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ARIANE TEIXEIRA DOS SANTOS

PEPTÍDEOS MODIFICADOS DERIVADOS DA CROTALICIDINA E CROTAMINA DO VENENO DE CASCAVEL E DO CNIDÁRIO MARINHO: ATIVIDADES E FARMACOLÓGICAS RECENTES

FORTALEZA-CE
2023

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Tese submetida à Coordenação do Programa de Pós-Graduação em Ciências Farmacêuticas, da Universidade Federal do Ceará como requisito parcial para obtenção do título de Doutor em Ciências Farmacêutica

Orientador: Prof. Dr. Gandhi Rádis Baptista

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Orientador: Prof. Dr. Gandhi Rádis Baptista

Aprovada em: 09 de agosto de 2023

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Até parece que foi ontem
Minha mocidade
Com diploma de sofrer
De outra Universidade
Minha fala nordestina
Quero esquecer o francês

RESUMO

A biotecnologia desempenha um papel importante na descoberta de novos e promissores alvos farmacológicos e isso tem permitido a descoberta de peptídeos bioativos. Os peptídeos de veneno têm ganhado destaque nos últimos anos devido à sua seletividade e capacidade de atuar em alvos específicos. Esta tese abrange dois focos distintos de pesquisa, nas quais peptídeos derivados do veneno de um cnidário marinho e de uma cascavel, foram investigados para aplicação em tecnológica de embriões e em neurofisiologia de canais iônicos. A primeira parte da tese avaliou o potencial dos peptídeos sintéticos derivados do transcriptoma de um cnidário Chi, PPA, SSB e análogos de peptídeos de serpente (SFR-Ctn[1-9], SFR-Ctn[16-24] e NRTP1) em relação à suas atividades sobre canais iônicos, citoproteção e padrões eletrofisiológicos. O objetivo foi investigar a modulação dos peptídeos sobre os canais iônicos e seu papel cito- e neuroprotetor. Por meio de técnicas eletrofisiológicas e reversão ou impedimento de citotoxicidade in vitro, os peptídeos exibiram uma importante capacidade de bloquear os canais iônicos, particularmente os canais para íons sódio e cálcio, solidado por análises *in silico* de ancoramento molecular. Ademais, foram observadas propriedades citoprotetoras e antioxidantes em células neuroblásticas e neuroendocrinas. A segunda parte se concentrou na investigação da ativação partenogenética por meio do peptídeo derivado da crotalicidina, o RhoB-Ctn[1-9]. O objetivo foi avaliar o potencial desse peptídeo modificado de induzir a partenogênese in vitro e sua capacidade de ativar embriões bovinos. Os resultados revelaram que o peptídeo RhoB-Ctn[1-9] possui uma capacidade de indução partenogenética, clivagem e a formação de blastocistos com eficiência. Utilizando a técnica de microscopia de fluorescência Lightsheet, foi visualizado a capacidade dos peptídeos em atravessar a zona pelúcida, a internalização e acumulação do RhoB-Ctn[1-9] no citoplasma das células em divisão, corroborando com os dados *in silico* via ancoramento molecular. Verificou-se que peptídeos de veneno estruturalmente modificados podem além de ter ações terapêuticas diretas, atuar como componentes adjuvantes em área diversas como na fertilização *in vitro* e modulação neurofisiológica. Essas descobertas fornecem exemplos e subsídios importantes para a biotecnologia, com implicações promissoras para futuras aplicações terapêuticas e desenvolvimento de estratégias inovadoras para estudos moleculares e celulares.

Palavras-chave: Peptídeos, Partenogênese, Eletrofisiologia, Neuroproteção.

ABSTRACT

Biotecnology plays an important role in the discovery of new and promising pharmacological targets, allowing for the identification of bioactive peptides. Venom peptides have gained prominence in recent years due to their selectivity and ability to target specific receptors. This thesis covers two distinct research focuses in which peptides derived from the venom of a marine cnidarian and a rattlesnake were investigated for their applications in embryo technology and ion channel neurophysiology. The first part of the thesis assessed the potential of synthetic peptides derived from the transcriptome of a cnidarian (Chi, PPA, SSB) and snake venom peptide analogs (SFR-Ctn[1-9], SFR-Ctn[16-24], and NRTP1) concerning their activities on ion channels, cytoprotection, and electrophysiological patterns. The objective was to investigate the modulation of peptides on ion channels and their cyto - and neuroprotective roles. Through electrophysiological techniques and in vitro cytotoxicity reversal or prevention, the peptides exhibited a significant ability to block ion channels, particularly sodium and calcium ion channels, supported by in silico molecular docking analyses. Additionally, cytoprotective and antioxidant properties were observed in neuroblast and neuroendocrine cells. The second part focused on investigating parthenogenetic activation using the peptide derived from crotalicidin, RhoB-Ctn[1-9]. The aim was to evaluate the potential of this modified peptide to induce in vitro parthenogenesis and activate bovine embryos. The results revealed that the RhoB-Ctn[1-9] peptide has the ability to induce parthenogenesis, cleavage, and blastocyst formation efficiently. Using Lightsheet fluorescence microscopy, the peptides' ability to penetrate the zona pellucida, internalize, and accumulate in the cytoplasm of dividing cells was visualized, confirming the *in silico* data via molecular docking. It was found that structurally modified venom peptides can not only have direct therapeutic actions but also act as adjunct components in various areas such as in vitro fertilization and neurophysiological modulation. These findings provide important examples and insights for biotechnology, with promising implications for future therapeutic applications and the development of innovative strategies for molecular and cellular studies.

Keywords: Peptides, Parthenogenesis, Electrophysiology, Neuroprotection.

LISTA DE ABREVIATURAS E SIGLAS

AMPs – peptídeos antimicrobianos

CPP – peptídeo de penetração celular

CRAMPs – peptídeos antimicrobianos relacionados à catelicidina

Ctn – Crotalicidina

Ctn [1-9] – Crotalicidina-[1-9]

Ctn [15-34] – Crotalicidina-[15-34]

IGF1– fator de crescimento semelhante à insulina 1

IL-10 – Interleucina 10

IL-6 – Interleucina 6

kDa – quilodaltons

NrTP1 – *Nucleolar targeting Peptides* 1

RhoB-Ctn[1-9] – Rodamina B – Crotalicidina [1-9]

SFR-Ctn[1-9] - Sulforodamina-Crotalicidina[1-9]

SFR-Ctn[16-24] – Sulforodamina – Crotalicidina [16-24]

TNF-α – fator de necrose tumoral alfa

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1. INTRODUÇÃO1

A biotecnologia tem impulsionado descobertas significativas de novos alvos terapêuticos e potenciais fármacos. O avanço das técnicas empregadas tem facilitado a identificação, a análise e o desenvolvimento de substâncias até então desconhecidas, que se mostraram promissoras como alvos farmacológicos (CHIEN et al., 2019). O emprego de sistemas automatizados como genômica, proteômica ou transcritômica permite analisar detalhadamente genes e proteínas de compostos altamente complexos, como os peptídeos bioativos naturais (ZHANG et al., 20222). Nesse contexto, o emprego da biotecnologia na indústria farmacêutica facilitou o desenvolvimento de métodos mais otimizados para o uso terapêutico. Os peptídeos puderam ser sintetizados com estruturas semelhantes àqueles encontrados na natureza ou, ainda, com modificações que aumentam sua estabilidade, eficácia e especificidade (CRAIK et al., 2013).

O interesse nos estudos dos peptídeos remonta o século XX, quando cientistas iniciaram a exploração de moléculas naturais bioativas para o tratamento de doenças originárias do déficit hormonal, como o tratamento do diabetes mellitus tipo 1 e 2 (BRUNO, MILLER, LIM, 2013). O marco farmacêutico no uso de moléculas peptídicas foi o desenvolvimento da insulina. Desde então, estas moléculas têm recebido grande atenção na indústria farmacêutica e, aproximadamente, 18,4% (1981-2019) dos fármacos aprovados pelo FDA são peptídeos ou proteínas (NEWMAN, CRAGG, 2020). Nos anos seguintes, a biotecnologia e seus avanços levaram os estudos de peptídeos a um novo patamar (CHEN et al,2020; WANG et al., 2022).

A natureza dispõe de uma vasta riqueza no que concerne à obtenção de moléculas peptídicas, tendo a possibilidade de obtê-las a partir de diversos organismos e habitats, tais como organismos marinhos e terrestres (HARVEY, 2014). Os animais peçonhentos são uma abundante e interessante fonte de compostos naturais bioativos, sendo encontrados em serpentes, aranhas e escorpiões dentre outros produtores de venenos. Alguns destes animais possuem toxinas capazes de gerar um quadro de desregulação da homeostase, que vai desde paralisia, choque anafilático, a problemas cardiovasculares severos, podendo ser fatal. Entretanto, as toxinas isoladas podem ser úteis na clínica médica, por possuírem ações farmacológicas diversas tais como analgésica, anticâncer, antimicrobiana e neuroprotetora (FILIPPENKOV et al., 2020).

Dentre as inúmeras fontes de obtenção de peptídeos, os organismos marinhos surgiram como uma importante fonte de compostos bioativos. Os peptídeos oriundos de organismos

marinhos têm emergido como uma fonte notável de compostos bioativos nos últimos anos (BLUNT et al., 2015). A vasta biodiversidade dos ecossistemas marinhos proporciona um rico reservatório de peptídeos com estruturas e propriedades diversas. Desde as regiões mais profundas dos oceanos até recifes de coral e zonas de marés, os organismos marinhos, como esponjas, moluscos e algas têm sido amplamente investigados quanto ao seu conteúdo peptídico (LIN et al., 2021). Estes peptídeos marinhos têm demonstrado uma ampla gama de atividades farmacológicas, incluindo efeitos antimicrobianos, antitumorais e imunomoduladores. Estas características têm despertado o interesse em pesquisas para investigar suas potenciais aplicações terapêuticas (NGO et al., 2012).

2. JUSTIFICATIVA

A biotecnologia de peptídeos desempenha um papel fundamental na pesquisa farmacêutica e no desenvolvimento de terapias inovadoras. Os peptídeos bioativos naturais têm uma vasta gama de atividades biológicas e por possuírem propriedades alvos farmacológicos específicos e mais seletivos a alvos moleculares, são potenciais agentes terapêuticos. Determinados compostos e substâncias isoladas têm sido amplamente estudados em virtude das suas capacidades de modular processos fisiológicos e patológicos específicos, sendo assim, objeto de interesse para a descoberta de novos medicamentos.

Nesse contexto, os peptídeos derivados de organismos marinhos e terrestres têm se destacado como fontes ricas em moléculas bioativas em virtude da sua biodiversidade. A evolução desses animais resultou em um amplo repertório de peptídeos com propriedades farmacológicas únicas e distintas. Estudos prévios têm apontado que esses peptídeos exibem atividades biológicas relevantes, abrangendo ações desde analgésicas a fibrinolíticas, bem como efeitos cardio - e neuroprotetores, que culminaram no desenvolvimento de fármacos que se encontram em uso clínico. A investigação de características específicas (atividade intrínseca) reveste-se de extrema importância para ampliar nosso entendimento acerca da biologia desses animais e para explorar o potencial terapêutico dos referidos peptídeos em diversas áreas da saúde.

Em particular, os estudos envolvendo os peptídeos derivados da crotalicidina (Ctn) e crotamina, mas também os peptídeos derivados de corais (peptídeos neuroativos de *Protopalythoa*) buscam o entendimento destas moléculas bioativas em diferentes processos biológicos. Por exemplo, a caracterização de suas propriedades de ativar ovócitos de mamíferos e induzir a partenogenese, especialmente mediadas por canais iônicos, pode fornecer informações valiosas sobre os mecanismos envolvidos na reprodução e no desenvolvimento embrionário. Paralelamente, a ação de canais iônicos permite obter mediadores farmacológicos que possuem propriedades imuno - e neuromoduladoras, com efeitos cito - e neuroprotetivos.

Portanto, a pesquisa sobre os peptídeos derivados do veneno de serpentes e de corais (cnidários marinhos), com ênfase nas atividades partenogenéticas e neuroprotetoras representa uma área promissora e relevante no campo da biotecnologia de peptídeos. Esses estudos não apenas contribuem para a ampliação do conhecimento sobre a biologia dessas espécies, mas também fornecem subsídios para a descoberta de novos medicamentos e adjuvantes para terapias inovadoras.

3. OBJETIVOS

3.1. Objetivo Geral

Realizar um *screening* de atividades biológicas de peptídeos sintéticos e modificados derivados de peptídeos bioativos de venenos, provenientes de uma espécie de cascavel (*Crotalus durissus terrificus*) e de um cnidário marinho (o antozoário *Protopalythoa variabilis*) utilizando modelos *in vitro* de embrião bovino, células neuronais e neuroendócrinas.

3.2. Objetivos Específicos

- Investigar se RhoB-Ctn[1-9] possui alguma ação embriotóxica;
- Avaliar se RhoB-Ctn[1-9] possui a capacidade de ativar *in vitro* a formação de embriões;
- Analisar por microscopia de fluorescência se RhoB-Ctn[1-9] tem a capacidade de atravessar a zona pelúcida;
- Estudar se os peptídeo Chi, PPA, SSB derivados de um peptídeo helicoidal do cnidário marinho *Protopalythoa*, e os peptídeos SFR-Ctn[1-9], SFR-Ctn[16-24] e NrTP1, derivados da crotalicidina e crotamina do veneno de cascavel modulam canais iônico para Ca^{2+}, Na^{+} e K^{+};
- Avaliar a ação do peptídeo PcActx no Ca^{2+} sob diferentes potenciais de ação;
- Quantificar o cálcio citosólico após a exposição do peptídeo Chi, PPA, SSB, SFRCtn[1-9], SFR-Ctn[16-24] e NrTP1;
- Averiguar a morte celular pelo peptídeo Chi, PPA, SSB, SFR-Ctn[1-9], SFRCtn[16-24] e NrTP1 através da atividade de LDH;
- Mensurar a viabilidade de células tratadas com o peptídeo Chi, PPA, SSB, SFRCtn[1-9], SFR-Ctn[16-24] e NrTP

4. REFERENCIAL TEÓRICO

4.1 Peptídeos bioativos naturais

Os peptídeos bioativos naturais são polímeros de aminoácidos de cadeia curta encontrados em organismos vivos, na qual tem como característica comum a atividade biológica. Estas macromoléculas podem ser obtidas de diferentes fontes, tais como plantas, animais marinhos, terrestres e microrganismos. Ademais, podem ser produzidos a partir de hidrólise enzimática ou química de proteínas endógenas (SÁNCHEZ, VÁZQUEZ, 2017). Os peptídeos bioativos apresentam uma cadeia peptídica constituída de 2 a 20 resíduos de aminoácidos e com pesos moleculares que variam de 0,4 a 2 kDa (ZAKY et al., 2021. A forma como estas substâncias são encontradas determinam suas propriedades bioativas, podendo assim se apresentarem com estruturas tridimensionais definidas, como os peptídeos cíclicos ou com ligações dissulfeto (DALY, CLARK, CRAIK, 2003).

Os peptídeos naturais têm despertado considerável interesse no meio científico devido à sua ampla gama de atividades biológicas. Essas substâncias possuem aplicações diversas, como melhorar as propriedades funcionais dos alimentos, serem utilizadas em cosméticos para a prevenção do envelhecimento, além de serem investigadas como fármacos para o tratamento de inúmeras doenças, incluindo câncer, diabetes e doenças cardiovasculares (XU et al., 2021).

Por exemplo, peptídeos bioativos que possuem propriedades antimicrobianas inibem efetivamente a proliferação de bactérias e fungos patogênicos (NONG, HSU, 2021). Os peptídeos antimicrobianos (AMPs) são uma das classes mais conhecidas de peptídeos biativos naturais, sendo produzidos por organismos como parte de sua resposta imunológica inata para combater agentes invasores. Os AMPs possuem atividade antimicrobiana de amplo espectro, o que significa que podem atingir uma ampla gama de bactérias, fungos e até mesmo vírus. Seu mecanismo único de ação envolve a interrupção da membrana celular do microrganismo, levando à lise celular e à morte (TORRENT, NOGUÉS, BOIX, 2012; MAHLAPUU ET AL., 2016).

Além dos peptídeos antimicrobianos, existem muitos outros peptídeos naturais com diversas funções. Alguns peptídeos atuam como fatores de crescimento, regulando a proliferação celular e a diferenciação (PAN et al., 2020). Por exemplo, o fator de crescimento semelhante à insulina 1 (IGF-1) promove o crescimento celular e a reparação tecidual. Outros peptídeos foram descobertos por exibir atividades antioxidantes, anticancerígenas, anti-

hipertensivas e imunomoduladoras (ZHANG et al., 2023). Além das atividades antimicrobianas, os peptídeos bioativos também apresentam efeitos antioxidantes potentes, combatendo radicais livres e reduzindo o estresse oxidativo (ZHANG et al., 2017). Esses peptídeos podem proteger contra diversas doenças crônicas associadas a danos oxidativos, como doenças cardiovasculares e câncer (LORENZO et al., 2018). Além disso, peptídeos bioativos têm mostrado propriedades imunomoduladoras ao regular a função das células imunes e modular respostas inflamatórias (KANG et al., 2019). Essa imunomodulação os tornam candidatos potenciais para o tratamento de doenças autoimunes e alergias. As atividades antioxidantes e imunomoduladoras dos peptídeos bioativos demonstraram potencial como agentes anticancerígenos. Vários peptídeos derivados de fontes naturais têm exibido efeitos citotóxicos seletivos contra várias linhagens de células cancerígenas (GHALAY et al., 2023). Por exemplo, peptídeos encontrados em organismos marinhos têm mostrado atividades anticancerígenas promissoras ao induzir a apoptose e inibir o crescimento tumoral (PAVLICEVIC, MAESTRI, MARMIROLI, 2020). Além disso, os peptídeos bioativos também podem regular diversas vias metabólicas, incluindo o metabolismo da glicose e dos lipídios, sugerindo seu uso potencial no controle de distúrbios metabólicos (AKBARIAN et al., 2022).

A capacidade dos peptídeos de interagir seletivamente com receptores específicos ou moléculas-alvo in vivo é a chave para manifestar esses efeitos. Alguns dos numerosos peptídeos atualmente disponíveis são capazes de se ligar seletivamente enzimas, canais iônicos ou receptores, modular sua atividade e provocar respostas biológicas específicas.

4.2 Peptídeos de venenos de animais

O uso de enzimas e polipeptídios com finalidades medicinais desperta bastante interesse devido à sua elevada especificidade e seletividade, o que reduz a probabilidade de interferir em processos celulares não relacionados ao alvo terapêutico. Os fármacos proteicos são compostos por polipeptídios bioativos com grande potencial terapêutico (RECÍO et al., 2017). Embora os venenos de animais possam causar efeitos nocivos graves, seus componentes sempre atraíram a atenção dos pesquisadores e têm sido amplamente estudados em busca de novas moléculas com atividade farmacológica (HARVEY, 2014). Um exemplo de molécula de sucesso a partir de toxinas de veneno é o captopril, que foi desenhado a partir de um penta-peptídeo isolado do veneno da serpente *Bothrops jararaca* sendo um dos principais fármacos utilizados no tratamento de hipertensão e insuficiência cardíaca (WAHEED, MOIN, CHOUDHARY, 2017).

Os efeitos causados por uma variedade de famílias de toxinas derivadas de viperídeos devem-se à rica e complexa mistura de componentes com diversas funções biológicas presentes no veneno. Isso abre grandes possibilidades para a descoberta de novos compostos, que trazem importantes contribuições terapêuticas e representam uma alternativa interessante para o estudo da toxicidade e dos mecanismos de ação dos poli-peptídeos farmacologicamente ativos no organismo (CALDERON et al., 2014; ZHANG, 2015; MUNITA, ARIAS, 2016; BONDARYK et al., 2017).

Portanto, mesmo sendo tóxicos, os venenos dessas famílias são estudados com o objetivo de identificar moléculas biologicamente ativas, principalmente peptídeos. Devido ao uso de alguns desses animais na medicina oriental tradicional para o tratamento de doenças inflamatórias, há um crescente interesse da comunidade científica na avaliação da atividade anti-inflamatória de peptídeos derivados de himenópteros e viperídeos (FALCÃO et al., 2014).

Diferentes toxinas provenientes de animais, como escorpiões, têm a capacidade de modular a atividade das células T e agir seletivamente sobre canais iônicos específicos de linfócitos T, como Kv1.3 e IKCa1, que são ativados por voltagem e Ca^{2+}, respectivamente (OLAMEDI- PORTUGAL et al., 2005). Assim, o potencial inibitório seletivo desses canais iônicos tem se destacado como possíveis compostos imunossupressores (HARTZELL et al., 2016).

No Brasil, as serpentes venenosas possuem peçonhas constituídas por proteínas biologicamente ativas, como enzimas, fosfolipases, toxinas e poli-peptídeos (LU et al., 2005). Essas enzimas possuem uma variedade de efeitos farmacológicos, incluindo atividades pró-inflamatórias, miotóxicas, neurotóxicas e antimicrobianas (SITPRIJA, 2012). O veneno das serpentes apresenta atividade proteolítica intrínseca e sua composição varia entre as diferentes famílias a que pertencem (FURTADO et al., 1991; SILVA et al., 2003). As vipericidinas, como a crotalicidina, batroxicidina, lutzicidina e lachesicidina, são exemplos de peptídeos antimicrobianos relacionados à catelicidina (CRAMPs) encontrados nas glândulas de veneno de víboras e cobras elapídeas, respectivamente (FALCÃO et al., 2014; WANG et al., 2008; ZHAO et al., 2008). As vipericidinas maduras e os CRAMPs apresentam alto grau de similaridade na sequência de aminoácidos, sendo que a maioria dos 34 resíduos são substituições idênticas ou estritamente conservadas. Esses peptídeos possuem uma carga líquida positiva devido à alta proporção de resíduos de lisina intercalados com resíduos de aminoácidos hidrofóbicos, conferindo-lhes um caráter anfipático, como observado em outros peptídeos ativos em membranas venenosas, como anoplin e mastoparan (KONNO et al., 2001; ROCHA et al., 2007). Os peptídeos das vipericidinas possuem propriedades antimicrobianas

potentes contra bactérias gram-negativas e fungos, além de apresentarem baixa citotoxicidade. Portanto, possuem um grande potencial como agentes antimicrobianos. Além disso, esses peptídeos demonstraram propriedades anti-inflamatórias, promovendo a diminuição da produção de citocinas pró-inflamatórias, como o fator de necrose tumoral alfa (TNF-α) e a interleucina-6 (IL-6), e o aumento da produção de citocinas anti-inflamatórias, como a interleucina-10 (IL-10), na presença de estímulos inflamatórios (FALCÃO et al., 2014).

4.2.1 Crotalicidina

A crotalicidina (Ctn) é um peptídeo originário da serpente *Crotalus durissus terrificus*, espécie de cascavel comumente encontrada na região centro-oeste e sudeste do Brasil. Esse peptídeo tem recebido uma atenção pela sua característica farmacológica. Apresentando características hidrofóbicas e catiônicas, o Ctn é um peptídeo de peso molecular 4 kDa que apresenta na sua estrutura primária de 34 resíduos de aminoácidos, sendo eles alanina, leucina e alguns resíduos catiônicos incluindo arginina e lisina (FALCÃO et al., 2015; BANDEIRA et al., 2018). Esta particular característica do Ctn faz com que a sua sequência primária seja mantida e preservada mesmo em diferentes espécies. Esse peptídeo adota uma α-hélice anfipática, comumente encontrada em muitos peptídeos antimicrobianos, o que permite que o Ctn interaja com a com membrana de uma forma eficiente (MORAES et al., 2022). O Ctn é um peptídeo que apresenta poderosas propriedades farmacológicas, sendo relatado principalmente pelos seus efeitos antimicrobianos. Em razão disso, os fragmentos desse peptídeo de 34 aminoácidos foram analisados objetivando encontrar regiões mais aprimoradas, culminando com a caraterização do peptídeo Ctn[15-34] (FALCÃO, et al. 2015 , PERÉZ-PEINADO, et al.,2018).

Posteriormente, fragmentos ainda menores do Ctn foram investigados: o Ctn-[1-9] é uma regiãoda crotalicidina que consiste em 9 aminoácidos (1KRFKKFFKK9), na qual adota uma estrutura conformacional tridimensional com características anfipáticas e ricas em lisina (LIMA et al., 2022). Quando modificado estruturalmente, as atividades farmacológicas desse peptídeo conjugado e não conjugado com o fluoróforo Rodamina B (RhoB) foi avaliada. Em ensaios biológicos, foi observado que o peptídeo Ctn-[1-9] (Crotalicidina-[1-9]) não exibiu qualquer efeito sobre a redução da proliferaçãode células tumorais, e também não demonstrou toxicidade em larvas de peixe zebra. Enquanto que a forma ligada do peptídeo com o composto fluorescente Rodamina B (RhoB-Ctn[1-9]), induziu uma diminuição na propagação de células tumorais MCF-7 e o aumento intracelular de cálcio (WANG et al., 2015). O ligante ao peptídeo

Ctn 1-9, a rodamina B, é um fluoróforo catiônico que possui carga variante, sua carga sofre alteração mediante o pH em que se encontra, podendo se comportar de forma neutra ou eletronicamente positiva (BIRTALAN et al., 2011). A capacidade ajustável com que os grupos químicos estão (associados ou dissociados) a rodamina B, conforme o valor de pH, pode fazer variar a atividade biológicado peptídeo (MORENO-VILLOSLADA et al., 2006).

A sequência repetitiva de nove resíduos é uma das principais características estruturais da crotalicidina e de outras vipericidinas; são repetições in tandem de 9 resíduos de aminoácidos Ctn-[1-9]-(1KRFKKFFKK9) e Ctn-[16-24]-(16KRLKKIFKK24) que ocorrem na crotalicidinas de cascavel e no peptídeo minimizado Ctn[15-34] (WANG et al., 2015. LIMA et al.,2022). O Ctn-[16-24] está relacionado a região C-terminal, que é caracterizado por ser uma sequência mais madura, além de ter uma menor hidrofobicidade quando comparada aos outros fragmentos da crotalicidina. Essa região C-terminal é onde está reservada uma forte atividade bactericida e essacaracterística pode justificar a capacidade de ser mais estável em determinadas condiçõesfisiológicas, tal como resistir a certo grau de degradação proteolítica e resistir por mais tempo nosoro sanguíneo (FALCÃO 2020 et al., 2022). Esta porção da crotalicidina apresenta uma atividadecontra bactérias gram-negativas, citotoxicidade nula em células eucarióticas sadias e toxicidade moderada em linhagens tumorais quando comparados com o Ctn completo (FALCÃO et al., 2015). Um outro fator importante da região C-terminal deste peptídeo é que tanto a versão conjugada ou não com Rodamina B é que a permeabilização membranar foi mais rápida com comparado ao Ctn completo nas suas duas versões (PÉREZ-PEINADO et al., 2018).

4.2.2 Crotamina

A crotamina é um peptídeo de natureza anfipática derivado de serpente encontrado em diferentes espécies de cascaveis sul-americanas. Esta substância possui um peso molecular de 5 quilodaltons (kDa), com 42 resíduos de aminoácidos, sendo 11 resíduos básicos, dos quais são 9 de lisina e 2 de arginina e 6 resíduos de cisteína que formam 3 ligações dissulfeto internas (Cys4- Cys36, Cys11Cys30 and Cys18-Cys37) (KERKIS et al., 2014). A estrutura α-hélices e outras características que lhe são atribuídas, confere ao peptídeo uma maior estabilidade estrutural, resistência contra a degradação enzimática pelas enzimas proteolíticas e desempenha uma capacidade de penetração celular e interação com células-alvo (CORONADO et al., 2013).

Esta mio - e neurotoxina é um peptídeo de veneno dotado da capacidade de interação com membranas carregadas negativamente. A conformação tridimensional da crotalicidina

(αβ1β2β3) assemelha-se com outras proteínas humanas que estão relacionadas à atividade antimicrobiana, tal como as β-defensinas. Embora apresente essa característica, a crotamina não tem uma forte ação antimicrobiana, tanto para Gram-positivas quanto para Gram-negativas. Estudos posteriores relataram uma ligeira ação antibacteriana contra algumas cepas, como *Escherichia coli* e *Staphylococcus aureus* (OGUIURA et al., 2011; YAMANE et al., 2013). Em contrapartida, a crotamina mostrou-se um potente agente anti-tumoral contra linhas de câncer pancreático e melanoma na concentração de 5 µg/mL e inofensivo para células normais (KERKIS et al., 2014). Previamente foi observado que este peptídeo é capaz de penetrar rapidamente em diferentes linhas de células, tais quais fibroblastos, linfoblastos, células de tronco embrionário e ovários (HAYASHI et al., 2008). Pesquisas paralelas ao estudo anti-câncer verificaram que este peptídeo não provocou toxicidade, além de ter corroborado com a depuração de glicose no sangue e redução das taxas de colesterol (CAMPEIRO et al., 2018). A ação anti-tumoral está intrinsecamente relacionada a liberação de cálcio pelo retículo endoplasmático e a perda do potencial de membrana mitocondrial (NASCIMENTO et al., 2012).

Estes estudos prévios mostraram a propriedade da crotamina como um peptídeo de penetração celular (CPP), sendo capaz de transportar cargas sem realizar ligações covalentes, nanocarreador de moléculas de DNA e RNA e atuando como uma CPP no tratamento para doenças renais (CAMPEIRO et al., 2018; FREITAS et al., 2014). Em geral, CPPs são moléculas catiônicas pequenas de até 30 resíduos que tem a capacidade de transportar até o interior de diferentes células, substâncias de peso variado, com baixa biodisponibilidade, biopolímeros (proteínas, peptídeos e ácidos nucleicos) e nanopartículas (EL-ANDALOUSSI et al., 2005; SIMEONI et al., 2004). A crotamina por ser caracterizada como um CPP tiveram suas regiões estruturais avaliadas e foram encontrados fragmentos então denominados nucleolar-targeting peptides (NrTPs).

Os NrTP é um grupo de peptídeos desenhados derivados da crotamina e pertencentes aos peptídeos de penetração celular (CPPs), mas que possuem uma melhoria em vários aspectos, sendo estas substâncias de toxicidade reduzida, capazes de transportar moléculas de alto peso molecular em células de mamíferos. O NrTP1 é, portanto, uma "mini-crotamina" resultante de um splicing entre as regiões N-terminal dos resíduos 1-9 e C-terminal dos resíduos 38-42 (RADIS-BAPTISTA, DE LA TORRE, ANDREU, 2012). Ensaios realizados demonstraram que esse peptídeo tem a capacidade de internalização e interação com membranas lipídicas linhas de células, sejam elas saudáveis ou tumorais, como carcinoma

mamário ou pancreático, mesmo sob diferentes condições e em baixas concentrações (RODRIGUES, ANDREU, SANTOS, 2015).

4.3 Peptídeos de cnidários marinho

Os oceanos constituem em média 70% de toda a superfície terrestre detendo uma grande riqueza e diversidade farmacológica biomarinha. No decorrer dos anos, seres marinhos evoluíram desenvolvendo mecanismos de defesa contra patógenos e mudanças no habitat marinho. Estas substâncias estão relacionadas a mecanismos de crescimento, defesa, reprodução e homeostase. A partir destes animais, peptídeos protetores foram isolados de animais marinhos vertebrados e invertebrados. Os peptídeos biomarinhos atuam como fontes de nitrogênio e podem ser obtidos apartir de diferentes seres, tais como moluscos, crustáceos, bactérias marinhas, fungos dentre outros (KIM, WIJESEKARA, 2010). Relevantes efeitos farmacológicos têm sido relatados a partir do isolamento de peptídeos derivados destes seres, dentre os quais antienvelhecimento, anticoagulante, antitumoral, anti-inflamatórios e antidiabético (NGO et al., 2012; MAYER et al.,2013; MASSO-SILVA et al., 2014; RANGEL et al., 2017; ANJUM et al., 2017).

Nos últimos anos, 13.000 novas moléculas bioativas foram identificadas em animais marinhos, sendo 3.000 pertencentes ao grupo Cnidaria (KAYAL et al., 2013; ARRUDA et al., 2021). O filo Cnidaria englobla anêmonas do mar, corais, águas-vivas e hidróides e constituído por animais diploblásticos. Estes seres possuem um conjunto de venenos que atuam como um mecanismo dedefesa e predação (JOUIAEI et al., 2015). Este filo é responsável por uma grande produção de compostos naturais nos trópicos com cerca de 28% da produção total, seguido das esponjas do mar com 33% (BLUNT et al., 2015). Tal como outros animais peçonhentos, o filo Cnidaria é caracterizado pela produção de toxinas que incluem em sua composição química, pequenas moléculas, peptídeos e proteínas (GERSSEN et al., 2010).

Os venenos de cnidários contém peptídeos que atuam a partir da modulação de canais iônicos envolvidos no potencial de ação do nervo, coração e músculo esquelético. Estes peptídeos possuem em torno de 10 a 60 aminoácidos e apresentam estruturas secundárias bem definidas que são estabilizadas por ligações dissulfeto altamente conservadas (LIAO et al., 2018). Embora já setenha investigado e identificado um grande volume destes peptídeos de organismos marinhos, seu mecanismo de ação, bem como sua possível atividade frente a mecanismos biológicos ainda é uma incógnita na literatura (KARTHIKEYAN et al., 2022). Estudos anteriores já abordaram a capacidade de antagonistas peptídicos derivados do veneno

do filo Cnidario desempenhar funções biológicas. Peptídeos derivados de anêmonas do mar, por exemplo, mostraram ser potentes bloqueadores de canais iônicos em concentrações inferiores a 10 μM (ANDREEV et al.,2008; KALINA ET AL., 2020).

4.3.1 O cnidário *Protopalythoa variabilis* e seus peptídeos bioativos

Os recifes de corais são habitats naturais para inúmeras espécies, que variam desde corais, crustáceos, pequenos peixes até comunidades microbianas. A *Protopalythoa variabilis* é um coral pertencente família Zoanthidae, no qual está inserido no filo Cnidaria. Esta espécie marinha é comumente encontrada em recifes de corais em zonas tropicais e subtropicais de águas mornas e rasas de zonas como Mar do Caribe, Indo-Pacífico e o litoral brasileiro, especificamente na região nordeste (DE ANDRADE et al, 2012). A família a qual pertence a *P. variabilis* produz inúmeros metabólitos secundários como alcaloides, peptídeos, terpenos, que são importantes a sobrevivência das espécies e apresentam consideráveis atividades farmacológicas (WANG et al.,2016).

A espécie *P. variabilis* ainda é pouco estudada no que concerne suas atividades farmacológicas. Através de análises transcriptômicas foi visto que esse coral apresenta importantes metabólitos secundários e polipeptídeos biologicamente ativos, além de enzimas hidrolases, oxidorredutases e transferases (HUANG et al., 2016). Ademais, estudos têm descoberto substâncias que, até então, não eram encontradas na natureza. O α-aminoácido lipídico é considerado um aminoácido artificial, no entanto cientistas o encontraram na P. varibilis e estudos comprovaram um interessante efeito pró-apoptótico e anti-proliferativo em linhas celulares tumorais HL-60 em concentrações a 5 μg/ml. Além disso, mostrou-se citotóxico em quantidade nanomolar (ng/mL) em células de glioblastoma, câncer de cólon e leucemia (WILKE et a., 2009). Posteriormente, um estudo transcriptômico desta espécie identificou proteínas de diferentes famílias de toxinas que incluem neuropeptídeos, estes com ações como bloqueador de canais de íons, inibidor do receptor nicotínico e muscarínico, além de toxinas hemostáticas e hemorrágicas, proteínas formadoras de poros, inibidores de proteases e proteínas de função mista (HUANG et al., 2016).

Destas análises transcriptômicas foi descoberto o peptídeo PpVα(KYWILNVPASVCDEYCWSQMLLYKKS-NH2), antozoário *P. varibilis* que possui uma estrutura α-hélice e forma de “V”. Previamente, um homólogo desse peptídeo havia sido encontrado primeiro na espécie *Palythoa caribaeorum* e, funcionalmente, esse peptídeo foi caracterizado como um bloqueador dos canais de sódio NaV1.7 (LAZCANO-PÉREZ et al.,

2016). Sabe-se que o mesmo peptídeo quando tem suas ligações dissulfeto modificadas podem apresentar atividades biológicas distintas (TIEtZE et al., 2012).

Nesse sentido, Liao e colaboradoradores (LIAO et al., 2019) modificaram a estrutura do PpVα de forma linear (KYWILNVPASVCDEYCWSQMLLYKKS-NH2), denominado aqui PPA) para uma com ligação dissulfeto (S-S) (KYWILNVPASVCDEYCWSQMLLYKKS-NH2, denominado aqui de SSB). Além desses, foi sintetizado um peptídeo quimérico (GELIKMKYWILNVPASVCDEYCWSQMLLYKKS-NH2), denominado aqui CHI). O estudo desses peptídeos mostrou que desempenham uma importante ação antiepilética, sendo capaz depromover a redução dos sintomas por meio da baixa expressão dos proto-oncogenes c-fos enpas4a, em modelo de peixe zebra (LIAO et al, 2019). Além disso, proporcionou uma redução demediadores do estresse oxidativo o que, por consequência, preveniu a perda de neuronais dopaminérgicas em peixes zebra.

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Exploring the neuromodulatory activity of modified animal venom peptides: *in vitro* electrophysiological evaluation and in sílico analysis

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ABSTRACT

Neurodegenerative diseases pose a significant challenge in the field of healthcare, and the search for effective treatments is of paramount importance. In this context, bioactive toxins have sparked interest due to their ability to modulate physiological and pathological processes. This study aimed to investigate the therapeutic potential of modified peptides derived from a marine coral (Chi, PPA and SSB), and a rattlesnake venom (SFR-Ctn[1-9], SFR-Ctn[16-24], and NRTP1) for their effects on ion channels. To assess the possible neuroprotective activity of these peptides, patch clamp techniques were employed to examine their behavior on sodium, calcium, and potassium ion channels, as well as their cytotoxic or neuroprotective effects on human neuroblastoma cells. The results revealed that these peptides exhibited a remarkable ability to block ion channels, with selective affinity for sodium channels, as confirmed by molecular docking analyses. Furthermore, these peptides demonstrated significant neuroprotective action, attenuating toxin-induced cellular damage. In particular, the peptides proved effective against the toxic effects of veratridine, an agent that interferes with sodium channel function, as well as oligomycin/rotenone, substances that affect mitochondrial function. These findings underscore the importance of peptides derived from animal venom as prospective pharmacological agents. The ability of these peptides to modulate ion channels, particularly sodium channels, suggests significant potential in attenuating neurodegenerative processes associated with dysfunctions of these channels. Moreover, the neuroprotective and

antioxidant actions of these peptides against specific toxins highlight their relevance in the context of oxidative stress and neuronal protection.

INTRODUCTION

Neurodegenerative diseases (ND) are a comprehensive group of over 600 conditions that lead to progressive and irreversible deterioration of the Nervous System (NS) in patients (TRIPPIER et al., 2013). According to data provided by the National Institute of Environmental Health Sciences (NIEHS), NDs affect a considerable number of individuals worldwide, with Parkinson's Disease (PD) and Alzheimer's Disease (AD) being the most prevalent, affecting approximately 66.2 million and 1.2 million American citizens, respectively (NIEHS, 2022). Inflammation, oxidative stress, and mainly disruptions in ion channel homeostasis are signaling pathways that play a significant role in the development of these diseases (WANG; JACKSON; XIE, 2016).

In the context of neurons, ions play essential roles in a wide range of physiological processes, ranging from gene transcription regulation, neuronal growth, cell survival, to differentiation (SUN et al., 2015). Based on these mechanisms of ionic balance in neuronal cells, numerous studies have focused on the development of new drugs or compounds that act as therapeutic agents targeting these mentioned therapeutic targets, especially due to the lack of specific therapies available for the clinical treatment of these patients (LAJARÍN-CUESTA et al., 2016).

In this regard, bioactive peptides have emerged as a promising source of new neuroprotective drugs due to their ability to modulate processes in the central nervous system. The numerous effects caused by a variety of toxin families result from the rich and complex mixture of components with diverse biological functions present in venom, which provides a great possibility for the discovery of new compounds, bringing important contributions to the population from a therapeutic perspective and as a highly interesting alternative for studying the toxicity and mechanisms of action of pharmacologically active (poly)peptides on the organism (CALDERON et al., 2014; ZHANG, 2015; MUNITA, ARIAS, 2016; BONDARYK et al., 2017).

In this context, the discovery of bioactive components, often produced by already known species, has had a significant impact on the development of alternative therapies, especially in marine and terrestrial organisms. These organisms inhabit a wide range of ecological niches, displaying unique metabolic adaptations with unparalleled structures, and

they possess a highly unexplored library of bioactive compounds (KARTHIKEYAN et al., 2022). The marine and terrestrial environments, housing a diverse array of animals, remain insufficiently explored areas. The vast biological diversity in these ecosystems necessitates in-depth studies to investigate and discover new pharmaceutical compounds that can effectively act on the reversal or mitigation of neurodegenerative diseases. Thus, our objective is to investigate the profile of peptides from the venom of Crotalus durrissus terrificus (a rattlesnake) and from the mat coral *Protopalythoa variabilis* as ion-channel modulators using cells in cultureand electrophysiological records.

METHODOLOGY

Peptides

Peptide sequences used in this study for the electrophysiology and cytoprotection experiments are from previous published works and are listed in table 1.

Peptide	Sequence	Characteristics
Chi-aSVP	GELIKMKYWILNVPASVCDEYCWSQMLLYKKS-NH2	Neuroprotector (LIAO et al., 2019)
Ppa-aSVP	KYWILNVPASVCDEYCWSQMLLYKKS-NH2	Neuroprotector (LIAO et al., 2019)
SSb-aSVP	KYWILNVPASVCDEYCWSQMLLYKKS-NH2 (C12-C16, S-S bond)	Neuroprotector (LIAO et al., 2019)
SFR-Ctn[1-9]	Sulforhodamine-KRFKKFFKK-NH2	CPP, Antimicrobial (this work)
SFR-Ctn[16- 24]	Sulforhodamine-KRLKKIFKK-NH2	CPP, Antimicrobial (this work)
NrTP-1	YKQCHKKGGKKGSG-NH2	CPP, nucleolar marker RADIS-BAPTISTA et al., 2008; RODRIGUES et al., 2011; RADIS-BAPTISTA et al., 2012;

Table 1. Sequences and characteristics of peptides investigated in this study.

Preparation of chromaffin cell cultures

All experiments were carried out in accordance with the guidelines established by National Council on Animal Care and the European Communities Council Directive (86/609/ECC); and were approved by the local Animal Care Committee of Universidad Autónoma de Madrid (ES280790000092). and were approved by the local Animal Care Committee of the Universidad Autónoma de Madrid. Bovine chromaffin cells (BCCs) were isolated from calf adrenal medullary tissues obtained from a local slaughterhouse, isolated according to Moro et al. (Moro *et al.*, 1990) with some modifications (De Pascual *et al.*, 2016), and plated at a density of $5x10^6$ cells per 5 mL of Dulbecco´s modified Eagle´s medium (DMEM) on 6-cm diameter Petri dishes (to study secretion) and on 12 mm diameter glass coverslips at a density of $5x10^4$ cells/coverslip, to study calcium currents (ICa) through voltage-activated calcium channels (VACCs). Cells were kept for 1-4 days at 37 ºC in a water-saturated incubator, in a 5% CO2/95% air atmosphere.

Recording of input calcium and sodium currents with patch-clamp technique

Input currents through voltage dependent calcium channels (ICa) and through voltage dependent sodium channels (INa), were recorded under the whole cell mode of the patch-clamp technique (Hamill et al., 1981). For patching the cells, pipettes of pproximately 2-5 MΩ resistance were pulled from borosilicate glassand lightly fire polished. Pipettes are filled with an internal solution (containing in mM: 100 CsCl, 14 EGTA, 20 TEA.Cl, 10 NaCl, 5 Mg-ATP, 0.3 Na-GTP, and 20 HEPES, pH 7.3CsOH) and introduced into a headstage coupled to an EPC-9 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany), allowing cancellation of capacitive transients and series resistance compensation.

Data were acquired with a sampling frequency of 20 kHz using the PULSE 8.74 software (HEKAElektronik, Lambrecht, Germany). The leakage current and capacitive components were removed using a P4 protocol and series resistance was compensated to 80%. The data analysis was performed using Igor Pro (Wavemetrics, Lake Oswego, OR) and PULSE (HEKAElektronik) and Origin 8.0 (Microcal) programs.

Cells were plated on coverslips and mounted in a Nikon Diaphot inverted microscope and were continuously perfused with a standard Tyrode solution (in mM): 137 NaCl, 1 MgCl2, 2 CaCl2, 10 HEPES, pH 7.4 with NaOH. External solutions were exchanged by a fast perfusion device consisting of a modified multi-barrelled pipette, the common outlet of which was positioned 50-100 μm from the cell. Control and test solutions were changed using miniature solenoid valves operated manually. Under these conditions the flow of liquid used is regulated

to 1 ml/min. For the measurement of I_{Na} cells were perfused with the same external solution (Tyrode solution) but containing 0 Ca^{2+}. Cells are held at -80 mV and 10 ms depolarizing pulses to -10 mV are applied for the recording of sodium currents. I_{Ca} was induced by 50 ms depolarizing pulses to +10 mV. All the experiments were performed under room temperature (24±2 ºC) and using cells with 2 to 4 days of culture.

Recording of output potassium currents in bovine chromaffin cells

All currents obtained in these experiments were recorded under the whole cell mode of the patch-clamp technique (Hamill et al., 1981). Cells are constantly perfused with a standard solution (containing in mM): 137 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, 10 glucoseand 10 HEPES, pH 7.4 with NaOH, and internally dialyzed with a solution of the following composition (in mM): 135 KCl, 10 NaCl, 10 HEPES,1 MgCl, 5 EGTA, pH7.4 with KOH. All experiments were done at room temperature (24-26 ºC). Electrophysiological data were carried out using an EPC-9amplifier under the control of PULSE software (HEKA Elektronik). The protocol used in these set of experiments consisted of a pre-pulse of 10 ms from -80 mV to +30 mV, followed by a more sustained depolarization (400 ms) at a voltage of +120 mV, given at intervals of 10 s. In doing so we can distinguish between the calcium dependent K^+ current (due to the pre-pulse) and the voltage dependent K^+ current (plateau).

Monitoring of cytosolic calcium levels

To monitor the changes of [Ca2+]c, cells were plated at a density of 2x105 cells per well into 96-well black plates, and the experiments were performed 48 h later for BCCs. Cells were loaded with a Krebs-HEPES solution containing 10 µM fluo-4-AM and 0.2% pluronic acid. Cells were incubated for 45 min at 37ºC in the dark. After this incubation period, cells were washed twice with the Krebs-HEPES solution (composition in mM:144 NaCl, 5.9 KCl, 1.2 MgCl2,11 glucose, 10 HEPES, 2 CaCl2, pH 7.4 with NaOH) at room temperature in the dark. Changes in fluorescence (excitation 485 nm, emission 520 nm) were measured using a fluorescent plate reader (Fluostar, BMG Labtech, Offenburg, Germany). Basal levels of fluorescence were monitored before adding the stimulation solution (35 mMK+) with an automatic dispenser. After stimulation of the cells, changes in fluorescence were measured for 60 s. To normalise fluo-4 signals, responses from each well were calibrated by measuring maximum and minimum fluorescence values. At the end of each experiment, 5% Triton X-100 (Fmax) was added followed by 2 M MnCl2 (Fmin). Data were calculated as a percentage of Fmax-Fmin.

Maintenance of SH-SY5Y cell line

For neuroprotection experiments, SH-SY5Y cell lines derived from human neuroblastoma were used as experimental models. This cell line was chosen because it is used as an *in vitro* model for Parkinson's disease research. Being a subline of SK-N-SH cells, this cell line exhibits catecholaminergic expression with activity of choline acetyltransferase, acetylcholinesterase, dopamine-β-hydroxylase, and tyrosine, in addition to releasing noradrenaline (BIEDLER et al., 1978; PÅLMAN et al., 1984; XICOY, WIERINGA, MARTENS, 2017). Furthermore, these cells can be maintained as neuroblastomas or induced and differentiated into neurons (LOPEZ-SUAREZ et al., 2022).

These cells from aliquots frozen in liquid nitrogen were suspended in DMEM/F-12 medium (Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12), supplemented with 10% SBF, 50 IU/mL penicillin and 50 μg/mL streptomycin, and cultured in cell culture bottles. Once its proliferation and confluence state have been achieved (usually 24- 48 hours after being cultured), the cell line expresses its most characteristic qualities and stops growing, so, at this time, passes were made to new bottles to continue favouring its

growth and avoid intraspecific competition. The cells were stored for 48 hours at 37ºC in an atmosphere saturated with humidity with 5% CO2 before being used.

Cell viability in the SH-SY5Y line

Once the cell lines contained in the culture vials were found in optimal conditions for use in the experiments, they were initially treated with 0.25% Trypsin with EDTA (1 mL) to get them to detach from the support and to be able to seed them in plates of 96 wells with a black flat bottom, used especially for fluorescence techniques, at a density of 4 x 104 cells per well. The cells were preserved for 24 hours at 37ºC in an atmosphere saturated with humidity with 5% CO2, until a correct cellular abundance was achieved with which to start the experiments. 24 hours after planting, and with the cells attached to the surface of the plate, the experiment began, which lasts 3 calendar days. In each plate, the experiment for each drug solution was developed in triplicate, being occupied 3 wells with the content of the same Eppendorf.

The protocol to be followed was determined by the laboratory itself, being summarized as follows: 200 μL were removed from each well (medium consumed by the cells) and 200 μL of the prepared solutions were incorporated (from here the media used will be free of serum).

For the validity of the experiments, it was necessary that the day after planting (at 24 hours), the cells were exposed only to treatment or positive control.

Within 48 hours of planting, the contents of the wells were renewed. This time, although the wells of the even columns were again replaced by the solutions incorporated the previous day, the wells of the odd columns were filled with 200 μL of the solutions provided with oligomycin / rotenone (O/R) (both cell death-inducing compounds). At 72 hours after planting, 200 μL of 10% resazurin were incorporated into each well, and after a period of action of 5 hours, cell viability was measured. Resazurin is a reagent that allows detecting cell viability from the conversion of a non-fluorescent stain (blue) to a fluorescent form (pink) because of redox reactions, so the fluorescence signal detected will be proportional to the number of living cells present in the well. The detection of fluorescence intensity, and therefore cell viability, was carried out by the multimode plate reader (FLUOstar). Molecular docking procedures

The crystallographic structure for human Na^{+} and Ca^{2+} channels was obtained from Protein Data Bank (PDB) with access code 5EK0 and 7WLJ, respectively (AHUJA et al., 2015). The chemical structure for the natural peptides Chi-aSVP, Ppa-aSVP, SSb-aSVP, and NrTP-1 was built and minimized in terms of energy by semi-empirical method, available in the Spartan'18 software (Wavefunction, Inc., Irvine, CA, USA) at pH 7.4. The blind docking calculations were performed with GOLD 2022.3 software (Cambridge Crystallographic Data Centre, Cambridge, CB2 1EZ, UK). Hydrogen atoms were added to the proteins following tautomeric states and ionization data inferred by GOLD 2022.3 software at pH 7.4. The standard ChemPLP was used as scoring function due to the lack of similar crystallographic ligands complexed with human Na^{+} and Ca^{2+} channels to redocking calculations. The Protein-Ligand Interaction Profiler (PLIP) webserver (https://plip-tool.biotec.tu-dresden.de/plip-web/plip/index) (ADASME et al., 2021) was used for the identification of protein-ligand interactions and both Root Mean Square Deviation (RMSD) value and 3D-figures were generated with PyMOL Molecular Graphics System 1.0 level software (Delano Scientific LLC software, Schrödinger, New York, NY, USA).

Statistical Analysis

The results were expressed as mean ± standard error of the mean (SEM) of the number of samples (n) analyzed, obtained from at least 3 distinct animals. Initially, statistical analysis was conducted using Student's t-test or analysis of variance (ANOVA), followed by Dunnett's multiple comparisons test to determine the statistical significance between the mean values.

The data were subjected to a normality test (D'Agostino and Pearson normality test), revealing that some parameters followed a normal distribution while others did not. Therefore, the Mann-Whitney test was applied in all instances. Statistical significance was established for p-values below 0.05. All analyses were performed using GraphPad Prism 6.01 software package.

RESULTS

Evaluation of Intracellular Calcium Ca^{2+} in a Population

Previous studies have revealed that analog peptides of crotamine, crotalicidin-derived, and *Protopalythoa variabilis* alpha-helical V-shaped peptide analogs affect calcium channels. It is well established that neurodegenerative diseases are related to the dysregulation of diverse channels but also calcium homeostasis. Likewise, as mentioned earlier, oxidative stress and reactive oxygen species play an important role in this process of disorders. Aiming at the evaluation of the effect of these peptides on intracellular calcium regulation, Fluo-4AM was used to measure changes in Ca^{2+} levels in bovine chromaffin cells, which serve as an *in vitro* model for human neuroendocrine signaling.

After obtaining the baseline fluorescence of the chromaffin cells, K+ (35 mM) was added for 10 seconds, resulting in an increase in fluorescence that persisted for 60 seconds. The following figures show the control group with an increase in fluorescence. When analyzing the peptides derived from corals chimeric peptide (CHI), alpha-helical V-shaped peptide (PPA), and single-stranded stabilized alpha-helical V-shaped peptide (SSB) at three concentrations (Figure 1), it was observed that these peptides block intracellular calcium release, as evidenced by the reduction in fluorescence. However, intracellular blockade is not dose-dependent, as the concentration of 1 µM of PPA reduced fluorescence by more than 50% ($P \leq 0.001$) compared to baseline, while the concentration of 10 µM of CHI reduced calcium by more than 60% ($P \leq 0.001$). On the other hand, SSB showed similar values of fluorescence reduction. IN case of the two analog peptides of snake venom-related peptides, the crotalicidin analogs (SFR-Ctn) showed slight differences between each other regarding Ca^{2+} release, where SFR-Ctn[16-24] at 10 µM demonstrated a greater reduction ($P \leq 0,01$) than SFR-Ctn[1-9] ($P \leq 0,001$) and even NRTP-1 ($P \leq 0,001$). The minimized crotamine-derived peptide fragment (NrTP-1) caused lower fluorescence readouts at the two lower concentrations (1 µM, $P \leq 0,001$; 3 µM, $P \leq 0,001$)

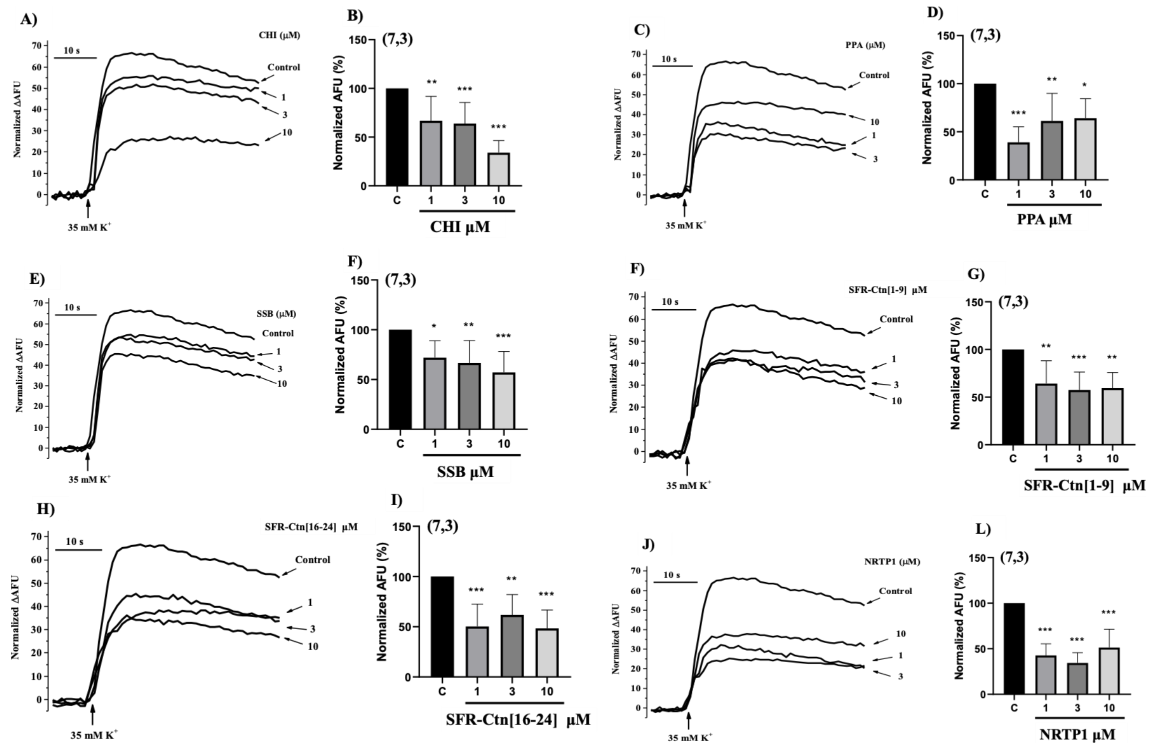

Figure 1. Effects of CHI, PPA, SSB, Ctn[1-9], SFR-Ctn[16-24], and NRTP1 on [Ca^{2+}] at concentrations of 1, 3, and 10 μM. (A, C, D, E, F, H, and J) Original trace of Fluo-4 fluorescence induced by K+ in the absence (control) or presence of the peptides (respectively). The control trace represents the absence of endogenous signals evoked by the peptides. The values were normalized to the maximum fluorescence increment produced with K^+ (35 mM). The data correspond to mean values ± S.E.M. in triplicates. AFU, arbitrary fluorescence units. Results were considered statistically significant when $P \leq 0.05$*, $P \leq 0.01$**, $P \leq 0.001$***.

Peptides	Concentrations (%)			
	Control	1	3	10
Chi-aSVP	54,99	49,09	48,97	25,40
Ppa-aSVP	66,56	35,55	3014	45,96
SSb-aSVP	66,19	54,83	52,65	45,46
SFRCtn[1-9]	66,07	44,65	41,41	41,39
SFR-Ctn[16-24]	66,19	43,93	36,19	34,82
NrTP-1	66,56	30,77	25, 24	37,29

Table 2: Values pertaining to the original Fluo-4 traces induced by K+ and treated with the peptides Chi-aSVP, Ppa-aSVP, SSb-aSVP, SFRCtn[1-9], SFR-Ctn[16-24], and NrTP-1 at concentrations of 1, 3, and 10 μM. The data represents the mean values obtained in triplicates.

Neuroprotective Effects of Peptide Toxins in Human Neuroblastoma

We investigated the neuroprotective potential of the peptides CHI, PPA, SSB, SFR-Ctn[1-9], SFR-Ctn[16-24], NRTP1 (Figure 2) in human neuroblastoma cells after using veratridine as an inducer of neuroinflammation. The cells were subjected to a protocol involving pre-incubation and co-incubation for 24 hours, resulting in a mortality rate of 50%. We performed triplicate experiments with different concentrations of each peptide and found that veratridine reduced cell viability by almost 50% compared to the baseline value. However, when the peptides were added along with veratridine, a significant increase in cell viability was observed, reaching an overall average of approximately 80% ($p < 0.001$). Furthermore, we observed the sodium channel blocker TTX almost completely reversed the cellular damage. Initially discovered in the pufferfish, tetrodotoxin is a neurotoxin that acts by blocking voltage-gated sodium channels, affecting six out of the nine subtypes of these channels (GONZÁLEZ-CANO et al., 2021). TTX acts on sodium channels through the interaction of the guanidine group and carboxylate residues, binding to the neurotoxin receptor-1 of the α subunit, located on the external surface of these channels, resulting in a blockade of sodium currents (FOZZARD, LIPKIND, 2010; BUCCIARELLI et al., 2021).

In this study, one of the aims were to investigate the presumable neuroprotective activity of different peptides on cultured human neuroblastoma (SH-SY5Y) cells as a model. Cell viability results were obtained using the MTT method after 24 hours of veratridine- induced stress in SH-SY5Y cells treated or not with peptides derived from coral (Chi, PPA, SSB) or snake (SFR-Ctn[1-9], SFR-Ctn[16-24], and NRTP1), in addition to the use of tetrodotoxin (TTX), a veratridine antagonist. Overall, all evaluated substances demonstrated a significant cytoprotective effect ($p < 0.005$) counteracting the cytotoxic effect of veratridine. Veratridine reduced cell viability by 49.21% compared to the baseline value ($p < 0.001$). On the other hand, the concentrations used, ranging from 1 to 3 and 10 µM, exhibited cytoprotective activity above 40% compared to the toxin. When compared to the reference substance, most peptides at a concentration of 10 µM showed similar inhibition values and increased cell viability superior to TTX.

An interesting detail is that the peptides SFR-Ctn[1-9], SFR-Ctn[16-24], and NRTP1 demonstrated a slight superior cytoprotective action toward SH-SY5Y compared to the coral-derived peptides CHI, PPA, and SSB. Although both groups exhibited cytoprotective effects reverting the veratridine cytotoxic effects, this difference was observed. Furthermore, it was found that the cell protective effect was not dose-dependent, even among the different peptides

evaluated. Additionally, the groups that received only the toxin-free peptides, at their different concentrations, showed increased cell viability compared to the baseline value. These results suggest that peptides derived from both corals and snakes have significant cytoprotective potential mediating by ion channel, reverting the cytotoxic effect caused by veratridine, a toxic substance capable of compromising cell viability in human neuroblastoma cultures.

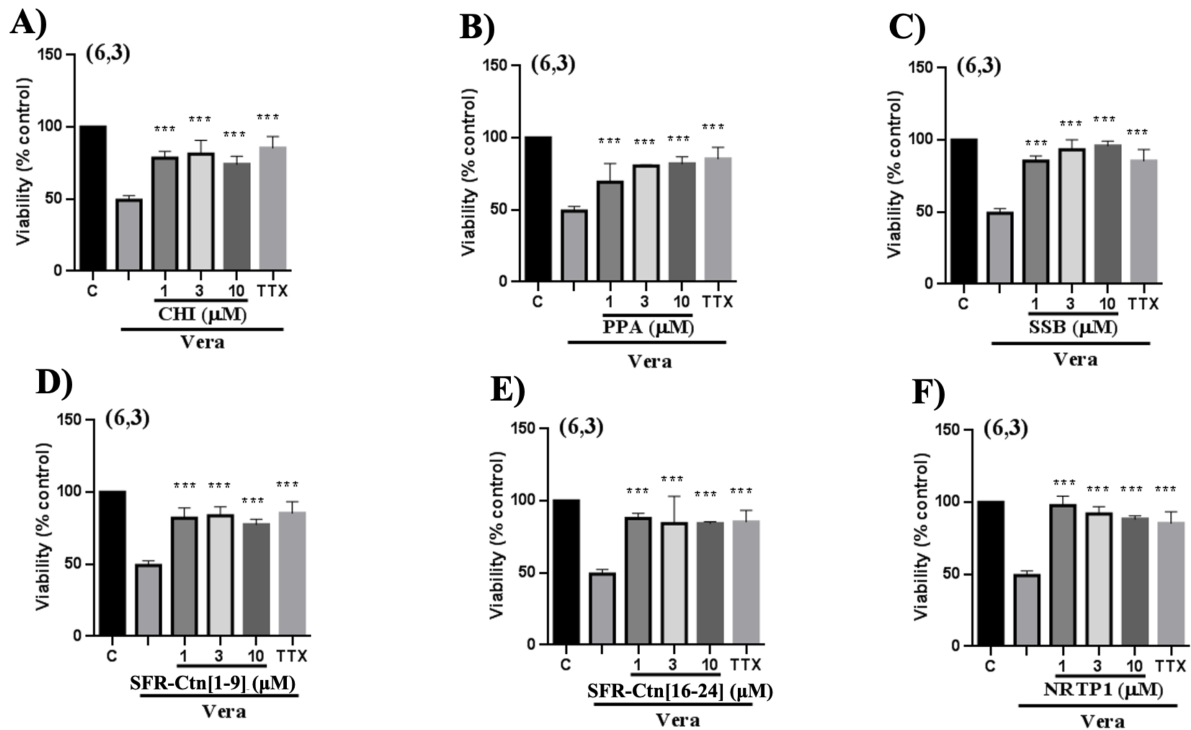

Figure 2. The cytoprotective effects of CHI, PPA, SSB, SFR-Ctn[1-9], SFR-Ctn[16-24], and NRFTP1 that revert the neurotoxicity of veratridine (Ver). (A) Experimental protocol consisting of two pre-incubation periods of 24 hours with the peptides at concentrations of 1, 3, and 10 µM, followed by a co-incubation period of 24 hours with the same peptides and veratridine. Statistical differences were determined in relation to the neuronal damage caused by veratridine using one-way analysis of variance (ANOVA) test. Results were considered statistically significant when $P \leq 0.05$*, $P \leq 0.01$**, $P \leq 0.001$***.

Peptides	Concentrations (%)					
	Control	VTD	TTX	1	3	10
Chi-aSVP				78,38	81,25	74,03
Ppa-aSVP				69,29	80,45	81,88
SSb-aSVP				85,32	93,23	99,40
SFRCtn[1-9]	100	49,21	85,41	81,92	83,68	77,59
SFR-Ctn[16-24]				98,77	84,33	84,25
NrTP-1	-	-	-	97,69	91,93	88,30

Table 3: Cytoprotection in SH-SY5Y cells against veratridine (VTD) toxicity. The values refer to the treatment with peptides Chi-aSVP, Ppa-aSVP, SSb-aSVP, SFRCtn[1-9], SFR-Ctn[16-24], and NrTP-1 at concentrations of 1, 3, and 10 µM, or tetrodotoxin (TTX). The data represents the mean values obtained in triplicates.

Cytoprotective Effect against Mitochondrial Stress

Following up the molecular studies, another test was conducted to investigate the cytoprotective effect of peptides against mitochondrial oxidative stress (Figure 3). For this purpose, a toxic mixture of oligomycin and rotenone was employed and tested on SH-SY5Y cells to measure lactate dehydrogenase (LDH) release. The combination of these substances results in the production of endogenous reactive oxygen species (ROS) due to the inhibition of mitochondrial respiratory chain complexes I and V (LIN et al., 2006). Oligomycin primarily targets the mitochondrial ATP synthase (complex V), which is responsible for the production of adenosine triphosphate (ATP) by mitochondria. When it binds to the F0 subunit of ATP synthase, oligomycin blocks the proton gradient, leading to a reduction in energy production (NICHOLLS et al., 2010). On the other hand, rotenone is a blocker of NADH dehydrogenase (complex I) and acts by binding to this complex, blocking the influx of electrons from NADH to the electron transport chain (CHEN et al., 2007).

The selected time point for analysis was 24 hours after the cells were exposed to the combination of mitochondrial disruptors and coral-derived peptides (CHI, PPA, SSB), snake-derived peptides (SFR-Ctn[1-9], SFR-Ctn[16-24], NRTP1), or the natural antioxidant

melatonin. After the predetermined time, it was observed that cells treated with oligomycin/rotenone exhibited a considerable increase oxidative products resulting from the inhibition of energy production release, resulting in a 61% decrease in cell viability compared to the baseline after 24 hours of exposure to the substances. In contrast, it was observed that the

different peptides did not show apparent toxicity and were able to maintain an average of 70% cell viability in cells exposed to oligo/rotenone.

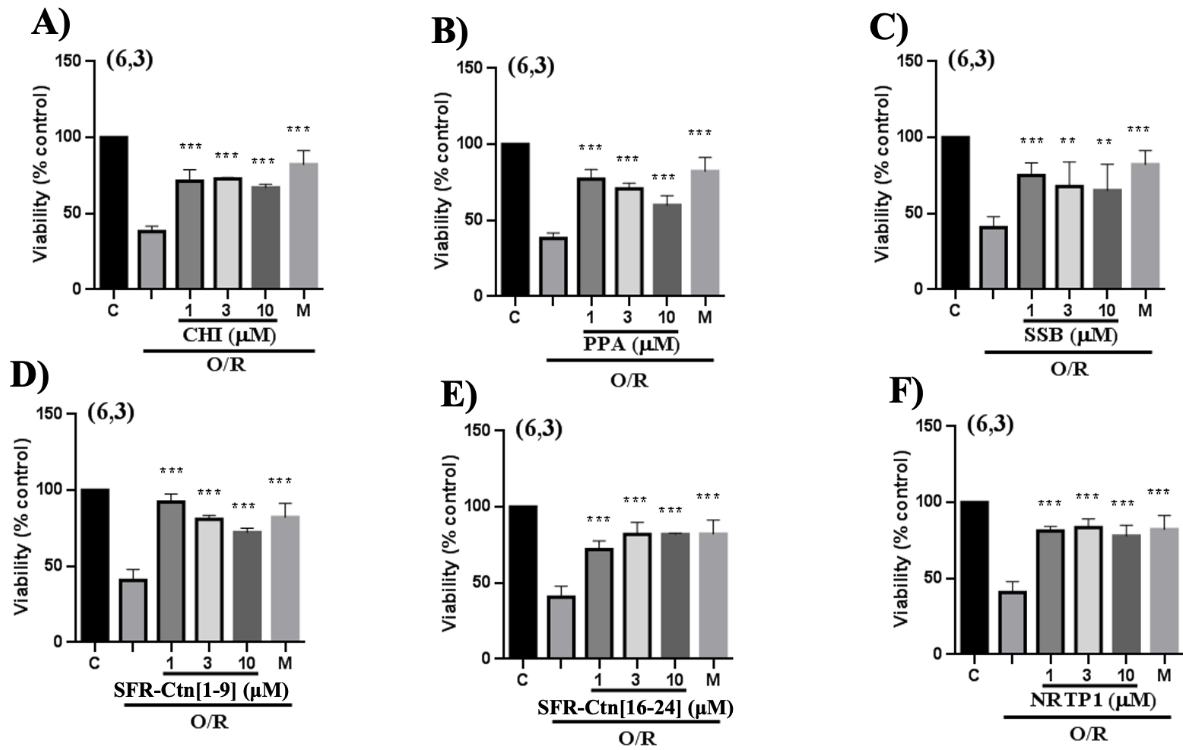

Figure 3. Peptides CHI, PPA, SSB, SFR-Ctn[1-9], SFR-Ctn[16-24], and NRTP1 protected against the neurotoxic effects of veratridine (Ver). (A) Experimental protocol consisting of two pre-incubation periods of 24 hours with the peptides at concentrations of 1, 3, and 10 µM, followed by a co-incubation period of 24 hours with the same peptides and veratridine. Statistical differences were determined in relation to the neuronal damage caused by veratridine using a one-way analysis of variance (ANOVA) test. Results were considered statistically significant when P ≤ 0.05*, P ≤ 0.01**, P ≤ 0.001***.

Peptides	**Cytoprotection (%)**					
	Control	**O/R**	**MLT**	**1** µM	**3** µM	**10** µM
Chi-aSVP	100	39	82	71	73	67
Ppa-aSVP				77	71	60
SSb-aSVP				75	68	65
SFR-Ctn[1-9]				91	81	72
SFR-Ctn[16-24]				72	82	82
NrTP-1				81	84	78

Table 4: Cytoprotection in SH-SY5Y cells against mitochondrial stress induced by oligomycin A and rotenone (O/R). The values refer to treatment with peptides Chi-aSVP, Ppa-aSVP, SSb-aSVP, SFRCtn[1-9], SFR-Ctn[16-24], and NrTP-1 at concentrations of 1, 3, and 10 μM, or melatonin (MLT). The data represents the mean values obtained in triplicates.

Time- and concentration-dependent blockade of voltage-dependent Ca^{2+} channel by Peptides

Chromaffin cells have been used to evaluate mechanisms related to calcium channels. These cells secrete catecholamines in response to the activation of specific calcium channels (LUKYANETZ, NEHER, 1999). In bovine chromaffin cells, the subtypes of P/Q channels (Cav2.2) represent 30 to 50%, N channels represent 25 to 30%, and L channels represent 15 to 20% (GARCÍA et al., 2006).It is worth noting that bovine adrenal medulla chromaffin cells have a highly cholinergic innervation and are sensitive to catecholamines, making them a suitable model for reproducing and evaluating action potential (DIEGO, 2010).

Depolarization tests (Figure 4) were performed on bovine chromaffin cells under constant voltage, where pulses ranging from 50 ms to 0 mV were applied with a time interval of 10 s, starting from a resting potential of -80 mV. To stimulate the internal calcium current, 10 mM of Ca^{2+} was used as a charge carrier. Once the cells were stabilized, they were perfused with coral-derived peptides (CHI, PPA, SSB) or snake-derived peptides (SFR-Ctn[1-9], SFR-Ctn[16-24], and NRTP1) for a period of 1 minute. To reduce data variations, the initial currents of each analyzed cell were normalized. The results in the following figures show the effects of different peptides and concentrations on the blockade of calcium channels. It can be observedthat snake peptides did not exhibit significant effects on calcium channels. When analyzing the effects of marine peptides, it is noticeable that these peptides displayed a dose-dependent blockade along with partial recovery of the cell upon compound removal. On average, these peptides blocked Ca^{2+} currents at a concentration of 10 μM by approximately 65-70% (CHI, PPPA, and SBB, $p \leq 0.001$), while with NrTP1 and Ctn-derived peptides, only SFR-Ctn[1-9] achieved a blockade of approximately 70%. The remaining substances did not show significant blocking effects.

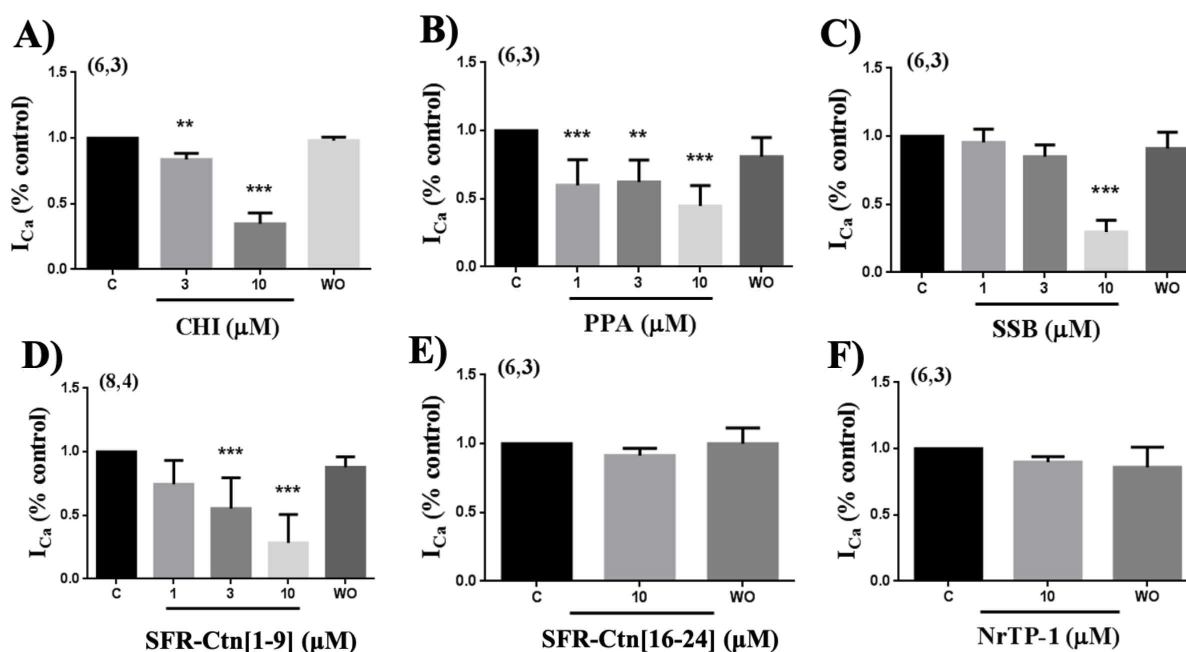

Figure 4. **Time- and concentration-dependent Blockade of ICa by peptides**

The graphs represent the peak currents initially obtained with 10 mM Ca^{2+} as the charge carrier. Once ICa was stabilized, cells were continuously superfused with the concentrations of CHI, PPA, SSB, SFR-Ctn[19], SFR-Ctn[16-24], and NRTP1 shown in A, B, C, D, E, and F. An isolated bovine chromaffin cell was used for each individual drug concentration. The graph data were normalized to the mean value of the control period and expressed as the mean ± S.E.M. of 7 experiments.

Peptides	**Blocked Ca+ Channel (%)**			**Type Channel**
	1 µM	3 µM	10µM	
Chi-aSVP	16,4%	25,9%	65,3%	Cav2.1 a1a
Ppa-aSVP	40,3%	37,75%	55,4%	Cav2.1 a1a
SSb-aSVP	4,6%	15,1%	70,4%	Cav2.1 a1a
SFR-Ctn[1-9]	-	-	10,1%	No block
SFR-Ctn[16-24]	25,5%	44,8%	71,8%	Cav2.1 a1a
NrTP-1	-	-	8,7%	No block

Table 5. Percentage of ICa channel block by the peptides. The values correspond to patch clamp tests conducted on single cells with Chi-aSVP, Ppa-aSVP, SSb-aSVP, SFRCtn[1-9], SFR-

Ctn[16-24], and NrTP-1 at concentrations of 1, 3, and 10 μM. The data represents the mean values obtained from 7 experiments.

Sodium currents INa are affected by peptides

As demonstrated in Figure 5, each chromaffin cell under voltage-clamp conditions received depolarization stimuli ranging from 10 ms to -10 mV with 10 intervals and a resting potential of -80 mV. During the testing period, no significant decline was observed in this current. In cases where a decline was observed, the corresponding cell was excluded from the analysis. Once the current reached stability, the cell was exposed to the peptides and perfused for 1 minute. Figure 5 (A, B, and C) shows the average data of sodium currents after perfusion with coral-derived peptides CHI, PPA, and SSB at different concentrations (1, 3, and 10 μM). Unlike the terrestrial peptides, the marine peptides demonstrated a considerable effect on this current, indicating that these peptides have a greater impact on sodium channels. Among all these peptides, CHI showed the highest blockade, with its highest concentration reducing sodium currents by 80%. Bovine chromaffin cells perfused for 1 minute with SSB and PPA blocked INa currents by 72% and 60%, respectively, at the highest concentration. None of the synthetic peptides derived from *C. durissus terrificus* venom peptides showed statistical significance greater than $P \leq 0.01$, remaining in the range of 25-31% blockade (SFR-Ctn[16-24] and SFR-Ctn[1-9], respectively) (Figure 5 D, E, and F).

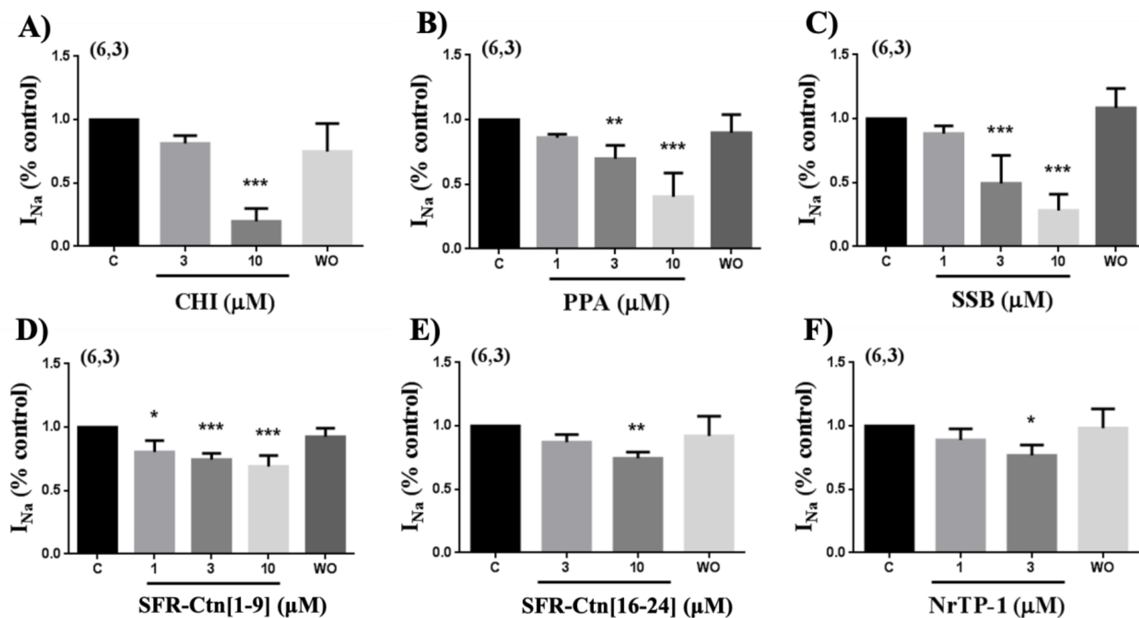

Figure 5. Depolarization test pulses of 10 ms to -10 mV from a holding potential of -80 mV were applied at 10 s intervals. The graphs represent the traces obtained under control condition and at the end of perfusion with a concentration of CHI, PPA, SSB, SFR-Ctn[19], SFR-Ctn[16-24], and NRTP1. Average results of the effects exerted by increasing peptide concentrations after 1 min of perfusion compared to control (INa measured immediately before introduction of the experimental medium). An isolated bovine chromaffin cell was used for each individual drug concentration. The graph data were normalized to the mean value of the control period and expressed as the mean ± S.E.M. of 7 experiments.

Peptides	**Blocked Channel (%)**		
	1 µM	3 µM	10 µM
CHI	13,6%	18,8%	77,6%
PPA	14 %	30,3%	59,8%
SSB	11,6%	50,8%	71,8%
NRTP1	11,1%	23,3%	-
SFR-Ctn[1-9]	19,4%	25,4%	31%
SFR-Ctn[16-24]	-	12,8%	25,6%

Table 6. Percentage values of the blockage of INA channels by the peptides. The data refers to patch clamp tests conducted on single cells with peptides Chi-aSVP, Ppa-aSVP, SSb-aSVP, SFRCtn[1-9], SFR-Ctn[16-24], and NrTP-1 at concentrations of 1, 3, and 10 µM. The data represents the mean values obtained from 7 experiments.

Peptides are inactive on potassium currents IKv

Bovine chromaffin cells and other cell types play a fundamental role in repolarization and action potential-related activities, with the essential involvement of K^+ ions (Solano et al., 1995; Lingle et al., 2018). In bovine chromaffin cells, K^+ currents are related to Ca^{2+}/voltage-dependent K^+ channels (IkvCa) and voltage-dependent K^+ channels (Ikv). (PLATT et al., 1995). In bovine adrenal cells, little is known about the specific subtypes of potassium channels that are found in these cells. In this study, tests were performed with peptides derived from *C. durissus terrificus* and *P. variabilis* (Figure 6) to evaluate their effect on voltage-dependent currents (IKv). To activate IKv currents, cells were subjected to 100 ms depolarizations to +120

mV from a resting potential of -80 mV, with 30 s intervals, in order to avoid the activation of Ca^{2+}-dependent potassium channels. After stabilization of chromaffin cells, increasing concentrations of Chi, PPA, SSB, SFR-Ctn[1-9], SFR-Ctn[16-24], and NRTP1 peptides were applied. Patch-clamp analyses revealed that all peptides did not show a significant blockade of IKv currents, except for SSB. At this concentration of 10 µM, SSB blocked this current by 30% ($p \leq 0.001$), with a slight recovery of the cell after the washout period.

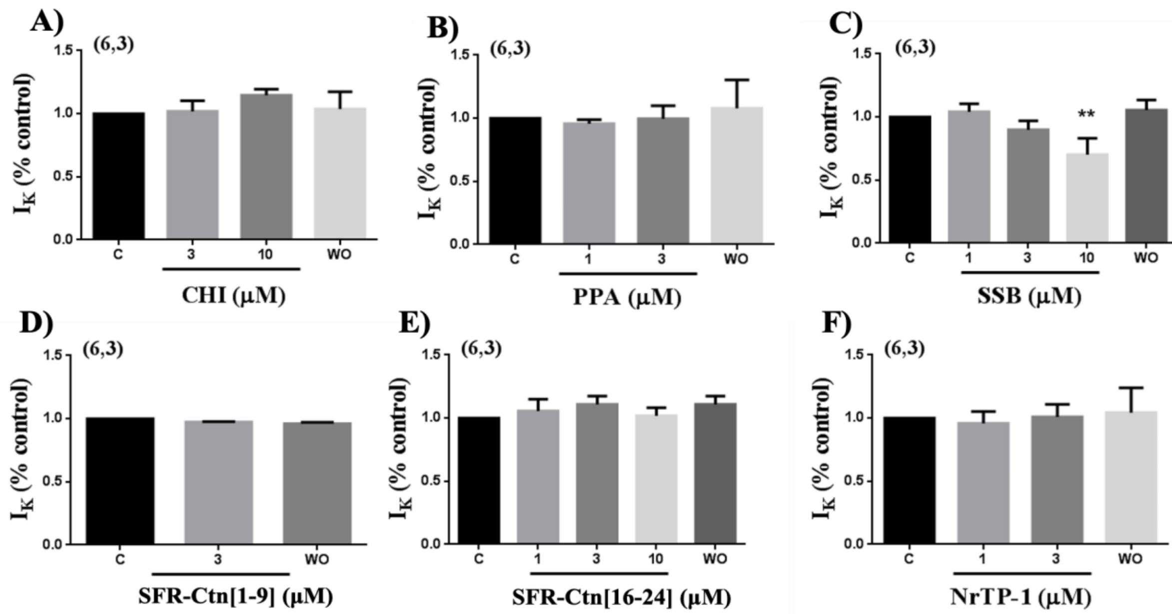

Figure 6. Partial inhibition of voltage-dependent K+ currents by CHI, PPA, SSB, SFRCtn[1-9], SFR-Ctn[16-24], and NRTP1. Graphs corresponding to the control condition and after 1 minute perfusion with the peptides. An isolated bovine chromaffin cell was used for each individual drug concentration. The graph data were normalized to the mean value of the control period and expressed as the mean ± S.E.M. of eight cells.

Peptides	**Blocked Channel (%)**		
	1 µM	3 µM	10 µM
CHI	+1,9%	+14,6%	+3,8%
PPA	4,3%	0,5%	-
SSB	+0,4%	10,2%	29,8%
NRTP1	4,1%	0%	-
SFR-Ctn[1-9]	-	2,6%	-
SFR-Ctn[16-24]	+0,55%	+1,7%	+1,8%

Table 7. Percentage values of the blockage of voltage-dependent potassium channels (Ikv) by the peptides. The data refers to patch clamp tests conducted on single cells with peptides Chi-aSVP, Ppa-aSVP, SSb-aSVP, SFRCtn[1-9], SFR-Ctn[16-24], and NrTP-1 at concentrations of1, 3, and 10 µM. The data represents the mean values obtained from 7 experiment. Molecular docking calculations

Molecular docking calculations

To have a molecular view of the interaction between the natural peptides with the human Na+- and Ca2+-channels, in silico calculations via blind docking were carried out at pH 7.4. In this case, was not in silico assayed the K+-channel due to the experimental data that indicated this channel was not the main target for the synthetic peptides. Since each pose obtained by GOLD 2022.3 software is considered the negative value of the sum of energy terms from mechanical-molecular type components, a more positive docking score value indicates better interaction. The docking score value (dimensionless) for the interaction between Na+-channel with Chi-aSVP (CHI), Ppa-aSVP (PPA), SSb-aSVP (SSB), and NrTP-1 was 86.1, 115.8, 112.7, and 122.5, respectively, while the corresponding for Ca2+-channel was 56.9, 91.8, 65.9, and 94.5, suggesting that Na+-channel is the main target for these synthetic peptides, corroborating with the experimental trend. Additionally, the last three peptides presented similar docking score values agreeing with the similarities in the inhibitory trend.

Despite the 3D structures of Na+- and Ca2+-channels having a high superposition with RMSD value of 2.364 dimensionless (Figure 7), these targets presented a significant difference in the docking score values, probably due to the differences in the electrostatic potential map of the channels that might influence in the interactive profile of the peptide (Figure 7). This was in silico evidenced through the differences of the binding poses, i.e., the natural peptides mainly interact into the surface of the voltage-sensor domains of Na+- channel (Figure 8), following the reported in silico results for the Palythoa neurotoxin (LIAO et al., 2014), while for Ca2+-channel, the synthetic peptides interact with the extracellular loops (ECLs) (Figure 9). For both channels the main intermolecular forces responsible for the stabilization of the association channels-peptides is hydrogen bonding and hydrophobic interactions.

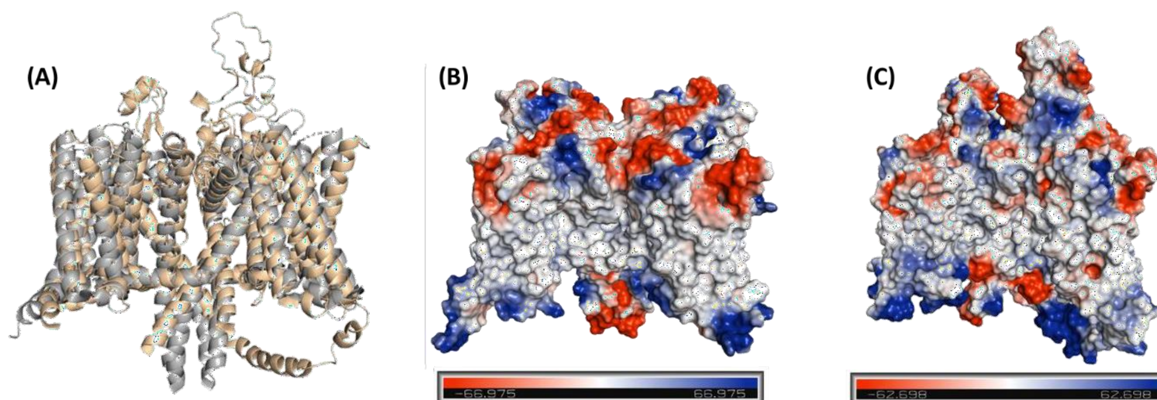

Figure 7. (A) Superposition of the crystallographic structure of the human Na^{+}- and Ca^{2+}-channels (PDB codes 5EK0 and 7WLJ, respectively with the corresponding colors gray and orange). The electrostatic potential map for the (B) Na^{+}-channel and (C) Ca^{2+}-channel at pH 7.4.

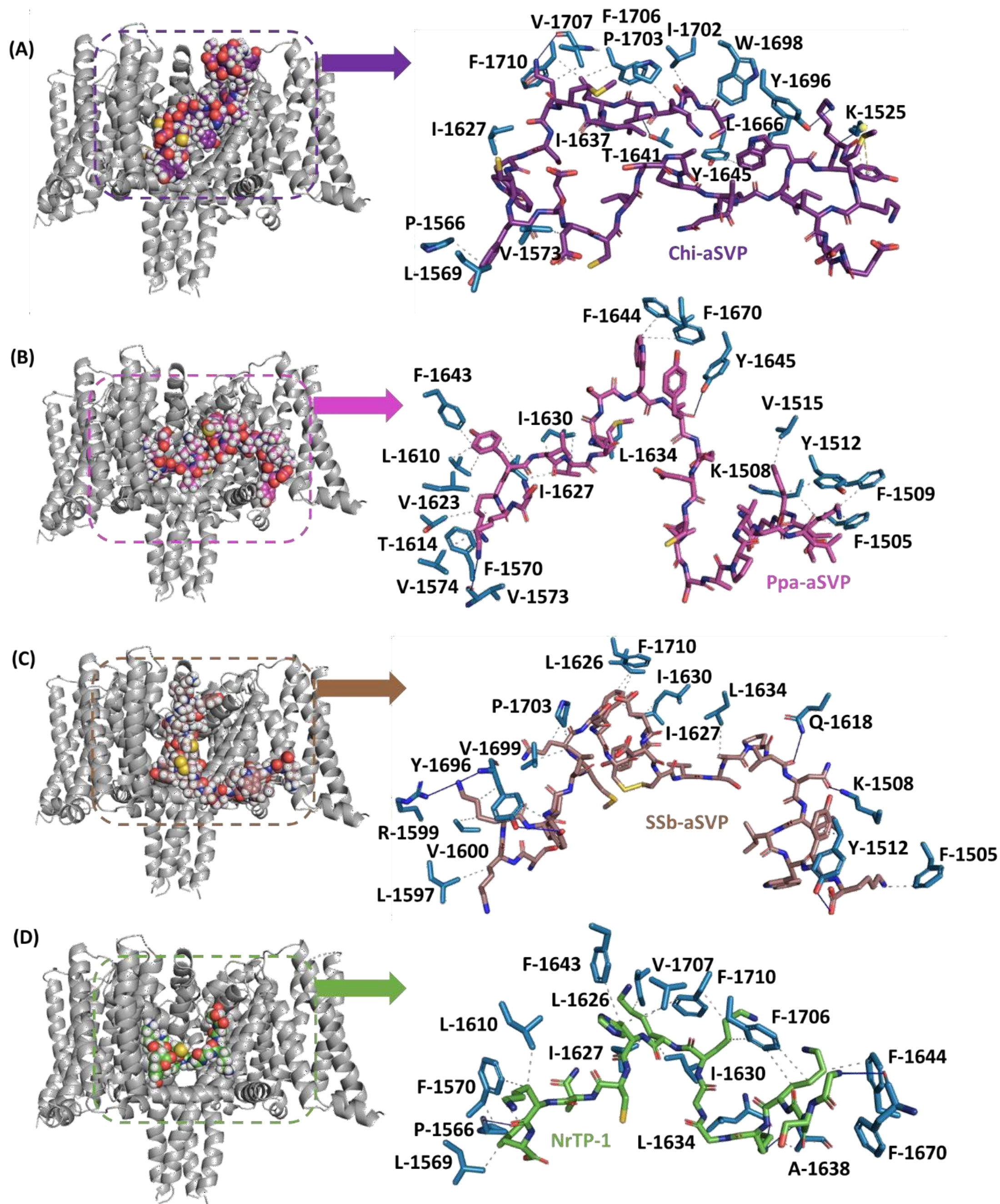

Figure 8. Best docking pose with the corresponding zoom representation for the interaction between the Na^{+}-channel with the natural peptides (A) Chi-aSVP, (B) Ppa-aSVP, (C) SSb-aSVP, and (D) NrTP-1 at pH 7.4. Selected amino acid residues, Chi-aSVP, Ppa-aSVP, SSb-aSVP, and NrTP-1 are in stick representation in duck blue, purple, pink, brown, and green, respectively. Elements' color: oxygen: red, sulfur: yellow, and nitrogen: dark blue. For better interpretation all hydrogen atoms were omitted. The hydrophobic interactions and hydrogen bonding were represented as black dots and blue lines, respectively.

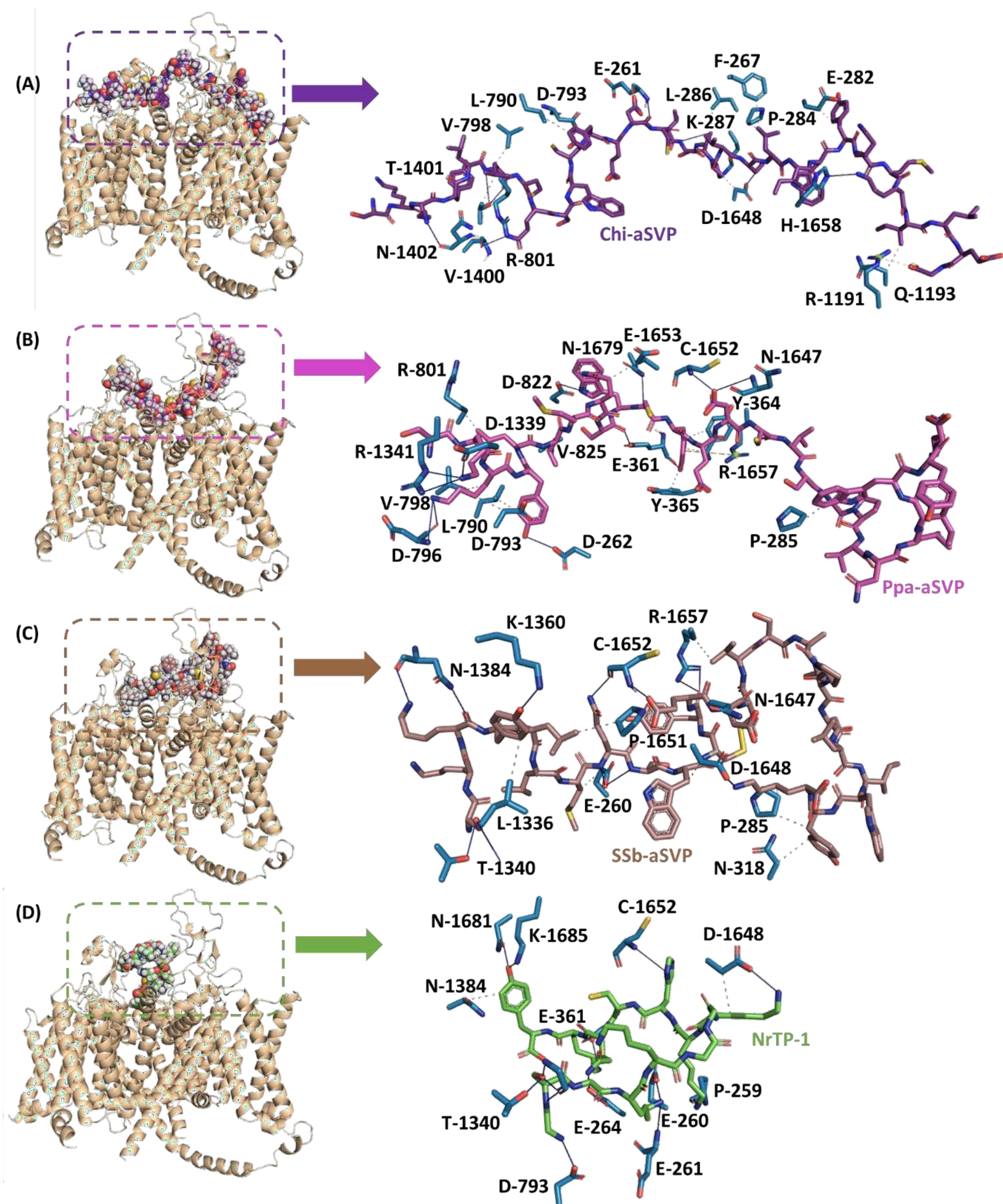

Figure 9. Best docking pose with the corresponding zoom representation for the interaction between the Ca^{2+}-channel with the natural peptides (A) Chi-aSVP, (B) Ppa-aSVP, (C) SSbaSVP, and (D) NrTP-1 at pH 7.4. Selected amino acid residues, Chi-aSVP, Ppa-aSVP, SSbaSVP, and NrTP-1 are in stick representation in duck blue, purple, pink, brown, and green, respectively. Elements' color: oxygen: red, sulfur: yellow, and nitrogen: dark blue. For better interpretation all hydrogen atoms were omitted. The hydrophobic interactions and hydrogen bonding were represented as black dots and blue lines, respectively.

DISCUSSION

There has been a steady increase in neurodegenerative diseases in recent years. Neurodegenerative disorders directly compromise the physical health and the quality of life of affected individuals. The medications currently available on the market are mainly to alleviate or delay the progression of symptoms. In this scenario, research related to improve the arsenal of therapies through more effective and specific manner to treat these disroders have been intensified. Here, we investigated the electrophysiological and cytoprotective profile of synthetic peptides derived from venom peptides of Protopalythoa coral and a rattlesnake.

The process of neurotoxicity is related to the excessive release of glutamate or inadequate removal of synaptic glutamate, resulting in overstimulation of AMPA and NMDA receptors, overload of calcium and sodium ions, and subsequent cell death (LAU, TYMIANSKI, 2010; ZUNDORF, REISER, 2011). Cells in culture were exposed to veratridine, a neurotoxic alkaloid agent that leads to sensitization and cell death in different models, including bovine chromaffin cells (MAROTO et al., 1994; NICOLAU et al., 2009). In vivo, this toxic substance acts by opening sodium channels to increase the influx of sodium ions. This effect leads to lymphatic vessel depolarization, increased smooth muscle contraction, and catecholamine secretion. Tetradotoxin is a specific blocker of sodium channels Na+ (NaV) that acts under the influence of veratridine when this toxin stimulates the opening of Na+ channels, thus increasing calcium accumulation inside the cells (CAMPOS et al., 2004; NEMOTO et al., 2013). Voltage-dependent sodium channels are organized into nine subtypes, namely Nav1.1 to Nav1.9. Certain channels are resistant to sodium channel blockers. For example, Nav1.5, Nav1.8, and Nav1.9 channels are resistant to TTX, while being able to block other channels (WOOD et al., 2004; ZHANG et al., 2018). Here we demonstrate that peptides from P. variabilis and rattlesnake exhibit cytoprotective action under the influence of veratridine, similar to the blocking action of TTX. These results suggest that the peptides possibly possess a mechanism similar to TTX, acting as blockers of sodium channels. Although there are nine subtypes of Na+ channels, bovine chromaffin cells contain only two subtypes: Nav1.3 and Nav1.7 (VANDAEL et al., 2015). The Nav1.7 subtype is considered the most plausible target for the action of peptides and other substances, both in voltage clamp studies and in cytoprotection, due to its higher concentration in neuroendocrine cells. On the other hand, Nav1.3 is present in peripheral neurons, and bovine chromaffin cells do not possess more than one subtype. Therefore, it is believed that in bovine chromaffin cells, the blockade of sodium channels occurs primarily through Nav1.7.

The organic toxins oligomycin and rotenone, when combined, can inhibit the respiratory chain coupled with phosphorylation in the mitochondria and disrupt the cellular metabolism. This occurs because oligomycin inhibits ATP synthase, interfering with ATP production in the mitochondria. Meanwhile, rotenone is known for being an inhibitor of complex I in the mitochondrial respiratory chain. Both toxins act on the mitochondria, disrupting ATP production, which leads to an increase in the production of reactive oxygen species (ROS) (PubChem Compound Database, 2021). Free radicals, one of the byproducts of ROS, can lead to oxidative stress, resulting in cellular damage. In this study, it was observed that crotamine- and crotalicidin-derived peptides (NrTP-1 and Ctn[1-9]), exhibited an antioxidant effect, acting in a dose-dependent manner and reversing the damage caused by oligomycin and rotenone, reversing the blockade in mitochondrial complexes I and V. Other peptides from the same species exhibited an anti-inflammatory and antioxidant profile against different inflammatory agents, likely due to the secretion of anti-inflammatory cytokines (CRUZ et al., 2018; SARTIM, MENALDO, SAMPAIO, 2018).

The toxins from P. variabilis exhibited a pharmacological profile that indicates their ability to reverse the opening of sodium channels and mitochondrial damage. This effect may be intrinsically linked to the fact that both peptides influence the nuclear factor erythroid 2-related factor (Nrf2) signaling pathway and the mitogen-activated protein kinase (MAPK) pathway, both of which have antioxidant actions (BANERJEE et al., 2021). Previously, it was reported that homologous peptides isolated from the parent species Palythoa caribaeorum suppressed the overproduction of reactive oxygen species (ROS), while Nrf2 and other antioxidants such as HO-1 and NQO1 accumulated in the cytoplasmic medium, whereas Keap1 was significantly reduced (WANG et al., 2022). Nrf2 functions by inducing enzymes and molecules with antioxidant action, while MAP kinases, including p38MAPK and ERK, regulate genes that act in cellular oxidative response (KYRIAKIS, AVRUCH, 2012; SAHA et al., 2022). Furthermore, a reversal of the neurotoxic effects caused by 6-hydroxydopamine (6- OHDA) was observed, wherein it prevented the production of oxidative stress byproducts and consequently reduced the loss of dopaminergic neurons (LIAO et al., 2018).

Furthermore, we found that marine peptides are cytotoxic and maintain cytoprotectiveand antioxidant action even under the influence of different toxins. These peptides showed reduction in their activities at a concentration of 12.5 μM (LIAO et al., 2019). It is evident that the production of reactive species leads to oxidative stress, which in turn disrupts ion channel and has been associated with various neurodegenerative diseases. Reactive oxygen species, through the oxidation of cysteine residues, alter the functionality of ion

channels, leading to an imbalance in ion homeostasis (KYSELYOV, MUALLEM, 2016). This study investigated the intracellular and electrophysiological impact of the peptides derived from venom peptides of *Protopalythoa variabilis* (CHI, PPA, and SSB) and *Crotalus durissus terrificus* (SFR-Ctn[1- 9], SFR-Ctn[16-24], NRFTP1).

Marine organisms have emerged as an important source of bioactive compounds. Marine peptides derived from marine organisms have become a notable source of bioactive compounds in recent years (BLUNT et al., 2015). The species P. variabilis is still poorly studied in relation to components with neurotoxic activities. Transcriptomic analysis identified proteins from different toxin families, including neuropeptides, which act as ion channel blockers, inhibitors of nicotinic and muscarinic receptors, as well as hemostatic and hemorrhagic toxins, pore-forming proteins, protease inhibitors, and proteins with mixed functions (HUANG et al., 2016; MORLIGHEM et al., 2018). Electrophysiological analyses show that sodium channels appear to be strongly influenced by these peptides, although there is a dose-dependent blockage observed in calcium channels, the blockage in Na+ channels is more evident.

It has become evident that Nav channels play a significant role in neuroinflammation and neurodegeneration. This is because nitric oxide, one of the byproducts of reactive oxygen species, among other factors, causes dysregulation in the function of the Na+/K+-ATPase (NKA) pump (NKA), leading to depolarization and the entry of calcium into the cell. The NKA enzyme is critical for the proper functioning of membrane potential and transmembrane flow of Ca2+ in excitatory neurons (ALBERS et al., 2012). The imbalance in Na+/K+-ATPase activity leads to the activation of the internal Na+ current, mediated by Nav 1.6, and subsequent exacerbated accumulation of this ion within the axon (PIVOVAROV et al., 2019). The overload of Na+ causes the Na+/Ca2+ pump to transfer excessive calcium into the axons, resulting in a series of damages (LEE et al., 2007). Thus, in order to maintain a cytoplasmic concentration of Ca2+, it is necessary to block Na+ influx.

Computational analyses have confirmed a strong binding of CHI, PPA, and SSB to NaV1.7 channels, which is consistent with the electrophysiological analyses conducted in this study. SSB has shown a stronger interaction than PPA, and the same pattern was observed in the electrophysiological currents for both peptides. The difference between these peptides lies in their disulfide bridges. SSB, which contains an S-S bridge, demonstrated higher efficacy than the linear peptide PPA. Disulfide bridges are commonly found in peptide toxins and have pharmacological advantages for the peptide stability and bioavailability. Although less effective, the marine peptides studied here still display a nearly 50% blockade of calcium

compared to the baseline. Analogues of *Protopalythoa variabilis* have also been found to block intracellular calcium influx mediated by capsaicin and various genes associated with calcium signaling (WANG et al., 2021).

Different toxins derived from animals have the ability to modulate T cell activity and selectively act on voltage-gated ion channels in T lymphocytes (Kv1.3) and calcium-activated channels Ca2+ (IKCa1) (CHANDY et al., 2004; OLAMENDI-PORTUGAL et al., 2005). Thus, the selective inhibition of these ion channels has emerged as potential immunosuppressive compounds (HARTZELL et al., 2016). In this study, we obtained an electrophysiological profile of venom peptides derived from crotamine and crotalicidin of *Crotalus durissus terrificus* and observed that these short peptides exhibit a higher affinity for calcium channels, although no significant difference was observed between them. One of the main structural characteristics of crotalicidin and other vipericidins is their tandem repeat of 9 amino acid residues Ctn-[1-9]-(1KRFKKFFKK9) and Ctn-[16-24]-(16KRLKKIFKK24) (BANDEIRA etal., 2018). The difference between these two repetitive sequences is the conservative replacement of the Phe for a Leu in position 3 and a Phe for Ile in position 5 of Ctn[16-24] Ctn[1-9] appears to indeed act on calcium channels when covalently coupled to rhodamine B by increasing the concentration of intracellular calcium (WANG et al., 2015). Furthermore, such a peptide (RhoB-Ctn[1-9]) enhanced at certain level the embryo activation in the absence of spermatozoa (SANTOS et al., 2023, submitted). In *in vitro* fertilization, the sperm activates intracellular calcium flow upon fertilizing the egg. In partenogenetic activation, the intracellular flux of calcium ions is required for embryo cleavage and development.Here, we report for the first time that these cathelicidin-dervied peptides (SFR-Ctn[1-9] and SFR- Ctn[16-24]) modulate by blocking calcium channels in a dose-dependent manner. However, As observed by electrophysiological analyses using the patch clamp technique, there were no significant differences in the binding of calcium channels and SFR-Ctn[1-9] and SFR-Ctn[16- 24], likely due to the fact that both peptides have similar conformations. The other cathelicidin NRTP1 showed a stronger interaction with sodium channels than with Ca2+. NrTP-1 is a derivative of crotamine belonging to the class of cell-penetrating peptides (CPPs) and it is the synthetic product of splicing the N-terminal regions of residues 1-9 and the C-terminal regions of residues 38-42. NrTPs exhibit improvements in various aspects, including reduced toxicity and the ability to transport high molecular weight molecules into mammalian cells (RADIS-BAPTISTA, DE LA TORRE, ANDREU, 2012). Previous studies indicated that full-size crotamine (34 amino acid residues) could act through voltage-gated sodium and potassium channels (RIZZI et al., 2007; PEIGNEUR et al., 2012.). As a result of crotamine injection, in

micr, it cause hindlimb paralysis. It is known that skeletical muscle have a higher density of sodium channels. Here, patch clamp tests did not show any effect of the crotamine-fragment NrTP1 on sodium channels (Nav1.1-Nav1.6). Our results did not demonstrate a significant effect on these channels. However, molecular docking showed that this short crotamine fragment, i.e., NrTP-1, can strongly interact with calcium channels.

Here, we conducted a screening with synthetic peptides derived from venom peptides from a marine animal (the mat coral Protopalythoa) and a rattlesnake terrestrial animal (rattlesnake). These two organisms from distinct ecosystems provide us with a multitude of substances to be evaluated and investigated for interacting with pharmacologicaly important targets, particularly, ion channels involved in several chronic disorders. In this study, it was observed that synthetic peptides designed from *Protopalythoa variabilis* neurotoxic peptide displayed a significant effect on sodium channels. It is known that many neurodegenerative diseases involve imbalances in sodium channels. Additionally, the partial blockade of cathelicidin-derived peptides indicates that these peptides may act through an alternative therapeutic pathway, such as in cardiac diseases where calcium channel disturbances are involved. This study opens the door for future research involving these designed peptides toward the improvement of neurodegenerative diseases and channelopathies.

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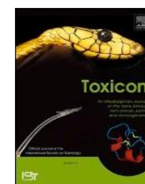

The anti-infective crotalicidin peptide analog RhoB-Ctn[1–9] is harmless to bovine oocytes and able to induce parthenogenesis in vitro

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In vitro parthenogenesis

ABSTRACT

Crotalicidin is a cathelicidin-related anti-infective (antimicrobial) peptide expressed in the venom glands of the South American rattlesnake *Crotalus durissus terrificus.* Congener peptides of crotalicidin, named vipericidins, are found in other pit vipers inhabiting South America. Crotalicidin is active against bacteria and pathogenic yeasts and has anti-proliferative activity for some cancer cells. The structural dissection of crotalicidin produced fragments (e.g., Ctn [15–34]) with multiple biological functionalities that mimic the native peptide. Another structural characteristic of crotalidicin and congeners is a unique repetitive stretch of amino acid sequences in tandem embedded in their primary structures. One of the encrypted vipericidn peptides (Ctn [1–9]) was synthesized, and the analog covalently conjugated with rhodamine B (RhoB-Ctn [1–9]) displayed considerable antimicrobial activity and selective cytotoxicity. Methods to evaluate antimicrobial peptides' toxicity include lysis of red blood cells (hemolysis) *in vitro* and cytotoxicity of healthy cultured cells (e.g., fibroblasts). Here, as a non-conventional model of toxicity, the bovine oocytes were exposed to two standardized concentrations of RhoB-Ctn [1–9], and embryo viability and development at its first stage of cleavage (division of cells) and blastocyst formation were evaluated. Oocytes treated with peptide at 10 and 40 μM induced cleavage rates of 44.94% and 51.53%, resulting in the formation of blastocysts of 7.07% and 11.73%, respectively. Light sheet microscopy and *in silico* prediction analysis indicated that RhoB-Ctn [1–9] peptide interacts with zona pellucida and internalizes into bovine oocytes and developing embryos. The ADMET prediction estimated good bioavailability of RhoB-Ctn [1–9]. In conclusion, the peptide appeared harmless to bovine oocytes and, remarkably, activated the parthenogenesis in vitro.

1. Introduction

Nature has a fantastic biodiversity with a handful of biomes, i.e., major ecological communities, with thousands of unique species potentially useful for drug discovery. Among the diverse chemical classes of biologically active compounds produced by living organisms, peptide toxins are significant since they offer a range of opportunities for the research and development of pharmaceuticals. Toxins from vertebrate and invertebrate animals play an essential role in the evolutionary history of organisms of different taxa (Yacoub et al., 2020). Venomous animals like snakes have complex toxin cocktails containing hundreds of structurally and functionally diverse molecules with particular biological activities (Oliveira et al., 2022). The use of enzymes and polypeptides for clinical and diagnostic purposes from animal venoms has

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attracted much interest due to their high selectivity and specificity for numerous molecular targets (Fox and Serrano, 2007; Herzig et al., 2020). Potency and target specificities are intrinsic properties of animal toxins that make them excellent drug leads for developing therapeutics (Fischer and Riedl, 2022). Thus, the pace of discovery in Toxinology is a hot topic in biological and natural product chemistry and life sciences.

Crotalicidin is a cathelicidin-related anti-infective (antimicrobial) peptide from the South American rattlesnake *Crotalus durissus terrificus* expressed in the venom glands, and it is active against bacteria and pathogenic yeasts. Crotalicidin and congener peptides from South American pit vipers belong to the group of so-called vipericidins (Falcao et al., 2014). Crotalicidin is linear, consisting of thirty-four amino acid residues and adopting a helical conformation in solution (Falcao et al., 2015). The structural dissection of crotalicidin through peptide design and synthesis produced fragments named Ctn [1–14] and Ctn [15–34] with variable biological activity compared with the native peptide (Aguiar et al., 2020; Cavalcante et al., 2017; de Aguiar et al., 2020; Pérez-Peinado et al., 2020). The fragment Ctn [15–34] preserved the antimicrobial and anti-proliferative activity of crotalicidin but is less hemolytic and cytotoxic to healthy cells (Falcao et al., 2015; Falcao and Radis-Baptista, 2020). Another primary structure particularity of crotalicidin, other vipericidins and Elapid venom cathelicidins is a unique repetitive stretch of amino acid sequences in tandem (Amer et al., 2010; de Latour et al., 2010; Falcao et al., 2014). The vipericidins' nonapeptide and analogs, derived from these repetitive stretches, were also synthesized and studied, as in the case of the encrypted vipericidin peptide (Ctn [1–9]); rhodamine B-conjugated peptide (RhoB-Ctn [1–9]), but not unlabeled peptide can kill breast cancer cells in vitro (Wang et al., 2015). The cytotoxic mechanism toward unhealthy cells appeared to involve the release of intracellular calcium. Despite these exciting findings, RhoB-Ctn [1–9] was toxic to zebrafish larvae (Wang et al., 2015). Against pathogenic yeast and drug-resistant bacteria, RhoB-Ctn [1–9] was efficient, and its activity was synergic with conventional antibiotics of clinical use (Lima et al., 2022).

As an estimate of cytotoxicity associated with biologically active peptides, particularly antimicrobial peptides, the *in vitro* level of hemolysis of red blood cells from numerous species usually is evaluated (Ruiz et al., 2014). In this respect, RhoB-Ctn [1–9] showed low to negligible cytotoxicity to human erythrocytes (Lima et al., 2022). Although *in vitro* hemolytic activity and cytotoxicity might correlate with peptide target selectivity *in vivo* and, consequently, with the therapeutic index in the development of antimicrobial peptides for clinical purposes (Greco et al., 2020), *in vivo* tests and assessment of other potential adverse physiological effects should be considered. Hence, it was interesting to investigate the biological effects of RhoB-Ctn [1–9] in bovine oocytes as a non-conventional toxicity model to confirm peptide safety in mammalian systems. In this regard, bovine oocytes have been used to test the action of chemicals as a model for reproductive toxicity screening (Asimaki et al., 2022). A mature, good-quality oocyte is identified by a spherical shape and a translucent, homogeneously colored cytoplasm. It is also accepted that good-quality oocytes are embedded in a well-expanded cumulus mass and surrounded by a radiant array of corona cell layers during ovulation (Coticchio et al., 2004). Since fertilization occurs only in structures in perfect morphological and functional conditions, mammalian oocytes can be used to assess reproductive toxicity (Chunjie et al., 2022; Yan et al., 2018).

This study reports the effect of RhoB-Ctn [1–9] on bovine oocytes in culture. RhoB-Ctn [1–9] was harmless to oocytes and can activate parthenogenesis *in vitro*. Additionally, light sheet microscopy analysis and *in silico* prediction of protein interaction indicated that RhoB-Ctn [1–9] binds to zona pellucida components and internalizes into the cells of the developing bovine embryo after translocation.

2. Material and methods

2.1. Chemicals and synthetic peptides

Unless otherwise indicated, all chemicals used in this study were from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA). The peptides were obtained by solid-phase peptide synthesis with a purity of $> 95\%$, as confirmed by mass spectrometry analysis (China Peptides Company, Ltd., Shanghai, China).

2.2. Recovery and in vitro maturation of the oocyte

Bovine ovaries were from a local slaughterhouse. After collection, ovaries were immediately transported to the laboratory in sterile NaCl solution (9 g/L), supplemented with antibiotics (Pentabiótico Veterinário, Fort Dodge, Campinas, Brazil). Seven hundred and ninety-six *cumulus*-oocyte complexes (COCs) were aspirated from follicles (2–8 mm) with an 18-gauge needle connected to a 10 mL disposable sterile syringe. COCs were inspected with a stereomicroscope (SMZ800, Nikon Co., Tokyo, Japan). Only COCs evaluated as classes I to III were selected for testing according to criteria previously determined for bovine (Leibfried and First, 1979). Class I COCs refer to complete cumulus cover with several compact cell layers; class II COCs have only partial cumulus cover, and class III has a darker cytoplasm indicative of follicular atresia. COCs classified as IV (denuded and irregular ooplasm) were discarded. For in vitro maturation, COCs were washed in TCM-199 medium with 10% (v/v) fetal bovine serum (Gibco, Grand Island, NY, USA), 20 μg/mL follicle-stimulating hormone, and luteinizing hormone (Pluset; Hertape-Calier, Spain), 0.2 mM sodium pyruvate, 0.1 mM cysteamine, 1 × antibiotic-antimycotic, 10 ng/mL epidermal growth factor, 1 μg/mL estradiol and 1 mM L-glutamine. COCs were incubated at 38.5 °C for 26 h in an atmosphere of 5% CO_2.

2.3. Oocyte activation and embryo culture

The in vitro matured COCs were stripped using a vortex for 2 min and selected by observing the first polar body. Then, 796 oocytes were distributed in four groups comprised of RhoB-Ctn [1–9] peptides at two different concentrations (10 and 40 μM), ionomycin (at 5 μM, positive control), and 6-Dimethylaminopurine (6-DMAP, negative control). In all groups, the oocyte exposure was for 4 min, followed by a 4 h-period of incubation in the presence of 6-DMAP (2 μM) in a synthetic oviductal fluid (SOF) medium. SOF medium contained: 0.1 M NaCl, 6 mM KCl, 2 mM $CaCl_2.2H_2O$, 1 mM KH_2PO_4, 1,5 mM $MgSO_4.7H_2O$, 20 mM $NaHCO_3$, 0.2 mM sodium pyruvate, 0.1% non-essential amino acid solution (MEM), 0.2% basal medium eagle (BME), 1x antibiotic-antimycotic, 0.8% BSA and 2.5% fetal bovine serum (Gibco, 37 Grand Island, NY, USA). After that, oocytes were washed three times in SOF medium and cultured for seven days in SOF medium at 38.5 °C, in a humid atmosphere of 5% CO_2, 5% O_2, and 90% N_2, under mineral oil. The oocyte cleavage and blastocyst formation rates were determined on the second and seventh day of incubation.

After 7 days of cell culture, the blastocysts obtained were removed and then washed in a drop of PBS 0.4% BSA and another drop of PBS + paraformaldehyde for 20 min in the latter. Soon after, the embryos were rinsed three times with a solution of PBS/BSA. Blastocysts were stained with 10 μM Hoechst 33,342 in PBS/BSA for 15 min at room temperature and then visualized in the light sheet microscopy (Fig. 1).

2.4. ADMET Predictor® in silico simulation

In silico physiological behavior of peptides and chemicals was analyzed to investigate their presumable ADMET (absorption, distribution, metabolism, and toxicity). The structures of chemicals and peptides

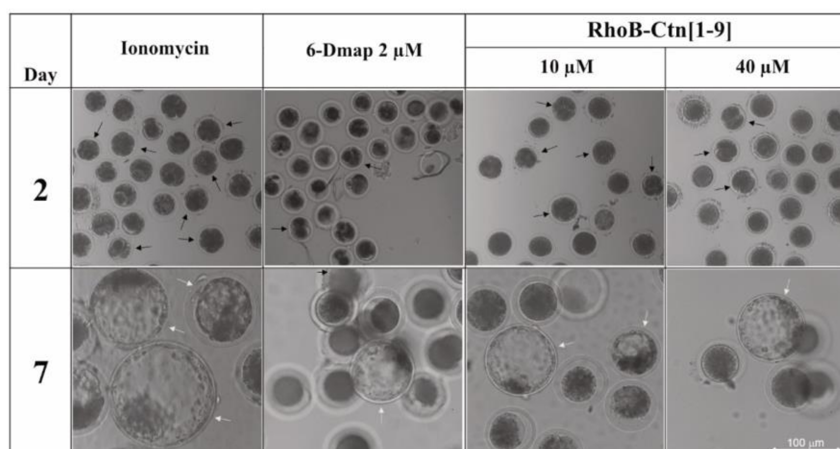

Fig. 1. Parthenogenetic activation effect of bovine oocytes after exposure to RhoB-Ctn [1–9], ionomycin, 6-DMAP, in the process of bovine embryo viability and development. Representative images of structures from the second and seventh days (2 and 7, in the figure) of culture after parthenogenetic activation (Magnification: 20×).

were designed using the MedChem DesignerTM software version 5.5 (Simulations Plus Inc., Lancaster, CA, USA) and analyzed in the ADMET Predictor® version 10.4 (Simulations Plus Inc., Lancaster, CA, USA). The ADMET Predictor® software simulates computational analyses according to the selected model. Data obtained were related to the lipophilicity (Mlog), toxicity (LD50, TD50, AST, ALT, tissue and respiratory toxicity), corneal and tissue permeability in rabbits, probability of crossing the blood-brain barrier (BBB), glycoprotein Substrate and inhibitory P, unbound fraction in human liver microsomes (S+fumic) and volume of distribution at steady state in humans (VD) and mouse model.

2.5. Light sheet microscopy analysis

For images of bovine oocytes and embryos, light sheet microscopy was used with the equipment Zeiss Lightsheet 7 (Carls Zeiss, Jena, Germany). Samples were analyzed with an average thickness of 1.44 µm in fast-track switch mode, 405 nm waveband, and magnification of 20 × with W Plan-Apochromat lens (water: 1.333). This wavelength matches the excitation maximum of rhodamine B fluorophore coupled to the peptide, and Hoechst 33,342, used for marking blastocysts. The entire oocyte and blastocyst image dataset was acquired under 5 × /0.1 foc and 10 × /0.2 foc optical illumination. Lighting was with a focused beam LED, with Axiocam 702 mono detection module, sCMOS, and pixel resolution 1216 × 1920, scale per pixel 0.38 µm × 0.34 µm x 0.34 µm. The central position of the image was x-230.90 µm and y-1.42 µm, and the z-stack generated 469 slices (161,419 µm). The microscope's ability to fuse from medium and maximum illumination to lateral illumination allows multidimensional imaging. The images were processed in light sheet seven Multiview Processing with the 3DXL software arivis Vision4D.

2.6. Molecular docking

The crystallographic structure of zona pellucida (ZP)-*N* domain of ZP3 protein, crystal form I (ZP3-ND) was from Protein Data Bank (PDB code 3D4C) (Monné et al., 2008). The *Bos taurus* (bovine) ZP2 protein structure model was obtained from AlphaFold Protein Structure Database (Jumper et al., 2021; Varadi et al., 2022). The chemical structures for RhoB-Ctn [1–9], ionomycin, and 6-DMAP were built, and the energy was minimized with density functional theory (DFT), method Becke-3-Lee Yang Parr (B3LYP) with standard 6-31G* basis set, available in the Spartan'18 software (Wavefunction, Inc., Irvine, CA, USA) (Shao et al., 2006).

The molecular docking calculations were with GOLD 2020.2 software (https://www.ccdc.cam.ac.uk/solutions/software/gold/) (Cambridge Crystallographic Data Center Software Ltd, Cambridge, UK) (Gareth et al., 1997; Hartshorn et al., 2007). Hydrogen atoms were added to the protein following tautomeric states and ionization data inferred by the software at pH 7.4. For ZP3-ND, a spherical cavity of 12 Å was used to explore all the protein structures in the docking calculations (blind docking). For comparison, the model of ZP2, a spherical cavity of 10 Å, was built in the center of the binding core to Ionomycin (Mikoshiba, 1997). The number of genetic operations (crossing, migration, mutation) during the search procedure was set at 100,000. *ChemPLP* was used as a scoring function, the default function of GOLD 2020.2 software. The figures of the docking predictions were generated with the PyMOL Delano Scientific LLC software (DeLano Scientific LLC: San Carlos, CA, USA) (Yuan et al., 2017).

2.7. Bioethics

All protocols used in the study were approved by the Committee of Animal Ethics of the State University of Ceará (CEUA/UECE, protocol number 2193327/2022).

3. Results

3.1. Cleavage and parthenogenetic activation as an indicator of the absence of embryotoxicity

Approximately eight hundred oocytes were cultured in an activation medium containing separately two concentrations of peptide solution, positive (ionomycin) and negative (6-DMAP) controls. No sign of degeneration was observed in bovine oocytes treated with RhoB-Ctn [1–9] at concentrations of 10 and 40 µM. The percentages of embryonic cleavage were 44.94% and 51.53%, respectively. Blastocyst formation occurred at 10 µM and 40 µM, corresponding to 7.07% and 11.73%, respectively. In the positive control, 5 µM ionomycin - a golden standard of oocyte activation, the cleavage reached 74.07%, and 41.11% activated oocytes progressed further to the stage of blastocysts' formation. With oocytes exposed to only 6-DMAP, the negative control, the cleavage percentage was 41.1%, forming only 13 blastocysts with altered morphology (Table 1 and Fig. 1).

Table 1

In vitro cleavage and development progress of bovine embryos after exposure of oocytes to RhoB-Ctn [1–9], ionomycin, and 6-DMAP.

Peptide and controls[a]	Number of analyzed oocytes	Cleavage (2 and 4 Cells)	Nr. Of Blastocysts
RhoB-Ctn [1–9] (10 μM)	198	89 (44.94%)	14 (7.07%)
RhoB-Ctn [1–9] (40 μM)	196	101 (51.53%)	23 (11.73%)
Ionomycin (5 μM)	270	200 (74.07%)	111 (41.11%)
6-DMAP (2 μM)	132	56 (42.42%)	13 (9.84%)[b]

[a] Amidated-peptides in aqueous solution; ionomycin, positive control; 6-DMAP, negative control.
[b] Blastocysts with altered morphology.

3.2. Analysis of the parthenogenetic activation effect with light sheet microscopy

Light sheet microscopy images confirmed that RhoB-Ctn [1–9] peptide is harmless to bovine oocytes and embryo, and this technique allowed for tracking the cellular localization of the fluorescent peptide in bovine oocytes. The morphology and quality (viability) of the embryos at the blastocyst stage were analyzed after 7 days of cultivation. Matured oocytes undergoing parthenogenetic activation could be observed with the light sheet slide attached to an incubator. This analysis also revealed the cellular uptake of the peptide into the oocyte exposed to both peptide concentrations. The light sheet microscopy images were obtained during the initial 5 min of parthenogenetic activation. The resultant images provided a three-dimensional (3-D) representation of the matured oocyte structure with excellent contrast. The intracellular accumulation of the peptide was observed through rhodamine fluorescence of oocytes exposed to both peptide concentrations (10 and 40 μM). With 40 μM RhoB-Ctn [1–9], the nucleus of oocytes showed a higher peptide accumulation Fig. 1. The average fluorescence intensity for 10 μM-treated parthenogenetic oocytes was 198.37, while with 40 μM, the relative intensity reached a value of 1069,036 (Fig. 3).

3.3. Blastocyst microscopy analysis

Images of light sheet analysis of cells in the blastocyst stage of embryo development stained with Hoechst 33,342 after 7 days of cell culture (embryos at the 6 to 8-cell stages) indicated that the embryonic structures displayed good morphology and viability. The data showed a higher rate of division and efficiency in parthenogenetically activated embryos exposed to 40 μM and 10 μM, and ionomycin-treated oocytes, in this latter case, as expected. When treated with 10 μM RhoB-Ctn [1–9], there was a significant difference in the number of embryo-dividing cells compared to ionomycin (Fig. 4). Furthermore, oocytes exposed to RhoB-Ctn [1–9] at a concentration of 40 μM exhibited better production of blastomeres in comparison with 10 μM Rho-Ctn [1–9]. Embryos at the blastocyst stage exposed to only 6-DMAP showed a proportion of cells similar to the peptide at lower concentrations. Parthenogenetic activation with the peptide indicated concentration-dependent induction of cell division in embryo development.

3.4. ADMET predictor in silico simulations

The intrinsic peptide properties related to absorption, excretion, and toxicity were predicted with the ADMET Predictor—specifically, the assessment of presumable lethality, genotoxicity, biodegradation, and sensitivity (Table 2).

The toxic effects of the peptide and control chemicals (ionomycin and 6-DMAP) in silico analyzed here presumably indicated the peptide RhoB-Ctn [1–9] exhibited potential harm to an organism. The predicted LD 50 and TD 50 values for RhoB-Ctn [1–9] were inferior to ionomycin and 6-DMAP. The data indicated that the peptide could cause liver alterations in rats like ionomycin and 6-DMAP, which presumably causes liver changes due to elevated aspartate and alanine aminotransferase activities. RhoB-Ctn [1–9] showed a lower tendency as a respiratory sensitizer than the controls.

In addition to toxicity, the peptide and chemical controls were analyzed regarding the insertion through lipoprotein barriers. The lipophilicity analyzed by MlogP varied from —1.641 (variant with greater lipophilicity, RhoB-Ctn [1–9]) to 3.245 (hydrophilic variant, ionomycin). Regarding the solubility of the compounds, the peptide has better solubility values in an aqueous environment; in contrast, the standard substance presents a more significant dissolution in a saline

Table 2

Pharmacokinetic results obtained by ADMET PREDICTOR® for RhoB-Ctn [1–9], ionomycin, and 6-DMAP.

ADMET Predictor Properties		Compounds		
		Rho-Ctn [1–9]	6-DMAP	Ionomycin
Toxicity	LD_{50}	107.385	836.958	996.553
	TD_{50}	0.326	4.753	153.329
	In Skin	414.075	1.822	395.94
	Respiratory	Sentizer (53%)	Sentizer (70%)	Sentizer (94%)
	AST	Elevated (84%)	Elevated (99%)	Elevated (42%)
	ALT	Elevated (89%)	Elevated (80%)	Elevated (56%)
Lipophilicity	Mlog	- 1.641	—0.397	3.245
Solubility	S + Sw (mg/mL)	6.438	0.784	0.011
	SolFactor	105.365	52.109	2625.87
Permeability	In Skin (cm s X10^7)	414.075	1.822	395.394
	In Cornea cm/s X10^7)	14.907	231.618	87.085
	BBB	Low	High (94%)	Low (97%)
Distribution	S + Fumic	0.668	0.906	0.320
	Pgp_substrate	Yes	No (67%)	Yes (97%)
	Pgp_ Inhibition	No	No (78%)	Yes (88%)
	VD (L/kg)	0.295	1.441	0.394

Notes: LD50: lethal dose, the quantity of a substance that causes the death of 50% of a test sample when ingested; TD50: median toxic dose, the dosage at which toxicity manifests in 50% of cases for a given drug or toxin. In Skin: Skin sensitivity; Respiratory: respiratory sensitivity. AST: aspartate aminotransferase; ALT: Alanine aminotransferase; Mlog: log of the octanol to water partition coefficient; S + SW: solubility in pure water; SolFactor: Salt solubility factor; In Skin: Skin permeability; BBB: Blood-brain barrier permeation; S + fumic: Fraction unbound in human liver microsomes; Pgp_substrate: Likelihood of intestinal efflux by P-gp transporter in human; Pgp_ Inhibition: Inhibition of the intestinal P-gp transporter in human; VD: Human volume of distribution.

solution. The peptide displayed a numerically superior permeability in tissues (414.075). At the same time, 6-DMAP presents a hypothetically better permeability in the corneas (231.618) and blood-brain barrier. Ionomycin seems to be a P-glycoprotein (P-gp) substrate and inhibitor, likewise RhoB-Ctn [1–9]. Predictive values showed that 6-DMAP displayed a higher blood distribution (VD) volume and fraction not linked to liver microsomes. Contrastively, ionomycin and RhoB-Ctn [1–9] exhibited a reduction in VD (0.394 and 0.295 L/kg, respectively) and S + fumic (Table 2).

3.5. Molecular docking

To investigate at the molecular level the presumable targets of RhoB-Ctn [1–9] in zona pellucida, molecular docking calculations were carried out with the surface proteins ZP3-ND and ZP2. Table 3 shows the docking score value for the top ten best docking runs related to the interaction ZP3-ND:RhoB-Ctn [1–9]/6-DMAP and ZP2:RhoB-Ctn [1–9]/ 6-DMAP/ionomycin. The solvent 6-DMAP was included in the docking procedure to evaluate its impact on the binding capacity to the targets while following the experimental methodology; Ionomycin was again used as a positive control for *in silico* calculations of ZP2 interaction. To the target ZP3-ND, the highest docking score values were obtained for RhoB-Ctn [1–9]. It was about 1.7-fold higher than 6-DMAP, indicating that the positively charged peptide and not the organic solvent interact preferentially with ZP3-ND. For ZP2, 6-DMAP did not interact appropriately with this protein compared with ionomycin and RhoB-Ctn [1–9], corroborating the observation of oocyte activation as seen experimentally through light sheet microscopy images. Molecular docking score values for ZP3-ND:RhoB-Ctn [1–9] are comparable to those obtained for ZP2:RhoB-Ctn [1–9], suggesting that the positively charged peptide might interact with both proteins; however, comparing the binding capacity of ZP2:RhoB-Ctn [1–9] with ZP2:ionomycin, the positive control interacts stronger than the peptide under study to ZP2. Probably, RhoB-Ctn [1–9] might permeabilize the membrane for the influx of ions from the medium; however, not in the same way as ionomycin – an ionophore, does for calcium ions and, for this reason, ionomycin is a better activator of oocytes than RhoB-Ctn [1–9] (Table 3).

Figs. 4 and 5 depict the docking interaction of each evaluated compound studied here with ZP3-ND and ZP2. At the same time, the corresponding primary amino acid residues and the type of intermolecular forces involved in such interactions are summarized in Tables 4 and 5. Molecular docking results suggested that van der Waals forces are the primary binding force that stabilizes the interactions of peptide and ionomycin with ZP proteins; additionally, hydrogen bonds were also observed to be involved in the interactions of ZP3-ND:RhoB-Ctn [1–9], ZP2:ionomycin, and ZP2:6-DMAP. For ZP3-ND the positively charged peptide interacts strongly with a negatively charged pocket of the protein resulting in more connected points than 6-DMAP. For ZP2, the positive-charged peptide interacts less favorably with a net positive pocket than ionomycin (a negatively charged ligand).

Table 3
The ten docking score values (dimensionless) for the interaction between ZP3-ND:RhoB-Ctn [1–9]/6-DMAP and ZP2: RhoB-Ctn [1–9]/6-DMAP/ionomycin.

Docking run	ZP3-ND:6-DMAP	ZP3-ND: RhoB-Ctn [1–9]	ZP2:6-DMAP	ZP2:RhoB-Ctn [1–9]	ZP2: ionomycin
1	36.3	10.7	22.5	28.8	41.9
2	36.4	21.5	25.1	29.4	43.9
3	37.1	28.8	25.4	44.6	51.3
4	37.2	32.3	25.9	50.1	52.6
5	37.3	41.6	26.0	53.9	53.5
6	37.9	63.6	26.5	55.5	56.2
7	38.0	65.5	26.8	58.6	57.2
8	38.1	66.8	26.9	64.0	68.6
9	38.5	68.0	27.2	65.2	78.1
10	38.6	68.3	27.3	66.5	79.4

Table 4
Data of molecular docking for the interaction between ZP3-ND and RhoB-Ctn [1–9] and 6-DMAP.

Compound	Amino acid residue	Interaction	Distance (Å)
RhoB-Ctn [1–9]	Asn-13	Van der Waals	2.80
	His-40	Hydrogen bonding	3.70
	Glu-45	Van der Waals	1.60
	Trp-63	Van der Waals	2.30
	Glu-154	Van der Waals	3.90
	Phe-157	Van der Waals	1.60
	Tyr-211	Van der Waals	3.30
	Trp-231	Van der Waals	2.50
	Ser-234	Van der Waals	3.30
	Lys-240	Van der Waals	3.10
	Lys-298	Van der Waals	2.00
	Asp-437	Hydrogen bonding	2.00
6-DMAP	Trp-63	Van der Waals	3.90
	Ala-64	Van der Waals	2.80
	Asp-66	Van der Waals	2.60
	Glu-112	Van der Waals	2.60
	Glu-154	Van der Waals	2.70
	Tyr-156	n-n	3.50
	Phe-157	Van der Waals	2.40
	Trp-231	Van der Waals	3.80
	Met-331	Van der Waals	3.00

Table 5
Data of molecular docking for the interaction between ZP2 and RhoB-Ctn [1–9], 6-DMAP, and ionomycin.

Compound	Amino acid residue	Interaction	Distance (Å)
RhoB-Ctn [1–9]	Arg-510	Van der Waals	3.80
	Arg-568	Van der Waals	3.20
	Lys-569	Van der Waals	2.20
	His-573	Van der Waals	2.80
	Lys-576	Van der Waals	2.70
	Arg-603	Van der Waals	3.80
	Lys-608	Van der Waals	2.00
	Asn-641	Van der Waals	2.50
	Ile-643	Van der Waals	3.20
	Leu-1119	Van der Waals	2.10
6-DMAP	Lys-569	Van der Waals	2.30
	Glu-572	Hydrogen bonding	1.90
	Lys-608	Van der Waals	3.80
Ionomycin	Arg-510	Van der Waals	2.40
	Arg-568	Van der Waals	1.90
	Lys-569	Van der Waals	1.30
	Gln-571	Hydrogen bonding	2.90
	Glu-572	Van der Waals	2.30
	His-573	Van der Waals	2.20
	Lys-576	Van der Waals	3.30
	Gln-577	Hydrogen bonding	2.20
	Asn-602	Hydrogen bonding	1.80
	Lys-604	Van der Waals	2.20

4. Discussion

Cathelicidins and cathelicidin-related antimicrobial peptides (CRAMPs) comprise a growing family of vertebrate peptides with anti-infective and anti-proliferative properties. Vipericidins are CRAMPs found in the venom glands of South American pit vipers, and crotalicidin, a CRAMP from South American rattlesnake *Crotalus durissu terrificus*, with its various derived peptides and analogs, is now one of the best-studied vipericidins. As reported in previous studies, crotalicidins and other vipericidin share high similarities with cathelicidins expressed in the tissues of Asian species of snakes (Falcao and Radis-Baptista, 2020; Radis-Baptista, 2015; van Hoek, 2014). An interesting structural feature in snake venom cathelicidins is a highly conserved in tandem repetitive stretches of nine residues, with the consensus sequence H_2N-KRhKKhFKK-COOH, where h is a hydrophobic amino acid residue. This nonapeptide is active when coupled with rhodamine B at its

N-terminal, while the unlabeled peptide is not (Lima et al., 2022; Wang et al., 2015). RhoB-Ctn [1–9] was the peptide analog that showed multiple activities against bacteria and pathogenic yeasts and exerted diverse biological effects on zebrafish larvae, with mild hemolytic effect *in vitro* with concentrations up to 50 μM (Lima et al., 2022; Wang et al., 2015). Rhodamine B is a cationic fluorophore with a pH-dependent variable charge (Birtalan et al., 2011) that might contribute to the multiple activities of the Ctn [1–9] peptide in the physiological milieu. The peptide termini extension with segments of positively charged amino acids in the N or C terminal increased, for instance, the magainin peptide's antimicrobial activity (Bessalle et al., 1992). However, adding a single lysine residue replacing rhodamine B fluorophore abolished the antimicrobial activity of RhoB-Ctn [1–9].

Interestingly, rhodamine B contributed not only to the effective antimicrobial activity of Ctn [1–9], as aforementioned, but also to tuning the properties of other peptides conjugated with a different fluorophore (Bucki et al., 2004; Kiss et al., 2018). Thus far, the purpose here was to investigate the RhoB-Ctn [1–9] effects on bovine embryos, which also serve as a non-conventional toxic model for peptide toxicity assays. The crotalicidin peptide fragment conjugated with rhodamine at its N-terminal (RhoB-Ctn [1–9]) was harmless to bovine oocytes and remarkably induced some level of parthenogenetic activation by promoting cleavage and the formation of blastocysts. The cleavage, i.e., the division of cells in the first stages of embryo development, and the formation of blastocysts occurred in both concentrations of peptide tested (10 and 40 μM) that did not cause any hazardous effect *in vitro*. The microscopic analyses confirmed the morphology of the embryos developed after peptide-induced bovine oocyte cleavage (Table 1 and Fig. 1). Parthenogenesis means "the development of the egg cells into a new individual without fertilization" (and, additionally, with or without the eventual development of an adult") (Mittwoch, 1978). Parthenogenesis can be a natural process, as seen in hymenopterans and reptiles, or artificial, induced by physical and chemical means. Observing induction of oocyte activation mediated by RhoB-Ctn [1–9] is worthy of note, and visualization of cleavage and blastocyst formation strongly indicates the parthenogenic process. After the passage of these embryonic stages, the morphology of inner cell mass is still a better predictive biomarker parameter of blastocyst viability (Sivanantham et al., 2022), with other additional biomarkers of embryo viability having not yet been disclosed, even for humans (Rosenwaks, 2017).

The fluorescence emitted by rhodamine B allowed for analyzing the cellular localization and uptake of the conjugated nonapeptide in oocytes. The use of light sheet technology here allowed for capturing images of the moment in which the oocytes undergo parthenogenetic activation. The nucleus of these structures evidenced a red fluorescence due to the accumulation of rhodamine B-labeled Ctn [1–9] peptide in this vital subcellular compartment. The peptide quickly interacted with the zona pellucida of bovine oocytes, entered the cytoplasm of dividing cells in the bovine embryo after trespassing the cell membrane and accumulated in the nucleus (Fig. 2). Interestingly, in other cell types and models, prolonged incubation of different crotalicidin-derived peptides, namely Ctn [15–34], is cytotoxic, causing a considerable reduction in the number of unhealthy or pathogenic cells (Falcao and Radis-Baptista, 2020). In agreement with the present experimental findings, images of the blastocysts of the bovine embryo obtained with the light sheet microscopy showed that the blastomeres' characteristics were unaltered when the oocytes were incubated with the peptide for up to seven days. Although RhoB-Ctn [1–9] was harmless to bovine oocytes and capable of activating the cleavage to the next embryo stage, the parthenogenetic activation was less effective than ionomycin – the golden standard of parthenogenetic activation.

Previously, snake venom proteins were studied for the potential activation of bovine oocytes. The experimental data indicated that bovine oocytes were responsive to disintegrins (i.e., kistrin, echistatin, and elegantin), as evidenced by their ability to induce parthenogenetic development up to the blastocyst stage, with a more significant effect observed at the 1 mg/mL concentration, compared to lower concentrations (White et al., 2007). Thus, compared with disintegrins, RhoB-Ctn [1–9] is a cationic peptide analog derived from a snake venom CRAMP, that can induce embryo activation at low concentrations.

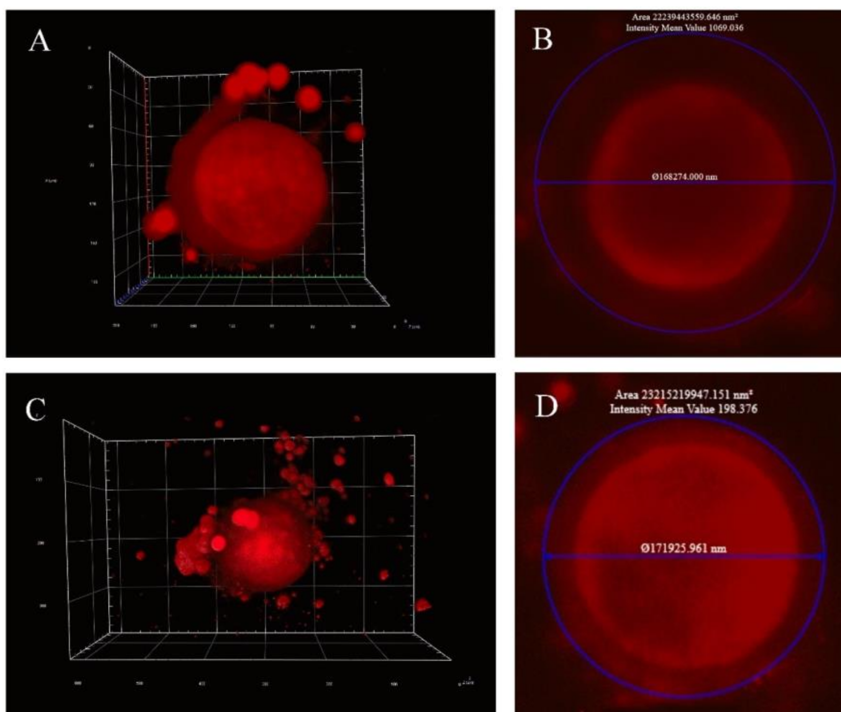

Fig. 2. RhoB-Ctn [1–9] peptide penetrates bovine *cumulus*-oocyte complexes (COCs). Snapshots from time-lapsed imaging Lightsheet 7 microscopy showing penetration of Ctn [1–9] (rhodamine B- labeled) into COCs and localization to the nucleus. A) 3-D image of peptide penetration into COCs RhoB-Ctn [1–9] (40 μM). B) Intensity of fluorescence emitted after penetration RhoB-Ctn [1–9] (40 μM). C) 3-D image of peptide penetration into COCs RhoB-Ctn [1–9] (40 μM). D) Intensity of fluorescence emitted after penetration RhoB-Ctn [1–9] (10 μM).

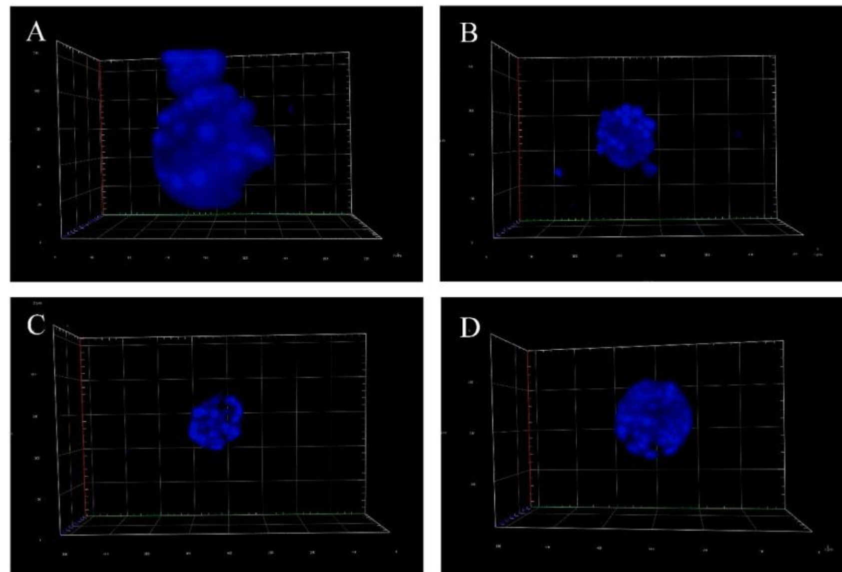

Fig. 3. Representative light sheet microscopy imaging of the bovine parthenogenetic embryos obtained after exposure to A) 5 μM Ionomicyn, B) 40 μM RhoB-Ctn [1–9], B) 10 μM RhoB-Ctn [1–9] rhodamine, and D) 6-DMAP. The embryos were stained with Hoechst 33,342.

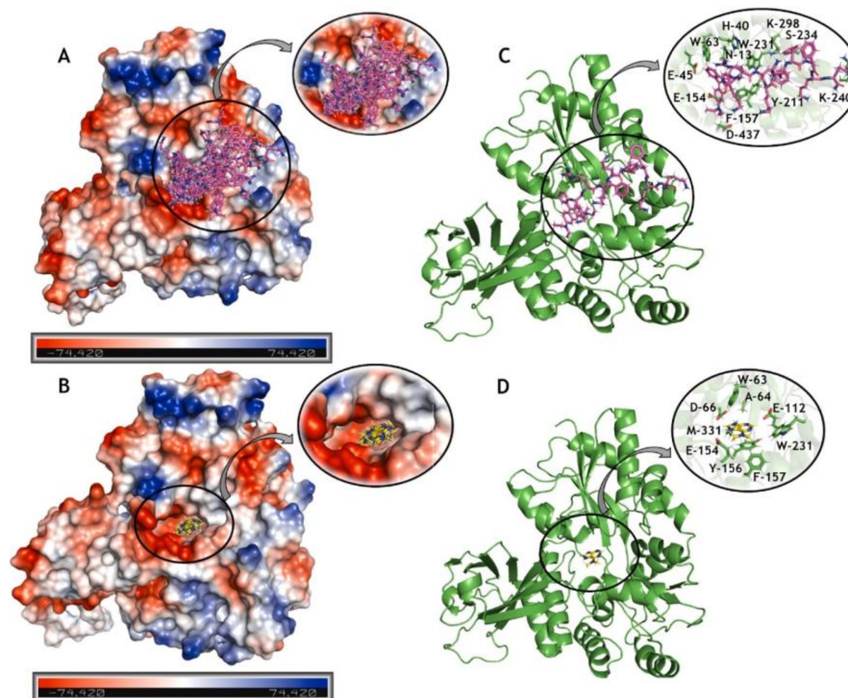

Fig. 4. (A, B) Electrostatic potential map for ZP3-ND with the superposition of the ten docking locations for RhoB-Ctn [1–9] and 6-DMAP, respectively. The best docking pose with the primary interactive amino acid residues as zoom representation for (C) RhoB-Ctn [1–9] and (D) 6-DMAP. Selected amino acid residues, RhoB-Ctn [1–9], and 6-DMAP are in green, pink, and yellow, respectively. 'Elements' colors: hydrogen is white, oxygen is red, and nitrogen is blue.

Predictive drug pharmacokinetics with *in silico* analysis contributes to identifying and optimizing small molecules with greater efficiency and safety (Cáceres et al., 2020; Effinger et al., 2018). Here, predictive analysis provided information on ADMET of RhoB-Ctn [1–9] peptide and docking analysis to estimate the interaction of the peptide with components of zona pellucida of bovine oocytes, as experimentally observed by microscopy. Despite the predictor algorithm indicating that RhoB-Ctn [1–9] displayed a lower value of acute toxicity, therefore, a hypothetically inferior tolerance than ionomycin and 6-DMAP, the peptide showed a safe performance at low concentrations in the bovine embryo culture medium for parthenogenetic activation.

Moreover, predictive data pointed out that RhoB-Ctn [1–9] has good lipophilicity, permeability, and biodistribution (Table 2). The lipophilicity and permeability were observed through fluorescent

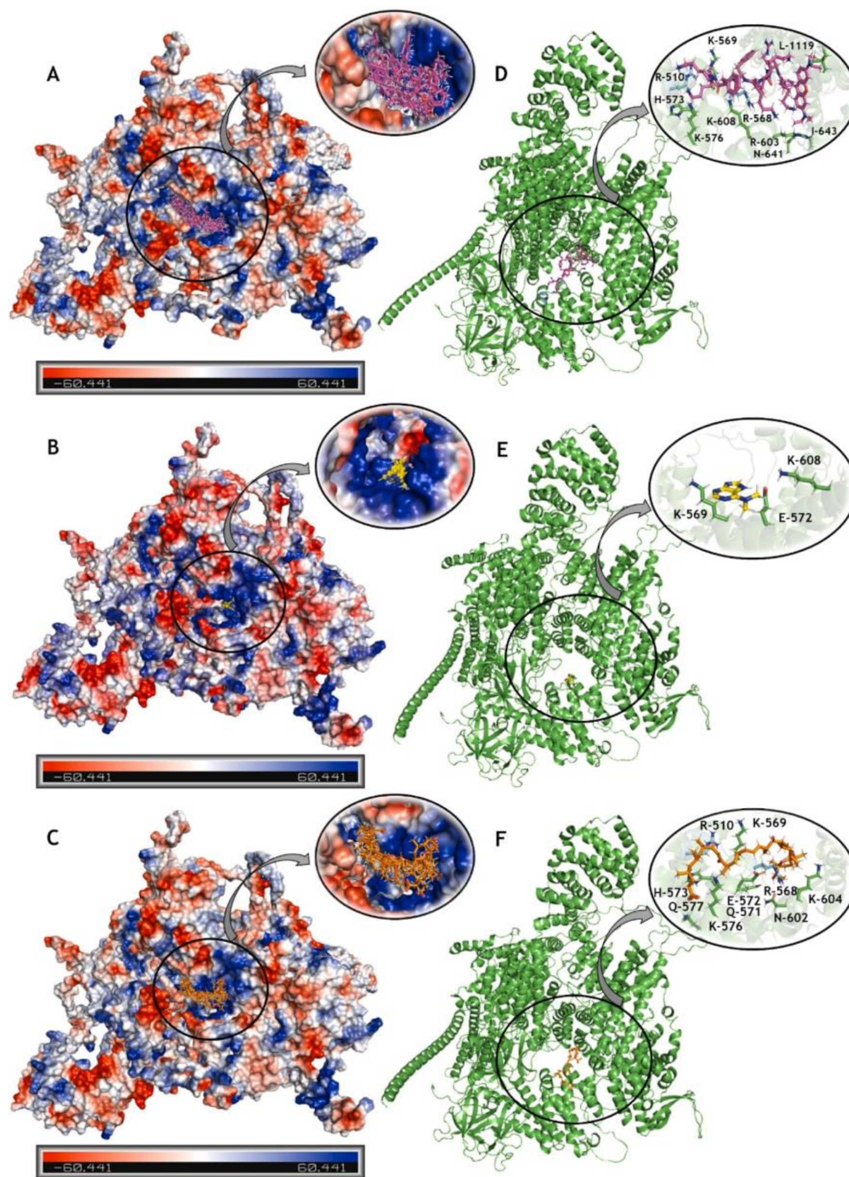

Fig. 5. (A, B, C) Electrostatic potential map for ZP2 with the superposition of the ten docking sites for RhoB-Ctn [1–9], 6-DMAP, and ionomycin, respectively. The best docking pose is indicated with the primary interactive amino acid residues as zoom representation for (D) RhoB-Ctn [1–9], (E) 6-DMAP, and (F) ionomycin (Tables 4 and 5). In green, pink, yellow, orange, and cyan, are the selected amino acid residues, RhoB-Ctn [1–9], 6-DMAP, ionomycin, and amino acid residues that are critical for specific binding, respectively. 'Elements' colors: hydrogen is white, oxygen is red, and nitrogen is blue.

microscopy of bovine embryos exposed to RhoB-Ctn [1–9] and the nuclear dye Hoechst 33,342. The peptide localized to cell membranes, cytoplasm, and the nucleus of the dividing oocytes (Fig. 2), without affecting the morphology of the developing embryos (Fig. 3). The docking analysis with RhoB-Ctn [1–9] and surface proteins of zona pellucida indicated that ZP proteins seem as possible targets for peptide interaction (Table 3). The extracellular matrix surrounding the eggs, the zona pellucida, is critical in fertilization. Preimplantation development encompasses the "free"-living period of mammalian embryogenesis, which culminates in forming a fluid-filled blastocyst structure. The main difference between the blastocyst and the other mammalian cells is the presence of the zona pellucida (ZP) in the first one (Watson, 1992). The ZP, an extracellular matrix of glycoprotein filaments, plays a crucial role in animal fertilization by acting as a gatekeeper for sperm. Its components polymerize using a standard ZP "domain" module consisting of two related immunoglobulin-like domains, ZP-N and ZP-C. The ZP module has also been recognized in other secreted proteins with different biological functions (Bokhove and Jovine, 2018). The glycoproteins ZP2 and ZP3 that are components of zona pellucida can act as a binding site for the spermatozoa and thus start the fertilization process (Hoodbhoy et al., 2006; Rankin et al., 1999). The docking score values for RhoB-Ctn [15–34] - or ionomycin, and ZP proteins have values of favorable energy in the biomacromolecule-ligand interaction (Martins et al., 2022), and both ionomycin and ZP3-ND:RhoB-Ctn [1–9] bind hypothetically to a region that was described as critical for the increase Ca^{2+} concentration through cellular uptake (Mikoshiba, 1997). Regardless of peptide, ionomycin, and 6-DMAP all appear interacting with both selected ZP glycoproteins, the docking data for 6-DMAP indicated that it does not have sufficient capacity to bind to zona pellucida (Tables 4 and 5).

It is reported that a combination of ionomycin and 6-DMAP can induce high activation rates of bovine oocytes and development to the blastocyst stage; however, they can cause DNA damage associated with carcinogenesis in the first cell cycle. For example, abnormalities and impaired development occur when oocytes are exposed to activation with 6-DMAP (Lan et al., 2005). Besides, different mechanisms and

chemicals can activate parthenogenesis *in vitro*, like the modulation of intracellular calcium concentration, culminating in the development of embryos (Pathak et al., 2017). Thus, future research must elucidate how RhoB-Ctn [1–9] and analogous vipericidin peptides can induce parthenogenetic activation without causing harm to the developing bovine embryos.

5. Conclusion

RhoB-Ctn [1–9] appears harmless to bovine oocytes and, remarkably, activates parthenogenesis *in vitro*. Light sheet microscopy and *in silico* prediction analysis indicated that peptides interact with zona pellucida (and ZP proteins) and internalize into bovine oocytes and developing embryos. RhoB-Ctn [1–9] and other vipericidin-encrypted peptides are promising templates for translating venom peptides into beneficial cell biology compounds.

Credit author statement

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Ethics approval

All protocols used in the study were approved by the Committee of Animal Ethics of the State University of Ceará, CE, Brazil (CEUA/UECE, protocol number 2193327/2022).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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7. CONCLUSÃO DA TESE

Nesse trabalho de tese, evidenciou-se a importância da biotecnologia de peptídeos como uma ferramenta fundamental para a descoberta de novas atividades farmacológicas para alvos específicos. Ao explorar os peptídeos de organismos que habitam os ecossistemas marinhos e terrestres, fica evidente modificar estruturas e investigar os peptídeos com tais modificações utilizando distintos de modelos *in vitro* e alvos moleculares de interesse.

Os resultados apresentados revelaram que os peptídeos derivados da crotalicidina, isto é, SFR-Ctn[1-9], SFR-Ctn[16-24] peptídeo derivado de serpentes, possuem múltiplas atividades biológicas, demonstrando a capacidade de desempenhar diferentes ações mesmo tendo estruturas químicas semelhantes entre os avaliados, mas que dependen de moléculas conjugadas como a rodamina-B (RhoB) e sulforodamina (SFR). Aqui no caso, SFR-Ctn[1-9], SFR-Ctn[16-24] interagem e inibem canais de sódio e cálcio. Ademais, confirmou-se que os peptídeos sintéticos derivados do peptídeo PpVα interage e bloqueia significativamente os canais de sódio e, dessa forma, atuam como agentes citoprotetores contra os efeitos citotóxicos da veratridina. Portanto, os peptídeos aqui avaliados apresentam múltiplas atividades, podendo serem investigado posteriormente para suas aplicações na reprodução *in vitro* e nas doenças neurodegenerativas e canalopatias. Estas novas descobertas, com os peptídeos descrito nessa tese, evidenciam o valor da biotecnologia de peptídeos e suas amplas aplicações em diversas áreas da pesquisa farmacêutica, sugerindo o desenvolvimento de insumos terapêuticos e adjuvantes em terapias inovadoras e mais eficazes no futuro.

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Anti-inflammatory activities of arthropod peptides: a systematic review

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Abstract

Peptides obtained from different animal species have gained importance recently due to research that aims to develop biopharmaceuticals with therapeutic potential. In this sense, arthropod venoms have drawn attention, not only because of their toxicity but mainly for the search for molecules with various bioactivities, including anti-inflammatory activity. The purpose of the present study is to gather data available in the literature on new peptides derived from arthropod species with anti-inflammatory potential. This systematic review followed the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines. Studies on peptides from arthropods that display anti-inflammatory activity were retrieved from PubMed, Scopus, Web of Science, and Google Scholar databases. The bibliographic research started in 2020 and searched papers without a limit on the publication date. The articles were analyzed using a search string containing the following terms: “Peptides” and “Anti-inflammatory”, in combinations such as “Ant”, “Bee”, “Wasp”, “Crab”, “Shrimp”, “Scorpion”, “Spider”, “Tick” and “Centipedes”. Besides, a search was carried out in the databases with the terms: “Peptides”, “Antitumor”, or “Anticancer”, and “Arthropods”. Articles that met the inclusion and exclusion criteria totalized 171, and these served for data extraction. Additionally, the present review included anti-inflammatory peptides with anticancer properties. Peptides with confirmed anti-inflammatory activity were from insects (ants, bees, and wasps), crustaceans (shrimp and crabs), arachnids (scorpions, spiders, and ticks), and centipedes. These arthropod peptides act mainly by decreasing pro-inflammatory cytokines as analyzed *in vitro* and *in vivo*. Some showed significant antineoplastic activity, working in essential cellular pathways against malignant neoplasms.

Keywords:
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Background

The use of enzymes and polypeptides for medicinal purposes has attracted considerable interest due to their high specificity and selectivity. They are also less likely to interfere with cellular processes that are not the aimed therapeutic targets. Protein drugs are composed of bioactive polypeptides with significant therapeutic potential [1]. Although animal venoms have toxic effects, they are extensively studied to find pharmacologically active molecules [2]. A known example of an isolated venom component that served as a template for developing the anti-hypertensive drug captopril belongs to the bradykinin-potentiating peptide (BPP) family found in the venom of *Bothrops jararaca* [3].

Arthropods comprise one of the largest groups of animals on Earth, with diverse species being venomous. These species contain complex mixtures of components in their venoms with various families of toxins that exert numerous biological effects on target organisms and systems, testified by a growing number of reported studies available in public databases. This kind of natural chemical and peptide library provides excellent potential for discovering new compounds and activities for alternative or adjuvant therapies based on the mimetic modulation of pharmacological activities of endogenous (poly)peptides in the body [4–6]. More than 400 toxins from various animals have activities reported in the literature, and around 3400 reported proteins are from arthropods [7].

Natural products comprise an essential source of bioactive substances, and they have contributed significantly to the manufacture of old and new drugs for diverse therapeutic purposes. In recent years, of all the molecules approved by the U. S. Food and Drug Administration (FDA), a third of them are natural products and derivatives from mammals and microbes [8]. However, arthropod venoms as sources of new pharmaceutically functional molecules are yet to be deeply explored [9]. Many arthropod venom peptides represent an opportunity by which venom components could be converted into "pharmaceutical gold" [10,11,12]. The production of a drug derived from venoms also includes the characterization of synthetic or recombinant peptide forms. Examples include peptides capable of modulating and/or regulating pain [13].

This review presents examples of peptides from various arthropod species, mainly focused on biologically active peptides found in arthropod venom with anti-inflammatory potential.

Methods

Investigation plan

This systematic review followed the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines [14]. The search of published articles on the topic of arthropod-derived peptides with anti-inflammatory activity was through PubMed, Scopus, Web of Science, and Google Scholar electronic databases. The bibliographic retrieval started in August 2020 and finished in March 2021. The search did not limit the date of publication. The publications were analyzed using a search string containing terms: "Peptides" and "Anti-inflammatory", in combinations such as "Ant", "Bee", "Wasp", "Crab", "Shrimp", "Scorpion", "Spider", "Tick" and "Centipede". In addition, a search was carried out in the databases with the terms: "Peptides", "Antitumor", or "Anticancer", and "Arthropods".

Selection of the literature

The studies were selected by the coauthors' ATS and GSC through Mendeley software (version 1803, 2020) and verified by GRB, ensuring the review work's inclusion. The selected literature adhered to the following criteria: full research articles that have been conducted *in vitro* or *in vivo* experimental studies and evaluated the anti-inflammatory effects of peptides derived from arthropod venoms or their crude extract. Besides, included in this review are ethnopharmacological data related to the topic covered. The criteria used to exclude studies were: repeated articles, editorials, letters to the editor, thesis, dissertations, reports, and articles that are out of the scope of this review.

Data collection

According to the required criteria, the studies selected for inclusion in this systematic review were chosen by the authors' ATS and GSC. The information collected from the literature contains the following information: authors, affiliation, year of publication, applied methodology, characterized compound, and main results.

Results

After searching the databases, 171 original and review articles were selected out of 769 published papers and utilized to prepare the current review. The flow diagram (Figure 1) depicts the details of the selection process in the databases. Also, general information was obtained, referring to the article's title, authorship, and publication year.

Reading the material in its entirety made it possible to identify specific information about the animal species involved in the study, the peptide structure identified as a potential anti-inflammatory agent, and the anti-inflammatory activity described more precisely. Table 1 summarizes the collection of this information.

Insect peptides

Ants

Insects possess a multitude of unexplored toxins with presumed potent biological activities. For instance, ants (Insecta class, Hymenoptera order, Formicidae family) are mostly venomous and express several types of peptides in their venoms, therefore emerging as an essential source of bioactive peptides [15]. Not so long ago, investigating the biological effects of isolated peptide toxins from insects was hampered by the size of these majorly tiny animals. With the advent of omics technology, the discovery and characterization of novel peptides progressed [16]. Initial studies aimed to unveil a way to alleviate the secondary effects

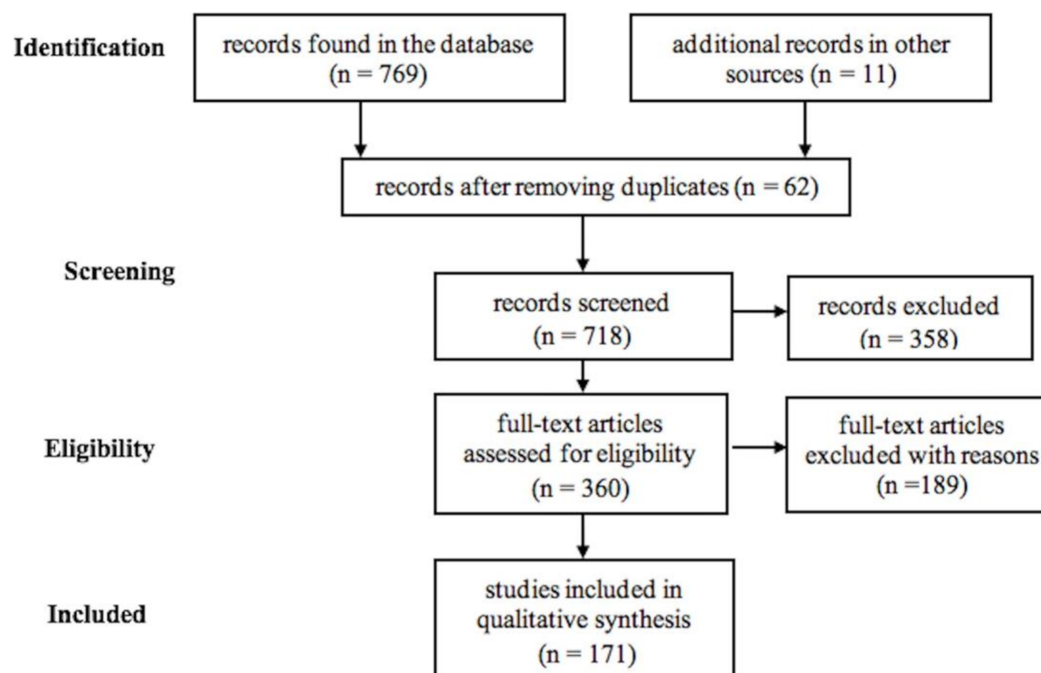

Figure 1. PRISMA flowchart showing the research design process of the study.

Table 1. Examples of peptides from the Uniprot database with anti-inflammatory activies.

Animal (Source)	Peptide	Access number	Activity as inflammatory mediator	Ref.
Insect				
Pseudomyrmex triplarinus	Pseudomyrmecitoxin-Pt1 subunit LS1	P0DSL7	Antidematogenic effect	[19-21]
	Pseudomyrmecitoxin-Pt1 subunit SS3	P0DSM1		
	Pseudomyrmecitoxin-Pt1 subunit LS2	P0DSL8		
	Pseudomyrmecitoxin-Pt1 subunit SS2	P0SDM0		
	U1-pseudomyrmecitoxin-Pt1 subunit SS1	P0DSL9		
Paraponera clavata	Delta-paraponeritoxin-Pc1a	P41736	Edema reduction, antinociceptive	[22]
Dinoponera quadríceps	Venom peptides (Extract)	C0HJK0	Suppression of inflammatory mediators	[23-26]
Brachyponera sennaarensisare	Venom peptides (Extract)	-	Regulate the expression of MHC-II, CD80 y CD-86, IFN-γ and IL-17	[28,29]
Pachycondyla sennaarensis	Venom peptides (Extract)	-	Regulate NF-kB, kinase IkB, TNF-α and Fas	[39]
Apis melifera	Venom peptides (Extract)	-	Reduction the levels of inflammatory mediators	[40-46]
	Phospolipase A2	P00630	Reduction of apoptotic levels mediated by Bcl-2 and Bcl-xL	[55-57]
	Melittin	P01501	Inactivation of NF-kB	[58-67]
	Apamin	P01500	Suppression Th2-related chemokines/Regulation the activation of the NF-kB, STATS 1 and 2 pathways	[69-71]
	Adolapin	-	Reduction of paw edema, the levels of prostaglandins, cyclooxygenase 2, in addition to inhibiting PLA2 activity	[72-74]

Table 1. Cont.

Animal (Source)	Peptide	Access number	Activity as inflammatory mediator	Ref.
Crustacean				
Protopolybia exígua	Mastoporan-1	P69034	Inhibition Toll-like receptor 4 (TLR4) mRNA, suppressionTNF-α and interleukin-6 (IL-6)	[80]
Nasonia vitripennis	Venom peptides (Extract)	-	ReductionIL-1β, IL-6 and NF-kB	[82,83]
Vespa magniflcca	-	P0CH47	Inhibition of the NF-kB pathway	[84]
Limulus polyphemus	Anti-lipopolysaccharide factor	P07086	Immunomodulatory activity	[67,68]
Penaeus monodon	Anti-lipopolysaccharide factor	B1NMC7	Disruption of the mitogen-activated protein (MAP) pathway by regulating and reducing the release of pro-inflammatory cytokines	[90-91]
Portunus trituberculatus	Anti-lipopolysaccharide factor	C0KJQ4	Immunomodulatory activity	[98-102]
	Anti-lipopolysaccharide factor isoform 4	H9MYY2		
	Anti-lipopolysaccharide factor isoform 5	-		
	Antilipopolysaccharide factor isoform 8	-		
Scylla paramamosain	Catalase	D0EVW7	Antioxidant potential	[105,106]
Scylla serrata	Anti-lipopolysaccharide factor	B5TTX7		
Charybdis natator	Crab leg	-	Modulating the NF-kB pathway	[107]
Arachnid				
Titius obscurus	Toxin To3	P60213	Suppression of TNF-α and IL-1β	[112]
	Toxin To4	P60215		
Tityus stigmurus	Hyaluronidase	P0C8X3	Reduction the migration of leukocytes and TNF-α release	[113]
Tityus serrulatus	Antimicrobial peptide TsAP-2	S6D3A7		
Mesobuthus martensii	Makatoxin-1	P56569	Reduction the production of inflammatory mediators such as nitric oxide (NO), TNF -α, IL-6 and IL-1β	[116]
	Potassium channel toxin alpha-KTx 3.6	Q9NII7	Suppress cytokine secretion	[119, 121, 122]
Heterometrus laoticus	Hetlaxin	C0HJN0	Act on Kv1.3 potassium channel	[123]
Heterosodra maculata	Delta-theraphotoxin-Hm1a	P60992	To control thehypersensitivity and chronic visceral pain	[125]
Phlogiellus sp.	Phlotoxin 1	P0DM14	Antinociceptive activity	[128]
Phoneutria nigriventer	Phα1β	P81789	Anti-inflammatory and antinociceptive	[132-135]
	Tx3-3	-		
	PnTx4			
	PhKv			
Pardosa astrigera	Lycotoxin-Pa4a	-	Suppresses nitric oxide, nitric oxide-induced synthase (iNOS), IL-1β, TNF-α	[136]
Ornithodoros savignyi	OsDef2	-	Inhibits the production of TNF-α and NO-induced	[139]
Hyalomma asiaticum	Hyalomin-A1	-	Inhibits the secretion of pro-inflammatory cytokines and increasing the secretion of IL-10	[140,141]
	Hyalomin-B1			
Rhipicephalus sanguineus	Evasin-1	P0C8E7	Inhibits cell of chemokines CCL3, CCL3L1, and CCL4 and CCL5	[142]
	Evasin 3	P0C8E8		
	Evasin 4	P0C8E9		
Amblyomma variegatum	Amphiregulin	-	Inhibitis the secretion of TNF-α, IL-1, IL-8, and IFN-γ	[143]
Chilopod				
Scolopendra subspinipes	Formyl peptide receptor 2	-	Inhibitis the release of pro-inflammatory cytokines and the recruitment of neutrophils in the joint	[147]
	Scolopendrasin IX	-	down-regulate the expression of pro-inflammatory mediators such as TNF-α and IL-6	

Source: Uniprot database.

caused by these animals' bites, with ants belonging to the genera Solenopsis, Pachycondyla spp, and Myrmecia the most studied [17, 18]. In crude and isolated forms, the characterization and verification of several bioactive peptides from the venom of *Pseudomyrmex* species, such as the mirmexin peptide, proved to have a potent antidematogenic activity [19–21]. As observed *in vivo,* poneratoxin, a 25-residue peptide from the bullet ant *Paraponera clavate*, and some Formicidae peptides, can reduce edema, besides their antinociceptive activity [22]. In the context of ethnopharmacology, there are reports about the topical use of macerated giant ants *Dinopera quadriceps* for the treatment of back pain and rheumatic cases [23]. These studies have shown that the crude extracts reduced paw edema, leukocyte migration, malonaldehyde, and nitrite content, ameliorating acute peritonitis *in vivo* and *in vitro*. This extract contained modulator molecules of cellular oxidant/antioxidant mechanisms involved in acute inflammation elicited by zymosan, but more specific mechanisms of action have not been described [24,25]. The crude venom of this species has the potential to reduce nociception and interleukin-1β (IL-1β), which suggests that it suppresses inflammatory mediators such as cyclooxygenase-2 (COX-2) and prostaglandin-2 (PGE-2) involved with pain [26,27]. The *Brachyponera sennaarensisare* (Samsum ant) ant-derived toxins modulate not only pain but also the immune response. The *B. sennaarensisare* toxins regulate the expression of MHC-II, CD80, and CD-86, as well as interferon- γ (IFN-γ) and interleukin-17 (IL-17), mediators that are involved in various chronic pathologies and cancer as demonstrated after in vivo tests [28]. Furthermore, these peptides can regulate the nuclear factor kappa B (NF-kB), kinase IkB upward, and suppress nuclear transcription factor-α (TNF-α) and the cell surface death receptor (Fas), although the mechanism involved in anti-inflammatory activity has not been fully elucidated [29,30].

Bees

Bees are part of the class Insecta, order Hymenoptera, family Apoidea, and clade Anthophilia. In Brazil, bee venom is commonly found and consists of various bioactive agents that induce allergic reactions when injected into the human body [31]. However, its use for medicinal purposes was documented approximately 6,000 years ago [32]. Bee venom therapy (BV) is a form of medicine native to ancient Greece and China [33]. In recent years, bee-based therapy has become a new treatment option. An increasing body of scientific evidence has demonstrated the therapeutic potential of bee venom [34]. In traditional medicine in Asia, BV was used in conjunction with acupuncture to treat some anti-inflammatory diseases. Furthermore, combination therapy can reduce inflammation in amyotrophic lateral sclerosis (ALS) due to the disease's side effects on the liver, kidney, and spleen [35]. Combination acupuncture and BV therapy (i.e., *Apis mellifera* crude venom) were also favorable to treat respiratory inflammation accompanied by leukocyte, myeloperoxidase (MPO), and IL-1 suppression, using a carrageenan-induced pleurisy mouse model [36].

The inflammation suppression mechanism of *European honey bee Apis mellifera* BV, observed in previous studies with animal models, also reduces the formation of atherosclerotic lesions by decreasing the intercellular adhesion molecule 1 (ICAM-1), vascular adhesion molecule- 1 (VCAM -1), and transforming growth factor-β1 (TGF-β1) [37]. Furthermore, the reduction of inflammation induced by apitoxins - a venom bee peptide (*A. mellifera*) component, is due to the decrease in apoptotic levels mediated by Bcl-2 and Bcl-xL and activating BCL2-associated X protein (Bax) and caspase-3 [38]. The application of bee venoms (*A. mellifera*) extends to reduce inflammatory lesions caused by the bacteria *Propionibacterium acnes* through decreasing TNF-α, interleukin-8 (IL-8), and IFN-γ, while also blocking the expression of Toll-like receptor 2 (TLR2) in human keratinocytes and monocytes [39].

Based on previous studies, bee venom toxins from *A. mellifera* and *A. cerana indica* act by regulating NF-kB signaling; the antiarthritic effect has been explored to reduce the levels of inflammatory mediators directly involved in the pathophysiology of rheumatoid arthritis, similarly to standard drugs such as methotrexate [40–46]. The compound bee venom's potential extends to reducing pain, acting as an antinociceptive agent by modulating the α2-adrenergic receptor and cyclooxygenase-2, accompanied by suppressing edema [47–51]. BV has a broad spectrum of activities. Its effects are not limited only to joint diseases and respiratory diseases, promoting an improvement in the allergic condition by suppressing inflammatory cytokines when tested in an allergic chronic rhinosinusitis mouse model [52].

Bee venom is a complex mixture that includes proteins and peptides such as melittin, apamin, phospholipase A2, phospholipase B, hyaluronidase, phosphatase, α-glucosidase, MDC peptide, and adolapin, among other minor components [53,54]. Secretory phospholipase A2 (PLA2- *Apis mellifera*), a prototype enzyme in bee venom, hydrolyze fatty acids while also having a role in protecting liver damage by producing anti-inflammatory cytokines in mice and reducing neuroinflammation by reducing phosphorylation of STAT3 and inflammatory mediators, including p-STAT3 [55,56]. Bee venom phospholipase A2 ameliorates amyloidogenesis and neuroinflammation by inhibiting signal transducer and activating the transcription-3 pathway in Tg2576 mice [57].

Melittin (*Apis mellifera*), one of the main peptides in bee venom, comprises 26 amino acid residues with an overall amphipathic character. Administration in high doses of this apitoxin can trigger an allergic reaction, causing local itching and pain. In low doses, it may have an anti-inflammatory role by inhibiting the enzymatic activity of PLA2. Synthetic melittin inhibited the enzymatic activity of secretory phospholipase A2 (PLA2) from various sources, including bee and snake venoms, bovine pancreas, and synovial fluid from rheumatoid arthritis patients. Based on melittin's hydrophobic nature and its capacity to bind to PLA2, melittin could act as a carrier for PLA2 to translocate it to the membrane. Melittin inhibits the bee venom PLA2 noncompetitively by binding to the enzyme

domain other than the catalytic site. [58]. The protective effect of melittin on inflammation and apoptosis was also observed in acute liver failure; the treatment with melittin attenuated the increase of inflammatory cytokines and significantly inhibited caspase expression Bax protein levels, as well as cytochrome *c* release in vivo [59,60].

Moreover, the JNK-dependent inactivation of NF-kB caused by melittin may prevent the release of inflammatory mediators involved in oxidative stress and the generation of pain [61]. Melittin-induced inhibition of this signaling pathway, which included the ERK and AkT cascade, and suppression of the inflammatory mediators upregulated in periodontitis, a chronic inflammatory disease, was observed in *P. gingivalis* LPS-stimulated human keratinocytes [62]. Melittin also reduced the release of pro-inflammatory cytokines by monocytes after contact with *P. acnes*. It is also an effective agent that prevents liver fibrosis by inhibiting inflammation by interrupting the NF-κB signaling pathway [63–64]. Moreover, melittin modulated inflammation, having better activity and less toxicity when associated with glutathione S-transferase while *in vitro*. When using doses that exceed the toxic concentration, it still retains its inflammatory properties [65]. A study reports its beneficial effect in treating inflammatory diseases, including skin inflammation, neuroinflammation, atherosclerosis, arthritis, and liver inflammation [66].

Apamine is another toxin that constitutes bee venom. It is an 18 amino acid-residue neurotoxic peptide. Despite its neurotoxicity, apamine helps treat Parkinson's disease or learning deficits [67]. Moreover, apamine, as an anti-inflammatory peptide, reduced the paw's volume and the haptoglobin and seromucoid contents *in vivo* [68,69]. This bee venom peptide was efficient in treating atopic dermatitis. The Apamin inhibits TNF-α- and IFN-γ-induced inflammatory cytokines and chemokines via suppressions of NF-κB signaling pathway and STAT in human keratinocytes [70]. Apamine showed anti-inflammatory effects in mice with gouty arthritis by inhibiting pro-inflammatory cytokine production and inflammasome formation [71].

Adolapin, from *A. mellifera* venom, is another bee venom peptide with potent anti-inflammatory effects but not as well studied as melittin. It reduces the edema of the paw in mice, the levels of prostaglandins, cyclooxygenase 2, in addition to inhibiting PLA2 activity. The anti-inflammatory activity of adolapin is evident in carrageenin models, prostaglandin, rat hind paw edemas, and adjuvant polyarthritis. The adolapin effects are presumably due to its capacity to inhibit the prostaglandin synthase system, following a biphasic dose-response relationship. Likely, among the central mechanisms, one involved an analgesic action of adolapin [72]. Peptide 401 (mast cell degranulating peptide – MCD peptide), with 22 amino acid residues, considered a potent degranulation factor for bee venom mast cells, substantially inhibited the edema caused in rats and attenuated the inflammatory process at the affected site [73,74].

Wasps

Like bees, wasps (Insecta, Hymenoptera, Apocrita) have complex mixtures of toxins in their venoms and have attracted interest as a potential arthropod source of bioactive substances. Wasps belong to the family Vespidae, and members include the genus Dolichovespula (wasp), Vespula (yellow wasps), and Polistes (paper wasps) [75]. When injected, the wasp toxins trigger local adverse effects such as pain, edema, erythema, and immune reactions such as anaphylaxis [76,77]. In general, wasps' venom comprises a cocktail of hydrophobic peptides, including amines, peptides, enzymes, allergens, and toxins [78,79]. For example, mastoparan is an amphipathic, 14-amino acid residue, and it was the first peptide isolated from wasps. This toxin is found in the genera *Vespa, Parapolybia, Protonectarina, Polistes, Protopolybia* [80].

Like bee venom, wasps' venoms have a considerable anti-inflammatory effect, shown in *in vitro* studies. These contain toxins that have the potential to inhibit Toll-like receptor 4 (TLR4) mRNA expression, in addition to suppressing TNF-α and interleukin-6 (IL-6) [81]. Although crude venoms contain several toxins that can trigger a toxic reaction, wasp venoms have powerful anti-inflammatory complexes, as is the case of the crude venom of the wasp *Nasonia vitripennis* (jewel wasp). The *N. vitripennis* crude venom reduced the expression of inflammatory cytokines directly involved in inflammatory processes mediated by IL-1β, IL-6, and NF-kB [82,83]. In an arthritis model, crude wasp venoms caused the inhibition of the NF-kB pathway. Likewise, *Vespa magnifica* (murder hornet) and other wasp species' crude venoms suppressed the expression of mediators involved in hyperalgesia and rheumatoid arthritis [84–88].

A study dealing with *Vespa tropica* (Greater banded hornet) showed that crude venom significantly reduced oxidative stress and the mouse microglial cell line activation, previously stimulated by LPS. Moreover, the peptides purified from the crude venom exhibited potential anti-inflammatory properties, targeting the p38 and MAPK pathways, causing the suppression of NF-κB phosphorylation in LPS-stimulated cells [89].

Crustacean peptides

Prawns/shrimps

Despite not being poisonous, shrimps (Crustacea, Malacostraca, Decapoda) were included here because they do not have an adaptive immune system and therefore rely on their innate immunity bioactive peptide components to deter invading pathogens. Antimicrobial peptides (AMP) are responsible for the immediate host response against invading bacteria, fungi, parasites, and, in some cases, they connect the innate and the adaptive immune response by modulating the expression and release of cytokines. The primary AMPs found in shrimp are grouped into three families of cationic peptides, namely, penaeidins, crustines, and anti-lipopolysaccharide factor (ALF) [90]. The ALF, firstly discovered in the horseshoe crab (*Limulus*

polyphemus), was followed by the identification in other crustacean species, like in the black tiger prawn *Penaeus monodon*, being designated SALF (Shrimp Anti-Factor Lipopolysaccharide) [90,91]. It is a precursor molecule with a signal sequence of 22 to 28 residues, followed by a mature peptide that contains two conserved cysteine residues. ALF's functional domain is named lipopolysaccharide-binding domain (LPS-BD) and contains the primary amino acids involved in recognizing and binding LPS and other components of Gram-positive bacteria and fungi [92].

P. monodon shrimp contain eleven ALF isoforms distributed in seven groups (Group A to Group G). Likewise, these isoforms can be found in the shrimp species *Farfantepenaeus aztecus* (brown shrimp), *L. vannamei* (pacific white shrimp or king prawn), and *Marsupenaeus japonicus* (known as the kuruma shrimp, kuruma prawn, or Japanese tiger prawn) [91,92]. LPS is an endotoxin present in the outer cell membrane of Gram-negative bacteria. When in contact with the host, it binds to pathogen recognition receptors that recognize this pathogen-associated molecular pattern (PAMP) and activates the signaling pathways that initiate the inflammatory process [93]. Recent studies show that SALF, besides antimicrobial activity, plays an essential role in neutralizing LPS and preventing its binding to the TLR-4 type Toll-like receptor (TLR). This peptide could inhibit or reduce the inflammatory response, disrupting the mitogen-activated protein (MAP) pathway by regulating and reducing the release of pro-inflammatory cytokines after *in vitro* tests with different cell lines [93–96].

Among studies about the efficacy of SALF as an anti-inflammatory agent, the effects of *Penaeus monodon* (giant tiger prawn) SALF on the production and release of tumor necrosis factor (TNF) were reported. This peptide showed suppression of inflammation in a dose-dependent manner in LPS-stimulated cervical cancer HeLa cells. Although the results have been promising, the mechanism involved in anti-inflammatory activity has not been fully elucidated [93]. The SALF peptides' protective role includes an anti-inflammatory effect in response to LPS, as observed in cervical cancer epithelial cells (HELA cells). SALF fragments inhibited inflammatory cytokines production, including TNF, interleukin IL-1 β, IL-6, IL-1, and monocyte chemoactive protein (MCP-1). SALF also suppressed IL-6, IL-8, IL-1, and MPC-1e mRNA levels and regulated vaginal epithelial cell immune responses through MAPK (mitogen-activated protein kinases) and NF-κβ (nuclear factor kappa B) pathways [93].

In addition to the SALF response to bacterial LPS, this peptide modulates the inflammatory responses provoked by the protozoan *Trichomonas vaginalis*, an etiological agent of Trichomoniasis that affects the cervicovaginal mucosa. When vaginal cells were subjected to stimulation by *T. vaginalis*, SALF inhibited the release of pro-inflammatory cytokines such as TNF-α, IL-6, IL-8, and MCP-1 through the MAPK pathways and NF-κβ [96]. These reports exemplify the promising profile of SALF as an anti-inflammatory agent.

Crabs

In recent years, marine organisms have attracted great interest due to their unique constituents with diverse bioactivities. These animals have hemolymph with potent antimicrobial peptides essential for their innate immunity. These peptides are valuable for biomedical applications [97]. Crabs (Crustacea, Malacostraca, Decapoda, Pleocyemata) have been investigated for the peptides' antimicrobial activity and their immunomodulatory effects. Purified peptides from various species of crabs such as LALF (The Atlantic horseshoe crab-*Limulus polyphemus*), M-ALF (kuruma shrimp-*Marsupenaeus Japonicus*), PtALF, PtALF4, PtALF5, and PtALF8 (horse crab-*Portunus trituberculatus*) showed an anti-lipopolysaccharide activity [98–103]. In another example, the β-1,3-glucan binding protein (β-GPB) from the rice paddy crab *Paratelphusa hydrodromus* can trigger an immune response against external aggressors. Additionally, β-GPB also exerts an antioxidant effect, reducing DPPH radicals, in a model of restraining the albumin's denaturation [104]. Regarding the antioxidant enzymatic profile, enzymes purified from distinct crab species showed an effective antioxidant potential by increasing the activity of superoxide dismutase (SOD) and catalase (CAT) [105,106]. Moreover, crab-derived peptides can restrain the inflammatory process by reducing inflammatory mediators' levels and modulating the NF-kB pathway, implicated in various inflammatory diseases [107]. Besides their role as an anti-inflammatory substance, these crustacean-derived peptides can exert antinociceptive effects, consequently playing a role in pain control as potent COX-2 reducers *in vitro* [108].

Arachnida peptides

Scorpions

Venom peptides from scorpion (Chelicerata, Arachnida, Scorpiones) distribute into two main groups: DBPs (disulfide-bridged peptides) and NDBPs (non-disulfide-bridged peptides). DBPs generally target ion channels. Most scorpion DBPs contain three to four disulfide bridges and interact with the Na+, K+, Ca2+, and Cl− channels. In comparison, the NDBP peptides are less abundantly distribute among scorpion venoms and exhibit multiple activities, such as insecticide, antiviral, antimicrobial, hemolytic, antiproliferative, bradykinin-enhancing, and immunomodulatory [109,110].

Dias and collaborators [111] analyzed 320 non-disulfide bond-containing peptides, of which 27 had their sequences assigned. Among them, thirteen peptides constituting novel toxins in *Tityus obscurus* venom (Amazonian black scorpion). As examples, ToAP3 (FIGMIPGLIGGLISAIK-NH2) and ToAP4 (FFSLIPSLIGGLVSAIK-NH2) NDBPs exerted their effect on immunomodulation and suppression of inflammatory mediators, such as TNF-α and IL-1β. Furthermore, ToAP3 and ToAP4 were associated with the modulation of antigen presentation. They reduced TNF-α and IL-1β at transcriptional and translational levels in bone marrow-derived macrophages (BMDM) and dendritic cells (BMDC). The reduction of TNF-α secretion

before LPS-inflammatory stimuli is associated with peptide interaction with TLR-4. ToAP4 increased MHC-II expression in BMDC, while ToAP3 decreased co-stimulatory molecules such as CD80 and CD86 [112]. Stigmurin, a cationic peptide from the scorpion *Tityus stigmurus* venom (scorpion from the family Buthidae found in Brazil) and TsAP-2 from the scorpion *Tityus serrulatus* venom (Brazilian yellow scorpion) both reduced the migration of leukocytes and TNF-α release, reducing the inflammatory process. Additionally, the fractions extracted from their respective crude venoms could modulate the expression of the cytokines IL-4, IL-6, IL-13, and IL-13, which are pro and anti-inflammatory [113].

The peptide Ts14 from *T. serrulatus* modulates critical events occurring in the fibrovascular tissue, i.e., it causes neovascularization, inflammatory cell recruitment, and extracellular matrix deposition induced by polyether-polyurethane sponge implants in mice. Consequently, Ts14 has therapeutic potential in wound healing and ischemic and inflammatory conditions. Furthermore, Ts14 reduced TNF-α levels and neutrophil infiltration, although stimulated macrophage infiltration into implants, as determined by myeloperoxidase (MPO) and N-acetyl-β-d-glucosaminidase (NAG) enzyme activities, respectively [114]. BotAF is a peptide derived from *Buthus occitanus tunetanus* (common yellow scorpion), another yellow scorpion species that comprises a long chain of 64 amino acid residues, with potent analgesic activity in rodents [115]. From the Chinese scorpion *Mesobuthus martensii* (Chinese scorpion), 35 scorpion oligopeptides (CMOs) were studied. Specifically, the peptide CMO-1 suppressed inflammation by reducing the production of inflammatory mediators such as nitric oxide (NO), TNF-α, IL-6, and IL-1β in RAW264.7 macrophages cells. Moreover, CMO-1 inhibited the degradation of IkBα and the nuclear translocation of p65.

It also suppressed NF-kβ activation and inhibited MAPK phosphorylation of ERK, JNK, and p38 [116]. The venom of another species of *Mesobuthus* (*Mesobuthus eupeus*- lesser Asian scorpion, the lesser Asian scorpion, or the mottled scorpion) was effective in treating CFA-induced arthritis, in which the edema reduction correlated with the reduction of arthritis [117]. Sc20 from the venom of *Scorpiops tibetanus* is also a potent anti-inflammatory and immunosuppressor. This peptide modulated two important pro-inflammatory factors: the secretion of TNF-α and IFN-γ, displaying a positive effect in delayed hypersensitivity. Similar peptide St20, the first disulfide-bridged toxin peptide from the scorpion *S. tibetanus*, showed immunosuppressive and anti-inflammatory activities, suggesting that it may be a novel source of venom peptides to treat human disease [118].

The voltage-gated Kv1.3 channel, expressed in memory-efficient T cells, is presently a recognized targeted drug for treating various autoimmune diseases. Scorpion venom possesses Kv1.3 channel peptide blockers that suppress cytokine secretion and alleviate disease in animal models of T-cell-mediated autoimmune diseases [119]. Thus, to improve the selectivity and activity of these scorpion venom peptides directed at regulating Kv1.3 potassium channels are currently undertaken. A remarkable example is the study of the scorpion toxin BmKTX, isolated from *M. martensii* [120]. Recently, BmKTX analogs such as ADWX-1, BmKTX-D33H, BmKTX-19, and BmKTX-196 demonstrated specific inhibition of the Kv1.3 channel. Most venom-derived peptides have not evolved to target specific mammalian receptors of therapeutic interest; therefore, preparing peptide analogs with higher potency toward specific targets is customary [119,120,121]. The Vm24 scorpion toxin also showed similar activity to the venom-peptides above, which are blockers of Kv1.3 channels, acting without affecting the T cells' viability and inhibiting the activation of CD25 and CD40L, as well as the cytokine secretion of pro-inflammatory IFN-γ and TNF [122].

Hetlaxin (ISCTGSKQCYDPCKKKTGCPNAKCMNKS-CKCYGC) is a DBPs, belonging to the scorpion alpha-toxin family, isolated from the *Heterometrus laoticus* venom (Vietnam forest scorpion), which possesses a high affinity to the Kv1.3 potassium channel. This isolated *H. laoticus* venom peptide exerted an anti-inflammatory effect similar or slightly superior to ketoprofen [123].

Spiders

Spiders (Chelicerata, Arachnida, Araneae) comprise one of the oldest living animals on Earth that surged approximately 300 million years ago and comprise the most significant number of living species (> 40,000) [124]. As in other arthropods, inoculation of their venom causes local discomfort, such as edema, and more severe deleterious effects, like ulcerations, acute renal failure, and even death in the worse cases [125,126]. Although arachnids venoms are harmfully toxic to humans, some venom peptides have beneficial bioactivities applicable to biomedicine. In general, arthropod-derived venom's biochemical targets are excitable neuronal receptors; these include ion channels like voltage-gated sodium channels (Nav) found in neurons, which allow the modulating of pain. Spider peptides that modulate such pharmacological targets serve as molecular templates for the development of analgesic drugs. For example, the Hm1a peptide purified from the venom of *Heterosodra maculate* (togo starburst baboon spider) can control the hypersensitivity in chronic visceral pain [127].

Phlotoxin 1 (Ph1Tx1) is a 34-residue toxin purified from *Phlogiellus spider* venom, a promising antinociceptive peptide with a high affinity for Pav [128]. The crude venom of *Phoneutria nigriventer* (armed spiders), besides its antineoplastic activity, can suppress the IFN-γ release and increase the expression of the anti-inflammatory cytokine IL-10. Phα1β, a peptide purified from the venom of *P. nigriventer*, has a significant role in the control of the CFA-induced chronic arthritis model. The Phα1β suppressed the inflammatory agent's side effects while the antinociceptive role acted as the antagonist of the TRAP1 channel [129–131]. Furthermore, other peptides such as Tx3-3, PnTx4, PhKv, and PhTx3-5 from the *P. nigriventer* venom have important antinociceptive properties as observed in the animal neuropathic inflammatory pain model [132–135].

Lycotoxin-Pa4a peptide from *Pardosa astrigera* venom displays immunomodulatory activity by increasing the expression of IL-10 and suppressing pro-inflammatory mediators such as nitric oxide, nitric oxide-induced synthase (iNOS), IL-1β, TNF-α, in addition to reducing COX-2. *In vitro* studies with an LPS-stimulated model demonstrated that this peptide could act as a potential antinociceptive modulator [136].

Ticks

Ticks are hematophagous arthropods that rely only on the innate defense to protect themselves against invading microorganisms. Biologically active molecules are also necessary to keep blood fluid during feeding and eliminate the host's defense mechanisms, such as vasoconstriction, forming a hemostatic plug, activating the coagulation cascade, and initiating inflammatory responses that lead to wound healing and tissue remodeling. Thus, some bioactive molecules have anticoagulant, antiplatelet, vasodilatory, anti-inflammatory, and immunomodulatory activity and are crucial to overcoming the host's hemostatic and immunological responses, allowing ticks to feed and develop [137].

Ornithodoros savignyi (sand tampan, African-eyed tampan, or Kalahari sand tampan) is a tick that parasites cattle and is endemic in arid and semi-arid regions of the African continent. This tick species express antimicrobial peptides (defensins) constitutively in various tissues at low levels and inductively during blood-feeding or in response to bacterial challenge. Defensins are cationic molecules with molecular masses of approximately 4 kDa containing cysteine residues forming three disulfide bonds [138]. Studies on *O. savignyi* resulted in the cloning and sequencing of defensin isoforms, OsDef1 and OsDef2, derived from the terminal carboxy region. Due to the bactericidal activity isoform 2, this peptide served as a model for the synthesis of the peptide Os (KGIRGYKGGYCKGAFKQTCKCY) and its analog Os-C (KGIRGYKGGY- KGAFKQT- K-Y), with 22 and 19 residues of amino acids, respectively [139]. Os peptides' mechanisms of action in bacterial cells' membrane involve their penetration into the cell and action on intracellular targets. As a result of these findings, Malan et al. [139] evaluated these peptides' effects in inflammatory conditions resulting from gram-negative bacteria infection. Thus, Os and Os-C's showed anti-inflammatory properties on Raw 264.7 macrophages stimulated by LPS and IFN-γ *in vitro*. Both peptides inhibited the production of TNF-α and NO-induced by LPS in RAW 264.7 cells without appreciable cytotoxic effects. In addition to anti-endotoxin activity and anti-inflammatory properties, Os eliminated NO directly, and both Os and Os-C peptides exhibited antioxidant activity, which together can reduce oxidative stress associated with inflammation [139].

Wu et al. identified two families of immunoregulatory peptides, hyalomin-A1 and hyalomin-B1, from the salivary glands of the *Hyalomma asiaticum* tick. The amino acid sequences of hyalomin-A1 and B1 correspond to the sequences QTPRTIGPPYT and TLRTTTGYWTTVEKGNGTTPAANSTEKGNRPYGR, respectively. Hyalomin-A1 and B1 act as immunoregulators, inhibiting the secretion of pro-inflammatory cytokines induced by LPS in vitro and increasing immunosuppressive cytokine, IL-10 [140]. Both hyalomin-A1 and B1 could quickly eliminate oxidants in a few seconds. Such antioxidant activities can contribute to immunoregulatory and anti-inflammatory abilities.

Furthermore, the results indicated that both hyalomin-A1 and B1 significantly suppressed the LPS-induced activation of the JNK subgroup of the MAPK signaling pathway by blocking JNK phosphorylation and, consequently, led to a reduction in MCP-1, IFN-γ, and tumor necrosis factor-α genes. The *in vivo* experiments identified that these peptides could inhibit the hind paw's inflammation in mice depending on the dose administered. These anti-inflammatory functions were significantly present after nine days of administration. At a dose of 5 mg/kg of body weight, the mice could recover to a normal state after 21 days of administration of hyalomin-A1 or B1 [141].

Ticks have another mechanism of escape from the host's defenses related to the presence of evasins, small cysteine-rich binding proteins secreted in their saliva. To neutralize chemokines and their signaling, ticks, such as *Rhipicephalus sanguineus* (commonly called the brown dog tick, kennel tick, or pantropical dog tick), secrete evasins [142]. Evasin-1 (P0C8E7) inhibits cell recruitment of chemokines CCL3, CCL3L1, and CCL4-mediated chemotaxis in L1.2/CCR5 transfectants *in vivo* and *in vitro*. Besides, it also inhibited CCL3-induced granulocyte recruitment in mice. Evasin-3 (P0C8E8) inhibits neutrophil recruitment and reduces inflammation. Treatment with this peptide resulted in inhibiting total cell accumulation in the synovial cavity in a mouse-induced arthritis model. Inhibition of neutrophil infiltration in the knee joint reduced induced hypernociception, reduced production of TNF-α in the periarticular tissues, and inhibition of leukocyte adhesion [142]. The peptide derived from the N-terminal region of evasin-4 (P0C8E9), which had an affinity with the chemokine CCL5, inhibited the activity of CCL5 in monocyte migration assays. This result suggests that evasin-4 derivatives can serve as a starting point for developing anti-inflammatory drugs [142].

Tian et al. [143] investigated the immunosuppressive peptide amphiregulin from the tick *Amblyomma variegatum* (the tropical bont tick). This peptide is composed of 40 amino acid residues (HLHMHGNGATQVFKPRLVLKCPNAAQLIQ-PGKLQRQLLLQ). In rat splenocytes, amphiregulin exerted significant anti-inflammatory effects by inhibiting the secretion of TNF-α, IL-1, IL-8, and IFN-γ *in vitro*. Compared to LPS, these inflammatory mediators' inhibition was significant in all tested peptide concentrations (2, 4, and 8 μg/mL). Amphiregulin showed substantial elimination of free radicals and antioxidant activities in specific concentrations (5, 10, and 20 μg/mL) *in vitro* and also significantly inhibited the paw inflammation induced by adjuvant mice *in vivo* [143].

Chilopod peptides

Centipede

Centipedes are part of the subphylum Myriapoda (class Chilopoda). *Scolopendra subspinipes mutilans* (Chinese red-

headed centipede) is a component of natural extract formulation widely used in traditional Chinese and Korean medicine to treat various conditions due to its anti-inflammatory, antimicrobial, and analgesic effects [144]. It is a stable extract of which studies report its neuroinflammatory activity and efficacy as a mitigating agent of inflammation in rheumatoid arthritis, as well as antitumor and immunostimulant [145,146]. From the venom of *Scolopendra subspinipes mutilans* (Chinese redhead), the formyl peptide receptor 2 (FPR2) peptide with a chemo-attractive property for FRP2 on the neutrophils' surface was isolated. Results evidenced the therapeutic effects of this peptide on rheumatoid arthritis by inhibiting the release of pro-inflammatory cytokines and the recruitment of neutrophils in the joint [147]. Scolopendrasin IX, another peptide isolated from the same centipede species, can down-regulate the expression of pro-inflammatory mediators such as TNF-α and IL-6, also having therapeutic effects against rheumatoid arthritis. In mouse neutrophils, peptides from this centipede species' venom have a high potential to control the inflammatory process due to their targeted effects. However, the mechanism of action has not been clarified yet [147].

Discussion

Peptides and antitumor activities

When there is a failure in the inflammatory process's control mechanism, the condition can evolve into chronic inflammation with consequent mutation and cell proliferation, thus creating an environment conducive to cancer development. In this context, numerous treatments rely on antineoplastic therapy, including chemotherapy, radiotherapy, and immunotherapy [148]. These therapeutic options can cause serious side effects and increase resistance to neoplastic cells, therefore continuous research intent to find new therapeutical options. Animal venoms have become an object of interest because they have specific and structurally stable components that can interact with and modulate their molecular targets, making them good therapeutic candidates [149].

Among the drugable candidates, peptides from different arthropod species can potentially control inflammatory processes and control malignant neoplasms [150]. For instance, among the various ant toxins, solenopsin A (derived from red imported fire ant- *Solenopsis invicta*) is a potent anti-angiogenic agent that inhibits the phosphorylation of Akt-1 and FOXO1a, a substrate of Akt, thus modulating the Akt signal transduction, phosphatidylinositol-3-kinase in mouse embryos (3T3-L1 and NIH3T3) and zebrafish [151]. In cell cultures of HepG2, MCF-7, and LoVo lines, this peptide proved to be an anti-angiogenic toxin that can reduce the levels of cytokines such as interleukin (IL) -1β, IL-6, IL-8, and NF-κB) [152]. Table 2 summarizes information regarding some venom peptides with antitumoral and anti-inflammatory activity.

In this line, the centipede glycosphingolipid peptide-7 from the millipede – *Parafontaria laminata armigera* exerts an antiproliferative effect on neoplastic cells and inhibits the focal adhesion kinase (FAK) pathway in addition to the signal-regulated kinase (Erk) 1 and 2, both involved in the proliferation of melanoma cells. This same peptide reduced proteins' expression related to oral squamous cell carcinoma (cyclin D1) [153]. Regarding bee venom, melittin (*Apis mellifera*) is undoubtedly one of the most multifunctional toxins. In the fight against neoplastic cells, melittin can bind calmodulin and prevent cell proliferation, inducing the death of neoplastic cells through the activation of caspases and metalloproteinases (MMPs) [154,155]. In cells transformed by an oncogene, melittin activates PLA2, which destroys cancer cells and comprises another mechanism that acts as an antineoplastic agent. Through the PLA2-dependent mechanism of activation, melittin is effective in leukemic cell lines that are even resistant to TNF-α [156, 157].

PLA2 (*Apis mellifera*) is a toxin that negatively regulates transduction pathways related to cell survival and tumor invasion. Moreover, treatment with this peptide decreased epidermal growth factor (EGFr) [158]. BV is efficient in killing K1735M2 and B16 melanoma cells, halting the cell cycle at the G1 stage and, therefore, inhibiting cancer cells' proliferation in a dose-dependent manner. Furthermore, BV treatment stimulated Bax production, a pro-apoptotic protein, and reduced the expression of Bcl-2, resulting in the formation of dimers with Bax and the consequent cell death [159, 160].

Mastoparan is a peptide isolated from wasp *Polybia paulista*, which alone can induce mitochondrial permeability; however, it does not have specificity in malignant cells. Though, when encapsulated in a liposome, this peptide could release cytochrome in human chronic myeloid leukemia cells [161]. Isolated from *Polybia paulista*, the Polybia MPI peptide has cytotoxicity against leukemic T lymphocytes, in addition to being able to reach the cells of the lipid membranes creating channels that provoke ionic permeabilization, depolarization, and consequent cell death [162]. Although spiders are a widespread species within the arthropod group, toxins that act as antineoplastic agents are understudied. Research has shown that the crude venom from *Macrothele raven* (Araneae, Hexathelidae) can arrest cancer cells via caspase 3 in treated cells, leading to the HeLa cell's cell death. In breast cancer cells, the crude venom of this species caused cell death, in addition to causing a cell arrest in the G2/M and G0/ G1 cycles [163, 164].

The toxins obtained from the Chinese bird spider *Haplopelma hainanum* showed antitumor activity in a liver cancer cell line, decreasing cell growth, mitochondrial membrane potential, in addition to stimulating the production of caspase 3 and 9 and inducing apoptosis through a dependent mitochondrial pathway [165].

Scorpion venoms have been a promising target in cancer treatment, the most interesting being the long-chain toxins that act on K +, Cl-, and ion channels. For example, human breast cancer MCF-7 cells treated with *Buthus matensii karsch* toxin extract could induce apoptosis by producing caspase 3 and down-regulating Bcl-2. In *in vitro* studies, gonoearrestide, a peptide found in the fat-tailed scorpion *Androctonus mauritanicus* and *A. australis*, was able to kill neoplastic cells by arresting the cell

Table 2. Examples of peptides from the Uniprot database with antineoplastic activities.

Animal (Source)	Peptide	Access number	Antitumoral activity	Ref.
Insect				
Solenopsis invicta	Solenopsin	–	Inhibits PIK3 activation, Akt and FOXO1 phosphorylation	[150-152]
Apis mellifera	Melitin	P01501	Activation of caspases, metalloproteinases and PLA2	[155-157]
	Phospholipase	P00630	Epidermal growth factor receptor (EGFr) reduction	[158]
	Bee venom	–	Reduction of Bcl-2 expression	[159,160]
Polybia paulista	Mastoparan 1	P0C1Q4	Induces mitochondrial permeability and cytochrome release	[161]
	Polybia MPI	–	Cytotoxicity against leukemic T lymphocytes	
Arachnid				
Macrothele raven	Macrothele raven venom	–	Antitumoral activity	[163,164]
Haplopelma haunanum	*Haplopelma haunanum* venom	-	Reduced cell growth and stimulation of the production of caspase 3 and 9	[167]
Crustacean				
Buthus matensii karsch	*Buthus matensii karsch* venom	-	Induce apoptosis by producing caspase 3 and down-regulating Bcl-2	[168]
Androctonus mauritanicus e *Androctonus australis*	Gonearrestide	–	Inhibition of cyclin-dependent kinase 4 (CDK4) and increased cell expression of cycle regulators and inhibitors (cyclin D3, p27, and p21)	[169]
Leiurus quinquestriatus	Chlorotoxin	P45639	Can bind endogenously to MMP-2 expressed in glioma cells	[170,171]
Parabuthus schlechteri	PBITx1	P60271	Selective toxin of the Na^+ channel	[172]
Chilopod				
Parafontaria laminata			Suppressive activity of the focal adhesion kinase pathway (FAK) and the kinase pathway regulated by the extracellular signal (ERK)	[153-154]

cycle in the G1 phase due to inhibition of cyclin-dependent kinase 4 (CDK4) and increased cell expression of cycle regulators and inhibitors cyclin D3, p27, and p21 [166]. Also, this species' venom was able to block the cell cycle from the G0/G1 phase to the S phase [167].

Chlorotoxin (Cltx) is found in the venom of the Palestine yellow scorpion *Leiurus quinquestriatus*. *In vitro* studies showed that Cltx binds to glioma cells without affecting normal cells; Cltx can bind endogenously to MMP-2 expressed in glioma cells, thus generating a loss of the gelatinase activity of the glioma and decreasing the expression of MMP2. PBITx1, extracted from the burrowing thick tail scorpion *Parabuthus schlechteri*, is a selective toxin of the Na+ channel and structurally similar to Cltx, suggesting that it could act on chloride channels and arrest cancer cells [168–170]. This synthesized peptide showed low toxicity in clinical trials, inhibiting angiogenesis, a possible candidate to combat gliomas [171].

Work limitations

Arthropods comprise a large phylum of invertebrate animals, and their particular biological and ecological characteristics vary according to each species. It is worth mentioning that numerous species have bioactive peptides in their venoms with anti-inflammatory activity, as observed in studies conducted *in vivo* and *in vitro*. Thus, we selected certain arthropods groups that provided more publications related to the theme when inquiring databases. We expected the present review to glimpse the theme and attract the audience's attention to this exciting research topic. A limitation of the study is about some elusive mechanisms of action of venom peptides reported by different laboratories that can be further explored for peptide drug development. Despite this, a handful of information allowed describing the peptides' significant "anti-inflammatory effects" from venom components of numerous arthropod species.

Conclusion

Considerable diversity of bioactive molecules under investigation can be developed as therapeutic agents to treat numerous human diseases. Various research groups have studied different peptides identified in arthropod venoms to unravel their potential as anti-inflammatory agents. The selected examples listed herein comprise peptides found in the venom and hemolymph of diverse species of arthropods. Included in this review were arthropods related to insects (ants, bees, and wasps), crustaceans (shrimp and crabs), arachnids (scorpions and spiders), and chilopods (centipedes), all of them containing in their venom peptides with important anti-inflammatory activity. Peptides derived from arthropod venoms act on different inflammatory pathways,

reducing pro-inflammatory cytokines both in *in vitro* and *in vivo* models. It is known that inflammation at an advanced stage can trigger malignant neoplasms and contribute to their exacerbation. Thus, multifunctional venom peptides that act on inflammatory pathways and pathways related to cancer deserve considerable attention in the present and future natural drug development programs. Consequently, arthropod venom peptides, which evolved over millions of years, comprise a rich source for discovering and developing peptides with potent pharmacological efficacy to treat inflammatory and malignant diseases. The disclosure of their specific mechanisms of action and application potential as therapeutic agents should continue in the years to come.

Abbreviations

ALF: anti-lipopolysaccharide factor; ALS: amyotrophic lateral sclerosis; BAX: BCL2-associated X protein; BCL: B-cell lymphoma; BV: bee venom therapy; CAT: catalase; CD: cluster of differentiation; CMO: scorpion oligopeptides; COX: cyclooxygenase; DBPs: disulfide-bridged peptides; DPPH: 2,2-diphenyl-1-picrylhydrazyl; ERK: extracellular signal-regulated kinase; FAZ: cell surface death receptor; FDA: U.S. Food and Drug Administration; FPR-2: formyl peptide receptor-2; ICAM-1: intercellular adhesion molecule 1; IFN- γ: interferon gama; IL-1: interleukin 1; IL-1β: interleukin beta; IL-4: interleukin 4; IL-6: interleukin 6; IL-8: interleukin 8; IL-10: interleukin 10; IL-13: interleukin 13; iNOS: nitric oxide-induced synthase; JNK: c-Jun N-terminal kinases; LALF: limulus anti-lipopolysaccharide factor; LPS-BD: lipopolysaccharide-binding domain; LPS: anti-lipopolysaccharide; M-ALF: marsupenaeus anti-lipopolysaccharide factor; MAP: mitogen-activated protein; MAPK: mitogen-activated protein kinase; MHC-II: major histocompatibility complex 2; MPC: monocyte chemoactive protein; MPO: myeloperoxidase; NDBPs: non-disulfide-bridged peptides; NF-kβ: nuclear factor kappa beta; PAM: antimicrobial peptides; PAMP: pathogen-associated molecular pattern; PRISMA: preferred reporting items for systematic reviews and meta-analysis; PGE: prostaglandin; PLA2: phospholipase A2; PtALF: portunus trituberculatus anti-lipopolysaccharide factor; SALF: shrimp anti-lipopolysaccharide factor; SOD: superoxide dismutase; TGF-β1: transforming growth factor-β1; TLR: toll-like receptor; TNF- α: nuclear transcription factor-alpha; TRAP1: transient receptor potential ankyrin; VCAM: vascular adhesion molecule; β-GPB: guanine nucleotide-binding protein subunit beta.

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Competing interests

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Authors' contributions

All authors collaborated with the preparation and revision of the manuscript. All authors read and approved the final manuscript.

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