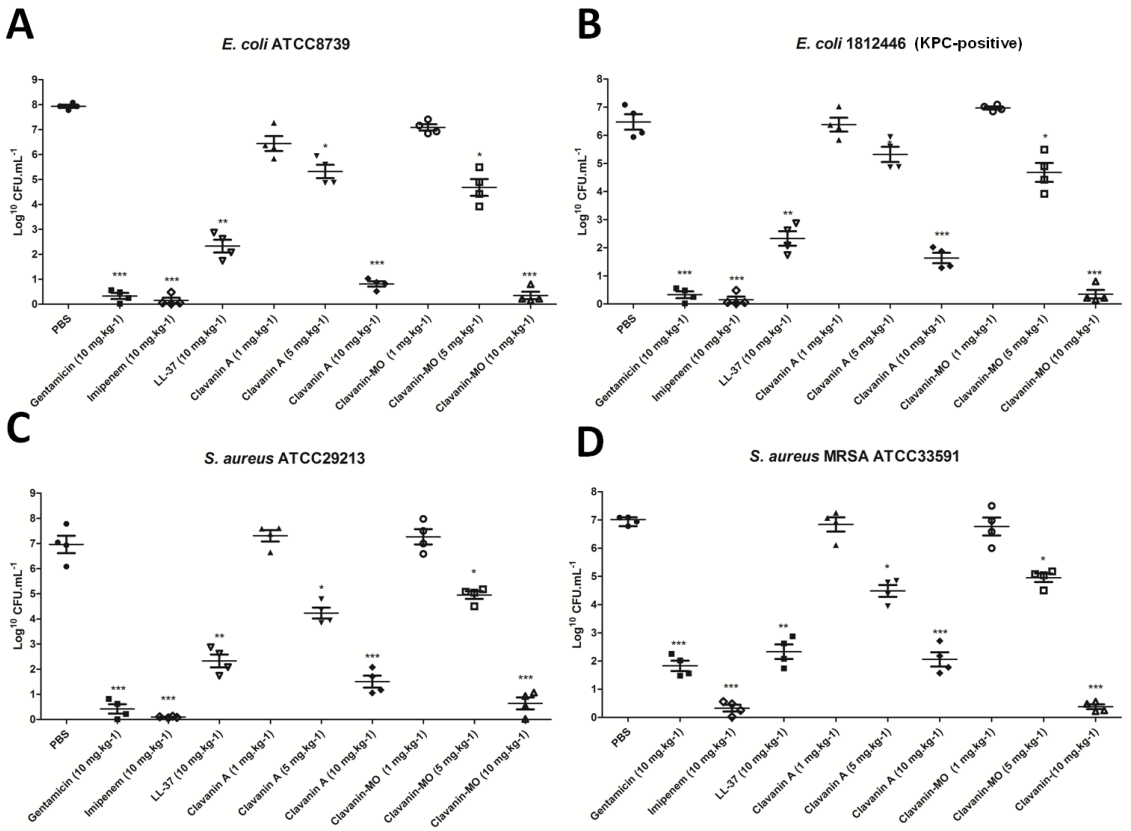


Figure 4. Bacterial counts in the peritoneal lavage after Gram-negative and Gram-positive infection. Evaluation of the protective activity of the peptides in murine models of infection with 2 x 10^7 CFU of *E. coli* ATCC 8739 and *E. coli* KPC-positive ID Nº. 1812446 and 2×10^9 CFU of *S. aureus* ATCC 29213 and *S. aureus* (MRSA) ATCC 33591. Mice were treated IP with a single dose of peptide (1. 5 and 10 mg.kg^{-1}) 3h after infection. The bacterial load was measured by counting bacteria in the peritoneal lavage 24 hours after infection. The bars represent the standard deviation. Statistical significance of differences between experimental groups of animals was determined using the Bonferroni test. Survival of mice was evaluated over time during 8 days. Survival data were compared with the PBS group. **P,0.01; ***P,0.001.



Clavanin A and clavanin-MO induce *in vivo* recruitment of mouse leukocytes

An important activity exerted by PAMs is acting on and migration of leukocytes to the infectious focus[219-222]. In his work, we examine whether the clavanin A and clavanin-MO induce the migration of leukocytes and if these peptides could induce a significant increase in the recruitment of leukocytes into the intraperitoneal cavity of healthy animals and infected with *E. coli*ATCC8739and *S. aureus* ATCC 29213. As can be seen in figure5, there was a substantial and significant increase in the number of leukocytes in the peritoneal fluid of healthy animals (Figure 5A) and infected with *E. coli* ATCC8739 (Figure 5B) and *S. aureus* ATCC 29213 (Figure 5C) treated with clavanin A and clavanin-MO 10 $mg.kg^{-1}$. The peak of leukocytes migration was observed 3 h after treatment with the peptides, for healthy animals and infected (Figures 5A, 5B and 5C).

In the *in vivo* assays clavanin A and clavanin-MO stimulated chemokines (MCP-1 and GM-CSF) and the cytokine IL-6 secretion (Figure 6A – 6D). We believe that recruitment of leukocytes is associated with increased levels of these molecules in the peritoneal lavage. No significant increase was detected in the secretion of pro-inflammatory cytokines IFN-γ, TNF-α, IL-1β, IL-12, and the regulatory cytokine IL-10 (Figure 6A – 6D).

Figure 5. Clavanin A and clavanin-MO induce migration of leukocytes into the peritoneal cavity of mice. (**A**) Leukocyte migration was evaluated into the peritoneal cavity of C57BL/6 mice uninfected, 30 min, 1, 2, 3, 4 and 24 h after treatment with 10 $mg.kg^{-1}$ of peptides. Mice are infected by *E. coli* ATCC 8739 (**B**) and *S. aureus* ATCC 29213 (**C**) 3 and 24 after treatment with 10 $mg.kg^{-1}$ of peptides, leukocyte migration was evaluated into the peritoneal cavity. Thioglycolate (TGA) 3% we used as a positive control. Data were expressed as mean ± standard deviation. Statistical analysis was performed using Bonferroni test. *** P <0.001 as significant compared to the control.

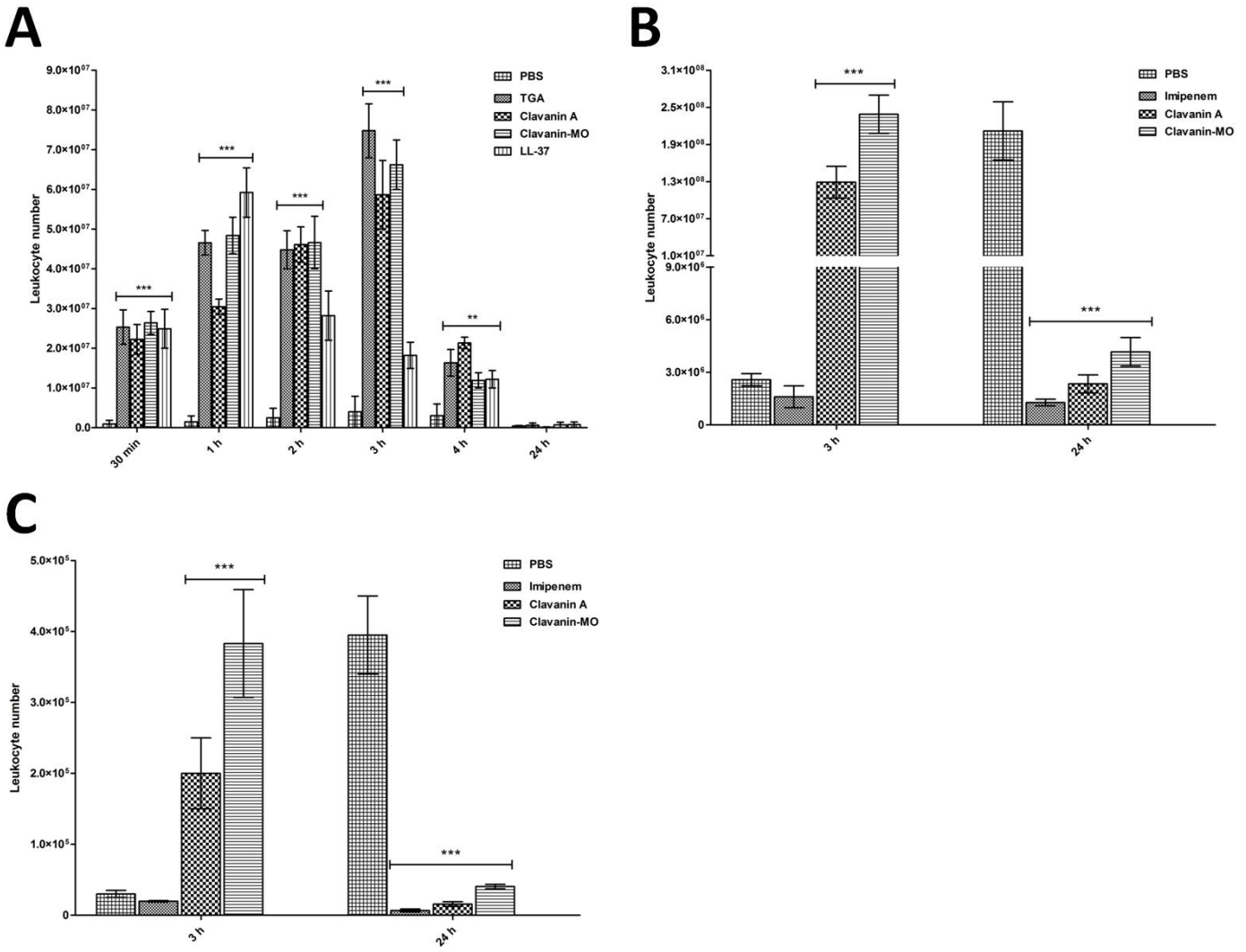
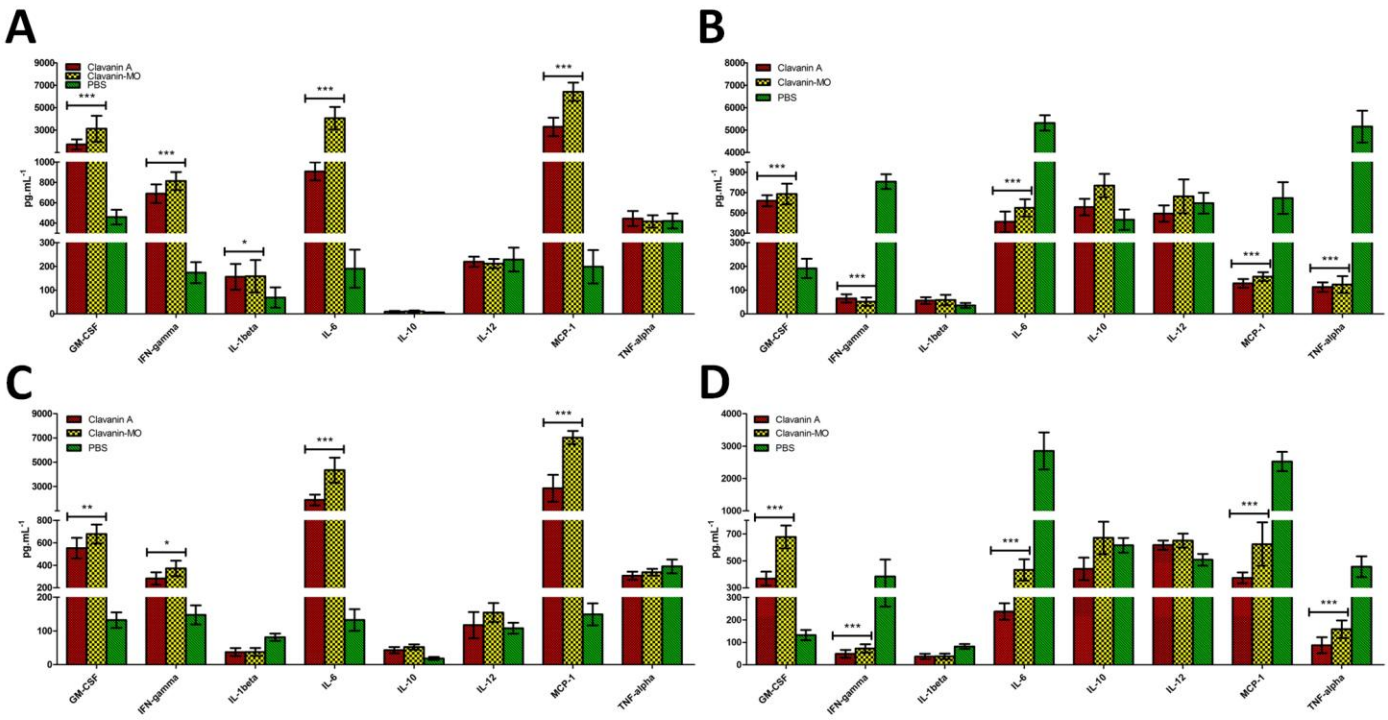


Figure 6. Effects of clavanin A and modified peptide clavanin-MO on the release of cytokines into the peritoneal cavity of mice infected with *E. coli* ATCC 8739 and *S. aureus* ATCC 29213 after treatment with the clavanin A and clavanin-MO. Cytokine release was evaluated into the peritoneal cavity of C57BL/6 mice infected by *E. coli ATCC 8739* treated with 10 $mg.kg^{-1}$ clavanin A and clavanin-MO, after 3 h (**A**) and 24 h (**C**) of administering of peptides and mice infected by *S. aureus* ATCC 29213, treated with 10 $mg.kg^{-1}$ clavanin A and clavanin-MO, after 3 h (**B**) and 24 h (**D**) of administering of peptides. PBS and imipenem (10 $mg.kg^{-1}$) were used as controls. Data were expressed as mean ± standard deviation. Statistical analysis was performed using Bonferroni test. *** P <0.001 as significant compared to the control.



Discussion

We observed that clavanin MO presents increased antibacterial action against different bacterial strains, including multi-drug resistant clinical isolates, when comapared to clavanin A. These results suggest that the increased antibacterial activity is possibly related to the addition of this small hydrophobic sequence of aminoacid (FLPII). Although clavanin-MO mechanism of action has not been determined, this small hydrophobic sequence may have increased specificity for bacterial membranes.

The sequence FLPII is conserved sequence in plant lipocalins[223-225]. Lipocalins interact with different types of membranes and organelles, and this interaction is mediated by this short hydrophobic domain (FLPII) located in these surface proteins[223-225]. It has been observed that increased hydrophobicity increases the microbicidal activity of AMPs, in addition to making AMPs less sensitive to salt, besides enhancing the affinity of peptides for biological membranes[205, 226-228]. However, highly hydrophobic PAMs were found to be less selective in its action, showing thereby an increased toxicity[229-232].

We also observed that to clavanin A and clavanin-MO was able to reduce the biofilm of KPC- producing *K. pneumoniae* 1825971. However no filamentation was detected among biofilms cells of this strain. Clavanin-MO may have a different action on biofilm structure when compared to clavanin A (Silva, O.N., Alves, E.S.F., la Fuente-Núñez, C., Mandal, S.M., Gaspar, D., Veiga, A.S., Castanho, M.A.R.B. Andrade, C.A.S., Nascimento, J.M., Fensterseifer, I.C.M., Ribeiro, S.M., Porto, W.F., Correa, J.R., Hancock, R.E.W., Korpole, S., Oliveira,

A.L., Liao, L.M., Franco, O.L.). More studies are necessary to investigate the differences about the antibiofilme action of these clavanins.

In this study, it was found that the clavanin A showed low cytotoxicity at all concentrations tested, despite of clavanin-MO be more hydrophobic than the clavanin A, the two peptides showed no acute toxicity and *in vitro* cytotoxicity. Although clavanin A was previously shown to promote the survival of 80% mice infected with *E. coli* ATCC 8739 and *S. aureus* ATCC 29213[133, 218].In the present we study we show that clavanin-MO showed activity similar to clavanin A in bacterial infections caused by susceptible bacteria, however, the animals infected with multiresistant *E. coli* and MRSA and plotted with clavanin-MO showed 20% survival longer than those treated with clavanin A. We have obtained compelling evidence that intraperitoneal treatment with clavanin A and clavanin-MO induced an inflammatory response in the abdominal cavity, thereby protecting the mice from death due to infection. Three hours after the injection of both peptides showed that there was a large migration of leukocytes into the peritoneal cavity. These findings corroborate the increase in the secretion of chemokines (MCP-1 and GM-CSF) and the cytokine IL-6 secretion. Clavanin A and clavanin-MO protect mice from acute septic shock, increasing the leukocyte migration in the first few hours after infection and inflammation reduced by a substantial decrease in a range of pro-inflammatory cytokines (IFN-γ, TNF-α, IL-1β, IL-12). This study therefore presents a new concept of multifunctional peptide[95, 214, 233], in this case the peptides involved in the defense against bacteria, and modulate inflammation, an ability that can be used therapeutically to compensate and normalize the excessive inflammatory

response seen in sepsis. Furthermore, a high antibacterial activity in the peritoneal cavity 24 h after treatment clavanin A and clavanin-MO, which we believe is due not only to the direct killing of bacteria but also due to the accumulation of leukocytes in the peritoneal cavity. Wherein, treatment with clavanin-MO promoted significantly better results than clavanin A. In this context, it is worth noting that many AMPs/host-defense peptides (HDPs) modulate the host immune system and protects you from lethal bacterial infections. The cathelicidin LL-37 exerts several immunomodulating activities, including recruitment of monocytes in in vivo models[109, 234].Immunomodulatory peptides IDR-HH2, IDR-1002, and IDR-1018 induce neutrophils migration and chemokine production, and suppresses the release of ROS and the production of inflammatory cytokines TNF-α and IL-10[221, 235, 236].

Previous studies have shown that the addition of small hydrophobic sequences is an easy and flexible approach of general applicability, by which the AMPs significantly increase the affinity to bacterial membranes, thereby enhancing the antibacterial activity *in vitro* and *in vivo*[206, 207, 209, 237]. In the present study, show that the addition of a small hydrophobic sequence (FLPII) at the C-terminus of clavanin A left the most active peptide. These results show that peptides end-tagging by hydrophobic amino acid are potential candidates for the development of molecules with more specific activity.

Supplementary Table 1. Evaluation of gross toxicity in mice treated with clavanin A and clavanin-MO. Each mouse was injected with a 0.5-ml solution of freshly prepared clavanin A and clavanin-MO in PBS. The doses of peptide administered per mouse were 0, 10, 30, 50 and 90 $mg.kg^{-1}$ of body weight. Animals were directly inspected for adverse effects for 30 min, and mortality was monitored for 6 h thereafter.

Dose ($mg.kg^{-1}$)	Imipenem – mice (n)	Clavanin A – mice (n)	Clavanin-MO – mice (n)
0	No effect	No effect	No effect
10	No effect	No effect	No effect
30	No effect	No effect (therapy dose)	No effect (therapy dose)
50	3, no effect; 2, toxicity level 1	No effect	No effect
90	3, toxicity level 2	3, no effect; 2 toxicity level 1	3, no effect; 2 toxicity level 1

Toxicity grading: level 1, narrowing of eyes; level 2, crouching and cuddling. Most mice recovered 2 h after treatment.

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6 CAPÍTULO III

Artigo submetido à revista ***Plos Pathogens***. *Impact factor: 8.057.*

Turning a toxic peptide from wasp venom into a therapeutic agent that provides broad-spectrum protection against lethal bacterial infections

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Introduction

The human body continuously interacts with different types of microorganisms, including commensal and pathogenic bacteria that colonize the skin and mucous membranes[1]. In most cases invading pathogens can be effectively eradicated by our immune system, thus preventing the development of an infection[1]. Lethal infections that were once incurable were treated after the discovery and subsequent marketing of antibiotics[238]. However, shortly after the introduction of penicillin in the market, *Staphylococcus aureus* resistant strains were observed. Currently various bacteria are resistant to antibiotics, leading to the emergence and spread of so-called "superbugs" which are resistant to almost all antibiotics available market[239, 240].

In hospital patients who use medical products such as catheters, endotracheal tubesand different prostheses, a higher incidence of systemic infections, since the surface thereof is common bacterial biofilm development have been observed[3]. Biofilms are of great importance to public health due the microorganisms associated with biofilms are less antimicrobial susceptibility. In clinical settings, biofilms are particularly problematic, since they tend to form in long-term devices and cause persistent infections and can lead to sepsis[65].

The investigation of new strategic therapies has been of great importance and the antimicrobial peptides (AMPs) are presented as excellent candidates due to its rapid antimicrobial activity and broad spectrum of action[175]. In our quest to find a suitable therapy for biofilm and systemic bacterial infections, we turned to the incremental modification of mastoparan-L. Mastoparan-L was initially isolated from the venom of wasp *Vespula lewisii*[241], showing important pharmacological applications such as antimicrobial and anti-inflammatory activity. However, both present certain levels of haemolytic and cytotoxic activity, promoting mast cell degranulation[144], limiting its use as a pharmacy. This study aimed to transform useless AMPs in potential pharmacies by reducing or avoiding toxicity against mammalian cells and increasing antibacterial activity against biofilms and planktonic cells.

Methods

Synthetic peptides

The peptides (mastoparan-L, mastoparan-MO and cathelicidin LL-37) were synthesized by Shanghai Hanhong Chemical (China) using the solid-phase with the N-9-fluorenylmethyloxycarbonyl (Fmoc) strategy and purified by high-performance liquid chromatography (HPLC)[177]. Peptide purity used in biologic assays was higher than 95%.

Bacterial strains and media

Strains used included clinical isolates *Escherichia coli* KPC-positive ID Nº.1812446[210], *Escherichia coli* multiresistant ID Nº.2101123[210] and carbapenemase-producing *Klebsiella pneumoniae* 1825971 (KPC971), as well as reference strains *Bacillus subtilis* ATCC6633, *Enterococcus faecalis* ATCC12953, *Staphylococcus aureus* ATCC29213, Methicillin-Resistant *Staphylococcus aureus* ATCC33591, *Streptococcus pyogenes* ATCC19615, *Escherichia coli* ATCC8739, *Klebsiella pneumoniae* ATCC13885, *Proteus mirabilis* ATCC25933, *Pseudomonas aeruginosa* ATCC 15442 and *Salmonella enterica* ATCC14028. Bacteria were plated on brain heart infusion broth (BHI) (Himedia, India) from a frozen stock. Following 24 h of incubation of the agar plate, three isolated colonies were transferred to 1 mL of BHI. The broth culture was incubated overnight (12–16 h) at 37 °C with shaking[242].

Antibacterial assays

Minimum inhibitory concentration (MIC) of peptides and antibiotics were evaluated using the broth microdilution technique in Mueller-Hinton Broth medium (MHB) with an initial inoculum of 5×10^5 cells in nontreated Polystyrene microtiter plates (Corning, USA) in accordance with Wiegand et al.[178]. The MIC was interpreted as the lowest concentration of peptide or antibiotic that completely inhibited the visible growth of bacteria after 12 h of incubation of the plates at 37°C. Each agent was tested in triplicate in at least three independent experiments[211].

Quantification of activity against biofilms

Biofilms were grown in BM2 glucose medium for 72 h, at 37°C in flow cell chambers with channel dimensions of 1x4x40 mm, as previously described by de la Fuente-Nunez et al.[243]. For the treatment of pre-formed biofilms, bacteria were allowed to develop structured 2-day-old biofilms prior to treatment with peptides for the following 24 h. Biofilm cells were then stained using the Live/Dead BacLight bacterial viability kit (Molecular Probes, Eugene, OR) and subsequently examined using a confocal laser scanning microscope (Olympus, Fluoview FV1000); three-dimensional reconstructions were generated using the Imaris software package (Bitplane AG).

Hemolysis assay

Hemolytic activity of peptides was determined by using fresh mouse red blood cells (mRBCs), by measuring the peptide-induced changes of the optical density (OD) at 540 nm (Victor X, Perkin-Elmer, Germany)[213], 100% lysis

was determined by analyzing the supernatant of erythrocytes that had been incubated with Triton X-100 (1%)[214].

Cell cytotoxicity assay

L929 mice fibroblasts (Rio de Janeiro Cell Bank) and SPCs were seeded in 96-well microtiter plates in a concentration of 1.0 x 10^5 cells per well, in DMEM medium, supplemented with different concentrations of tested peptides (1-600 µM). After 48 h incubation, a thiazolyl blue tetrazolium bromide (MTT) protocol was performed. Briefly, 60% of the medium was removed, and 10 µL of MTT (5 $mg.mL^{-1}$) (Sigma, USA) solution was added to each well and the plate was incubated for 4 h, in 5% CO_2, at 37 ºC. The blue formazan product generated was dissolved by the addition of 100 µL of 100% DMSO (Mallinckrodt, Germany) per well. Plates were then gently swirled for 5 min, at room temperature, to dissolve the precipitate. The absorbance was monitored at 575 nm using a microplate spectrophotometer (Bio-Tek, USA). Cytotoxicity was determined as a percentage of the maximum value after subtracting the background. The results were expressed as the percentage of each sample compared to the negative control (PBS buffer, pH 7.4) and cell culture was incubated in a lysis buffer (10 mM Tris, pH 7.4, 1 mM ethylenediamine tetraacetic acid (EDTA), and 0.1% Triton X-100)[178].

Animals

Six-week-old female C57BL/6 mice were used in this study. Animals were provided by the Central Bioterium of the USP Campus in Ribeirão Preto.

All animals were housed in individual cages under a constant temperature (22 °C) and humidity with a 12-h light/dark cycle and had access to food and water *ad libitum* throughout the study. The mice were euthanized by CO_2 at the end of the experiments. All procedures, care, and handling of the animals were approved by the Ethics Committee of the Catholic University of Brasilia number 005/13.

Acute toxicity

Acute toxicity assay was performed based on the work of Navon-Venezia and co-workers 2002[183]. The experiment was performed by intraperitoneal (i.p.) injection of the tested peptides to groups of 10 C57BL/6 mice. Each mouse was injected with a 0.5.mL solution of freshly prepared of peptides in PBS. The doses of peptide administered per mouse were 0, 10, 30, 50, 70 and 90 $mg.kg^{-1}$ of body weight. Animals were directly inspected for adverse effects for 6 h, and mortality was monitored for 7 days thereafter. Differences between groups were analysed using the Fisher exact test (differences were considered to be statistically significant when the P value was <0.05)[211].

Murine systemic infection model

Briefly, *E. coli* ATCC8739 and *E. coli 1812446* or *S. aureus* ATCC 29213 and *S. aureus* (MRSA) ATCC33591 were plated on tryptic soy agar plus 5% sheep blood (blood agar, Himedia, India) from a frozen stock. Following 24 h of incubation of the agar plate, three isolated colonies were transferred to 1 mL of BHI. The broth culture was incubated overnight (12–16 h) at 37°C with

shaking[242]. Based on preliminary experiments to determine a challenge dose of *E. coli* ATCC8739 and *E. coli* KPC-positive ID Nº. *1812446* or *S. aureus* ATCC 29213 and *S. aureus* (MRSA) ATCC33591 that resulted in consistent systemic infection without rapidly killing the mice (data not shown), the broth-grown bacteria were diluted to ~2 x 10^4 (cfu/mouse in phosphate buffered saline – PBS) for Gram-negative bacteria and 2×10^9 (cfu/mouse in PBS) for Gram-positive bacteria. Mice were challenged with either strain of *E. coli* ATCC8739 and *E. coli 1812446* or *S. aureus* ATCC 29213 and *S. aureus* (MRSA) ATCC33591 in a final volume of 200 µL of PBS by intraperitoneal injection (i.p.). The day of challenge was designated as day 1 of the experiment. Three hour after bacterial injection, mice (n=5) were treated, intraperitoneal, with different concentrations of peptides (1, 5 or 10 $mg.kg^{-1}$), LL-37, gentamicin and imipenem 10 $mg.kg^{-1}$ or PBS for 8 days[211, 215].For bacterial load evaluation, 5 mice/group were used. Animals were humanely killed 24 h after bacterial administration. To detect bacterial colonization, the peritoneal lavage were collected under sterile conditions. Peritoneal lavage was obtained by washing the cavity with 5 ml of sterile PBS. Samples were diluted serially, and 100 µl of each dilution was spread in duplicate on appropriate agar plates for the count of developed colonies[216].

Statistical analysis

Data are present as mean ± SD of all samples. Statistical significance of fatality rates between different groups was analysed by Kaplan–Meier test. The other data were submitted to one-way analysis of variance

(ANOVA) followed by Tukey test. Values of $p<0.05$ were considered statistically significant. GraphPad Prism software v5.0 (GraphPad Software, USA) was used for all statistical analyses[211].

Results

Design of modified peptide

AMPs have been known for many years, but very few are used in medical practice, for although some AMPs show significant activity in vitro, some peptides lose this activity under physiological conditions, and present high cytotoxicity for eukaryotic cells[175, 244]. Mastoparan-L highly cytotoxic (<10 mM)[161], in order to avoid such problem mastoparan-MO were yielded based on mastoparan-L. Five residues apolar amino acids were added at the C-terminal region peptide (FLPII). This short sequence tag were obtained after comparison with peptides presents in AMPer databases[245] (Table 1). Such modification is intended reduce the cytotoxic activity by preserving the microbicidal activity of mastoparan-L.

Table 1. Some primary sequence of peptides that were used as a basis for reaching small apolar amino acid (FLPII) added in the C-terminal region of peptide.

Peptide	Sequence
Brevinin-1BLb	FLPIIAGVAAKVLPKIFCAISKKC
Brevinin-1BLc	FLPIIAGIAAKFLPKIFCTISKKC
Brevinin-1CHa	FLPIIAGVAAKVLPKLFCAITKKC
Brevinin-1Pa	FLPIIAGVAAKVFPKIFCAISKKC
Brevinin-1Pb	FLPIIAGIAAKVFPKIFCAISKKC

Brevinin-1Pc	FLPIIASVAAKVFSKIFCAISKKC
Brevinin-1Pd	FLPIIASVAANVFSKIFCAISKKC
Brevinin-1Pe	FLPIIASVAAKVFPKIFCAISKKC
Brevinin-1Pf	FLPIIAGIAAKFLPKIFCAISKKC
Brevinin-1Pk	FLPIIAGVAAKVFPKIFCTISKKC
Brevinin-1Pl	FLPIIAGMAAKFLPKIFCAISKKC
Brevinin-1SPb	FLPIIAGMAAKVICAITKKC
Brevinin-1Yb	FLPIIAGAAAKVVQKIFCAISKKC
Brevinin-1Yc	FLPIIAGAAAKVVEKIFCAISKKC
Temporin-1AUa	FLPIIGQLLSGLL
Temporin-1BYa	FLPIIAKVLSGLL
Temporin-1DRc	FLPIIASVLSSLL
Temporin-1TGa	FLPIIGKLLSGIL
Temporin-PRb	FLPIITNLLGKLL
Vespid chemotactic peptide L	FLPIIAKLVSGLL
Vespid chemotactic peptide M	FLPIIGKLLSGLL
Vespid chemotactic peptide X	FLPIIAKLLGGLL

Determination of *in vitro* antibacterial activity

In order to evaluate the antimicrobial activity MIC values of mastoparan-L and modified peptide mastoparan-MO were determined against clinical isolates reference strains (Table 2). Mastoparan-MO presenting better activity against Gram-positive bacteria tested in this study (Table 2). Mastoparan-MO presenting MIC of 2.0 µM against *S. pyogenes*, 3.0 µM for *E. faecalis*, 3.5 for *B. subtilis* and *S. aureus* and 12.5 µM for MRSA. MICs results

showed by modified peptide mastoparan-MO was lower (approximately 50%) than that presented by mastoparan-L.

Table 2. Antibacterial activity of mastoparan-L and modified peptide mastoparan-MO. Bacteria were cultured in the presence of different peptide concentrations. Gentamicin and cathelicidin LL-37 were used as positive control. Data are representative of three independent experiments.

Microorganisms	MIC (μM)			
	Mastoparan-L	Mastoparan-MO	Gentamicin	Imipenem
Gram-positive				
B. subtilis ATCC6633	12.0	3.5	6.0	0.5
E. faecalis ATCC12953	3.5	3.0	6.0	0.5
S. aureus ATCC29213	7.0	3.5	1.5	0.5
S. aureus (MRSA) ATCC33591	7.0	12.5	40.0	10.0
S. aureus MRSA #SAP0017	21.5	3.8	40.0	10.0
S. pyogenes ATCC19615	3.5	2.0	24.0	0.5
Gram-negative				
E. coli ATCC8739	7.0	6.0	0.7	1.0
E. coli KPC-positive (1812446)	7.0	10.0	46.0	6.7
E. coli (2101123)	7.0	10.0	53.0	7.5
E. coli 0157	21.5	3.8	40.0	6.0
K. pneumoniae ATCC13885	11.0	11.0	1.5	1.5
K. pneumoniae 1825971 (KPC971)	21.5	31.0	55.0	10.0
P. mirabilis ATCC25933	15.0	11.0	6.0	2.0
P. aeruginosa ATCC 15442	15.0	10.0	1.5	1.5
S. enterica ATCC14028	15.0	11.0	6.0	2.0

Hemolysis and cell toxicity assays

After bactericidal activities, the hemoglobin release from mice erythrocytes exposed was measured by using different peptides concentrations. As shown in Table 3, even at concentrations as high as 1000 μM of mastoparan-MO presented minimal hemolysis against red blood cells (10%

maximum hemolysis observed). In contrast, a completely lysed red blood cells treated with mastoparan-L (100% hemolysis) at a significantly lower concentration of 10 µM (Table 3) was observed. Moreover, the cytotoxic effects of peptides on L929 RAW264.7 cells and monocytes were evaluated by MTT assay. As shown in Table 3, mastoparan-MO were non-toxic to mammalian cells tested up to 400 µM concentrations (12.5% maximum toxicity observed), values at least 10x higher than the MIC of the peptides. Moreover, mastoparan-L showed high toxicity even at bacteriostatic concentration (10 µM).

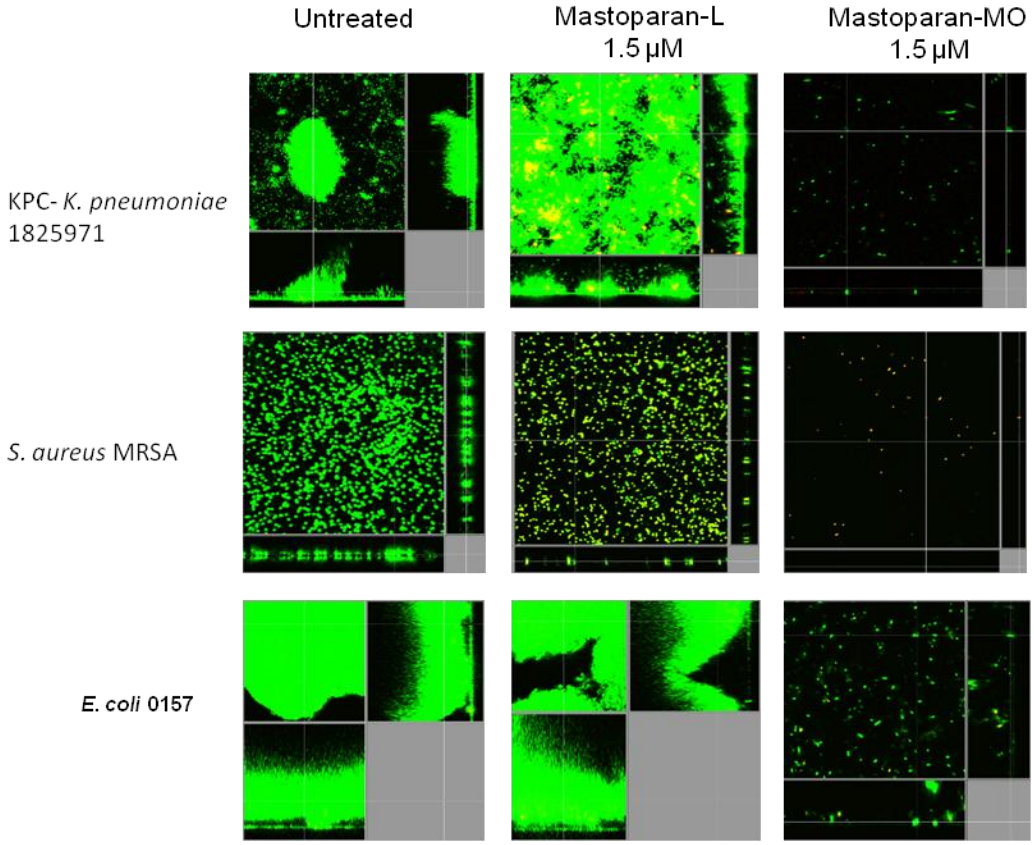
Table 3. Evaluation of cytotoxic activity of mastoparan-L and modified peptide mastoparan-MO. In assays evaluating cytotoxic activity of the peptides against human red blood cells (hRBCs) 1% Triton X-100 served as positive control (100% hemolysis). The release of hemoglobin was measured at 540 nm and is expressed as% hemolysis. In assays evaluating the cytotoxic activity of the peptides against L929 and 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.

Cells type	[peptides] (µM)				
	Mastoparan- L	Mastoparan MO	LL-37	Gentamicin	Imipenem
hRBCs	7	> 400	87	> 400	> 400
L929	10	> 400	95	> 400	> 400
RAW 264.7	10	> 400	100	> 400	> 400

Modified peptides as a potent broad-spectrum anti-biofilm agent

We observed that 1.5 µM of mastoparan-MO eliminated the biofilm formed by the multidrug resistant KpC 1825971 and MRSA, and *E. coli* 0157 (Figure 1).

Figure 1. Effect of Mastoparan and Mastoparan-MO on pre-formed biofilms of KPC- *K.pneumoniae* 1825971, *S. aureus* MRSA and *E. coli* 0157. Biofilms were grown in the flow cell system. Treatments were applied for 24 h on 2-days old biofilms. After this time, bacteria were stained green with the all bacteria stain Syto-9 and red with the dead-bacteria stain propidium iodide (merge shows as yellow to red) prior to confocal imaging. Each panel shows reconstructions from the top in the large panel and sides in the right and bottom panels (xy, yz and xz dimensions).



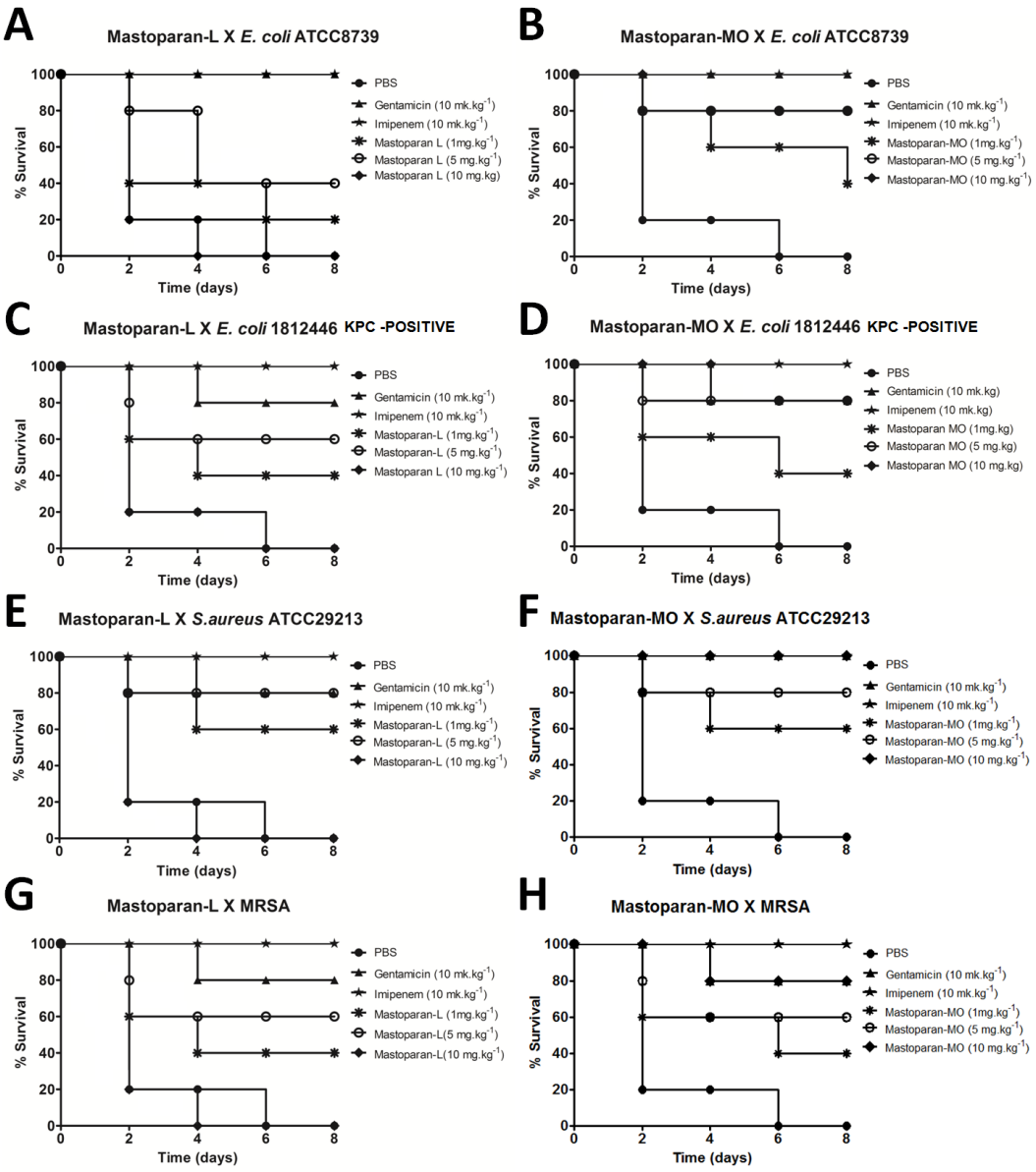
Determination of *in vivo* antibacterial activity

In order to assess whether the antibacterial activity *in vitro* translates our peptides *in vivo* activity, determine the antibacterial activity in infected mice with lethal amounts of bacteria. For that four different bacterial species were used: *E. coli* ATCC8739 and *E. coli 1812446* or *S. aureus* ATCC29213 and *S. aureus* (MRSA) ATCC33591[183, 242]. The smallest number of bacteria causing 100% lethal infection (LD100) after i.p. injection was ~$2x10^4$ for *E. coli* ATCC8739 and *E. coli* 1812446 and ~$2x10^9$ for *S. aureus* ATCC29213 and *S.*

aureus (MRSA) ATCC33591 (data not shown). Bacterial LD100 killed mice in 20–24 h. Mice were infected with the LD100 of bacteria and treated three hours later with the mastoparan-L and modified peptide mastoparan-MO by i.p. administration.

After infection with *E. coli* ATCC8739 and multidrug resistant *E. coli* 1812446 mastoparan-MO protected 80% of animals from sepsis (8-days survival) when administered daily at a concentration of 10 $mg.kg^{-1}$ (Fig. 2B and 2D respectively). Mice treated with mastoparan-L (5 $mg.kg^{-1}$) and infected by *E. coli* ATCC8739 and multidrug resistant *E. coli* 1812446 had a survival rate of 40 and 60% (Fig. 2A and 2C respectively). Mice infected with *S. aureus* ATCC29213 mastoparan-MO protected 100% of animals when administered at a daily dose at a concentration of 10 mg.kg-1 (Fig 2F); mice treated with mastoparan-L (5 $mg.kg^{-1}$) had a 80% survival rate (Fig. 2E). Finally, *S. aureus* MRSA ATCC33591 was used to challenge the mice. Modified peptide mastoparan-MO administered at daily doses in concentrations of 10 $mg.kg^{-1}$ protected 80 % of animals (Fig. 2H). Mice infected with *S. aureus* MRSA ATCC33591 and treated with mastoparan-L (5 $mg.kg^{-1}$) showed 60% survival (Fig. 2G).

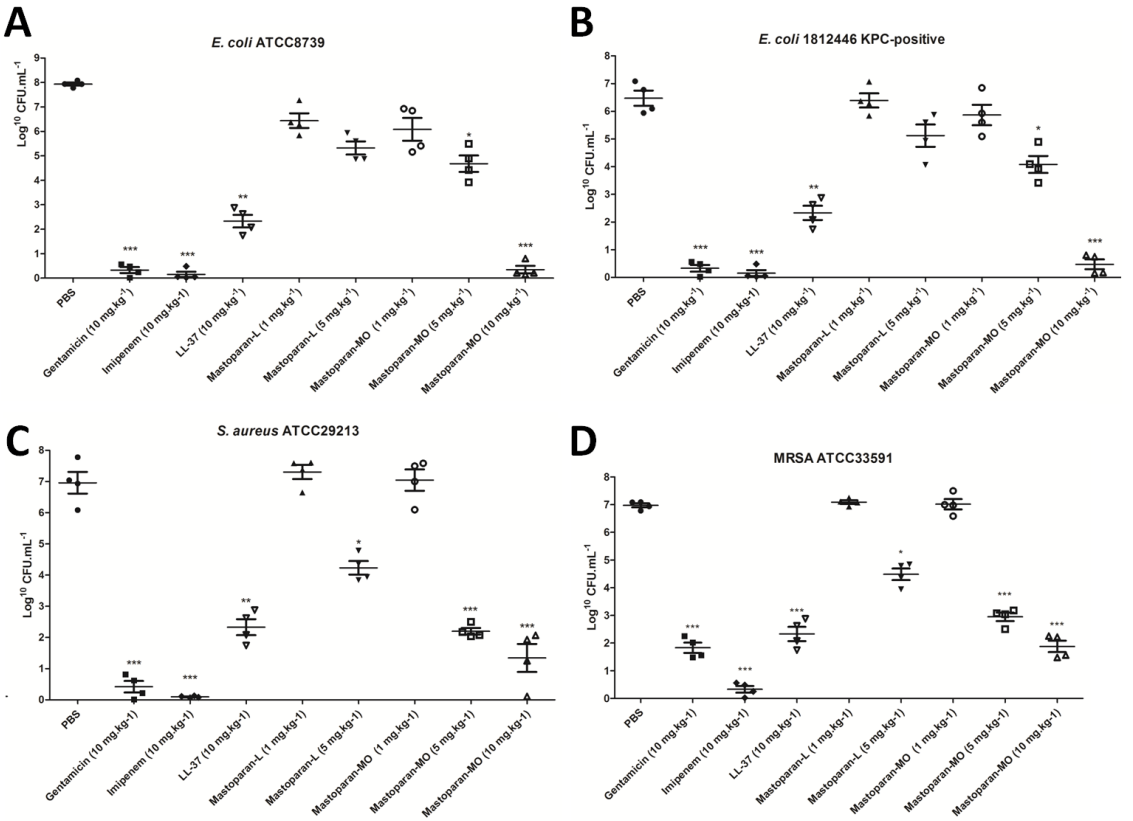
Figure 2. Protective activity of mastoparan-L and mastoparan-MO in mice infected. The mice were infected with 2 x 10^7 CFU of *E. coli* ATCC 8739 and *E. coli* KPC-positive ID Nº. 1812446 and 2×10^9 CFU of *S. aureus* ATCC 29213 and *S. aureus* (MRSA) ATCC 33591. Mice were treated I.P. with a single dose of peptides (1. 5 and 10 mg.kg^{-1}) 3h after infection. The bars represent the standard deviation. Statistical significance of differences between experimental groups of animals was determined using the Bonferroni test. Survival of mice was evaluated over time during 8 days. Survival data were compared with the PBS group. **P, 0.01; ***P, 0.001.



Efficacy of peptides in reducing bacterial load in a mouse sepsis model

Antibacterial activity *in vivo* of mastoparan-L and modified peptide mastoparan-MO was also assessed by bacterial counts in the peritoneal fluid after infection with LD100 of *E. coli* ATCC8739, *E. coli* 1812446, *S. aureus* ATCC29213 and *S. aureus* (MRSA) ATCC33591. 24 h after infection, bacterial counts in the peritoneal fluid were significantly lower than in controls.

Figure 3. Bacterial counts in the peritoneal lavage after Gram-negative and Gram-positive infection after treatment of mastoparan-L and mastoparan-MO. Evaluation of the protective activity of the peptides in murine models of infection with 2 x 10^7 CFU of *E. coli* ATCC 8739 and *E. coli* KPC-positive ID Nº. 1812446 and 2×10^9 CFU of *S. aureus* ATCC 29213 and *S. aureus* (MRSA) ATCC 33591. Mice were treated IP with a single dose of peptides (1. 5 and 10 mg.kg^{-1}) 3h after infection. The bacterial load was measured by counting bacteria in the peritoneal lavage 24 hours after infection. The bars represent the standard deviation. Statistical significance of differences between experimental groups of animals was determined using the Bonferroni test. Survival of mice was evaluated over time during 8 days. Survival data were compared with the PBS group. **P,0.01; ***P,0.001.



Efficacy of peptides *in vivo* toxicity

Mastoparan-MO did not caused apparent signs of toxicity in animals intraperitoneal treated with a dose of 90 $mg.kg^{-1}$ of peptide, 9 times the maximum reported in this article. The animals treated with mastoparan-L10 $mg.kg^{-1}$ had a survival rate of 20% in the first two days, and 100% mortality within 2 h after administration of higher doses (>10 $mg.kg^{-1}$) (Supplementary Table).

Discussion

Numerous studies have shown that the AMPs may be the next line of compounds in combating multidrug-resistant strains.[246] Here we modify known AMPs mastoparan-L, in order to reduce the toxicity of mastoparan-L, with the aim of developing analogues in order to increase the *in vivo* activity and reduce the toxicity.

The peptide obtained mastoparan-MO show a reliable antibacterial *in vitro* and *in vivo* activity against clinically relevant Gram-positive and Gram-negative strains, including resistant bacteria MRSA and *E. coli* KPC-positive. An interesting finding were obtained with mastoparan-MO, which showed *in vitro* antibacterial activity close to or better than mastoparan-L, but also reduced by more than 10X the hemolytic activity against blood cells and fibroblasts.

One of the bottlenecks in the therapeutic use of AMPs is the toxicity against mammalian cells. In recent years, many researchers have been devoted to eliminating the toxicity of AMPs in their therapeutic dose while maintaining a high antibacterial efficacy. The sheep myeloid antimicrobial

peptide-29 (SMAP-29) had some amino acid residues altered in an attempt to reduce the hemolytic activity, where two of the analogues had antibacterial efficacy comparable with SMAP-29 against *B. anthracis*, and was reduced hemolytic activity (2.3-2.6 fold over SMAP-29)[247]. In another study, Papo et al.[248] modified some α-helical AMPs by replacing 35% of L-amino acids by D-amino acids and found that this modification eliminated the hemolytic activity and has the AMPs are not sensitive to proteinases[249]. In another study by Nell et al.[32], They removed neutral amino acids Asn and Gln, and adding positively charged residues (Arg two units) in the primary sequence of LL-37, the newly generated peptide (P60) showed less cytotoxic to eukaryotic cells and has been successfully used for nasal applications against MRSA[249, 250]. As can be seen in the aforementioned studies, the rational design of AMPs is an important tool for reducing the toxicity of AMPs against mammalian cells and increase of antibacterial activity *in vivo*. The use of this powerful tool may permit the use of the AMPs in the treatment of systemic bacterial infections.

The systemic bacterial infections are a major cause of mortality and increased hospital costs and, despite the efforts that have been made by public health authorities in recent decades, systemic infections continue to increase year after year, due in particular to increase in antibiotic-resistant microorganisms available.[251-253] Various AMPs have been reported to modulate the host's response to systemic bacterial infections, including LL-37 which acts as a potent mediator of inflammation, mice and protects mice from lethal effects of infections caused by Gram-negative and Gram-negative bacteria.[254] In the present work mastoparan-MO protected mice significantly in an experimental

model of systemic bacterial infection caused by *S. aures* and *E. coli*, including a strain of MRSA and *E. coli* KPC-positive, animals treated with the peptide showed a survival rate that reached 80%. Furthermore, *in vivo* toxicity tests showed that the mastoparan-MO are not toxic in the tested concentrations (9X greater than the therapeutic dose).

Mastoparan-MO prevented the growth of planktonic bacteria, leading to the mature biofilm eradication of pathogenic Gram-negative and Gram-positive these two peptides show promise as therapeutic agents against systemic infections and bacterial biofilms caused by a variety of bacteria.

Supplementary Table 1. Evaluation of gross toxicity in mice treated with mastoparan-L and mastoparan-MO. Each mouse was injected with a 0.5-ml solution of freshly prepared mastoparan-L and mastoparan-MO in PBS. The doses of peptide administered per mouse were 0, 1, 5, 10, 30, 50 and 90 $mg.kg^{-1}$ of body weight. Animals were directly inspected for adverse effects for 30 min, and mortality was monitored for 6 h thereafter.

Dose ($mg.kg^{-1}$)	Imipenem – mice (n)	Mastoparan-L – mice (n)	mastoparan-MO – mice (n)
0	No effect	No effect	No effect
1	No effect	No effect	No effect
5	No effect	3, no effect; 2 toxicity level 1	No effect
10	No effect	5 animals death	No effect
30	No effect	-	No effect (therapy dose)
50	3, no effect; 2, toxicity level 1	-	No effect
90	3, toxicity level 2	-	3, no effect; 2 toxicity level 1

Toxicity grading: level 1, narrowing of eyes; level 2, crouching and cuddling. Most mice recovered 2 h after treatment.

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7 CAPÍTULO IV

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

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Abstract

Peptide rational design was here used to guide the creation of two novel short β-lactamase inhibitors, here named dBLIP-1 and -2, with five amino acid residues length. Molecular modeling associated to 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.

Keywords: β-lactamase inhibitors, bacterial resistance, drug design, *in vivo* model.

Introduction

Antimicrobial resistance is not a new problem, but the number of resistant organisms and lethal outbreaks is unprecedented[1-3]. 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 difficult[4,5]. Among the antibacterial therapies, the most often used antibiotics commonly consist of lactam, including penicillins and cephalosporins[6]. 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 treatment[7]. 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 ineffective[8]. 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 ring[9,10].

Results and Discussion

The 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.

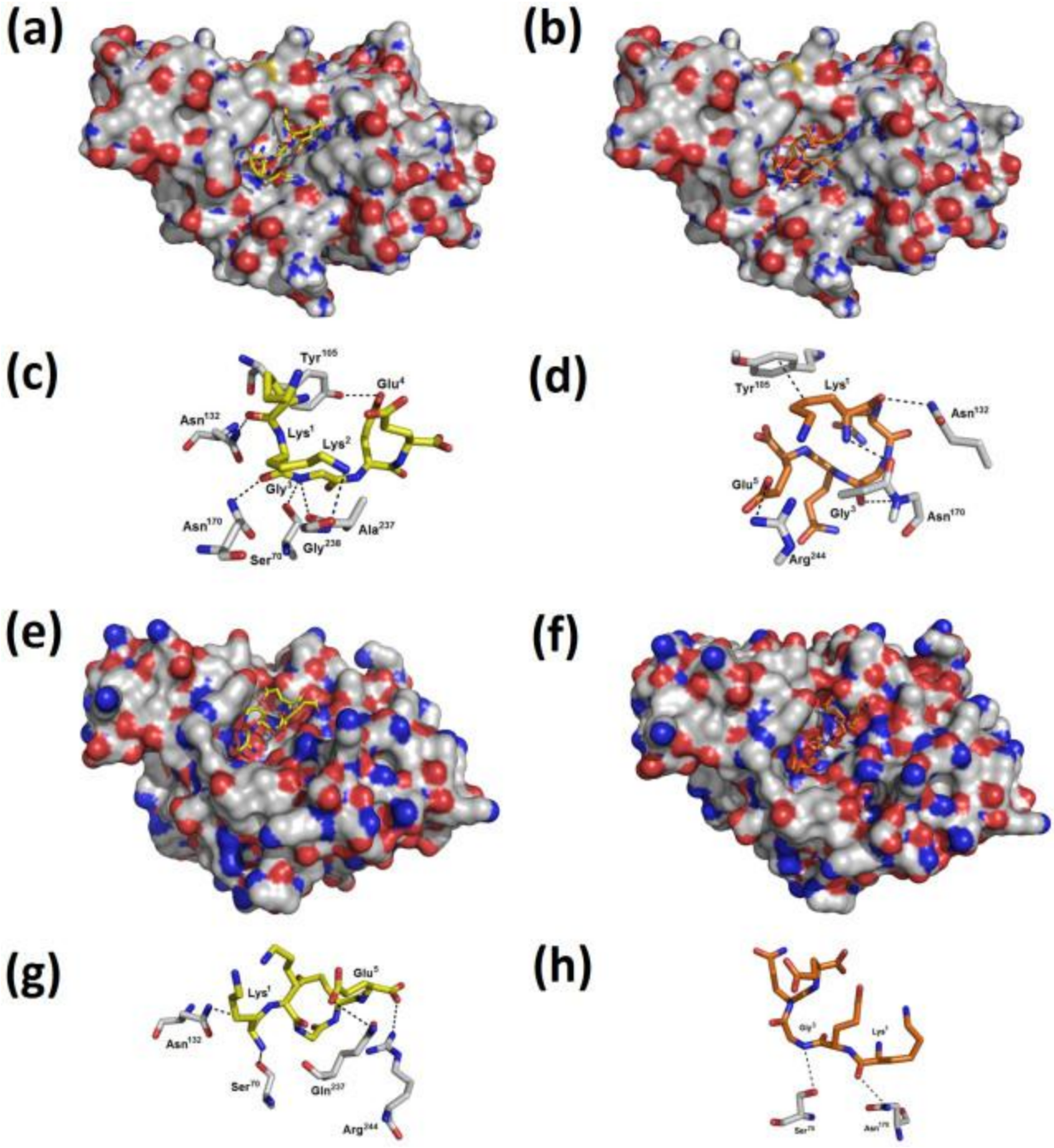
In order to set out the peptide-enzyme interactions more clearly, theoretical models of dBLIP-1 and -2 were constructed. Procheck summary of dBLIP-1 and -2 showed that, for both peptides, 100 % of amino acid residues are located in the most favorable regions in the Ramachandran plot. In addition, the 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 (**Fig. 1C**). The interactions observed for dBLIP-1 were between the backbone oxygen atom (O) of lysines (Lys^1 and Lys^2) and the hydrogen (2HD2 and 1HD2) of Asn^{132} and Asn^{170} forming, respectively, two hydrogen bonds with distance of 2.65 and 2.5 Å. Gly^3 (N and O atom) participated in two hydrogen bond interactions between Ser^{70} (HG) and Ala^{237} (HN), with distances of 2.6 and 3.1 Å, respectively. The last hydrogen bond observed was between Glu^4 (OE1) and Tyr^{105} (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 Lys^1 and oxygen atoms of Ser^{235} (OG) and Ala^{237} (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 *S. aureus* 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 Tyr^{105}. Another interaction observed was between the oxygen atom (OE1) of Glu^5 and the nitrogen atom (NH2) of Arg^{244} with a distance of 3.2 Å, forming an electrostatic interaction. dBLIP-1 presented interactions between the backbone oxygen atom (O) of lysines Lys^1 and the nitrogen atom (ND2) of Asn^{132}, forming a hydrogen bond with distance of 3.45 Å. The nitrogen atom of Lys^1 also participated in hydrogen bond interaction between the oxygen atom of Ser^{70} (OG), with a distances of 3.47 Å. The last interaction observed was between the oxygen atom of Glu^5 (O) and nitrogen of the amine group of Arg^{244} (NH2), forming an electrostatic interaction with distances of 3.43Å. Two hydrogen bonds were observed:

one between the oxygen atom (O) Lys^{1} and hydrogen of nitrogen atom of Asn^{170} (ND2), with a distance of 3.42, and the other between hydrogen of nitrogen atom of Gly^{3} (N) and oxygen atom (OG2) of Ser^{70} with a distance of 3.6 Å (**Fig. 1h**). Similar data were observed for other β-lactamase inhibitors[10,11] in which the presence of cationic and hydrophilic residues seems to be essential for the inhibition process.

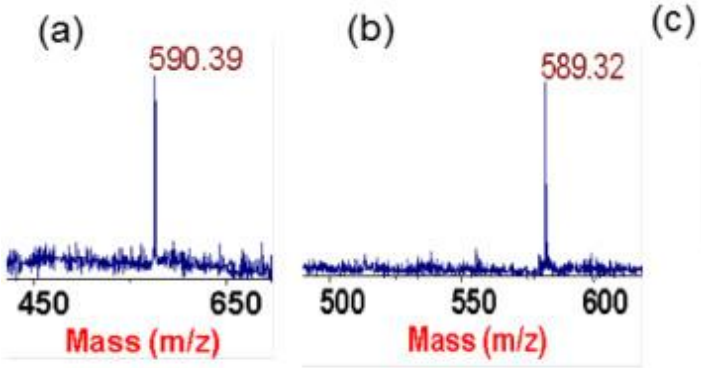
Figure 1. Docking studies of dBLIP-1 and dBLIP-2 and β-lactamases from two different bacterial sources. Structural complementarity between dBLIP-1 (a) and dBLIP-2 (b) toward β-lactamase from *Escherichia coli*. In detail, the non-covalent interactions (dotted lines) of dBLIP-1 (c) and dBLIP-2 (D) and *E. coli* β-lactamase catalytic site. Structural complementarity between dBLIP-1 (e) and dBLIP-2 (f) with β-lactamase from *Staphylococcus aureus*. In detail, the non-covalent interactions (dotted lines) of dBLIP-1 (g) and dBLIP-2 (h) and *S. aureus* β-lactamase catalytic site.



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 checked the identity with MALDI ToF MS analysis after HPLC separation (**Fig. 2a and 2b**). Peptides were also synthesized for authentication 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 $\mu g.mL^{-1}$ concentration (**Fig. 2c**). dBLIP-1 demonstrated greater affinity for the β-lactamase enzyme by calorimetry assays when compared to dBLIP-2. The binding affinity (*K*) and binding stoichiometry 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 titration curves for the two peptides and their stoichiometry at the endpoint are shown (**Fig. 2d-2f**). In both cases, complexation took place, as indicated by their binding isotherm. The line through the simulated 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, ΔH and ΔS are listed in **Supplementary Table 1.** The interaction processes are enthalpy-driven and based upon van der Waals interactions, along with H-bonding, because the values of ΔH and ΔS were negative[12]. 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, for better improve information about

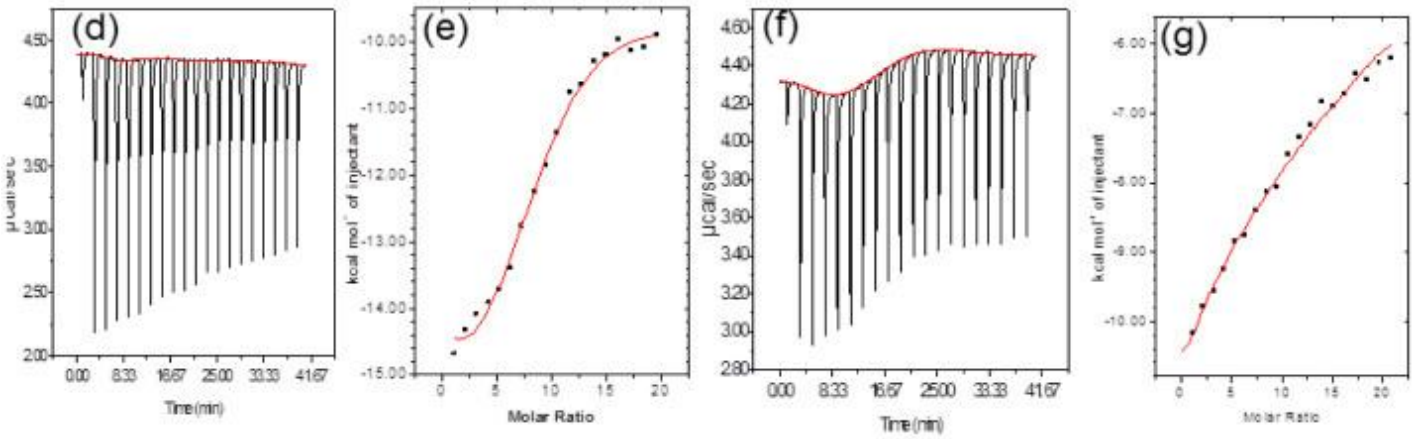
interactions among dBLIPs with enzyme, the free energy value were evaluated through docking studies being observed that dBLIP-1 toward the β-lactamase from *E. coli* interactions presented -5.8 Kcal.mol^{-1}, while the energy observed for dBLIP-2 toward the same β-lactamase was -5.4 Kcal.mol^{-1}, demonstrating 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.

Figure 2. Binding and inhibitory activity of *Bacillus cereus* 569 β-lactamase (Calbiochem, UK) by using dBLIP-1 and -2. MALDI-ToF Mass spectra of purified dBLIP-1 (a) and dBLIP-2 (b). *In vitro* study for inhibition (IC50) of β-lactamase activity by dBLIP-1 and dBLIP-2, (c). ITC bonds measurement between dBLIP-1 and 2. Figures show the raw data obtained from ITC binding experiment between β-lactamase with dBLIP-1 (d) and dBLIP-2 (f). Figure representing the integration of raw heat associated data for dBLIP-1 (e) and dBLIP-2 (g). These data have been corrected by subtraction of appropriate blank experiments and then fitted with nonlinear regression; data derived after analysis have been listed in Supplementary Table 1. The binding thermodynamics for these experiments are listed in the methods section.



(c)

	IC_{50}(μM)	K_m (μM)
dBLIP-1	186.34±6.52	412.48±12.36
dBLIP-2	152.80±8.46	403.18±8.78



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 $\mu g.mL^{-1}$, 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. Interestingly, 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.[10,13]

Table 1. Antibiotic sensitivity profile of clinical isolates. (R = Resistance; S= susceptible; and I = Intermediate). AMP: Ampicillin; AMX: Amoxicillin; CEP: Cephalothin; CZ: Cefazolin; CX: Cefoxitin; CAZ: Ceftazidime; CTR: Ceftriaxone; CTX: Cefotaxime; NA: Nalidixic acid; CIP: Ciprofloxacin; NX: Norfloxacin; OF: Ofloxacin; LE: Levofloxacin; DO: Doxycycline; C: Chloramphenicol; COT: Cotrimoxazole; GEN: Gentamicin; AK: Amikacin; IMP: Imipenem; NIT: Nitrofurantoin.

Isolates	AMP	AMX	CEP	CZ	CX	CAZ	CTR	CTX	NA	CIP	NX	OF	LE	DO	C	COT	GEN	AK	IMP	NIT
ID N^0 2101123 *E. coli*	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	S	S	S	S
ID N^0 6881 *E. coli*	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	R	R	R	R	S
ID N^0 1812446 *E. coli (blaKPC)*	R	-	R	-	-	R	R	R	-	R	-	-	R	-	-	R	R	S	R	R
ID N^0 6817 *P. aeruginosa*	R	R	R	R	R	R	R	R	R	R	R	R	S	R	S	R	S	S	R	R
ID N^0 7314 *S. aureus*	R	R	R	R	S	R	R	R	R	R	R	S	R	R	S	R	S	S	S	R
ID N^0 ATCC33591 *S. aureus*	R	R	-	R	-	-	R	-	-	S	S	-	S	-	-	S	S	-	R	S
ID N^0 6591 *B. cereus*	R	R	R	R	R	R	R	R	R	R	S	S	S	S	R	S	S	R	S	S

Table 2. Susceptibility of β-lactamase positive clinical bacterial isolates to different commercial antibiotics in the absence of dBLIPs. (a) and in the presence of dBLIP1 **(b)** and dBLIP2 **(c)**. Amoxycillin (AMX); ampicillin (AMP), cefotaxime (CTX) and gentamicin (GEN).

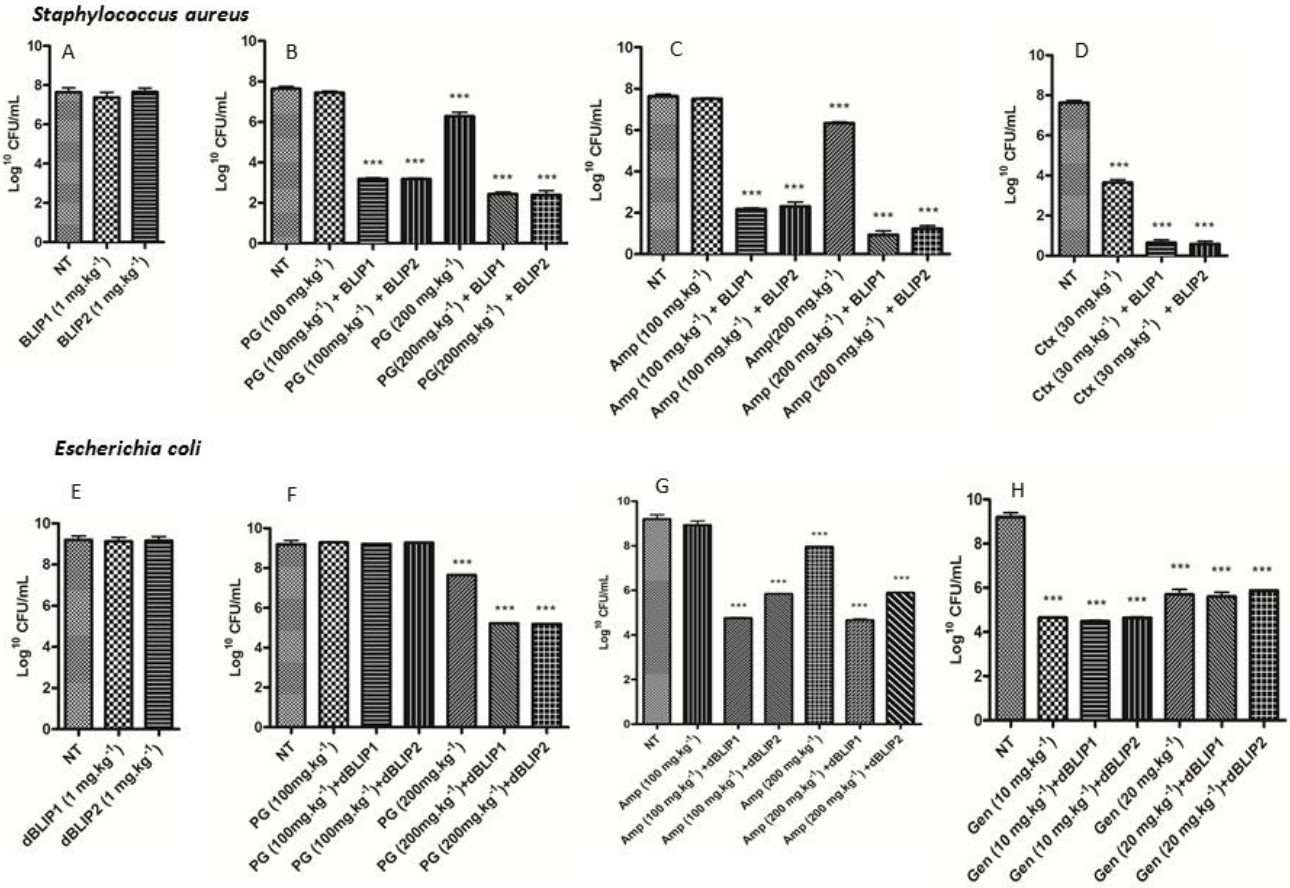
	MIC ($\mu g.mL^{-1}$)[a]				MIC ($\mu g.mL^{-1}$)[b]				MIC ($\mu g.mL^{-1}$)[c]			
Microorganisms	**AMX**	**AMP**	**CTX**	**GEN**	**AMX**	**AMP**	**CTX**	**GEN**	**AMX**	**AMP**	**CTX**	**GEN**
E. coli 6881	128	128	64	32	32	32	8	4	16	32	8	4
E. coli 2101123	256	>512	128	256	8	256	8	128	8	256	8	128
E. coli 1812446	256	>512	256	512	16	512	16	128	16	512	16	128
P. aeruginosa 6817	512	512	64	32	32	32	8	8	16	32	8	8
B. cereus 6591	256	256	64	32	8	16	8	4	8	8	8	4
S. aureus 7314	512	512	128	64	32	32	8	8	16	32	8	8
S. aureus ATCC33591	512	512	128	64	32	128	8	8	32	128	8	8
E. coli (TEM-1)	128	1024	32	32	32	128	4	4	32	128	4	4

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 (**Table 3**), the efficacy of such compounds was investigated in mouse models of bacteremia and sepsis caused by *Escherichia coli* and also *Staphylococcus aureus* clinical isolates with high resistance to different antibiotics. 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.

Table 3. *In vitro* evaluation of cytotoxic activity of dBLIPs in combination of 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.

	Cell type	
Treatments ($\mu g.mL^{-1}$)	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

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**) and gentamicin (GEM). Bars represent means and SDs from three to six independent experiments. Results are shown as mean ± SD from triplicate measurements. *p, 0.05; **p , 0.01;***p , 0.001; comparison by ANOVA with Tukey's post hoc test.



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 antibiotics 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* infection, penicillin and ampicillin alone (**Fig. 3b and 3c**) at a standard dose of 100 $mg.kg^{-1}$ led to a similar CFU to the untreated control. When the dose was increased to 200 $mg.kg^{-1}$ a lower reduction of CFU, of 10^8 to 10^6, was obtained when compared to untreated controls. Furthermore, treatment with commercial antibiotics associated with dBLIP-1 and -2 led to a CFU count decrease reaching ~ 10^8 to 10^2 when animals were treated with ampicillin and penicillin in combination with dBLIP-1 and -2.

In contrast, cefotaxime treatment led to an efficient reduction in CFU (**Fig. 3d**). However, when cefatoxime 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 did not showed any direct bactericidal effect as had been previously observed for *E. coli* (**Fig. 3e**). In this case, penicillin and ampicillin alone (**Figs. 3f and 3g**) at a standard dose of 100 $mg.kg^{-1}$ led to a similar CFU to the untreated control. When the dose was increased to 200 $mg.kg^{-1}$ a lower reduction in CFU was obtained when compared to untreated controls. 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.

Gentamicin 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 this 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 dBLIPs 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, dBLIPs 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, leading to a reduction in resistance and opening the market for novel products.

Methods

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

Peptide designing and molecular modelling

The rational design for synthetic peptides construction was mainly based on pocket volume that included distances between the amino acid residues that compose the β-lactamases conserved motif (KSG), Ser^{130} (adjacent nucleophilic amino acid) and several adjacent amino acid residues around of the catalytic triad (Lys^{73}, Ser^{130} and Glu^{166}). Firstly an isosceles trapezoid was designed inside of catalytic enzyme pocket. The distances encountered inside of this geometric form with dimensions of approximately 12 (major base) x 9 (minor base) x 13 (sides) Å in a total of

13 $Å^2$ (**Supplementary Fig. 3**). Peptides were designed with ideal lengths to fit and interact with catalytic triad and neighbour's amino acid residues localized around the catalytic site inside of trapezoid. The peptides were designed with less than five amino acid residues, flexible (presence of glycines) and soluble (presence of amino acid residues hydrophilic 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 utilized to rank the better peptides to be synthesized were the results of energy being the values below -4.0 $Kcal.mol^{-1}$ discarded. Five of seven peptides constructed and analysed were discarded being four them presented moderated or no activity toward β-lactamases (data not shown). dBLIP-1 and -2 presented values of -5.8 and -5.4 $Kcal.mol^{-1}$ toward β-lactamase from *E. coli*, respectively and values of -4.8 and -4.8 $Kcal.mol^{-1}$ toward β-lactamase from *S. aureus*, respectively. The three-dimensional models for dBLIP-1 and dBLIP-2 were constructed based on the structures of 2je8 and 3pxi (pdb code), which presented 100 % of identity for both peptides[18,19]. Fifty theoretical tridimensional peptide structures were constructed by using Modeller v.9.8[20,21] for each peptide. Final models were evaluated using PROCHECK for analysis of stereochemical quality. The peptide structures were visualized and investigated on Delano Scientific's PyMOL http://pymol.sourceforge.net/[22]. To calculate the grand average of hydropathicity, named GRAVY, ProtParam[23] was used to evaluate physical-chemical parameters for a given amino acid sequence.

***In silico* lactamase interactions**

All docking calculations were performed using the AUTODOCK 4.2 program. Docking simulations of both peptides (dBLIP-1 and dBLIP-2) were performed for two β-lactamases (EC 3.5.2.6), pdb code 1zg4 from *Escherichia coli* and pdb code 3blm from *Staphylococcus aureus*[24,25]. All hydrogen atoms were added by using the AutoDockTool[26]. Grid maps were calculated with 20 x 15 x 15 for both dBLIP-1 and -2 tested against *E. coli* enzyme; for the enzyme from *S. aureus* the grid was calculated with 35 x 35 x 15 for both dBLIP-1 and -2, and in all tests the spacing centre was 1.0 Å on the catalytic pocket of both enzymes. In order to understand the competitive *in vitro* inhibition observed, the simulation was optimized in a reduced region around the catalytic pocket. A Larmarckian genetic algorithm was used as the search method to find the best peptide–protein complex. Fifty docking runs were performed for each peptide, where the maximum freedom to side chains was unlocked due to length of the peptides. The generated structures were ranked in two steps: firstly a cluster with the best models with lowest free energy (below -4.0 kcal.mol^{-1}), and secondly with a root-mean-square deviation (RMSD), for all atoms docked in the serine proteinase catalytic pocket, showing tolerance of 4Å, as recommended for blind docking. The program PyMOL http://pymol.sourceforge.net/[22] was used to characterize peptide-protein interactions.

Solid phase synthesis of peptides

dBLIP-1 and dBLIP-2 were prepared by solid-phase peptide synthesis using HBTU activation procedure for Boc chemistry. Both dBLIP-1 and dBLIP-2

were synthesized from the UTMB peptide synthesis core facility (Galveston, USA) and Peptide 2.0 (USA) to authenticate the activity.

Isothermal Titration Calorimetry

To measure the binding affinity of β-lactamase with inhibitor peptides (dBLIP-1 and dBLIP-2), isothermal titration calorimetry (ITC) was performed using iTC200 Systems (GE Healthcare, USA) coupled with non-reactive Hastelloy® cells for chemical resistance. All pure samples were dissolved against phosphate buffer (pH 7.5). The same buffer degassed prior to titration, and experiments were performed at 25°C. Isothermal interactions between proteins and peptides were measured by titrating over 20 injections using 40 µL peptide solution (1 mM) and proteins in sample cell with a concentration of 5 µM of 200 µL. Experiments were repeated three times.

Identification of different β-lactamase classes among clinical isolates

The identification of different β-lactamase classes present in clinical isolates were done following the method described by Upadhyay and cooworkers (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, cefotaxime (30µg) and cefoxitin (30µg) disks were placed 20 mm apart from centre to centre. Isolates showing blunting of the cefotaxime zone of inhibition adjacent to the cefoxitin 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 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), positive strain was determined if the increase in inhibition zone with imipenem and EDTA disk was ≥ 7 mm, 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 $\mu g.mL^{-1}$ 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 × [(c-r)/c], where *c* is the activity in control samples incubated without inhibitor and *r* is the remaining activity in samples incubated with 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 10.0 [27].

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 clinically isolated bacterial strains were determined by using Kirby-Bauer disk diffusion method in accordance to CLSI guidelines[28]. All the strains were isolated from patients associated with keratitis, and species identifications were confirmed by Vitek 2 system (bioMérieux, France). Bacteria were cultured in Mueller Hinton Broth (Himedia, India) at 37°C.

Chromogenic nitrocefin β-lactamase assays in clinical isolates

Detection of β-lactamase in the clinical isolates was determined by using nitrocefin assays[29] at different time intervals (1, 10, and 30 min).

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentrations of selected antibiotics (amoxicillin, ampicillin, cefotaxime, and gentamicin) and in combination with 128 $\mu g.mL^{-1}$ 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 [28]. The concentrations of each antibiotic used a range from 0.5 to 1024 $\mu g.mL^{-1}$. 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% CO^2 at 37 ºC[29].

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 further evaluated by 3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazoliumbromide (MTT; Sigma) (5 $mg.mL^{-1}$ in phosphate buffered saline (PBS)) in three replicates by using RAW 264.7 cells. The results were expressed as the percentage of each sample compared to the negative control (PBS buffer, pH 7.4) and positive control [mastoparan-L (1-100 $\mu g.mL^{-1}$)] [29-32].

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 mastoparano-L (1-100 $\mu g.mL^{-1}$)[33].

Animals

C57BL/6 mice weighing 18 to 22 g were used in this study. Animals were provided by the Central Biotery belonging to USP Campus Hall in Ribeirão Preto. Animals were housed in separate cages in 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 CO^2 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) 2×10^5 $CFU.mL^{-1}$, previously cultured as described by Steinstraesser and co-workers[34]. 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 of 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 $CFU.mL^{-1}$ [34,35].

Murine non-lethal *Escherichia coli* infection model

Non-lethal bacteremia was produced in C57BL/6 mice with an i.p. inoculation of 200 µl of bacterial suspension containing 2.5 × 108 CFU of *E. coli* 1812446 clinical strain. One hour after the *E. coli* 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 gentamicin (10 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 Mueller Hinton Agar (Himedia, India) plates. The plates were incubated overnight at 37 °C, and the bacterial colonies were counted on the following day. The bacterial limit count detected by this method was 100 CFU.ml^{-1} [36].

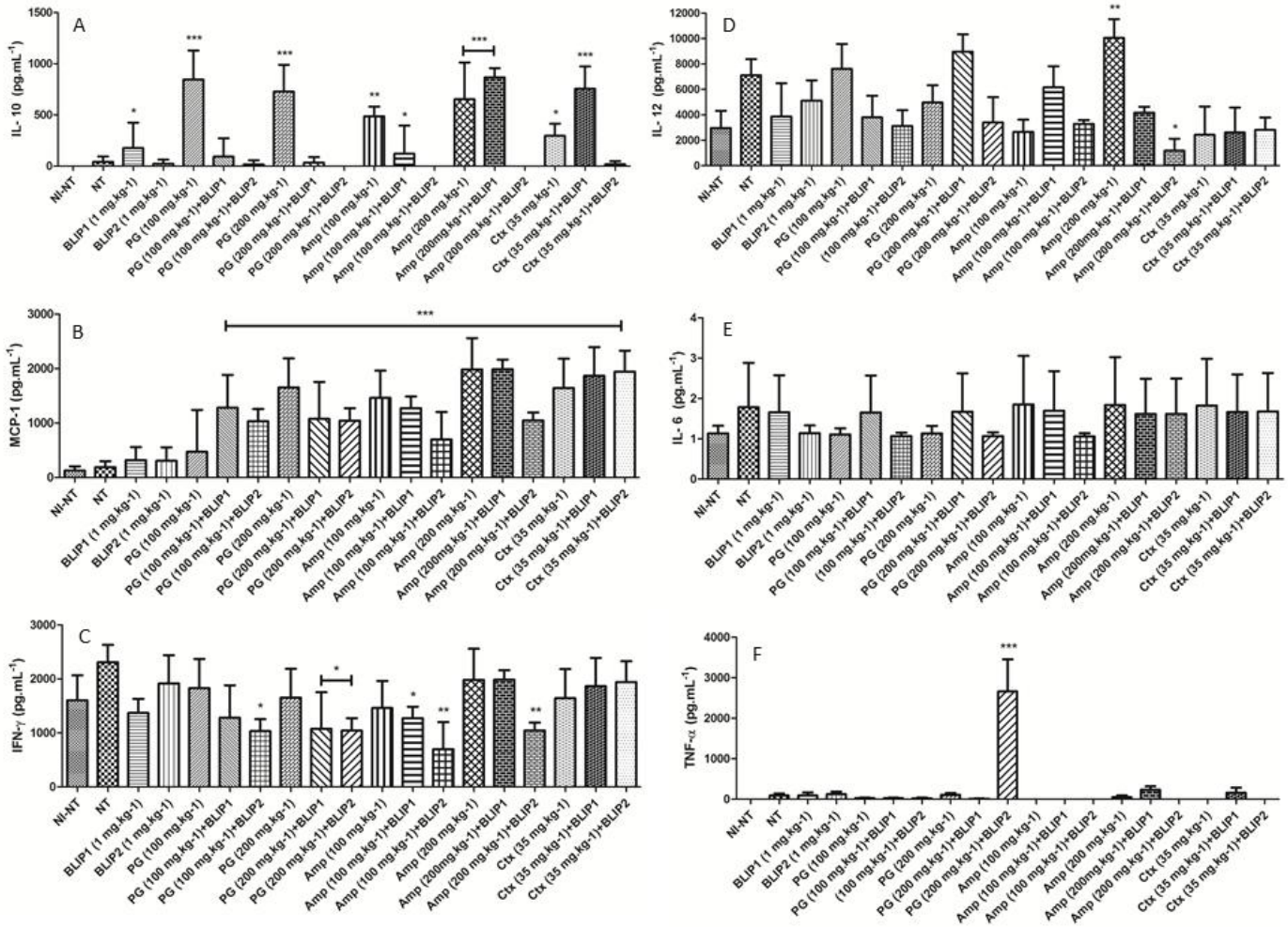
Cytokine assay

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.

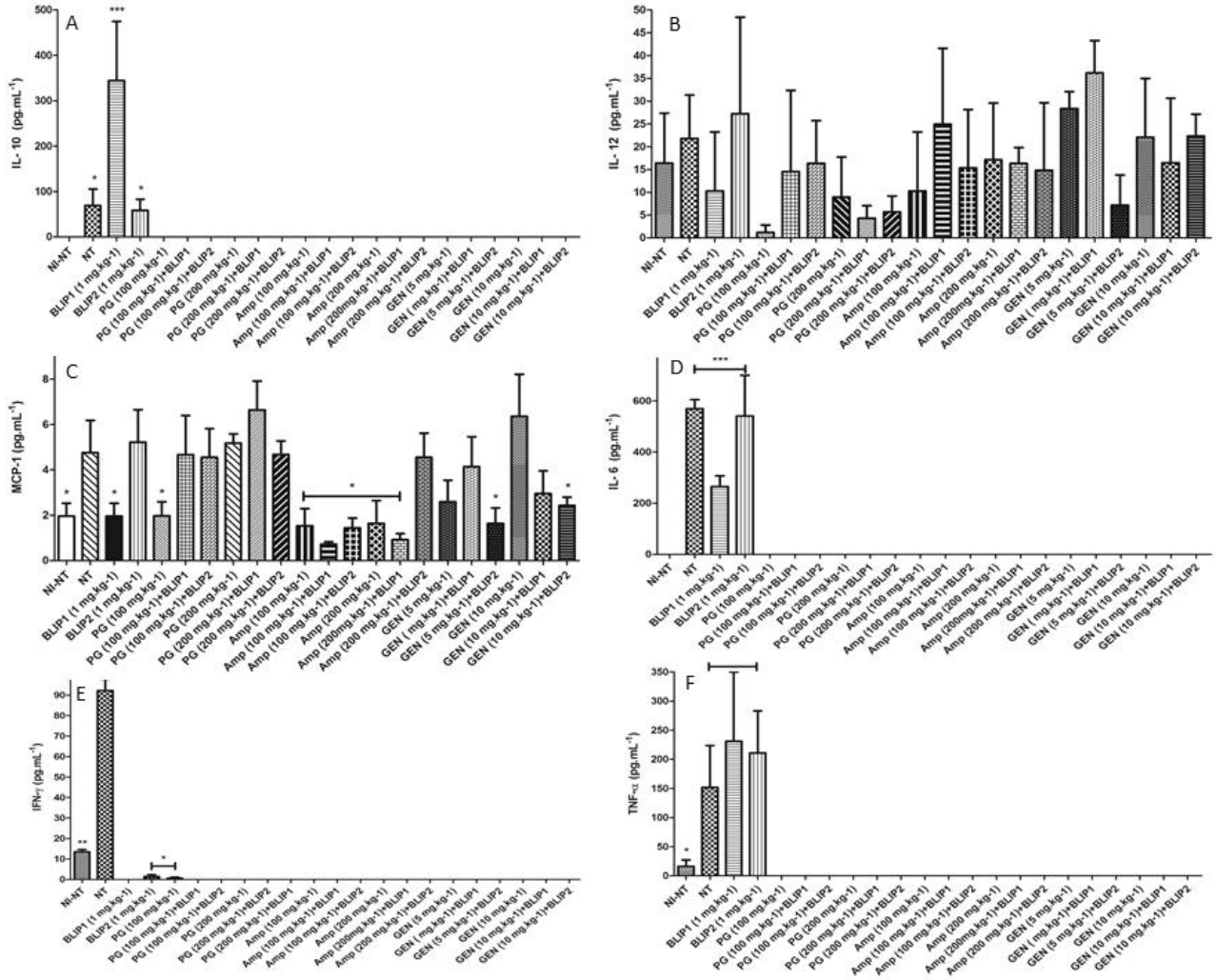
Statistical analysis

Data are presented as mean ± SD of all samples. Statistical significance of fatality rates between different groups was performed by Kaplan–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.

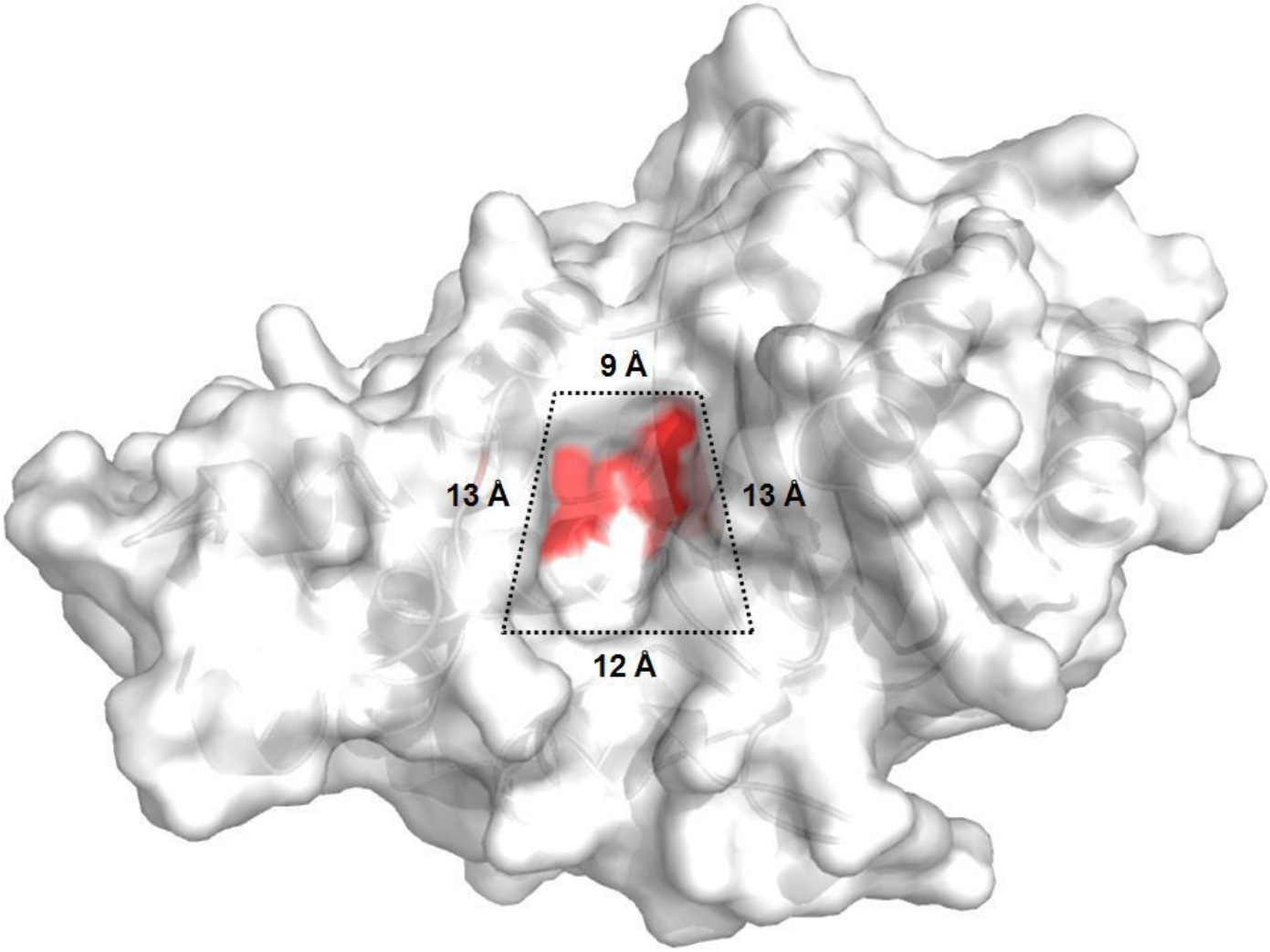
Supplementary Figure 1. dBLIP-1 and dBLIP-2 effects on immune response of mice *in vivo* model under *Staphylococcus aureus* infection. Determination of IL-10 (**A**), MCP-1 (**B**), IFN-g (**C**), IL-12 (**D**), IL-6 (**E**) and TNF-α (**F**) in *S. aureus* groups treated with dBLIP 1 and 2 alone or in combination with (PG) penicillin, (AMP) ampicillin and (CFX) cefotaxime. Bars represent means and SEs from three to six independent experiments. Results are shown as mean ± SD from triplicate measurements. *p, 0.05; **p , 0.01;***p , 0.001; comparison by ANOVA with Tukey's post hoc test.



Supplementary Figure 2. dBLIP-1 and dBLIP-2 effects on immune response of mice *in vivo* model under *Escherichia coli* infection. Determination of IL-10 (**A**), MCP-1 (**B**), IFN-g (**C**), IL-12 (**D**), IL-6 (**E**) and TNF-α (**F**) in *E. coli* groups treated with dBLIP 1 and 2 alone or in combination with (PG) penicillin, (AMP) ampicillin and (GEN) gentamicine. Bars represent means and SD from three to six independent experiments. Results are shown as mean ± SD from triplicate measurements. *p, 0.05; **p , 0.01;***p, 0.001; comparison by ANOVA with Tukey's post hoc test.



Supplementary Figure 3. Three-dimensional structure of *E. coli* β-lactamase firstly utilized for peptide designe. The trapezoid highlight (dotted lines) represents the catalytic pocket area (red region) and neighbor regions used for docking analysis.



Supplementary Table 2. Data derived after fitting the raw heat associated data with nonlinear regression.

Parameters	dBLIP-1	dBLIP-2
K	$6.33 \pm 0.52E^{4}\ M^{-1}$	$4.5 \pm 0.18E^{4}\ M^{-1}$
ΔH	$-12.9 \pm 0.56E^{4}$ cal.mol^{-1}	$-35.7 \pm 0.82E^{3}$ cal.mol^{-1}
ΔS	-415 cal/mol/deg	-98.6 cal/mol/deg

Supplementary Table 3. Susceptibility of β-lactamase positive clinical bacterial isolates to different commercial antibiotics in the absence of dBLIPs (a) and in the presence of dBLIP1 (b) and dBLIP2 (c). Amoxycillin (AMX); ampicillin (AMP), cefotaxime (CFT) and gentamicin (GEN).

Clinical isolates	Beta-lactamases	
ID No 2101123 *E. coli*	AmpC	ESBL
ID No 6881 *E. coli*	AmpC	MBL
ID No 1812446 *E. coli (blaKPC)*	AmpC	MBL
ID No 6817 *P. aeruginosa*	AmpC	ESBL
ID No 7314 *S. aureus*	AmpC	ESBL
ID No ATCC33591 *S. aureus*	Ampc	TEM-1 like β-lactamase
ID No 6591 *B. cereus*	AmpC	ESBL

Supplementary Table 4. Peptides rationally designed evaluated against β-lactamases. Assays were *in vitro* performed toward β-lactamases from *E. coli* and *S. aureus* showing the presence (+)or absence of inhibition activity (-).

Peptides	Sequence	Activity
dBLIP-1	KKGEE	+
dBLIP-2	KQGQE	+
dBLIP-3	KNGNE	-
dBLIP-4	KNPNE	-
dBLIP-5	KQPQE	-
dBLIP-6	KGPGE	-
dBLIP-7	KGPAE	-

Supporting Information Available

The data of immunomodulatory response as well the calorimetric assays could be found in supporting information. This material is available free of charge via the Internet at http://pubs.acs.org.

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Authors' 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.F.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.

Competing financial interests

The authors declare no competing financial interests.

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8 DISCUSSÃO

Os antibióticos indubitavelmente têm melhorado a expectativa de vida humana. No entanto, a resistência a múltiplos antibióticos tornou-se comum, sugerindo que estamos à beira de voltar para a era pré-antibiótica. Neste sentido, a necessidade de novos agentes antibacterianos tem sido reconhecida como uma das principais necessidades médicas contemporâneas para enfrentar com sucesso a resistência bacteriana.[255]

Neste cenário os PAMs são considerados moléculas promissoras no desenvolvimento de novos antibióticos. A descoberta de PAMs remonta a 1939, quando Dubos[256, 257] extraiu um agente antimicrobiano isolado de *Bacillus brevis*. O extrato deste microrganismo protegeu camundongos da infecção por pneumococos ou estreptococos.[258] O fracionamento deste extrato identificou um PAM denominado gramicidina.[258] A injeção intraperitoneal da gramicidina apresentou efeitos tóxicos aos animais. Em 1941, um outro PAM conhecido como tirocidina foi isolado, e verificou-se que o mesmo podia ser eficaz contra bactérias Gram-negativas e positivas.[259] No entanto, a tirocidina exibiu toxicidade para eritrócitos humano.[260]

Os PAMs são conhecidos há muitos anos, mas pouquíssimos são utilizados na prática médica, pois embora alguns PAMs apresentem significativa atividade *in vitro*, alguns peptídeos perdem esta atividade sob condições fisiológicas, além de apresentar alta citotoxicidade.[261, 262] No presente estudo mostramos que o peptídeo clavanina A é ativo em altas concentrações de sal e em pH ácido, apresentando um amplo espectro de atividade contra bactérias Gram-positivas, incluindo MRSA, bactérias Gram-negativas e fungos.[130, 176]

No presente estudo, demonstramos que a clavanina A exibe uma ampla atividade antibacteriana *in* vitro[130, 176], bem como, esse peptídeo não apresenta nenhuma atividade citotóxica contra células de mamíferos, e em ensaios de toxicidade aguda nenhuma reação adversa foi observada. A clavanina A possui uma potente atividade, atuando na cura de feridas *in* vivo, onde eliminou as unidades formadoras de colônias de *S. aureus* ATCC29213

em um modelo de infecção de ferida experimental. Em um estudo realizado por Malmsten *et al.*[196], foi comprovada a eficácia de um peptídeo derivado de uma sequência rica em arginina, leucina e prolina (concentração utilizada 1000 μM), em um modelo *ex vivo* de ferida cirúrgica em pele de suínos infectada com *S. aureus* ATCC29213 e *P. aeruginosa* 15159 (isolado clínico de ulcera crônica). No presente estudo, com 75 μM de clavanina A eliminou-se 99% das bactérias, demonstrando dados similares aos de Malmsten *et al*.[196], e com uma potência treze vezes maior. Em outro estudo, a administração tópica do PAM halocidina HG1 mostrou clara atividade antimicrobiana em um modelo de ferida cirúrgica infectada com MRSA, em camundongos.[197]

Recentemente, um lipopeptídeo cíclico obtido da fermentação do *Streptomyces pristinaspiralis* foi aprovado para uso clínico no Brasil, a daptomicina, cuja principal indicação clínica é no conjunto de infecções, principalmente tópicas, causadas por estafilococos resistentes à oxacilina e os enterococos. Além disso, também mostra-se potente contra bactérias resistentes à vancomicina e linezolida.[263, 264] Apesar de apresentar atividade *in vitro* contra pneumococo, a daptomicina pode ser inativada pelo surfactante pulmonar, não podendo dessa maneira ser utilizada no tratamento de pneumonia.[263, 264]

A destruição da barreira mecânica do corpo durante uma infecção da pele pode aumentar a susceptibilidade do doente à uma infecção bacteriana sistêmica.[265] No presente estudo foi demonstrado que animais infectados sistemicamente com *E. coli* ATCC8739 e *S. aureus* ATCC29213, e tratados com 10 $mg.kg^{-1}$ de clavanina A apresentaram uma sobrevida de 80 % após 7 dias de tratamento. Tendo em vista o aumento no relato de cepas bacterianas resistentes aos antibióticos disponíveis no mercado, os achados aqui descritos são de grande importância, uma vez que os PAMs voltaram a ser objeto de estudo das companhias farmacêuticas, sendo que vários PAMs encontram-se em fase desenvolvimento.[266, 267] Até o momento, cerca de 100 peptídeos estão no mercado, movimentando cerca de US$6 bilhões de dólares ao ano.[268, 269]

Os achados positivos no presente trabalho mostram que a clavanina A apresenta um grande potencial para o tratamento de infecções bacterianas

sistêmicas. Os dados aqui apresentados, sugerem que a proteção dos camundongos foi devido à eliminação das bactérias. Estudos prévios mostraram que PAMs carregados positivamente podem ligar-se ao LPS, neutralizando a resposta inflamatória mediada por LPS (isto é, a produção de citocinas).[270, 271] Em nosso estudo foi demonstrado que os camundongos infectados por *E. coli* e tratados com clavanin A, apresentaram redução de citocinas pró-inflamatórias (Cap. I Fig. 5A – 5D). Esses achados levantam duas hipóteses, (I) a clavanina A neutraliza o LPS liberado das bactérias mortas, e assim reduz a liberação de citocinas pró-inflamatórias, (II) no mecanismo de ação proposto para a clavanina A em pH ligeiramente ácido, a morte das bactérias ocorre sem o rompimento da membrana citoplasmática,[131] o que explicaria a redução nas UFC 24 horas após a infecção (Cap. I Fig. Suplementar 2), acompanhada de uma redução nas citocinas pró-inflamatórias.

Os achados *in vitro* e *in vivo* sobre a clavanina A indicam que esse peptídeo pode servir de molde para o desenho racional de uma terapia eficaz à base de peptídeos para o tratamento de feridas infectas e infecções bacterianas sistêmicas, entretanto, a utilização do mesmo como produto seria inviável, atualmente, uma vez que a atividade apresentada pelo mesmo é próxima à apresentada pelos antibióticos convencionais. Deste modo, a viabilidade econômica para a comercialização deste PAM tem sido inviável, uma vez que o custo de produção é alto, quando comparado aos antibióticos convencionais (a tonelada de penicilina custa aproximadamente R$240.000,00 – duzentos e quarenta mil reais – enquanto, 1 grama de clavanina A custa aproximadamente R$100.000,00 – cem mil reais).[272]

Afim de reduzir os custos referentes à produção dos PAMs, desde os anos 1990, vários métodos de desenho racional de PAMs tem sido desenvolvidos, a fim de gerar análogos com atividade melhorada, reduzindo assim as limitações e aumentando as vantagens quanto ao uso dos PAMs.[153, 249, 273-281] O desenho racional de PAMs consiste em uma abordagem moderna para o desenvolvimento de antibióticos, e caracterização detalhada do alvo, um vez que antibióticos que tenham como alvo especifico o patógeno e que não interajam com o hospedeiro são de extrema importância, a fim de reduzir ou

eliminar o efeitos adversos, tendo como base o princípio da toxicidade seletiva.[152] O principal obstáculo quanto à utilização comercial dos PAMs como mencionado outrora, é sua citotoxicidade para as células de mamífero. No entanto, para que sejam utilizados comercialmente, é de extrema importância dissociar a toxicidade para as células de mamífero da atividade antimicrobiana. Esta redução pode ser alcançada através do aumento da atividade antimicrobiana ou redução da atividade hemolítica, o ideal seria ambos.[152] O desenho racional de PAMs tem sido utilizado afim de revolver estes problemas, através de substituições de aminoácidos, incluindo a substituição de L-aminoácidos por D-aminoácidos, e identificação de padrões de aminoácidos responsáveis pela atividade antimicrobiana. Estas substituições podem promover alterações na anfipaticidade/hidrofobicidade, levando a uma redução da citotoxicidade dos peptídeos para células de mamífero, sem alterar a atividade antimicrobiana, além de deixar os PAMs menos susceptíveis à degradação proteolítica.[152, 282]

No presente estudo, criamos dois análogos dos PAMs clavanina A e mastoparano-L , através da adição de cinco resíduos de aminoácidos apolares (FLPII) na região C-terminal dos referidos peptídeos, com base na ideia de que a composição de aminoácidos desempenham um papel importante na desestabilização da bicamada lipídica. A adição dessa *tag,* reduziu significativamente a toxicidade do análogo do mastoparano-L (mastoparano-MO) em mais de dez vezes, mantendo entretanto a notável atividade antibacteriana *in vitro* e *in vivo* de amplo espectro. Adicionalmente, foram eliminadas bactérias resistentes a antibióticos como *E. coli* KPC positiva e multirresistente, *K. pneumoniae* KPC positiva além de MRSA, tanto em ensaios *in vitro* como *in vivo*. Sendo necessário observar que, o mastoparano-MO apresentou uma importante atividade antibacteriana *in vivo*, protegendo 80% dos animais infectados com *E. coli* ATCC8739, *E. coli* 1812446 (KPC positiva), *S. aureus* MRSA ATCC33591 e 100 % dos animais infectados com *S. aureus* ATCC29213 (Cap. II, Figs. D, H, L e P respectivamente). Enquanto que o mastoparano-L protegeu até 80% dos animais infectados com *S. aureus* ATCC29213 quando administrado na dose de 5 $mg.kg^{-1}$ mas, quando

administrado na dose de 10 $mg.kg^{-1}$ promoveu a morte de todos os animais em decorrência da sua forte atividade toxicológica (cap. II Tabela Suplementar 1). Além disso, o mastoparano-MO apresentou uma atividade melhorada frente aos biofilmes bacterianos, eliminando completamente biofilmes maduros com concentrações 4 vezes menores (8 µM para a mastoparano-MO, enquanto o apresentado pelo mastoparano-L foi 32 µM) (Cap. II, Fig. 1).

A adição da *tag* na sequência primária da clavanina A, não potencializou a atividade antibacteriana *in vitro* (sobre células planctônicas) e *in vivo*, uma vez que a clavanina MO apresentou o mesmo perfil de atividade da clavanina A, com CIM igual ou muito próxima à da sequência molde (Cap. II Tabela 2) e a sobrevida foi semelhante ou muito próxima à apresentada pelos animais tratados com a clavanina A (Cap. II, Figs. 2 B, F, J e N). Entretanto, a clavanin MO apresentou uma atividade 50% melhor em relação a sequência molde na eliminação de biofilmes maduros (Cap. II Fig. 1).

A grande diversidade estrutural das PAMs torna difícil compreender a relação estrutura-atividade para projetar PAMs competitivos. Numa tentativa de melhorar a atividade antimicrobiana de um peptídeo, várias estratégias de otimização da sequência primária têm sido adotadas, como por exemplo a ciclização,[283] aumento da carga positiva ou hidrofobicidade.[284] Em estudos recentes, os pesquisadores investigaram o efeito de diferentes resíduos de aminoácidos na atividade antibacteriana e seletividade, sendo que, em um desses estudos, foi observado que a substituição de arginina por lisina aumentou a atividade antibacteriana e reduziu a atividade hemolítica do peptídeo tritrpticina,[285] apesar desses dois resíduos serem carregados positivamente em ambiente neutro a carga total da arginina é ligeiramente mais elevada (174,2) que da lisina (146,19). Em outros estudos foi observado que os resíduos de triptofano estão envolvidos na atividade hemolítica em vários PAMs. A substituição do triptofano por fenilalanina na sequência primária da indolicidina[286] e triptofano por leucina na sequência primária da melitina[287] reduziu significativamente a atividade hemolítica, mas manteve a atividade antibacteriana dos mesmos.

Vários trabalhos que estudam a relação estrutura-função de PAMs todos os resultados sugerem que a atividade de AMP é determinada por uma combinação sutil de fatores tais como sequência, a hidrofobicidade e a posição dos resíduos de aminoácidos.[284, 288-291]. No entanto, apesar de estudos extensivos, pouco se sabe sobre a base molecular subjacente a seletividade de PAMs para atacar células bacterianas em vez de células de mamíferos. Uma indicação geral parece impossível de ser obtida, devido à complexidade do alvo e o mecanismo de ação envolvido.

No presente estudo a modificação incremental afetou ligeiramente a hidofobicidade dos peptídeos mastoparano-L (0,05), mastoparano-MO (0,17), clavanina A (0,15) e clavanina-MO (0,21), esses dados sugerem que uma hidrofobicidade mais baixa parece estar relacionada com a atividade citotóxica dos peptídeos, tendo em vista que a clavanina A e clavanina-MO apresentam valores próximos, não foram observadas diferenças significativas na toxicidade desses peptídeos (Cap. II Tabela 2).

Os dados aqui apresentados indicam um grande potencial antibacteriano *in vitro* e *in vivo* da clavanina-MO e mastoparano-MO, sendo moléculas efetivas inclusive na eliminação de biofilmes maduros. Entretanto ambos os peptídeos apresentam uma sequência primária relativamente longa (clavanina-MO 28 e mastoparano-MO 19 resíduos de aminoácidos). Esta propriedade inviabiliza a síntese química dessas moléculas, uma vez que o ideal seriam sequências com no máximo 10 resíduos. A redução do comprimento de um peptídeo sem afetar a atividade é uma característica altamente desejada, uma vez que diminui o custo de produção.[274] Além disso, a clavanina-MO e o mastoparano-MO, como a maioria dos antibióticos em uso clínico, tem amplo espectro de atividade. Embora tal atividade seja vantajosa devido ao fato de serem utilizadas para o tratamento de infecções bacterianas sem diagnóstico preciso, eles também acabam eliminando bactérias comensais, assim, podem perturbar a flora normal e criar numerosos efeitos secundários adversos. Muitas destas complicações pós-antibioticoterapia poderiam ser evitadas através do uso de agentes antimicrobianos específicos.[292]

Na última década, nosso grupo vem trabalhando no desenvolvimento de compostos antibacterianos alvo especifico com sequência primária inferior a dez resíduos de aminoácidos, através do desenho racional de peptídeos. Os estudos iniciais levaram a criação de dois inibidores de β-lactamase (dBLIP-1 e dBLIP-2). As infecções causadas por bactérias resistentes a antibióticos foram listadas como doenças principais prioritárias pela Organização Mundial de Saúde (OMS) em termos de ameaça à saúde pública global. Sendo que o mecanismo mais comum de resistência bacteriana aos antibióticos é a produção de β-lactamases, uma vez que os antibióticos β-lactâmicos, como as penicilinas e cefalosporinas estão entre os agentes antimicrobianos mais utilizados.[62, 63] Um método alternativo para combater a resistência mediada por β-lactamase foi a utilização de inibidores β-lactamase – ácido clavulânico e sulbactam – sendo o ácido clavulânico o mais utilizado,[60] entretanto, já foram relatadas cepas resistentes a esses inibidores,[60, 293-298] existindo a necessidade de desenvolver novos inibidores.

Os inibidores de β-lactamases concebidos no presente estudo foram testados com sucesso em ensaios *in vitro* e *in vivo*, inibindo β-lactamases das classes A e C, quando utilizados em combinação com antibióticos β-lactâmicos. Quando testados isoladamente esses peptídeos não apresentaram qualquer atividade antibacteriana, além disso, a dosagem de citocinas do soro de animais tratados com os dBLIPs sugere que esses peptídeos não causam efeitos colaterais, e que a atividade deletéria só está relacionada com a inibição das β-lactamases. Os dBLIP parecem ser uma ferramenta poderosa para superar infecções bacterianas resistentes, uma ameaça crescente e difundida para a saúde pública em vários países. Eles podem ajudar, a ressuscitar os inibidores de β-lactamase,[299] podendo ser úteis como aditivos para antibióticos comerciais. Trabalhos anteriores relataram o desenho de peptídeos com atividade semelhante,[169, 300, 301] entretanto, os dBLIPs são os menores peptídeos inibidores de β-lactamases descritos até o presente, tornando-os notáveis candidatos a chegar ao competitivo mercado farmacêutico, tendo em vista o seu pequeno tamanho.

O presente trabalho trouxe achados importantes que lançam luz na luta contra as bactérias multirresistentes, uma vez que, desenvolvemos dois peptídeos (clavanina-MO e mastoparano-MO) altamente potentes na eliminação de biofilmes bacterianos maduros que apesar de sua sequência longa para a síntese química, poderiam ser facilmente obtidos através da expressão heteróloga, ao mesmo tempo criamos dois peptídeos pequenos (cinco resíduos de aminoácidos cada) que se mostraram altamente eficazes na eliminação de bactérias produtoras de β-lactamases, quando utilizados em combinação com antibióticos β-lactâmicos.

9 CONSIDERAÇÕES FINAIS

Os achados do presente estudo mostraram que:

- ✓ A clavanina A mostrou ser eficiente na eliminação de *S. aureus* em um modelo de infecção de ferida e impediu o início da sepse e, assim, aumentou a sobrevida dos camundongos infectados em um modelo de infecção bacteriana sistêmica;
- ✓ A modificação incremental na sequência da clavanina A alterou moderadamente a atividade *in vitro* e *in vivo* em comparação com a sequência molde, exceto na eliminação do biofilme bacteriano *in vitro* onde a clavanina-MO foi quatro vezes melhor que a clavanina A;
- ✓ O mastoparano MO teve uma redução em mais de dez vezes na atividade citotóxica quando comparado com o mastoparano-L, mantendo contudo a atividade antibacteriana com CIM comparáveis aos do grupo controle;
- ✓ O mastoparano-MO nos ensaios *in vivo*, promoveu a sobrevida de 80% dos animais infectados com bactérias resistentes (MRSA e *E. coli* KPC positiva);
- ✓ O mastoparano-MO eliminou eficientemente biofilmes bacterianos maduros, sendo oito vezes mais efetivo que a sequência molde;
- ✓ Os dois inibidores (dBLIPs 1 e 2) – criados através do desenho racional de peptídeos – em combinação com antibióticos convencionais foram eficazes na eliminação de *E. coli* e *S. aureus* que expressam β-lactamases em um modelo murino de infecção sistêmica.

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ANEXO I

LISTA DE ARTIGOS NÃO INCLUSOS NA TESE

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